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New Vaccine Technologies, Adjuvants and Delivery System

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ABSTRACT

Immunization represents the most cost-effective means to achieve the prevention of infectious diseases. Vaccines successfully protected humans from serious infections resulting in worldwide eradication of smallpox and regional elimination of poliomyelitis. New technologies continue to provide many effective biological products for disease control.

Various techniques including recombinant DNA approaches, peptide synthesis chemistry, and immunomodulation have been applied to the development of new vaccines and improvement of existing vaccines. These technologies have constructed avirulent and attenuated strains to be used as live vaccines, as well as provided more effective manufacturing methods to produce protein antigens, bacterial polysaccharides (PS) and PS-protein conjugate vaccines.

Adjuvant is an agent added to biologics that augments specific immune responses to antigens. Adjuvants can be divided into several classes including aluminum salts, surface-active agents, bacterial derivatives and slow-release materials. Nonionic block copolymer as a multiple emulsion, and proteinoid microspheres were also developed for use in various vaccines.

The ideal vaccine delivery system depends on the capabilities of the controlled delivery system to achieve optimum concentration of antigens, while maintaining vaccine stability under physiological conditions. Microspheres were used for controlled release of antigens whereas peptides of monoclonal antibodies were applied for enhancement of mucosal IgA antibody formation.

INTRODUCTION

The development of vaccines for prevention of diseases is one of the great accomplishments in medicine. Biotechnology has provided many new drugs and biological products for the pharmaceutical industry, enhanced medical treatment against diseases, and improved diagnostic reagents. Vaccines can successfully protect humans from many serious epidemic diseases. Worldwide eradication of smallpox and regional elimination of poliomyelitis, cholera, as well as malaria have been achieved. However, many infectious diseases still cause problems for mankind, Many microorganisms became resistant to various antibiotics over the last 30 years. We continue to need effective therapeutic medicines for treatment but frequently turn to immunization as a cost-effective approach for prevention and control of diseases.

Since 1980, many different proteins with important pharmacological activities and vaccines have been synthesized and manufactured by

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new technologies. The applications of biotechnology involve recombinant DNA technology, chemical synthesis, and immunologic methods. Biotechnology and its products have provided a major impact on many aspects of our lives and contribute to the progress of pharmaceutical sciences. At present there are more than 11 recombinant protein drugs and vaccines licensed by the U.S. FDA for use in therapy. Further, more than 130 biotechnology products overall are in various stages of development and clinical trial.

During the past several years significant vaccine developments have occurred to achieve the goal of prevention of specific diseases. In 1986, the hepatitis B virus vaccine was developed by the application of recombinant DNA technique. New conjugate vaccines against *Haemophilus influenzae* type b (Haemophilus b) are now widely used in the United States and European countries[1,2]. As a result, the incidence of Haemophilus b diseases in young children in the United States declined from approximately 60 cases/100,000 population to 1/100,000 in the last two years[3]. The acellular pertussis vaccine has been used in Japan for more than 10 years[4]. In the United States, acellular pertussis vaccine is now combined with diphtheria and tetanus toxoid vaccines for immunization of young children[5]. New vaccines are also available and under development for prevention of encapsulated bacterial diseases including: meningococcus, group B streptococcus, *Klebsiella, Salmonella typhi* polysaccharide (PS) or PS-protein conjugate vaccines.

Many vaccines used at present are effective. However, there are a few deficiencies which need to be addressed. These deficiencies include inducing systemic and local adverse reactions, and requiring more efficient manufacturing techniques and delivery system. An ideal vaccine should be able to elicit high and longlasting protective immunity, evoke minimal adverse reactions, be available, inexpensive, stable, and convenient for immunization.

Recently, various methods of chemical synthesis and biotechnology have produced many antigens. Although these synthesized antigens can provide specific immunologic determinants to be recognized by the immune system, they usually induce low antibody responses. Thus, it is necessary to apply immunostimulating substances such as adjuvants as well as an effective delivery system to stimulate high antibody responses and enhance the defense mechanisms of host resistance.

NEW TECHNOLOGIES APPLIED TO VACCINE DEVELOPMENT

Today's vaccine research and development are complex processes, requiring a multidisciplinary field in chemistry, molecular biology, and immunology. New strategies for preparation of antigens are being applied in the following areas:

I. *Recombinant DNA technology.*

Recombinant DNA technology is applied extensively in the identification and isolation of antigens. This technology has made the study of pathogenic organisms much safer, by being able to clone the specific genes and express their translated antigen products. Thus, persons need not come in direct contact with disease causing organisms or potentially contaminated antigens from the pathogenic organisms.

The application of recombinant DNA techniques has made possible the production of safer immunologically protective molecules in higher yields. For example, by mutation of a single amino acid of the diphtheria toxin gene, a nontoxic antigen, CRM197 was produced. CRM197 contains a single glycine to glutamic acid change in position 52 that makes the fragment A of diphtheria toxin unable to bind NAD+and, therefore, enzymatically inactive[6,7]. Being nontoxic but otherwise identical with diphtheria toxin, it is the best candidate for a new vaccine against diphtheria. It can also serve as a protein carrier

for bacterial polysaccharideprotein conjugate vaccines.

Recombinant DNA technology has facilitated the construction of avirulent and attenuated strains that can be used as live vaccines, e. g. the genes related to the virulence properties of cholera toxin subunit A were deleted to form a safe and protective strain[8,9]. The virulent strains of *Salmonella* have been attenuated by mutation in the *galE* gene or in various *aro* genes which are related to their toxicity[10,11]. These mutations facilitate the stimulation of the immune system, but prevent the organism from causing disease.

There are three considerations that are important for making a decision about the system of expression for most recombinant-DNA vaccines and other biological products: (i) the immunogenicity or biological activity of the expressed protein, (ii) the feasibility or large-scale production processes, and (iii) the purity and safety of the final product[12]. For example, the immunogenicities of the yeast-and mammalian-derived hepatitis B surface antigen (HBsAg) are comparable. However, the recombinant HBsAg can be produced in yeast cells at higher yield and thus lower costs than in mammalian cells. Therefore, yeast is the preferred expression system for the production of hepatitis B vaccine. The hepatitis B vaccine (Recombivax HB, Merck Sharp & Dohme, 1986) was manufactured by recombinant DNA technology, using the following procedures[13].

1. Genetic material (DNA) is extracted from the hepatitis virus.

2. The S gene that encodes for HBsAg is identified and isolated from viral genomic DNA and inserted into a plasmid vector.

3. The vector that contains the specific gene is cloned into yeast cells.

4. Yeast cells are grown to produce large amounts of HBsAg.

5. The HBsAg is isolated from yeast cells and purified by chemical and chromatographic methods.

6. The HBsAg preparation is combined with preservative and aluminum hydroxide adjuvant to form the final vaccine.

An alternative strategy for hepatitis B vaccine uses live recombinant vectors, such as vaccinia virus or adenovirus[14-16] to introduce HBsAg into the body as the result of an innocuous viral infection.

II. *Chemical synthesis.*

Since most protective antigens are proteins, the knowledge of protein chemistry has made significant contributions to the vaccine development. The antigen-combining sites on immune cell receptors recognize only a small part of a polypeptide antigen, about 3-5 amino acids for antibody epitopes and about 15-20 amino acids for T-cell epitopes. Such peptides are easily prepared by the current peptide synthesis techniques. If the primary sequence of an antigen is elucidated, the B-cell epitopes on the antigen can be identified by synthesizing possible overlapping peptides and, then, reacting these peptides with a wide range of specific antibodies. This approach has been applied to the identification of protective epitopes on the merozoite surface antigen for the development of a malaria vaccine [17-20].

Another application of peptide synthesis to vaccine development is the synthesis of known B - and T-cell epitopes for use as peptide vaccines. Furthermore, techniques in synthetic chemistry have prepared some effective bacterial polysaccharideprotein conjugate vaccines. Haemophilus b conjugate vaccines from three U.S. manufacturers have been licensed and widely used for immunization in young children[21,22].

Traditional chemical techniques have been used for isolation and purification of protein antigens, and development of new adjuvant formulations.

III. *Immunological methods.*

Monoclonal antibody preparations have

been widely used in antigen identification and isolation. They are also used as effective probes to detect antigenic and epitopic variability.

Another immunological application is the use of anti-idiotypes as vaccines. Anti-idiotypes are antibody molecules that are mirror images of antibodies that recognize a protective epitope of a disease-causing organism. They contain a structure that is immunologically identical with the protective epitope for the disease-causing organism [23-25]. However, the anti-idiotype vaccines usually produce low antibody responses and need conjugation with a protein carrier to enhance their immunogenicity. The use of lymphokines or cytokines as immunostimulating agents has enhanced the immune response to many antigens[26-28].

ADJUVANTS FOR IMMUNOSTIMULATION

Adjuvant is an agent that augments the specific immune response to antigens. Some subunit antigens and peptides produced by recombinant DNA technology and peptide synthesis are not potent immunogens, and their application as vaccines will become practical only if they are used with adjuvants that enhance their respective antibody responses. Adjuvants can influence the titer, duration, isotype and avidity of antibody. The generation of cell-mediated immunity, mucosal immunity and memory are improved with adjuvants[29].

Most vaccines consist of either attenuated live pathogens or killed organisms. Newly available component vaccines contain pure components of proteins or polysaccharides. The essential components of a vaccine should include the delivery vehicle such as water-in-oil emulsion and adjuvant, e.g. aluminum hydroxide gel.

Adjuvants can be divided into several classes[30,31]:

I. *Aluminum salts.*

Aluminum salts such as aluminum hydroxide and phosphate have been widely used for immunization[32]. They are the only adjuvants licensed by a regulatory agency for use in veterinary and human vaccines. The hydrated form of the aluminum gel adsorbs protein antigen due to the electropositive net charge of the alum compounds below pH 9. The adsorption of antigen to the alum forms a deposit that releases antigen slowly, thus extending the antibody response.

II. *Surface-active agents.*

Saponins and Quil A, which is a purified component of saponin, have been used in experimental and veterinary vaccines. However, saponin induces some adverse reactions, such as causing irritation, inflammation, binding to cholesterol, and lysis of cells. The less toxic immunestimulating complex (iscom) preparations which contain membrane proteins and low amount of Quil A in the presence of a detergent, Triton X-100, have been reported to increase antibody responses to many viral membrane protein antigens, e.g. hepatitis B virus and cytomegalovirus, etc.[33-36]. Dimethyl dioctadecyl ammonium bromide (DDA) is a quaternary amine with adjuvant activity applied in the BCG vaccine[37,38].

III. *Bacterial products and their derivatives.*

The classic bacterial adjuvant, Freund's complete adjuvant (FCA), consisting of killed mycobacteria in mineral oil and Arlacel A, stimulates both cell-mediated and humoral immunity. Freund's incomplete adjuvant (FIA), which lacks the mycobacteria, stimulates only humoral immunity. However, the use of FCA is restricted to animals because it causes granuloma formation at the injection site, and possible carcinogenicity by both the mineral oil and the Arlacel A emulsifier in FCA make it unsafe for human use [39,40].

N-acetylmuramyl-L-alanine-D-isoglutamine (muramyl dipeptides, MDP) preparations are represented as the adjuvant-active component of the mycobacterial cell wall[41,42]. Synthetic MDP

and its analogues have been used to induce both cell-mediated and humoral immune responses and applied in the BCG and other bacterial vaccines.

Monophosphoryl lipid A (MPL) was synthesized by removing the phosphate moiety from the C-1 position of the toxic diphosphoryl lipid A of *Salmonella*. The MPL is less toxic than the diphosphoryl molecule, but retains much of the adjuvant and mitogenic activity. It also increases nonspecific protection against bacterial infections[43]. The adjuvant activity of MPL is enhanced by the addition of bacterial cell wall derivatives and trehalose dimycolate (TDM), which is a lipid component of mycobacteria[44].

IV. *Vehicles and slow-release materials.*

Liposomes, which are preparations of phospholipid bilayers separated by aqueous compartments, act as vehicles for the delivery of water- or lipid-soluble antigens. They cause mobilization of antigens into draining lymph nodes at the site of injection, and interact with macrophages[45]. Moreover, liposomes form a deposit that releases the antigen slowly over a period of days to weeks[46].

Several formulations of water-in-oil emulsions containing metabolizable oils have been developed. Adjuvant 65, a preparation of peanut oil, aluminum monostearate stabilizer, and Arlacel A emulsifier, was reported to be a safe and potent adjuvant in humans when combined with influenza vaccine[47,48]. Squalene, a natural unsaturated oil that is an intermediate in cholesterol biosynthesis, functions as a potent vehicle to induce immunostimulatory activity[49].

In addition, cytokines such as interleukin-1 (IL-1) and IL-2, and interferon-γ also exhibit adjuvant activity[26-28]. The mechanisms of action of adjuvants are listed in Table 1.

Recently, several adjuvants have been developed for the induction of high-titer, long-lasting systemic and mucosal antibody responses and may be applicable as effective oral vaccine delivery vehicles.

Table 1. Mechanism of action of adjuvant.

1. Activation of macrophage.
Adjuvants, such as muramyl dipeptide (MDP), aluminum hydroxide, and LPS, induce activation of macrophages. These cells display the class II major histocompatibility complex (MHC) receptor, which is critical for antigen presentation. Macrophages also produce interleukin-1.
2. Physicochemical properties as amphipathic surface active substances. For example, Saponin, immunostimulating complex (Iscom), trehalose dimicolate (TDM).
3. Activation of helper T cells to release lymphokines, e.g. LPS.

Nonionic block copolymers are simple linear chains or polymers of hydrophobic polyoxypropylene flanked by two chains of hydrophilic polyoxyethylene (Figure 1)[50,51]. They are adhesive molecules which bind antigens and host components to hydrophobic surfaces by a combination of hydrophobic and hyprogen bonding interactions. This binding results in antigen presentation to immune cells and augments the expression of class II antigen by macrophages[52].

A microparticulate stabilized water-in-oil emulsion of a block copolymer (TiterMax) was found to induce higher antibody titers to a leuteinizing hormone releasing hormone conjugated to bovine serum albumin (LHRH-BSA) following a single injection in rabbits than two injections of Freund's complete adjuvant[53]. When mice were immunized with 20 mg of plasma derived hepatitis B surface antigen mixed in the block copolymer adjuvant, they induced 10^4 to 10^5 mIU/ml antibody titers 28 days after injection. Protection from lethal malaria has been achieved in outbred ICR mice by single injection

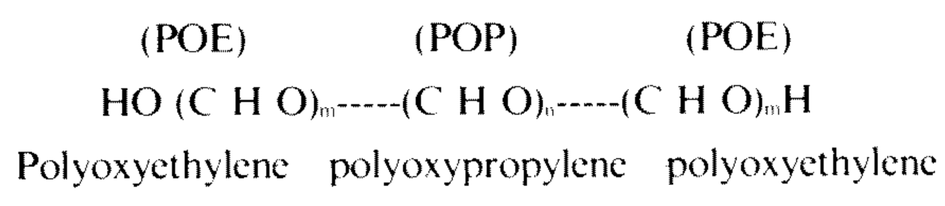

Figure 1. Structure of nonionic block copolymer.

of copolymer adjuvant mixed with 100 ug *P. yoelii* blood stage antigen. The immunized mice were challenged with 10,000 malaria-infected red blood cells on day 30. More than 85% survival rate was observed in the immunized mice, while the control group showed less than 20% survival rate[54,55].

The block copolymer is effective in producing a multiple emulsion that is stable at frozen or room temperature for months. The multiple water-in-oil-in-water (w/o/w) emulsion, made with the metabolizable oil squalene and block copolymer, produces a high efficiency of delivery of protein antigen protected from digestion in the stomach and upper gastrointestinal tract to reach the intestine. Mice given orally three doses of a multiple emulsion of TNP-hen egg albumin produced serum IgG antibody which persisted for months. They also produced secretory IgA antibody detectable in intestinal secretions[56].

ORAL DELIVERY SYSTEM FOR VACCINES

Most infectious microorganisms, such as those causing influenza, diarrhea, and gonorrhea, enter the body through the mucosal epithelium of tissues[57]. Among the defense mechanisms present on the mucosal surfaces, IgA antibody secreted into the mucous fluid is the most important component produced from the immune system. Secretory IgA binds to infectious agents in the mucous fluids, neutralizes them, and inhibits their entry into epithelial cells.

For increasing mucosal IgA production to enhance the protective effects of protein immunogens, several approaches have been pursued: (a) the development of effective systems that can deliver the intact proteins to the mucosal surfaces of small intestine for stimulating IgA-rich lymphoid cells, and (b) the development of chemical agents that induce the proliferation of IgA-expressing B-cells. An unique monoclonal antibody designated as *migis*-α antibody is specific for membrane-bound IgA (mIgA) on the surface of IgA-expressing B cells. It recognizes the transmembrane-anchor peptide extending from the C-terminus of membrane bound a chain (*migis*-α peptide), and binds specifically to mIgA-expressing B-cells.

Since mIgA is the antigen receptor of IgA-expressing B-cells, the modulation of mIgA will cause cell activation and proliferation of B-cells. The antigen receptor-mediated activation of B-cells involves the crosslinking and aggregation of the antigen receptors. Thus, the binding Fab fragment of *migis*-α antibody to polymer backbones, such as dextran or Ficoll will form crosslinking and aggregation of mIgA molecules on the surface of B-cells. Furthermore, these antibodies can be used as a specific carrier to deliver certain stimulatory factors, e.g. IL-6 to mIgA-expressing B-cells. Therefore, a chemical agent or system that enhances the production of IgA antibody can be applied to the development of oral vaccines.

The use of proteinoid microspheres for oral immunization provides the possibility of delivering high local concentrations of antigen and protection from low pH and proteolytic enzymes in the stomach[58]. Proteinoid material was synthesized by the thermal condensation of aromatic and acidic amino acids and dissolved in deionized water. The antigen such as influenzae M protein in citric acid solution was mixed with proteinoid material to form microspheres. A single enteric dose (1 mg/rat) of influenzae M1 antigen in proteinoid microspheres induced a significant primary antibody response to M1 antigen in rats. In contrast, little or no response was observed in uncapsulated M1 antigen[59,60]. These results may have important application towards the development of an oral vaccine for influenza. Furthermore, parotid protein, taurine and lithium were used as safe and effective adjuvants for oral rubella and influenzae vaccines [61].

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新疫苗技術、佐劑與接種方式

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摘　要

免疫接種爲達成預防感染疾病最經濟有效之方式。疫苗成功地使人類防禦遭受感染，從全球根絕痘瘡，並消滅地域性小兒麻痺症。新科技繼續生產許多有效的生物製劑以控制疾病。

各種技術，包括遺傳因子重組，蛋白質合成化學，與免疫調節，應用於新疫苗的開發與對現用疫苗的改進。這些科技創製了非活性與活性弱化的菌株疫苗，並且供給更爲有效的製造方法來生產蛋白質抗原，細菌性多糖類，與多糖類～蛋白質結合疫苗。

佐劑爲加進於疫苗製劑之化合物以增進對抗原的特異性免疫反應。佐劑可分爲幾類，包括鋁化合物、表面活性劑、細菌性化合物，和遲延性釋放物質。非離子性重聚化合物所做成的複合性乳化劑，似蛋白質微粒化合體可應用於各種疫苗的佐劑。

理想的疫苗接種需要能控制其接種方式達到適當的抗原濃度，並在生理狀況下維持疫苗的安定性。微粒性化合體可應用於抗原的控制釋放，而單一同源抗體之胜類可增進黏膜性IgA抗體之生成。