Pharm Res (2011) 28:986–1012
DOI 10.1007/s11095-010-0314-4

EXPERT REVIEW

Progress Towards a Needle-Free Hepatitis B Vaccine

Filipa Lebre • Gerrit Borchard • Maria Conceição Pedroso de Lima • Olga Borges

Received: 11 June 2010 / Accepted: 27 October 2010 / Published online: 19 November 2010
© Springer Science+Business Media, LLC 2010

ABSTRACT Hepatitis B virus (HBV) infection is a worldwide public health problem. Vaccination is the most efficient way to prevent hepatitis B. Despite the success of the currently available vaccine, there is a clear need for the development of new generation of HBV vaccines. Needle-free immunization is an attractive approach for mass immunization campaigns, since avoiding the use of needles reduces the risk of needle-borne diseases and prevents needle-stick injuries and pain, thus augmenting patient compliance and eliminating the need for trained medical personnel. Moreover, this kind of immunization was shown to induce good systemic as well as mucosal immunological responses, which is important for the creation of both a prophylactic and therapeutic vaccine. In order to produce a better, safer, more efficient and more suitable vaccine, adjuvants have been used. In this article, several adjuvants tested over the years for their potential to help create a needle-free vaccine against HBV are reviewed.

KEY WORDS adjuvant · hepatitis B antigen · needle-free vaccine

F. Lebre • M. C. P. de Lima • O. Borges (✉)
Center for Neuroscience and Cell Biology, University of Coimbra
Coimbra, Portugal
e-mail: olga@ci.uc.pt

F. Lebre • O. Borges
Faculty of Pharmacy, University of Coimbra
Pólo das Ciências da Saúde Azinhaga de Santa Comba
3000-548, Coimbra, Portugal

G. Borchard
School of Pharmaceutical Sciences, University of Geneva 30
Quai Ernest Ansermet 30
1211, Geneva, Switzerland

M. C. P. de Lima
Department of Life Sciences, University of Coimbra
3004-517, Coimbra, Portugal

INTRODUCTION

Hepatitis B is one of the most common infectious diseases in the world. It is estimated that 40% of the world's population has had contact with or are carriers of hepatitis B virus f(HBV), which corresponds to an estimated 350 million HBV carriers (1). About 600,000 persons die each year due to the acute or chronic consequences of hepatitis B (2). Despite the availability of effective HBV vaccines, hepatitis B remains a major global problem. This situation is particularly serious in developing countries, especially since a significant percentage of the population does not have access to the vaccine or does not return for the required booster doses. A number of approaches are being tested in order to minimize this problem, and needle-free immunization appears to be the most attractive alternative.

Hepatitis B virus infection has a worldwide distribution, however, at different levels of prevalence. According to the World Health Organization (WHO), the prevalence of chronic HBV infection is relatively low in North America, Australia, Northern and Western Europe and New Zealand (less than 2% of the general population). Areas of high prevalence (≥ 8%) include most of Asia (except Japan and India), the Amazon, the southern parts of Eastern and Central Europe and sub-Saharan Africa. The other areas of the globe show an intermediate HBV infection prevalence (Fig. 1).

Hepatitis B is transmitted among persons by several routes, which include direct contact with the blood or body fluids of an infected person (blood transfusions, sharing equipment for injecting drug use, or unprotected sex with an infected person) or from an infected mother to her baby at birth. The patterns of transmission vary among different parts of the world. In areas of high endemicity, perinatal and person-to-person transmission are the major transmission routes, and the majority of the infections occur at early

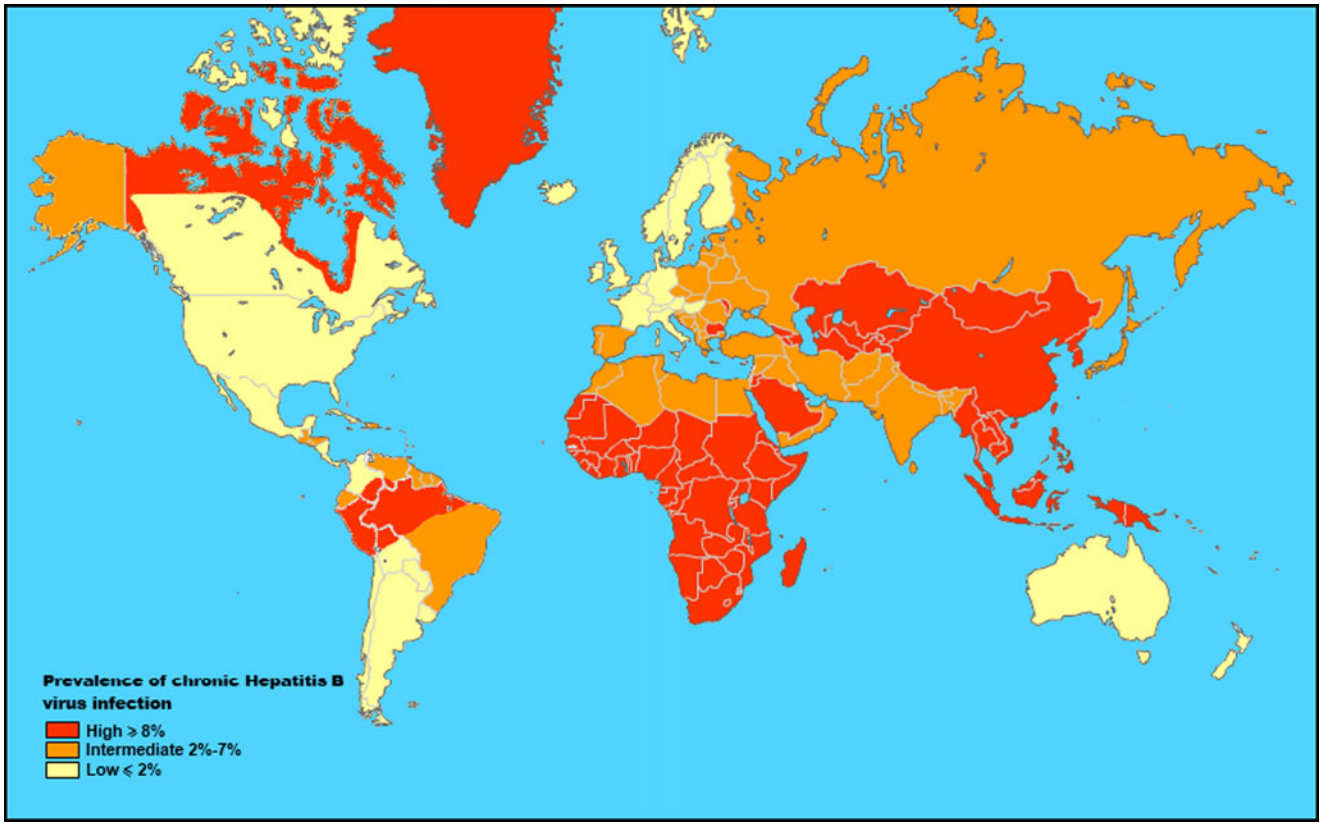

Fig. 1 Prevalence of chronic Hepatitis B virus infection (Adapted from *Center for Disease Control and Prevention Travelers' Health Yellow Book*).

ages. In low prevalence areas, the transmission essentially happens via unprotected sexual intercourse and injected drug use (2,3). The incubation period of HBV varies from 1 to 4 months after exposure. HBV causes both acute and chronic infections, and between one-third and one-quarter of persons infected chronically with HBV are expected to develop long-term consequences, such as cirrhosis, liver failure or hepatocellular carcinoma (3–5).

In 1981 FDA approved the first hepatitis B vaccine which consisted of the surface antigen of the hepatitis B (HBsAg) virus present in the blood of human carriers of the infection. This plasma-derived vaccine successfully immunized millions of individuals worldwide until it was replaced by a recombinant vaccine due to safety concerns. The currently available vaccine was introduced in the market in 1986 and represents the world's first subunit vaccine, the world's first licensed vaccine against human cancer and the world's first recombinant expressed vaccine. The gene encoding the HBsAg was expressed in yeast cells grown in bioreactors, and the protein was collected and induced to refold by chemical treatment to yield virus-like particles subsequently formulated for injection (6).

A great disadvantage of parenteral vaccines is that the immune response produced is mainly systemic, and little or no mucosal immunity is elicited. Since mucosal surfaces are the main entry site for virus and bacteria, mucosal immunization would provide the first line of defense, stimulating the secretion of IgA that avoids the attachment of the infectious pathogens to the mucosa. Furthermore, although hepatitis B vaccines already in the market are considered safe and effective, in order to maintain their efficiency, vaccines must be administered in three doses, which reduces patient compliance. In addition, trained medical personal and special vaccine storage conditions are needed, which may pose a problem especially in developing countries. Taking these facts into account, needle-free vaccination could have a big impact on the efficacy of immunization against HBV worldwide.

Although no well-established classification of adjuvants concerning needle-free immunization exists, for the sake of clarity in this article they will be divided into two major groups: delivery systems and immunopotentiators. Under the delivery system group, a description will be made for adjuvants designed for mucosal (like oral and nasal routes) immunization and some types of topical immunization.

Mucosal vaccination has been the common generic name attributed to the oral, intranasal, pulmonary, rectal and vaginal routes of vaccine administration. Mucosa with a combined surface of about 400 m^2 (8) are undoubtedly the major site of entry for most pathogens. Therefore, these

vulnerable surfaces are associated with a large and highly specialized innate and adaptive mucosal immune system that protects the surface and the body against potential destructive agents and harmful substances from the environment. In a healthy human adult, this local immune system contributes almost 80% of all immune cells (9). These immune cells accumulate in a particular mucosa or circulate between various mucosa-associated lymphoid tissues (MALT), which together form the largest mammalian lymphoid organ system (8).

Mucosal vaccines are giving rise to some expectations as highly desirable from various perspectives. As noted above, one of the most significant advantages is the incising evidence that local mucosal immune response is important for protection against early infection, mainly for diseases originating on mucosal surfaces, such as sexually transmitted HBV. It is generally accepted that mucosal immune response is theoretically more efficiently induced by the administration of vaccines at mucosal surfaces, while injected vaccines are generally poor inducers of mucosal immunity and, therefore, may be less effective against infections at mucosal surfaces (7). Injected vaccines generally tend to induce only systemic immune response, and the antibodies produced in this kind of response are not capable of offering protection at mucosa level. Moreover, mucosal immunization tends to be considered as an attractive substitute to parenteral immunization as it may stimulate both humoral and cell-mediated immune response and induce mucosal and systemic immunity simultaneously (10). Cellular and humoral immune responses to HBV antigens are thought to play a crucial role in the elimination of virus by the host. On one hand, humoral immune response leads to defense against infection. On the other hand, cellular immune response, associated with the activation of the CD8+ cytotoxic T lymphocytes (CTL) has been reported to be one of the key factors contributing to virus elimination from infected hepatocytes playing, as a consequence, an important role in the pathogenesis and in the reduction of the severity of hepatitis and the succeeding development of chronic liver disease (11–14). CTL can protect the organism against intracellular pathogens, because they can lyse the infected cells and secrete important cytokines (15). Most non-replicating vaccines administered by intramuscular injection are unable to induce this type of CTL-mediated immune response.

As mentioned above, some types of topical vaccine administration can also be included in the needle-free immunization group. Skin is one of the largest immune organs; it is considered an immunologically attractive target for immunization and a promising vaccination route. The skin is full of antigen-presenting cells (APCs), such as Langerhan cells (LCs) in the epidermis and dermal dendritic cells (DDCs) in dermis (Fig. 2) (16–18). Their main function is to capture and process antigen and present it to other cells of the immune system, which then activate a specific T-cell-mediated immune response (17,18). Antigen-presenting cells can stimulate T-lymphocytes and B-lymphocytes, thus making the skin a highly efficient location for the beginning of a cellular and humoral immune response (19–21). Unlike the skin, muscle tissue lacks large quantities of APCs, and, therefore, intramuscular (i.m.) injections fail to elicit a sufficiently good cell-mediated immune response (16).

RECENT ADVANCES IN THE DEVELOPMENT OF A NEEDLE-FREE HEPATITIS B VACCINE

Needle-free immunization is attracting increased attention by the scientific community because of some advantages associated with it (22,23). However, although needle-free vaccines have some attractive features when compared to parenteral immunization, they still present some disadvantages. Vaccines administered mucosally encounter the same host defense barriers as microbial pathogens and other foreign macromolecules: they are diluted in mucosal secretions, detained in mucus gels, attacked by acid,

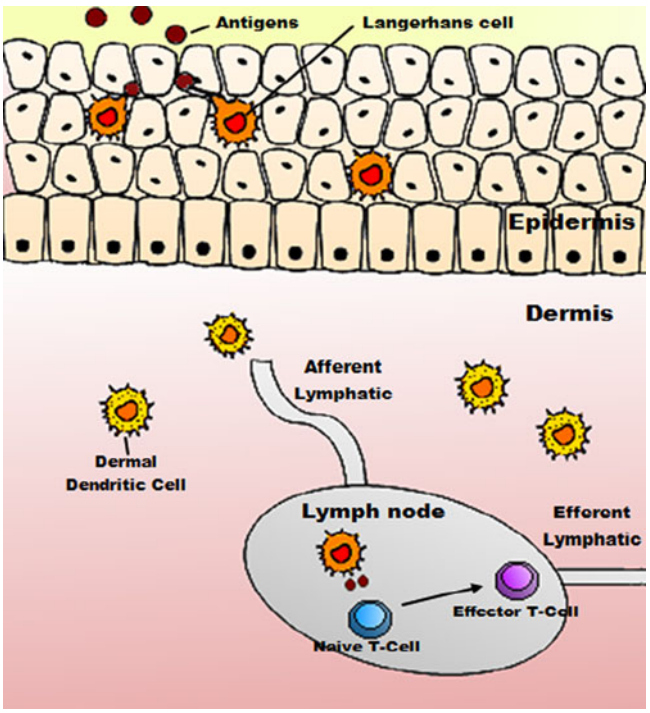

Fig. 2 The skin is full of antigen-presenting cells (APCs), such as Langerhans cells (LCs), in the epidermis and dermal dendritic cells (DDCs) in dermis. Their main function is to capture and process antigen and present it to naïve T-Cells, which then activate a specific T-cell-mediated response.

proteases and nucleases and barred by epithelial barriers. Therefore, it is estimated that large doses of antigen would be required in order to achieve an efficient transport and uptake by MALT, where antigens can be processed and presented (24). In addition, it is also necessary to overcome the problem of immunological tolerance mechanism. Mucosal surfaces are in a permanent state of alert, but they adapt to the presence of foreign microorganisms. Vaccines that produce a strong immune response if injected in sterile tissues such as muscle could be ignored when administered through mucosal surfaces (7). This state of unresponsive or so-called immunological tolerance is dependent on the route, frequency of antigen administration and dose (25,26) and has been appointed as one of the biggest challenges for mucosal vaccine development. Nasal immunization presents some other disadvantages. One of the most important limitations of nasal immunization is the general rapid clearance of the vaccine formulation in the mucosal surface owing to the mucociliary clearance. Also, new evidence suggest the existence of antigen transfer to neuronal tissue via olfactory bulb, present in the nasal cavity, which can lead to potential side effects (27,28). Furthermore, some nasal vaccine systems were shown to induce some serious clinical manifestations, such as Bell's palsy syndrome. It was reported that the inactivated intranasal influenza vaccine used in Switzerland during 2000/01 increased the risk of Bell's palsy among vaccinated individuals. In contrast, no significant risk of the adverse event was found to be associated with the parenteral influenza vaccine (27,28). Finally, topical immunization faces some difficulties in view of the fact that skin forms a protective barrier that is difficult to penetrate.

Adjuvants have traditionally been defined as agents added to vaccine formulations that enhance the immunogenicity of antigens *in vivo*. A proposed revision of this definition (29) divides adjuvants into two classes: delivery systems and immunopotentiators, based on their dominant mechanism of action. Both delivery systems and immunopotentiators are able to augment the antigen-specific immune response *in vivo*, and it is becoming common to use them together to develop multi-component adjuvants with the potential to act synergistically. Many adjuvants were tested in order to create an efficient needle-free vaccine against hepatitis B (Tables I, II, III and IV)

Delivery Systems

Delivery systems have two main functions: first, helping to protect the vaccine while inside the body; second, helping its targeting to the immune cells. In recent years several approaches have been designed and tested in order to develop an effective delivery system. For clarity reasons, in this text, delivery systems for mucosal and for topical immunization will be described separately.

Delivery Systems for Mucosal Immunization

Lipid Particles. The ability of liposomes to act as adjuvants was first described in 1974 by Allison and Gregoriadis. Since then, they have been extensively studied. Liposomes are composed of naturally occurring, biodegradable lipids organized in bilayers surrounding an aqueous core (Fig. 3a) (30). A great variety of substances can be associated to liposomes, regardless of their solubility, charge, size or shape, as long as they do not interfere with liposome formation (31). Although they can promote antigenic responses to various antigens, there are some problems related to their physical and chemical stability. In order to overcome these limitations, researchers developed different approaches.

Lipospheres, also known as solid lipid microparticles (SLMs), are relatively more stable than liposomes at room temperature and, thus, more suitable to act as vesicle carriers. These particles consist of a solid fat core based on naturally occurring lipids and stabilized by a layer of surfactant molecules on the surface (32). SLMs combine the benefits of liposomes and polymeric microparticles, and at the same time avoid several of their drawbacks, such as instability, toxicity, biodegradability problems and production costs (32–34). Moreover, they are excellent in controlling and sustaining drug release efficiency.

Saraf *et al.* prepared and optimized lipospheres for intranasal delivery of HBsAg (35). Researchers tested two sets of lipid particles, one containing the cationic polymer stearylamine in the formulation (LMST) and the other lacking this polymer (LM). LMST was shown to be more rapidly taken up by the alveolar macrophages and revealed considerable stronger mucoadhesion than LM. Researchers proposed that stearylamine imparts cationic charge onto the particle surface, which may lead to a stronger interaction with negatively charged sialic groups of the mucus, which may also explain the localized uptake of LMST observed by the same authors. Immunological studies were conducted by immunizing mice with different formulations (LM, LMST, Alum-HBsAg and plain HBsAg) through intranasal (i.n.) or intramuscular routes. In general, LMST formulation proved to be the best. In fact, mice vaccinated with LMST (Table I) developed strong systemic and mucosal immunity. IgG and IgA levels induced by lipid microparticles without stearylamine were always lower than the ones induced by the particles that contained the cationic polymer. The better mucoadhesion property displayed by LMST was again pointed out as the reason for these superior results, showing that the use of stearylamine in the lipidic formulation further helps to enhance the adjuvant capacity of SLMs as delivery system.

Table I Delivery Systems for Mucosal Immunization

Adjuvant	Type of vaccine	Phase	Immunization type	Dose	Immunization schedule	Immunity		Results	Reference
Lipid microparticles	Sub-unit vaccine	Pre-clinical studies in mice	Intranasal	10 μg HBsAg	$t = 1$st day $t = 2$nd day $t = 3$rd day $t = 21$st day (boost)	IgG sIgA	+ +	Mice developed strong systemic and mucosal immunity.	(35)
Chitosan-coated liposomes	DNA vaccine	Pre-clinical studies in mice	Intranasal	100 μg pDNA	$t = 0$ weeks $t = 2$ weeks (boost)	IgG sIgA INF-γ IL-2	+ + + +	Vaccine induced good systemic, mucosal and cell-mediated immunity.	(36)
Bilosomes	Sub-unit vaccine	Pre-clinical studies in mice	Oral	3 groups: B1–10 μg HBsAg B2–20 μg HBsAg B3–50 μg HBsAg	$t = 1$st day $t = 2$nd day $t = 3$rd day $t = 21$st day (boost)	IgG sIgA	+ +	Higher dose of vaccine (50 μg) elicited anti-HBsAg titers similar to the ones obtained with commercial vaccine.	(43)
Mannosylated niosomes	DNA vaccine	Pre-clinical studies in mice	Oral	100 μg of pDNA	$t = 0$ weeks $t = 3$ weeks (boost)	IgG sIgA INF-γ IL-2	+ + + +	Delivery system induced considerable humoral and cellular immune response.	(47)
Nanoemulsion	Sub-unit vaccine	Pre-clinical studies in mice, rats and guinea pigs	Intranasal	5 μg (rats & guinea pigs) 20 μg HBsAg (all)	$t = 0$ weeks $t = 6$ weeks (boost)	IgG sIgA INF- γ TNF-α IL-4,5,10	+ + + + +	Vaccine was immunogenic in all species tested; good Th1-type cytokine production.	(48)
Supramolecular biovector (SMBV™)	Sub-unit vaccine	Pre-clinical studies in mice	Intranasal	0.2 μg and/or 1 μg and/or 3 μg and/or 10 μg according to the assay	$t = 0$ weeks $t = 3$ weeks (boost) $t = 6$ weeks (boost) (not all groups received it)	IgG sIgA CTL	+ + +	Mice immunized with antigen formulated with SMBV™ developed strong systemic, mucosal and cellular immunity.	(54)
Alginate-coated chitosan nanoparticles + CpG ODN	Sub-unit vaccine	Pre-clinical studies in mice	Oral	10 μg HBsAg + 10 μg CpG ODN	$t = 0$ weeks $t = 3$ weeks (boost)	IgG sIgA INF-γ IL-2	+ + + +	Humoral and cellular immune response were better induced in mice vaccinated with the formulation combining the delivery system and the immunopotentiator.	(68)
Alginate-coated chitosan nanoparticles + CpG ODN	Sub-unit vaccine	Pre-clinical studies in mice	Intranasal	10 μg HBsAg + 10 μg CpG ODN	$t = 0$ weeks $t = 3$ weeks (boost) $t = 6$ weeks (boost)	IgG sIgA INF-γ	+ + +	Delivery system in combination with immunopotentiator in solution gave rise to systemic and mucosal immunity.	(70)
Chitosan nanoparticles	DNA vaccine	Pre-clinical studies in mice	Intranasal	50 μg of plasmid loaded chitosan nanoparticles	$t = 0$ weeks $t = 2$ weeks (boost)	IgG sIgA INF-γ IL-2	+ + + +	Delivery system elicited good humoral, mucosal and cellular responses.	(74)

Table I (continued)

Adjuvant	Type of vaccine	Phase	Immunization type	Dose	Immunization schedule	Immunity		Results	Reference
PLGA microparticles	DNA vaccine	Pre-clinical studies in mice	Oral	20 μg or 200 μg of plasmid-loaded PLGA microparticles	$t=0$ weeks	IgG sIgA CTL INF-γ	+ + +/− +	Mice developed systemic, mucosal and cellular immune responses in a dose-dependent manner.	(86)
Surface-modified PLGA microspheres	Sub-unit vaccine	Pre-clinical studies in mice	Intranasal	10 μg HBsAg	$t=0$ weeks $t=4$ weeks (boost)	IgG sIgA INF-γ IL-2	+ + + +	Vaccine induced good systemic, mucosal and cell-mediated immunity. Surface-modified formulation elicited higher immune response compared to the non-modified.	(80)
PLG microparticles	Sub-unit vaccine	Pre-clinical studies in mice	Oral	100 μg of BCEM	$t=0$ weeks	IgG IgM	+ +	Vaccine induced good systemic immune response. Mice were i.m. injected with HBsAg, which lead to a rapid and vigorous production of antibodies.	(88)
M-cell-targeted PLGA nanoparticles	Sub-unit vaccine	Pre-clinical studies in mice	Oral	10 μg HBsAg	$t=1$st day $t=2$nd day $t=3$rd day $t=21$st day (boost)	IgG sIgA INF-γ IL-2	+ + + +	Delivery system elicited good humoral, mucosal and cellular responses. M-targeted stabilized formulation elicited higher immune response compared to the non-targeted.	(91)
Triblock copolymer-based nanoparticles	Sub-unit vaccine	Pre-clinical studies in mice	Intranasal	10 μg HBsAg	$t=0$ weeks	IgG sIgA	+ +	Copolymer nanoparticles elicited good systemic and mucosal immunity.	(78)
Salmonella typhimurium	Sub-unit vaccine	Pre-clinical studies in mice	Intranasal	$1–1\times10^8$ CFU of bacterial suspension	$t=0$ weeks	IgG sIgA	+ +	Higher doses induced better systemic and mucosal immune response. Nasal administration proved more efficient than oral immunization.	(111)
Salmonella typhimurium	DNA vaccine	Pre-clinical studies in mice	Oral	6×10^9 bacterial cells	$t=0$ weeks	IgG CTL INF-γ IL-4	– + + +	Mice developed a strong cellular response but weak humoral immune response, potential use as therapeutic vaccine.	(112)

Table II Edible Vaccines

Adjuvant	Type of vaccine	Phase	Immunization type	Dose	Immunization schedule	Immunity	Results	Reference
Lettuce	Edible vaccine	Phase I clinical trials in humans	Oral	≈ 0.1–0.5 μg HBsAg/100 g fresh tissue	$t = 0$ months (200 g fresh tissue) $t = 2$ months (150 g fresh tissue)	IgG +/−	2 out of 3 volunteers developed protective levels against HBV. Expression levels of the antigen in lettuce were very low.	(120)
Potato	Edible vaccine	Pre-clinical studies in mice	Oral	5.5 μg HBsAg (1.1 μg HBsAg/g fresh tuber) + 10 μg CT	$t = 0$ weeks $t = 1$ weeks $t = 2$ weeks $t = 10$ weeks (i.p. boost with 0.5 μg of commercial vaccine)	IgG +	Mice fed transgenic potato tuber developed a primary immune response greatly boosted by a subimmunogenic dose of commercial vaccine, low amount HBsAg expressed in potatoes.	(122)
Potato	Edible vaccine	Pre-clinical studies in mice	Oral	5 g tuber (8.35 μg HBsAg/g tuber) + 10 μg CT	Experiment 1: $t = 0.1.2$ weeks $t = 16$ weeks (i.p boost with 0.5 μg of commercial vaccine) Experiment 2: $t = 0$ weeks (i.p immunization with 0.5 μg of commercial vaccine) $t = 5,6,7$ weeks	IgG +	Combination of oral immunization with an edible vaccine and parenteral immunization resulted in good antibody response. Oral immunization without CT resulted in much limited antibody response. Cooking the potatoes significantly reduces immunogenicity.	(121)
Potato	Edible vaccine	Phase I clinical trials in humans	Oral	100–110 g tuber (≈8.5 μg HBsAg/g tuber)	Group 2: $t = 0$ and 28 day (transgenic tuber) $t = 14$ day (placebo tuber) Group 3: $t = 0$, 14 and 28 day (transgenic tuber)	IgG +	In ~ 60% of the volunteers, serum anti-HBs titers increased after eating two or three doses of transgenic potato tuber.	(118)
Cherry tomatillo	Edible vaccine	Pre-clinical studies in mice	Oral	20 g transgenic tomatillo (≈ 1 μg HBsAg)	Experiment 1: Every day for 4 weeks $t = 4$-week parenteral immunization with 0.5 μg of commercial vaccine Experiment 2: $t = 0$-week parenteral immunization with 2 μg of commercial vaccine Boost with transgenic tomatillo when serum antibody levels ↘ to OD < 1.0	IgG +/−	Only when mice were first immunized with a single shot of commercial vaccine and boosted with transgenic tomatillo, later an immune response was elicited. Reverse protocol failed to develop any immune response. Low levels of antigen expression in cherry tomatillo.	(117)

Table III Delivery Systems for Topical Immunization

Adjuvant	Type of vaccine	Phase	Immunization type	Dose	Immunization schedule	Immunity	Results	Reference
PMED	DNA vaccine	Pre-clinical studies in pigs	Topical	0.25 μg DNA 0.5 μg DNA 1.5 μg DNA	$t=0$ week $t=8$ weeks (boost) $t=16$ weeks (boost)	IgG +	Pigs Immunized with higher doses (2nd and 3rd groups) developed comparable humoral response to commercial vaccine.	(136)
PMED	DNA vaccine	Phase I clinical trials in humans	Topical	0.25 μg pDNA	$t=0$ day $t=56$ day	IgG +/−	Only 1 of 7 volunteers developed protective antibody levels.	(135)
PMED	DNA vaccine	Phase I clinical trials in humans	Topical	1 μg DNA 2 μg DNA 4 μg DNA	$t=0$ week $t=8$ weeks (boost) $t=16$ weeks (boost)	IgG + CTL +	All volunteers developed protective antibody responses as well as detectable cell-mediated immune response.	(132)
PMED	DNA vaccine	Phase I clinical trials in humans	Topical	4 μg DNA	$t=0$ week $t=8$ weeks (boost) $t=16$ weeks (boost)	IgG +	Vaccine was able to elicit antibody responses in 12 of 16 subjects that had previously failed to acceptably respond to 3–9 doses of commercial vaccine.	(133)
EPI	Sub-unit vaccine	Pre-dinical studies in mice	Topical	2 μg HBsAg	$t=0$ day $t=28$ day	IgG + CTL +	EPI was able to elicit humoral as well as cell-mediated immunity.	(138)
EPI	Sub-unit vaccine	Pre-dinical studies in mice	Topical	Varying doses (0.05–2 μg HBsAg + 0.1–20 μg CpG DNA)	$t=0$ day $t=28$ day (some studies)	IgG + IL-4 + INF-γ + (w/CpG)	Delivery system induced considerable humoral and cellular immune responses especially with the addition of the immunopotentiator that further enhanced EPI capacities.	(137)
Niosomes	DNA vaccine	Pre-dinical studies in mice	Topical	100 μg pDNA	$t=1$ day $t=14$ day	IgG + INF-γ + IL-2 +	Niosomes were capable of inducing cellular and humoral responses but with lower results compared to i.m. immunization of naked DNA.	(21)
Elastic liposomes	Sub-unit vaccine	Pre-dinical studies in mice	Topical	10 μg HBsAg	$t=1$ day $t=14$ day	sIgA + IgG +	Mice developed strong systemic and mucosal immunity.	(153)
Cationic transfersomes	DNA vaccine	Pre-dinical studies in mice	Topical	100 μg pDNA	$t=1$ day $t=14$ day	IgG + INF-γ + IL-2 +	Vaccine formulation elicited clinical protective antibody levels and strong cellular response in mice.	(156)
Ethosomes	Sub-unit vaccine	Pre-clinical studies in mice	Topical	10 μg HBsAg	$t=1$ day $t=14$ day	IgG + sIgA + Th1 +	Good systemic and mucosal humoral immune responses were observed. Ethosomes elicited a predominant Th1-like immunity.	(160)

Table IV Immunopotentiators

Adjuvant	Type of vaccine	Phase	Immunization type	Dose	Immunization schedule	Immunity		Results	Reference
Recombinant Cholera Toxin B	Sub-unit vaccine	Pre-clinical studies in mice	Intranasal	1 or 5 μg HBsAg + 10 μg rCTB	$t = 1$ day $t = 14$ day $t = 21$ day $t = 28$ day	IgG sIgA	+ +	Addition of rCTB elevated systemic and mucosal immune responses.	(175)
CpG ODN	Sub-unit vaccine	Pre-clinical studies in mice	Intranasal	1 or 10 μg HBsAg + 1 or 10 μg CpG and/or 1 or 10 μg CT	$t = 0$ weeks $t = 8$ weeks (some mice)	IgG sIgA CTL	+ +/− +	Immunization with formulations containing CpG induced high systemic response in all mice and strong mucosal immune response (w/higher CpG dose). CpG and CT act synergistically.	(73,189)
CpG ODN	Sub-unit vaccine	Pre-clinical studies in mice	Oral	100 μg HBsAg + 50 or 100 or 500 μg CpG	$t = 0$ day $t = 7$ day $t = 14$ day	IgG sIgA INF-γ IL-4 IL-5	+ + + + +	Combination of the high doses of antigen with the immunopotentiator resulted in high systemic, mucosal and cell-mediated immune responses in contrast to antigen alone.	(185)

Modified Liposomes. A recent study evaluated the potential of surface-modified liposomes for nasal immunization (36). The authors prepared glycol chitosan-coated liposomes (Fig. 3c) and loaded them with DNA containing pRc/CMV-HBs(S). Chitosan is known for its mucoadhesive properties (37); thus, the addition of this polymer to the formulation is advantageous for creating an intranasal formulation with prolonged local retention. To test the immunological response, mice were immunized with glycol chitosan-coated liposomes loaded with DNA, as well as with naked DNA (i.n. and i.m.), uncoated liposomes (i.n.) and conventional alum-based vaccine (i.m.). Immunization with the novel delivery system resulted in lower anti-HBsAg titers compared to intramuscular immunizations with either plain DNA or conventional vaccine. Nevertheless, all mice developed protective levels of antibodies after a couple of weeks. On the other hand, only glycol chitosan-treated mice had high levels of sIgA in nasal, salivary and vaginal secretions, while i.m. immunization failed to elicit any significant mucosal immune response. In addition, glycol chitosan-coated liposomes induced high levels of IFN-γ and IL-2, indicative of strong cell-mediated immune response with a Th1-like profile (Table I). In all immunological assays, coated liposomes tended to produce superior responses compared to uncoated ones, emphasizing the idea that mucoadhesive properties of chitosan are important for i.n. administration. As expected, recombinant alum-based vaccine induced a strong humoral response but failed to elicit a good mucosal and cell-mediated immune response, which would be important for developing not only a prophylactic vaccine but with therapeutic properties, whereas surface-modified liposome formulation produced an overall better immunological response.

Bilosomes. Lipid vesicles are especially vulnerable to the detergent effect of interstitial bile salts that leads to membrane disruption and vesicle lysis (38). In order to overcome this problem, Margaret Conacher and coworkers developed bilosomes (39). The preparation of bilosomes is based on nonionic surfactant vesicle (NISV) technology, which involves the generation of liposome-like structures of extremely low toxicity that are capable of stimulating humoral and cellular immune responses and include bile salts in their formulation, which stabilize vesicles and prevent premature release of the antigen (Fig. 3b) (39,40).

Bilosomes showed potential as carrier systems for oral immunization (39,41,42) and, therefore, were tested to develop an oral vaccine for hepatitis B (43). They proved to be stable in simulated gastric fluid (SGF), as well as in simulated intestinal fluid (SIF) and at different bile salt concentrations. Antibody titers after oral immunization (Table I) were compared with titers obtained after i.m. immunization. Results showed that mice receiving the

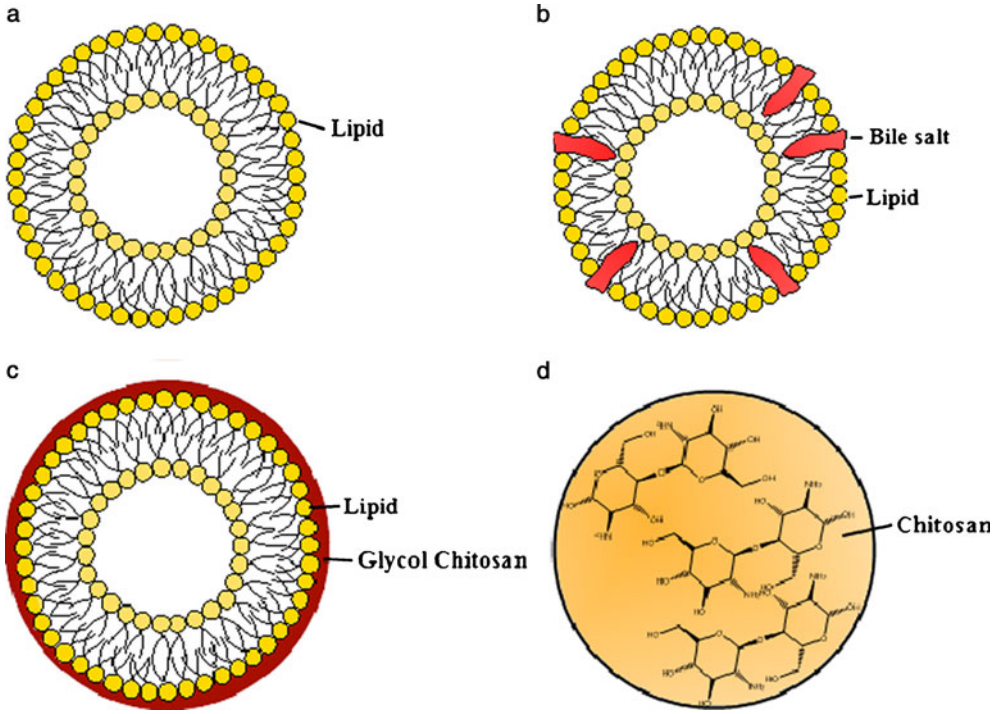

Fig. 3 Some delivery systems tested for mucosal immunization against hepatitis B. **a**: liposome; **b**: bilosome; **c**: modified liposome; **d**: chitosan nanoparticle.

highest oral antigen dose (50 μg) achieved anti-HBsAg titers similar to ones obtained with the alum-based vaccine. Moreover, the authors showed that all orally administered formulations generated higher sIgA levels, while conventional alum-based vaccine failed to elicit a mucosal immune response. Despite the high HBsAg dose administered, the results revealed the potential of this formulation for HBsAg oral vaccination.

Mannosylated Niosomes. Niosomes are uni- or multilamellar vesicles formed from synthetic, non-ionic surfactants of the alkyl or dialkyl polyglycerol ether class, offering an alternative to liposomes as drug carriers because of their increased chemical stability (44). The adjuvant capacity of these non-ionic surfactant vesicles has been shown for a long time, (45,46), but they have only recently been used to create a new delivery system for a HBV oral vaccine. In order to overcome the problem associated with the vulnerability of these vesicles to bile salt and enzymatic degradation (47), Jain and coworkers improved the plain niosome formulation by coating them with O-palmitoyl mannan (OPM), which confers protection and targeting specificity to the mannose receptors expressed on macrophages and dendritic cells (47). Plain and coated niosomes were compared in terms of their stability in both SGF and SIF, demonstrating that the uncoated formulation released a significantly higher amount of encapsulated DNA. Immunological response was assayed after oral immunization with naked DNA, pDNA-loaded plain and OPM-coated niosomes. DNA vaccines require more time to elicit higher antibody levels because DNA needs to be transfected and translated before the antigen is expressed. OPM-coated niosomes induced higher anti-HBsAg titers compared to plain niosomes, possibly because of the protection and targeting effect conferred by the OPM coating. In spite of the higher doses of pDNA administered (100 μg), when compared with that used in previous studies, mucosally applied formulations induced statistically lower antibody levels compared to i.m. immunization. In contrast, and similar to what was observed in previous studies, i.m. formulations failed to elicit detectable sIgA levels, while OPM-coated and plain niosomes efficiently induced the release of sIgA. Cellular responses (IL-2 and IFN-γ levels) were only present in mice vaccinated with DNA vaccines.

Nanoemulsion. A nanoemulsion-based hepatitis B vaccine was recently tested on nasal mucosa (48). Nanoemulsions are water-in-oil formulations stabilized by small amounts of surfactant. They proved to have wide biocidal efficacy against bacteria, enveloped viruses, and fungi (49), and their potential as non-toxic mucosal adjuvants for other antigens has been demonstrated (50–53). Researchers evaluated the immune response obtained with the vaccine formulation composed of a mixture of 20 μg recombinant hepatitis B surface antigen and nanoemulsion (NE) (HBsAg-NE). Vaccine adjuvanted with aluminum hydroxide (intramuscular) was used as a positive control. Both vaccines produced equivalent protective levels of anti-HBsAg IgG, but the alum-based vaccine produced higher levels of IgG1 subclass antibodies associated with a Th2 response, while HBsAg-NE produced mostly IgG2b (and some IgG2a antibodies) related to a Th1 response. Mucosal and cellular

responses were characterized in bronchioalveolar lavage (BAL). The nanoemulsion-based vaccine induced considerable mucosal immunity as well as high production of Th1-type cytokines (IFN-γ and TNF-α). The immunogenicity of the vaccine was also tested in rats and guinea pigs, with both species developing high anti-HBsAg IgG titers. Authors claimed that HBsAg-NE is a safe, stable and effective mucosal adjuvant.

Cationic Particles. Researchers tested the ability of supramolecular biovector (SMBV™) cationic nanoparticles to work as delivery systems for HBsAg (54). SMBVs™ particles were first described in the mid-1990s (55), and they are composed of a hydrophilic internal core surrounded by a lipophilic external layer (56,57). This structure allows the entrapment of various substances, such as antigens, facilitating their delivery to APCs. SMBVs™ were found to be highly muco-resident particularly in nasal mucosa (58). In order to test the potential of SMBVs™ nanoparticles, humoral, mucosal and cellular immune responses were evaluated following intranasal vaccination of mice with different antigen doses (1, 3, 10 μg) either alone or in association with SMBV™ nanoparticles. After three immunizations, all SMBV™ formulations produced high levels of specific serum IgG in contrast to free HBsAg, which elicit small antibody titers. Similar results were obtained with sIgA antibodies and CTL response. Anti-HBsAg IgG1 and IgG2 isotypes were determined by the same authors demonstrating a mixed Th1/Th2 profile.

Chitosan Nanoparticles. The term *chitosan* is applied to a family of deacetylated chitins and is the only largely available cationic polysaccharide. It has been considered a non-toxic, biodegradable and biocompatible polymer (Fig. 4) (59), so extensive research has been directed towards its use in medical applications such as drug and vaccine delivery (60–63). One major advantage of this polymer is its ability to easily produce nanoparticles under mild conditions without the application of harmful organic solvents. This has been one of the main reasons for its wide applicability to the encapsulation of different molecules such as DNA and antigens. Chitosan is also known to be mucoadhesive (37). At physiological pH, sialic acid molecules present in mucus have a negative charge, and, as a consequence, positively charged chitosan exerts strong electrostatic interaction with them. Moreover, its ability to stimulate cells of the immune system has been shown in numerous studies (64–67). These unique features make chitosan an attractive polymer to create a novel delivery system for a mucosal vaccine against HBV (Fig. 3d).

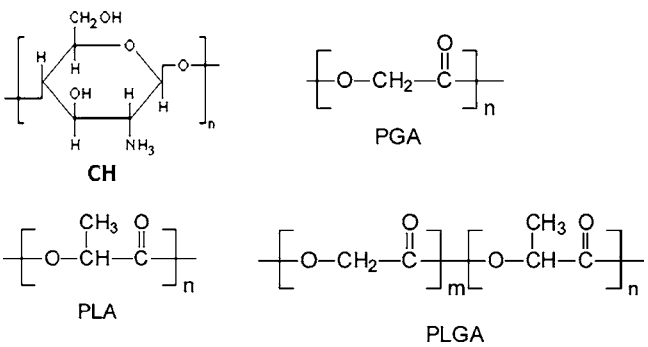

Fig. 4 Some polymers tested for the development of a needle-free delivery system against HBV. Chitosan (CH), poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and poly(lactide-co-glycolic acid) (PLGA).

Alginate-coated chitosan nanoparticles were synthesized and tested for their ability to work as a delivery system for mucosal immunization against HBV (68–70). Alginate is a biodegradable and biocompatible natural polysaccharide polymer with negative charge that demonstrated modifying antigen release from chitosan nanoparticles. The delivery system is composed of a chitosan core, to which the antigen was adsorbed, and was subsequently coated with sodium alginate, all under mild conditions. To further improve the immunological capacities of the novel delivery system, CpG motifs were added to the formulation. Alginate-coated chitosan nanoparticles have been shown to be non-toxic, and their ability to be taken up by M-cells of Peyer's patches was demonstrated in rats (71). Immunological studies were conducted in different treatment groups. Only some mice orally immunized with alginate-coated nanoparticles loaded with 10 μg of HBsAg, with or without the addition of CpG (10 μg), were able to elicit protective antibody levels after a booster dose. The addition of CpG seemed to be beneficial, as it augmented the number of responder mice, but only when the immunopotentiator was encapsulated inside the nanoparticles. IgG subclass profiling revealed that immunization with HBsAg-loaded nanoparticles induced a Th2-like profile, or a mixed Th1/Th2-like response after boost. On the other hand, when CpG motifs were co-administered while inside the nanoparticles a Th1-like profile was elicited, due to CpG capacity of re-directing Th bias (72,73). Mice immunized with plain antigen failed to elicit a mucosal immunological response, even when co-administered with CpG ODN, while in all the other treatment groups, detectable sIgA levels were induced.

Subsequent studies were carried out by an other laboratory using a similar delivery system, this time for nasal administration of HBsAg (70). Systemic, mucosal and cellular immune responses were evaluated. Animals were immunized with 15 μl of different formulations. Subcutaneous (s.c.) administration of a current licensed vaccine was used as positive control. Mice parenterally vaccinated elicited the highest anti-HBsAg IgG titers but failed to develop detectable sIgA levels in nasal and vaginal washes,

as well as in feces. Upon nasal immunization, only animals vaccinated with formulations containing CpG were able to produce a systemic immune response, suggesting that an immunopotentiator is required for effective immunization by the nasal route with alginate-coated nanoparticles. Conversely to the previous report, this time higher IgG titers were detected in animal groups nasally immunized with formulations where CpG was not associated with the nanoparticles. Evaluation of IgG subclasses demonstrated a predominant Th2-like response in seroconverted mice vaccinated with the commercial vaccine *versus* a mainly Th1-like immune response elicited by the other two groups. Regarding mucosal immunization, all treatment groups developed quantifiable sIgA levels with the exception of the above-mentioned s.c. immunized and untreated mice, further demonstrating that mucosal immunization was needed to elicit a mucosal immune response. Quantification of IFN-γ showed that all nasally vaccinated animals showed higher interferon production compared to the control group. Overall, the best results were obtained upon nasal administration of chitosan nanoparticles loaded with the antigen plus CpG in solution or with plain HBsAg co-administered with the immunopotentiator in phosphate buffer saline (PBS). Authors considered the results still not fully conclusive and that improvement of the delivery system was required.

A different research group also worked with chitosan nanoparticles but loaded with DNA encoding surface protein of HBV (74). This group also chose the intranasal route of administration, bearing in mind chitosan's mucoadhesive properties. To test the potential of the nanoparticles, immunization studies were conducted, mice being immunized with different formulations and by different routes. Higher anti-HBsAg titers were elicited after i.m. immunization with either recombinant alum-based vaccine or plain DNA in comparison to i.n. immunization with chitosan nanoparticles loaded with DNA or naked DNA. Nevertheless, nasal administration of plasmid DNA-loaded chitosan nanoparticles was able to elicit seroprotective levels in all mice. In contrast, sIgA levels were induced only upon intranasal immunization, while i.m. formulations failed to elicit them. Statistically higher levels were produced in mice immunized with DNA loaded in chitosan nanoparticles rather than DNA alone. Authors suggested that chitosan-DNA nanoparticles adhere to nasal or gastrointestinal mucosa, are more easily transported to MALT and, thus, might be taken up by M-cells and induce strong mucosal immune responses. IFN-γ and IL-2 levels revealed a dominant Th1-like profile, which is advantageous for the eradication of HBV. In general, chitosan nanoparticles loaded with DNA elicited good humoral, mucosal and cellular responses, further demonstrating the potential of this polymer to create an effective delivery system for mucosal immunization against HBV.

Polymers of Lactic and Glycolic Acids. A number of different polymers have been utilized in formulating nanoparticle drug carriers. Among them are the homo- and copolymers of lactic and glycolic acids: poly(lactic acid) (PLA), poly (glycolic acid) (PGA) and poly(lactide-co-glycolic acid) (PLGA) (Fig. 4).

These polymers have several attractive features such as being non-toxic for humans, tissue compatible, and biodegradable and having an adjustable degradation rate (75–78), as well as an adjuvant effect on antibody induction (79), although they are not deprived of disadvantages. The synthesis of particles using these polymers requires the use of organic solvents and high shear stress, which affects the antigen integrity. Also, the low pH caused by the degradation of the polymer affects the antigen (77,80–82). Researchers all over the world are trying to overcome these disadvantages and produce improved and more efficient vaccine delivery systems.

PLGA particles demonstrated good results in eliciting not only systemic but also mucosal immune response after oral delivery to viruses such as HIV (83,84) and rotavirus (85). In this regard, it is important to mention that Xiao-Wen He and coworkers developed PLGA microparticles containing DNA encoding HBsAg for oral immunization (86) and proposed that they act by protecting the antigen from degradation and by promoting a direct delivery to APCs, thus enhancing uptake by APC. They compared antibody (IgG and IgA), IFN-γ and CTL levels after i.m. or oral delivery of naked or PLGA-encapsulated DNA. When orally administered, naked DNA showed minor immunogenic response, because DNA was exposed to the harsh environment of the stomach and thus degraded, demonstrating the need for protection when given by the oral route. A dose of 20 μg of DNA encapsulated in the PLGA microparticles was able to induce HBsAg-specific antibodies to levels comparable to those induced by i.m. injection of 200 μg of naked DNA. Considerably higher levels were induced when DNA loading of the nanoparticles was increased to 200 μg. Also, fecal sIgA was detected in the stool of mice immunized with PLGA-encapsulated DNA in opposition to mice immunized i.m., where no IgA response was detected. These two facts proved that this kind of vesicular carrier is able to elicit both systemic and mucosal immune responses. It is accepted that a Th1-like response is important for the elimination of the virus from infected cells, and it is associated with CTL activity and IFN-γ production (87). Induction of IFN-γ to the antigen was only achieved when mice were immunized orally with PLGA nanoparticles encapsulating the antigen, whereas the levels achieved with i.m. immunization were almost

negligible. Results were similar to the ones obtained with CTL, where a much stronger response was reached by oral immunization rather than by i.m. Overall, the results show the potential of PLGA microparticles for oral vaccination, as they were able to induce mucosal and systemic cellular and humoral responses.

Another approach using PLGA as a polymer for the development of a novel vaccine carrier system involved the development of surface-modified DL-lactide/glycolide co-polymer microspheres with chitosan (80) for nasal administration. Researchers improved previous PLGA formulations by the addition of trehalose, which is a stabilizer of the proteins. As mentioned before, the preparation of PLGA nanoparticles may require the use of organic solvents, which may affect the antigen integrity. Trehalose acted as a shield preventing protein from coming into contact with the solvent. The addition of chitosan to the surface of the PLGA microspheres also has advantages: it resulted in the shift of the zeta potential from negative to positive, which promoted bioadhesion of the positively charged microspheres to the mucin network in the nasal cavity and thus reduced the nasal clearance rate. Serum of mice immunized with different formulations was collected, and anti-HBsAg levels were determined, showing that nasal administration of modified PLGA microspheres with or without cholera toxin B (CTB), after a booster dose, resulted in levels similar to those obtained with alum-HBsAg vaccine injected subcutaneously. Furthermore, when IgA, IL-2 and IFN-γ levels were measured in different secretions (vaginal, nasal, salivary and serum secretions), they were negligible after s.c. immunization with alum-adsorbed HBsAg vaccine. In contrast, modified PLGA microspheres showed significant levels of those compounds. The authors concluded that modified PLGA microspheres had potential as vesicular carriers for HBsAg, as they were able to protect HBsAg until uptake, eliciting good mucosal, cellular and humoral responses after intranasal administration and demonstrating a Th1-like cytokine profile important for the treatment of HBV infections. Nevertheless, they required at least two administrations to be effective. On the other hand, unmodified microspheres were not able to elicit such good results, most likely due to their faster clearance rate. CTB showed no additional benefit.

Rajkannan and co-workers developed a different carrier for hepatitis B oral vaccine using B-cell epitope-loaded poly DL-lactide-co-glycolide (PLG) microparticles (BCEM) (88). B-cell epitopes are antigenic regions of a protein that is recognized by immunoglobulin molecules. Authors highlighted the capacity of PLG microparticles to act as a depot for the antigen, which allows prolonged and pulsatile release of encapsulated antigens. The release profile of the antigen depends on the degradation rate of PLG microparticles that varies according to the physical characteristics of the polymer. BCEM showed a triphasic release profile that researchers found valuable, as it gives primary immunization effects and contributes to the development of a single dose vaccine. BCEM are taken up by M-cells and then transported to Peyer's patches, where they are retained and gradually release the antigen. This retention appears to be essential for persistent stimulation of memory B-cells and for maintaining antibody titers over extended periods of time (89). Immunological tests compared BCEM against B-cell epitope peptide (BCEP) alone. Mice were orally immunized with 100 μg of either BCEM or BCEP, and after a single administration both formulations were able to induce IgG anti-HB antibodies in a similar way. At the onset, BCEP elicited higher IgG titers, reaching a maximum around the fourth week. BCEM IgG titers were initially lower, reaching their maximum by week 6 and then remaining constantly higher than the ones obtained with BCEP, demonstrating that PLG microencapsulation of BCEP leads to slow release of peptide, which elicited a prolonged immune response and ultimately making the need of booster doses unnecessary. After a period of time, mice were i.m. infected with HBsAg, which led to a rapid and vigorous production of antibodies in the group of mice previously immunized with BCEM due to the induction of a secondary immune response. Mice that were immunized with BCEP were only able to elicit a gradual and weaker response.

Another group of researchers proposed a different carrier, also based on PLGA particles (90,91). Lectin anchored-stabilized biodegradable nanoparticles are PLGA nanoparticles with lectin from *Arachis hypogaea* (PNA) anchored to the surface and were shown to present some enhancement as compared to simple PLGA nanoparticles. This vesicular carrier combines two strategies for enhancing immune response. On one hand, PLGA nanoparticles function as a protection of the fragile antigen to the harsh environment, preventing its destruction; on the other hand, lectins target vaccines specifically to M-cells. Lectins are defined as proteins which specifically and reversibly bind carbohydrates. Lectin receptors are expressed on innumerous types of cells, namely a specialized epithelial cell called M-cell, which is responsible for uptake and transport of antigens. Lectins allow specific targeting of the PLGA nanoparticles to these areas of antigen uptake and, thus, have a greater chance of inducing stronger immune responses. These PLGA nanoparticles also had the same drawbacks of previously synthesized PLGA-based carriers. In this regard, researchers used trehalose as a protein stabilizer and also added $Mg(OH)_2$ that acted by neutralizing the acidity within the particles, generated by polyester hydrolysis of PLGA, conferring an overall protection to the antigen. Immunological response was assayed in mice after i.m. and oral administration of different formulations (91).

To evaluate the generated immune response, serum anti-HBsAg, mucosal sIgA and serum IL-2 and IFN-γ levels were determined. Results showed that formulations containing lectin had higher antibody titers, which can be attributed to its targeting capacity. In addition, the formulations containing trehalose and $Mg(OH)_2$ showed higher immune responses, most likely due to the protective capacity of the two agents towards the antigen. Moreover, serum anti-HBsAg titers obtained after oral immunization with the stabilized lectinized nanoparticles were found to be equivalent to those observed after i.m administration of alum-HBsAg. As expected, mucosal IgA levels were negligible for the intramuscular administration. The highest levels of IgA were achieved with the stabilized lectinized nanoparticles. Similar results were obtained with IL-2 and IFN-γ levels, indicative of a Th1 immune profile. Overall, the described results were considered very promising.

Recently, an Indian group described the use of poly (lactic) acid (PLA) to synthesize new vesicular carriers to encapsulate HBsAg for mucosal vaccination against hepatitis B (78). PLA is a well-known polymer, similar to PLGA, with attractive features for the development of vaccine delivery systems (76,78). In this study, researchers used different combinations of PLA with another polymer, poly (ethylene glycol) (PEG) to synthesize copolymers blocks. PEG has been shown to confer mucoadhesion and long circulation properties to PLA (78). Different AB, ABA and BAB (PLA as block-A and PEG as block-B) block-based nanoparticles were prepared and tested to optimize their properties. *In vitro* release profiles of optimized AB, ABA and BAB nanoparticles showed a triphasic release profile, very advantageous for vaccine delivery, as it mimics the three-shot schedule of the conventional vaccine. Immunological studies with the optimized formulations were performed, and results were compared with those of plain PLA nanoparticles and alum-based vaccine. Mice immunized with the conventional alum-based vaccine presented the highest serum anti-HBsAg titers, but required a booster on day 30. Conversely, AB, ABA and BAB formulations only needed a single administration to present comparative results, which may be due to their triphasic release profile *in vitro*. The initial release acted as a primer and resulted in the generation of memory cells; a subsequent release then had the effect of a booster dose that potentiated the IgG titers in the blood. PLA nanoparticles showed the lowest immunological response, probably because of the degradation of the antigen that was not as well protected as in the other formulations. IgA levels were significantly higher in mice nasally vaccinated with copolymer nanoparticles, both at the administration site and distant mucosal surfaces. Intramuscular injection of the conventional vaccine did not elicit significant IgA levels. IgG1 and IgG2 levels were also measured. Block copolymers gave a mix Th1/Th2 response, in contrast to alum-based vaccine that failed to generate Th1 immunity. In general, the results showed the potential of copolymer-based nanoparticles as mucosal vaccine delivery systems, and, among them, BAB nanoparticles were found to be the most promising, being superior to other copolymer-based formulations.

Salmonella. For many centuries, diseases such as smallpox, cholera and diphtheria killed millions of people. There was no way of preventing infectious diseases until the late 1700s, when Edward Jenner developed the first vaccine with a naturally attenuated virus to protect against smallpox infection. Jenner's technique of inoculating with cowpox to protect against smallpox rapidly spread across Europe, but only more than a century later Louis Pasteur established the basis of modern live attenuated vaccine technology. Although these vaccines proved to be effective, there were some safety issues related to the method of preparation and the possibility of it to revert to a virulent form. Over the years, many advances have been made towards a safer vaccine, and a new generation of rational attenuated vaccines was designed.

Bacterial vectors represent an alternative approach to synthetic (non-living) delivery systems for mammalian cell gene and protein delivery. This technology was first described in the early 1980s (92). Attenuated strains can induce immune responses against antigens that are only expressed *in vivo* and are not present in an inactivated vaccine preparation. Moreover, the ability of most attenuated vaccines to replicate in the host results in the elicitation of strong and long-lasting immune responses, which mimic those stimulated by natural infections (93). There are intrinsic difficulties in this approach, namely the expression of virulence factors which are responsible for the main limitation associated with the design of live vaccines. Therefore, the ultimate goal will be the creation of steady immunogenic recombinant strains that express sufficient quantities of antigen *in vivo* and that offer no threat to the vaccinated individual and the environment.

One commonly studied live bacterial vector is *Salmonella*. Recombinant *Salmonella* strains are attenuated and become no longer pathogenic, but retain their immunogenicity, making them attractive carriers to deliver antigens for mucosal immunization (94–96). *Salmonella* invades the host by crossing the intestinal mucosa via M-cells and colonizing GALT (97,98). In fact, a benefit of this bacterial vector is that it replicates and expresses the antigen in the intestine, thus delivering it to the mucosal immune system, where local and systemic humoral and cellular responses are strongly stimulated (93,99,100).

Schodel and coworkers investigated the use of *Salmonella* as a bacterial carrier to create a mucosal vaccine for

hepatitis B (101–110). The group developed expression systems coding for hybrid HBcAg particles displaying epitopes of the preS1 and preS2 regions in non-virulent *S. typhimurium* (mice) and *S. typhi* (humans) vaccine strains and was able to prove that, in animals, recombinant salmonellae induced an immune response against HBV when delivered orally (102,105–107). The same authors also tried to establish which route of administration (oral, nasal, rectal and vaginal) was the best to induce the strongest systemic, as well as mucosal, immunity (108). Results showed that, although *Salmonella* vaccine was successful in eliciting an immune response after immunization by all routes, the nasal route proved to be the most efficient at inducing both systemic and mucosal immune responses. Nevertheless, oral and rectal route were chosen to continue studies in human volunteers (101,110). Results were disappointing when the *S. typhi*-based vaccine failed to elicit good antibody responses in both phase I clinical trials, suggesting that additional work would be required.

Further studies were carried out in animals using *Salmonella*-based vaccines to create a hepatitis B mucosal vaccine. Nardelli-Haefliger *et al.* used attenuated *Salmonella typhimurium* strains expressing the hepatitis B nucleocapsid as a vaccine system (111). They found that nasal administration of attenuated strains to mice was more efficient in inducing antibody response than oral vaccination, but the nasal route resulted in more adverse effects. Interestingly, when reducing the dose, nasal vaccination was still efficient, while oral vaccination was ineffective.

Woo *et al.* and Bo-jian Zheng *et al.* evaluated the feasibility of *S. typhimurium* delivering plasmid-encoded hepatitis B surface antigen (HBsAg) in eliciting effective immune responses in a mouse model (112,113). The immunogenicity of the DNA vaccine carried in *S. typhimurium* was compared to recombinant HBsAg vaccines and naked DNA vaccines (i.m). Results showed that live attenuated *S. typhimurium* DNA vaccine was able to elicit a strong CTL response, considerably better than the one induced by the recombinant protein vaccine. This was not unexpected, since in DNA vaccines part of the antigen generated is cleaved inside the APCs and presented by the MHC class I pathway, while in recombinant HBsAg vaccine the antigen is generally presented via MHC class II pathway. Quite unexpected was the observation of a weak antibody response elicited by the live attenuated *S. typhimurium* DNA vaccine, especially in comparison with the i.m. DNA vaccine. Authors believe that it may be due to the fact that *S. typhimurium* selectively infects MALT and only little HBsAg is presented through the MHC class II pathway. They concluded that the rather deficient humoral but strong cellular response could make the vaccine a possible candidate for a therapeutic vaccine for chronic HBV carriers as well as for non-responders who do not develop an antibody response to the conventional recombinant protein vaccine.

Edible Vaccines. Another interesting approach is the use of transgenic plants as a delivery system for oral vaccines. There are many studies involving the use of plants modified to express HBV surface antigen. These expression systems have some advantages when compared to others, such as low cost of large-scale production, no need for special storage conditions and lack of contamination with animal pathogens (114–116). Some researchers are using plants not only for the HBsAg production, but also as delivery systems for direct human consumption. In addition to the previous advantages, plant-derived vaccines present some benefits over the traditional ones: they are cheaper because there is no need for purification of the antigen, which is a very expensive step (115); the bioencapsulation of the antigen can protect it against enzymatic digestion (114,117); and the administration is easier, since it does not require the use of specialized equipment and trained staff. Only a few plants have already been tested as edible delivery systems for oral immunization against hepatitis B (Table II).

According to Stephen Streatfield, some aspects should be taken into consideration when developing a plant-derived vaccine First, it is necessary to choose the appropriate expression system, because not all plants are good candidates for plant-derived vaccines, since they are unpalatable to humans (114,118). It is also important to choose a resistant plant where the antigen can be stable for long periods of time without requiring cold conservation and which can preferably be dehydrated to avoid rapid protein degradation (114). Second, the antigen concentration must be high enough to achieve protective doses. Oral vaccines, unlike parenteral ones, are exposed to enzymatic degradation, so the oral dose of antigen required may need to be higher to achieve the same effect (114). Third, the antigen levels expressed by different plants should be homogeneous; therefore, we can be sure that we are giving the same dose to different subjects. Finally, the last aspect is the antigen accumulation location within the plant. Expression levels of the antigen may vary among the different plant parts, and since not all plant parts are edible, it is essential that the major accumulation site suits human consumption.

HBsAg was the first viral antigen chosen to be produced in transgenic plants, initially in tobacco (119). Although the antigen could be expressed in this system, the levels of expression were low ($\approx$ 0. 01% of the soluble leaf protein), and the plant tissue was not adequate for eating.

An edible vaccine for hepatitis only appeared years later with the expression of HBsAg in lettuce, as described by Kapusta *et al.* (120). The transgenic lettuce leaves were fed to three human volunteers twice, and results obtained from sera of the volunteers after the first feeding showed no

relevant levels of HBsAg-specific IgG. However, sera collected after the second feeding revealed HBsAg-specific antibodies, and in two volunteers those levels where higher than 10 international units per liter (IU/L), which is indicative of protection in humans. Although this study suggested that humans could be immunized using edible vaccines (120), the expression levels of the antigen in lettuce were again very low, and again the plant tissue was not adequate for large-scale vaccines, as the leaves needed to be immediately processed. Therefore, other options were considered.

Recombinant hepatitis B surface antigen was also expressed in potato tubers (118,121,122). Potatoes have been frequently used as convenient model systems for edible vaccines because transgenic strains are easy to produce (123). In the first study using potatoes as an oral vaccine for HBsAg, (122) mice were fed with 5 g of transgenic HBsAg potato tubers, expressing 1.1 μg HBsAg/g tuber, plus 10 μg cholera toxin (CT), used as an adjuvant. Mice were able to develop an antibody response that reached 73 IU/L three weeks after the last three doses and then declined. At week ten, an intraperitoneal boost using the commercial vaccine (0.5 μg/mouse) was administered, resulting in a clear immunogenic response, most likely due to immune memory cells formed after oral immunization. Authors highlighted that the value of the results was limited by the low amount of HBsAg expressed in potatoes and by the quantity of tubers mice were able to ingest. Although they were able to produce tubers that expressed higher quantities of the antigen, those lines grew badly, suggesting that high levels of HBsAg may be toxic to the plant. Although researchers found that it was necessary to improve those aspects as higher doses of antigen could induce higher antibody levels, the results were promising and paved the way to further studies (121). Immunogenicity of the edible vaccine was compared to that of an orally delivered yeast-derived HBsAg, usually found in commercial vaccines. No serum HBsAg-specific antibodies were found in mice immunized with two doses of 150 μg of yeast derived HBsAg mix with 10 μg CT. An immunogenic response was only elicited when mice were boosted with a subimmunogenic dose of the recombinant vaccine. In contrast, mice fed with 5 g of potatoes (approximately 42 μg HBsAg/dose) and also using 10 μg of CT as an adjuvant were able to elicit a primary antibody response that peaked up to 103 IU/L four weeks after the third dose. Also, a parenteral boost of a subimmunogenic dose of alum-adsorbed yeast-derived rHBsAg vaccine was able to elicit a memory response, but much stronger than the one obtained with the orally delivered yeast-derived vaccine. Authors believe that these results were related to the fact that in potatoes HBsAg is retained within vesicular structures that naturally protect the antigen from degradation in the gut, and, therefore, some of the antigen particles were able to be released near the Peyer's patches and elicit a mucosal immune response. Mice fed with non-transformed potatoes showed no primary or secondary responses. In reverse experiments, mice were first immunized with a single subimmunogenic dose of the recombinant vaccine, which elicited no primary antibody response, and then five weeks later were fed with transgenic tubers plus CT for three weeks, leading to rapid increase of antibody levels. Results might guide towards a new strategy where a single parenteral immunization is combined with additional orally delivered doses of potato edible vaccine. Authors also emphasize that such promising results were only possible in the presence of CT as an adjuvant, since using just the recombinant potato tuber resulted in much lower antibody titers, suggesting the need for an appropriate human mucosal adjuvant for oral delivery and with uncooked potatoes, because boiling the samples before feeding the mice reduced the immunogenicity of the vaccine considerably. This represents a major drawback, as most people find raw potatoes unpalatable.

Phase I clinical trials were further conducted (118). Forty-two previously parenterally vaccinated healthcare workers were enrolled in a placebo-controlled, double blind trial. The volunteers were divided into three groups (Table II). As expected, none of the individuals ingesting non-transgenic potatoes elicited an immune response during the study. On the other hand, 60% of the volunteers that ate transgenic potatoes had their serum anti-HBs titers increased. Despite the promising results, no additional studies with this vaccine have been released.

Other systems were also tested in order to create an improved edible vaccine. One of those was cherry tomatillo (117). Tomatillo is an interesting alternative to potato, as it is a relatively efficient transformation system that can be eaten raw, in contrast to potatoes (123). The expression levels of the antigen varied within different organs of the plant and were found to be much higher in leaves than in other organs, yet levels of recombinant HBsAg per total soluble protein (rHBsAg/TSP) were superior in the fruit than in the leaf. Mice were immunized orally by feeding them with transgenic cherry tomatillo and i.m. boosted in the fourth week with a subimmunogenic dose of the commercial vaccine. Considerable antibody levels were not observed in any of the mice. In contrast, when mice were first immunized with a single shot of commercial vaccine and then weeks later orally immunized with transgenic tomatillo, an immediate strong recall immune response was developed. Authors believe that the absence of response in mice primarily immunized with transgenic tomatillo was due to low levels of antigen expression, and the results were promising, although some issues still need to be addressed. In fact, plant-produced HBV vaccine appeared to be very promising, since it may represent an

economically viable alternative vaccination strategy for developing countries. However, as Edward Rybicki says, "the idealistic vision of a plant-provisioned arsenal of vaccines for poor people may still be far away: it may take commercial exploitation of the lower-hanging fruits to bring in both the production/processing base, and industry and public acceptance of the technology" (124). Possibly, the key event that leads to the decrease of interest in plant-based vaccines was the incident involving ProdiGene Crop. In 2002, regulators found a small amount of "volunteer" corn engineered to express transmissible gastroenteritis virus capsid protein to produce a pig vaccine, growing in soybean and corn fields. The "volunteer" corn had sprouted from seeds left over from a corn field used for the production of pharmaceuticals that was being grown under contract by ProdiGene. As a result, about 500,000 bushels of soybeans and 155 acres of corn plants had to be destroyed. Although a minuscule amount of volunteer transgenic corn would have posed no real danger to the food supply, the Food and Drug Administration put stronger regulations in place to keep transgenic pharmaceutical crops out of food (124).

It is probable that no edible vaccine for human use will be approved any time soon. Researchers are now focusing their interest in plants on creating novel viral expression systems for new HBV vaccines, and some plants have already been tested, including bananas (125), *Nicotiana benthamiana* leaves (126,127), rice seeds (128), *Nicotiana tabacum* and *Arabidopsis thaliana* (129) and lettuce (130).

Delivery Systems for Topical Immunization

According to Mahor, topical immunization (TI), or transcutaneous immunization (TCI), is a novel and needle-free strategy involving vaccine delivery through topical application of antigen and adjuvant(s) directly or via a suitable carrier system on intact skin (131). The skin is a complicated structure, made up of several layers. The superficial region is known as *stratum corneum* and is the primary protective site against percutaneous absorption of compounds. Underlying it is the viable epidermis that is mainly composed of keratinocytes. The innermost layer is the dermis and consists of blood vessels, nerves, elastin fibers, collagen, lymphatic channels, hair follicles, oil and sweat glands that serve as a support for the upper skin layers (16). As mentioned before, the skin is full of antigen-presenting cells, making it an immunologically active site and an attractive vaccination route.

TI is achieved by physical, chemical or vesicular approaches (Table III). The pursuit for a better HBV vaccine led researchers to exploit some of the mechanisms involving these strategies, such as particle-mediated epidermal delivery (132–136) and epidermal powder immunization (137,138) (physical approaches), and niosomes, and highly deformable liposomes, and ethosomes (vesicular approaches).

Particle-Mediated Epidermal Delivery. One approach to skin immunization is particle-mediated epidermal delivery (PMED). PMED is a needle-free technology by which DNA-coated gold microparticles are used to transfect target tissues using a device called *gene gun* (139). The gene gun DNA vaccine strategy utilizes a helium jet to accelerate DNA-coated gold particles into to the epidermis, which is under constant immune surveillance and is the body's first defense against pathogens. This type of transfer method has several advantages: easy and quick procedure of transfection, availability for all types of cells, little volume of DNA, and many target cells hit by a single shot, different from the microinjection method, and higher efficiency of gene transfection rate than attenuated by electroporation or lipofection. However, gene gun immunization also has some disadvantages (140). First, only small quantities of DNA can be loaded onto the golden beads because they can clump when high amounts are used. This can lead to reduced efficiency of the plasmid delivered and consequently result in the need for multiple shots. Also, gold beads are difficult to handle after coating, and, finally, the gene-guns themselves are not simple to use.

In the early 1990s a gene gun mediated-DNA vaccine for HBV was developed by Fuller *et col.* (141). Gene guns were originally designed for plant transformation, but were considered to be a promising way to deliver DNA plasmids encoding HBV antigens directly to cells (136). DNA vaccines are a good alternative to protein vaccines because they are able to induce T-cell response. During the research, Fuller and coworkers found that 95% of the immunized BALB/c mice were able to reach antibody titers higher than 10 IU/L after a boost immunization. Anti-HBsAg antibody titers decreased over time after some weeks but remained high enough during the study time. Tests with the epidermal DNA vaccine were also performed in pigs, mainly because of their similarity to humans in terms of skin physiology. The results demonstrated (Table III) that small doses of DNA vaccine were able to elicit protective levels of HBsAg-specific antibody similar to those obtained with the commercial vaccine (136).

As a result of the potential of gene gun mediated-DNA vaccines, phase I clinical trials were carried out to test the safety, tolerability and immunogenicity of the vaccine in healthy humans (132,134,135).

The first trial involved seven healthy adult volunteers that were hepatitis B core antibody negative (135) (Table III). No significant adverse effects were noticed. One of the volunteers developed a strong antibody response after the

first immunization, probably due to pre-exposure to HBV. All the other subjects, including a previous seropositive individual, failed to elicit humoral responses, most probably due to the low DNA dosage. Four seronegative volunteers later received three doses of the commercial vaccine and developed vigorous antibody responses, suggesting that the hepatitis B DNA vaccine given by a gene delivery system may induce a booster response, but higher doses might be needed to induce a primary immune response.

The second trial involved twelve hepatitis-naïve volunteers divided into three dosage groups (Table III) (132). The gold particles coated with DNA were delivered using a PowderJect™ XR1 device to the inner aspect of the upper arm. After the second immunization, 75% of the volunteers (nine in twelve) had seroconverted, but only three showed protective levels of the antibody. By the time of the third immunization all twelve volunteers had seroconverted with anti-HBsAg levels varying from 10 up to 5000 IU/L. Results suggested that DNA dose only affects the seroconversion rate rather than the geometric mean titer (GMT). Volunteers with larger time intervals between immunizations developed higher seroprotective levels and faster than other individuals. Although it may not be significant, researchers believe that an extended immunization interval is vital for optimal immune induction as also suggested from other studies (142). Some volunteers (the ones whose antibody titers were below 100 IU/L after three months of the last immunization) received a single boost with the commercial vaccine, all successively developing a strong immune response. Cellular response was also evaluated, showing that the DNA vaccine elicited, in some cases, a mixed Th1/Th2-like immune response, but mainly Th1-like response. In terms of safety and tolerability, the adverse effects reported were mild to moderate local inflammation that disappeared within days or weeks.

A clinical trial in subjects of low to non-responsiveness to commercial hepatitis B protein vaccines was conducted to determine if particle-mediated DNA vaccines could be similarly used to overcome vaccine non-responsiveness in humans (133). It is well known that conventional vaccines fail to elicit protective antibody responses in a percentage of vaccine recipients. Using a DNA vaccine against hepatitis B administered by PMED, researchers were able to elicit antibody responses in twelve of the sixteen subjects that had previously failed to acceptably respond to three to nine doses of the commercial vaccine. Although twelve volunteers demonstrated a response to the DNA vaccine, by the end of the study (280 or 392 days, depending on the group the subjects were in) only seven still had protective levels. It is important to highlight that the subjects of the study were pre-selected as being low to non-responders to the conventional vaccine, which could have had an impact on the overall result. This study also showed that a single DNA vaccine dose was sufficient to boost a pre-existing hepatitis B antibody response as subsequent clinical trial support (134).

The latest study served mainly to test the safety and efficacy of a commercial prototype device (ND5.5) for PMED *versus* the previous clinical research device (XR-1) (134). The two devices were shown to be safe and well tolerated with no difference in magnitude of antibody responses, cell-mediated response (CMR) or proportion of responders between them.

The results obtained illustrate the potential of particle-mediated epidermal delivery in eliciting good cellular and humoral immune responses and suggest that it may be an attractive alternative approach for prophylactic vaccination against hepatitis B. Clinical trials are now underway for therapeutic PMED DNA vaccines for HBV (139).

Physical Approaches: Epidermal Powder Immunization. Topical immunization demonstrated good results not only with DNA vaccines but also with conventional sub-unit vaccines (137,138). Epidermal powder immunization (EPI) is very similar to PMED. It is a needle-free technology for delivery of the antigen to the top layers of the skin using helium gas-released gene guns to propel the vaccine powder into the epidermis (143).

Chen and coworkers used EPI in the development of a new hepatitis B vaccine (138), demonstrating its ability to target not only the epidermis but, in particular, the Langerhans cells that later migrated to draining lymph nodes. BALB/c mice were immunized with 2 μg of HBsAg to study the humoral and cellular immune response; mice i.m. injected with 2 μg of HBsAg were used as control. EPI was able to elicit primary and secondary antibody responses with IgG titers similar to the ones obtained after i.m injection. However, EPI induced CTL responses that the control group failed to elicit. Further studies, performed by same group, compared EPI (2 μg HBsAg) with PMED (2 μg DNA) immunization, and both methods were able to induce primary and secondary responses as well as CTL responses in a similar way. The authors believe this to be correlated to the intracellular delivery of the antigen to LCs that may lead to a MHC class-I restricted antigen presentation and to a cross-priming effect by antigens delivered to the keratinocytes (144,145). Overall, the results showed that EPI can be considered as a viable alternative to create not only a prophylactic but also a therapeutic vaccine.

Additional studies from the previous group tried to further improve the vaccine by adding synthetic CpG oligonucleotides (CpG DNA) (137), which present many effects that contribute to their adjuvant activity (146–150). The obtained results suggest that EPI is a better method to deliver HBsAg when compared to unadjuvanted HBsAg and as good as the alum-adjuvanted commercial vaccine,

therefore being able to overcome the need for an adjuvant. Nevertheless, the use of CpG DNA can further induce the immune response. Antibody titers obtained using EPI and CpG DNA in the same formulation were statistically higher than those induced by the same dose of alum-adjuvanted HBsAg and comparable to those induced by i.m. injection of 20 μg HBsAg/50 μg $Al(OH)_3$. Increasing doses of CpG resulted in increasing antibody titers in EPI delivery but not in i.m. injection. The use of CpG DNA also affected the cytokine profile. For EPI, the IgG1/IgG2a ratio correlated inversely to the quantity of CpG present in the vaccine formulation. CpG adjuvanted formulation helped stimulate the production of TNF-α, IFN-γ, IL-6 and IL-12, demonstrating a Th1-like immune profile in contrast to the one revealed by alum-adjuvanted recombinant vaccine.

Vesicular Approaches. Topical immunization via vesicular systems is a subject of increasing interest for a growing number of researchers. Vesicular carriers have the advantage of providing controlled delivery to the target tissue via different pathways such as keratinolytic, transfollicular or pilosebaceous routes.

In the late 1990s, Fan *et al.* described the use of naked plasmid vector of hepatitis B surface antigen for topical immunization via hair follicles (151). The authors showed that an aqueous solution of naked DNA applied topically to the skin was able to induce IgG class antibodies at a titer 34.4% of that involving intramuscular polypeptide vaccination. Even though it is possible to induce an immune response using naked DNA, it has been proven that its uptake by APC is minimal. Also, naked DNA is vulnerable to hydrolysis by enzymes present in the interstitial space and therefore needs to be protected, thus making vesicular carriers an even more appealing approach (131).

Vesicular Approaches: Niosomes. Niosomes can be used as topical carriers for immunoagents aiming at transdermal delivery (21). They are considered a better alternative to liposomes because they are cheap, highly pure, uniform in content, very stable and easy to store (21,131).

The potential of niosomes as efficient vesicular carriers for the delivery of plasmid DNA encoding HBsAg through topical route has been studied (21). Serum antibody titers and interleukin-2 and interferon-γ levels of topical immunized mice with niosomes were compared to those of i.m. (recombinant and DNA vaccines) and topically (with naked DNA and with liposomes) immunized mice. Results from these studies showed that niosomes were capable of inducing strong cellular and humoral responses, although a lower response was observed when compared to i.m. immunization of naked DNA. However, encapsulation of the plasmid DNA within the niosomes resulted in improved immunological responses in comparison to topically applied naked DNA, probably due to the enhanced transport and protection of plasmid DNA across the skin, which leads to a more efficient APC presentation. Niosomes demonstrated to be slightly better than liposomes, and the authors believed that this finding might be explained by the composition of the niosomes. In this regard, the surfactants present in their formulation serve as penetration enhancers by raising the fluidity and reducing the barrier property of stratum corneum. The overall results supported the potential of niosomes as topical vaccine delivery systems.

Vesicular Approaches: Highly Deformable Liposomes. Deformable vesicles like elastic liposomes or transfersomes have been found to be more effective in enhancing drug transport compared to rigid vesicles like conventional liposomes (152). Elastic liposomes are composed of a mixture of lipids and biocompatible membrane softeners (152); the elasticity of the vesicle depends on the combination of these two components (152,153). The advantage of this system is due not only to its ultradeformability but also to the sensitivity to water gradient throughout the skin (154,155). The potential of elastic liposomes as vesicle carriers for HBsAg was first described by Mishra *et al.* (153). These authors prepared and characterized this system and performed *ex vivo* and *in vivo* studies. Mice were immunized topically using HBsAg-loaded elastic liposomes and i.m. by HBsAg injection. IgG levels of both kinds of immunization were very similar, while IgA levels in serum were much higher in mice topically immunized, suggesting the potential of this formulation in enhancing mucosal immunity in addition to systemic response. Elastic liposomes were demonstrated to exhibit good topical carrier characteristics (high entrapment efficiency, enhanced penetration and effective immunoadjuvant properties) (153). Further studies were carried out with murine dendritic cells (DC), which involved evaluation of their *in vivo* uptake of HBsAg-loaded elastic liposomes and capacity of generating a T-cell-dependent immune response (17). Elastic liposomes were quickly internalized by DC and were shown to be non-toxic. A robust Th1-like immune response was generated, with a marked increase of IL-2, IFN-γ and TNF-α levels as compared to control. Th2 cytokines levels also recorded a slight, but not significant, increase.

Another group also worked with elastic vesicles, referring to them as cationic transfersomes (156). These vesicular carriers were prepared using two main components—a cationic lipid (DOTMA) and sodium deoxycholate—that confer transfersomes their ultra-deformable characteristic, which enables them to efficiently transfer molecules across the stratum corneum. Transfersomes were optimized and loaded with DNA encoding HBsAg, and the immune response was studied after different routes of administration

(i.m. HBsAg, i.m. DNA, topical naked DNA, topical optimized transfersome formulation). As expected, mice i.m. immunized with HBsAg elicited initial higher levels of anti-HBsAg that started to decline after the fourth week. On the other hand, mice that received an i.m. injection of DNA encoding HBsAg elicited the highest and well-sustained antibody titers after an initial period of time required for transfection and translation of the antigen. Topically vaccinated mice with the optimized transfersome formulation elicited lower, but clinically protective, levels of antibody. Also, DNA formulations were able to induce a stronger cellular response important to eliminate the virus from the host, with comparable values between the topical and i.m. immunized groups. In all cases, the transfersome formulation proved to be better than naked DNA for topical immunization.

Vesicular Approaches: Ethosomes. Ethosomes are novel and attractive vesicular carriers for topical immunization that were first developed by Touitou *et al.* (157). Essentially, they are lipid vesicles with a high content of ethanol (131), which is a well-known permeation enhancer, since it fluidizes both lipid and bilayer of the stratum corneum, allowing a more effective delivery of active substances through the skin than conventional liposomes (131,158,159).

The potential of ethosomes as a delivery system for topical immunization against hepatitis B was recently assayed by Mishra *et al.* (160). These researchers prepared and characterized antigen-loaded ethosomes, which exhibited a better permeation profile when compared to conventional liposomes and plain antigen and showed to be efficiently internalized by DCs. Pulsed DCs elicited a predominant Th1-like immune response with two- to five-fold increase in IL-2, IFN-γ and TNF-α levels, but no significant increase in Th2-like cytokines levels was reported. Mice immunized with the ethosome formulation induced better and stronger humoral and mucosal immune responses.

Immunopotentiators

Cholera Toxin, Heat-Labile Enterotoxin and Derivates

Two of the most potent immunopotentiators available are the bacterial toxins secreted from *Escherichia coli* and *Vibrio cholerae*, which are called heat-labile enterotoxin (LT) and cholera toxin (CT). They are commonly used in animal models (161–163) and are considered too toxic for human use, because these molecules are respectively responsible for traveler's diarrhea and cholera. To overcome this problem two solutions have been found: the use of the non-toxic B subunit that lacks enzymatic activity, which has led to some diverse results (162,164–167), or the use of detoxified mutants that have little or no enzymatic activity with significantly less toxicity but some adjuvanticity (168–174).

In the year 2001, a Japanese group published an article describing how they tested the use of recombinant cholera toxin B subunit (rCTB) as an adjuvant for an intranasal hepatitis B vaccine (175). Recombinant CTB proved to be an effective mucosal adjuvant for HBsAg (Table IV). Intranasal administration of three doses of HBsAg combined with rCTB showed statistically higher IgG levels when compared to HBsAg alone. Recombinant CTB elevated not only the systemic response but also mucosal immune response in all sites examined (nasal cavity, lungs, saliva, small intestines, large intestines, vagina). Co-administration of rCTB an HBsAg resulted in a mixed Th1/Th2-like immune response.

CpG Motifs

The need to find an alternative to cholera toxin and *Escherichia coli* heat-labile enterotoxin also led to the development of a new class of immunopotentiators, the CpG motifs.

According to Krieg's definition (176), CpG motifs are DNA oligodeoxynucleotides sequences that include an unmethylated cytosine-guanosine sequence and certain flanking nucleotides, which have been found to induce innate immune responses through interaction with Toll-like receptor 9 (TLR9). There are three major classes of CpG ODN—A-, B- and C-classes that are structurally and phenotypically distinct. B-class CpG-ODN has been frequently used in animal studies due to the strong B-cell activation and capacity to induce potent Th1-type immune response. The same B-class CpG has also been shown to be a safe and efficacious vaccine adjuvant in humans (177,178). Although most cell types have the capacity to internalize CpG ODN via endocytosis (146), only those cells that express the TLR9 are activated. In humans, only B-cells and plasmacytoid dendritic cells (pDCs) are able to express the TLR9, whereas in mice, TLR9 is also found on myeloid dendritic cells (mDCs), macrophages and monocytes (179). Within minutes after exposure to CpG ODN, these cells take up the CpG ODN into endosomal compartments where the interaction with the TLR9 occurs (180). This leads to the activation of cell signaling pathways comprehensively described by McCluskie (179).

CpG ODN has been shown to be an effective mucosal adjuvant after the administration to different mucosal surfaces such as respiratory tract (73,181,182), the genito-urinary tract (183) and the gastrointestinal tract (184,185) in combination with different antigens including the hepatitis B antigen (73,186). Co-administration of CpG ODN with other immunopotentiators or delivery systems has been considered to be useful, especially because of being a strong

Th1 profile inducer, which has been shown to be able to dominate the Th2 bias associated with other adjuvants (72,73).

McCluskie *et al.* were the first to describe the use of CpG DNA as a mucosal adjuvant against HBsAg (73). They measured the anti-HBs titers of mice immunized by intranasal inhalation of HBsAg alone or in combination with CT and/or CpG-ODN. Immunization with HBsAg alone resulted in no or low anti-HBs IgG and IgA titers in most mice. On the other hand, mice immunized with HBsAg in combination with CpG induced high anti-HBs IgG titers in all mice, which were comparable to those elicited when CT was used. CpG and CT demonstrated to have a synergistic effect, since the immune response generated was five to ten times higher than with each adjuvant alone. No IgA was detected in mice that received the lower dose of HBsAg, even when CpG or CT were mixed; low IgA titers were detected with CpG/CT that increased after boost. With the higher antigen dose, IgA was detected in the presence of CT and CpG, either alone or in combination. A synergistic effect was also noted by the authors, which was explained by the fact that both CpG and CT activate B-cells, but by different mechanisms (187,188). This effect offers the possibility of using lower doses of CT in addition to CpG to elicit stronger immune responses. CpG and CT induce different IgG isotypes, with CpG having a more Th1-like response and CT a Th2-like response.

The same group further investigated the potential of CpG as a mucosal adjuvant (189). As found before, both systemic (humoral and cellular) and mucosal immune responses were induced following mucosal delivery of HBsAg with CpG and/or CT, but not with the antigen alone; the synergistic effect between CT and CpG was once again reported. In addition, new data showed that higher doses or boosting additionally enhanced the immune response.

Based on the promising results with the CpG when HBsAg was delivered by intranasal immunization (73,189–191), McCluskie and coworkers investigated its potential as an adjuvant for oral immunization (185). The group demonstrated that CpG was an effective oral adjuvant, as it was able to enhance both systemic and mucosal immune responses. Mice were immunized by oral administration of HBsAg alone or in combination with different doses of CpG (Table IV) or CT (10 μg). Immunization with the antigen alone failed to elicit detectable levels of anti-HBs IgG, but induced high antibody levels in combination with CpG. Ten μg of CT could only induce the same or worse immune responses, and similar results were obtained for IgA levels, with no apparent dose-response effect. CpG also enhanced T-cell proliferation and CTL activity, and proved to induce better immune responses when co-administered with CT, as a result of their synergistic action. Even though the combination of CpG with CT allows the use of lower doses of the toxin, these concentrations might still be too toxic for human use. Concerned with this fact, McCluskie *et al.* also tested the immune responses elicited by the use of CT or LT and by their non-toxic version (B subunit of CT and LTK63, respectively) (192), as well as CpG, alone or combined, using small or large volumes. CpG, CT and LT were shown to induce same or higher antibody titres when compared to CTB or LTK63.

To test the possibility that some enzymatic activity is needed to obtain a synergistic effect with CpG, McCluskie *et al.* used different genetically detoxified mutants of LT that have varying levels of residual enzymatic activity (193). Mice were intranasally or orally immunized with HBsAg alone or by co-administrated with CpG ODN and/or LT or its mutants. Although all adjuvants were able to increase Ag-specific immune response, they varied in terms of adjuvant activity. LT, LTR192G and LTA69G elicited strong immune responses (IgG, IgA and CTL). Weaker responses were obtained with the other LT-derivates (LTB, LTE112K and LTS61F). Overall, adjuvants with higher enzymatic activities had the best adjuvant effect in contrast to those with less or no enzymatic activity. An interesting finding was that no synergistic effect was seen when CpG was combined with any of the LT-derivates; only a shift in antibody isotype was observed. When administered alone, CpG ODN gave predominantly Th1-like response, and LT as well as its derivates a Th2-like response, while co-administrated they produced a mix response.

CpG motifs are considered to be a promising tool in the development of better and more potent needle-free HBV vaccines.

CONCLUDING REMARKS

The development of novel vaccine adjuvants with optimized delivery platforms is becoming as important as the development of the novel vaccine itself. Presently, most of the vaccines are given by intramuscular injection, which requires the use of needles that are painful and potentially dangerous, and require expensive trained medical personnel, making them unsuitable for mass vaccination campaigns, especially in developing countries. Moreover, with few exceptions, parenteral immunization failed to induce mucosal immune responses important for protection against some pathogens. Recently, many researchers have focused their interest on needle-free technologies for immunization, including a variety of approaches for mucosal and topical immunization. Although several strategies have been proposed, some with very promising results, there is not an approved needle-free vaccine against HBV so far, most

likely because regulatory entities tend to adopt a cautious approach towards novel adjuvants and administration routes in terms of safety in humans.

This review shows that although much progress has been made regarding the development of needle-free immunization against HBV, optimization of such approach requires further work. In this regard, oral administration of the hepatitis B antigen-loaded particles has proven to be efficient in all the delivery systems described in this review; however, in almost all the cases, three consecutive days of administration were necessary followed by a boost three weeks later. The exception to this regime was presented by a single administration followed by the boost with a formulation that includes the association of an immunopotentiator to the delivery system. This strategy deserves an investment in future research in order to obtain simplified oral immunization regimes. Additionally, the amount of antigen administered by oral route is always high when compared with the traditional parenteral routes, which may be a discouraging issue to bring those delivery systems to clinical trials. More efficient formulations with enhanced adjuvant properties are needed for the mucosal surfaces in order to overcome the high antigen doses normally required. In this regard, one of the strategies would be the association of an immunopotentiator to an optimized delivery system. Optimized delivery systems should not only target antigens to mucosal associated immune system, but also transmit a signal to immune cells to allow the transported antigen to be recognized. The great challenge would, therefore, be to efficiently modulate the oral tolerance mechanism.

It is important to emphasize that the administration of the vaccine by topical route, particularly pDNA-loaded particle-mediated epidermal delivery (PMED) is already in phase 1 clinical trials with very promising results using very small doses of the plasmid, which may constitute an additional attraction for commercial reasons.

ACKNOWLEDGMENTS

The authors are grateful to Portuguese Ministry of Science and Technology for its support with the grant SFRH/BD/64046/2009 and to GlaxoSmithKline Portuguese Foundation for supporting the work of the group in the area of the mucosal hepatitis B vaccine.

REFERENCES

1. Mauss S, Berg T, Rockstroh J, Sarrazin C, Wedemeyer H. Hepatology: a clinical textbook. Duesseldorf: Flying Publisher; 2009.
2. WHO http://www.who.int/mediacentre/factsheets/fs204/en/. accessed: 28-05-2009.
3. Flying Publisher, Mauss S, Berg T, Rockstroh J, Sarrazin C, Wedemeyer H. Hepatology: A clinical textbook. Duesseldorf: Flying Publisher; 2009.
4. Mahoney F, Kane M. Hepatitis B vaccine in "Vaccines third ed." Pensilvania: Saunders Company.
5. Yuen M, Lai C. Treatment of chronic hepatitis B. Lancet Infect Dis. 2001;1(4):232–41.
6. Sitrin R, Wampler D. Hepatitis B vaccines in clinical practice. New York: Dekker, M; 1992.
7. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. Nat Rev Immunol. 2006;6(2):148–58.
8. Goldsby A, Kindt T, Osborne B, Kuby J. Cells and organs of the immune system. Immunology: W H Freeman & Co.; 2002. p. 50.
9. Holmgren J, Czerkinsky C. Mucosal immunity and vaccines. Nat Med. 2005;11(4 Suppl):S45–53.
10. Vajdy M, O'Hagan DT. Microparticles for intranasal immunization. Adv Drug Deliv Rev. 2001;51(1–3):127–41.
11. Kagi D, Hengartner H. Different roles for cytotoxic T cells in the control of infections with cytopathic *versus* noncytopathic viruses. Curr Opin Immunol. 1996;8(4):472–7.
12. Kurts C, Robinson BW, Knolle PA. Cross-priming in health and disease. Nat Rev Immunol. Jun;10(6):403–14.
13. Wieland SF, Chisari FV. Stealth and cunning: hepatitis B and hepatitis C viruses. J Virol. 2005;79(15):9369–80.
14. Baumert TF, Thimme R, von Weizsacker F. Pathogenesis of hepatitis B virus infection. World J Gastroenterol. 2007;13(1):82–90.
15. Cullen SP, Martin SJ. Mechanisms of granule-dependent killing. Cell Death Differ. 2008;15(2):251–62.
16. Combadiere B, Mahe B. Particle-based vaccines for transcutaneous vaccination. Comp Immunol Microbiol Infect Dis. 2008;31(2–3):293–315.
17. Mishra D, Mishra PK, Dubey V, Dabadghao S, Jain NK. Evaluation of uptake and generation of immune response by murine dendritic cells pulsed with hepatitis B surface antigen-loaded elastic liposomes. Vaccine. 2007;25(39–40):6939–44.
18. Toebak MJ, Gibbs S, Bruynzeel DP, Scheper RJ, Rustemeyer T. Dendritic cells: biology of the skin. Contact Dermat. 2009;60(1):2–20.
19. Warger T, Schild H, Rechtsteiner G. Initiation of adaptive immune responses by transcutaneous immunization. Immunol Lett. 2007;109(1):13–20.
20. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature. 1998;392(6673):245–52.
21. Vyas SP, Singh RP, Jain S, Mishra V, Mahor S, Singh P, *et al.* Non-ionic surfactant based vesicles (niosomes) for non-invasive topical genetic immunization against hepatitis B. Int J Pharm. 2005;296(1–2):80–6.
22. Mitragotri S. Immunization without needles. Nat Rev Immunol. 2005;5(12):905–16.
23. Giudice EL, Campbell JD. Needle-free vaccine delivery. Adv Drug Deliv Rev. 2006;58(1):68–89.
24. Borges O, Lebre F, Bento D, Borchard G, Junginger HE. Mucosal vaccines: recent progress in understanding the natural barriers. Pharm Res. Feb;27(2):211–23.
25. Dubois B, Goubier A, Joubert G, Kaiserlian D. Oral tolerance and regulation of mucosal immunity. Cell Mol Life Sci. 2005;62(12):1322–32.
26. Tsuji NM, Kosaka A. Oral tolerance: intestinal homeostasis and antigen-specific regulatory T cells. Trends Immunol. 2008;29(11):532–40.
27. Kiyono H, Fukuyama S. NALT- *versus* Peyer's-patch-mediated mucosal immunity. Nat Rev Immunol. 2004;4(9):699–710.
28. Csaba N, Garcia-Fuentes M, Alonso MJ. Nanoparticles for nasal vaccination. Adv Drug Deliv Rev. 2009;61(2):140–57.

29. O'Hagan DT, Rappuoli R. Novel approaches to vaccine delivery. Pharm Res. 2004;21(9):1519–30.
30. Smyth Templeton N. Liposomal delivery of nucleic acids *in vivo*. DNA Cell Biol. 2002;21(12):857–67.
31. Gregoriadis G, Gursel I, Gursel M, McCormack B. Liposomes as immunological adjuvants and vaccine carriers. J Control Release. 1996;41:49–56.
32. Jaspart S, Piel G, Delattre L, Evrard B. Solid lipid microparticles: formulation, preparation, characterization, drug release and applications. Expert Opin Drug Deliv. 2005;2 (1):75–87.
33. Scalia S, Tursilli R, Sala N, Iannuccelli V. Encapsulation in lipospheres of the complex between butyl methoxydibenzoylmethane and hydroxypropyl-â-cyclodextrin. Int J Pharm. 2006;320:79–85.
34. Trotta M, Cavalli R, Carlotti ME, Battaglia L, Debernardi F. Solid lipid micro-particles carrying insulin formed by solvent-in-water emulsion-diffusion technique. Int J Pharm. 2005;288 (2):281–8.
35. Saraf S, Mishra D, Asthana A, Jain R, Singh S, Jain NK. Lipid microparticles for mucosal immunization against hepatitis B. Vaccine. 2006;24(1):45–56.
36. Khatri K, Goyal AK, Gupta PN, Mishra N, Mehta A, Vyas SP. Surface modified liposomes for nasal delivery of DNA vaccine. Vaccine. 2008;26(18):2225–33.
37. Deacon MP, McGurk S, Roberts CJ, Williams PM, Tendler SJ, Davies MC, *et al.* Atomic force microscopy of gastric mucin and chitosan mucoadhesive systems. Biochem J. 2000;348(Pt 3):557–63.
38. Schubert R, Jaroni H, Schoelmerich J, Schmidt KH. Studies on the mechanism of bile salt-induced liposomal membrane damage. Digestion. 1983;28(3):181–90.
39. Conacher M, Alexander J, Brewer JM. Oral immunisation with peptide and protein antigens by formulation in lipid vesicles incorporating bile salts (bilosomes). Vaccine. 2001;19(20–22):2965–74.
40. Senior K. Bilosomes: the answer to oral vaccine delivery? Drug Discov Today. 2001;6(20):1031–2.
41. Mann JF, Scales HE, Shakir E, Alexander J, Carter KC, Mullen AB, *et al.* Oral delivery of tetanus toxoid using vesicles containing bile salts (bilosomes) induces significant systemic and mucosal immunity. Methods. 2006;38(2):90–5.
42. Singh P, Prabakaran D, Jain S, Mishra V, Jaganathan KS, Vyas SP. Cholera toxin B subunit conjugated bile salt stabilized vesicles (bilosomes) for oral immunization. Int J Pharm. 2004;278(2):379–90.
43. Shukla A, Khatri K, Gupta PN, Goyal AK, Mehta A, Vyas SP. Oral immunization against hepatitis B using bile salt stabilized vesicles (bilosomes). J Pharm Pharm Sci. 2008;11 (1):59–66.
44. Conacher M., Alexander J, Brewer JM. Niosomes as immunological adjuvants. In: Synthetic surfactant vesicles. Singapore: International Publishers Distributors Ltd; 2000.
45. Brewer JM, Alexander J. The adjuvant activity of non-ionic surfactant vesicles (niosomes) on the BALB/c humoral response to bovine serum albumin. Immunology. 1992;75(4):570–5.
46. Brewer JM, Tetley L, Richmond J, Liew FY, Alexander J. Lipid vesicle size determines the Th1 or Th2 response to entrapped antigen. J Immunol. 1998;161(8):4000–7.
47. Jain S, Singh P, Mishra V, Vyas SP. Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against hepatitis B. Immunol Lett. 2005;101(1):41–9.
48. Makidon PE, Bielinska AU, Nigavekar SS, Janczak KW, Knowlton J, Scott AJ, *et al.* Pre-clinical evaluation of a novel nanoemulsion-based hepatitis B mucosal vaccine. PLoS ONE. 2008;3(8):e2954.
49. Hamouda T, Myc A, Donovan B, Shih AY, Reuter JD, Baker Jr JR. A novel surfactant nanoemulsion with a unique non-irritant topical antimicrobial activity against bacteria, enveloped viruses and fungi. Microbiol Res. 2001;156(1):1–7.
50. Bielinska AU, Janczak KW, Landers JJ, Markovitz DM, Montefiori DC, Baker Jr JR. Nasal immunization with a recombinant HIV gp120 and nanoemulsion adjuvant produces Th1 polarized responses and neutralizing antibodies to primary HIV type 1 isolates. AIDS Res Hum Retroviruses. 2008;24 (2):271–81.
51. Donovan BW, Reuter JD, Cao Z, Myc A, Johnson KJ, Baker Jr JR. Prevention of murine influenza A virus pneumonitis by surfactant nano-emulsions. Antivir Chem Chemother. 2000;11 (1):41–9.
52. Myc A, Kukowska-Latallo JF, Bielinska AU, Cao P, Myc PP, Janczak K, *et al.* Development of immune response that protects mice from viral pneumonitis after a single intranasal immunization with influenza A virus and nanoemulsion. Vaccine. 2003;21 (25–26):3801–14.
53. Bielinska AU, Janczak KW, Landers JJ, Makidon P, Sower LE, Peterson JW, *et al.* Mucosal immunization with a novel nanoemulsion-based recombinant anthrax protective antigen vaccine protects against Bacillus anthracis spore challenge. Infect Immun. 2007;75(8):4020–9.
54. Debin A, Kravtzoff R, Santiago JV, Cazales L, Sperandio S, Melber K, *et al.* Intranasal immunization with recombinant antigens associated with new cationic particles induces strong mucosal as well as systemic antibody and CTL responses. Vaccine. 2002;20(21–22):2752–63.
55. Peyrot M, Sautereau AM, Rabanel JM, Nguyen F, Tocanne JF, Samain D. Supramolecular biovectors (SMBV): a new family of nanoparticulate drug delivery systems. Synthesis and structural characterization. Int J Pharm. 1994;102(1–3):25–33.
56. De Miguel I, Ioualalen K, Bonnefous M, Peyrot M, Nguyen F, Cervilla M, *et al.* Synthesis and characterization of supramolecular biovector (SMBV) specifically designed for the entrapment of ionic molecules. Biochim Biophys Acta. 1995;1237(1):49–58.
57. von Hoegen P. Synthetic biomimetic supra molecular Biovector (SMBV) particles for nasal vaccine delivery. Adv Drug Deliv Rev. 2001;51(1–3):113–25.
58. Kravtzoff RFA, De Miguel I, Perkins A, Major M, Betbeder D, *et al.* Nasal residence time evaluation of cationic biovector in human volunteers. Proc Int Symp Control Release Bioact Mater. 1998;25:818–9.
59. van der Lubben IM, Verhoef JC, Borchard G, Junginger HE. Chitosan for mucosal vaccination. Adv Drug Deliv Rev. 2001;52 (2):139–44.
60. Baldrick P. The safety of chitosan as a pharmaceutical excipient. Regul Toxicol Pharmacol. Apr;56(3):290–9.
61. Panos I, Acosta N, Heras A. New drug delivery systems based on chitosan. Curr Drug Discov Technol. 2008;5(4):333–41.
62. Shaji J, Jain V, Lodha S. Chitosan: a novel pharmaceutical excipient. Int J Pharm Appl Sci. 2010;1(1):11–28.
63. Kang ML, Cho CS, Yoo HS. Application of chitosan microspheres for nasal delivery of vaccines. Biotechnol Adv. 2009;27 (6):857–65.
64. Shibata Y, Foster LA, Metzger WJ, Myrvik QN. Alveolar macrophage priming by intravenous administration of chitin particles, polymers of N-acetyl-D-glucosamine, in mice. Infect Immun. 1997;65(5):1734–41.
65. Babensee JE, Paranjpe A. Differential levels of dendritic cell maturation on different biomaterials used in combination products. J Biomed Mater Res A. 2005;74(4):503–10.
66. Borges O, Borchard G, de Sousa A, Junginger HE, Cordeiro-da-Silva A. Induction of lymphocytes activated marker CD69

following exposure to chitosan and alginate biopolymers. Int J Pharm. 2007;337(1–2):254–64.
67. Zaharoff DA, Rogers CJ, Hance KW, Schlom J, Greiner JW. Chitosan solution enhances both humoral and cell-mediated immune responses to subcutaneous vaccination. Vaccine. 2007;25(11):2085–94.
68. Borges O, Tavares J, de Sousa A, Borchard G, Junginger HE, Cordeiro-da-Silva A. Evaluation of the immune response following a short oral vaccination schedule with hepatitis B antigen encapsulated into alginate-coated chitosan nanoparticles. Eur J Pharm Sci. 2007;32(4–5):278–90.
69. Borges O, Borchard G, Verhoef JC, de Sousa A, Junginger HE. Preparation of coated nanoparticles for a new mucosal vaccine delivery system. Int J Pharm. 2005;299(1–2):155–66.
70. Borges O, Cordeiro-da-Silva A, Tavares J, Santarem N, de Sousa A, Borchard G, *et al.* Immune response by nasal delivery of hepatitis B surface antigen and codelivery of a CpG ODN in alginate coated chitosan nanoparticles. Eur J Pharm Biopharm. 2008;69(2):405–16.
71. Borges O, Cordeiro-da-Silva A, Romeijn SG, Amidi M, de Sousa A, Borchard G, *et al.* Uptake studies in rat Peyer's patches, cytotoxicity and release studies of alginate coated chitosan nanoparticles for mucosal vaccination. J Control Release. 2006;114(3):348–58.
72. Weeratna R, Brazolot Millan C, McCluskie MJ, Davis HL. CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. FEMS Immunol Med Microbiol. 2001;32(1):65–71.
73. McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. J Immunol. 1998;161(9):4463–6.
74. Khatri K, Goyal AK, Gupta PN, Mishra N, Vyas SP. Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. Int J Pharm. 2008;354(1–2):235–41.
75. Giteau A, Venier-Julienne MC, Aubert-Pouessel A, Benoit JP. How to achieve sustained and complete protein release from PLGA-based microparticles? Int J Pharm. 2008;350(1–2):14–26.
76. Avgoustakis K. Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: preparation, properties and possible applications in drug delivery. Curr Drug Deliv. 2004;1(4):321–33.
77. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly(D, L-lactide-co-glycolide) and its derivatives. J Control Release. 2008;125(3):193–209.
78. Jain AK, Goyal AK, Gupta PN, Khatri K, Mishra N, Mehta A, *et al.* Synthesis, characterization and evaluation of novel triblock copolymer based nanoparticles for vaccine delivery against hepatitis B. J Control Release. 2009;136(2):161–9.
79. Gupta RK, Singh M, O'Hagan DT. Poly(lactide-co-glycolide) microparticles for the development of single-dose controlled-release vaccines. Adv Drug Deliv Rev. 1998;32(3):225–46.
80. Jaganathan KS, Vyas SP. Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant hepatitis B antigen administered intranasally. Vaccine. 2006;24(19):4201–11.
81. Ravi Kumar MN, Bakowsky U, Lehr CM. Preparation and characterization of cationic PLGA nanospheres as DNA carriers. Biomaterials. 2004;25(10):1771–7.
82. Shenderova A, Burke TG, Schwendeman SP. The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins. Pharm Res. 1999;16(2):241–8.
83. Kaneko H, Bednarek I, Wierzbicki A, Kiszka I, Dmochowski M, Wasik TJ, *et al.* Oral DNA vaccination promotes mucosal and systemic immune responses to HIV envelope glycoprotein. Virology. 2000;267(1):8–16.
84. Sharpe S, Hanke T, Tinsley-Bown A, Dennis M, Dowall S, McMichael A, *et al.* Mucosal immunization with PLGA-microencapsulated DNA primes a SIV-specific CTL response revealed by boosting with cognate recombinant modified vaccinia virus Ankara. Virology. 2003;313(1):13–21.
85. Herrmann JE, Chen SC, Jones DH, Tinsley-Bown A, Fynan EF, Greenberg HB, *et al.* Immune responses and protection obtained by oral immunization with rotavirus VP4 and VP7 DNA vaccines encapsulated in microparticles. Virology. 1999;259 (1):148–53.
86. He XW, Wang F, Jiang L, Li J, Liu SK, Xiao ZY, *et al.* Induction of mucosal and systemic immune response by single-dose oral immunization with biodegradable microparticles containing DNA encoding HBsAg. J Gen Virol. 2005;86(Pt 3):601–10.
87. Rico MA, Quiroga JA, Subira D, Castanon S, Esteban JM, Pardo M, *et al.* Hepatitis B virus-specific T-cell proliferation and cytokine secretion in chronic hepatitis B e antibody-positive patients treated with ribavirin and interferon alpha. Hepatology. 2001;33(1):295–300.
88. Rajkannan R, Dhanaraju MD, Gopinath D, Selvaraj D, Jayakumar R. Development of hepatitis B oral vaccine using B-cell epitope loaded PLG microparticles. Vaccine. 2006;24 (24):5149–57.
89. Coombes AG, Lavelle EC, Davis SS. Biodegradable lamellar particles of poly(lactide) induce sustained immune responses to a single dose of adsorbed protein. Vaccine. 1999;17(19):2410–22.
90. Gupta PN, Mahor S, Rawat A, Khatri K, Goyal A, Vyas SP. Lectin anchored stabilized biodegradable nanoparticles for oral immunization 1. Development and *in vitro* evaluation. Int J Pharm. 2006;318(1–2):163–73.
91. Gupta P, Khatri K, Goyal A, Mishra N, Vyas SP. M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B. J Drug Target. 2007;15(10):701–13.
92. Formal SB, Baron LS, Kopecko DJ, Washington O, Powell C, Life CA. Construction of a potential bivalent vaccine strain: introduction of Shigella sonnei form I antigen genes into the galE Salmonella typhi Ty21a typhoid vaccine strain. Infect Immun. 1981;34(3):746–50.
93. Shata MT, Stevceva L, Agwale S, Lewis GK, Hone DM. Recent advances with recombinant bacterial vaccine vectors. Mol Med Today. 2000;6(2):66–71.
94. Curtiss 3rd R, Nakayama K, Kelly SM. Recombinant avirulent Salmonella vaccine strains with stable maintenance and high level expression of cloned genes *in vivo*. Immunol Invest. 1989;18 (1-4):583–96.
95. Fairweather NF, Chatfield SN, Charles IG, Roberts M, Lipscombe M, Li LJ, *et al.* Use of live attenuated bacteria to stimulate immunity. Res Microbiol. 1990;141(7-8):769–73.
96. Darji A, Guzman CA, Gerstel B, Wachholz P, Timmis KN, Wehland J, *et al.* Oral somatic transgene vaccination using attenuated S. typhimurium. Cell. 1997;91(6):765–75.
97. Jones BD, Ghori N, Falkow S. Salmonella typhimurium initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. J Exp Med. 1994;180 (1):15–23.
98. Clark MA, Jepson MA, Simmons NL, Hirst BH. Preferential interaction of Salmonella typhimurium with mouse Peyer's patch M cells. Res Microbiol. 1994;145(7):543–52.
99. Kraehenbuhl JP, Neutra MR. Molecular and cellular basis of immune protection of mucosal surfaces. Physiol Rev. 1992;72 (4):853–79.
100. McDermott MR, Bienenstock J. Evidence for a common mucosal immunologic system. I. Migration of B immunoblasts into intestinal, respiratory, and genital tissues. J Immunol. 1979;122(5):1892–8.

101. Nardelli-Haefliger D, Kraehenbuhl JP, Curtiss 3rd R, Schodel F, Potts A, Kelly S, *et al.* Oral and rectal immunization of adult female volunteers with a recombinant attenuated Salmonella typhi vaccine strain. Infect Immun. 1996;64(12):5219–24.
102. Schodel F, Kelly SM, Peterson D, Milich D, Hughes J, Tinge S, *et al.* Development of recombinant Salmonellae expressing hybrid hepatitis B virus core particles as candidate oral vaccines. Dev Biol Stand. 1994;82:151–8.
103. Schodel F, Will H. Expression of hepatitis B virus antigens in attenuated Salmonellae for oral immunization. Res Microbiol. 1990;141(7-8):831–7.
104. Schodel F, Enders G, Jung MC, Will H. Recognition of a hepatitis B virus nucleocapsid T-cell epitope expressed as a fusion protein with the subunit B of Escherichia coli heat labile enterotoxin in attenuated salmonellae. Vaccine. 1990;8(6):569–72.
105. Schodel F, Milich DR, Will H. Hepatitis B virus nucleocapsid/pre-S2 fusion proteins expressed in attenuated Salmonella for oral vaccination. J Immunol. 1990;145(12):4317–21.
106. Schodel F, Peterson D, Hughes J, Milich DR. A virulent Salmonella expressing hybrid hepatitis B virus core/pre-S genes for oral vaccination. Vaccine. 1993;11(2):143–8.
107. Schodel F, Kelly SM, Peterson DL, Milich DR, Curtiss 3rd R. Hybrid hepatitis B virus core-pre-S proteins synthesized in avirulent Salmonella typhimurium and Salmonella typhi for oral vaccination. Infect Immun. 1994;62(5):1669–76.
108. Hopkins S, Kraehenbuhl JP, Schodel F, Potts A, Peterson D, de Grandi P, *et al.* A recombinant Salmonella typhimurium vaccine induces local immunity by four different routes of immunization. Infect Immun. 1995;63(9):3279–86.
109. Schodel F, Kelly S, Tinge S, Hopkins S, Peterson D, Milich D, *et al.* Hybrid hepatitis B virus core antigen as a vaccine carrier moiety. II. Expression in avirulent Salmonella spp. for mucosal immunization. Adv Exp Med Biol. 1996;397:15–21.
110. Tacket CO, Kelly SM, Schodel F, Losonsky G, Nataro JP, Edelman R, *et al.* Safety and immunogenicity in humans of an attenuated Salmonella typhi vaccine vector strain expressing plasmid-encoded hepatitis B antigens stabilized by the Asd-balanced lethal vector system. Infect Immun. 1997;65(8):3381–5.
111. Nardelli-Haefliger D, Benyacoub J, Lemoine R, Hopkins-Donaldson S, Potts A, Hartman F, *et al.* Nasal vaccination with attenuated Salmonella typhimurium strains expressing the Hepatitis B nucleocapsid: dose response analysis. Vaccine. 2001;19(20–22):2854–61.
112. Woo PC, Wong LP, Zheng BJ, Yuen KY. Unique immunogenicity of hepatitis B virus DNA vaccine presented by live-attenuated Salmonella typhimurium. Vaccine. 2001;19(20–22):2945–54.
113. Zheng B, Woo PC, Ng M, Tsoi H, Wong L, Yuen K. A crucial role of macrophages in the immune responses to oral DNA vaccination against hepatitis B virus in a murine model. Vaccine. 2001;20(1–2):140–7.
114. Streatfield SJ. Oral hepatitis B vaccine candidates produced and delivered in plant material. Immunol Cell Biol. 2005;83(3):257–62.
115. Shchelkunov SN, Salyaev RK, Pozdnyakov SG, Rekoslavskaya NI, Nesterov AE, Ryzhova TS, *et al.* Immunogenicity of a novel, bivalent, plant-based oral vaccine against hepatitis B and human immunodeficiency viruses. Biotechnol Lett. 2006;28(13):959–67.
116. Thanavala Y, Lugade AA. Oral transgenic plant-based vaccine for hepatitis B. Immunol Res. Mar;46(1-3):4–11.
117. Gao Y, Ma Y, Li M, Cheng T, Li SW, Zhang J, *et al.* Oral immunization of animals with transgenic cherry tomatillo expressing HBsAg. World J Gastroenterol. 2003;9(5):996–1002.
118. Thanavala Y, Mahoney M, Pal S, Scott A, Richter L, Natarajan N, *et al.* Immunogenicity in humans of an edible vaccine for hepatitis B. Proc Natl Acad Sci USA. 2005;102(9):3378–82.
119. Mason HS, Lam DM, Arntzen CJ. Expression of hepatitis B surface antigen in transgenic plants. Proc Natl Acad Sci USA. 1992;89(24):11745–9.
120. Kapusta J, Modelska A, Figlerowicz M, Pniewski T, Letellier M, Lisowa O, *et al.* A plant-derived edible vaccine against hepatitis B virus. FASEB J. 1999;13(13):1796–9.
121. Kong Q, Richter L, Yang YF, Arntzen CJ, Mason HS, Thanavala Y. Oral immunization with hepatitis B surface antigen expressed in transgenic plants. Proc Natl Acad Sci USA. 2001;98(20):11539–44.
122. Richter LJ, Thanavala Y, Arntzen CJ, Mason HS. Production of hepatitis B surface antigen in transgenic plants for oral immunization. Nat Biotechnol. 2000;18(11):1167–71.
123. Mason HS, Warzecha H, Mor T, Arntzen CJ. Edible plant vaccines: applications for prophylactic and therapeutic molecular medicine. Trends Mol Med. 2002;8(7):324–9.
124. Rybicki EP. Plant-produced vaccines: promise and reality. Drug Discov Today. 2009;14(1–2):16–24.
125. Sunil Kumar GB, Ganapathi TR, Revathi CJ, Srinivas L, Bapat VA. Expression of hepatitis B surface antigen in transgenic banana plants and NT- I cell line of tobacco. Planta. 2005;222:484–93.
126. Huang Z, Elkin G, Maloney BJ, Beuhner N, Arntzen CJ, Thanavala Y, *et al.* Virus-like particle expression and assembly in plants: hepatitis B and Norwalk viruses. Vaccine. 2005;23(15):1851–8.
127. Huang Z, Santi L, LePore K, Kilbourne J, Arntzen CJ, Mason HS. Rapid, high-level production of hepatitis B core antigen in plant leaf and its immunogenicity in mice. Vaccine. 2006;24(14):2506–13.
128. Qian B, Shen H, Liang W, Guo X, Zhang C, Wang Y, *et al.* Immunogenicity of recombinant hepatitis B virus surface antigen fused with preS1 epitopes expressed in rice seeds. Transgenic Res. 2008;17(4):621–31.
129. Greco R, Michel M, Guetard D, Cervantes-Gonzalez M, Pelucchi N, Wain-Hobson S, *et al.* Production of recombinant HIV-1/HBV virus-like particles in Nicotiana tabacum and Arabidopsis thaliana plants for a bivalent plant-based vaccine. Vaccine. 2007;25(49):8228–40.
130. Marcondes J, Hansen E. Transgenic lettuce seedlings carrying hepatitis B virus antigen HBsAg. Braz J Infect Dis. 2008;12(6):469–71.
131. Mahor S, Gupta PN, Rawat A, Vyas SP. A needle-free approach for topical immunization: antigen delivery via vesicular carrier system(s). Curr Med Chem. 2007;14(27):2898–910.
132. 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(7–8):764–78.
133. Rottinghaus ST, Poland GA, Jacobson RM, Barr LJ, Roy MJ. Hepatitis B DNA vaccine induces protective antibody responses in human non-responders to conventional vaccination. Vaccine. 2003;21(31):4604–8.
134. Roberts LK, Barr LJ, Fuller DH, McMahon CW, Leese PT, Jones S. Clinical safety and efficacy of a powdered Hepatitis B nucleic acid vaccine delivered to the epidermis by a commercial prototype device. Vaccine. 2005;23(40):4867–78.
135. Tacket CO, Roy MJ, Widera G, Swain WF, Broome S, Edelman R. Phase 1 safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. Vaccine. 1999;17(22):2826–9.
136. Fuller JT, Macklin M, Drape B, McCabe D, Swain WF, Haynes JR, et al. Gene-gun-mediated DNA immunization with HBsAg: efficacy in small and large animals. In: Brown F, Burton D, Norrby E, editors. Vaccines 97: molecular approaches to the

control of infectious diseases. Cold Spring Harbor Laboratory Press; 1997. p. 157-61.
137. Osorio JE, Zuleger CL, Burger M, Chu Q, Payne LG, Chen D. Immune responses to hepatitis B surface antigen following epidermal powder immunization. Immunol Cell Biol. 2003;81 (1):52–8.
138. Chen D, Weis K, Chu Q, Erickson C, Endres R, Lively C, *et al.* Epidermal powder immunization induces both Cytotoxic T-Lymphocyte and antibody responses to protein antigens of influenza and hepatitis B viruses. J Virol. 2001;75(23):11630–40.
139. Fuller DH, Loudon P, Schmaljohn C. Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases. Methods. 2006;40(1):86–97.
140. Babiuk LA, Babiuk SL, Baca-Estrada M. Novel vaccines strategies. Advances in Virus Research: Academic Press; 2002. p. 55.
141. Fuller JT, Fuller DH, McCabe D, Haynes JR, Widera G. Gene-gun-mediated DNA immunization with HBV antigens: immune responses in mice, monkeys and pigs. In: Brown F, editor. Vaccines 96 : molecular approaches to the control of infectious diseases. Cold Spring Harbor Laboratory Press; 1996. p. 87.
142. Leitner WW, Seguin MC, Ballou WR, Seitz JP, Schultz AM, Sheehy MJ, et al. Immune responses induced by intramuscular or gene gun injection of protective deoxyribonucleic acid vaccines that express the circumsporozoite protein from Plasmodium berghei malaria parasites. J Immunol. 1997;159.
143. Maa YF, Ameri M, Shu C, Zuleger CL, Che J, Osorio JE, *et al.* Hepatitis-B surface antigen (HBsAg) powder formulation: process and stability assessment. Curr Drug Deliv. 2007;4(1):57–67.
144. Fu TM, Ulmer JB, Caulfield MJ, Deck RR, Friedman A, Wang S, *et al.* Priming of cytotoxic T lymphocytes by DNA vaccines: requirement for professional antigen presenting cells and evidence for antigen transfer from myocytes. Mol Med. 1997;3 (6):362–71.
145. Ulmer JB, Deck RR, Dewitt CM, Donnhly JI, Liu MA. Generation of MHC class I-restricted cytotoxic T lymphocytes by expression of a viral protein in muscle cells: antigen presentation by non-muscle cells. Immunology. 1996;89(1):59–67.
146. Krieg AM, Yi AK, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, *et al.* CpG motifs in bacterial DNA trigger direct B-cell activation. Nature. 1995;374(6522):546–9.
147. Yi AK, Klinman DM, Martin TL, Matson S, Krieg AM. Rapid immune activation by CpG motifs in bacterial DNA. Systemic induction of IL-6 transcription through an antioxidant-sensitive pathway. J Immunol. 1996;157(12):5394–402.
148. Klinman DM, Yi AK, Beaucage SL, Conover J, Krieg AM. CpG motifs present in bacteria DNA rapidly induce lymphocytes to secrete interleukin 6, interleukin 12, and interferon gamma. Proc Natl Acad Sci USA. 1996;93(7):2879–83.
149. Sparwasser T, Koch ES, Vabulas RM, Heeg K, Lipford GB, Ellwart JW, *et al.* Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol. 1998;28(6):2045–54.
150. Davis HL, Weeratna R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol. 1998;160(2):870–6.
151. Fan H, Lin Q, Morrissey GR, Khavari PA. Immunization via hair follicles by topical application of naked DNA to normal skin. Nat Biotechnol. 1999;17(9):870–2.
152. Mishra D, Garg M, Dubey V, Jain S, Jain NK. Elastic liposomes mediated transdermal delivery of an anti-hypertensive agent: propranolol hydrochloride. J Pharm Sci. 2007;96(1):145–55.
153. Mishra D, Dubey V, Asthana A, Saraf DK, Jain NK. Elastic liposomes mediated transcutaneous immunization against Hepatitis B. Vaccine. 2006;24(22):4847–55.
154. Gompper G, Kroll DM. Driven transport of fluid vesicles through narrow pores. Phys Rev E Stat Phys Plasmas Fluids Relat Interdiscip Top. 1995;52(4):4198–208.
155. Cevc G, Blume G. Lipid vesicles penetrate into intact skin owing to the transdermal osmotic gradients and hydration force. Biochim Biophys Acta. 1992;1104(1):226–32.
156. Mahor S, Rawat A, Dubey PK, Gupta PN, Khatri K, Goyal AK, *et al.* Cationic transfersomes based topical genetic vaccine against hepatitis B. Int J Pharm. 2007;340(1–2):13–9.
157. Touitou E, Alkabes M, Dayan N. Ethosomes: novel vesicular carriers for enhanced skin delivery. Pharm Res. 1997;14:S305–6.
158. Williams A. Transdermal and topical drug delivery. Pharmaceutical Press; 2005.
159. Dubey V, Mishra D, Nahar M, Jain NK. Vesicles as tools for the modulation of skin permeability. Expert Opin Drug Deliv. 2007;4(6):579–93.
160. Mishra D, Mishra PK, Dubey V, Nahar M, Dabadghao S, Jain NK. Systemic and mucosal immune response induced by transcutaneous immunization using Hepatitis B surface antigen-loaded modified liposomes. Eur J Pharm Sci. 2008;33(4–5):424–33.
161. Lycke N, Tsuji T, Holmgren J. The adjuvant effect of Vibrio cholerae and Escherichia coli heat-labile enterotoxins is linked to their ADP-ribosyltransferase activity. Eur J Immunol. 1992;22 (9):2277–81.
162. Holmgren J, Lycke N, Czerkinsky C. Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems. Vaccine. 1993;11(12):1179–84.
163. Spangler BD. Structure and function of cholera toxin and the related Escherichia coli heat-labile enterotoxin. Microbiol Rev. 1992;56(4):622–47.
164. Yamamoto S, Takeda Y, Yamamoto M, Kurazono H, Imaoka K, Yamamoto M, *et al.* Mutants in the ADP-ribosyltransferase cleft of cholera toxin lack diarrheagenicity but retain adjuvanticity. J Exp Med. 1997;185(7):1203–10.
165. Verweija R, Haana L, Holtropa M, Agsteribbea E, Brandsb R, Scharrenburgb G, *et al.* Musosal immunoadjuvant activity of recombinant Escherichia coli heatlabile enterotoxin and its B subunit: induction of systemic IgG and secretory IgA responses in mice by intranasal immunization with influenza virus surface antigen. Vaccine. 1998;16(20):2069–76.
166. Yamamoto S, Kiyono H, Yamamoto M, Imaoka K, Fujihashi K, Van Ginkel FW, *et al.* A nontoxic mutant of cholera toxin elicits Th2-type responses for enhanced mucosal immunity. Proc Natl Acad Sci USA. 1997;94(10):5267–72.
167. Tochikubo K, Isaka M, Yasuda Y, Kozuka S, Matano K, Miura Y, *et al.* Recombinant cholera toxin B subunit acts as an adjuvant for the mucosal and systemic responses of mice to mucosally co-administered bovine serum albumin. Vaccine. 1998;16(2-3):150–5.
168. Komase K, Tamura S, Matsuo K, Watanabe K, Hattori N, Odaka A, *et al.* Mutants of Escherichia coli heat-labile enterotoxin as an adjuvant for nasal influenza vaccine. Vaccine. 1998;16(2-3):248–54.
169. Douce G, Giuliani MM, Giannelli V, Pizza MG, Rappuoli R, Dougan G. Mucosal immunogenicity of genetically detoxified derivatives of heat labile toxin from Escherichia coli. Vaccine. 1998;16(11–12):1065–73.
170. Chong C, Friberg M, Clements JD. LT(R192G), a non-toxic mutant of the heat-labile enterotoxin of Escherichia coli, elicits enhanced humoral and cellular immune responses associated with protection against lethal oral challenge with Salmonella spp. Vaccine. 1998;16(7):732–40.

171. Rappuoli R, Douce G, Dougan G, Pizza M. Genetic detoxification of bacterial toxins: a new approach to vaccine development. Int Arch Allergy Immunol. 1995;108(4):327–33.
172. Fontana MR, Manetti R, Giannelli V, Magagnoli C, Marchini A, Olivieri R, *et al.* Construction of nontoxic derivatives of cholera toxin and characterization of the immunological response against the A subunit. Infect Immun. 1995;63(6):2356–60.
173. Di Tommaso A, Saletti G, Pizza M, Rappuoli R, Dougan G, Abrignani S, *et al.* Induction of antigen-specific antibodies in vaginal secretions by using a nontoxic mutant of heat-labile enterotoxin as a mucosal adjuvant. Infect Immun. 1996;64(3):974–9.
174. Giuliani MM, Del Giudice G, Giannelli V, Dougan G, Douce G, Rappuoli R, *et al.* Mucosal adjuvanticity and immunogenicity of LTR72, a novel mutant of Escherichia coli heat-labile enterotoxin with partial knockout of ADP-ribosyltransferase activity. J Exp Med. 1998;187(7):1123–32.
175. Isaka M, Yasuda Y, Mizokami M, Kozuka S, Taniguchi T, Matano K, *et al.* Mucosal immunization against hepatitis B virus by intranasal co-administration of recombinant hepatitis B surface antigen and recombinant cholera toxin B subunit as an adjuvant. Vaccine. 2001;19(11–12):1460–6.
176. Krieg AM. Therapeutic potential of Toll-like receptor 9 activation. Nat Rev Drug Discov. 2006;5(6):471–84.
177. Cooper CL, Davis HL, Angel JB, Morris ML, Elfer SM, Seguin I, *et al.* CPG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults. AIDS. 2005;19(14):1473–9.
178. Cooper C, Davis H, Morris M, Efler S, Adhami M, Krieg A, *et al.* CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B® HBV vaccine in healthy adults: a double-blind phase I/II study. J Clin Immunol. 2004;24(6):693–701.
179. McCluskie MJ, Weeratna R. Cpg oligodeoxynucletides as vaccine adjuvants in "Immunopotentiators in Modern Vaccines ". Academic Press; 2006.
180. Ahmad-Nejad P, Hacker H, Rutz M, Bauer S, Vabulas RM, Wagner H. Bacterial CpG-DNA and lipopolysaccharides activate Toll-like receptors at distinct cellular compartments. Eur J Immunol. 2002;32(7):1958–68.
181. Gallichan WS, Woolstencroft RN, Guarasci T, McCluskie MJ, Davis HL, Rosenthal KL. Intranasal immunization with CpG oligodeoxynucleotides as an adjuvant dramatically increases IgA and protection against herpes simplex virus-2 in the genital tract. J Immunol. 2001;166(5):3451–7.
182. McCluskie MJ, Davis HL. Oral, intrarectal and intranasal immunizations using CpG and non-CpG oligodeoxynucleotides as adjuvants. Vaccine. 2000;19(4–5):413–22.
183. Kwant A, Rosenthal KL. Intravaginal immunization with viral subunit protein plus CpG oligodeoxynucleotides induces protective immunity against HSV-2. Vaccine. 2004;22(23–24):3098–104.
184. Eastcott JW, Holmberg CJ, Dewhirst FE, Esch TR, Smith DJ, Taubman MA. Oligonucleotide containing CpG motifs enhances immune response to mucosally or systemically administered tetanus toxoid. Vaccine. 2001;19(13–14):1636–42.
185. McCluskie MJ, Weeratna RD, Krieg AM, Davis HL. CpG DNA is an effective oral adjuvant to protein antigens in mice. Vaccine. 2000;19(7–8):950–7.
186. McCluskie MJ, Weeratna RD, Payette PJ, Davis HL. Parenteral and mucosal prime-boost immunization strategies in mice with hepatitis B surface antigen and CpG DNA. FEMS Immunol Med Microbiol. 2002;32(3):179–85.
187. Munoz E, Zubiaga A, Merrow M, Sauter N, Huber B. Cholera toxin discriminates between T helper 1 and 2 cells in T cell receptor-mediated activation: role of cAMP in T cell proliferation. J Exp Med. 1990;172:95–103.
188. Klinman D, Yi A, Beaucage S, Conover J, Krieg A. CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFN-ã. Proc Natl Acad Sci USA. 1996;93:2879.
189. McCluskie MJ, Davis HL. CpG DNA as mucosal adjuvant. Vaccine. 1999;18(3–4):231–7.
190. Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine. 1998;16(11-12):1216–24.
191. Horner AA, Ronaghy A, Cheng PM, Nguyen MD, Cho HJ, Broide D, *et al.* Immunostimulatory DNA is a potent mucosal adjuvant. Cell Immunol. 1998;190(1):77–82.
192. McCluskie MJ, Weeratna RD, Davis HL. Intranasal immunization of mice with CpG DNA induces strong systemic and mucosal responses that are influenced by other mucosal adjuvants and antigen distribution. Mol Med. 2000;6(10):867–77.
193. McCluskie MJ, Weeratna RD, Clements JD, Davis HL. Mucosal immunization of mice using CpG DNA and/or mutants of the heat-labile enterotoxin of Escherichia coli as adjuvants. Vaccine. 2001;19(27):3759–68.