C H A P T E R

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Trends in Nonparenteral Delivery of Biologics, Vaccines and Cancer Therapies

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O U T L I N E

INTRODUCTION

Although more than 200 years have passed since Edward Jenner conducted the first human clinical trial of smallpox vaccine in 1796, the vaccination routes remain unchanged; subcutaneous and intramuscular remain the two main routes of vaccinations worldwide.[1] These administration routes are one of the factors that render vaccines unaffordable and unavailable in Third-World countries. The hurdles encountered include refrigeration, transport, and the need for health professionals to administer the vaccine, all which have led to higher cost. All of the shortcomings of traditional vaccinations have become a deterrent in our effort to improve world health standards. In order to ameliorate these problems, alternative vaccination routes such as oral, nasal, or buccal offer new hope. Among the alternative routes, the oral route is preferred as a result of its advantages over the other routes.

The concept of oral vaccines has been explored by numerous researchers, and oral vaccines have begun to emerge in the market, including the Polio Sabin™ oral vaccine, and Dukoral™ oral vaccine for traveler's diarrhea and cholera. The advantages of oral vaccines include improved patient compliance, ease of administration, and lower cost of production and transportation. They also serve as an alternative to the needle phobic, including young children and the elderly. More importantly, oral vaccines are capable of inducing both mucosal and systemic immunity. Recent studies have suggested that in order to produce a more effective vaccine, both systemic and mucosal immunity has to be induced.[2] Nevertheless, the oral delivery of proteins and vaccines faces major physiological barriers such as the acidic environment in the stomach and the enzymatic environment throughout the intestine, as well as formulation challenges such as stability and targeting issues.

Despite these challenges, significant levels of success are being witnessed in the veterinary field for oral delivery of *Mycobacterium pneumonia* vaccines.[3] Most biologics or vaccines generally consist of a large molecule consisting of a peptide, protein, or a conjugate. Unlike the delivery of small molecules, these products have special considerations that make it a challenge to deliver them to the site of action or its receptor. The most important consideration is the stability of the active protein or peptide in the formulation and maintaining its basic structure and its tertiary structure to obtain optimal effect. The ideal delivery system would protect the protein, peptide, or antigen from the physiological conditions encountered during

administration and allow it to retain its structure (both primary and tertiary) until it reaches its site of action.

In an attempt to deliver biologics and vaccines orally, numerous strategies have been carried out by scientists around the world using various vehicles such as microparticles, liposomes, virus-like particles (VLPs), lectins, and immune stimulating complexes (ISCOMs).[4] In this chapter, we discuss various nonparenteral routes that have been employed for the delivery of these products. Nonparenteral routes offer ease of administration of the biologic formulation in nonprofessional settings and promise greater patient compliance.

FORMULATION ASPECTS OF BIOLOGICS AND VACCINES

Protein Structure

The formulation of biologics including proteins is difficult without an understanding of their structures and physicochemical properties. Proteins and peptides are formed by binding of various amino acids. There are 20 naturally occurring amino acids, and they differ only in their side chains. Proteins contain more than 50 amino acids, whereas peptides contain fewer than 20 amino acids. The four levels of protein structures are primary, secondary, tertiary, and quaternary. The primary structure includes the sequence of covalently bonded amino acids, and it is dictated by the sequence of deoxyribonucleic acid (DNA). Secondary structures consist of α-helixes, β-sheets, random coils, β-bends, and small loops of the polypeptide chain. The tertiary structure of proteins includes the overall packing in space of various elements of secondary structures, and quaternary structures represent the specific associations of separate protein chains that form a well-defined structure.[5] The primary structure of protein dictates its folding process. Generally, the overall shape is spherical with polar groups on the surface and hydrophobic groups buried in the interior. The fold structure of proteins is stabilized by both noncovalent and covalent forces. These forces include disulfide bridges between cysteine residues, hydrogen bonding, salt bridges between ionic groups, and hydrophobic interactions between side chains of amino acid residues.

Formulation of Biologics

It is evident from the above discussion that proteins have very intricate structures, which make the formulation of proteins a challenging task. In order to successfully formulate a protein, its stability and bioactivity must be maintained over its shelf life (1.5 to 2 years) and until it reaches the intended target. Any subtle changes in the secondary, tertiary, or quaternary structure will lead to physical instabilities such as denaturation, aggregation, precipitation, and adsorption. In addition, hydrolysis, deamidation, oxidation, disulfide exchange, β-elimination, and racemization will lead to chemical instability of proteins. Hence, all of these degradation pathways need to be avoided during the formulation process, and a strategy to minimize the probability of their induction should be in place. These strategies include reducing thermal stress by using the lowest temperature possible while spray drying and storing the protein at 4°C at pH 5.0 to 7.0. Harsh chemicals such as strong acids, bases, and organic solvents will disrupt the higher order structure of proteins, so their use should

be avoided or minimized during the formulation process. Aqueous systems should be used rather than organic solvents. Additionally, any energy input such as shaking, vortexing, sonication, temperature increase, radiation, or ultrasound or changes in pH, ionic strength, salt concentration, buffer type, and solvent composition should be minimized to prevent the development of aggregation and eventually other degradation pathways.

Sluzky et al.[6] found significantly higher aggregations of insulin solutions in vials with overhead space and with the addition of Teflon® beads compared to aggregations of insulin in fully filled vials. This is due to the creation of air–water interphase in the partially filled vial, and Teflon beads create hydrophobic surfaces that induce aggregation.[6] Also, it has been demonstrated that vortexing causes aggregation of human growth hormone.[2] Another stress that proteins undergo during the manufacturing process is lyophilization. In lyophilization, the proteins will be exposed to moisture and elevated temperatures. Optimal moisture content actually is essential for protein stability; however, when moisture content increases above the monolayer level, other reactants at the vicinity will be mobilized, leading to aggregation, especially at elevated temperature during lyophilization.[2] For this reason, cryoprotectants such as trehalose and sucrose and lyoprotectants such as polyethylene glycol (PEG) are commonly used to stabilize the protein during lyophilization. Cryoprotectants are preferentially excluded from the protein, whereas lyoprotectants act as a water substitute and hence stabilize the folded structure of proteins. Surfactants such as Polysorbate 20, 40, 60, and 80 stabilize protein by preferential adsorption at the interphase, whereas preservatives such as phenol, *m*-cresol, chlorobutanol, and parabens reduce microbial contamination.

Precaution should also be taken in the selection of vials and any container for proteins because untreated surface will induce adsorption of the proteins. Siliconized and type I glass vials should be used in preference to untreated plastic vials to avoid the leaching of chemicals from the wall of the vials and induction of adsorption.

Microparticles

Among the many vehicles for successful delivery of biologics are microparticles. Microparticles are bead-like particles with a diameter greater than 1.0 µm, and they are mostly spherical in shape. Microparticulate delivery systems have been used for a wide range of drugs, including neoplastic agents, vaccines, inactivated bacteria, proteins, and peptides.[7–9]

The matrix of microparticles can be categorized into two classes; monolithic and reservoir. In the monolithic matrix the active agent is dispersed homogeneously within the polymer matrix, whereas in the reservoir matrix the drug is surrounded by the polymer matrix in the mononuclear or polynuclear state (core). The monolithic matrix has the advantage of avoiding the risk of dose dumping due to the rupture of the membrane of the reservoir matrix. Drug release from a monolithic matrix may depend on the solubility of the drug in the matrix, as well as on the porosity and tortuosity of the polymer network.[10]

The microparticulate delivery system offers the ability and flexibility to allow it to be formulated into sustained or controlled-release systems with organ or tissue targeting capability. Microparticles have been shown to selectively target drugs to an organ or diseased site.[11,12] Microparticles also possess the ability to sustain or control the release of various drugs.[13] Additionally, microparticles act as immunological adjuvants in light of the fact that they are particulate in nature.[14] Furthermore, stealth microparticles can be produced with the addition

of polyethylene glycol (PEG), thus enabling the chemical attachment of PEG chains to a broad range of substances. This pegylation process increases the half-life of circulation, improves drug solubility and stability, and reduces immunogenicity.[8]

Conventional chemotherapy in cancer treatment has been widely used in the clinic but it is associated with significant systemic toxicity and the bigger problem of reoccurrence of the tumor. Immunotherapies have been recently developed to boost the immune system to detect cancer and prevent its reoccurrence, thus improving the patient's quality of life. This chapter talks about a few immunological strategies and cancer vaccines that have been researched and could be potential therapies in the future.

We will first discuss the various nonparenteral routes of administration of these therapies, the associated immunological structures in each of these routes, and the mechanistic pathways by which they act. Later in the chapter we provide specific examples.

ROUTES FOR ADMINISTRATION OF BIOLOGICS, VACCINES, AND CANCER THERAPIES

Oral Route

The oral route of administration is an attractive mode of immunization because of its ease of administration, low manufacturing cost, and high patient compliance. Intestinal Peyer's patches are the predominant sites for oral administration.[15] In the case of particles, the uptake depends on various factors such as size, charge, and hydrophobicity.[16,17] For oral delivery, it has been reported that particles of size less than 5 µm with positive charge and a hydrophobic nature can preferentially enter the Peyer's patch of the small intestine.[18] Orally delivered vaccines, especially particulate antigens, are recognized and sampled by microfold (M) cells in Peyer's patches. This is followed by transport of the particles to underlying follicles and to professional antigen-presenting cells (APCs), such as dendritic cells and macrophages. These APCs can phagocytose the particles, process them, and present them on both major histocompatibility complex (MHC) class I (through cross priming) and MHC class II molecules; as a result, both T and B cells can be triggered, as shown in Figure 5.1.[19,20]
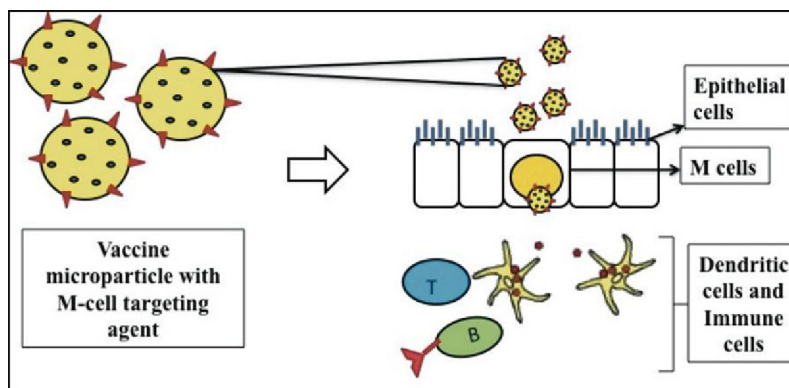
FIGURE 5.1   Schematic of vaccine microparticle uptake by microfold (M) cells of Peyer's patches in small intestine.

The major hurdle in oral delivery is protecting the active biological entity from acidic and enzymatic degradation in the gastrointestinal tract. Another obstacle to be considered while designing an oral vaccine or biologic is the probability of oral tolerance.[21] Low particle uptake and gastric degradation products of antigens can cause oral tolerance. One of the ways to avoid these issues is to formulate microparticles with enteric coating polymers.

Various studies have been reported describing the use of enteric coating polymers such as Eudragit® L 100, S 100, and L 100-55; cellulose acetate phthalate; and hydroxylpropyl methylcellulose phthalate/acetate succinate as polymers for particulate delivery vehicles.[22] These polymers are soluble at pH 5.5 and above and thus can render protection to antigens or active in gastric media. Oral delivery of vaccine antigens or biologics using such polymeric microparticles offers remarkable advantages over others such as induction of mucosal as well as systemic immune response, protection of antigen from gastric degradation, prolonged presentation of antigen to the immune system, and obviation of the need of vaccine adjuvants because microparticles themselves can act as self-adjuvants.[3,14]

Strategically designed particulate delivery systems incorporate enteric polymers to protect the biological entity from harsh gastric conditions and to target ligands to enhance its uptake from M cells of the Peyer's patches in the small intestine. M cells, or microfold cells, act as sampling ports for any foreign entities encountered in the small intestine. These M cells house various dendritic cells and immune cells. For oral vaccines, particles are sampled by M cells, processed by dendritic cells or antigen-presenting cells, and presented on MHC class I or class II molecules. The antigens are further recognized by the immune cells in the vicinity, leading to the cascade of an immune response as shown in Figure 5.1. The immune response also includes a humoral response by plasma B cells that leads to production of antibodies and their class switching. The role of B cells has been debatable in the past, but a recent study by Mahmoud et al.[23] showed that humoral immunity is important in addition to cell-mediated immunity in the prognosis of cancer. Thus, this route can trigger both humoral and cell-mediated immune responses to various cancer vaccines or biologics. The use of microparticles for cancer therapies and the delivery of biologics via the oral route are discussed further later in this chapter.

Buccal Route

The oral route of drug delivery is the most preferred route by both the patient and clinician; however, there are drawbacks associated with it such as hepatic first-pass metabolism and enzymatic degradation in the gastrointestinal tract. For these reasons, other mucosal sites are being considered as alternatives to the oral route. These alternative mucosal routes of drug delivery (e.g., nasal, rectal, vaginal, ocular, buccal, sublingual) offer advantages over the peroral route with respect to bypassing first-pass metabolism and enzymatic degradation in the gastrointestinal tract.

The nasal route of drug administration has been studied[24–29] and has reached commercial status with drugs such as luteinizing hormone-releasing hormone (LHRH)[30,31] and calcitonin.[32–34] However, disadvantages such as irritation to nasal mucosa and damage to the ciliary action of the nasal cavity due to chronic use of nasal dosage forms limit the use of these drug delivery systems. Large intra- and intersubject variability can also affect drug absorption from this site. Similarly, the rectal, vaginal, and ocular sites offer advantages over the oral

route but at the same time have poor patient compliance. The oral cavity, on the other hand, is much more accepted by patients; it is permeable with a rich blood supply and is robust, and it recovers quickly after stress or damage. Buccal or sublingual delivery can help to avoid first-pass metabolism and presystemic elimination in the gastrointestinal tract.

Overview of Oral Mucosa

The oral mucosa consists of an outermost layer of stratified squamous epithelium below which lies a basement membrane and a lamina propria (Figure 5.2). This is similar to stratified squamous epithelia found elsewhere in the body as it has a basal cell layer and a number of differentiating intermediate layers. The epithelial layers increase in size and become flatter as they travel from basal to superficial layers.[3]

The turnover time for the buccal epithelium has been estimated at five to six days,[35] which is probably representative of the oral mucosa as a whole. The oral mucosal thickness varies depending on the site. The buccal mucosa measures at 500 to 800 µm, whereas the mucosal thickness of the hard and soft palates, the floor of the mouth, the ventral tongue, and the gingivae measures at about 100 to 200 µm. The composition of the epithelium also varies depending on the site in the oral cavity. The mucosae of areas subject to mechanical stress (the gingivae and hard palate) are keratinized similar to the epidermis. The mucosae of the soft palate, sublingual, and buccal regions, however, are not keratinized.[35] The keratinized epithelia are relatively impermeable to water. In contrast, non-keratinized epithelia, such as
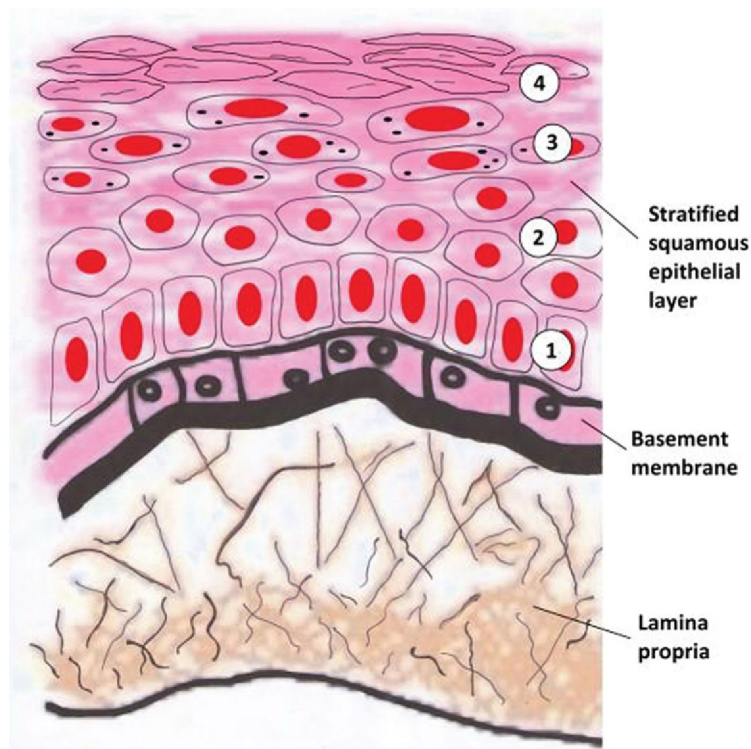

FIGURE 5.2 Structure of oral mucosa.

the floor of the mouth and the buccal epithelia, do not contain acylceramides and only have small amounts of ceramide.[36,37] They also contain small amounts of neutral but polar lipids, mainly cholesterol sulfate and glucosyl ceramides. These epithelia have been found to be considerably more permeable to water than keratinized epithelia.[35–37]

Immunology of the Buccal Cavity

It is important for a vaccine against bacterial or viral pathogens to elicit mucosal immunity, as these pathogens primarily attack the mucosal surfaces of the body. Also, the administration of a vaccine by any of the mucosal sites—oral, nasal, vaginal, or rectal—has proven to be effective and patient compliant. However, there is still a lack of vaccines that can be delivered by these routes, probably due to the challenges associated with development of an effective and stable vaccine that can be delivered by these routes. One of the mucosal routes of drug delivery that has been largely neglected for vaccine delivery is the buccal route

The mucosal surface of the oral cavity can be an ideal site for vaccination because of its easy accessibility and good antigen presentation. The buccal cavity is rich with dendritic cells similar to Langerhans cells which are a type of antigen-presenting cell. Also, a high density of T lymphocytes and mucosal-associated lymphoid tissue such as tonsils, salivary glands, Waldeyer's ring, and pharyngeal lymphoid tissue is present in the buccal mucosa. Hence, buccal immunization can help to elicit both mucosal and systemic immunity.[38,39]

Etchart et al.[40] reported that immunization with DNA injected into the buccal mucosa of mice induced a measles virus hemagglutinin-specific cytotoxic lymphocyte response in the spleen. It was also seen by Lundholm et al.[41] that pDNA administered to the oral cheek of mice using a jet injection induced production of immunoglobulin A (IgA), signifying a mucosal immune response. Wang et al.[42] reported that mucosal delivery of a melanoma vaccine in a hamster model helped treat oral melanoma and distant skin lesions. All of these studies demonstrated that buccal immunization is possible and can be very effective at the same time.

After having established the efficacy of buccal vaccination, the next step is to develop a robust and effective delivery system. Only liquid formulations containing the vaccine and adjuvant have been studied in animals so far. Hence, there is a need to develop a delivery system with prolonged residence on the buccal mucosa coupled with retention of the efficacy and stability of the vaccine antigen and adjuvant.

Pulmonary Route

Pulmonary delivery has been around for a long time and has its origins in the early civilizations; however, it has become increasingly popular and is being increasingly explored as an alternative means for the systemic delivery of drugs, including a large number of proteins and peptides. Since the latter part of the 20th century, pulmonary delivery has been used for the local delivery of drugs for diseases such as asthma and chronic obstructive pulmonary disease (COPD), but more recently it is also being considered as a viable alternative for the systemic delivery of both small and large molecules.[43]

Biologics are most certainly replacing conventional small molecule drugs as the therapy for the future. Their potency and specificity give them an edge, making them more popular with pharmaceutical and biotechnological industries. However, there are challenges associated with their delivery because of their inherent properties such as high molecular weight,

hydrophilicity, and instability, which is why they are predominantly administered by parenteral routes so as to ensure their direct entry into systemic circulation. In order to improve their patient compliance, many non-invasive routes of delivery are being explored, and pulmonary delivery is one of them.[44]

Pulmonary delivery has emerged as a promising alternative because of the unique anatomical features of the lungs. The lungs provide a large absorptive surface area (up to 100 m^2), an extremely thin (0.1 to 0.2 μm) absorptive mucosal membrane, and excellent vascularization, but it is challenging to incorporate the drug in a formulation that will deliver it to such a depth in the lungs that will permit permeation of the drug into blood circulation. The respiratory system is equipped with a series of filters designed to protect the lungs from undesirable environmental material and to keep the lungs clean. This factor must be taken into consideration for a particular therapy to be sufficiently effective. Low efficiency and a large variability have been the major factors that have prevented this route of administration from gaining complete acceptance. Some of the factors that affect the deposition of the drug in the alveolar regions of the lung are uptake by alveolar macrophages and proteolytic degradation.

A number of formulation parameters are unique to pulmonary delivery, such as the aerodynamic particle behavior (particle size, density, hygroscopicity, shape, and surface charge), breathing pattern of the patient (flow rate, ventilation volume), airway anatomy, and morphometry of the patient. Irrespective of the type of drug being delivered (small molecule or large molecule), the above factors have to be considered when preparing a formulation for pulmonary delivery.[45]

Metered dose inhalers (MDIs) and dry powder inhalers (DPIs) have been traditionally used for the delivery of measured doses to the lung and are commonly used to deliver drugs to the lung for the treatment of diseases such as asthma or COPD. Another commercially available system, the AERx® Pulmonary Delivery System by Aradigm uses a bolus of aerosol particles that can be delivered at a certain time during inspiration. The bolus is produced by a piston that empties a small liquid reservoir into the inhalation air. Other devices, including the AKITA® inhalation system (Activaero) and the ProDose™ system (Profile Therapeutics), also use the bolus inhalation technique to deposit particles in the lungs. The bolus delivery technique can help to increase particle deposition in the lungs. Nectar Therapeutics, along with Pfizer, came up with Exubera® for the inhalable delivery of insulin for the treatment of diabetes. This form of insulin was expected to replace all forms of injected insulin but the product lasted on the market for only little over a year after being introduced in September 2006. It was soon recalled, as it was cost ineffective and provided the same efficacy as injected insulin. Since then, a number of other companies (e.g., MannKind Corporation, Aerogen, Dance Biopharm) have been attempting to develop a form of inhalable insulin that is less expensive to produce than Exubera.

Besides insulin, other proteins or peptides whose delivery is being attempted by the pulmonary route are hormones (e.g., insulin, calcitonin, growth hormone, somatostatin, thyroid-stimulating hormone, follicle-stimulating hormone), growth factors (e.g., granulocyte–monocyte colony-stimulating factor, granulocyte colony-stimulating factor), various interleukins, and heparin. Many of the problems associated with the stability and absorption of macromolecules are now being resolved, and these products are currently showing promise in clinical studies. This proves that, in spite of the large number of challenges that pulmonary delivery of biologics faced at the start, progress has been made in leaps and bounds, thus proving its potential.[46]

Transdermal Route

The concept of immunization evolves with knowledge of the working of the body's defense system. Vaccines are traditionally made by using live, attenuated or fragments of pathogens. The transdermal route has been explored to deliver vaccines non-invasively through the skin, thus offering a number of benefits of needle-free delivery.

Transdermal vaccine delivery (TVD) is achieved by directly applying the vaccine antigen topically. The antigen-presenting cells in the epidermis and dermis, better known as Langerhans cells, interact with the antigen to initiate an adaptive immune response by migrating from the skin to the draining lymph nodes, where they activate naïve T cells and B cells.[47] This technology, however, is limited due to the fact that vaccines are large-molecular-weight antigens which, in many instances, find it difficult to cross the outer barrier of the skin: the stratum corneum. Moreover, topically applied macromolecules may get lodged in the hair follicles and sweat ducts, preventing the vaccine from reaching an effective concentration in the skin.

Numerous methods have been successfully used to bypass the stratum corneum such as chemical enhancers, electroporation, ultrasound, iontophoresis, and the use of microneedles. These methods have the potential to increase skin permeability to a large number of therapeutic substances including macromolecules such as insulin and vaccines.[48] Such methods increase transdermal transport through the use of (1) chemical enhancers, which increase drug solubility; (2) increased diffusion coefficients (chemical enhancers, ultrasound, electroporation); and (3) external driving forces (ultrasound, iontophoresis, electroporation).[49] The stratum corneum is the outermost layer of the skin, with a thickness of 10 to 20 μm; it protects against microbes, fluids, and foreign materials. It is also the most significant barrier for transdermal delivery of many compounds, including vaccines.[50]

Much research has been carried out in the field of transdermal vaccine delivery in recent years. Bhowmik et al.[51] formulated a microparticulate whole cell lysate vaccine composed of melanoma cell line antigens, in a biodegradable matrix of bovine serum albumin (BSA) and other sustained-release polymers. The particles ranged in size from 0.165 to 1.5 μm. This formulation was delivered transdermally by creating microchannels in the skin of four- to six-week-old DBA-2/J mice, using the DermaRoller®, a device that uses microneedle technology. *In vivo* studies showed that the vaccine produced an increase in immune response. A significant increase in the IgG antibody titers was observed which may have been the result of uptake and presentation of antigen by the Langerhans cells in the skin. Also, the vaccinated mice showed no palpable tumor even when observed for 35 days after tumor induction. Thus, transdermal microparticle delivery of vaccines opens up new avenues for vaccine administration with the use of rather non-invasive methodologies that require further modifications and testing in a clinical setting.

Ongoing research is exploring drug delivery systems such as organic nanoparticles and hydrogels, polymer-based particles, solid–lipid nanoparticles, and liposomes, as well as other lipid-based vesicles for transcutaneous vaccine delivery.[48] Nanoparticles were shown to be a promising carrier for transcutaneous vaccine. These are advantageous because their small size allows them to penetrate through the hair follicles and cause the vaccine antigens to target the antigen-presenting cells and augment the immune response. Additionally, their interaction with skin lipids results in the generation of transient and reversible openings of the

stratum corneum.[52] Polymers such as polylactic acid, polylactic-*co*-glycolic acid, and chitosan have shown promise in transdermal immunization by eliciting strong immune responses.[53,54] Liposomes are other exciting carrier system for delivery of vaccines via the transdermal route. The lipid-based vesicles of liposomes, especially elastic liposome, may change the bioactive permeation kinetics due to an impaired barrier function of the stratum corneum, helpful for the skin penetration.[55] Liposomes are generally composed of phosphatidylcholine and a surfactant and contain a lipid bilayer enveloping an aqueous compartment, which has a large capacity for loading drugs or vaccines. Mishra et al.[56] have demonstrated enhanced immunity against antigen using elastic liposomes loaded with hepatitis B surface antigen (HBsAg).

Research in the area of developing novel nanosized formulations may be helpful with regard to stabilizing the antigen and protecting it from disrupting lipids in the stratum corneum. These properties are of primary importance for the delivery of vaccines through the skin. However, the development of such novel nanoscale systems is limited by their low efficiency in eliciting a robust immune response. Clinical trials for transcutaneous immunization usually rely on microneedles or needle-free skin patches. Research and clinical trials are concentrated on transdermal vaccines for infectious diseases such as influenza, with cancer immunization taking a back seat. The development of future vaccine delivery strategies requires further investigation into the transdermal route because research work published thus far has shown great promise.

CHEMOTHERAPY VERSUS IMMUNOTHERAPY

Chemotherapy

Chemotherapy, the most common treatment for cancer, uses anticancer drugs to kill cancerous tissue. In chemotherapy, a single drug or a combination of drugs can be used for a particular cancer or along with treatments such as surgery and radiation. Chemotherapy may be used to achieve total remission, prevent reoccurrence of the cancer, or slow down metastasis.

Because chemotherapeutic drugs are generally given orally or as injections, not only could these drugs exert their cytotoxic effect on the cancer cells and the healthy cells around the cancer, but they could also reach other parts of the body and cause mild to devastating side effects. Side effects of chemotherapeutic drugs can range from mild symptoms such as fatigue and loss of appetite to severe effects such as vomiting or neutropenia, leading to a poor quality of life. Moreover, drugs administered by the intravenous route could cause such serious effects as extravasation. Although much progress has been made in alleviating the side effects caused by chemotherapy, there is a need for improved therapies that are less damaging to the patient.

Immunotherapy

Cancer immunotherapy is designed to modify the host's immune system or utilize the components of the immune system as cancer treatment. Immunotherapy may be preferred over the traditional chemotherapy because the treatment offers less severe side effects and

sometimes may be targeted therapy. Immunological products that have received regulatory approval are either single anticancer agents or in combination with chemotherapy. These products include cytokines such as interferon-α and interleukin-2; the monoclonal antibodies trastuzumab, bevacizumab, and ipilimumab; and others such as the anticancer cell-based therapy sipuleucel-T.

When cell-mediated immune response is enhanced against tumor cells it offers various advantages over targeted therapies, particularly the generation of a long-term-memory lymphocyte population patrolling the body to attack metastases before they become visible by traditional imaging modalities.[57]

A significant interest in the development of therapeutic cancer vaccines over the last decade has led to an improvement in overall survival of cancer patients in several clinical trials. As a result, two active immunotherapy agents, sipuleucel-T and ipilimumab, have been approved by the U.S. Food and Drug Administration (FDA) for the treatment of prostate cancer and melanoma, respectively. GVAX® cellular vaccine (Cell Genesysis, Inc.) is another active immunotherapy agent that targets prostate cancer and has been well studied in various clinical trials. van den Eertwegh et al.[58] studied the combination of two active immunotherapy approaches (GVAX and ipilimumab) for the treatment of metastatic castration-resistant prostate cancer. Whereas GVAX is designed to amplify the antitumor response specific to prostate cancer cells, ipilimumab contributes to T-cell activation. Thus, the authors presented the possibility of augmenting antitumor T-cell activity in two different ways.

The immunotherapeutic agent ipilimumab has helped address a significant unmet need in the treatment of advanced melanoma. Ipilimumab is a fully human monoclonal antibody that targets cytotoxic T-lymphocyte antigen-4 (CTLA-4), thereby augmenting antitumor immune responses. Ipilimumab (at a dose of 10 mg per kilogram) in combination with dacarbazine, as compared with dacarbazine plus placebo, improved overall survival in patients with previously untreated metastatic melanoma. Ipilimumab was recently approved by the FDA for the treatment of metastatic melanoma.[59]

In a pivotal Phase III trial, treatment with sipuleucel-T (provenge, dendreon), an autologous cellular vaccine consisting of activated antigen-presenting cells loaded with prostatic acid phosphatase (PAP), gave a median overall survival of 25.8 months compared with 21.7 months for placebo-treated patients, resulting in a 22% relative reduction in the risk of death.[60]

Classification of Cancer Immunotherapy

Cancer immunotherapy can be classified as two types: active and passive. Active immunotherapies that can stimulate the immune system can be further subdivided into specific immunotherapy including prophylactic or therapeutic vaccines. Nonspecific immunotherapy consists of components of the immune system such as cytokines and immune adjuvants. Passive immunotherapies are not used to boost the immune system. The passive transfers of monoclonal antibodies can increase antitumor immunity by either inhibition of immune check points or augmentation of stimulatory signals. Adoptive cell therapy involves infusion of tumor-reactive cells that are autologous tumor-infiltrating lymphocytes, T-cell receptor gene-modified lymphocytes, or chimeric antigen receptors that are recombinant receptors providing antigen-binding and T-cell activation functions.[61]

Vaccines and Active Specific Immunotherapy

Prevention or treatment with a cancer vaccine, or active specific immunotherapy, is a very attractive therapeutic option because the mechanism of action is eventually an enhanced endogenous immune response against the host's malignancy.

CURRENT IMMUNE THERAPIES

Monoclonal Antibodies

Antibodies provide protection to the immune system from microorganisms, and therapy with monoclonal antibodies targeted specifically to tumors has proven to be one of the successful forms of immune therapy in cancer. Antibodies exert their role by binding to their targets, through several effector mechanisms, including steric inhibition and neutralization, complement activation, and activation of cell-mediated cytotoxicity.

Monoclonal antibody therapy is relatively nontoxic with lesser side effects than chemotherapy when the antibodies are bound to the tumor cells.[62–64] Nine monoclonal antibodies, targeting six tumor-associated proteins, are clinically approved for the treatment of cancer. For example, rituximab, the most widely used monoclonal antibody, is now used in combination with cyclophosphamide, doxorubicin, vincristine, and prednisone for non-Hodgkin's lymphoma and has shown complete remission in patients compared with chemotherapy alone.[65–66] Monoclonal antibodies (ibritumomab tiuxetan and 131I tositumomab) have been used to target tumors directly by conjugating them to radioactive isotypes or toxic chemicals as in the case of gemcitumab.[65,67]

Cytokines

Cytokines, proteins secreted by the immune cells when stimulated by antigen, may be given systemically in certain cancers. IL-2 is a potent mediator in antiviral therapy and is used in advanced melanoma and renal cell carcinoma, both cancers that are resistant to chemotherapy alone.[68–70] Tumor necrosis factor α (TNF-α) is another cytokine used for the treatment of soft tissue carcinomas of the limb and melanoma.[71]

Cancer Vaccines

The purpose of cancer vaccines is to initiate an active immune response toward a tumor. There are several types of cancer vaccines in development: adenoviral, dendritic cell, tumor cell, adoptive T-cell transfer, and peptide.[72] Antigens from tumors and antigen-presenting cells may be used as potential vaccine approaches to enhance a preexisting antitumor immune response, or, perhaps in some cases, to induce an antitumor immune response that did not exist earlier. There are many potential sources of tumor antigens, including purified or synthesized tumor cell surface molecules, which may be peptides or proteins, cells or lysates derived from fresh or cryopreserved autologous tumor samples (actually a mixture of normal and malignant cells), and cells or lysates of allogeneic or autologous tumor cell lines. There are a variety of methods by which tumor antigens can be presented including as purified antigen, via heat

shock proteins, in viruses or DNA, by antigen-presenting cells (APCs) such as dendritic cells, or as the idiotypes of monoclonal antibodies (mAbs) that have been selected by their tumor antigen recognition. There are numerous molecules that might be useful as adjuvants to enhance the immunogenicity of a vaccine. Cancer vaccines may be given by different routes: subcutaneous, intradermal, intramuscular, or intravenous.

Although the oral route is the most acceptable route of administration among patients, the formulation of an oral vaccine faces a number of challenges. Still, many researchers are focused on the aspect of formulating vaccines using novel delivery systems such as microparticles and nanoparticles. This chapter touches upon some of the research carried out in our laboratory on oral microparticulate vaccine or biologics delivery. It had long been assumed that if cancer vaccines could elicit a strong enough immune response they could overcome tumor-induced immune suppression, but after poor clinical results for so many promising vaccines it is now being realized that immunogenicity is not enough. In addition to a strong vaccine, tumor-induced immunosuppression must be actively reduced, and this may be achieved through combination with the arsenal of chemotherapy agents already in use.[73] In a Phase III trial for sipileucel-T (Provenge®, Dendreon Corporation), the first autologous vaccine consisted of activated dendritic cells comprised of granulocyte–macrophage colony-stimulating factor (GM-CSF) and prostatic acid phosphatase (PAP) approved by the FDA for treatment of advanced prostate cancer. This vaccine included a large proportion of antigen-presenting cells, which were infused back into the patient to stimulate antitumor T-cell responses.[74] The results showed higher median overall survival compared to placebo-treated patients, resulting in a 22% relative reduction in the risk of death, but it failed to show benefit in progression-free survival, and tumor regressions were rare.[60,61] This is an example of a personalized vaccine.

COMBINATION OF IMMUNOTHERAPY AND CHEMOTHERAPY

Chemotherapies can help improve the efficacy of cancer vaccines by exerting various effects on the immune system; however, there are several pitfalls to consider. Chemotherapeutic regimens are different for different cancers, stage of cancer, and patient characteristics. Adding cancer vaccines into the program introduces another layer of complexity. Indeed, several studies looking at vaccine chemotherapy combinations highlighted the fact that chemotherapies must be carefully dosed and delivered at particular times in relation to the vaccine for optimal effect.[75,76] Although substantial research has combined chemotherapies and vaccines in mouse models, information from human studies is rare.

Due to the active role the immune system plays in tumor clearance, it is likely that the benefits of cancer vaccines will be best observed in patients with early, untreated disease.[73] Gemcitabine, cyclophosphamide, and dacarbazine (or temozolomide, which is metabolized to dacarbazine *in vivo*[77]) in particular have been used. So far, no studies report the safety risks associate with chemotherapy and immunotherapy.

Recent clinical studies on combination treatments have proven chemotherapy given after vaccination is a better treatment strategy than chemotherapy pretreatment or simultaneous administration. A clinical study published by Antonia et al.[78] showed results that indicated

that patients with extensive stage small cell lung cancer were essentially more responsive to second-line chemotherapy treatment after vaccination with dendritic cells transduced p53 via adenoviral vector.

In a Phase II study, a personalized peptide vaccine when combined with the anticancer drug gemcitabine to treat advanced pancreatic cancer, produced a response rate of 67% for both cellular and humoral responses.[79]

MELITAC is a peptide cancer vaccine administered after cyclophosphamide in a resected stage IIB to IV melanoma Phase I/II study, which showed that cyclophosphamide provided no detectable improvement in CD4 or CD8 T-cell responses or in clinical outcome.[67]

In addition to chemotherapy, other studies have been attempted wherein the vaccine response was improved by inactivating Treg cells through the specific targeting of the T-cell CTLA-4 receptor with a monoclonal antibody such as ipilimumab (Yervoy®, Bristol-Myers Squibb). Preliminary clinical trials suggest that administering a therapeutic vaccine followed by ipilimumab enhances immune responses and tumor reduction in prostate and ovarian cancers as well as melanoma.[80]

Similarly, in the transgenic murine prostate cancer model, a tumor shrinkage effect was observed with combination therapy of whole cell GM-CSF-secreting vaccine (GVAX) and low-dose cyclophosphamide one to two days before immunization with vaccine alone. This effect was associated with a reduced Treg population in the tumor and its lymph nodes, as well as stimulation of dendritic cell activity.[81]

Apart from chemotherapy and vaccine combinations, novel antibodies targeting certain cancers may also be used in conjunction with certain cancer vaccines and anticancer drugs. For example, anti-CTLA-4 antibody administered before a cell-based, GM-CSF-secreting vaccine (GVAX) showed a remarkable increase in the effector CD8 T-cell response.

Hence, it is possible to boost the therapeutic advantage of vaccines in combination with chemotherapy as seen in some of the cases mentioned above. However, research in this area many pose a problem as far as the chemotherapeutic aspect is concerned because the dose and the time of administration differ with different chemotherapeutic regimens for various cancers. Traditionally, patients in the last stages of cancer having limited therapeutic alternatives have been tested with experimental therapies. In these cases, such patients deteriorate with the disease and previous chemotherapies may be less likely to mount strong responses to a vaccine, thus they may not be ideal candidates.[80] Many questions remain unanswered and require further investigation. Many experimental strategies will have yet to be tried out to determine an effective combination that results in maximum therapeutic benefit with minimum side effects.

In our lab we have used particulate-based delivery systems in both the micrometer range and nanometer range delivered via the above discussed routes of administration. The particulate delivery system in general offers many advantages compared to traditional methods of drug delivery. Some of them include:

- Small and large molecules accommodated
- Multidrug therapy using one particle
- Stable delivery system for bioactive molecules
- Easy manufacturing and scale-up
- Elimination of cold-chain requirements.

MICROPARTICLE-BASED DELIVERY SYSTEMS FOR NONPARENTERAL DELIVERY OF BACTERIAL (MENINGOCOCCAL) VACCINES

Need for Micro/Nanoparticulate Meningococcal Vaccines

Neisseria meningitidis is a leading cause of bacterial meningitis and sepsis in young children and young adults in the United States and is associated with a high mortality rate.[82,83] Childhood vaccination has been shown to induce a herd immunity effect by reducing nasopharyngeal carriage.[84–86] The current available polysaccharide-based meningococcal vaccines are licensed for use in adolescents and adults but are expensive. Therefore, utilizing micro-/nanotechnology to explore novel meningococcal vaccine formulations that boost innate and adaptive immunity and offer protection is very important. Another important aspect to be noted is that micro-/nanoparticles could facilitate the oral, transdermal, nasal, or buccal delivery of vaccines. This could prove to be an incredible boon when dealing with epidemics in Sub-Saharan African countries where the need for trained medical personnel could be effectively avoided. Needle-free vaccination has gained importance when dealing with countries where human immunodeficiency virus (HIV) is a significant concern and has improved the safety of vaccination.

Microparticle Vaccines for Polysaccharide Based Meningococcal Antigens

Capsular polysaccharides (CPS) are a major virulence factor in meningococcal infections and form the basis for serogroup designation and protective vaccines. CPS is anchored in the outer membrane through a 1,2-diacylglycerol moiety[87] and functions to protect the bacteria from complement-mediated killing while also inhibiting phagocytosis by professional phagocytes.[88,89]

Ubale et al.[90] attempted to formulate a microparticulate meningococcal vaccine to serve as a sustained-release system administered by the oral/buccal route. The vaccine formulation consisted of meningococcal CPS polymers (Serogroup A) and/or adjuvant (kdtA) encapsulated in albumin-based biodegradable matrix microparticles that mimic the chemical conjugation process of CPS to a protein carrier, thus enhancing antigen uptake via albumin receptors and eliciting a T-cell-dependent immune response.[8,12]

The ability of the CPS-loaded microparticles to induce cytokine and chemokine release from macrophages was investigated in an *in vitro* cell culture model of THP-1 human macrophage-like cells. Dose-dependent release of TNF-α (Figure 5.3) from THP-1cells exposed to meningococcal CPS-loaded microparticles, but not empty microparticles, was observed. The cytokine release reflects the recognition and immunostimulatory activity of the polysaccharide antigen in the microparticle matrix. Taken together, the data suggested that CPS-loaded microparticles are recognized by macrophages and the encapsulation in BSA matrix did not hamper the immunostimulatory activity of CPS.

Autophagy, an ancient homeostasis mechanism for macromolecule degradation, recently has been recognized to play a role in host defense and antigen presentation.[91] It was seen that CPS-loaded microparticles but not the empty microparticles strongly induced autophagic vacuoles in a dose-dependent manner (Figure 5.4). The innate immune recognition of these
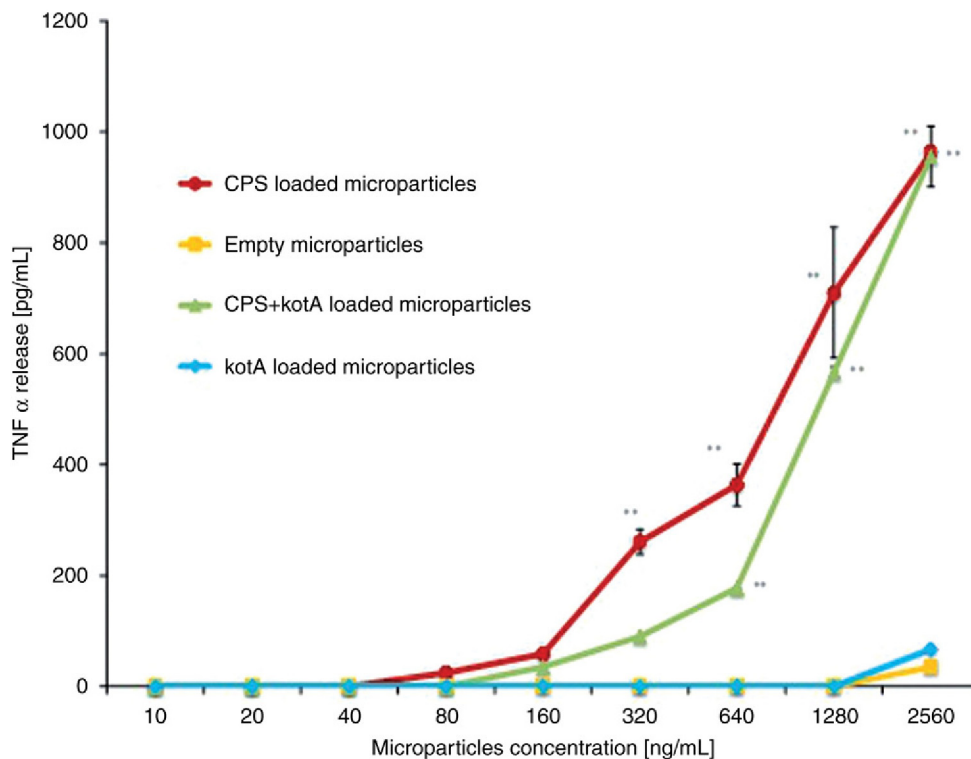
FIGURE 5.3   TNF-α release induced by microparticulate meningococcal vaccine from THP-1 cells.

vaccine-loaded microparticles is the first step and prerequisite for eliciting adaptive immune responses.

Conclusions

This case study briefly describes the use of micro-/nanoparticles as an effective alternative to the nonparenteral delivery of bacterial vaccines. This paves the way for more research and improvement in this field of vaccine delivery.

INFLUENZA VACCINE

Influenza viruses cause a highly contagious disease in the human respiratory tract, such as the nose, lungs, and throat. Symptoms of influenza infection are fever, sore throat, headache, cough, and fatigue.[92] During the influenza epidemic of 1918 the deadly virus killed approximately 50 million people globally.[93] In 2009, a new strain of influenza virus (H1N1) emerged and spread quickly throughout the world, causing the first influenza pandemic of the 21st century.
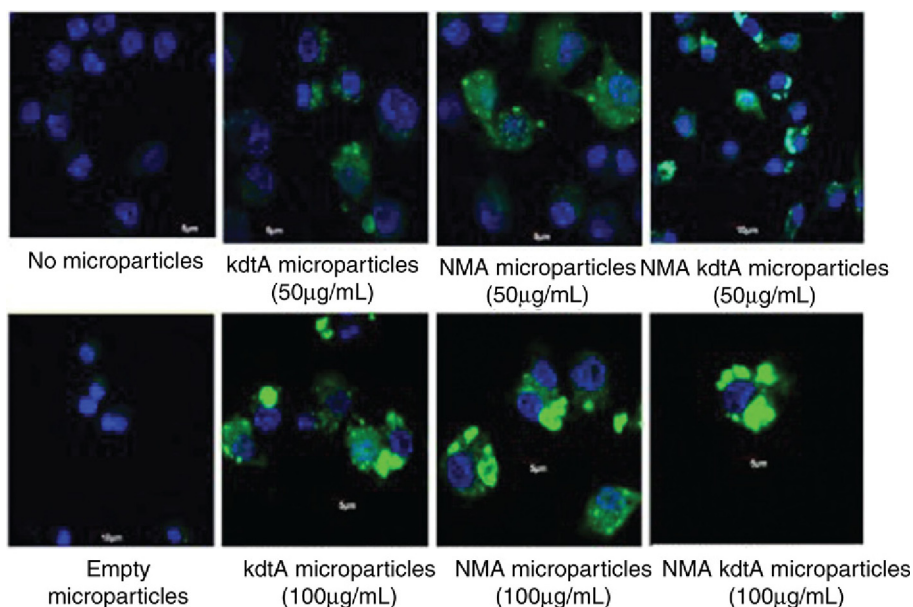
FIGURE 5.4   Autophagy induction by microparticulate meningococcal vaccine from RAW cells.

There are two types of influenza vaccines: the "flu shot" and the nasal-spray flu vaccine.[92] Flu shots contain inactivated influenza virus, while the nasal-spray flu vaccine (FluMist®) is made with live attenuated flu viruses. Oral administration is a potential route for vaccination because there is no need for trained medical personnel for administration, thus leading to increased coverage of vaccination, as seen in case of oral polio vaccination.[94] Development of a novel influenza oral vaccine will have a significant impact on improving public health.[95] In one case study, microparticulate vaccine was developed by using inactivated influenza (A/PR/34/8 H1N1) virus as an antigen, which was incorporated into a polymer matrix (Eudragit® S).[96] For *in vivo* testing, BALB/c mice were immunized with two doses within a three-week interval. Serum samples was analyzed and proved that microparticulate vaccine enhanced antigen-specific antibodies (Figure 5.5). Also, these mice were challenged with heterologous influenza viruses, and the results showed a significant induction level of protection via this technology.[96]

Another trend of developing influenza vaccine is to minimize the number of doses. One innovative approach is to broaden protective efficacy by using the extracellular domain of matrix protein 2 (M2e) antigen, which is common among influenza A viruses.[97] This group replaced the hyperimmunogenic region of *Salmonella enterica* serovar *typhimurium* flagellin (FliC) with four repeats of M2e (4.M2e-tFliC) and fused it to a membrane anchor from influenza virus hemagglutinin to establish a membrane-anchored fusion protein. The fusion protein was then integrated into influenza M1 virus-like particles. The *in vivo* studies showed a strong antigen-specific antibody in case of intramuscular and intranasal administrations.[97]
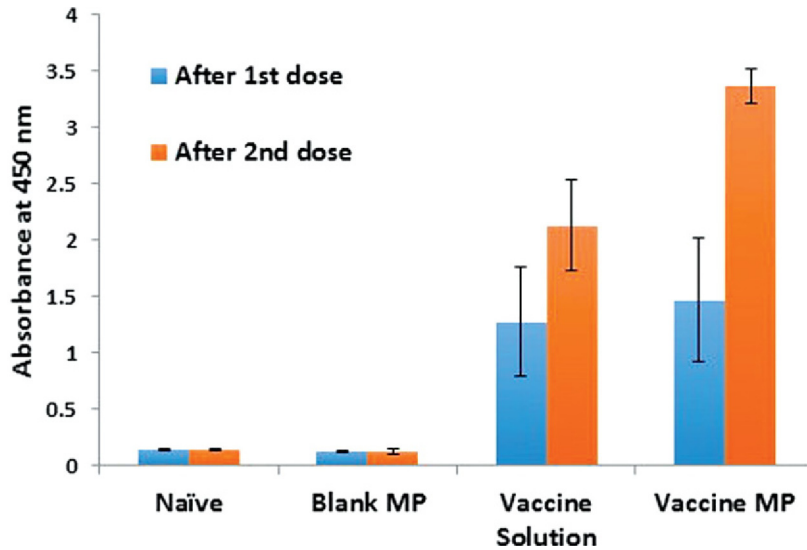
FIGURE 5.5 Serum IgG levels in BALB/c mice. Mice ($n = 6$) were vaccinated with prime followed by booster doses at 0 and 5th week, respectively. Serum samples (1:100 dilution) were analyzed at the third and seventh week. Statistical significance was calculated by two-way ANOVA comparing the results between different groups at different time points ($p < 0.0001$).

Developing influenza vaccine via the transdermal route has been investigated using microneedle technology. Influenza virus-like particles delivered through microchannels under skin showed an induction of protection against influenza infection and proved a long-tem protective efficacy.[98] In this study, influenza virus-specific total IgG and hemagglutination inhibition titers were maintained at high levels for over one year after vaccination. In addition, the levels of lung IgG and IgA antibody responses were higher compared to intramuscular administration.

Overall, many innovative technologies have been applied to develop enhanced influenza vaccines. Diverse antigens (inactivated viruses, live attenuated viruses, proteins and virus-like particles) have been studied along with various routes of administrations (transdermal, intranasal, intramuscular, and oral). These novel approaches would be promising influenza vaccines for better protection against influenza viruses and could substantially reduce the burden for public health in the future.

HUMAN PAPILLOMAVIRUS VACCINE

Human papillomavirus (HPV) is a sexually transmitted virus that causes genital warts and leads to cancer of the cervix, vulva, vagina, penis, oropharynx, and throat.[99] Approximately 600,000 women were diagnosed with cervical cancer in 2010, and that number could rise to 700,000 new cases annually by 2020 in the absence of intervention.[100] There are at least

100 distinct types of HPV, including more than 30 HPV types that can develop infection in humans. Therefore, HPV vaccination is strongly recommended for females and males, especially for girls ages 9 to 26.

Currently, two prophylactic HPV vaccines have been commercialized worldwide: the bivalent vaccines Cervarix® (GlaxoSmithKline) and Gardasil® (Merck).[101102] However, both of these vaccines have to be injected and are very expensive (approximately $450 for a course of three doses); consequently, they are not affordable for people from developing countries.

There are numerous novel approaches to HPV vaccination with regard to different routes of administration (intranasal, oral, or transdermal) and types of antigen (virus-liked particles, DNA, or proteins). In one study, a protein-based HPV prophylactic cancer vaccine was tested in C57BL/6 mice; it was administered intranasally with a combination of E6/E7 antigens and Toll-like receptor 5 (FlaB).[103] The results showed that this combination elicited a very strong antigen-specific cytotoxic T-lymphocyte activity and antigen-specific interferon production from spleenocytes and cervical lymph node cells.[103] Such a novel approach could prove to be a very effective prophylactic HPV vaccine in the future.

DNA-based vaccines are another excellent alternative for targeting HPV infection and cervical cancer. For example, pcDNA3.1-HPV16E7 recombinant vector was used as an antigen, which passed through microchannels under skin by using microneedle arrays.[104] In this study, after BALB/c mice were immunized with one prime followed by two boosters every two weeks, serum and lymphocytes were collected to detect the functions of humoral and cellular immunities. The results proved the DNA vaccine is a promising platform for transdermal delivery via microneedles to induce specific antibodies for *in vitro* and *in vivo* studies.[104]

Recently, a nine-valent vaccine in HPV (HPV 6/11/16/18/31/33/45/52/58) has been proposed for better protection against HPV infection and cervical cancer.[105] If the nine-valent vaccine induces immunity as strong as that of Gardasil® or Cervarix®, world incidence rates could be dramatically reduced.[105]

In summary, many novel approaches for alternatives of HPV vaccines have been proposed to improve efficacy and reduce costs. Various routes of administration have been tested along with different types of antigen including protein, peptide, DNA, and virus-liked particles. A variety of adjuvants was incorporated with antigen to stimulate both humoral and cellular immune responses against HPV infection and cervical cancer.

CANCER VACCINE CASE STUDIES

Prostate Cancer Vaccines

The prostate gland is part of the male reproductive system located below the bladder and in front of the rectum. The National Cancer Institute (NCI) reported approximately 238,000 cases of prostate cancer in 2013 alone. It is the second leading cause of cancer-related deaths in America and Europe.[106] The primary goal of cancer immunotherapy is destruction of tumors by the immune system. There are several strategies to elicit an effective antitumor response, including designing vaccines that target specific tumor epitopes and modulating the activity of regulatory T cells that dampen any underlying immune response.[107] Many prostate-specific antigens are known to be immunogenic; one such antigen is the prostate-specific antigen (PSA), which also serves as a marker for disease progression.

Oral Particulate Prostate Cancer Vaccines

Nanoparticles and microparticles have been at the forefront of drug delivery. In recent years, investigators have focused on using these platforms to improve several aspects of vaccine delivery. Particulate vaccines offer a distinct advantage over antigen solutions themselves. They protect the antigen from the *in vivo* environment, improve uptake of the antigen, reduce the number of doses for primary immunization, and can be used to target specific cell populations for enhanced efficacy.[108] Several studies in our laboratory have proved the efficacy of oral microparticulate systems to elicit immune response against tumors.[109–111] The mechanism of immune system activation by the oral route is explained in the earlier section of this chapter. Our laboratory has also explored several targeting ligands that target M cells in order to enhance site-specific uptake of microparticles. Microparticles are formulated using the spray-drying technology with enteric polymers that fall under the Generally Regarded as Safe (GRAS) category classified by the FDA. Preliminary studies in our laboratory evaluated the efficacy of a microparticulate whole cell lysate vaccine of TRAMP-C2 prostate cancer cells as a prophylactic cancer vaccine therapy. It was observed that tumor growth was significantly retarded in vaccinated mice compared to the control group.[111] Recently, Chiriva-Internati et al.[112] isolated and tested AKAP-4 as a target for prostate cancer-specific immunotherapy. Our lab is currently exploring the efficacy of therapeutic oral microparticulate vaccines for prostate cancer. Preliminary studies indicate that AKAP-4 along with a whole cell lysate microparticulate vaccine system could efficiently induce antitumor immune responses, leading to a significant reduction in tumor growth *in vivo*.

Transdermal Delivery of Particulate Prostate Cancer Vaccines

The use of transdermal drug delivery for the delivery of oligonucleotides, proteins, peptides, and inactivated viruses is growing steadily. Microneedles are micron-sized needles with lengths up to 1 μm. Microneedles pierce the upper layer of skin for local or systemic delivery of small molecule drugs or biologics.[113] Delivery of vaccines to skin has been established as a promising target to elicit an immune response.[114] Our laboratory studied the efficacy of whole cell lysate vaccine microparticles with murine prostate cancer cells (TRAMP C2) on C57BL/6 mice. Vaccination was performed by the transdermal route. Mice were challenged with live murine prostate cancer cells following immunization and tumor growth was monitored for 8 weeks. Results from the study proved that mice vaccinated by the transdermal route elicited delayed tumor growth that was significant compared to control groups (Figure 5.6). Mechanistic studies revealed an increase in CD4$^+$ T cells and B cells in vaccinated mice compared to control. Thus, it was observed that both innate and adaptive immune responses were activated following transdermal vaccination which delayed tumor growth for vaccinated mice.

Breast Cancer Vaccine

Breast cancer is one of the most common type of cancer in females all over the world. According to the Centers for Disease Control and Prevention (CDC), about 211,731 cases were reported in 2009 and about 40,676 women died because of breast cancer.[115] In the past decade, there has been exponential growth in the research being conducted on immunotherapy.
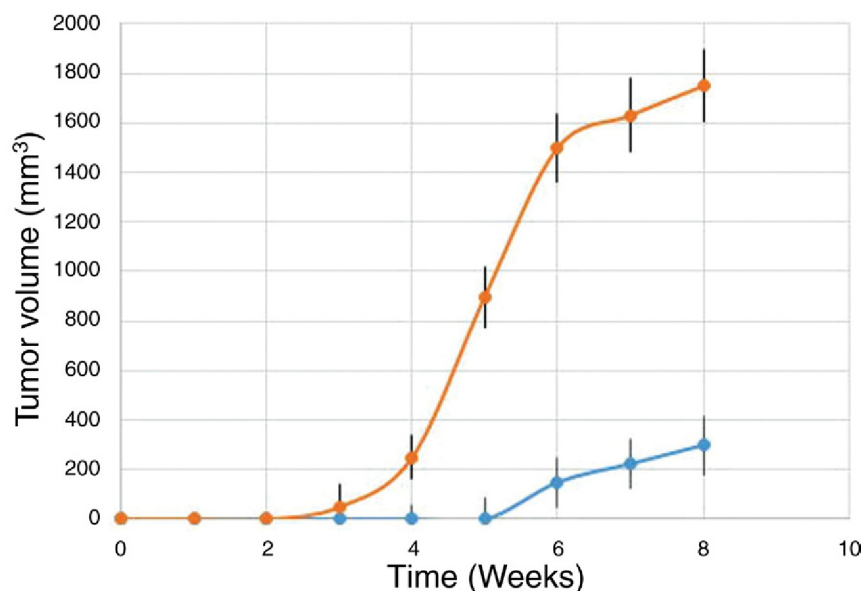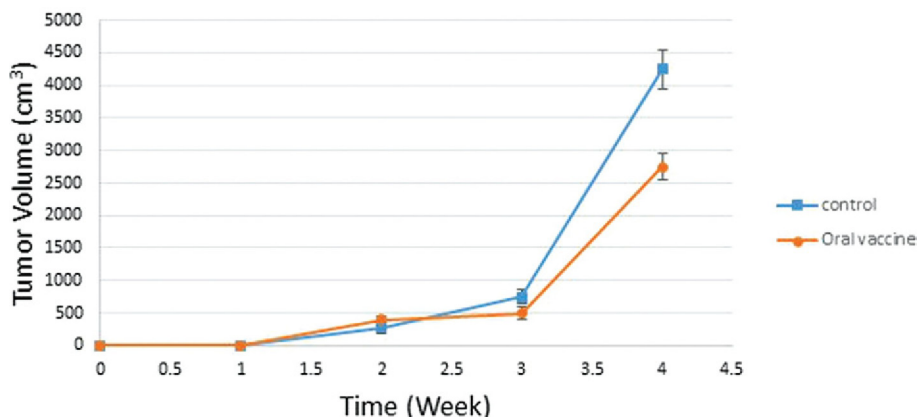
FIGURE 5.6 This graph compares the mean tumor volume of vaccinated group via the transdermal route and the naïve group following 10-week vaccination regimen. C57BL/6 mice were challenged with live 5×10^6 prostate cancer cells subcutaneously and the tumor growth was monitored for 8 weeks. Significant delay in tumor growth was observed in the vaccinated group compared to the naive ($p < 0.05$). Blue line, vaccinated group; orange line, control group.

Immunotherapy works by inducing or enhancing the body's immune system to identify and reject tumor. Several breast cancer antigens such as human epidermal growth factor receptor 2 (HER2), mucin 1 (MUC1), human telomerase reverse transcriptase (hTERT), tumor protein 53 (p53), and cancer embryonic antigen (CEA) have been used to prepare these vaccines where these tumor-associated antigens (TAAs) were represented by whole cell extracts/dendritic cells or DNA motifs.[116]

Oral and Transdermal Breast Cancer Vaccine

Whole cell vaccine was formulated into microparticles using enteric coating polymers. The microparticle formulation is optimized by using a different combination and concentration of polymers. These microparticles were administered both orally and transdermally. *In vitro* and *in vivo* studies were carried out to evaluate the humoral and cellular response against the vaccine loaded microparticles.

In vitro experiments showed that spray-dried microparticles provided protection against gastric conditions and controlled the release for about six hours. Vaccine-loaded particles were nontoxic to normal cells. The majority of the particles were in the size range of 1 to 5 μm. *In vivo* studies proved that both oral and transdermal groups were able to prevent and delay tumor growth compared to the control groups receiving blank microparticles. Flow cytometry results for the immune organs revealed that animals receiving the vaccine showed higher CD4+ T cells, CD4+, CD161+, and CD8+ levels than the control groups. A

FIGURE 5.7 This graph compares the mean tumor volume of vaccinated group via oral route and the control group following a 10-week vaccination regimen. BALB/c mice were challenged with live breast cancer cells subcutaneously and the tumor growth was monitored for four weeks. Significant delay in tumor growth was observed in the vaccinated group compared to the control group ($p < 0.05$).

higher expression of these markers shows that the vaccine particles were able to induce a better immune response to fight against the cancer cells. Efficacy of vaccine microparticles could be seen with the tumor volume data (Figure 5.7).[109] Therapeutic applications of vaccine microparticles are currently being evaluated. The therapeutic efficacy of these vaccine microparticles is reportedly enhanced when given in combination with cytotoxic drug and adjuvants.

Melanoma Cancer Vaccine

Melanoma is a dangerous form of skin cancer characterized by malignant proliferation of the melanocytes. It is one of the leading causes of death and its rate of occurrence in the world is progressively increasing as people are exposed to larger amounts of damaging ultraviolet rays due to the vanishing ozone layer. The early stages of melanoma are localized and treatable to a large extent, but with time the tumor metastasizes to the visceral organs, making it more difficult to treat.

In a recent study carried out by the American Cancer Society for melanoma located near where it started, the five-year survival rate is 98%. The five-year survival rates for melanoma that has spread to the nearby lymph nodes or to other parts of the body are 62% and 15%, respectively. Melanoma accounts for only about 5% of skin cancer cases, but it is the cause of more than 75% skin cancer deaths.

The standard and approved treatments for melanoma include surgery, targeted therapy, radiation, chemotherapy and treatment with biologics. The current treatment approved by the FDA includes IFN-α and dacarbazine and requires supplementation with other treatment options such as surgery and radiation. In an extensive study conducted by the Mayo Clinic for determining the efficiency of chemotherapeutic agents, only 10 of 503 melanoma cancer patients achieved complete remission.[117]

Patients who have undergone surgery to completely remove tumors and who are at a high risk for relapse are systemically given high doses of pegylated IFN-α-2b, which is approved for the adjuvant treatment. Prospective, randomized, controlled trials with both agents have not shown an increase in overall survival when compared with observation.[118,119]

The novel monoclonal antibodies ipilimumab and vemurafenib were approved by the FDA in 2011, as they demonstrated an improvement in overall survival in international, randomized trials in patients with advanced melanoma.[120] These single agents are rarely curative; however, clinical trials that incorporate these agents are testing combinations in an attempt to prevent development of drug resistance. Vemurafenib is a selective BRAF V600E kinase inhibitor, and its indication is limited to patients with a demonstrated *BRAF* V600E mutation by an FDA-approved test.[121] Interleukin-2 (IL-2) was approved by the FDA in 1998 on the basis of durable complete response (CR) rates in a minority of patients (0 to 8%) with previously treated metastatic melanoma in eight Phase I and II studies.[122–124]

The National Cancer Institute mentions interferon and interleukin-2 as types of biologic therapy used to treat melanoma. Interferon slows tumor progression by affecting the division of cancer cells. IL-2 improves the growth and activity of many immune cells, especially lymphocytes. Lymphocytes can attack and kill cancer cells. Tumor necrosis factor (TNF) therapy is another type of biologic therapy used in conjunction with other treatments for melanoma. TNF is a cytokine released by white blood cells in response to an antigen or infection.

The success of prophylactic vaccines against infectious diseases has increased interest of researchers to explore the feasibility of using vaccines against cancer. The John Wayne Cancer Institute (Santa Clara, CA) developed a vaccination regimen in the form of a living whole cell melanoma vaccine called CancerVax. This vaccine includes a mixture of irradiated melanoma associated antigens (MAA) derived from allogenic tumor cell lines to treat melanoma.[125] Results from this vaccination study showed that the median survival rate of melanoma patients was significantly higher when compared to patients receiving other forms of treatment. This in turn has led to interest in the development of a vaccine that would trigger the immune system to stimulate the cytotoxic cells and inhibit the tumor growth.[126]

In recent years, research has been focused on developing novel vaccines such as whole cell vaccines, dendritic cell vaccines, peptide vaccines, DNA vaccines, viral vaccines, and ganglioside vaccines for either the prevention or treatment of melanoma. Melanoma vaccines that have been tested have shown poor clinical responses, thus no effective vaccine has been developed to date.[127] All of the vaccines tested, however, are given by the parenteral route.

Oral vaccines are currently being investigated for their efficacy in stimulating the mucosal as well as systemic immunity. The mucosal route of entry and initiation of primary immune response is well established where pathogens and other invasive microbes enter the host system via regions in the small intestine. Bernadette et al.[128] described a study wherein they formulated a novel prophylactic oral microparticulate vaccine composed of whole cell lysate for melanoma using the spray-drying technique. Surface morphology of microparticles evaluated by scanning electron microscopy (SEM) showed that the particles had a spherical surface with a size distribution of around 1 to 2 μm (Figure 5.8). They used an M-cell targeting ligand called aleuria aurantia lectin (ALL) that improved the uptake into the Peyer's patches of the small intestine. The results suggested that melanoma antigen microparticles were able to significantly increase the antibody titers in the mice for groups containing the lectin ligand in comparison to the other groups involved in the study. The serum IgG response of orally immunized mice with AAL ligand in the vaccine was compared with the oral group without
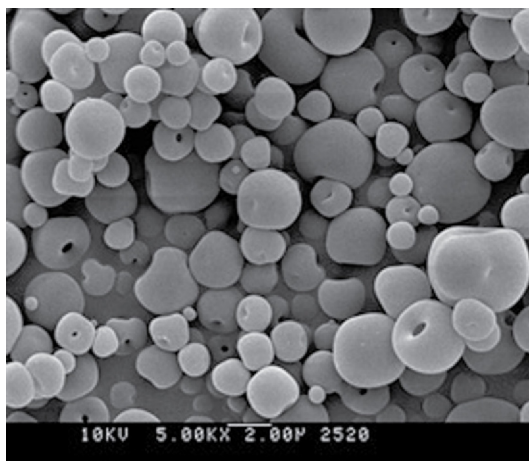
FIGURE 5.8   Surface morphology of microparticles showed that the particles had a spherical surface with a size distribution of around 1.2 ± 0.5 μm.

AAL and showed a significant increase in serum IgG titer. The results of the tumor challenge study showed that the group vaccinated with AAL-associated microparticles showed the highest level of protection against tumor development. Thus, in the future, when designing oral particulate vaccines, AAL may be incorporated in the formulation as the targeting ligand.

The major challenge in designing a successful vaccine is the delivery of antigens to the part of the immune system where maximum stimulation and proliferation of the professional immunopotent cells can be achieved. In a previous study with model antigens, it was shown that microparticles are capable of exhibiting potent serum antibody responses to entrapped antigens following oral administration.[129]

Figure 5.9 represents the cytotoxic evaluation of the melanoma-antigen microparticles in an *in vitro* cell culture model. The data show more than 80% cell viability measured as an
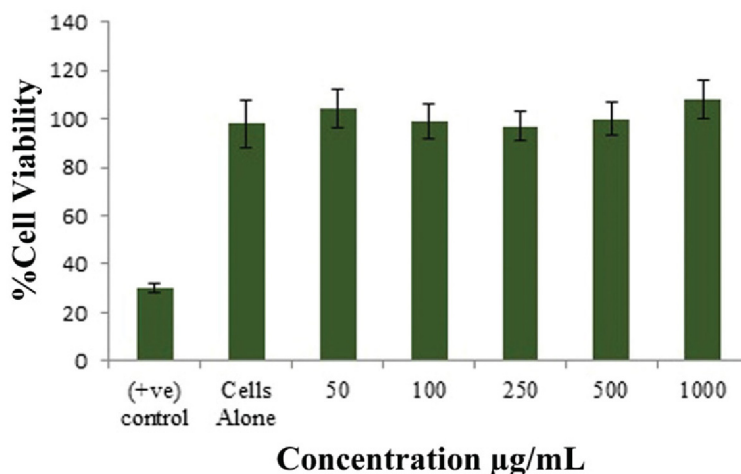
FIGURE 5.9   *In vitro* cytotoxicity assay of melanoma vaccine loaded microparticles.

I. NOVEL THERAPEUTIC APPROACHES

average of $n = 3$ (+ SE) after 24 hours of incubation with the microparticles. The microparticle formulations were added at doses of 0.05, 0.1, 0.25, 0.5, and 1 mg/mL in 0.2 mL of complete cell culture media. Cells alone in media was used as the negative control, and atropine sulfate (cytotoxic agent) was the positive control, which resulted in cell death.

Studies are currently aimed at combining chemotherapy and novel immunotherapies, including vaccines, cytokines, and anti-CTLA4 antibodies. Hundreds of combination therapies are currently undergoing clinical trials. Vaccine therapy still remains an experimental therapy in patients with metastatic melanoma.

The vaccine delivery by the formulation demonstrated in the investigation described here opens up new avenues for vaccine administration by more patient-convenient routes for cancer therapy. Further research is needed, although a future therapy for advanced melanoma is probably a multimodal approach including vaccines, adjuvants, and negative costimulatory blockade.

Ovarian Cancer Vaccine

Among the gynecological cancers, ovarian cancer is the most lethal and the fifth leading cause of cancer-related deaths in women globally and more specifically in the United States. According to the data from the CDC, each year around 20,000 women in the US are diagnosed with ovarian cancer and 15,000 women sadly die each year due to ovarian cancer. The major reason for high mortality is that in more than 70% of women with ovarian cancer are diagnosed with advanced disease.[130]

Many vaccines have made it to the clinical trials but most of them have not progressed beyond Phase I/II studies. Although antigen-specific responses are obtained using various approaches of immunization, there is a lack of consistency in the benefits.[131,132] Here, we discuss an oral vaccination with microparticles containing the ovarian cancer antigens that can prevent or retard ovarian cancer growth. A murine ovarian cancer cell line, ID8, was used as a source of antigens as it correlates closely to human ovarian cancer cell lines in signaling pathways and results in development of tumor in mice models similar to human ovarian cancer.[133] Whole cell lysate, which is a very promising approach, provides a pool of tumor-associated antigens (TAAs) which include both $CD8^+$ and $CD4^+$ T cells. It also overcomes the drawbacks associated with using a single antigen or epitope vaccine.

Like other studies, to protect the antigen from the gastric conditions, enteric polymers such as methacrylic copolymers FS 30D and hydroxyl methylcellulose succinate (HPMCAS) were used to make the microparticles along with AAL for its M-cell targeting property using the spray-dryer method (Figure 5.10). In addition, inclusion of immunostimulatory molecules such as IL-2 and IL-12 was evaluated in order to enhance the overall potency of the formulated vaccines.[134,135] Characterization of the vaccine particles was done. The immunogenicity of the microparticulate vaccine was evaluated using C57BL/6 female mice model. Three different formulations such as placebo, vaccine, and vaccine with interleukins were evaluated for their efficacy. A tumor challenge study was carried out in which one week after the last vaccine was administered the mice were challenged with 1×10^7 live ID8 cells subcutaneously. Tumor volume was checked and a reduction in tumor volume was indicative of the success of the vaccine formulation.[136,137]

The morphological characterization of the particles showed that the particles were crumbled, collapsed, and irregular in shape within a micrometer size range having a net positive
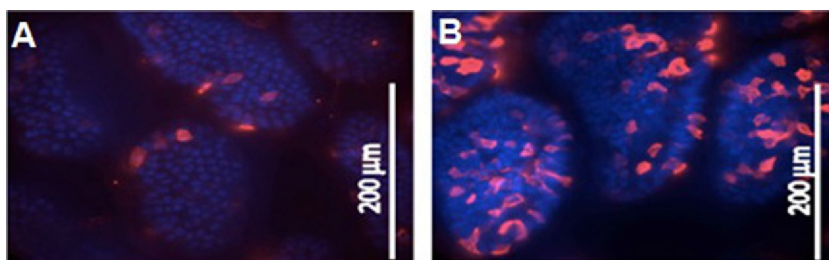
FIGURE 5.10   Increased uptake of particles by M cells (SEM images) in Peyer's patches using AAL. (A) Particles without AAL; (B) particles with AAL.

charge. The immunization studies showed that in the group of mice with the placebo particles the tumor growth was very rapid. In the vaccinated group, there was a sixfold tumor suppression as compared to the placebo. However, there was not much difference between the vaccine group and the group with interleukins, which could possibly be due to low concentrations of the interleukins.

To access the humoral immune response (B-cell mediated) the serum samples were collected before each dose and were analyzed by enzyme-linked immunosorbent assay (ELISA) for the IgG subtypes IgG_1 and IgG_{2a}. The results from the ELISA showed increased IgG titer in vaccinated mice as opposed to nonvaccinated mice. Thus, both the subtypes of IgGs indicate that a mixed Th1 and Th2 type response was generated in the vaccine-only group. But, a Th2 type response was triggered among the interleukin group. This difference could be attributed to the effect of interleukins on the Peyer's patches which has been previously reported as a strategy for increasing the immune response for oral vaccine. Similar studies were carried out by Marinaro et al.[135] where IL-12 was given orally to mice immunized with oral tetanus toxoid and cholera toxin. Their studies showed that oral IL-12 resulted in a TH2 response to the oral vaccine, and a TH1 response occurred when IL-12 was given via the intraperitoneal route.

To check for T-cell populations, lymphatic organs such as the spleen and the lymph nodes were collected. $CD4^+$ and $CD8^+$ levels were elevated in vaccinated mice as compared to the placebo group. The $CD4^+$ T-cell population was found to be elevated in the spleen cells of the vaccine + interleukin group when compared to the spleen cells of the vaccine-alone group. The B-cell population was determined in the spleen, lymph nodes, and bone marrow. For B-cell populations in spleen cells, there were elevated levels in the vaccine + interleukin group when compared to placebo and vaccine alone. B cells in bone marrow were found to be elevated in the vaccine + interleukin group compared to the placebo or vaccine-alone groups.[110]

In summary, the B-cell population was found to be more elevated in the bone marrow and spleen cells of the vaccine + interleukin group compared to the vaccine-alone group. It was higher in the lymph nodes of the vaccine-alone group as well as the vaccine + interleukin group when compared to placebo. It was found that $CD8^+$ and $CD4^+$ T cells were expanded in mice treated with vaccine with and without interleukins when compared to non-vaccinated mice (Figure 5.11). However, when a comparison was made between the vaccine-along group and the vaccine + interleukin groups, the B-cell populations in bone marrow and spleen along with $CD4^+$ T cells in spleen were found to be elevated in the vaccine + interleukin group.[110]
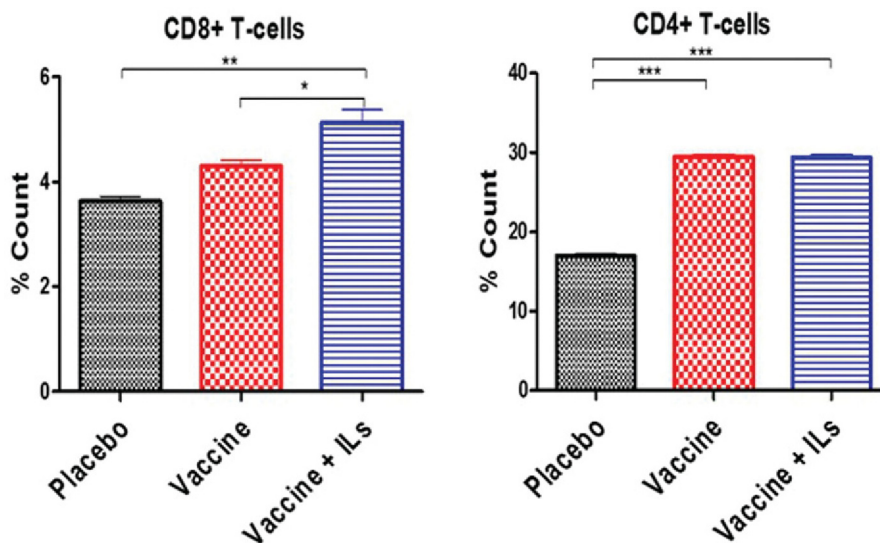
FIGURE 5.11   CD8$^+$ T cells and CD4$^+$ T cells in spleen and lymph nodes ($n = 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Thus, there was overall stimulation of the humoral and cellular response upon administration of the vaccine, indicating the success of the vaccine. These results also correlate with the tumor volume reduction in the mice. Thus, this study demonstrated the efficacy of the microparticulate vaccine containing the whole cell lysate. Oral vaccine delivery could be an effective and attractive mode of immunization because of its ease of administration, low cost of manufacturing, and most importantly patient compliance.

OVERALL SUMMARY AND CONCLUSIONS

Today, there are still increasing numbers of new vaccines being developed to prevent different evolving diseases, including H5N1 bird flu, acquired immunodeficiency syndrome (AIDS), severe acute respiratory syndrome (SARS), and others. Nevertheless, the main vaccination routes are still the subcutaneous and intramuscular routes. These administration methods render vaccination unaffordable in many areas of Third-World countries. The use of a spray dryer and the formulation and development processes performed demonstrated the feasibility for scale-up and manufacture.

One of the hurdles of oral proteins or vaccines delivery is the harsh acidic environment in the stomach. To overcome this obstacle, enteric coating polymers and various combinational matrices have been used to protect the proteins. Such protection should be sufficient to ensure successful delivery of the encapsulated biologics or vaccines. Another feature of the formulations developed is their sustained- or controlled-release properties achieved by employing different combinations of polymers. This feature can be tailored to achieve desired release profiles by adjusting the compositions of the polymers used.

In light of the fact that proteins are very sensitive to any subtle changes in their microenvironment, therefore, the stability of encapsulated proteins must be evaluated. To achieve that, the physicochemical characteristics of the formulations developed were analyzed using bioactivity assays, Fourier transform infrared (FTIR) spectroscopy, differential scanning calorimetry (DSC), particle sizes analyzers, and the Zetasizer. Additionally, FTIR and DSC studies performed before and after microencapsulation showed no major changes in their native structures. These results suggest that the formulation developed can be used as an effective vehicle for delivery of proteins and vaccine.

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