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Developments in High-Yield System Expressed Vaccines and Immunotherapy

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Abstract

Conventional vaccine production techniques are outdated, leaving the world defenseless to viruses and pathogens. Successful protection necessitates the innovation of strategies that can generate an induced defensive humoral and cellular response with: ease of mass production, nominal side-effects, and controlled design specificity, all while being cost effective. Fortunately, technology exists to facilitate such advances in this billion dollar industry and this review is focused on recent publications and patents which hold promise to revolutionize the fight against pathogenic illnesses.

Keywords

Cell surface protein display; viral vectors; antigen delivery systems; influenza vaccine; immunotherapy; vaccine production

I. INTRODUCTION

The race to revolutionize current vaccination production strategies is sparked on the emergence of new highly pathogenic organism. The development and modification of molecular vaccine design techniques have just recently been applied to generating specific vaccine products with high-yield production potential. The needs for such innovations are clear; in the case of high mortality influenza pandemic, swift vaccination of the entire global population is required to avoid worldwide devastation. In these types of cases, the pandemic strains have little or no immunological memory in the population. Furthermore, it may require two dosages of a vaccine to induce protective immunity in naïve individuals. Dated egg-based vaccine production, will likely founder to these needs leaving mankind unprepared, exposed, and diseased [1]. Concern grows at the possibility of avian influenza H5N1 strain outbreaks, where hen egg-derived vaccine production techniques are experiencing setbacks. Pandemic preparedness is essential, yet little commercial progress has been made towards that need. Models developed by the World Health Organization (WHO) indicate that despite knowledge of the need for generation of vaccine stockpiles a change in operation is unlikely for at least a decade more [2].

For decades, the basic technology for vaccine production has remained the same, focused on the growth of large volumes of targeted microbes which are inactivated, concentrated, and then purified before used as vaccines [3]. While antigen-based vaccines have been explored. These vaccines usually do not induce broad and long-term immune protection, requiring

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follow-up vaccinations to booster immunity. It also requires reformulation to cover new viral subtypes. On the other hand, the short shelf-life of vaccines and difficulties in growing certain microbial in cell culture also impair our fight against virus infection.

To aid in the generation of high-levels of vaccine products, successful new recombinant DNA technologies have significant potential and value for vaccine production. Therefore, a review of pertinent patent applications for generation of DNA, epitope, surface displayed protein, and vector encoded antigen for vaccines are discussed. Also, trends in journal publications are described to indicate the current state of the art and opportunities in the development of high-efficiency vaccine systems.

Current State of the Art of Vaccine Production

Several methods of vaccine production exist along with their own sets of advantages and disadvantages in production time and principle investments. The most widely used technique is egg-based vaccine production, which can be jeopardized by unforeseen fertilized egg-supply, challenged by the ability for virus to successfully proliferate within the eggs, and mutations in viral proteins throughout production. In March 2007, the WHO called for international support for a stockpile of a safe and effective egg-based inactivated H5N1 influenza vaccine (IIV) that would benefit developing countries [3]. The destructiveness of a viral strain causing high pathogenicity zoonotic infections or even those which have low pathogenicity but acquire a highly pathogenic phenotype by mutations or by recombination at the HA cleavage site by either genetic RNA reassortment between virus types or by co-infections has frightening potential [4]. A new H1N1 swine-associated virus is circulating along with the current other influenza A and B forms. Also, several new avian subtypes have traversed species barriers from domestic poultry to humans since 1996, and have caused pathogenesis ranging from mild to death [5]. These avian subtypes include: H5N1, H7N1, H7N2, H7N3, H7N7, and H9N2. Any of these new subtypes could adapt to increase their virulence in the naïve human population.

Opportunities in Vaccine Development

Researchers have deciphered information concerning protein structures associated with pathogenesis. Increased investigations in cell-mediated and humoral immunity pathways open the door to a combination of different prevention and treatment strategies designed specifically towards the progressive global control and eradication of these diseases. Small-scaled cell culture strategies are being optimized for safer and large-scale production of molecular vaccine antigens. The capabilities for each system are being reported, many with pharmaceutical applications targeting a decrease in purification costs and increased immune specificity. Concoctions made from polypeptides and polynucleotide or through the expression of antigens and mimotopes produce antibodies which have an increased affinity and heightened protection against subsequent exposure. These technologies open doors to the development of rationally designed immunological protection through vaccination that once was thought to be unobtainable.

Classically, the concentration and purification of the antigen has been achieved through aluminum hydroxide gel absorption or polyethylene glycol precipitation [6]. These methods are being replaced by industrial ultra-filtration and chromatography, because they allow the removal of cellular protein contaminants and viral non-structural proteins which may cause allergic reactions. High-yield antigen expression and purification possibilities include: genetic engineering manipulations in cell culture techniques ranging from bacterial systems to plants. Large-scale production of antigens through bioreactor further enhances the molecular expression capabilities of vaccines developed from cell cultures. The grandiose goal of global immunization by an optimal design formulation includes: unrefrigerated

storage, needle-free administration routes with few dosages, along with the ease of manufacturing. The matching of such goals and emerging sequencing, expressing, and purification technology presents a challenging opportunity in an emerging field of creatively engineered vaccine products.

COMPONENTS IN VACCINE DESIGN

Vaccine Design

Vaccine mechanism of action exploits the body's innate and adaptive immunity to induce a protective immune response against the delivered antigen particles. Advances in engineering materials for immunomodulation also contribute to prophylactic vaccine formulations and immune therapeutics [4]. An area of vaccine research is to induce antibodies against multiple targets to achieve effective protection against a specific pathogen, which often has multiple natural virulence factors. Currently, the majority of vaccine strategies only utilize a single immunodominant component [7]. Most vaccines are defined as a preparation of microbial antigen, usually combined with adjuvant, to induce protective immunity against microbial infections, by stimulating the development of antibodies, effector and memory T cells. In these cases, the antigen may be in the form of live but virulent microorganisms, killed microorganisms, purified macromolecular components of a microorganism, or a plasmid that expresses a microbial antigen. Most vaccines induce humoral immunity with these antigens, but stimulation of cell-mediated immunity has an attractive value [8]. For the manufacturing of a vaccine against a virus, cell culture systems are often exploited to produce virus particles in a large-scale [9]. Vaccine production systems such as egg-based vaccine production systems must be properly equipped to protect both the manufacturer and public from highly infectious strains of the rapidly mutating viruses. Production of conventional egg-derived vaccines is cumbersome and often necessitates revision of the strains, which leads to vaccine shortages and false protection. Pitfalls of animal or even insect cell methods are often due to contamination occurring throughout cultivation, antigen purification is cumbersome, and the possibility of the particles from the systems causing side effects.

Vaccine immunogenic components must be antigenically conserved and usually exposed on surface of the infectious agent. Viral surface peptides of wide spread vaccine development interest include: HA, NA, and M2 domains [1]. The bacterial agent E-surface exposed protein is a commonly used polypeptide capable of raising an immune response. This component is a studied target in diseases where there are no commercial vaccines available, like in non-typable *Haemophilus Influenza* (NTHi) where common protein subunits exist in all encapsulated (Hib) and NTHi type pathogens [8]. Recombinant proteins, synthetic peptides, protein-polysaccharide conjugates and plasmid DNA have potential to generate a wide range of novel vaccines through the genetically guided advances in production, expression, and method of action of the pathogenic factors. Target proteins of interest may also include hormones, hormone analogs, enzymes, enzyme inhibitors, signaling proteins or parts of, antibodies or parts of, single chain antibodies, binding proteins, binding domains, peptides, antigens, adhesion proteins, structural proteins, toxin proteins, cytokines, transcription regulators, blood coagulation factors, and plant defense-inducing proteins [10].

Live Whole Reassortant Virus Vaccines

Live attenuated and chimeric vaccines require extensive preclinical and clinical characterization. Also, genetically modified organisms have to comply with additional and specific regulations to get the licensure of new vaccines [11]. Live-attenuated vaccines like live polio and yellow fever have decades of proven efficacy and trusted safety records. Still concern exists since, such as viruses and their vaccine derivatives, have the ability to spread

in the host. Attenuated vaccines have low and short duration of viremia and critical safeguards protecting against the replication and dissemination of the viruses in the environment through recombination with cocirculating strains.

Influenza viruses cause seasonal epidemics of 3–5 million cases of severe illnesses and approximately 500,000 deaths each year [5]. They are single-stranded RNA type viruses belonging to the family Orthomyxoviridae. The seasonal human influenza vaccines currently licensed contains an inactivated split of human influenza H1N1, H3N2, and influenza B viral antigens to promote hemagglutinin (HA) and neuraminidase (NA) subtype and strain specific functional antibody protection [4]. Influenza vaccines generated by using reverse genetic technology are now licensed for used in humans in the United States for avian-linked H5N1.

Whole virus vaccine production challenges include: potential virus leakage from the production facility, short shelf life of formulated product, short duration of immunity, and requirement of dozens of antigens to address viral antigenic diversity [1]. In addition, laboratory biosafety concerns of the pathogens necessitate biocontainment level three, with investigators wearing powered air purifying respiratory equipment and protective clothing. The formalin-inactivated mixtures either contain alum adjuvant or without alum. When injected, alum works as an adjuvant to promote inflammation to draw immune cells to administration site [4]. Additionally, serious problems have been associated with inactivated vaccines including the incomplete viral antigen inactivation by formaldehyde treatment [6]. Binary ethylenimine (BEI) inactivation of antigens has been more successful in this respect.

High-growth reassortant virus candidate vaccines are made by combining the surface viral protein genes of low pathogenicity parent strains with high amino acid sequence homology to the targeted isolate, with internal genes of a model type virus [4]. The Center for Disease Control (CDC) has developed a Eurasian H7N7/PR8 reassortant virus that was entered into phase-I clinical trial in the United States using classical genetic reassortment methods. This vaccine candidate virus contains low pathogenic subunits of the H7 hemagglutinin from A/mallard/Netherlands/12/2000 (H7N3) and N7 from A/mallard/Netherlands/2/2000 (H10N7), and the standard internal genes of A/Puerto Rico/8/34 human strain (H1N1). The reassortment phase with the model virus Joh/PR8, provided a high-growth property of the internal genes. The influenza viruses were propagated in the allantoic cavity of 10 day old embryonated chicken eggs, and grew to high titers within 48 hours of infections to yield 2048 unit hemagglutination titer.

Sanofi Pasteur has developed chimeric vaccines by using the ChimeriVax technology developed at St. Louis University [11]. Live-attenuated chimeric viruses were constructed by replacing the genes coding for the Premembrane (prM) and Envelope (E) proteins from yellow fever virus (LFV) with those of heterologous flaviviruses (ChimeriVaxTM technology). The construct uses the stable YFV RNA virus genome with a low polymerase error rate and the seed lot strategy with monitoring of the plaque size to maintain the attenuated viral population. By use of this technology, a West Nile Vaccine, Japanese encephalitis, and dengue vaccines have been generated and all have successfully completed preclinical trials.

Immunogenic Expression

Biological materials are being engineered to present antigens through specific intracellular pathways, giving designers control in the way the rationally designed antigens can be presented to lymphocytes [12]. Rationally designed vaccines are specifically created at the level of the amino acid sequence using biochemical protein properties as guides [4]. In short, the expression system uses DNA sequences incorporating a coding sequence coding for a

desired polypeptide and appropriate control sequences to promote and regulate expression on the operably joined sequence [13].

The global antibody market is projected to grow into a 34 billion dollar market this year [14]. Replacement of polyclonal antibodies with single or multiple monoclonal antibodies is an area of interest to manufacture safe and highly potent antibodies to target infections where current systems struggle like in hepatitis C infections [15]. Potential antigen candidate categories for vaccine formulations for safe, robust, portable, scalable cultivation processes include: lipopolysaccharide, surface polysaccharides, polysaccharide-protein conjugates, flagella, outer membrane proteins, pili, whole formalin-killed pathogenic cells, live-attenuated *P. aeruginosa* and *salmonella* enteric strains expressing pathogenic antigens and DNA sequences [16]. Mammalian cell line monoclonal antibody production start-up is focused on the production costs of the bioreactor titer.

It is important to monitor both the biological response of the raising of antibodies (humoral immunity) and elicitation of adaptive immune responses (T-cell or B-cell mediated responses) in response to an antigen or immunogen [13]. Often immunogenic response is only detected as hemagglutinin (HA) specific antibody response is widely used to monitor vaccine effectiveness in CD 4+ T helper cells and in turn the optimization of CD8+ T cell responses [5]. Cloning and expression of recombinant protein expression and/or display of proteins with increasing specific activity in a vaccine boosts its therapeutic effectiveness and immunogenicity.

Massive immunization campaigns and current state of the art in vaccine development is focused on attenuated pathogenic bacteria or virus concoctions. In bacteria, an antigen can be secreted and expressed inside a cell, on a cell membrane, or outside a cell and transferred to a host cell [8]. Attenuated and inactivated bacterial and viral vaccines induce immunoprotection through a composition of either intact nonpathogenic microbes or by killing the microbe while retaining its immunogenicity. Attenuated vaccines elicit both innate and adaptive immune responses. Empirically derived attenuated vaccines are obtained through passages, with the emergence of mutants with potentially increased or decreased virulence being unavoidable across passages, underlying the safety which has to be strictly controlled [11]. Attenuation has also been induced through introduction of specific attenuating mutations into the wild type by site directed mutagenesis. Modern system approaches involve temperature-sensitive and deletion mutations which move away from the common technique of using repeated cell passages to select for this quality [8].

Purified subunit vaccines are compiled from purified antigens that are generated from microbes or inactivated toxins and usually administered with adjuvant. An antitoxin vaccine targeting the circulating HIV-1 tat protein which is crucial for maintaining HIV replication reacts with the immunodominantly conserved B cell epitope of TAT [17]. This synthetically generated self-adjuvanting lipopeptide anti-Tat epitope vaccine (TUTI-16) induces neutralizing antibodies to Tat when administered in equimolar amounts of the variant regions of amino acid peptides (wobble peptides), which may hold promise for immunological suppression of HIV replication.

Synthetic antigen vaccine production is a promising technology which has the potential to manufacture large quantities of protein antigens that are specific to a microbial infectious agent [1]. By identification of the most immunogenic microbial antigen or epitopes, and sequencing the nucleotide sequence data, researchers can use this genetic information to create synthetic antigenic proteins. Successful sequencing of these epitopes has led to patents of these genetic codes with some of the most valuable ones being surface exposed and is highly antigenically conserved. The patents information on these sequences describes

the encoded polypeptides, polynucleotides, and recombinant materials and methods for production.

One example is U.S. patent 20090246219, where it discloses the sequence information of the *Haemophilus influenza*, (Pfeiffer's bacillus), surface exposed protein E [8]. This patent reveals the potential of nontypical *Haemophilus influenza* vaccine production from use of the protein data. US patent 6299880 discloses a polynucleotide sequence of a cell surface protein of *Staphylococcus aureus* identifiable during infection. Sequences such as these have anti-bacterial targeting and therapy potential [18]. Encoded polypeptides can be used as cell surface markers which in turn can be expressed as components of vaccines to generate protective memory against the organisms or to generate antibodies to inhibit the binding to prevent microbial adhesion of host tissue leading to pathogenesis.

Introduction of genes encoding for microbial antigens into a noncytopathic virus to infect individuals with it with goals of induction of full immune response is the hypothesis of the invention revealed in the US patent 20050249752A1 [10]. It describes a vector for anti-hpv vaccine and transformed microorganism by the vector. Examples of employment of these vector types are valuable in highly variable pathogens such as: herpes simplex virus, hepatitis virus, foot and mouth disease, HPV and rotavirus.

To decrease the possible selection of antigenic variants while retaining immunogenicity, empty capsid vaccines have been developed so that they contain the entire repertoire of immunogenic sites on the intact particle minus the nucleic acid [6]. These empty viral capsids can be synthesized and assembled in cell culture, or administered as a vector where the capsids are expressed and assembled in the host potentially inducing widespread immunity. A notable application of this strategy has been demonstrated in the successful delivery of the Foot and Mouth Disease virus capsid sequence with a recombinant human adenovirus type-5 in cattle and swine.

Viral antigen delivery systems are categorized in attenuated virus, DNA viral vectors, and RNA viral vectors [19]. DNA viral vectors allow simultaneous expression of multiple antigenic determinants. Viral vectors that have been used as antigenic delivery systems include: poxviruses, herpesvirus, adenovirus, and baculovirus. RNA viral vector studies include those with paramyxovirus, rhabdovirus, bunyavirus, alphavirus, coronaviruses, retrovirus, and flavivirus.

The DNA vaccine approach leads to a strong and lasting complete immune response through the inoculation of a plasmid expressing a protein antigen. This type of vaccine is effective without the addition of adjuvant because the plasmid itself acts as a stimulant, but clinical safety of these vaccines must be carefully considered. DNA vaccines offer the immunotherapy of diseases like tumors, because they have the capability of inducing cytotoxic T effector lymphocyte response for antigen-specific apoptosis of infected cells [20]. Modifications in the targeted antigen encoding oncogene have increased their immunogenicity. These DNA immunizations are administered through a gene gun. Antitumor effect of a modified E6 human papilloma virus oncogene by fusion with GUS and site directed mutagenesis with the goal of destroying the alternative splicing in the E6 gene or by reducing the ability of the targeted mRNA to induce p53 degradation was evaluated. The modified E7GGG gene fused either with the wild type or modified GUS with E6 gene both demonstrated immunogenicity.

The universal vaccine production goal is to grant protection against a diverse span of pathogenic subtypes by inducing the targeting shared highly conserved antigenic determinants [5]. Successful formulations of such vaccines would be immensely valuable against worldwide pandemics. The most conserved regions of viruses are those of their

internal protein, with many studies focusing on the NP and M proteins. Alexander *et al.* report that vaccination with conserved CD4+ influenza-specific epitopes may establish memory T cells to prime the immune system against infection of live virus or subsequent vaccinations [5]. This induction of cell-mediated immunity would mediate control of extent and length of infection and prevent death. Their design is of a HLA-DR epitope-based universal influenza plasmid DNA vaccine which was derived from highly conserved protein regions. This targeting of conserved epitopes may allow protection against both circulating and emerging new influenza viruses. They used epitope derivatives of the following internal proteins of influenza A: M1, M2, NP, PB1, PB2, PA, NS1, and NS2. Their live DNA vaccine construct delivered the epitopes of multiple epitopes at once to show possibility of conserved region priming to give cross-subtype protection.

The patent US 20020048816 describes a mechanism of expression of surface layer proteins in *Bacillus sphaericus*, and *Lactococcus lactis* [13]. The microbes donate proteinaceous surface arrays (s-layers) comprising of an expressed fusion polypeptide which contain a fused exogenous, heterologous antigenic polypeptide. The inventors provide a recombinant DNA (rDNA) molecule having an s-layer protein (SLP) system that expresses and presents a fused SLP with an antigenic peptide extracellularly. The antigenic peptides that can be used in this mechanism can be the antigenic determinant of a pathogen from a virus, bacterium, fungus, yeast, or parasite as well as a polypeptide subunit from a toxin. *Bacillus sphaericus* was reported as having an efficient SLP presentation system because of its high level of growth and lengthily expression of the fusion polypeptide.

US patent 20060269541A1 provides a vaccine strategy for protection against *Streptococcus agalactiae* infection through the identification of an amino acid sequence for a hyperimmune serum reactive antigen in these bacteria [21]. This bacterium's serotypes are responsible for the majority of invasive diseases worldwide, therefore necessitating a significant amount of antibiotic treatment. The development of vaccines that provide immune protection against bacterial infections will reduce the excessive antibiotic usage in a treated population. Discoveries in this area will help fight against antibiotic resistant bacteria and its expanding population.

Another aspect of immunotherapy is the administration of antibodies through passive immunization. Passive immunizations are used to rapidly treat fatal diseases, and are short-lived with no memory protection from future exposures. Attempts to use human intravenous immunoglobulin preparations for prevention are spurred on by monoclonal antibody production techniques, and this transfer of specific antibodies has potential to be applied to virtually any expressible antigenic sequence [21].

Transgenic Production Systems

Recombinant proteins are produced using some common protein expression systems such as bacteria, yeast, and baculovirus protein expression systems [9]. Microbial systems offer the advantage due to their low cost for production. The most widely used yeast expression system has been focused on *Saccharomyces cerevisiae*, but recent trends are auditioning other yeast strains in the search for the best microbe expressed antigen factory. The methylotrophic yeast, *Pichia pastoris* utilizes economical carbon sources to reach high density cell cultures needed for large-scale production of vaccines. Expressed proteins in *P. pastoris* have high levels of glycosylation which may enhance or in some cases hinder the efficacy of vaccines. Fungal-type N-linked glycosylation in peptides may be immunogenic or may lack the effector function, making post translational modifications in antibodies are necessary in antibody development. To date, there have been reported a library of glycoengineered *P. pastoris* strains that have been "humanized" to overcome this limitation and this technology is held by GlycoFi Inc which is a subsidiary of Merck and Co Inc [22].

To increase vaccine yield with the goal of producing an oral vaccine, foods such as tomatoes and potatoes have been used to produce the antigens or used as an edible vegetable transformant. There are still no licensed plant-produced human vaccines on the market even though plants have been extensively studied. This may be in part due to the limitations of their expression levels, purification, and commercial challenges. However, progress is being made in this area. The expression of heterologous proteins at high yields has been reported through plant viral vectors [23]. Their scalable transient expression technology is based on using two different viral vectors (derived from both tobacco mosaic virus and potato virus X), each expressing different polypeptides, delivered by *Agrobacterium* to *Nicotiana benthamiana* leaves. Their technology could be used on an industrial scale in events requiring rapid response.

Cell Surface Display

Cell surface display is the expression of proteins or peptides by joining them with a suitable surface anchoring motif on the surface of microbial cells [24]. The displayed protein (passenger protein) is fused with an anchoring motif (carrier protein) by one of three ways: N-terminal fusion, C-terminal fusion, or sandwich fusion. Classifications of these cell surface anchoring carriers include: secretory protein, surface organelle protein, lipoprotein, and cell outer membrane protein [9]. This technology has been applied to gram negative and positive bacteria, molds, yeasts, and animal cells.

Surface expressed live vaccines generate a large and long lasting immune response, because a non-pathogenic host can be made to express the target antigen continuously while proliferating, which in turn stimulates both humoral and cell-mediated immunity [10]. These vaccines have been capable of stimulating an immune response even through oral administration [25]. US patent number 20070105150A1 disclosed pharmaceutical potential compositions which comprise of an expression vector of an outer membrane protein (FadL) of *E. coli* which can effectively express target proteins or peptides (lipase in this design) and stably display them outside the cell [10]. The advantage of using FadL is that it has 10 fusion points (#9 being the focus of this invention) and allows expressed enzymes to have lengthy activity. The inventive method can be useful in live recombinant vaccines, since it allows large amounts of the desired proteins to be expressed functionally and exogenously displayed by a surface anchoring motif.

Another multiple display surface immunogen expression system design has been worked in flagellum display. The bacterial filament of flagellum is the major extracellular part of the motility organelle consisting of 20,000 FliC molecules at the distal end of it and ending with a capping structure of five FliD molecules (HAP2) [26]. HAP2 variable region has been successfully used in the displaying of multiple foreign peptides. In a study in *E. coli*, three effector molecules were displayed in a multi-hybrid surface display system directly attached to the hook cap in the absence of FliC.

The strategy disclosed in EPO516286A1 has hepatitis B virus (HBV) surface proteins which have reduced host carbohydrate content [27]. HBV surface proteins were expressed in a recombinant yeast host which is unable to glycosylate its own proteins providing a reduced amount of entrapped carbohydrate content while expressing HBV surface protein particles from Dane particles giving the subtype of HBsAg. This patent currently licensed by Merck, where they manufacture the vaccine, Recombivax HB from their technologies. Similarly, Merck also has patent #EP0511855A1, which is for an HBsAg escape mutant vaccine that uses epitope variants of HBV surface protein produced in recombinant yeast without the ability to glycosylate proteins reducing their carbohydrate content [28].

Another vaccine vector patent US20090117151A1, used for the preparation of a vaccine for the treatment or prevention of disease, specifically targeting cervical cancer [9]. Their technique employed the concept of cellular molecule surface display, which is where a microbe expresses a protein with an anchoring motif to present an exogenous protein on its cellular surface. This mechanism has a wide range of application for industrial use where the assortment of the displayed protein can be designed with genetic engineering techniques and various carrier types can be used. Their recombinant live vaccine design focused on exploiting the non-pathogenic bacteria surface as an adjuvant in conjunction with abundantly expressed exogenous surface antigen. This invention was specific for the human papilloma virion capsid proteins (from the genes HPV L1 or HPV L2 and tumor associated protein genes HPV E6 or HPV E7), and the pgs A-C genes encoding poly-gamma-glutamate synthetase complex in *Bacillus* sp. Strains. These transformed cells can be used as a vaccine with the microbial transformant carrying the antigen on its surface, or by using crude or purified extract of cell membrane components after microbial disruption.

Autotransporter display systems and fimbrial display systems have been thought to be promising mechanisms for the insertion and display of heterologous target antigens on live bacterial vaccines [20]. An advantage to the autotransporter display system is its ability to export and display larger peptides. A study compared the two mechanisms in *Salmonella enterica* (Serovar Typhimurium) as vectors to deliver foreign antigens as live vaccines. In these *Salmonella* vaccines, N-terminal fusion of MisL autotransporter protein to antigenic determinants of *Plasmodium falciparum* was compared to the 987P fimbrial display system FasA using the *Salmonella* pgtE vaccine vector. They reported that the fimbrial system induced a higher systemic specific IgG titer.

Another valuable approach to develop live vaccines is to display the virulence factors from the targeted pathogens on the cell surface of *S. cerevisiae* [2]. *S. cerevisiae* has a status as GRAS (generally regarded as safe), and offers many advantages such as: easy and inexpensive cultivation, available surface display system, and regenerative and adjuvant function. The yeast surface display system has been used to immobilize and display both antibodies and antigens [23]. *Camelidae* antibodies have been successfully displayed on the surface of *S. cerevisiae* to bind to specific antigens. When antigens are surface expressed they are accessible to the immune system to induce a protective immune response against the target fragments. The potential live vaccine to protect marine fish has been designed using *S. cerevisiae* mating system surface display (EBY100/pYD1 from Invitrogen) of extracellular hemolysin produced from *Vibrio har-veyi* (which is a serious bacterial pathogen of marine animals) [2].

Bacteriophage T4 has been used in the designing of a multicomponent antigen display and delivery system [7]. Their design reported a single T4 bacteriophage preparation using two non-essential outer capsid proteins, Hoc and Soc, to codisplay antigens to elicit immunological response. A phage display technique has also been used to target the Beta-amyloid peptide deposition in Alzheimer's disease [29]. This live or dead vaccine design anchors the self-anti-beta-amyloid (aggregating epitope) antibodies to filamentous recombinant phages' major (p8) or minor (p3) coat proteins.

Another expression host candidate is baculovirus which infects insect cells in nature or mammalian cells in culture by receptor mediated endocytosis of an envelope glycoprotein, gp64 [30]. The cytoplasmic domain of gp64 joins with the budding nucleocapsids to incorporate gp64 into the virion. The surface display of exogenous peptides has been generated by inserting a heterologous peptide between the N-terminal signal peptide and mature domain of gp64. Once expressed it is translocated to the plasma membrane and displayed on the baculoviral envelope. This system has been used as a potential vaccine

delivery platform using a universal baculovirus surface display system to different foreign proteins on the envelope of the baculovirus [26]. Here, the baculovirus itself acts as an adjuvant and the virus allows the rapid generation of antigens in high titer stocks without the need for purification. In addition, baculoviruses have a good biosafety profile. The researchers constructed recombinant baculoviruses that have potential as vaccine candidates because they induced immunogenicity against the displayed avian reovirus proteins [26].

Enhancements in Vaccine Formulations

Optimization of vaccines is another focused area of research and progression open for innovations. This increased efficiency in the effectiveness to induce a protective immune-enhancing response may be created through: coupling with carriers, acceptable adjuvant, vehicles, excipients, binders, carriers, preservatives, buffering agents, emulsifying agents, wetting agents, and transfection facilitating compounds.

Antigen are often poorly immunogenic when administered alone, so carriers are employed to increase their visibility to immune cells. These carriers may cause cross-reactions, which can decrease the specificity of a generated antibody response and its immunological memory. The engineering of filamentous bacteriophages by decreasing their surface complexity has shown positive results for focusing antibody specificity to the antigens attached to these highly immunogenic particles [31]. These types of conjugate vaccines create a covalent linkage between a specific antigen, (protein or non-protein), and an immunogenic protein carrier to induce a lengthy immune response against a region or domain of a complex pathogen. A traditional carrier in vaccines is ovalbumin, and current enhancers include proteins, mixed particle enhancers and enhancers that target engulfment of the immunogen by phagocytic cells.

Vaccine designers also have options in the route of administration of their immunogenic concoctions. Intramuscular, intranasal, topical, and oral vaccinations each have their own advantages and purposes of dispersing its material. With determinants set by the action of antibody secreting cells and generation of long-term B cell memory, studies to quantify the optimal route of administration are recorded. In one study, an inactivated influenza virus vaccination was compared in intramuscular (i.m.) verses intranasal (i.n.) administered in mice [32]. Their analysis supported the established reports that i.m. administration produces a stronger virus-specific IgG response compared to i.n. administration of a slightly increased antigen dose. More understood is the i.m. vaccination induction of immunity which is thought to be maintained in bone marrow antibody secreting cells. This allows a strong secondary B cell response through circulation and transudation, and is increased by the adjuvant effect of tissue trauma and ease of delivery of particles to the immune system. The i.n. administration provides a high IgA-mediated antiviral barrier at the mucosa in airways, compared to the i.m. vaccination. Future studies may reveal more insight to the particular advantage of localization of these memory cell populations, and important dosage requirements to strengthen the specific responses.

Vaccine adjuvant and immunomodulators help obtain satisfactory immunogenicity of coadministered antigens. The inclusion of an adjuvant enhances immunological sensitivity in memory and in span to allow antigen sparing and reduction of needed dosages [33]. Tested adjuvant includes synthetic emulsions (oil-in-water), alum (aluminum hydroxide gel or Aluminum phosphate), lipid formulations, and cytokine-based adjuvant like type one interferon [6,32]. Adjuvant is under scrutiny in the areas in monitoring of resultant adverse effects and their safety profile before licensing. The only adjuvant approved in the United States is alum, which has been used in vaccine formulations since the 1920s [33]. Recently in other countries, the approval of other vaccine adjuvant in human use include: MF59

(squalene-based-oil-in-water emulsion), ASO3 (oil-in-water emulsion), and a combination of monophosphoryl lipid A (MPL) and aluminum hydroxide (ASO4) has been licensed.

Alum is the most widely used adjuvant increasing the efficacy of the vaccines by increasing inflammation and activating complement cascade, possibly creating particulate multivalency, and allowing increased exposure of antigen because of its lengthen release into the injection site [6] MF59 has been used in more than 20 countries within the last decade with influenza antigens HA and NA to enhance uptake by APCs (antigen presenting cells) also by TLR-independent mechanisms similar to Alum [33]. MPL is a bacterial component lipopolysaccharide which acts on the highly expressed Toll-like receptor-4 on APCs inducing cell-mediated immunity. CpG oligonucleotides have been successful in targeting TLR-9 in human trials. Areas in experimentation of immunostimulatory substances may include: bacterial products, mineral salts, emulsions, interleukin-12, microparticles, plasmid DNA, stimulators, cytokines, saponins and liposomes [21,33]. Adjuvant is now being modeled after the natural resultant cytokine environment during infection to activate the immune system [12].

The engineering of materials for enhancing antigen uptake by APCs are designed for recognition and recruitment [12]. Anti-DEC205 antibodies fused with an antigen or bio-material particle have been used to successfully target dendritic cell endocytic receptors. Chemoattracting APCs to the delivery site has been demonstrated by gradients that release a mixture of cytokines, pathogen-associated molecular patterns (PAMPs), and antigen.

CURRENT & FUTURE DEVELOPMENTS

For those seeking an intellectual challenge in a field where the optimal framework in molecular genetics, biological pathogenesis, and targetable aspects of the immune system are awaiting manipulation to improve upon vaccine strategies in the spirit of inventions or in the development of novel approaches for the rational design of vaccines the foundation has been laid. One can hope that worldwide programs of vaccination will continue to control or eradicate some of the infectious diseases that burden society, especially if vaccine dosages are available and costs are economical. Through the introduction of new vaccine technologies as summarized in Fig. (1), the logical design of vaccines with increasingly efficient immune system stimulation is already creating a new generation of vaccine formulations. This new generation of vaccines may prove to not only allow the simultaneous expression of antigens, but as in the case with surface display eradicate the need for extensive purification steps and selective design may provide the host organism to act as an adjuvant itself. There is sure to be a plethora of fit-to-purpose molecular design of vaccine products thoughtfully designed for its targeted pathogenesis.

As with any breakthrough, there may be downsides in the vaccine industry with some reluctance to invest in additional production techniques and their capacity unless a demand justifies their expenses. The vaccine industry is an already profitable region of pharmaceutical development and start up costs in production of molecularly designed vaccines is substantial. Thankfully, the expressed production in recombinant microbes is being investigated to find optimal expression conditions. It is possible that future developments in these expression vessels will help provide long lasting protection even against highly variable antigenic subtypes which may pharmacologically rendering these vaccines invaluable.

Most of the patents lay claim to their invention being used as a vaccine for pharmaceutical prevention or treatment or for use in immobilization, screening, or diagnostic detection techniques. Basically, designing a vaccine in any of their mechanisms would simply entail knowing the coding sequence of an antigenic determinant of a pathogen, cloning it into a

plasmid with a proper promoter, and selection mechanism and signal peptide to make up a desired expression vector. Proper techniques can be used to insert and express the vector in the desired microbial host. In addition, high-yield expression can be focused on the desired specific protein expression by employing inducible promoter sequences. High-yield antigen production can be achieved by use of cell culture techniques like bioreactors which optimize the microbes' desirable expression conditions. Next, test for correct protein expression. Then either the cell culture solution can be purified or microbes can be used alive, attenuated, or crude, and test for an induced immune response. If successful, then a lethal trial is next. If everything goes according to plan, the focus would be optimization of the vaccine dosage and increased production efficiency.

This is an exciting time to be researching antigenic production in cell culture techniques. The advent of cDNA technologies and knowledge about functional genomics allows the introduction of specific changes in the genome to improve immunogenicity and the evaluation of positive characteristics to induce protection to prevent and control disease [3]. Furthermore, with more and more recombinant vaccines being approved by the FDA and with protein expression techniques becoming commonplace (generating optimal protein production strategies), research has never been so close to creating protection and therapy against such a wide scope of pathogens. It is only a matter of time before vaccine development and the expectations there of change dramatically.

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Fig. 1.
Outline of different approaches to develop high-yield vaccine expression systems.