

**THE CHALLENGE OF PROVIDING AN EFFECTIVE, BUT TECHNICALLY COMPLEX,  
VACCINE TO THE DEVELOPING WORLD.**

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PREVENAR® is arguably one of the most successful vaccines ever developed. It is designed to provide protective immunity against the seven serotypes of *Streptococcus pneumoniae* most commonly associated with invasive pneumococcal disease. Universal immunization of infants with PREVENAR was introduced in the United States in 2000. Since then, the U.S. has witnessed a greater than 98% decline in disease caused by the seven pneumococcal serotypes in the vaccine and, as a result of herd immunity, a greater than 50% decline of disease in adults. Currently the vaccine is registered in over 80 countries and, in a number of these, is included in the national immunization program. Yet, in spite of the vaccine's success, pneumococcal disease remains the largest cause of vaccine-preventable death of children in the developing world. An imperative exists to bring PREVENAR to developing world countries. However, a significant challenge facing such an effort is the vaccine's complexity. It is composed of seven purified polysaccharides, each covalently conjugated onto a carrier protein, and specifically combined into a single formulation. The complexity is compounded for the second-generation vaccine, currently in phase 3 development, which is designed to expand serotype coverage to include an additional six serotypes. The latter are particularly important for protection against pneumococcal disease in the developing world. The production and manufacturing control of the vaccines is complex and involves multiple worldwide manufacturing sites. These represent potentially substantial impediments facing the provision of the hundreds of millions of vaccine doses that are needed. Nonetheless, given the vaccine's effectiveness and medical importance, the challenges must be overcome. The present discussion will specifically define the challenges and will provide a context for evaluating potential solutions.

## **HUMAN MONOCLONAL ANTIBODIES AND ANALYTIC VACCINOLOGY**

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Following appropriate priming by infection or vaccination, memory B cells and serum antibody levels are sustained for a lifetime conferring immediate protection upon secondary encounter with the pathogen. I will first discuss the differential requirements for activation of human naïve and memory B cells and describe two methods that can be used to interrogate the human memory B cell repertoire. The first is based on limiting dilution analysis of polyclonally stimulated mononuclear cells. Using this method, we measured the frequency and fine specificity of memory B cells in serial samples under steady state conditions and after vaccination. The second method is based on the efficient immortalization and cloning of memory B cells. Using this method we have been able to isolate from the human memory repertoire several potent and broadly neutralizing monoclonal antibodies against viruses such as SARS, Dengue, H5N1, HCMV and HIV-1. I will discuss how such antibodies can be used not only to provide immediate protection, but also as probes for epitope discovery and vaccine design.

## **UTILIZING INFLUENZA VACCINATION TO RAPIDLY CLONE HIGH AFFINITY HUMAN MONOCLONAL ANTIBODIES AND TO TEST THE CONCEPT OF ORIGINAL ANTIGENIC SIN**

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Pre-existing neutralizing antibody provides a first line of defence against pathogens. For influenza virus, annual vaccinations are given to maintain protective levels of antibody against the currently circulating strains. We show that a booster vaccination induced a rapid and robust influenza specific IgG antibody-secreting cell (ASC) response that accounted for up to 6% of peripheral blood B cells at the peak. Importantly, as much as 80% of purified ASCs were influenza specific at the peak of the response. This ASC response was characterized by a highly restricted B-cell receptor (BCR) repertoire that in some donors was dominated by only a few B-cell clones. This pauciclonal response, however, showed extensive intraclonal diversification from accumulated somatic mutations. We used the immunoglobulin variable regions isolated from sorted single ASCs at the peak of the response to produce over 50 human monoclonal antibodies (mAbs) that bound to the three influenza vaccine strains with high affinity. This strategy demonstrates that we can generate multiple high affinity mAbs from humans within a month after vaccination. The panel of influenza-virus-specific human mAbs allowed us to address the issue of original antigenic sin (OAS): the phenomenon where the induced antibody shows higher affinity to a previously encountered influenza virus strain compared with the virus strain present in the vaccine. However, we found that most of the influenza-virus-specific mAbs showed the highest affinity for the current vaccine strain. Thus, OAS does not seem to be a common occurrence in normal, healthy adults receiving influenza vaccination.

## ANTI-VIRAL IMMUNE RESPONSES IN LYMPH NODES

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Cell migration and coordinated cell-cell interactions are hallmark features of the immune response to antigen challenge. Recent advances in real-time *in vivo* imaging technology have added a new dimension to our efforts to understand the dynamics and complex interplay of the key cellular players in the steady state and during ongoing immune responses. In particular, multiphoton intravital microscopy (MP-IVM) allows prolonged three-dimensional observations of highly dynamic events that occur hundreds of micrometers below the surface of solid tissues in living animals. The capacity to visualize the cellular dynamics in skin-draining lymph nodes is of particular importance because these organs are the primary sites of immune recognition of transcutaneously administered antigens, including vaccines.

We have recently developed a MP-IVM model in mouse popliteal lymph nodes, which has allowed us to analyze, in real time, how T and B cells respond to cognate antigens derived from the skin and subcutaneous tissue. Specifically, we have examined how follicular lymph node B cells become first exposed to viral particles after intradermal injection. Within minutes after injection, intact viral particles accumulated on a discrete population of CD169+ macrophages that populated the floor of the subcapsular sinus (SCS). Using MP-IVM, immunohistochemistry and electron microscopy, we found that these cells were critical in presenting antigens to virus-specific B cells in the underlying follicles. Moreover, upon selective depletion of lymph node-resident macrophages by subcutaneous injection of clodronate liposomes, the downstream lymph nodes were no longer able to prevent the systemic spread of lymph-borne virus, indicating that the SCS macrophages play a dual role as critical antigen-presenting cells for follicular B cells and as innate “flypaper” for skin-derived lymph-borne pathogens.

**USING VACCINES TO REVEAL HUMAN IMMUNE SYSTEM FUNCTION AND THE  
PATHOGENESIS OF ZONOTIC DISEASES**

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(Abstract not received in time for printing)

## FIRST CLINICAL EFFICACY TRIAL OF AN ADENOVIRUS TYPE 5-BASED HIV-1 VACCINE: THE STEP STUDY

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**Background:** Recent activities in HIV-1 vaccine R&D have focused on vaccine candidates capable of inducing HIV-specific T cell immune responses. Such vaccines have shown partial success in some animal models, but this concept has not been previously tested in humans.

**Methods:** A collaborative study between Merck, the HIV Vaccine Trials Network, and US National Institute of Allergy and Infectious Diseases was initiated in 2004. This phase II, randomized, multi-center, double-blind, placebo-controlled test-of-concept study enrolled 3000 HIV seronegative volunteers at high risk of acquiring HIV in regions of the world where clade B predominates. Volunteers were randomized (1:1) to receive 3 injections (Day 1, Week 4 and Week 26) of either the MRKAd5 HIV-1 gag/pol/nef vaccine (a replication defective Ad5 vector) or placebo. Randomization was pre-stratified by gender, baseline Ad5 titer, and study site. Volunteers were tested at 6-month intervals for HIV acquisition. In volunteers who became HIV infected, plasma HIV viral load and CD4 cell counts were measured at multiple time points post-diagnosis. Serum, plasma and PBMC were collected at multiple time points in all volunteers for laboratory analyses.

**Results:** In September 2007 an independent DSMB reviewed data from a planned interim analysis in volunteers with low (<200) baseline Ad5 neutralizing antibody titers (the primary analysis population). Using a modified 'intent-to-treat' approach, in this stratum, there were 24 infections among the 741 vaccinees compared to 21 infections among the 762 placebo recipients. Among those who became HIV infected, the geometric mean plasma HIV RNA level was ~40,000 copies/mL in the vaccine group compared to ~26,000 copies/mL in the placebo group. Lack of efficacy did not appear to be explained by lack of vaccine-induced immune responses in vaccinees. Post-hoc analyses demonstrated that the trend towards more infections in the vaccine group was more pronounced in volunteers with high (>200) baseline Ad5 titers (21 infections in 392 vaccinees vs. 9 in 386 placebo recipients).

**Conclusions:** There was no evidence that vaccination prevented infection or lowered viral load setpoint; in fact, there were more infections in the vaccine group. Investigations are under way to understand if these results are explained by immune responses induced by the vaccine, by differences in the study populations or, since the numbers are relatively small, simply due to chance. Because of the implications for the development of other CMI based vaccines, it will be important to understand the potential mechanisms underlying these results.

## QUALITY ISSUES: THE GOOD-ENOUGH VACCINE

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Most vaccines cannot be treated as well characterised chemical entities but they are among the most successful medical interventions. Confidence in their effect is based on properties that may or may not be correctly identified as markers of clinical safety or efficacy but which are related to the consistency of production. Vaccines against Human Papilloma Virus and poliomyelitis will be used as examples.

## NEW METHODS FOR DETECTING ADVENTITIOUS AGENTS

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Two factors have driven new approaches to adventitious agent testing in vaccine production; the need for more rapid assays to test bulk product and the requirement to thoroughly exclude latent adventitious agents in new cell substrates.

European and US regulatory authorities have opened the door to PCR based testing methods to detect mycoplasma contamination, facilitating rapid screening. However, direct PCR methods suffer from two disadvantages, often only a small volume from the fermenter is tested and secondly the assays do not distinguish between viable organisms and contaminating mollicute DNA that can be introduced from animal and animal free reagents like serum and plant hydrolysates respectively. We have developed a hybrid culture-PCR method "HyMy" that provides the advantages of culture while shortening the assay period from 28 days to 14 days. In the next phase we will introduce hybrid culture and molecular detection systems for both bacteria and viruses.

One of the tools we are investigating for the rapid detection of viruses are cells engineered with beta-lactamase constructs linked to interferon response elements. Exposure of the indicator cells to interferon from virus infected cells leads to conditional expression of beta-lactamase. This enzyme will cleave appropriate FRET substrates leading to a blue fluorescence within the cells at an early stage in the infection cycle.

The recent acceptance of tumorigenic cells, like MDCK cells, as substrates for the production of prophylactic vaccines has been a major change in the industry. It has prompted an unprecedented level of investigation of the cells for adventitious agents, particular for viruses that might be oncogenic or latent. While specific viruses can be excluded by QPCR analysis, the potential for unknown agents remains. We have developed degenerate PCR systems for the detection of all known polyomaviruses and herpesviruses and demonstrated the utility of this approach through the identification of new herpesviruses in pigs and primates. We are now investigating the use of oligonucleotide arrays coupled to random PCR reactions to detect a wider range of potential contaminants in these new cell substrates.



## TOWARDS A THERAPEUTIC HEPATITIS C VACCINE – A PRECLINICAL AND CLINICAL LEARNING CURVE

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Novel approaches for treatment of chronic HCV infection are urgently needed. To this end, we have comprehensively identified disease relevant viral T-cell epitopes derived from therapy responders and spontaneous resolvers. Based on this analysis, we designed a prototypic peptide vaccine, IC41 that is containing CD8 and CD4 T-cell epitopes and poly-L-arginine as adjuvant. Correlation of immune and RNA response showed that the single parameter correlating best with RNA decline was induction of CD8 CTLs, above a critical threshold. Responses were dominated by T-cells directed against the viral NS3 protein, and in one patient a viral amino acid exchange of a vaccine epitope resulted in reduced epitope recognition that emerged prior RNA rebound. The observed mutational T-cell escape corroborates a causal relationship of T cell induction and HCV RNA decline. Further pre-clinical (HLA transgenic mice) and clinical trials (healthy volunteers) identified an optimized application schedule of our vaccine with significant stronger CD8 responses and broader simultaneous responses against CTL epitopes. Based on this study a novel trial in chronic HCV genotype 1 patients naïve to ST was initiated. The data obtained shows that the primary endpoint set for this study, namely a statistically significant sustained HCV-RNA decline, has been met. In the second week after the final vaccination, a 40 % reduction of viral load was observed in comparison to the baseline prior to vaccination. The therapeutic effect of the vaccine on the viral load is small, but found to be significant when data was submitted for rigorous statistical analysis ( $p=0.0010$ ). The results are especially significant in the light of the observation that viral load reduction is increasing with the number of vaccinations. The study included patients with various levels of viral load. In the subset of patients ( $N=25$  with high viral load ( $> 2$  million copies/ml) before treatment, a statistically significant ( $p=0.0001$ ) average decline of 60 % (0.4 log) was achieved. Thus, it seems that the therapeutic effect is more pronounced when the patients' immune system is unable to keep the viral load under control. With this data, we will move forward with a second generation vaccine that includes further T-cell epitopes and where poly-L-arginine will be replaced by IC31TM, a novel adjuvant that has shown to induce in preclinical and clinical settings a sustained and superior level of pivotal T-cells. The previous data and the design of the novel vaccine will be discussed in my presentation.

## **DELIVERY DEVICES AND APPROACHES FOR PRE-CLINICAL AND CLINICAL HIV IMMUNIZATION**

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*Preclinical delivery methods.* Vaccine delivery methods are essential in humans but may not be identical to those effective in animals. DNA vaccines were used to investigate different forms of deliveries (1-8). In mice and macaques skin delivery (id by biojector, syrijet or gold beads) and mucosal delivery (nasal, oral, rectal) appear more potent than intramuscular or subcutaneous administration. In a transgenic murine model we have shown complete protection after mucosal or dermal gene vaccination and HIV challenge. Plasmid DNA encoding HIV antigens was delivered intradermally by electroporation. This resulted in a very rapid induction of potent immune responses.

*Clinical studies.* Both mucosal (oral by syrijet) and intradermal (by biojector) administration of genetic vaccines have shown capacity to induce potent immunity, indicating that it is possible to significantly reduce the amount of DNA needed for intramuscular immunization. The use of an HIV DNA vaccine prime followed by modified vaccinia Ankara carrying multiple HIV genes gave very high antigen-specific and broad response rates in a recent phase 1 clinical trial (5-6). In an ongoing study of infected individuals, DNA is delivered to abraded skin, aided by transfecting agent and occlusive dressing (8). The use of the skin delivery pathway may help both systemic and mucosal immunity, by inducing a persistent systemic immunity. The broad representation of different subtype genes of HIV combined with the widely distributed skin delivery may contribute to potent immunogenicity in humans.

## FLAVIVIRUS CAPSID DELETION MUTANTS AS A NEW VACCINE APPROACH

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Flaviviruses are a group of arthropod-transmitted pathogens of major and ever-increasing, medical importance. Among the most relevant human pathogens are yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), the dengue viruses (DV), and tick-borne encephalitis virus (TBEV). In the absence of any available specific antiviral drugs, disease prevention by vaccination represents the most important and effective measurement to combat flavivirus diseases.

In the past, both live attenuated vaccines (against YFV and JEV in China) and inactivated whole virus vaccines (JEV and TBEV) have proven very successful to reduce disease burden. Nevertheless, new vaccination strategies are in big demand to meet the many challenges imposed by flavivirus infections. The rapid invasion of North America by West Nile virus illustrates the potential of these pathogens to establish themselves in new geographical regions and to infect a multitude of host organisms. The geographical expansion of endemic areas, socioeconomic changes in, and the rising travel activities to such areas steadily increases the number of people at risk of infection. A particular problem for vaccine development is imposed by the difficult immunopathogenesis that is seen with successive dengue virus infections, which has hampered attempts to develop dengue vaccines.

We have explored the possibility of using genetically engineered flavivirus mutants with specific deletions in the capsid protein gene as a new vaccine approach. Remarkably, replication competent viral strains can be obtained which lack up to one third of the amino acid sequence of this structural protein. Due to this modification, an increased proportion of non-infectious, but highly immunogenic subviral particles are produced. This approach has been shown for TBEV and WNV to generate apathogenic vaccine strains and is likely applicable also to other flaviviruses. In the mouse model, capsid deletion mutants achieved a higher attenuation index (ratio of 50%-lethal dose to 50%-protective dose) than observed for any other attenuating principle tested in the same system and induced a solid, protective immune response. The introduction of even larger deletion mutations into the capsid gene yielded mutants which could not be propagated in cell culture, but represent self-replicating RNAs which can undergo major steps of the viral life cycle *in vivo*. Significantly, these RNA replicons produce ample amounts of highly immunogenic subviral particles. We have explored in the mouse model the application of such self-replicating RNA as a new type of nucleic acid vaccine. This approach combines features of classical live vaccines, such as the *in vivo* production of RNA replication intermediates and all relevant viral antigens, with the advantages of a nucleic acid vaccine. The absence of viral spread in the host in this replicon approach is a relevant safety feature. Capsid deletion mutants hold promise to overcome the particular challenges to vaccine development imposed by dengue viruses.

## **VIRAL VECTORS – COUPLING INNATE SIGNALS TO ANTIGEN EXPRESSION AND PRESENTATION**

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Several viral vectors and DNA vaccines are being developed as vehicles for recombinant vaccines and many of them have already entered clinical trials. Results from both preclinical studies and clinical trials have indicated that vectors expressing similar or identical inserts generate qualitatively different immune responses. These findings probably bear on the fact the different vectors target different innate immune pathways thus resulting dissimilar acquired responses. Furthermore, it is becoming evident that e.g. measurements of only quantitative T cell responses do not necessarily correlate to levels of protection for a certain vaccine. For example, in the P1A mouse tumor model animals immunized with a recombinant adenovirus (Ad5) vector expressing the P1A antigen generated significantly higher T cell responses than did animals that had been immunized with a recombinant alphavirus vector (Semliki Forest virus – SFV) based vaccine expressing exactly the same P1A antigen. However, upon tumor challenge the animals that had been immunized with the alphavirus vaccine showed significantly better protection than did the corresponding Ad5 immunized animals. Analysis of the SFV induced memory T cells indicated that their capacity to expand upon recall was much greater than the Ad5 induced T cells. As also heterologous prime-boost regimens using at least two kinds of vectors are being contemplated careful analysis of the qualities of different vectors need to be assessed separately and in combination. These aspects will be discussed in light of several examples from preclinical and clinical trials.

**VEEV REPLICON-BASED VACCINES USED IN HETEROLOGOUS PRIME BOOST STRATEGIES INDUCE LIFELONG PROTECTION FROM PROSTATE CANCER AND THERAPY OF CERVICAL CANCER IN MICE AND ROBUST CELL-MEDIATED IMMUNITY IN RHESUS MACAQUES**

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Therapeutic HPV vaccines, based upon platforms of Venezuelan equine encephalitis virus replicon particles (VRP) and attenuated recombinant Vesicular Stomatitis Virus (VSV) vectors, both expressing mutated E7-E6 fusion proteins from the high-risk HPV16 and 18 genotypes, were tested in various homologous or heterologous prime-boost regimens in mice and in Rhesus macaques to assess levels of immunogenicity and anti-tumor immunity. Anti-tumor immunity was assessed by prophylactic and therapeutic vaccination with the HPV16 E7-E6 coding vectors in mice against HPV16-transformed tumors. Full protection from tumor challenge was observed after immunization with all three VRP/VRP, VSV/VSV, and VRP/VSV regimens. Therapeutic immunization of tumor-bearing mice showed 75% rejection of tumors in mice treated with VRP/VRP or VSV/VSV regimens and 100% rejection in mice treated with VRP/VSV combination strategies. Rhesus macaques vaccinated intramuscularly with three doses of VRP four weeks apart and boosted once with VSV showed very robust and sustained antigen-specific IFN gamma and IL-2 ELISPOT responses against HPV E6 and E7 peptides. In contrast to mice, only modest responses were detected after three doses of VRP alone or two doses of VSV alone in the macaques. In a separate TRAMP mouse prostate cancer model VRP in combination with DNA based vaccines both coding for the prostate cancer associated antigens PSCA or STEAP were able to induce lifelong protection against prostate cancer development when the male mice were vaccinated at an age of 8 weeks, which is the stage at which they have developed prostate intraepithelial neoplasia. All control vaccinated mice had succumbed of prostate cancer within a year but of the DNA prime VRP boost immunized mice 90% were alive and apparently healthy at month 12 and 65% at month 18. In conclusion, these strong in vivo anti-tumor responses both in cervical cancer and prostate cancer models and the unprecedented high cellular immune responses in non-human primates after heterologous VRP prime and VSV boost or in mice DNA prime and VRP boost provide strong justification for further development of the VRP platform for therapeutic anti-tumor vaccines delivery.

## **EFFICIENT AND ECONOMICAL INFLUENZA VACCINES INCORPORATING A TOLL-LIKE RECEPTOR LIGAND**

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Pathogens display and produce a variety of molecules that are unique and not found in higher order animals. Examples include lipopolysaccharide, unmethylated CpG DNA, double-stranded RNA, and flagellins. Collectively, these motifs are called Pathogen-Associated Molecular Patterns, or PAMPs. Nature has evolved a system of receptors that recognize the various classes of PAMPs, and one major category of these receptors is known as the Toll-Like Receptors or TLRs. TLRs are expressed on the surface of, or on membrane-bounded vesicles inside, cells capable of processing and presenting antigens to T-cells. Uptake of a pathogen by an antigen-presenting cell via a TLR results in highly efficient processing and presentation of the antigens of the pathogen. Our laboratory has adapted this TLR-mediated antigen uptake process for the design of vaccines against influenza. We fuse the genetic sequence for flagellin, the ligand for TLR5, in-frame with the sequence for influenza antigens. We have two independent, but complementary approaches to influenza vaccination. The first vaccine targets the ectodomain of the highly conserved ion channel protein, M2e. M2e is small (24aa), present in low copy number, and poorly immunogenic in the context of natural infection. A vaccine comprising flagellin fused to four copies of M2e provides complete protection against death and severe disease in mice challenged with an LD90 dose of live virus. This vaccine has been shown to be safe and highly immunogenic in humans. The second vaccine, directed at the globular head moiety of hemagglutinin, elicits potent neutralizing antibody responses and provides complete protection against disease and death in mice challenged with an LD90 dose of live virus. Both vaccines are produced by standard rDNA methods in *E. coli* at very high yield and low cost. Production levels, antigen per volume of starting fluids, are approximately three logs higher than the current egg-based system, making the distribution of both pandemic and seasonal vaccines world wide a workable possibility.

## **FLUBLOK, A HIGH DOSE RECOMBINANT INFLUENZA VACCINE**

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Influenza vaccine effectiveness of inactivated vaccines needs to be improved particularly when vaccine strains and circulating strains are poorly matched not only for those at high risk. For example, the 2007-2008 inactivated influenza vaccine provided only 44% vaccine effectiveness according to a recent CDC Morbidity and Mortality Weekly Report (April 18, 2008, Vol 57, No 15).

Four simple ways to improve vaccine performance are: 1) Increase the number of doses, 2) Increase the hemagglutinin content; 3) Use an adjuvant or 4) Vary the type of preparation (whole virus vaccine, subvirion versus subunit).

A recombinant HA vaccine produced in cell culture using the baculovirus vectors system provides an attractive alternative to the current egg-based influenza vaccine (TIV) manufacturing process and presents the possibility for safe and expeditious vaccine production. The high purity of the antigen enables administration at higher doses without a significant increase in side-effects in human subjects.

The HA genes from the annual World Health Organization recommended strains are cloned, expressed and purified using a general purification process. An overview of the expression technology used to make the annual adjustments will be provided. The insect cell - baculovirus production technology is a modern solution for rapid viral or parasitic antigen production and that this technology is particularly suitable for influenza where annual adjustment of the vaccine is required.

The safety, immunogenicity and efficacy results from four recent clinical studies conducted in different populations (ages 18 - 49; 49- 64 and 65 and older) will be presented. The highly purified protein vaccine, administered at three times higher antigen content than TIV is well tolerated and results in stronger immunogenicity, a long lasting immune response and provides cross protection against drift influenza viruses.

**CHALLENGES AND SOLUTIONS FOR THE NEXT GENERATION OF VACCINES:  
DEVELOPMENT OF CELL CULTURE-BASED LIVE ATTENUATED INFLUENZA VACCINE**

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To rapidly meet the public demand for influenza vaccines in the event of a pandemic influenza outbreak and with the support of a \$170 million award by the US federal government, MedImmune has intensified its R&D efforts to replace eggs as the production substrate with cell-based manufacturing for its commercial flu vaccine, FluMist®. The cell substrate under development is an MDCK cell line developed at MedImmune. Vaccine viruses produced from these cells maintain the characteristic phenotypes of FluMist® including cold-adaptation, temperature sensitivity and attenuation in a ferret animal model. Here we report our progress in characterization of the host cells and productivity improvement. In order to maximize the productivity of the cells used for vaccine manufacturing, we screened over 2500 cell clones that originated from an ATCC MDCK cell bank, optimized the serum-free cell growth conditions for a dozen selected cell clones and finally produced a single master cell bank. We conducted extensive cell bank testing that consists of a panel of 32 product specification tests, 20 cell line characterization assays and 8 tumorigenicity and oncogenicity tests. Using this cloned MDCK cell line we have developed an influenza vaccine production platform process that is adaptable to various cell culture vessels. This production platform has been used to efficiently produce cold-adapted vaccine strains in both conventional stir tank bioreactors and disposable bioreactors (Single Use Bioreactors). We have produced cold-adapted influenza viruses up to 8.9 log<sub>10</sub> fluorescent focus units per milliliter of harvest fluid and have shown that the platform process is applicable to the production of a broad range of currently circulating cold-adapted seasonal vaccine strains and several cold-adapted prototypic pandemic influenza viruses (H5, H7 and H9). We will share our experience and scientific approaches to address regulatory concerns about MDCK cell characterization, including tumorigenicity and oncogenicity, and our methods which have increased virus productivity by more than 300-fold.



## **TOWARD THE DEVELOPMENT OF A SARS VACCINE**

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The pandemic potential and pathogenicity of SARS coronavirus (SARS-CoV), as well as the absence of effective licensed drugs, highlights the need to develop an effective SARS vaccine. Preliminary data have indicated that a human monoclonal antibody directed toward the spike envelope glycoprotein of SARS-CoV can neutralize virus in vitro and provide protection from live virus challenge in animal models. Two established vaccine technologies were evaluated for their ability to elicit protective neutralizing antibodies. First, SARS-CoV was grown in cell culture, isolated and inactivated by beta propiolactone treatment. This killed vaccine preparation induced neutralizing antibodies in mice, rabbits and ferrets, and conferred protection in ferrets against live SARS CoV challenge. Second, two forms of recombinant spike glycoprotein were produced in CHO cells and purified to near homogeneity. A full-length form was expressed as a trimer on the cell surface, and a truncated form was expressed as a monomer and secreted from the cells. Both recombinant proteins were recognized by the SARS-CoV neutralizing monoclonal antibody, suggesting that important neutralization epitopes were preserved and exposed in the proteins. Moreover, when formulated with the MF59 adjuvant, both proteins were able to induce neutralizing antibodies in mice, rabbits and ferrets. However, the truncated form of the spike glycoprotein was selected for further development, based on better productivity in CHO cells and superior immunogenicity in animal models. In efficacy studies in ferrets, the recombinant subunit truncated spike glycoprotein plus MF59 was protective against live SARS CoV challenge. In collaboration with the NIH, the subunit vaccine is progressing toward a phase I clinical trial.

## **ANALYTICAL AND MANUFACTURING CHALLENGES IN PREPARATION OF BACTERIAL POLYSACCHARIDE CONJUGATES**

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There are now highly effective conjugate vaccines against several invasive bacterial diseases of children including those caused by *Haemophilus influenzae*, pneumococci and meningococci. The commonality is that protective immunity to these diseases is directed against capsular polysaccharides (PS). However, PSs must be covalently attached to a protein to render them sufficiently immunogenic in young children.

To produce a conjugate vaccine, the purified PS must first be chemically modified to generate reactive groups that can be covalently linked to the carrier protein. Prior to activation and conjugation, generation of size reduced PS can facilitate conjugate purification, because it facilitates later removal of unconjugated PS. There are two commonly used methods for PS activation; periodate oxidation between adjacent carbons carrying hydroxyl groups, and cyanlation of hydroxyls. A serious potential problem with periodate activation is induction of conformational changes in the PS by opening of sugar ring structures.

There are a number of quality control tests that are common to most PS-protein conjugates. The purity of the PS and carrier protein must be assured. Low molecular weight impurities in the protein may result in inefficient conjugation. The Ps should be of known molecular size distribution before and activation. Two critical measures after conjugate purification are the PS to protein ratio and the percent non-conjugated saccharide (free saccharide). The latter two measures may be considered to constitute the potency test.

From a production standpoint, yield and conjugate stability are critical considerations. Typically, less than 20% of the activated PS becomes conjugated. Conjugate yield can be improved using some newer conjugation methods, whereby highly reactive groups are generated on both the PS and carrier protein. Using this approach yields can approximate 50%. Stability of the bulk and formulated conjugate must be followed. The two major measures used to follow stability are changes in molecular size and percent free (unbound) PS. Animal potency tests have proven to be insensitive to changes in conjugate potency during storage. Some manufacturing processes can yield relatively unstable conjugates.

## DEVELOPMENT OF VALIDATED ASSAYS FOR MEASURING THE HUMAN ANTIBODY RESPONSE TO POLYSACCHARIDE CONJUGATE VACCINES

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At present conjugate vaccines (CV) against *H. influenzae* type b (Hib), *N. meningitis* (Men) groups A, C W135 and Y, and 7 serotypes of *S. pneumoniae* (Pnc) are available. The methods used in the evaluation of the CVs measure concentration, avidity and functional activity of anti-polysaccharide (PS) antibodies.

Serum bactericidal activity (SBA) has been accepted as the method of choice for evaluating the efficacy of MenCV. The basis for this decision was the study of Goldschneider et al in 1960's, which showed correlation of SBA titer (using human complement, hSBA) of lower than 4 and acquisition of meningococcal infection in army recruits. Later, the correlate was re-evaluated after the introduction of group C MenCV in the UK by using baby rabbit complement (rSBA); a titer of 8 or higher was associated with short term protection. Additional indicators include demonstration of 4-fold or greater rise in SBA titer and a titer of 4 or greater by hSBA. Antibody concentration measured by EIA standardized and validated so that it correlates with the SBA is a useful adjunct. For evaluation of long-term protection, avidity assays for demonstration of immunological memory have been used. The reagents and references for meningococcal assays are available from NIBSC, Potters Bar, England.

The first HibCVs were licensed based on their efficacy in phase III trials. After that non-inferior immune response as compared to a licensed product has been accepted as a criterion for licensure. During the first trials with plain PS vaccine radio immunoassay (RIA) was used, and the antibody concentrations of 0.15 and 1.00 µg/ml were introduced as threshold concentrations for short and long-term protection, respectively. Presently EIA correlating with RIA has been used for measuring responses to CVs. In the 1980's and 1990's extensive interlaboratory comparisons and standardization of RABA and EIA took place, and at present the QC samples, reference serum and antigens are available from NIBSC and/or US FDA. No internationally standardized assays for measuring avidity and function of antibodies are available.

For evaluation of PncCVs standardised and validated EIA should be used. The recent developments in EIAs have included the neutralization of unspecific antibodies by using cell wall (CWPS) and type 22F polysaccharide. In a WHO Workshop held in 2000 a well-characterized EIA protocol was chosen as a reference or benchmark assay. The sera for the calibration of EIA are available from the WHO Pneumococcal Reference Laboratory at Institute of Child Health, London, the UK, reference serum from US FDA and the antigens from ATCC (PS) and SSI, Denmark (CWPS). Interlaboratory standardization of an assay measuring opsonophagocytic activity (OPA) of antibodies is ongoing.

## THE HUMAN IMMUNE RESPONSE TO POLYSACCHARIDES AND CONJUGATES

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Encapsulated gram positive and gram negative bacteria colonise the human nasopharynx and are important causes of human disease. *Haemophilus influenzae* type b (Hib), *Neisseria meningitidis* Group C and *Streptococcus pneumoniae* are examples of such pathogens responsible for meningitis, pneumonia and other syndromes. Antibodies to the capsular polysaccharides surrounding these pathogens are known to mediate protection in humans, yet vaccines derived from purified capsule (which behaves as a T cell independent antigen) are not immunogenic or protective in those most at risk ie the young and the elderly. Conjugate vaccines, where the polysaccharide is conjugated to a protein carrier, have revolutionised this field by rendering the sugar moiety immunogenic even in the very young. The T cell dependent nature of the immune response to the conjugate is also associated with the phenomenon of “boostability” rendering the polysaccharide immunogenic in primed infants who would otherwise not respond. Widespread use of such vaccines has demonstrated their high impact on reducing infectious disease thanks to both direct and indirect effectiveness.

**THE IMPACT OF POLYSACCHARIDE CONJUGATE VACCINES**

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(Abstract not received in time for printing)

## **REGULATORY ISSUES ASSOCIATED WITH THE DEVELOPMENT OF A COMPREHENSIVE MENINGOCOCCAL VACCINE**

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*Neisseria meningitidis* is a major cause of bacterial meningitis and septicaemia throughout the world. Polysaccharide conjugates, arguably the most important vaccine development of the late 20th century, have provided a partial solution to the prevention of meningococcal disease. However, there is no vaccine that will offer protection against meningococci expressing a group B polysaccharide capsule. The poor immunogenicity of the group B polysaccharide and its structural similarity to glycosylated host antigens has largely deterred the development of a group B conjugate. Instead, emphasis has been on the use of outer membrane vesicles (OMVs) and the identification of potential vaccine antigens using various “omics” techniques. This presentation will review some of the key issues in the evaluation the MenC conjugates and look ahead to the challenges posed by protein-based vaccine formulations.

## **TB VACCINE DEVELOPMENT AND MANUFACTURING**

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(Presented by Walter Kallaur)

There are currently around 2 billion people infected with TB with over 8 million new infections and almost 1.6 million deaths each year. Multiple (MDR) and extreme (XDR) antibiotic resistance is spreading. Although BCG is one of the worlds most widely used vaccines it does not prevent pulmonary TB and has not curbed the worldwide pandemic. A new effective vaccine is the only hope for elimination of TB. Modification of BCG by recombinant means to make it more immunogenic and safer has been accomplished by introducing endosome escape through expression of perfringolysin, introduction of pro-apoptotic factors such as Nuo G knockout, incorporation of GMCSF cytokine expression and over-expression of TB antigens from various stages of the TB organisms life cycle. New techniques for fermentation and stabilization have been developed for this new generation of rBCG vaccines. A cGMP facility capable of producing the worlds need (300 million doses/year) of bulk rBCG vaccine has been built and validated. These rBCGs when used as primes for boost with non-replicating viral vectors have provided non-human primate cellular immunogenicity and protection from challenge with TB. BCG prime followed by viral vector boost has induced high levels of CD4+ and CD8+ T cell immunity in humans. The human immunogenicity of these prime boost regimens makes this approach feasible for other diseases requiring cellular immunogenicity such as Malaria and HIV. A network of Vaccine trial sites for Phase II proof of concept testing in infants (Cape Town S.A.) and Phase III efficacy testing for licensure in infants and adolescents/adults are being developed in South Africa, Kenya, Uganda and India. It is anticipated that licensure of the first new TB vaccines in over 80 years will occur in the 2015-16 time frame.

## **EVALUATING NOVEL CELL SUBSTRATES FOR USE IN VACCINE MANUFACTURE**

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Basic principles for evaluating cell substrates will be presented from a regulatory perspective. Various novel cell substrate types, including tumorigenic cells and insect cells, will be used as practical examples. New technologies that could improve assurance that products manufactured in novel cell substrates are safe will also be discussed.



## EPITOPE-DRIVEN GAIA HIV VACCINE DEVELOPMENT: AN UPDATE

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**Introduction.** We set out to develop a globally-relevant epitope-based HIV vaccine composed of CTL and helper T-cell epitopes that are highly conserved and immunogenic over a broad range of HLA backgrounds.

**Methods.** All HIV protein sequences deposited in GenBank (1995-2002) were screened to create a non-redundant database. Entries containing sequences >60% of the nominal protein length were searched for conserved 9-mer segments using Conservatrix. 9-mers conserved in at least 5% of database sequences were analyzed by EpiMatrix for predicted binding affinity to six HLA Class I and eight HLA Class II DR archetype alleles. Class II DR epitopes were extended to 20-25 amino acids in length to create immunogenic consensus sequences (ICS) that are enriched for overlapping conserved 9-mers with broad HLA coverage. Candidate HIV vaccine epitopes were ranked by conservation and by putative affinity for HLA as measured by EpiMatrix z-score. Initial evaluations of selected epitopes in ex vivo (human) and in vivo (mouse) studies have been performed in Mali, Thailand and the USA.

**Results.** 5,494 highly conserved 9-mers were mined from a non-redundant database of 10,199 HIV protein sequences. Epitopes predicted to bind HLA class I and class II were generally more broadly conserved than those selected for other epitope-based vaccines (>70%, compared to Epimmune's 40%). Interferon-gamma ELISpot assays performed in Providence, RI using PBMCs obtained from HIV-infected subjects confirmed the antigenicity of both Class I (85% A2, 29% of A3, 67% of B7, 20% of A24) and Class II ICS (95%) epitopes. Similar assays performed in Mali and Thailand confirmed cross-clade recognition of these epitopes. Immunization of HLA A24 and DR1 transgenic mice with multi-epitope vaccine formulations elicited robust epitope-specific T cell responses to 3 out of 40 A24 and 7 out of 24 ICS epitopes tested.

**Conclusions.** Computational tools can be used to efficiently map epitopes and to incorporate validated epitopes into vaccines. The GAIA HIV vaccine epitopes elicit robust, epitope-specific T helper and CTL responses in HLA transgenic mice. These findings will serve as a springboard for optimal immunogen engineering and targeting to boost multi-epitope vaccine immunogenicity and efficacy.

## THE IMPROVED ISCOMATRIX® ADJUVANT

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The ISCOMATRIX® adjuvant is CSL's proprietary biological vaccine adjuvant that has been extensively refined and developed over a number of years. It is comprised of ISCOPREP® saponin, cholesterol and phospholipid which combine to form cage-like structures typically 40nm in diameter. It can then be formulated with virtually any antigen to produce an ISCOMATRIX® vaccine. A range of ISCOMATRIX® vaccines have been tested in clinical trials and have been generally safe and well tolerated as well as immunogenic, generating both antibody and T cell responses. In recent years the focus at CSL has been on improving the ISCOMATRIX® adjuvant which meets the ever increasing regulatory standards for components of human vaccines, whilst maintaining the ability to induce strong immune responses. The result is an ISCOMATRIX® adjuvant that is well defined, has minimal impurities and does not contain any materials of animal origin. Improvements have also been made to the methods of manufacture to ensure the product can be reliably produced at any relevant scale. Another area of recent activity has been to increase the understanding of the mechanisms of action. The ISCOMATRIX® adjuvant has both antigen delivery and immunomodulatory capabilities that combine to provide enhanced and accelerated immune responses. The improvements to the ISCOMATRIX® adjuvant and enhanced understanding of the science underpinning the mechanism of action should ensure that the ISCOMATRIX® adjuvant will be an integral component in the development of novel human vaccines that will facilitate manipulation of the body's own immune system to prevent and/or treat diseases that to date have been refractory to vaccination.

## ADJUVANTED PLASMID DNA-BASED VACCINES

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Over the past few years, plasmid DNA prophylactic vaccines have reached licensure for veterinary applications against viral infections such as infectious hematopoietic necrosis virus in farmed salmon in Canada and West Nile virus in horses in the United States. In addition, a therapeutic canine melanoma vaccine has received conditional approval in the US. Although promising immunological results have also been reported in recent human clinical trials with unadjuvanted DNA vaccines, for instance against Ebola and West Nile virus, a number of approaches are being evaluated to further enhance the potency of DNA vaccines, including formulation of DNA with adjuvants and administration of DNA with devices. This presentation reports on a cationic lipid-based adjuvant, Vaxfectin®, which has shown enhanced immune responses in a number of published animal studies using different routes of administration, with the potential of dose-sparing activity for DNA vaccines. In particular, the nonclinical development of a pandemic influenza DNA vaccine encoding conserved antigens and formulated with Vaxfectin® will be presented. Plasmid DNA vaccines encoding hemagglutinin and conserved influenza antigens represent an alternative approach that may overcome several of the limitations of current licensed vaccines against seasonal and pandemic influenza, including dependence on eggs for vaccine production, a relatively long manufacturing cycle, the need for yearly updates of vaccine composition, the need for annual revaccination, reduced efficacy against HA-mismatched strains and reduced overall efficacy in the elderly. Pandemic influenza DNA vaccine candidates are currently being evaluated in two clinical trials by intramuscular needle and needle-free administration and the studies design will be presented. The application of Vaxfectin® as an adjuvant for protein-based vaccines will also be discussed and data supporting enhanced immunogenicity and dose-sparing effect of Vaxfectin® will be presented.

## EXPLOITING GLYCOENGINEERED YEAST FOR THE DEVELOPMENT OF NEXT GENERATION VACCINES

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Victoria Copeland, Stefan Wildt, Natarajan Sethuraman,

GlycoFi, Inc has developed engineered humanized yeast strains capable of producing glycoproteins with human-like glycosylation. Unlike proteins obtained from mammalian cell culture, which can be quite heterogeneous, GlycoFi's engineered yeast secrete glycoproteins with exceptional glycan uniformity. The ability to produce a glycoprotein with a predetermined structure opens the door to systematically investigate glycosylation-dependent structure activity relationships and to create specific glycoforms with improved therapeutic properties. By using a variety of engineered yeast strains, a protein can be expressed with various glycoforms allowing for elucidation and/or development of glycosylation-mediated functions. GlycoFi's technology further encompasses a combinatorial library for high throughput screening of fusion proteins and promoters providing optimization of heterologous protein expression in yeast. For proteins that prove difficult to express, this innovative method has proven to be quite successful in optimizing the conditions for expression in a yeast host. Historically, viral subunit vaccine research and development often requires production of difficult to express glycoproteins. Proper folding, high-level secretion, and function can all be affected by N-glycosylation, and the nature of N-glycosylation can differ dramatically in many heterologous protein production hosts from that of the native protein. Application of GlycoFi's humanized yeast technology can provide a range of benefits to improve the lead identification and optimization of subunit vaccines. Furthermore, manufacture of subunit vaccines with highly immunogenic N-glycans, such as alpha-1,3-galactose can now be contemplated.

## ENHANCING VACCINE IMMUNOGENICITY THROUGH USE OF CPG TLR9 AGONISTS AND OTHER ADJUVANTS

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Synthetic oligodeoxynucleotides containing CpG motifs (CpG ODN) are ligands for Toll-like Receptor 9 (TLR9) that is found in the endosomal compartment of human B cells and plasmacytoid dendritic (pDC) cells. Direct activation of B cells and pDC through TLR9 can lead to potent innate immune activation. In addition to innate immune activation, in the presence of an antigen, CpG ODN can promote the induction of strong Th1 biased immune responses. Stimulation of B cells by CpG ODN in the presence of antigen can selectively enhance the development of antigen-specific antibodies, especially of the isotype associated with Th1-like immune responses (e.g., IgG2a in mice). Following CpG ODN stimulation, both B cells and DC can effectively present antigen to T cells. CpG-induced antigen presentation taking place in a Th1-like cytokine milieu can lead to induction of strong Th1 biased immune responses consisting of cell-mediated as well as humoral immunity.

According to preclinical data CpG ODN are effective in augmenting the kinetics and strength of both antibody and cell-mediated responses to virtually all types of antigens. They are effective adjuvants in hyporesponsive populations and in neonates even in the presence of maternally derived antibodies. CpG ODN are also effective adjuvants in mucosal vaccines and promote the induction of strong IgA responses both at local and distal mucosal sites. Use of CpG ODN as adjuvants allows antigen dose sparing and earlier boosting. Furthermore, CpG ODN can be used in combination with a wide variety of other adjuvants with the combinations showing either synergistic or additive effects.

CPG 7909 (VaxImmune) induced faster and stronger titers of higher avidity antibodies to a commercial hepatitis B vaccine (Engerix-B, GlaxoSmithKline) in normal volunteers. Similar results were found in a subsequent trial conducted in HIV infected subjects who failed to respond to previous vaccination with proportion of subjects remaining seroprotected being significantly greater in the CpG group compared to the controls even 5 years later. In other Phase I clinical studies, enhancement of the strength and kinetics of antibody responses were found with addition of CPG 7909 to either the Biothrax whole killed AVA anthrax vaccine (Emergent), or recombinant malaria vaccines containing either *Plasmodium falciparum* AMA1 or MSP1 adsorbed to alum (Malaria Vaccine Development Branch, NIAID).

**QUALITY ASSESSMENT OF CERVARIX™, GSK'S CERVICAL CANCER VACCINE ,  
MANUFACTURED WITH THE BACULOVIRUS EXPRESSION VECTOR SYSTEM (BEVS)**

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Cervarix™, GlaxoSmithKline Biologicals' (GSK) cervical cancer vaccine is a recombinant vaccine composed of human papillomavirus (HPV)-16/18 L1 virus-like particles (VLP) adjuvanted with the proprietary AS04 adjuvant system. Cervarix™ has been shown in phase II clinical trials to provide sustained antibody responses for HPV-16 and 18 and prevention of abnormal cytology and cervical neoplasia up to 5.5 years [Gall 2007, Harper 2007]. In phase III clinical trials, the cervical cancer vaccine has been shown highly efficacious against high grade cervical neoplasia caused by HPV-16 and HPV-18 in a broad population of women and to prevent 6-month persistent infections against non-vaccine HPV types (HPV-45 and 31) [Paavonen 2007].

GSKs HPV vaccine antigens were manufactured with the baculovirus expression vector system (BEVS) using recombinant baculoviruses genetically engineered to carry the capsid HPV-16 and HPV-18 L1 gene and express the L1 proteins in a qualified cell line derived from *Trichoplusia ni*.

The use of the BEVS for prophylactic HPV antigen vaccine production prompted the development of a specific quality control and characterisation testing programme. Classical quality control in combination with BEVS adapted testing (e.g specific permissive cell lines and PCR) at different levels of the vaccine manufacture substantiates the quality of the BEVS for human vaccine antigen production.

Highly purified, well-characterized and immunogenic L1 VLPs are obtained following protein extraction and purification through a multistage process. The structural integrity and similarity of the L1 VLPs to the native HPV virions was documented through morphological and antigenic analysis.

Overall, quality assessment results show that the BEVS technology can be used to manufacture controlled, well-characterized, safe and scalable HPV vaccine. These results demonstrate that GSKs BEVS technology is an effective expression system for the large scale manufacturing of efficacious, safe, and immunogenic HPV recombinant vaccines.

## **CHARACTERIZATION OF CONJUGATE VACCINES**

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Conjugate vaccines are produced by covalently attaching an antigen to a carrier protein in order to improve the effective immune response of the vaccine. The resulting antigen-to-carrier protein conjugate are often quite complex relative to a typical therapeutic protein or glycoprotein. Recent innovations in analytical technologies have allowed for more detailed characterization of vaccine conjugates and their intermediates. Analytical characterization of a conjugate vaccine against *Streptococcus pneumoniae* will be presented.

## **CHARACTERIZATION OF ADENOVIRAL VECTOR-BASED VACCINES**

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Recombinant replication-deficient adenoviral vectors have been used as a platform for vaccine development against various targets at Merck. In order to evaluate the safety and efficacy, and support process development of these candidate vaccines, we have developed an array of analytical methods to characterize various properties of the vaccines, including purity, identity, potency, and mass. Results from those key assays will be shown to illustrate various attributes of process intermediates, purified bulk, and final-container products. Data from the product characterization, stability studies, and process monitoring will be presented. Furthermore, the correlation between the results of in vitro cell based potency assays and human immunogenic response observed in the clinic will be reviewed.



## **FOCUS ON AGGREGATION: CAUSES, IMPACT, AND CHARACTERIZATION**

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Aggregation is often a significant degradation pathway for biologics. For vaccines, on the other hand, some aggregation (self-association) may be necessary for activity (as in VLPs), so reproducibility of such desirable association may be critical for product quality. This talk will review the types and causes of aggregation, and why characterization methods in addition to SEC are often requested by the regulators. Advanced methods for characterization of aggregation will be described, including analytical ultracentrifugation (AUC), light scattering (SEC-MALLS), and FFF. The advantages and disadvantages of these techniques will be discussed, along with real-world examples and applications.

## **DEVELOPING IN VITRO POTENCY ASSAYS TO MONITOR PROCESS DEVELOPMENT AND STABILITY FOR SUBUNIT VACCINE**

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Potency is one of most important quality attributes for bio products, especially for vaccine products. Functional assays such as plaque neutralization assays are commonly available for a number of viral vaccine candidates. However, there is no effective potency assay platform technology available for bacterial subunit vaccine programs. Here we will describe the development of platform technology that can be utilized for all bacterial vaccines. This technology comprises of selecting appropriate biologically relevant functional monoclonal antibodies directed against the conformational epitopes of a vaccine antigen coupled with homogenous immunoassay techniques. Based on ICH guidelines, all 6 qualification-parameters including specificity, linearity, repeatability, precision, accuracy, and robustness have been evaluated. Using the developed potency assay, we were able to monitor conformational structure of recombinant proteins during process and formulation changes. We'll present the assay format and show examples how these assays are used in the evaluation of process and formulation development as well as monitoring the stability of these products.

## **CHALLENGE OF FILING 4 NEW VACCINES AT ONCE**

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Lessons learned during recent regulatory review and approval of four vaccines will be reviewed. Overlapping and concurrent reviews of these four applications by multiple agencies worldwide required planning and sequencing of activities to support key milestones during regulatory review, such as FDA Advisory Committee Meetings and responses to questions in the EU and other agencies. Obtaining regulatory agency concurrence with development plans at appropriate stages of development was a key enabler of successful review. Important areas for early agency concurrence included: clinical endpoint validation, clinical protocol design, statistical analysis plan and criteria for success, assay validation, and analytical development plans for product characterization and release testing. Agreement should be obtained with the approach to endpoint validation prior to pivotal efficacy trials; this is particularly important for novel clinical endpoints. Similarly, for vaccines where licensure relies on comparisons of immunogenicity, agreement should be obtained regarding assay validation and criteria for success prior to finalizing the design of phase III trials. Gaining agreement with the rationale and approach to analytical development and product characterization, and ultimately release testing and comparability protocols, may be facilitated by early discussions with regulators. Development of risk management plans should start well before submission of the marketing application and include a careful assessment of the capacity of potential investigators to support completion of post-licensure study commitments within agreed upon timeframes.

## **PATH: NARROWING THE IMMUNIZATION GAP**

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Dr. Boslego's presentation, PATH: Narrowing the Immunization Gap, will illuminate the breadth of work on vaccines and immunization at PATH and show how nongovernmental organizations (NGO) can impact global public health. The presentation will discuss how an NGO can increase availability of vaccines for developing world populations, work to decrease the lag time in adoption of a vaccine between the developed and developing worlds, and work to further the development of a vaccine candidate through research and development. These topics will be highlighted through examples of PATH projects on vaccines and immunization.

## VIRAL VECTORS FOR GENETIC VACCINATION: STRATEGIES, VECTOR DESIGN AND PRODUCTION

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We have developed different strategies for gene therapy treatment as well as optimized vector production and purification systems for large scale virus production. Our first strategy consists of using first and third generation adenovirus vectors (also called “gutless” or helper dependant), which have been completely stripped of the sequences coding for the viral proteins and have dramatically reduced immunogenicity. Adenoviral vectors ( $\Delta$  E1, E3) have been used to induce inhibition of the mitochondrial aldehyde dehydrogenase gene using an antisense mechanism. The two genes responsible for enzymes that metabolize ethanol and oxidize acetaldehyde greatly influence the development of alcohol dependence. Rat lines bred as alcohol drinkers decreased their ethanol intake by 50-60% after treatment with the adenovirus vectors. A second strategy, that avoids possible activation of oncogenes, considers ex-vivo treatment of bone marrow cells where the appropriate gene is integrated into the genome.

Culture optimization and scale-up for the HEK293 cell line used for the production of adenovirus vectors has been achieved by first adapting the cells to serum-free media and secondly by growing them in suspension where they form aggregates with increased cell concentration. Adenovirus production after infection was therefore increased, resulting in higher specific glucose consumption and lactate accumulation rates compared to the growth phase. We applied media design tools and Metabolic Flux Analysis (MFA) to compare the metabolic states of cells during growth and adenovirus production and to optimize culture media according to the metabolic demand of the cells in terms of glucose and glutamine concentrations. This allows us to obtain higher maximum cell concentrations and increased adenovirus production by minimizing the production of metabolites that can have an inhibitory effect on cell growth. MFA results allowed us to determine how these changes in composition affected the way cells distribute their nutrient resources during cell growth and virus production. Virus purification was successfully achieved using chromatography and Aqueous Two-Phase Systems (ATPS).

## **CONTROLLING IN VITRO ASSEMBLY OF VIRUS-LIKE PARTICLES: FROM THEORY TO EXAMPLE**

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Virus capsid self-assembly is critical for virus biology and has potential as a target for antivirals. Virus-like particles (VLPs) are now vaccines and have potential as platforms for extra-viral antigens and as decoys. Expression systems do not always yield VLPs adequate for a given application. In vitro reassembly is a powerful strategy that can be facilitated by an understanding of the physical chemistry of self-assembly and the biochemistry of the specific viral proteins. In general, successful assembly is based on a nucleated cascade of weak association reactions. Unsuccessful reactions tend to have strong interactions leading to kinetically trapped aggregates. Though individual interactions are weak, closed spherical particles can persist in a metastable manner due to hysteresis to dissociation derived from their lack of "loose" ends. With hepatitis B virus, an important pathogen in its own right and a potential platform for epitopes, these principles are demonstrated with successful assembly reactions and in (putative) drug-induced misassembly. With human papilloma virus type 16, for which VLPs are a component of antiviral vaccines, we find that weak interactions lead to uniform and stable particles whereas strong interactions lead to the predicted kinetic trap. These observations suggest a general strategy for identifying reassembly conditions.

## **VIRUS LIKE PARTICLES AS VACCINE PLATFORMS**

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Virus-like particles (VLPs) can be considered as dense repetitive arrays of one or more protein subunits with properties that are highly advantageous for use as stand-alone vaccines or as platforms for the induction of antibody responses against heterologous antigens. In this talk, I will discuss the how VLPs have been used to increase the immunogenicity of diverse target antigens, including self-antigens. I will give an overview of the techniques for presenting diverse target antigens on VLPs including our recent development of a platform for vaccine development based on the VLPs of RNA bacteriophage MS2. This new platform serves for the engineered display of specific peptide sequences, but will also allow the construction of random peptide libraries from which specific binding activities can be recovered by affinity selection. I will also present some recent data that demonstrates how both antigen valency and the availability of T cell help play quantitative roles in the induction of antibody responses. For foreign antigens, we have shown that these factors are important in determining the magnitude of the antibody response. For self-antigens, antigen multivalency and the availability of T help are critical factors that modulate the ability to induce anti-self responses in the face of B cell tolerance mechanisms.

## **ALTERNATE ROTAVIRUS VACCINES: INACTIVATED VIRUS AND VLPs**

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Rotavirus is the leading cause of severe dehydrating diarrhea in young children worldwide resulting in approximately 600,000 deaths annually. The majority of these deaths occur in sub-Saharan Africa and Asia. Two licensed live attenuated oral rotavirus vaccines are highly efficacious in preventing diarrhea in developed countries but these vaccines have not yet been tested in countries with high mortality rates from rotavirus infection. Historically, live oral vaccines are much less efficacious in these settings, which is attributed to multiple factors including, prevalence of (i) maternal antibodies, (ii) pathogenic organisms in the intestine, and (iii) malnourishment. Therefore, it is unlikely that the live rotavirus vaccines will be highly efficacious in sub-Saharan Africa and Asia leaving a need to develop alternate rotavirus vaccine strategies that would be safe and efficacious in these settings. Vaccines administered by intramuscular (IM) route are an alternate vaccination strategy that may work well in such settings. Maternal vaccination of pregnant mothers by this route may also provide a means to protect young children from severe rotavirus infections. We utilized three animal models of rotavirus infection (mice, rabbits, cows) to assess in pre-clinical studies whether IM immunization with VLPs or inactivated virus induced active or passive protection from rotavirus infection. Multiple formulations of virus-like particles (VLPs) were produced using the baculovirus expression system. In mice and rabbits, active immunization with VLPs or inactivated virus, induced high levels of rotavirus-specific antibodies in both the serum and the intestine and vaccinated animals were highly protected from rotavirus infection. In mice, protection was mediated by rotavirus-specific IgA in the intestine. Importantly, VLPs induced broader and stronger heterotypic neutralizing antibody responses than live oral rotavirus infection, suggesting that VLPs might induce broader and stronger heterotypic protection. In cows, VLPs induced passive protection through the milk. Multiple new genotypes of rotavirus have emerged in recent years and in some developing countries these strains have become prevalent. A VLP vaccine would be advantageous because VLPs based on these new strains could be developed more rapidly and development of locale-specific vaccines would be feasible. Therefore, VLPs administered intramuscularly provide a promising alternate rotavirus vaccine candidate that may prove efficacious in those developing countries where the rotavirus mortality rates are high.



## **TOWARDS A RESPIRATORY SYNCYTIAL VIRUS VACCINE USING RECOMBINANT F PROTEIN TRANSIENTLY EXPRESSED IN MAMMALIAN CELLS**

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Respiratory syncytial virus (RSV) is regarded as the most important cause of viral lower respiratory tract illness during infancy and early childhood worldwide. It is also recognized as a significant problem in the elderly. However, no RSV vaccine is currently available. Early studies demonstrated the efficacy of the RSV-F protein (RSV-F) for inducing high titers of neutralizing antibodies, but the purification of RSV-F from virus is difficult and hampered by considerable safety concerns.

We are developing an RSV vaccine based on the transient expression of recombinant RSV-F in mammalian cells and its formulation into immunostimulating reconstituted influenza virosomes (IRIVs). Transient gene expression in suspension cultures of mammalian cells at volumes exceeding laboratory-scale operations (Baldi et al., *Biotechnol Lett.* 2007, 29(5):677-84) is a technology that allows the production of properly folded and glycosylated proteins in a considerably shorter time than from stable cell lines, significantly reducing costs for research and development. The codon-optimized RSV-F gene was transiently expressed in HEK293 cells adapted to serum-free suspension growth. The presence of the protein on the cell surface was confirmed by immunostaining. The production process was optimized in 10 mL cultures within ventilated, orbitally shaken 50 ml tubes as developed by ExcellGene SA (TubeSpin®). The optimized procedure was then performed in actively ventilated, orbitally shaken 20 L plastic biotainers, yielding up to 20 mg/L of recombinant RSV-F or about  $1 \times 10^7$  copies of RSV-F trimer per cell. Recombinant RSV-F was purified by affinity chromatography, formulated into virosomes, and administered to Balb/C mice for immunogenicity studies. Recombinant RSV-F production and purification processes are currently in development for scale-up in order to prepare sufficient material for a challenge study in cotton rats and for clinical trials in humans. If successful, this would be the first vaccine generated with a recombinant protein produced by large-scale transient gene expression in mammalian cells. Results of the optimization study, as well as the in vivo animal experiments, will be presented and discussed.

## **A SELECTIVE RECOVERY METHODOLOGY FOR THE CLARIFICATION OF LIPID-ENVELOPE VIRUS-LIKE PARTICLES FROM *S. CEREVISIAE***

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The recovery of virus-like particles (VLPs) expressed in *Saccharomyces cerevisiae* requires a multistage operation and the purification process is particularly challenged by the high levels of contaminants in the process stream. Lipid-envelope VLPs often remain localized on cell organelles after expression and the recovery process requires the use of a detergent to release the VLPs into the product stream. This study demonstrates the potentials of a clarification strategy for lipoprotein VLPs whereby an additional centrifugation step is introduced after cell disruption to allow selective VLP recovery during the detergent step. The studies, using the Hepatitis B Surface Antigen (HBsAg) as the model VLP, showed that almost all the VLP product remain associated with the pellet fraction. Detergent treatment of the pellet fraction achieved HBsAg product recovery of over 95% which is consistent with the findings of Chi et al, 1994. This approach allows the elimination of cytosolic material in the supernatant fraction containing bulk cell protein, lipid and nucleic acids contaminants, giving a product enrichment factor of ~3. Investigations on homogenisation conditions showed that the efficiency of the selective recovery approach could be further enhanced by the use of moderate homogenisation pressures in the region of 400 bar. Under these pressure conditions, better recovery of active HBsAg was observed as well as an improvement in lipid clarification. This is because minimal cell fragmentation at lower pressures leads to reduced co-liberation of host lipid contaminants during the detergent step. The value to the selective recovery methodology to a downstream hydrophobic interaction chromatography (HIC) process was also demonstrated. Owing to the reduced level of contaminants, product capture improved resulting in higher chromatography step yield.

Reference: Chi, W., Ku, C.H., Chang, C., Tsai, J.; Two-step cell disruption for extraction of membrane-associated recombinant protein from *Saccharomyces cerevisiae*. *Ann N Y Acad. Sci.*, 1994, 2, 721: 365-73

## **CONSIDERATIONS FOR USE OF THE 'ANIMAL EFFICACY RULE'**

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In 2002, US FDA published a Final Rule amending its regulations to allow appropriate studies in animals in certain cases to provide substantial evidence of the effectiveness for biological products (including vaccines) and new drugs (Federal Register 2002;67:37988-98; <http://www.fda.gov/cber/rules/humeffic.pdf>). This rule (sometimes referred to as the “animal efficacy rule”) will apply only when adequate and well-controlled clinical trials in humans cannot be ethically conducted and clinical field efficacy studies are not feasible. Under this Rule, animal data can be used to provide evidence of efficacy only if all of the following criteria are met: (1) There is a reasonably well understood pathophysiological mechanism of toxicity of the substance and its prevention or substantial reduction by the product; (2) The effect is demonstrated in more than one animal species (some exceptions) expected to react with a response predictive for humans; (3) The animal study endpoint is clearly related to the desired benefit in humans; and (4) Relevant data (e.g., kinetics) allow selection of an effective human dose. This rule will not apply if approval can be based on standards described elsewhere in FDA regulations. Also, vaccine immunogenicity and safety data will be needed from human clinical trials.

## **DE-RISKING VACCINE DEVELOPMENT: CORRELATES OF SUCCESS**

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The Wellcome Trust is an independent research-funding charity with the mission to foster and promote research with the aim of improving human and animal health. We support R&D across a diverse range of technologies, including vaccination, diagnostics, therapeutics and regenerative medicine. In relation to vaccination, we fund all stages of the research process, from basic investigations into infection and immunology, to product-oriented R&D. Through our Translation Award funding scheme, institutions or companies can seek support for product development at key stages of the product life-cycle where the aim is to ensure innovative medical technologies will be developed to satisfy an unmet medical need. Normally, our support for product R&D focuses on the demonstration of proof-of-concept, with the expectation that the research can be advanced to a stage at which it will attract further funding from the investment community, industry, or product-development Public-Private Partnerships. Examples of vaccine projects that have secured Translation Award funding will be presented.

## **AGE1.CR – A WELL CHARACTERIZED CELL SUBSTRATE DESIGNED FOR PRODUCTION VECTOR BASED VACCINES**

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The permanent cell lines AGE1.CR and its derivative AGE1.CR.pIX were developed by transfection of primary cells of a muscovy duck embryo with defined combinations of immortalizing genes (E1A, E1B 55k and E1B 19K). The focused biochemical approach allowed us to use defined tissue samples from a single duck embryo rather than a mixture of cells from a pool of embryos – thereby reducing the risk of contamination and facilitating documentation. The approach is consistent with the “defined risk” guidelines issued by the FDA for the generation of new cell substrates for application in the production of live vaccines because the immortalizing genes are known and traceable. Stable expression of the E1 transgenes for over 3 years in the absence of selection pressure indicates that the transgenes (rather than a spontaneous event) are responsible for the immortal phenotype of the developed cells. Master cell banks were established and extensively tested following EMEA and FDA guidelines taking the 2006 Draft Guidance for Industry into account. The cell lines are adapted to growth as a single-cell suspension or in aggregates facilitating virus production in fermenters (stir tank, Wave) without the requirement for animal-derived components in the media. A peak doubling time of 25h and a maximum cell density of  $9 \times 10^6$  cells/ml provide the basis for efficient production processes. As a permanent cell line AGE1.CR offers the opportunity for stable transfection of additional genes. We show how introduction of the adenovirus pIX gene modulates cell metabolism and enhances susceptibility to attenuated virus strains and vectors. Transfer of essential viral functions into the cell line provides the basis for a production platform for inactivated viral vectors.

## EMERGING DISEASES, ZOOSES, AND VACCINES TO CONTROL THEM

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Disease control is more complex when facing emerging or re-emerging infections, particularly zoonotic ones; for instance, the emergence of canine parvovirus posed a serious threat to animal health. The first step was to vaccinate dogs with a vaccine directed against feline panleukopaemia, since the two causative viruses are antigenically nearly identical. This first step was rapidly followed by the development of vaccines, either inactivated or attenuated, specifically directed against canine parvovirus. Developing vaccines is more problematic when facing outbreaks of diseases caused by viruses showing broad antigenic diversity, such as Foot-and-mouth disease virus or Bluetongue virus (in this latter case disease control is even more difficult due to the fact that the infection is transmitted by a *Culicoides* vector (biting midges)). Following the first outbreak of bluetongue in northern Europe it was two years before inactivated vaccines against serotype 8 of Bluetongue virus were available. However, in North America, the spectacular spread of West Nile virus infection, another vector-transmitted disease, in humans and horses was rapidly followed by the development of several vaccines for horses, including a DNA-based vaccine. One solution to being ready to vaccinate in the face of an outbreak is to stockpile vaccines, as exemplified by the storing of Foot-and-mouth virus vaccines as concentrated antigens. Stockpiling is also envisaged in preparation for the possible pandemic of avian influenza H5N1 in humans and to mitigate the risk of bio-agro-terrorism. For the time being, however, efforts should be focused on eradicating the infection at the animal source by vaccination to prevent human exposure. One of the best examples of this concept is wildlife vaccination against rabies. Sometimes, animals are vaccinated against certain infections not principally for their own sake, but to prevent human contamination. For instance animal vaccination may be used to prevent food poisoning, e.g. a vaccine against *Escherichia coli* 0157:H7 has recently been conditionally approved for cattle in the United States. A large number of pathogens are zoonotic and infect multiple animal species. Moreover, approximately 75% of all diseases, including zoonoses, which have emerged in the last few decades are of wildlife origin. Animal vaccination will continue, therefore, to play an important role in safeguarding animal and human healths in the future.

## **AVIAN INFLUENZA VACCINE DEVELOPMENT: APPLICATION TECHNOLOGY PLATFORMS, FIELD USE AND PREDICTORS OF PROTECTION**

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Vaccines against avian influenza (AI) began over 100 years ago as experimentally produced products, but commercial application did not occur until: 1) a reliable method was developed to grow and titer the virus (i.e. embryonating chicken eggs), 2) an efficient and predictable method was developed to inactivate the virus without destroying antigenic epitopes (i.e. formalin inactivation), and 3) a method was developed to enhance the immune response (i.e. oil emulsion adjuvants). Commercial usage of vaccines began in 1979 with limited usage of inactivated oil-emulsion vaccines for low pathogenicity avian influenza (LPAI) in meat and breeder turkeys. AI vaccine usage increased in the mid-1990s with H9N2 LPAI in developing countries, and the first usage of AI vaccines against high pathogenicity (HP) AI occurred following large scale outbreaks of H5N1 HPAI in Mexico and H7N3 in Pakistan. For the latter two epizootics, over 4 billion doses have been used in 10 years. However, the greatest quantity of AI vaccine used has been in response to the emergence of the H5N1 HPAI epizootic in Asia, Africa and Europe. The actual quantity of vaccine used is unknown, but from conservative estimates the amount has exceeded 30 billion doses since 2002. Over the past 40 years, AI vaccines have been primarily based on LPAI and HPAI viruses used in inactivated, oil emulsified vaccines. Recently, fowl poxvirus and avian paramyxovirus type 1 vectored vaccines with AI H5 gene inserts have been developed and licensed in some countries. Advances in biotechnologies may overcome some existing limitations and result in vaccines that can be grown in tissue culture systems for more rapid vaccine production; provide optimized protection as the result of closer genetic relationship to field viruses through rapid changing of AI hemagglutinin gene insert through cassette concept; can be mass applied by aerosol, drinking water or in ovo administration; and provide easier strategies for identifying infected birds within vaccinated populations. These rising technologies include AI viruses with partial gene deletions, AI-ND virus chimeras and vectored vaccines using adenoviruses, Marek's disease, or sub-unit vaccines. An additional benefit of some vectored technologies is a broad application in multiple animal species. For example, fowl poxvirus-vectored vaccines have shown efficacy in chickens, geese and cats. Their potential use in the field will also be determined on the requirement for low cost vaccines to be economically competitive. In addition, the vectored vaccines can be used to enhance immune response using a prime-boost two vaccine application protocol.

## **USE OF ALTERNATE HOSTS IN THE MODELING OF IMMUNE PROFILING AND VACCINE RECOGNITION**

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Advances in molecular biology and immunology have resulted in the rapid identification of specific antigens of many pathogens that can induce protective immune responses in mice. However, it is becoming well known that these data cannot always be translated to success in large animals or humans. This is especially the case in neonates – those most vulnerable to infections. Thus the choice of animal models for such studies is extremely critical. It is our belief that the most effective animal models will those in which the pathogenesis of the infection agent is identical or very similar to what happens in humans (i.e., a natural infection). The collateral advantage of using appropriate animal models is the system can also lay the foundation for protecting the animal species from the disease of interest. However, even with the best model and the best vaccine antigens if a vaccine is not formulated and delivered properly, the vaccine will not achieve its full potential. The current presentation will describe the use of large animal models (pig and calf) to complement mouse models in testing a series of novel immune modulators (CpG and host defence peptides) in combination with the delivery vehicle (poly phosphazene) to enhance the magnitude and modulate the quality of immune response to a variety of different vaccine antigens. We will demonstrate that these formulations can be delivered intranasally to induce mucosal immune responses as well as being able to develop appropriate immune responses that protect animals from infection.



## USE OF PLASMID DNA VACCINE TO TREAT MELANOMA IN DOGS

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(Presented by Robert Nordgren)

Immune responses to antigens found on cancer are usually constrained by tolerance to the largely 'self' molecules which are presented. Immunization using homologous antigens from a different species, so-called xenogeneic immunization, is one means to overcome the immune system's reluctance to adequately recognize these targets. We have developed plasmid DNA vaccines encoding human and mouse tyrosinase, a prototypical differentiation antigen expressed by most malignant melanomas and normal melanocytes. Preclinical mouse studies showed the injection of human tyrosinase DNA vaccines in C57BL/6 was able to induce CD8+ T cell and antibody responses to mouse tyrosinase capable of mediating protection from syngeneic tumor challenge. Based on these data, a clinical trial program was developed at the Animal Medical Center of NY in which dogs with spontaneous melanoma were injected with a series of 4 doses of human tyrosinase DNA vaccine using a needle-free delivery system. Initial results showed a significant prolongation in the expected survival of a cohort of 9 dogs with advanced melanoma. Further trials confirmed that vaccination resulted in longer-than-expected survival of dogs in the minimal residual disease setting and even occasional regressions of large metastases. Conditional licensure was granted to Merial in 2007 for the use of vaccine in canine melanoma. Human clinical trials of the mouse tyrosinase vaccine have proceeded with preliminary showing the ability of xenogeneic DNA immunization to induce CD8+ T cell responses in human melanoma patients. Vaccination in both humans and dogs has been well tolerated, with transient injection site reaction being the most common adverse event. This program has demonstrated the importance of testing novel biologics in a realistic animal model and also the significant potential for collaboration between human and veterinary cancer investigators.

## **PROCESS INTENSIFICATION FOR LARGE SCALE MANUFACTURING ISSUES.**

### **CASE STUDY - INNOVATIVE TECHNOLOGIES APPLIED TO HUMAN INFLUENZA VACCINES**

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The demand for influenza vaccines is increasing all over the world. The manufacturing paradigm of virus production is moving from embryonated eggs to replication on mammalian cell lines. Due to the market pressure, the targeted production scales lie in the range of 1500L to 5000L.

This presentation focuses on process intensification techniques that Artelis is currently investigating and developing to face the manufacturing issues related with these challenging scales.

More specifically, process intensification is implemented by following two axes.

Firstly, the volumetric yield of viral production is increased by 20 to 100 fold compared to traditional technologies, thanks to the implementation of high cell density fixed bed cell culture. This patented technology allows staying at small scales for manufacturing, from 25L to 100L. Secondly, disposable single-use components and systems are implemented for all the unit operations into the process.

This combined strategy leads to the design of a plastic factory, characterized by a dramatic reduction of space requirements, investment costs and delivery time. Quantitative evaluations will be presented.

The advantages of this way to manufacture were assessed in terms of direct and indirect economical impacts.

Investment costs are reduced by at least 50%, operational costs are decreased by at least 30%, time-to-market can be decreased by 2 years. Impacts on risks (safety issues, delivery issues) and pipeline management were also assessed.

Finally, some thoughts will be shared about the issues related to the supply of ready-to-use culture media and disposables for a plastic factory, as well as the chance for developing countries to incorporate their own vaccine production thanks to plastic factories.

## MULTIVALENT VACCINES FOR CONTROL AND ERADICATION OF FMD

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Foot and mouth disease (FMD) is a highly infectious viral disease that affects food producing animals such as cattle, pigs and sheep. The FMD status given by the OIE has a huge financial impact on economies all over the world. Control and eradication programs rely heavily on the development of marker vaccines that enable Differentiation of Infected from Vaccinated Animals (DIVA). In infected animals viral replication leads to the synthesis of antibodies against structural and non-structural proteins. In animals vaccinated with inactivated virions only antibodies against structural proteins are synthesized provided the vaccine does not contain residual non-structural proteins derived from viral replication during the vaccine manufacturing process. Various process options such as ultrafiltration, precipitation and chromatography have been proposed and implemented to separate structural from non-structural proteins. A polyethylene glycol precipitation-based approach provides a robust and simple platform which we have successfully scaled up to 11000 litres. The process step is suitable for purification of different FMD virus strains (A Arg 2000, A Arg 2001, A24 Cruzeiro, C3 Indaial, O1 Campos, O Taiwan 97) produced from cultures of suspension adapted BHK21 cells. Field trials in cattle subjected to multivalent multiple-dose immunization schemes demonstrated the antigenic purity of the product.

## PROTECTIVE IMMUNE RESPONSES TO PATHOGENIC INFLUENZA USING CONSENSUS DNA IMMUNOGENS AND CONSTANT CURRENT ELECTROPORATION

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The development of novel technologies for immunizing against emerging viral threats presents a formidable and critical challenge to the bio-pharmaceutical industry. Recently developed consensus DNA immunogens to pathogenic avian H5N1 influenza have the potential to induce highly cross-reactive immune responses against different viral subtypes and clades. Using our proprietary manufacturing process, a single vial multivalent, high concentration vaccine has been produced in large quantities. Three consensus constructs were produced: pH5HA (a consensus of 16 clade 1 H5N1 hemagglutinin sequences); pM2eNP (a fusion of the N-terminal domain of the M2 ion channel with the nucleoprotein antigen, designed from over 40 influenza A sequences); pN1NA (designed from over 40 N1-neuraminidase sequences). Using the CELLECTRA™ constant-current electroporation device to deliver low doses of DNA immunogens, we examined the induction of protective immune responses against highly pathogenic avian influenza viruses. Four groups of ferrets were immunized and electroporated with pVax (controls); pH5HA only; pM2eNP only; or pH5HA, pM2eNP, and pN1NA. Following lethal A/Vietnam/1203/2004 influenza challenge, 100% survival was observed in each vaccinated group (0% in the control). Maximum loss of body weight in each group were measured: control animals lost  $24.7 \pm 1.5\%$  of their body weight, pH5HA vaccinated animals lost  $12.8 \pm 5.9\%$  ( $p = 0.049$  vs. control), pM2eNP vaccinated animals lost  $17.4 \pm 0.9\%$  ( $p = 0.003$  vs. control) and combination vaccinated animals lost  $12.1 \pm 2.6\%$  ( $p = 0.003$  vs. control). All vaccinated groups displayed a statistically significant reduction in viral shedding by day 5 ( $p < 0.01$  comparing control group to vaccinated groups). In the pM2eNP vaccinated group, with protection based solely on cellular immunity, there was a 90% reduction in mean viral load. These studies were extended into a primate model of vaccination, demonstrating the ability of our consensus sequences to induce cellular immune responses in addition to protective titers of antibody ( $HI > 1:40$ ) following only two immunizations in rhesus macaques. These studies demonstrate that the manufacture, formulation and delivery of protective vaccines for H5N1 influenza can be done efficiently and effectively. Furthermore, delivery of these consensus DNA immunogens can both elicit protective immune responses and show protection post-challenge. These studies show promise for this vaccine regimen in human clinical trials.

## **INTENSIFYING THE PRODUCTIVITY OF A RECOMBINANT AD35 MANUFACTURING PROCESS USING THE PER.C6® CELL SUBSTRATE**

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Crucell and The Aeras Global TB Foundation are collaborating in the development of a recombinant adenovirus (rAd) based TB vaccine for which Phase I studies have been initiated at the end of 2006. The vaccine manufacturing process supporting Phase I clinical trials have a productivity of 700 doses/liter bioreactor volume at a dose of 10E11 virus particles. At this productivity, at least 150 batches at 1000L scale, or at least 15 batches at 10,000L scale will be required to provide a minimum of 100-200,000,000 doses to assure an immediate global impact on disease burden immediately after licensure.

Given the uncertainties attendant to the CAPEX commitment required to develop a facility for a 10,000-liter bioreactor process and the unprecedented need to develop a viral vaccine manufacturing process at 10,000L scale under BSL 2 conditions, our approach is to focus instead on an intensification effort on the rAd35 manufacturing process. Accordingly, we have set as an objective, a 10-20 fold intensification of the Upstream Manufacturing Process to be realized through increases in both unit and volumetric productivities. The process intensification will be developed by the use of scale down models that allow for a high throughput analysis of cell culture and infectivity variables.

This presentation will review our efforts to understand the determinations of unit productivity of rAd35 replication in PER.C6® cells and to boost volumetric productivities as well through an understanding and circumvention of the cell density effect which formerly imposed productivity limits on rAd35 manufacturing processes.

## **AFFINITY CHROMATOGRAPHY OF CELL CULTURE DERIVED VACCINIA VIRUS**

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Smallpox is an acute, highly infectious viral disease unique to humans with a mortality rate around 25%. It is caused by the Variola virus that belongs to the family of Poxviruses. Smallpox was responsible for an estimated 300-500 million deaths in the 20th century. Following successful vaccination campaigns through the 19th and 20th centuries, the World Health Organization (WHO) certified the eradication of smallpox in 1980. After the eradication, the compulsory vaccination was abandoned – with the result that about half of the world's population is not vaccinated. This represents a potential threat in the case of a deliberate release of Variola virus as an act of bioterrorism. Consequently, several governments are ordering stock piles of smallpox vaccines to protect their populations from this remote, but extremely grave threat. MVA-BN® is a third generation smallpox vaccine based on the Modified Vaccinia Ankara (MVA) virus which demonstrates superior safety compared to traditional smallpox vaccines based on native Vaccinia virus (VV) strains. In addition, re-engineered VV represent as a robust vector a platform technology for vaccine delivery systems as e.g. in the case of HIV, Dengue fever, Japanese encephalitis and cancer.

Traditionally, VV- and MVA virus based vaccines have been grown in primary chicken embryo fibroblast cultures and purified either by sucrose cushion or sucrose gradient centrifugation, or by ultrafiltration. However, a potential shift from primary to continuous cell cultures would impose stricter requirements regarding the purity level of the vaccines, and a new generation of vaccine manufacturing processes is needed that include more sophisticated and innovative downstream techniques for purification.

Here, we report the development of an affinity chromatography of cell culture derived VV after an initial host cell homogenization and clearance centrifugation. The Vaccinia viral envelope protein A27L is known to bind to heparin.. Based on this, small scale chromatography experiments with heparinized polymer beads and cellulose sulfate beads in addition to heparinized cellulose membranes have been conducted. There we have found that membrane adsorbers are superior over bead based chromatography media in terms of efficiency and productivity. Subsequent studies compared ion exchange membrane adsorbers with a heparinized membrane adsorber. The results indicate that the overall performance of the affinity chromatography in terms of virus capturing and contaminant removal is better than any of the tested ion exchange membrane adsorbers. Hence, membrane affinity chromatography represents a valuable choice to capture VV particles in addition to the general advantages of membrane chromatography.

## **TROUBLE-SHOOTING FERMENTATION AND PRIMARY RECOVERY MANUFACTURING ISSUES IN ORDER TO OPTIMIZE ANTIGEN EXPRESSION USED AS VACCINE CANDIDATES AGAINST INFECTIOUS DISEASES**

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As a process is scaled up, there are several considerations that must be employed both during fermentation and recovery of the product. Some key empirical parameters such as power/volume ratios, mass-transfer coefficient (KLa) and mixing time are considered. The KLa value is kept constant upon scale-up to maintain similar mass-transfer of oxygen at the larger production scale. One way of keeping the same KLa value as the small scale is to improve the power input by increasing the number of Rushton impellers or improving mixing by changing the impellers to low-shear Hydrofoil propellers within the large-scale reactor. In E.coli fermentation, it is shown that oxygen transfer rate at the production scale was improved by increasing the number of flat-bladed impellers rather than changing the impeller type. Due to the nature of the bacteria, shearing was not an issue. Both biomass and antigen concentration increased as a result. Although the transfer rate is increased using 3 flat-bladed impellers instead of 2 at the production scale, the overall productivity is still around 72 % in comparison to the yield achieved at the smaller bench-scale. To improve the productivity at the larger-scale, a lower feeding rate was adopted at the production scale. By feeding the substrate at a lower feed rate, the culture was less oxygen starved and had a greater chance of growing and producing product. The strategy was effective as the product was expressed up to 21 hours of induction using IPTG. Both hydrodynamic and physiological considerations will promote success of the process upon scale-up.

A number of filtration technologies (micro-filtration, static filtration and ultra-filtration) for harvesting the culture are discussed. A process consisting of cross-flow micro-filtration, homogenization and batch clarification using ion-exchange resins has been effective in recovering the protein. The majority of the cost of the batch adsorption operation is the cost of the anion-exchange Big beads (\$700/ Liter), which is much less than purchasing a filtration unit and cassettes (approximately \$150,000) or a centrifuge (\$300,000) for clarification. In addition, as the beads are cleaned and re-used again, the cost of operating the batch adsorption method is lowered and the disposable cost can be reduced significantly in comparison to using depth filters to clarify the antigen. Also, the batch adsorption process can achieve equal to or greater than 90 % protein recovery which is equal to or better than conventional micro-filtration methods. Hence, the clarification procedure of cell concentration step using a 0.2 um micro-filtration, homogenization and batch clarification using big beads is an attractive and cost-effective tool in the clarification of protein antigens for manufacturing.

## TECHNOLOGY TRANSFER AND PROCESS SCALE-UP

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In a broad sense, Technology Transfer is defined as the transfer of know-how and experience from an originator to a recipient. In reality it is a highly complex process, which usually plays a key role in efforts to successfully commercialize new vaccine candidates. Each Technology Transfer has some unique features. Being able to identify these features, process subtleties and other key components of the technology transfer can allow a focus on the major risks of the transfer.

In the development of Prevenar 13, several types of technology transfers were completed. Selected Case Studies from these transfers and key lessons learned will be presented.



## PREDICTIVE MODELING IN ROTAVIRUS-LIKE PARTICLES PRODUCTION: IMPROVING UPSTREAM AND DOWNSTREAM PROCESSING DESIGN

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Optimisation of rotavirus-like particle (RLP) production in insect cells is extremely complex. Multiplicity of infection (MOI), individual protein production (VP<sub>2</sub>, VP<sub>6</sub> and VP<sub>7</sub>) and correspondent assembly into a triple layered particle (RLP) are major challenges for the upstream processing. Concomitantly, the downstream processing of RLPs faces several setbacks due to the complexity of the cell cultured bulk imposing a number of purification steps. Thus, strategies to suppress product-derived contaminants (trimers of VP<sub>6</sub> and double layered particles – DLP) negatively impacting the overall process were designed based on novel mathematical and analytical tools. Such a rational approach integrating up- and downstream phases will be shown to improve global yields.

To maximise product synthesis, the starting point consisted in evaluating the effect of MOI on final VP<sub>2</sub> protein synthesis (used as a model for the remaining VPs). A stochastic/structured mathematical model developed in our group, combining an explicit stochastic infection process with intracellular mass action kinetics, was used. Final productivities were shown to be a compromise between high intracellular VP<sub>2</sub> templates, obtained at high MOIs, and high infected cell densities, obtained at low MOIs. In fact, an optimal midterm MOI between 0.01 and 1 pfu.cell<sup>-1</sup> maximised process productivity. This strategy, further applied to the remaining VPs, will allow the establishment of optimal operational conditions for maximisation of RLPs production yields confirming the relevance of mathematical models in process development. Moreover, these results highlight the relevance of having accurate analyticals for viral stock titers estimation as MOI variations may compromise process optimisation. Several titration methods were compared. The techniques that better combined all important variables (accuracy, cost per titration, titration time and labor intensity) and therefore proved reliable for titer estimation were the TCID<sub>50</sub>, MTT and flow cytometric assays with intra-variabilities and costs per titration of 19% and 13.5€, respectively.

Complementarily to this upstream optimisation, the design of an efficient downstream process route is equally important. A novel strategy using anion-exchange membrane chromatography (Sartobind™ D membrane adsorber from Sartorius Stedim Biotech) was evaluated as these permitted high fluxes, rapid processing, little buffer consumption and a validation-free environment. Screening studies performed to elucidate the effect of the ionic strength and the pH of the equilibration buffer on the RLP capture efficiency allowed for the definition of best operational conditions. Moreover, steric mass action formalism was implemented for the prediction of the adsorption/elution profile of RLPs demonstrating to be an important mathematical tool for process optimisation. At the end, global downstream processing yields were improved by 46%.

Mathematical tools were here demonstrated to play a determinant role in optimising RLPs production. The predictive capacity of the models presented show their utility in both up- and downstream phases reducing labor time and process costs, and improving yields.

## **IMPLEMENTATION OF DISPOSABLE TECHNOLOGY IN VACCINES MANUFACTURING: AN APPROACH TO EXTRACTABLES/LEACHABLES STUDIES**

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Disposable technology is being increasingly used within vaccine manufacturing , and the scope of available technologies makes it applicable to more complex applications. In particular, it is more and more used in formulation and filling applications . However, more than their stainless steel counterparts, single-use systems draw concerns about extractables and leachables from organic-based plastics and elastomers from which some compounds may migrate to finished products. Therefore, it is imperative to develop suitable and convenient methods to study extractables/leachables from single-use systems in order to address regulatory requirements. This talk presents a systematic study of extractables from single-use systems into water and ethanol via novel concepts, practical design, and analytical detection using advanced techniques. In order to tackle the fairly complex systems, we studied the components first, which included filters, connectors, and biocontainers, and then examined the whole single-use systems comprised of filter, tubing, connectors, and biocontainers. This approach greatly simplified the identification of the extractable compounds from the whole systems. The test design was based on actual process conditions using a worst-case scenario. The complete extractables results were obtained using validated analytical methods, including non-volatile residue measurement and FTIR for qualitative assessment, GC/MS for volatile/semi-volatile compounds, derivatization GC/MS for organic acids, HPLC/UV, LC/MS and LC/MS/MS for nonvolatile and heat-sensitive compounds, and ICP/MS for inorganic elemental analysis.

## THE DEVELOPMENT OF AN INACTIVATED JE VACCINE FOR ENDEMIC COUNTRIES

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### INTERCELL AND BIOLOGICAL E: A PROMISING PARTNERSHIP

Intercell AG, a Vienna based fast growing biotechnology company with a focus on the design and development of novel vaccines had strategically partnered in early 2005 with Biological E. Ltd. an Indian pharmaceutical drug manufacturing company based at Hyderabad for the development, manufacture and sales of its Japanese Encephalitis vaccine in Asian endemic market through technology transfer.

This alliance is anticipated to broaden by combining the strength of Intercell's Antigen Identification Program with Biological E's experience as a leading Asian vaccine manufacturer and as an active partner in the National Immunization Program of India.

### IC51: THE SMART VACCINE

IC51 a new purified inactivated JE SA<sub>14</sub>-14-2 virus vaccine with a two dose schedule (0, 28 day) would represent an improvement over the currently licensed three dose JE vaccines. Pivotal Phase III trials on JE IC51 world over have demonstrated a favorable safety and immunogenicity profile.

### JE PHASE-II CLINICAL TRIAL

#### Study Description and Objectives

This open label, phase II, single centre study was conducted in India (Bangalore). 60 healthy children of either sex in the age group of 1 to 3 years were recruited and randomised into 3 treatment groups in a 2:2:1 ratio to receive either 3mcg or 6mcg dose of IC51 or JenceVac<sup>TM</sup>.

The Primary Objective of the study was to evaluate the immune response to 3 mcg and 6 mcg dose of IC51 administered in a two dose schedule for optimal dose identification. The Secondary Objective was to assess safety and reactogenicity of both the IC51 doses and to evaluate their immunogenicity in comparison with JenceVac<sup>TM</sup>.

#### Study Summary

There were no significant differences in immunogenicity between all the three groups. Both IC51 dose groups appeared to have a lower rate of adverse events compared to JenceVac<sup>TM</sup>.

#### Study Conclusions

- Both IC51 groups are equally immunogenic and safe compared to JenceVac<sup>TM</sup>.
- IC51 showed an appealing safety and immunogenicity profile at the full and half adult dose in children aged 1 to 3 yrs.
- For further development of the IC51 Phase-III pediatric program this data supports the use of the 3 mcg dose in children below 3 years of age.

The encouraging results of this clinical trial have paved way to the Phase-III trial which will follow shortly. Intercell AG and Biological E. Ltd are excited to accelerate this program and make this vaccine available to the endemic countries.

## VACCINE DEVELOPMENT FOR DEVELOPING COUNTRIES – REGULATORY APPROACH IN THE EUROPEAN UNION

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In 2004, the European Commission made a substantial effort to overcome the numerous still existing obstacles that hinder the introduction and use of efficacious and safe medicinal products in developing countries. For four years now, through article 58 of Regulation (EC) 726/2004 the EU has been in a position to respond to the need to protect public health and to give scientific assistance via the EMEA to non-EU countries in the context of cooperation with WHO whilst at the same time allowing rapid access of important new medical products to those countries.

Article 58 entitles the EMEA's scientific committee CHMP to provide scientific opinions on products that are intended for use outside the EU. Important aspects of a specific EMEA guideline on issues regarding the development of the CHMP scientific opinion assessment report (AR) according to article 58 will be presented. Importantly, the AR contains only conclusions based on the same quality, safety and efficacy criteria applied to the assessment of vaccines intended for use in the EU.

Medicines eligible for this procedure are used to treat or prevent diseases of major public interest. This includes vaccines used in the WHO Expanded Programme on Immunisation or vaccines for protection against other public health priority infectious diseases largely limited to developing countries. Research for the development of these vaccines as well as their manufacturing and performance of clinical trials is primarily conducted by industrialized nations; while the vaccines themselves have the greatest impact on the social, economic, and political development of developing countries. This is not always ideal and has sometimes led to obstacles to vaccine introduction into third world countries. But in recent years we have fortunately been able to register an increasing number of important vaccine manufacturers in developing countries. However, the NCAs in many developing countries have still been considered by WHO to be weak with regards to a state of the art assessment of licensing applications for vaccines. WHO has been eager in trying to improve the vaccine evaluation capacities of these countries. The EU tries with the help of the article 58 procedure to circumvent this obstacle.

In 2007, a European manufacturer submitted the first application file to the EMEA under Article 58 for a combined vaccine against diphtheria, tetanus, pertussis, hepatitis B, Haemophilus influenzae type b, Neisseria meningitides serogroups A and C. The vaccine was to be used exclusively in markets outside the EU, primarily in Sub-Saharan Africa. Two other applications for vaccine candidates designed specifically to meet a pressing public health threat in Africa will be submitted within a short time frame. The outcome of this first application for a heptavalent vaccine to be administered in the classical EPI will be briefly discussed in this presentation.

## **DEVELOPING VACCINES FOR NEGLECTED DISEASES**

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Vaccines represent an extremely cost-effective public health intervention. Historically, due to the lack of market incentives, there has been insufficient investment in vaccines for parasitic diseases that primarily affect the most impoverished. However recently the R&D landscape has been changing as governments and foundations increase their investments in product development through both 'push' and 'pull' mechanisms, and new technologies and increasing biological insight create opportunities to tackle these challenging diseases. A general overview of the mission of the Bill & Melinda Gates Foundation and some specific examples of product development activities the foundation supports will be presented.