International Journal of Applied Pharmaceutics

Vol 1 Issue 1, 2009

ADVANCES IN VACCINATION: A REVIEW

SWARNALI DAS[a]*, ROHITAS DESHMUKH[b],

[a]School of Pharmacy and Technology Management, NMIMS University, Mumbai, Maharastra, India

[b]Sri Shankaracharya Institute of Pharmacy, Durg, Chattisgarh, India

E-mail address: swarnali4u@rediffmail.com or swarnali34@gmail.com

ABSTRACT

The search for methods of vaccine delivery not requiring a needle and syringe has been accelerated by recent concerns regarding pandemic disease, bioterrorism, and disease eradication campaigns. In this article, we summarize the rationale behind the advances of vaccination techniques. Needle-free vaccine delivery could aid in these mass vaccinations by increasing ease and speed of delivery, and by offering improved safety and compliance, decreasing costs, and reducing pain associated with vaccinations. Needle-free vaccination includes all methods for delivering vaccines that do not require a needle and syringe for administration.

New and promising technologies include using live attenuated bacteria as vectors for foreign antigens and transgenic plant "edible" vaccines. Both nasal and aerosol vaccines are easy to deliver and are painless. Many of the technologies described here require further clinical studies to ensure safety and efficacy, but they still offer promising vaccine delivery methods.

INTRODUCTION

Immunization is a means of providing protective shield to the body, *i.e.* immunization or vaccination is a prophylactic approach through which the body is shielded or made strong enough to fight against any incoming pathogenic invasion. Thus immunization generates a force that fights with various micro organisms and their products (metabolic as well as non-metabolic), which enter through different routes in the body. The immunology is a multidisciplinary science that involves different approaches of vaccination.

Immunization is a two-century-old science of prophylaxis. It came into existence in 1796 when Jenner studied that inoculation of cowpox virus prevents small pox in human. After this discovery the cow pox vaccination came into clinical practice worldwide in 19th century[1]. The science of immunization peaked to new heights in late 19th century to early 20th century and during World War II. After postulation of germ theory by Louis Pasteur, vaccinology never looked back. The advent of tissue culture techniques revolutionized the immunization approaches. A number of new vaccines with different approaches like live/attenuated bacterial or viral vaccines, killed bacterial suspension, toxins produced by bacterial toxoids, rickettsial suspension have been developed.

The process of distributing and administrating vaccines is referred to as vaccination. Vaccination is a form of immunization. Vaccination can be achieved through various routes of administration, including oral, nasal, intramuscular (IM), subcutaneous (SC), and intradermal (ID). It is well documented that the route of administration can impact the type of immune response. The majority of commercial vaccines are administered by IM or SC routes. In almost all cases, they are administered by conventional injection with a syringe and needle,

although high velocity liquid jet-injectors have had some success.

Vaccines represent an invaluable contribution of biotechnology as they provide protection against even such diseases for which effective cures are not yet available. The effectiveness of vaccines may be appreciated from the fact that small pox, once a dreaded disease the world over, has been completely eradicated from the world. Vaccines are used for active immunization as a prophylactic measure against some infectious diseases. They provide partial or complete protection for months or years [2].

An ideal vaccine

- It should not be toxic or pathogenic, i.e., it should be safe.
- It should have very low levels of side effects in normal individuals.
- It should not cause problems in individuals with impaired immune system.
- It should not spread either within the vaccinated individual or to other individuals (live vaccines).
- Should produce long lasting humoral and cellular immunities.
- The technique of vaccination should be simple.
- The vaccine should be cheap so that it is generally
- It should not contaminate the environment.
- It should be effective in affordable.

So far, such an ideal vaccine has not been developed. [3]

Need for new vaccines

There are certain infectious diseases for which no vaccines are available yet e.g. HIV, tuberculosis, malaria, *Neisseria meningitides* Type B, etc. Using traditional approaches to vaccine development, these kinds of diseases are found to be extremely difficult to control. Thus, there is a clear need for the development of new vaccines against these kinds of diseases. New vaccines are required to protect against the influenza virus and antimicrobial resistant organisms. Threat of bioterrorism is also an important reason for the development of new vaccines. [4]

There are various emerging infectious diseases, including West Nile, SARS, and Ebola & Hanta, which, if not controlled, would lead to a mass destruction of humankind. New vaccines have to be developed for the prevention of these diseases. There are various micro organisms which cause chronic diseases e.g. Hepatitis B & C viruses cause Hepato cellular carcinoma, Human papilloma virus causes cervical, anal & vulvar cancer & Epstein-Barr virus causes Burkitt lymphoma. These chronic diseases can be prevented only by novel vaccines. Vaccines are the potential therapeutic agents which can be used to treat established infections.

Thus, novel vaccine delivery technologies will be required to enable the development of these new vaccines. Traditional vaccines, although highly effective and relatively easy to produce at low cost, suffer from the following limitations:

- In many cases, live vaccines have to be used since killed pathogen vaccines are ineffective.
- Live vaccines are generally based on cultured animal cells; hence expensive tissue culture set up is essential.
- Live vaccines are heat labile.

- Traditional vaccines carry a variable risk of disease development due to the occasional presence of active virus particles or reversion to virulence after replication in the vaccinated individuals.
- In many cases, they are difficult to produce, e.g., hepatitis B virus does not grow in high titre in cultured cells.

These limitations have prompted the development of new vaccines, which are rather costly, at least for the present. [3]

Novel approaches to vaccine delivery

The concept of "vaccine delivery" can be expanded to include a range of devices and physical delivery systems that are designed to allow immunization using non-invasive routes. In recent years, a growing interest in the development of needle-free vaccine delivery systems has emerged. Independent laboratories have demonstrated needle-free immunization to macromolecules, including protein- and DNA-based antigens.

Although the currently available vaccines represent an outstanding success story in modern medicine and have had a dramatic effect on morbidity and mortality worldwide, it is clear that improvements are required in the current vaccine delivery technologies. Improvements are required to enable the successful development of vaccines against infectious diseases that have so far proven difficult to control with conventional approaches. Improvements may include the addition of novel injectable adjuvants or the use of novel routes of delivery, including mucosal immunization. Mucosal delivery may be required to provide protection against pathogens that infect at mucosal sites, including sexually transmitted diseases. Alternatively, novel approaches to delivery, including mucosal administration, may be used to improve compliance for existing vaccines. Of particular interest for safer mass immunization campaigns are needle-free delivery devices, which would avoid problems due to needle, re-use in many parts of the world and would avoid needle-stick injuries. [5]

Recently, jet injector devices have been developed, which are capable of delivering liquid vaccines into the skin using high pressure. Although, the majority of needle-free devices have delivered liquid vaccines, liquids are generally less stable than powders and are vulnerable to freezing. Therefore, needle-free dry powder vaccines are considered the optimal approach for large scale use, since these would be resistant to temperature fluctuations and should have greater stability.

High-pressure needle-free devices are being used for the delivery of liquid DNA vaccines in clinical trials. [6] A "gene gun" is a DNA vaccine coated onto gold beads and is delivered directly into the epidermis. [7]

An alternative needle-free approach to vaccine delivery, involves the use of microprojection arrays, designed to painlessly disrupt the outer layers of the skin to allow the vaccine access to epidermal langerhans cells. Perhaps the most attractive needle-free approach to vaccine delivery currently being explored involves transcutaneous immunization, through topical application of vaccine patches.

There has been a shift away from the use of whole pathogens or inactivated subunits, toward the use of recombinant purified proteins. This has resulted in the need to develop novel adjuvants and delivery systems to improve the immunogenicity of these antigens. Optimal new generation vaccines, particularly from a safety perspective,

will contain recombinant protein antigens, purified synthetic immuno potentiators, which represent well-defined PAMPs (pathogen-associated molecular patterns), and a delivery system designed to ensure that both the antigen and the adjuvant are targeted efficiently to APC (antigen presenting cells). It is clear that novel adjuvant and delivery technologies will be required to enable the successful development of vaccines against diseases that have not yielded to traditional approaches.

Hence, it is clear that needle-free vaccine delivery technologies will prove most easy to apply for novel vaccines, particularly if efficacy is dependent on a route of delivery. Alternatively, novel needle-free delivery approaches may become established to improve patient compliance for annual vaccination (e.g. influenza) or to protect against a potential bio- terrorist attack, or an emerging pandemic strain of influenza. In addition, novel delivery may achieve market success through improving the convenience of immunization or due to minimizing pain.

NEEDLE-FREE VACCINE DELIVERY

Importance and need

- The World Health Organization (WHO) estimates that 12 billion injections are given annually, and that 5% of these are immunisations.
- The development of needle-free delivery systems for vaccines has been named one of the Grand Challenges in Global Health.
- Needles are associated with an increased risk of infection, especially in developing countries, where there are problems with needles being reused and issues with waste management and disposal.
- Needle free delivery systems would make vaccines easier to deliver.
- Compliance would be significantly improved, with people more likely to avoid an injected vaccine because of fear and pain associated with the needle, especially children.
- Needle-free vaccines can reduce cost of immunization as they can be delivered without medical intervention.
- Topical vaccines are cheaper and easier to transport and store, than injectable vaccines, which generally require refrigeration.
- Needle stick injuries are a significant problem in both developed and developing countries. It is estimated that 5 in every 100 injections worldwide result in a needle stick accident. The introduction of needle-less vaccines would significantly reduce the risk and incidence to health care workers.
- Some progress has been made with oral polio, cholera and rotavirus vaccines. But diphtheria, tetanus, pertussis, varicella, measles, mumps, rubella, tuberculosis and yellow fever are all still injectable vaccines.
- The current market for vaccines is worth approximately $9 billion globally with a 1012% annual growth rate.

Delivery options

The search for methods of vaccine delivery not requiring a needle and syringe has been accelerated by recent concerns regarding pandemic disease, bioterrorism, and disease eradication campaigns. There are several methods currently in use and under development, focusing on needle-free injection devices, transcutaneous immunization, and mucosal immunization. Jet injectors are needle-free devices that deliver liquid vaccine through a nozzle orifice and penetrate the skin with a high-speed narrow

stream. They generate improved or equivalent immune responses compared with needle and syringe. Powder injection, a form of jet injection using vaccines in powder form, may obviate the need for the "cold chain." Transcutaneous immunization involves applying vaccine antigen and adjuvant to the skin, using a patch or "microneedles," and can induce both systemic and mucosal immunity. Mucosal immunization has thus far been focused on oral, nasal, and aerosol vaccines. Promising newer technologies in oral vaccination include using attenuated bacteria as vectors and transgenic plant "edible" vaccines. Improved knowledge regarding the immune system and its responses to vaccination continues to inform vaccine technologies for needle-free vaccine delivery. [8]

According to the routes of administration, the needle-free vaccine delivery options can be broadly categorized into:

i. Nasal vaccine delivery
ii. Topical vaccine delivery
iii. Oral vaccine delivery

I. NASAL VACCINE DELIVERY

Intranasal immunization has emerged as a promising and attractive vaccination route since:

- Mucosal surface is one of the major entry pathways for many pathogens.
- Nasal immunization can induce both mucosal and systemic responses. Due to the dissemination of the immune cells in the mucosal immune system, intranasal administration may also induce immune response at distant mucosal sites.
- Nasal vaccines are easier and cheaper to administer to large population and will benefit the vaccine distribution in developing countries.
- Nasal immunization does not require needles which is a potential source of infection.

The major advantage of nasally administered vaccine is its ability to induce mucosal IgA that provides a local defensive mechanism against the pathogens entering the human body through the mucosal surface. The acceptance of nasally administered vaccine will mainly depend on the vaccine's efficacy. The research and development in this area has achieved many accomplishments. However, the road to approval is still long and expensive. The Example of nasal vaccine is Live cold-adapted trivalent influenza vaccine FluMist™.

FluMist™, Medimmune, Gaithersburg, MD, is a needle-free flu vaccine — a gentle nasal mist delivered into the nose, where the flu virus usually enters the body. FluMist is a live weakened influenza virus vaccine approved for the prevention of certain types of influenza disease in children, adolescents and adults 2-49 years of age against influenza disease caused by influenza virus subtypes A and type B contained in the vaccine.[9] FluMist is for intranasal administration only. It is administered via a prefilled single-use device that sprays vaccine into the nostrils, and was licensed by the FDA in 2003 for use in healthy persons aged 5 to 49 years [10]. This vaccine has been studied in adults and children as young as 15 months for efficacy, immuno genicity, and safety. Among children the vaccine is safe, well tolerated, and up to 93% effective against culture-confirmed influenza[11].

One strategy to increase immuno genicity is to incorporate adjuvants in

the vaccine formulation. Immunological adjuvant is defined as "substance used in combination with a specific antigen that produced more robust immune response than the antigen alone". Adjuvants can be categorized into two classes based on their mechanism of action: vaccine delivery and immuno stimulatory adjuvant. Vaccine delivery adjuvants increase immune response by targeting the antigen into the antigen presenting cells (APC). The immuno stimulatory adjuvant activates immune response by stimulating the release of cytokines or the expression of the co - stimulatory molecules on APC. The use of both types of adjuvants is being explored for use in the nasal vaccine system.

The goal of the use of these adjuvants is to increase effectiveness of antigen delivery to the immune system. The adjuvants achieve their goals by:

- Increasing permeability or penetration through epithelium
- Taking advantage of the bioadhesive characteristic of the adjuvant. Adhesion to the mucosal membrane increase the retention time which in turn increase the opportunity of the antigen to reach the immune system.
- Enhancing the antigen uptake by designing a physical characteristic of the adjuvant to optimize mucosal immune system's recognition and processing.

Immune response following intranasal vaccination

The development of effective adjuvants depends on a good understanding of the immune response. Human nose is part of respiratory system covered with mucous membrane lining. The defensive mechanism of these mucosal lining predominantly involves a group of lymphoid tissues known as mucosal associated lymphoid tissue (MALT). The lymphoid tissues specifically related to the respiratory tract can be divided into three groups:

- Larynx-associated lymphoid tissue (LALT)
- Bronchus-associated lymphoid tissue (BALT)
- Nose-associated lymphoid tissue (NALT)

NALT is an organized system consisting of B cells, T cells and antigen presenting cells (dendritic cells and macrophages) which are covered by an epithelial layer containing M cells. When antigen is present on the mucosal system specialized cells called M cells transport the antigen across the epithelium [12].

When vaccine is delivered intranasally, the antigen is administered on the mucosal surface. The interaction of the antigen with the mucosal immune system is highly dependent on the nature of the antigen. Soluble antigen may be able to penetrate the nasal epithelium and interact with antigen presenting cells (APC) such as macrophages and dendritic cells. The APC migrate to the lymph node where the antigen is presented to the T cells as a start of the activation of the immune response cascade. If the antigen is in the form of particles, the antigen is transported to the NALT by the M cells through phagocytosis. The NALT is also drained to the lymph node where the antigen processing will occur.

A nasal spray vaccine for SARS has been developed and tested successfully in monkeys. The new nasal vaccine requires single dose and is delivered directly into the respiratory tract, the site of SARS attack. The vaccine uses a small piece of the virus's DNA to stimulate the body's immune system to mount a protective response.[13] A small

piece of DNA from the SARS virus that codes for a protein normally found on its outer surface is inserted into a weakened version of a virus that causes respiratory diseases like pneumonia in humans. The vaccine in its current form would be most effective in young children, since most adults already have some immunity against the common viruses that cause pneumonia.

Aerosol immunization

Intranasal immunization has been successfully achieved by aerosols because they are safe, effective, non-immunogenic, economical, easy to administer. The Example of aerosol vaccine is Tuberculosis vaccine. It is an aerosol vaccine -- under development through a collaboration between Harvard University and the international not-for-profit Medicine in Need (MEND). It could provide a low-cost, needle-free TB treatment that is highly stable at room temperature. It consists of heat killed or formalin killed *Mycobacterium tuberculosis* bacteria. The vaccine is spray dried instead of being freeze dried. Traditional TB vaccines are freeze dried, requiring refrigerated storage and transportation and a source of clean water to reconstitute the vaccine for injection. Spray-dried vaccines do not need refrigeration or water to be used. It shows the successful immunization of guinea pigs.[14]

New Delivery Systems for Aerosol Immunization:

i. **Jet Nebulizers:** Jet Nebulizers have gained immense popularity in the last few years. Some of their features are:

- It has been evaluated in humans with good results
- It is portable with rechargeable batteries
- It is successfully used in mass campaigns
- It has low cost (approx US$ 70-100)
- It can be used by trained non-health staff
- The dosage is not precisely known
- The basic and modern model need to be licensed
- Its additional pre-clinical trials are in progress

ii. **Ultrasonic Nebulizers:** These needle-free devices, designed to deliver drugs intranasally have attained broad acceptance with the medical community and patients. Some of their features include:

- It is portable
- It has rechargeable batteries
- Its cost is approx US $ 200-300
- It can deliver up to 100 doses/hour
- It is more suitable for campaigns
- Its trials in animals is in progress
- It is not yet tested in humans
- Its licensing is in procedure

II. TOPICAL VACCINE DELIVERY

Topical immunization is a simple, painless and economical approach to vaccination. This strategy may allow the development of vaccine that could be administered by individuals without specialized training or equipment. The genetic mode of immunization can be useful in immunizing against a number of diseases by single vaccine, *i.e.* different antigens can be encoded on a single DNA. The topical vaccination can thus be a genuine approach by which discomfort related to all other routes such as cellular toxicity in case of

intramuscular injection and degradation of antigen by oral route can be avoided. By means of topical immunization, safe, easy and relatively cheaper immunity can be obtained using different novel delivery systems.

Topical immunization is a novel immunization strategy by which antigens and adjuvants are applied topically to intact skin to induce potent antibody and cell-mediated responses. Among various approaches for topical immunization, the vesicular approach is gaining wide attention. Proteineous antigen alone or in combination with conventional bioactive carriers could not penetrate through the intact skin. Hence, specially designed, deformable lipid vesicles called transfersomes have been used for the non-invasive delivery of tetanus toxoid (TT).

The topical route is now considered as preferred route for delivery of immunogenic substances since it is having an edge over other routes:

a. It prevents unnecessary invasion to body.

b. It prevents or bypasses the problems related to degradation of peptidal vaccines as in case of oral route.

c. It drains the antigens or carrier associated antigens to the lymphatic system and hence to lymph nodes.

d. It prevents unnecessary toxicity encountered in case of immunization by other routes.

For topical immunization skin is the target site. Various routes within the skin are exploited for the delivery/targeting of antigen to the specialized cells. These include follicular pathway, normal pores present in the skin, lamellar lipid bodies and through corneocytes. Skin can normally allow the molecules not greater than 500Da to penetrate and at fairly low rate, when applied epicutaneously. Therefore, for large molecules, some specialized carrier systems are needed to transport them across the skin in immunologically active form.

The skin is exploited as a route for immunization, *i.e.* topical immunization because it shows specific (immunity) as well as non-specific (inflammation) responses for foreign substances. These responses are a result of presence of immunocompetent cells within the skin, which include Langerhan's cells (LC), Dendritic epidermal T-cells and epidermotropic lymphocytes. The mast cells also represent the immunocompetent cells of dermis. Other cells present in the skin are resident antigen presenting cells and transient inflammatory lymphoid cells (*e.g.*, polymorphonucleocytes, monocytes and lymphocytes). Skin consists of SALT (skin associated lymphoid tissue) responsible for the specific and non-specific responses. The SALT composed of the epidermal antigen presenting cells (APC) and migratory T-lymphocytes in circulation, which have avidity for the epidermal tissues. The existence of SALT in the skin is supported by the cytokinins, which have capacity to regulate the immune responses.The antigens that come in contact with the epidermis and hence in contact with the antigen presenting cells are taken to the lymph nodes by means of the lymphatics, because migratory T-cells are attracted towards the peripheral lymph nodes. After binding to high endothelial venules (HEV) they enter into the lymph nodes. The accumulation of T-lymphocytes gives rise to immunological response.

The non-invasive approaches are both numerous and inventive. They range from high voltage and laser light pulses,

sound wave utilization to the abrasive action of fine sand. They can be categorized as:

1. Physical - Iontophoresis, Gene gun, Laser pulse, Ultra sound waves

2. Chemical - Permeation enhancer e.g., DMF, Azones, DMSO

3. Vesicular - Liposomes, Niosomes, Transfersomes

The physical approaches such as electroporation can be used for the transfer of bioactive molecules across stratum corneum. Electroporation is used for the delivery of gene to the keratinocytes for immunization as well as for gene therapy without compromising the viability of the cells. The majority of protocols to increase the permeability of the epidermis (*i.e.*,stratum corneum) include utilization of the chemicals such as - surfactants, alcohols and polyols. They increase the permeability of the stratum corneum by any of the following mechanisms or combination of them:

a. Increasing the fluidity of skin lipids

b. Hydrating the polar pathways

c. Opening heterogeneous multilaminate path ways

d. Keratolytic action

The chemicals used, do not increase permeability of bioactive molecules to the desired extent. Only up to five times, the permeability can be enhanced by this method. However, permeability is still less in case of high molecular weight molecules. The chronic use of these chemicals for permeability enhancement may have dangerous side effects. [15]

The vesicular approach is gaining a wide acceptance nowadays for topical immunization, which includes the utilization of vesicles, virosomes and reconstituted viral envelop since they are efficient in transfer of immunogens (DNA and antigens) across the intact skin. These vesicular carriers are targeted through different pathways in the skin, *i.e*. either through keratinocytes or through follicles.

The vesicles that enhance skin permeability of bioactives include liposomes, niosomes, transfersomes, reconstituted sandai virus envelop (RSVE), adenovirus vector, herpes simplex virus (HSV) and amplicon vector.

- **Liposomes:** Liposomes have been studied extensively for topical (dermal) delivery of various immunoactive agents. Liposomes promote the antigenic response of various bacterial, viral and tumor cell antigens. This inherent immunoadjuvant action of liposomes depends upon their structural characteristics, which control their fate in the body .[16]

- **Niosomes:** Niosomes are nonionic surfactant based vesicles that can be utilized as a topical carrier for immunogens (Antigens or DNA) for dermal or transdermal delivery. Niosomes of decycloethyleneoleylether are found to fuse with the corneocytes. This fusion to corneocytes and formation of lipid stocks indicate that niosomes are most promising vesicular carriers for transdermal delivery of lipophilic molecules. [17]

- **Transferosomes:**

Transfersomes are specially designed lipid surfactant vesicles for transdermal or topical delivery of bioactive molecules. They are ultradeformable carrier system having high capacity of changing their shape and passing through the natural pores in the stratum corneum. They are highly efficacious in transferring the bioactive molecules

across the stratum corneum. They can pass through the small pores present in the skin having diameter five times less than their own diameter. [18]

- **Viral vectors:** Viral vector is another class of topical vaccine carriers. They can be utilized for epidermal transfer of the DNA or other suitable antigen. These include adenovirus vector and HSV amplican vector. Reconstituted viral vectors or virosomes have also been utilized for intracellular targeting of encapsulated DNA/antigen. The reconstituted sandai virus envelops (RSVE) can be applied topically for efficient gene or antigen transfer. [19]

Adjuvants for topical immunization

Adjuvants are substances of great importance as they help to enhance the immune response when mixed and administered with the antigen. Adjuvants are often used to boost the immune response when an antigen has low immunogenicity or when small amount of antigen is available, limiting the immunizing dose. [20]

TABLE 1: SHOWS VARIOUS ADJUVANTS USED FOR TOPICAL IMMUNIZATION

ADJUVANTS	ACTION
Bacterial DNA with unmethylated CpG dinucleotide	Stimulate the innate immune system to produce array of immunostimulating cytokines and induction of NK cells activity
Immunostimulating sequence containing oligodeoxy nucleotide (ISSODNs)	Maximum cell mediated response
Liposomes	Potentiation of humoral response
Calcium and aluminium phosphate	Increased antibody titre by about 100 fold or more
Lipopolysaccharides (LPS) and muramyldipeptide	Increased affinity and number of peptide antigen specific T cells secretory IFN-□ and IL-2
Freund's complete & incomplete adjuvants	Enhances intensity of humoral and cellular response
Co-administration of DNA vaccine with plasmid coding for chemokinins, cytokines or co-stimulating molecules	Enhanced cytotoxic T cell mediated responses
Formulation of DNA with cationic lipids or experimental adjuvants as monophosphoryl lipids	Cationic lipid facilitate intracellular trafficking and adjuvant action
SBAS-4 (Alum + 3-O-deacylated monophosphoryl lipid A)	Activation of macrophages

Transcutaneous immunization

Transcutaneous immunization (TCI) is a new method of vaccination that utilizes a topical application of an adjuvant and vaccine antigen to intact skin to induce an immune response.[21] It combines the advantages of needle-free delivery while targeting the immunologically rich milieu of the skin. This simple technique is reported to induce robust systemic

and mucosal antibodies against vaccine antigens in animal models. Safe application of a patch containing heat-labile enterotoxin (LT, derived from *Escherichia coli*) to humans, resulting in robust LT-antibody responses have been reported. These findings indicate that TCI is feasible for human immunization, and suggest that TCI may enhance efficacy as well as improve vaccine delivery.

Bacterial products such as LT and cholera toxin (CT) are members of a class of potent molecules known as adjuvants used to enhance immune responses to vaccine components. When applied to the skin of animals, both CT and LT induce systemic and mucosal immune responses to themselves and to co-administered antigens such as diphtheria and tetanus toxoids that are administered along with these adjuvants. In animal studies, adjuvants such as CT and LT are essential for the induction of robust immune responses via the skin.

CT and LT act as adjuvants and antigens [22], inducing antibodies against CT and LT when applied to the skin [23]. Antibodies against toxins contribute to protection against human diarrheal disease and, in mice; both serum and mucosal IgG and IgA antibodies against toxins can be detected in response to TCI.

Adjuvants are required for the induction of potent immune responses to co-administered antigens by TCI; the main adjuvants are ADP-ribosylating enterotoxins that include cholera toxin (CT) and the heat-labile enterotoxin of enterotoxigenic *Escherichia coli* (LT). TCI is not limited to ADP-ribosylating enterotoxins as the sole source of compounds available with adjuvant properties active in the context of the skin. Many other molecules have adjuvant activity when applied to the skin [24]. In their native form, CT and LT cannot be readily administered orally in humans due to their enterotoxicity, but they have been shown to be safe in animal and human skin immunization studies.

A. Patch Vaccine

Transcutaneous immunization is usually accomplished by using a patch (such as those manufactured by IOMAI Corporation, Gaithersburg, MD) or similar means to deliver both vaccine antigens and adjuvants. Adjuvants are immunostimulating compounds used to augment the immune system's response to vaccine antigens. The clinical study found that volunteers who received the vaccine before being exposed to high levels of enterotoxigenic *E. coli* (ETEC) bacteria had less severe diarrhea and were significantly less likely to require intravenous fluids than patients who were not vaccinated.[25]

B. Epidermal powder immunization (epi)

Epidermal powder immunization (EPI) is a technology that offers a tool to manipulate the LCs and the potential to harness the immune reactions towards prevention and treatment of infectious diseases and immune disorders. EPI was developed to target antigens to LCs in vivo. EPI has its roots in a technology that was developed in the early 1990s for genetically engineering plants and then was adapted for DNA immunization. EPI delivers antigens in the form of microscopic particles to the epidermis using a needle-free powder delivery system (or PowderJect device) and elicits broad immune responses. [26]

Many traditional vaccines such as proteins, peptides, polysaccharides,

inactivated pathogens, etc. are suitable for EPI [27]. Vaccine powders can be prepared by coating antigens onto 1-2 mm gold particles or embedding them into 20-50 mm particles using sugar excipients (trehalose, mannitol, sucrose, or combinations). The driving force of the device is pressured helium gas. Actuation of the device causes the release of helium gas, which accelerates the vaccine particles to high velocity that penetrates the stratum corneum and land in the LC rich viable epidermis. In addition to targeting antigen to the LC rich epidermis, EPI offers the advantage of pain-free delivery. This is because the sensory nerve endings in the epidermis are far less dense than deeper tissues such as dermis and muscle.

Potential applications of EPI

- **Infectious diseases:** EPI can elicit high levels of serum antibody, which are important for prophylactic immunization against infection by extracellular bacteria and many viruses. Mucosal antibodies are particularly important because these antibodies may prevent pathogens from gaining entry to the deeper tissue.

- **Cancer immunotherapy:** EPI represents an in vivo technique for developing DC (Dendritic cells)-based immunotherapy. It directly delivers antigens to the cytosol of the LCs and elicits both antibody and cellular immune responses. EPI is suitable for delivering tumour cell lysates, purified antigen, and other immuno stimulating agents (e.g.GM-CSF). It is technically much more suitable for commercialization when compared with ex vivo DC-based approach. [28]

- **Immunotherapy for allergic diseases:** Allergic diseases including ACD, asthma, and hay fever are commonly treated with corticosteroids and antihistamine drugs. EPI using antigen and an appropriate adjuvant (CpG DNA, saponin, etc.) promoted strong Th1 responses in an animal model, suggesting that it may be possible to reprogram the immune system of the sensitized individual and offers a more effective means of allergy immunotherapy. [29]

C. Needle-less injecting devices

a. Jet injectors

The need for an easy-to-use, reliable, rapidly deployable needle-free injector has been identified that provides protection against pre-injection vaccine contamination and that eliminates the risk of cross-infection. This safe and effective way to rapidly vaccinate large numbers of people is needed, not only for mass immunization campaigns in developing countries, but also to prepare for the possibility of bio terror attacks and disease pandemics world-wide. The reason for this need is that injections often come with the risk of cross-infection and other types of infection due to:

- Reuse of disposable needles and syringes
- Reuse of sterilizable needles and syringes without proper sterilization
- Improper disposal of contaminated sharps
- Accidental needle sticks
- Advance filling of multiple syringes (often done with the intent to increase efficiency and speed, because syringe filling is a very slow process).

Unsafe injection and disposal practices put the patient, the healthcare worker and the community at risk.

A jet injector is a type of medical injecting syringe that uses a high-pressure narrow jet of the injection liquid instead of a hypodermic needle to

penetrate the epidermis. It is powered by compressed air or gas, either by a pressure hose from a large cylinder, or from a built-in gas cartridge or small cylinder. Some are multi-shot, and some are one-shot.

They are used by diabetics to inject insulin as an alternative to needle syringes, though they are still not very common. In the Star Trek franchise, and sometimes in other fictional scenarios and occasionally in the real world, it is called a hypospray. Jet injectors deliver insulin beneath the skin using a high pressure jet through a tiny opening at the head of the injector. The opening is typically a fraction of the diameter of a needle. Proponents of jet injectors argue that the mist produced by the jet injector results in a better dispersal pattern of the insulin beneath the skin and is therefore less traumatic. Some also claim that jet injectors are less painful than needles.

Advantages of jet injectors:

- They eliminate the risk of cross contamination.
- Different settings allow adjustment for different skin types.
- They are portable.
- They are less expensive than syringes, since they have to be bought once.
- They provide greater precision.
- There is no syringe waste with jet injectors, reducing medical waste.
- The absence of a needle helps those who are afraid of needles.

Disadvantages of jet injectors:

- They are larger than syringes and are thus harder to carry around while out.
- They have to be sterilized, usually on a weekly basis.
- They are not suitable for everybody, e.g., people who are very thin or who are visually impaired.
- People taking anti-coagulant, those with haemophilia and those on dialysis cannot use jet injector as it could cause a bleeding problem.
- Bruising and pain is a common complaint with jet injectors.

b. Needle-less injections

A needle-less hypodermic jet injection system includes a hand-held injector, and an energizing or cocking unit for use with the injector to prepare it for administering an injection. The hand-held unit includes a cartridge which provides a cylinder of liquid medication to be injected, an injection orifice, and an injection piston forceful movement of which causes an injection jet of medication to be expelled from the orifice. A power unit of the injector provides for forceful movement of the injection piston when a trigger is actuated. After being used to effect an injection, the injector is interfaced with an energizer unit which cocks the power unit preparatory to the next injection.

The syringe squirts a very fine jet of liquid at sufficient pressure to penetrate tissue. The initial pressure has to be high to penetrate the skin, followed by a lower pressure to pump the drug in. There are two aspects to the research in which engineers are involved: one is to make an instrument that is both safe and effective when manufactured in large quantities as a mass production item, and the second is to understand what is happening when the drug is injected.

Some attractive features of needle-less hypodermic jet injection system are:

a. Provides a needle-less hypodermic jet injection device which includes a pre-filled, single use injection cartridge.

b. Provides a needle-less hypodermic jet injection system which includes a hand-held injection device or injector part which is pre-energized and carries an injection cartridge with medication cylinder, injection nozzle, and injection ram; and which makes use of a separate component of the system to energize the hand piece injector.

c. Provides such a needle-less hypodermic jet injector device which includes an injection hand piece powering the injection cartridge, and which hand piece is quickly re-energized by interfacing the hand piece with a cocking or energizing mechanism.

d. Provides such a needle-less injection device which allows the hand piece to be re-energized by interfacing the hand piece with, for example, a motor-driven re-energizing mechanism, or with a human-powered (i.e., manually or with a pedal, for example) re-energizing mechanism.

A needle-less hypodermic jet injection system includes a hand-held injector carrying an injection cartridge with medication cylinder, injection nozzle, and injection ram; the hand-held injector including a power unit which is pre-energized prior to each injection; and a trigger mechanism allowing the energy stored in the power unit to be released and applied in driving the injection ram to cause the injection; and an energizing mechanism with which the hand-held injector interfaces to either be re-energized automatically or by the application of force and displacement applied by a person.

D. Microprojection array patch technology

Microprojection array patch technology is being developed to increase the number of drugs that can be transdermally delivered through the skin. Upon application, the microprojections create superficial pathways through the transport barrier of the skin (stratum corneum) to facilitate hydrophilic and macromolecule delivery.

Microprojection arrays having a plurality of stratum corneum-piercing microprojections are used to intradermally deliver an antigenic agent and immune response augmenting adjuvant to induce a potent immune response in mammals, particularly in humans. Preferably, the antigenic agent comprises a vaccine antigen in which antigens is typically in the form of proteins, polysaccharides, oligosaccharides, lipoproteins and/or weakened or killed viruses. Particularly preferred antigenic agents for use with this technology include hepatitis virus, pneumonia vaccine, flu vaccine, chicken pox vaccine, small pox vaccine, rabies vaccine, and pertusis vaccine.

The immune response augmenting adjuvant is preferably selected from those materials which are known to augment the mammal's immune response to antigens and which do not promote adverse skin reactions in the patient. Most preferred is Gerbu adjuvant: N-acetyglucosamine-(beta-1-4)-N-acetylmuramyl-L-alanyl-D-glutamine (GMDP). The reservoir containing the antigenic agent and the immune response augmenting adjuvant can be a gel material, preferably in the form of a thin film laminated to the microprojection array, but more preferably is a material which is applied

as a coating directly onto the microprojections. Most preferably the coating is applied only on the skin piercing tips of the microprojections.

In use, the microprojection array is applied to the skin of an animal to be vaccinated and the array is pressed against the animal's skin causing the microprojections to pierce the outermost layer (i.e., the stratum corneum layer) of the skin. Most preferably, the microprojection array is applied to the skin of an animal to be vaccinated using an applicator which impacts the microprojection array against the skin, causing the microprojections to pierce the skin. The microprojections may be fabricated in different configurations and/or shapes e.g. needles, hollow needles, blades, pins, punches etc. For intradermal delivery of the antigenic agent and the adjuvant, the microprojects should pierce through the stratum corneum and into the underlying epidermis and dermis layers of the skin. Preferably, the microprojects do not penetrate the skin to a depth which causes significant bleeding. To avoid bleeding, the microprojections should pierce the skin to a depth of less than about 400 μm, preferably less than about 200 μm. The microprojections create superficial pathways through the stratum corneum to facilitate permeation of the antigenic agent and the adjuvant. Antigen dose and depth of microprojection penetration are easily controlled. This intradermal vaccine and method of vaccinating animals has broad applicability for a wide variety of therapeutic vaccines to improve efficacy, and convenience of use.

Two recent studies have evaluated the ability of microneedles to deliver vaccine transcutaneously. In one, the skin of hairless guinea pigs was penetrated using the Macroflux® microprojection array system (ALZA Corp., Mountain View, CA), which has projections 330 μm long, and 190 microprojections per cm^2, administered by a 1 or 2 cm^2 patch . In this study, microneedles coated with a dry film of ovalbumin antigen penetrated the guinea pig skin to an average depth of 100 μm.[30] Others have shown that trans-epidermal water loss after application to human volunteer skin is greatest using 200-μm micro needles. In animal models, the expression of genes introduced as vaccines given by microneedles was similar to that seen following intramuscular or intradermal injections. In considering solid microneedles for immunization, one of the expected challenges which needs to be addressed in future studies is the issue of dose uniformity. With both solid and hollow microneedles, more clinical studies in humans are needed to demonstrate the safety and efficacy of this promising method of vaccine delivery.

IV. ORAL VACCINE DELIVERY

Oral vaccines have a distinct advantage over traditional injected vaccines. Oral vaccines stimulate both systemic and mucosal immune responses, while injected vaccines only lead to serum antibody production. Stimulating an immune response at the mucosal sites (such as the nose and mouth) is very desirable, because many pathogens enter the body at these sites. If an immune response occurs at the mucosal sites, pathogens can be prevented from even entering the body. The Sabin polio vaccine, which has been instrumental in achieving the World Health Organization's polio eradication goal, is

one of the most well known oral vaccines. Other oral vaccines that are currently licensed in the United States are the Ty21atyphoid vaccine, which is administered to travellers, and the relatively new rotavirus vaccine. These vaccines are composed of live attenuated organisms; the pathogens used to make them can replicate in the body to the extent required to stimulate an immune response, but they cannot cause the symptoms of the disease.

Edible vaccines

Fruits and vegetables are now commonly being used to express the disease antigens. Oral vaccination with transgenic fruits and vegetables has several advantages. The cells walls of fruits and vegetables are a built in protection system for the antigens. Fruits and vegetables can be reliably and cheaply produced in the area where they are needed, and they do not need to be refrigerated. Their administration is safe, simple, and painless, and does not require trained personnel. In addition, clinical trials have shown that vaccination with transgenic vegetables stimulates both mucosal and serum immunity.

Development of edible vaccines:

Plants are currently made into edible vaccines through the process of transformation. In this process, foreign DNA is permanently integrated into the DNA of the plant. The first step of transformation involves creating a binary vector, which is a plasmid with two genes. The two genes that are inserted into the plasmid are the gene that codes for the disease antigen and a kanamycin resistance gene. The binary vector is then inserted into a soil bacterium called *Agrobacterium tumefaciens*. Normally, *Agrobacterium tumefaciens* contains a tumour-inducing (Ti) plasmid. In the *Agrobacterium tumefaciens* used to make transgenic plants, the Ti plasmid is inactivated so that it is only capable of integrating DNA into the host cell's genome; it can no longer cause tumours.

After the binary vector is inserted into the *Agrobacterium tumefaciens*, a leaf from the plant that is to be transformed is cut and exposed to it. The Ti plasmid in the *Agrobacterium tumefaciens* acts to randomly integrate the two genes from the binary vector into the plant's genome. The plant cells are then exposed to a plate of kanamycin. Any plant cells that have not been effectively transformed will not have the gene for kanamycin resistance, and they will die. The plant cells that have been effectively transformed are allowed to grow into calluses that sprout shoots and roots. The calluses are then planted, and they grow into transgenic plants.

Candidates used for transformation into edible vaccines:

Potatoes, tomatoes, and bananas are currently the top three candidates for transformation into edible vaccines. Each of these three vectors has advantages and disadvantages.

- Potatoes are easily transformed and propagated. One problem with potatoes is that many people do not like to eat them raw, and cooking them denatures the antigens, effectively destroying their ability to induce an immune response. In addition, potatoes may not grow well in the parts of the world where the vaccine is needed.
- The tomato is a popular candidate because it is also easily transformed. Three other factors in the tomato's favour are the fact that it grows relatively quickly, it can be eaten raw, and it has the highest level of vitamin A among the three candidates. Vitamin A regulates transcription of the

genes for antibody synthesis; this had led to speculation that its administration along with vaccines may boost immune response.

- The final favourite is the banana, which has been advanced as a candidate because of its popularity with children, who are the main targets of the vaccines being developed. Bananas are also inexpensive to produce, and they are normally eaten raw. In addition, bananas are native to many developing countries. One drawback to bananas is that they take twelve months to bear fruit after they are transformed.

When a potato, tomato, or banana containing a disease antigen is ingested, parts of it are taken up by components called M cell in the intestinal lining. The M cells pass the antigens to antigen-presenting cells such as macrophages and B cells. The macrophages display the antigens on their surface. As a result of interacting with the antigen-presenting macrophage, a helper T cell produces cytokines. These cytokines activate B cells, causing them to proliferate and divide. The B cells divide into plasma cells and memory cells. Plasma cells produce antibodies, while memory cells remain in the body indefinitely, ready to differentiate into plasma cells if the antigen is ever detected again in the body.

The problem faced by the developers of edible vaccines may actually have a beneficial application. An immune response known as "oral tolerance" usually prevents the development of immune responses to antigens that are eaten; the failure of this system leads to the development of food allergies. There are two possible implications of oral tolerance. One worry is that people will develop an allergy to the fruit or vegetable expressing the foreign antigen. Another concern is that body will become tolerant to antigens delivered by the oral route and will fail to mount an immune response against them. It is this concern, however, that has sparked a new avenue in edible vaccine research. Researchers hope to take advantage of the oral tolerance response to use edible vaccines to treat autoimmune diseases such as Type I diabetes, multiple sclerosis, and rheumatoid arthritis.

Liposomes

Liposomes are artificial, spherical, closed vesicles consisting of one or more lipid bilayer(s). Liposomes made from ester phospholipids have been studied extensively over the last three decades as artificial membrane models. Considerable interest has been generated for applications of liposomes in medicine, including their use as diagnostic reagents, as carrier vehicles in vaccine formulations, or as delivery systems for drugs, genes, or cancer imaging agents.

- **Archaeosomes:** They are novel liposomes prepared from the membrane lipids of Archaeobacteria (Archaea). Polar glycerolipids make up the bulk of the membrane lipids, with the remaining neutral lipids being primarily squalenes and other hydrocarbons. Ether glycerolipids extracted from various archaeobacteria were formulated into liposomes (archaeosomes) possessing strong adjuvant properties. Mice of varying genetic backgrounds, immunized by different parenteral routes with bovine serum albumin (BSA) entrapped in archaeosomes (~200-nm vesicles), demonstrated markedly enhanced serum anti-BSA antibody titers. [31]

- **Liposome-entrapped DNA oral vaccines:** Oral vaccine containing liposomes and complexes or, preferably

entrapped, DNA operatively encoding an antigen in which the liposomes are formed from components including cationic compounds and zwitterionic phospholipids have been reported. The hydrophobic groups within the liposome forming components must include at least one group which is saturated. This is believed to raise the transition temperature, rendering the liposomes more stable when delivered orally. The compositions have been found to give detectable increased in IgA levels, secreted immunoglobulins of importance in efficacious oral vaccine delivery. [32]

Nanoparticles

Peptides and proteins remain poorly bioavailable upon oral administration. One of the most promising strategies to improve their oral delivery relies on their association with colloidal carriers, e.g. polymeric nanoparticles, stable in gastrointestinal tract, protective for encapsulated substances and able to modulate physicochemical characteristics, drug release and biological behavior. Encouraging results upon in vivo testing are reported but low bioavailability and lack of control on absorbed dose slow down products development. Vaccines are certainly the most promising applications for orally delivered nanoparticles. The presence of RGD on nanoparticles allows the targeting of β1 integrins at the apical surface of human M cells and the enhancement of an immune response after oral immunization. RGD-labelling of nanoparticles significantly increased their transport by co-cultures, due to interactions between the RGD ligand and the β_1 intregrins detected at the apical surface of co-cultures. [33]

Recombinant oral vaccine

Recombinant vaccines formulated for oral use (i.e. encapsulated in biodegradable polymers) will provide multiple advantages over conventional vaccines [34, 35]. Combining encapsulated vaccines with nutritious foods will be more convenient and acceptable to use, and will be easier to administer periodically as discussed in the previous section. Large-scale agricultural production of recombinant vaccine antigens and functional recombinant human antibodies is feasible and can yield quantities of product sufficient to satisfy virtually any requirement, including "edible" vaccines. [36]

FUTURE PROSPECTS

Since its discovery in 1796 by Edward Jenner, vaccines have been an integral aspect of therapeutics, combating a number of infectious diseases with remarkable success. In recent years, due to rapid advances in proteomics, genomics, biotechnology and immunology and the plethora of knowledge amassed in related fields, it is fair to expect vaccine development to progress at an exponential pace. However, even in the 21st century, we are still struggling in our efforts to eradicate fatal diseases such as AIDS, malaria and hepatitis C due, in part, to the absence of effective vaccines against these diseases. Vaccine development faces major challenges both technologically and economically.

Since vaccines are administered to millions of infants annually, it is clear that the level of scrutiny of vaccines will continue to be intense. Therefore, the safety hurdles applied to new vaccines and delivery approaches will be high, with rigorous evaluations. Any new delivery technology will need to prove beyond reasonable doubt that vaccine potency is not impaired in comparison to the established vaccine. Delivery of vaccines through needle-free technologies would take the field of

immunization to new heights. It would help in the eradication of various fatal diseases. Using these techniques, people having needle-phobias would be vaccinated easily.

Newer vaccines that are stable, economical, require fewer doses and can be administered using needle free systems are a worldwide priority. An ideal theoretical vaccine may not be cogent unless formulated and delivered aptly. Delivery of vaccines via oral, intranasal, transcutaneous and intradermal routes will decrease the risk of needle-borne diseases and may eliminate the need for trained personnel and sterile equipment. Crucial to the success of a vaccine is the delivery strategy that is to be employed. Currently, various techniques involving DNA vaccines, adjuvants, microparticles and transgenic plants are being developed and evaluated. Although, no major breakthrough is in prospect, these systems have potential and will take immunization to a new technological level.

CONCLUSION

There have been a number of significant achievements in technologies to express and deliver vaccines. Needle-free vaccination includes a variety of approaches for mucosal immunization, and patches and devices allowing delivery of vaccines through or into the skin. Although some mucosal vaccines are commercially available, needle-free approaches involving devices have so far failed to achieve broad acceptance, despite their potential advantages. There are concerns that the efficacy of existing vaccines might be impaired if they were to be administered by alternative routes, or using novel devices.

Mucosal vaccination is an attractive alternative to parenteral vaccination. Intranasal route as well as oral route of administration is currently being pursued. Topical immunization is a simple, painless and economical approach to vaccination. DNA vaccination includes targeting of naked DNA to the hair follicles for topical immunization. Topical and transdermal delivery is a field of interest for researchers to deliver the drugs, bioactive molecules (enzymes, DNA, RNA) and immunogens. Microprojection array patch technology is being developed to increase the number of drugs that can be transdermally delivered through the skin. Jet injectors utilize a high-pressure narrow jet of the injection liquid to penetrate the epidermis. Oral vaccines can stimulate both systemic and mucosal immune responses.

While new vaccines may be added to the list of those already recommended, introducing needle-free delivery approaches for existing vaccines will be exceptionally difficult. In addition to the significant hurdle of proving that the novel approach is safe, it will also be necessary to undertake studies to prove that the new technology does not negatively impact the potency of the vaccine. Novel devices and routes of delivery may have a particularly important role to play in situations where chronic administration of vaccine products is required and ease of administration may become crucial to enable effective patient compliance in these situations.

REFERENCES

1. Singh RP, Singh P, Mishra V, Prabakaran D, Vyas SP. Vesicular Systems For Non-Invasive Topical Immunization: Rationale And Prospects, Indian Journal Of Pharmacology, 2002; 34: 301-310.

2. Halvorsen R. The truth about vaccines: how we are used as guinea pigs without knowing it. London: Gibson Square Books Ltd; 2007.
3. Singh BD. Biotechnology, Kalyani Publishers, 1998; 392-394.
4. Valiente NM, O'Hagan D, Ulmer J. Innate Immunity and biodefence vaccines. Cell microbiology, 2003; 5: 755-760.
5. O'Hagan DT, Rappuoli R. Novel approaches to vaccine delivery. Pharmaceutical Research, 2004; 21(9): 1519-1530.
6. Epstein Je, Gorak Ej, Charoenvit Y, Ruobing W, Freydberg N, Osinowo O, *et al*. Safety, Tolerability and lack of antibody responses after administration of a PfCSP DNA malaria vaccine via needle or needle-free jet injection, and comparison of intramuscular and combination intramuscular/ intradermal routes. Hum. Gene Ther., 2002; 13: 1551-1560.
7. Roy MJ, Wu MS, Barr LJ, Fuller JT, Tussey LG, Speller S, *et al*. Induction of antigen-specific CD8+ T cells, T helper cells, and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine. Vaccine, 2000; 19: 764-778.
8. Mitragotri S, Immunization without needles: reviews, Nature Reviews Immunology, 2005; 5: 905-916.
9. www.medimmune.com/pdf/products/flumist_pi.pdf
10. Harper SA, Fukuda K, Cox NJ, Bridges CB. Using live, attenuated influenza vaccine for prevention and control of influenza: supplemental recommendations of the Advisory Committee on Immunization Practices (ACIP), MMWR Recomm. Rep, 2003; 52: 1–8.
11. Belshe RB, Mendelman PM, Treanor J, King J, Gruber WC, *et al*. The efficacy of live attenuated, cold-adapted, trivalent, intranasal influenza virus vaccine in children, N. Engl. J. Me, 1998; 338:1405–1412.
12. Neutra MR, Frey A. Epithelial M cells: gateways for mucosal infection and immunization. Cell, 1996; 86: 345-8.
13. Ukreyev A, Lamirande EW, Bucholz UJ, *et al*. Mucosal immunization of African green monkeys (*Cercopithecus aethiops*) with an attenuated parainfluenza virus expressing the SARS coronavirus spike protein for the prevention of SARS. Lancet, 2004; 363(9427): 2122-7.
14. www.emednews.org/2008/03/15/aerosol-tuberculosis-vaccine
15. Cevc G, Schatzlein A, Blume G. Transdermal drug carrier: Basic properties, optimization and transfer efficiency in case of epicutaneously applied peptides. J Control Rel, 1995; 36: 3-16.
16. Gregoriadis G, Gursel I, Gursel M, McCormack B. Liposomes as immunological adjuvants and vaccine carriers. J Control Rel, 1996; 41: 49-56.
17. Hafland HEJ, Boustra J, Bodde HE, Junginger HE. Nonionic surfactant vesicles in transdermal formulations: Controlled release and in vitro effect on human. Pharm Res, 1989; 6: S178-S179.
18. Paul A, Cevc G, Bachhawat BK. Transdermal immunization with an integral membrane component, gap junction protein, by means of ultradeformable drug carrier transfersomes. Vaccine, 1998; 16: 188-95.
19. Bo lu, Federoff HJ, Wang Y, Goldsmith AL, Scoff G. Topical application of viral vector for epidermal gene transfer. J Invest Dermatol, 1997; 108: 803-8.
20. Gregoriadis G. Liposomes as immunological adjuvants. In: Stewart DES's The theory and application of adjuvants. Chichester: John Wiley, 1994; 145-69.
21. Glenn GM, Rao M, Matyas GR, Alving CR. Skin immunization made possible by cholera toxin. Nature, 1998; 391: 851.
22. Snider DP. The mucosal adjuvant activities of ADP-ribosylating bacterial enterotoxins. Crit. Rev. Immunol., 1995; 15: 317-348.
23. Glenn GM, Scharton-Kersten T, Vassell R, Matyas G & Alving CR. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. Infect. Immun., 1999; 67: 1100-1106.
24. Scharton-Kersten T, Vassell J, Hagan DO, Alving CR, Glenn GM *et al*. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants. Infect. Immun., 2000; 68: 5306–5313.
25. www.medicalnewstoday.com
26. Chen D, Endres RL, Erickson, CA, Weis KF, McGregor MW, Kawaoka Y, Payne LG. Epidermal immunization by a needle-free powder delivery technology: Immunogenicity of influenza vaccine and protection in mice. Nat Med, 2000; 6: 1187–90.
27. Chen D, Erickson CA, Endres RL, Periwal SB, Chu Q, Shu C, Maa YF, Payne LG. Adjuvantation of epidermal powder immunization. Vaccine, 2001; 19: 2908–17.
28. Moingeon P. Cancer vaccines. Vaccine, 2001; 19: 1305–26.
29. Wohlleben G, Erb KJ, Barnes P. Atopic disorders: a vaccine around the corner. Trends Immunol., 2001; 22(11): 618–26.
30. James AM, Michel C, Juanita J, Wendy AY, Margaret B, Kofi N, Peter ED. Macroflux® Microprojection Array Patch Technology: A New and Efficient Approach for Intracutaneous

Immunization. Pharmaceutical Research, 2002; 19(1).
31. Lakshmi K, Chantal JD, Patel GB, Sprott GD. Archaeosome Vaccine Adjuvants Induce Strong Humoral, Cell-Mediated, and Memory Responses: Comparison to Conventional Liposomes and Alum. Infection and Immunity, 2000; 68 (1): 54-63.
32. Reyes-Sandoval A, Ertl HCJ. DNA Vaccines. Curr. Mol. Med. 2001; 1: 217-243.
33. Garinot M, Fiévez V, Pourcelle V, Stoffelbach F, Rieux A, Plapied L, *et al*. PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination. Journal of Controlled Release, 2007; 120 (3): 195-204.
34. Morriss W, Steinhoff MC, Russell PK. Potential of polymer microencapsulation technology for vaccine innovation. Vaccine, 1994; 12:5-11.
35. Michalek SM, Eldridge JH, Curtiss R, Rosenthal KL. Antigen delivery systems: new approaches to mucosal immunization. In: Handbook of Mucosal Immunology, P.L. Ogra, J. Mestecky, M.E. Lamm, W. Strober, J.R. McGhee, and J. Bienenstock, editors. Boston: Academic Press, Inc. 1994. p. 373-380.
36. Anderson AO. New Technologies for Producing Systemic and Mucosal Immunity by Oral Immunization: Immunoprophylaxis in Meals, Ready-to-Eat, in Emerging Technologies for Nutrition Research, National Academy Press, Washington, D.C., 1997. p. 451-500.