

Delivery technologies for human vaccines

Philippe Moingeon, Charles de Taisne and Jeffrey Almond

Aventis Pasteur SA, Research and Development, Marcy l'Etoile, France

There is currently intense research activity aimed at the development of new delivery systems for vaccines. The goal is to identify optimal methods for presenting target antigens to the immune system in a manner that will elicit immune responses appropriate for protection against, or treatment of, a specific disease. Several different approaches to this general goal have been developed, some are empirical and remain poorly understood, others are more rational, being based, for example, on mimicking natural infections *in vivo* or on targeting particular features of the immune system. This article will review three categories of delivery systems: (i) adjuvants and formulations; (ii) antigen vectors, including live attenuated micro-organisms and synthetic vectors; and (iii) novel devices for vaccine administration. The review will be restricted to late stage developments in the field of human vaccination.

Why do we need innovative delivery technologies?

There are currently several factors that are creating pressure to improve delivery systems for vaccines. First, in the current regulatory environment, there is a growing requirement to develop vaccines that are very well defined in molecular terms. Thus, as opposed to using whole-inactivated pathogens presenting a complex range of antigens, most newly developed vaccines are rather based on selected target antigens. In some cases these may be single molecules, or even fragments thereof, derived from an infectious micro-organism, a tumour cell, an allergen or an auto-antigen. The target molecule may be administered as a purified protein or as a peptide(s), or may be expressed from plasmid DNA or a recombinant virus. Often, such molecular vaccines are poorly immunogenic, implying a need for an adjuvant, a specific formulation or a vector system of enhanced immunogenicity¹. Second, although in the past most vaccines have been designed to stimulate antibody responses against surface molecules of bacteria or viruses, new generation vaccines are increasingly designed to elicit cellular immune responses, especially of the Th1 type. Such responses are considered paramount for targeting chronic infectious diseases that may have an intracellular stage

*Correspondence to:
Dr Philippe Moingeon,
Aventis Pasteur SA,
Campus Mérieux, 1541
Avenue Marcel Mérieux,
69280 Marcy l'Etoile,
France*

Table 1 Main categories of adjuvants and formulations evaluated in humans¹⁻¹¹

Adjuvant/formulations	Pathogen (antigen)	Trial results
MINERAL SALTS		
Aluminium salts (hydroxide, phosphate, alum)	Numerous antigens	Licensed for human use. Induction of strong antibody responses ⁴
Calcium phosphate	DT	Was found to be better than Al(OH) ₃ in a booster trial
SBAS-4/ASO4 (alum + MPL)	HBV (HBs antigen), HSV (gD)	Increased antibody titres and lymphoproliferative responses when compared with alum, increased seroconversion rate after 2 immunizations ⁵
EMULSIONS		
MF59 (stabilized squalene/sater)	Flu (split trivalent)	Component of a licensed influenza vaccine. Increase vaccine immunogenicity in young adults and in elderly (HAI titre). Safe (only mild local reactions), even after repeated injections in elderly
	HBV(rPreS2-S)	More immunogenic than alum-adsorbed licensed hepatitis B vaccine
	HSV-2 (rgB + rgD)	Prophylactic vaccination: humoral and cellular immunity after 3 injections is superior to natural immunity after HSV-2 infection. A therapeutic vaccination trial in patients with recurrent genital herpes showed no improvement in rate of recurrence but both severity and duration of 1st outbreak were reduced
	HIV1 (gp120), CMV (rgB)	Improved immunogenicity over alum
MF59 + MTP-PE	Flu (trivalent split), HIV1 (env)	MTP-PE increases reactivity, with no overall improvement in terms of immunogenicity (equivalent to MF59)
QS21 (purified saponin from Quillaja saponaria)	Malaria (SPf), HIV (gp120), melanoma, pneumo conj	Some local reactions. Enhanced antibody responses. Limited cellular responses in humans, despite good results obtained in animal models. QS21 enhances by 2-fold the booster effect (antibody response) of second dose of conjugate polysaccharide vaccine against <i>Neisseria pneumoniae</i> ^{4,5}
SBAS-2/ASO2 (squalene/water + MPL + QS21)	Malaria (RTS,S)	High anti-CSP titres (better than with squalene/water or with MPL + alum) after 3 immunizations. Short-lived protection (less than 6 months) of 7 out of 8 naive individuals against challenge (infected mosquito bites). RTS,S-specific lymphoproliferative and antibody responses but no induction of CD8 ⁺ CTLs ⁷
Incomplete Freund adjuvant (IFA, stabilized water/Drakeol)	HIV-1 (rgp120)	Increased seroconversion rate in seronegative subjects after single immunization (superior to MPL + QS21 or alum). Strong cell-mediated immunity (T-cell proliferation; superior to MPL + QS21), but no CD8 ⁺ CTLs. No detectable neutralizing antibodies against primary isolates
	gp120-depleted inactivated HIV-1	REMUNE vaccine. Increased anti-p24 titres and DTH responses. In seropositive subjects: increased lymphoproliferation and β -chemokine (Rantes, MIP-1 α , MIP-1 β) production following p24 stimulation
Montanide ISA51 (stabilized water/Drakeol)	Melanoma (gp100)	Induction of T-cell responses (evaluated by ELISPOT/IFN- γ production) against gp100 HLA A2 restricted epitopes
Montanide ISA720 (stabilized water/squalene)	HIV-1 (Tat toxoid)	Well tolerated. Increased anti-Tat antibody titres in 100% of the subjects. DTH response and lymphoproliferation to Tat in 50% of the subjects
	Malaria (MSP1, MSP2, Resa AMA1)	Well tolerated (minor local effects – tenderness, swelling and discomfort of use). Low antibody responses (equivalent to alum, despite superior antibody responses observed in animals). Strong lymphoproliferation ⁸

continued on next page

Table 1 (continued) Main categories of adjuvants and formulations evaluated in humans¹⁻¹¹

Adjuvant/formulations	Pathogen (antigen)	Trial results
NATURAL/SYNTHETIC BACTERIAL PRODUCTS		
Monophosphoryl lipid A (MPL)	Various antigens	Well tolerated in humans when administered in association with bacterial antigens or TAAs. Limited increase of cellular responses ⁵
Detox (stabilized squalene/water + MPL + CWS)	Malaria (R32NS18)	Some side-effects in malaria naive individuals (tenderness, induration, oedema + malaise and fever). Induction of anti-CSP antibodies after 3 immunizations (better than alum). Protection of 2/11 naive individuals against challenge with infected mosquitoes ⁹
RC-529 (synthetic MPL-like acylated monosaccharide)	Melanoma cell lysates	Induction of cellular and humoral responses against melanoma associated antigens. Increase in survival in patients with metastatic melanoma. Vaccine (Melacine) has been registered for this indication in Canada
OM-174 (lipid A derivative, <i>E. coli</i>), OM triacyl	HBV (HBs)	Th1 and mucosal adjuvant in mice. Found to enhance, in association with alum, antibody responses against HBs antigen in humans (faster and stronger seroconversion) ⁵
	Malaria (CSP), cancer	OM-174 was found to be safe in a phase I study in cancer patients (i.m. route). OM triacyl adjuvants are synthetic analogues based on a common triacyl motif, which induce maturation of human dendritic cells <i>in vitro</i> ⁵
Holotoxins (CT, PT, LT)	Various antigens	Utilization of detoxified bacterial toxins (mutated toxins or B subunits) devoided of ADP-ribosyltransferase activity. Enhancement of serous and mucosal IgA production. On-going evaluation of CT and LT as adjuvants in patch-based transcutaneous immunization. A flu vaccine with LT mutants is about to be tested intranasally in humans ^{3,5}
CpG oligonucleotides	Hepatitis B (HBs)	Act as potent Th1 adjuvants in mice, chimpanzees and orang utangs. Two phase I trials conducted in humans (in association with alum) have shown enhanced antibody responses against the HBs antigen. CTL responses not documented. Based on the motif and chemical backbone, three classes of oligonucleotides are now defined with respect to their distinct capacity to activate either human B-, NK- or dendritic cells <i>in vitro</i>
IMMUNOADJUVANTS		
Cytokines (IL-2, IL-12, GM-CSF)	TAAs, malaria (CSP, MSP1), hepatitis A and B	Utilization of cytokines as recombinant proteins, with limitations including short biological half-life and some severe toxicity (vascular leak syndrome, hepatotoxicity for IL-2 and IL-12, respectively). Enhancement of antibody responses with GM-CSF. More recently, utilization of recombinant vectors expressing locally (intratumorally) immunostimulatory cytokines (e.g. poxviruses) ¹
Accessory molecules (B7.1)	Colorectal cancer (CEA)	The accessory molecule (B7.1), which provides co-stimulatory signals to T lymphocytes, has been included in association with the CEA antigen within the canarypox vector ALVAC, thereby enhancing cellular responses ¹
PARTICULATE FORMULATIONS		
Liposomes (DMP/Cchol)	Flu (monovalent split)	Well tolerated. No increase in antibody titres (equivalent to vaccine alone). Slight increase in CD8 ⁺ CTL response
DC Chol	<i>H. pylori</i> (urease)	Despite enhanced antibody and Th2/Th1 responses in animal models, no significant enhancement of cellular immune responses in humans ⁵
Virosomes	Hepatitis A, flu	Well tolerated. Rapid seroconversion leading to protective anti-hepatitis A or anti-influenza virus antibodies ¹⁰
ISCOMS (structured complex of saponins and lipids)	Flu (trivalent split), HPV16 (E6/E7)	Increase of influenza-specific CD8 ⁺ CTL response (when compared with flu vaccine alone) ¹¹
PLGA	TT	PLGA particles were shown to elicit Th1 (presentation of CTL epitopes) and Th2 responses in mice. On-going trial with the tetanus toxoid: a difficulty is to prepare GMP-grade PLGA particles under aseptic conditions
CSP, <i>P. falciparum</i> circumsporozoite; CWS, cell wall skeleton from <i>Mycobacterium phlei</i> ; DT, diphtheria toxoid; MTP-PE, muramyl tripeptide dipalmitoyl phosphatidyl ethanolamine; PLGA, poly-(D,L)-lactide-co-glycolic acid; TAAs, tumour associated antigens; TT, tetanus toxoid.		

(associated for example with HIV1, herpes viruses, hepatitis C virus, *Helicobacter pylori*, *Plasmodium falciparum*, *Mycobacterium tuberculosis*), but also for the development of therapeutic vaccines against cancer, autoimmune diseases or allergies². New vaccines are also being developed to elicit mucosal immune responses in humans, for example to protect against pathogens such as influenza virus, HIV1, HSV or human oncogenic or wart-associated papilloma viruses. Unlike most of the traditional vaccines, these efforts require the recruitment of cellular or mucosal immune effector mechanisms and necessitate the exploration of new routes of administration, new formulations, and new adjuvant systems³. Third, improving vaccine administration generally, either for the physician, or more importantly for the customer, towards pain-free and safe needle-less devices is likely to represent a major driver in the future vaccine market.

Adjuvant and formulation systems

Main adjuvant categories

Adjuvants encompass a highly heterogeneous group of substances capable of increasing or modulating humoral and/or cellular immune responses. They include mineral compounds (*e.g.* aluminium hydroxide or aluminium phosphate), water-in-oil or oil-in-water emulsions (*e.g.* incomplete Freund's adjuvant [IFA] or MF59, respectively), chemically or genetically detoxified bacterial toxins, such as the cholera toxin (CT) or lymphotoxin (LT) from *Escherichia coli*, saponins (QuilA, QS21), muramyl di- or tripeptides and derivatives (MTP-PE), copolymers, ISCOMS, cytokines, CpG oligonucleotides, and combinations thereof (Table 1)^{1,4-6}. Some of these adjuvants may facilitate long-term persistence of the antigen at the injection site (the so-called 'depot' effect). Others may target antigen presenting cells (APCs) by presenting antigens in a particulate state, or may specifically elicit the production of a pattern of cytokines relevant to the induction of a Th1 or Th2 response^{1,4}.

Efficient enhancement of antibody responses has been possible in humans for many decades through the use of aluminium salts⁴. By contrast, enhancement of the magnitude and duration of cellular (Th1) immune responses has been more difficult to achieve, even if some lymphoproliferative and, to a much lower extent, cytotoxic T-cell responses have been observed with selected antigen-adjuvant combinations (Table 1)⁶⁻¹¹. To elicit mucosal immunity, many approaches have exploited soluble holotoxins mixed with antigen, such as the holotoxin from *Vibrio cholerae* (CT), from *E. coli* (heat labile LT), or *Bordetella pertussis* (PT). In humans, CT is highly toxic, therefore attempts have

focused on the use of the CTB subunit, which can bind to the widely expressed GM1 ganglioside, but lacks the toxic ADP-ribosyltransferase activity associated with the A subunit. More recently, genetically detoxified toxins, which lack the ADP ribosyltransferase activity but retain most of their adjuvant properties have been developed⁵.

Cytokines such as IL-2, GM-CSF, IL-12 and accessory molecules such as B7.1 have also been tested as immunoadjuvants in humans, mostly in cancer patients, with mixed results, both in terms of safety and immunogenicity (Table 1).

Antigen particulate formulations

Apart from simply admixing the antigen with the adjuvant, formulation strategies may aim to facilitate the capture and the entry of the antigen into antigen presenting cells. For example, formulating T-cell antigens, expressed as peptides, proteins, plasmid DNA or even RNA into cationic liposomes appears to increase CTL responses *in vivo* in animal models^{12,13}. Liposomes are artificial, spherical, closed vesicles which consist of one or more lipid bilayers. Liposome-encapsulated antigens are delivered more efficiently to the cytoplasm of APCs, presumably as a result of membrane fusion. Usually, liposomes are made from ester phospholipids. More recently, polar phospholipids from archaebacteria have also been used, leading to so-called 'archeosomes'¹⁴. The latter are based on regularly branched phytanyl chains, with 20 or 40 carbon length. Archeosomes demonstrate better stabilities to high temperature, alkaline pH, serum proteins, when compared with conventional liposomes. Other formulations being explored include spherulites (multilamellar vesicles made of biocompatible amphiphiles) and transfersomes (highly deformable vesicles which can deliver small molecules non-invasively through the skin)^{13,15}. One liposome-based approach has proven successful in humans: in this approach, antigens derived from the hepatitis A or influenza virus have been incorporated into a mixture of natural and synthetic phospholipids, called virosomes (Table 1). Such vaccines were shown to be well tolerated and to induce both a 100% seroconversion rate and high antibody titres within 2 weeks¹⁰.

Other exploratory approaches consist of attaching the antigen to small particles. Non-ionic block co-polymers synthesized from ethylene oxide and propylene oxide can be produced, with varying surfactant characteristics. Other antigen formulations, based on poly-(L)-lactide or alginate microspheres, appear, in animal models, to enhance immune responses (both antibody and T-cell lymphoproliferative, and mucosal immunity)¹². Such formulations also facilitate phagocytosis and fluid

phase internalization of the antigen by macrophages and dendritic cells, with subsequent transfer into the class I and class II presentation pathways, with up to a 1000–10,000-fold increased efficiency as compared to soluble antigen¹². Microparticulate antigen presentation systems, when given orally, deliver the antigen to the mucosal surface, where they are captured by specialized microfold or M cells, prior to transfer to Peyer's patches, thereby inducing mucosal immunity³. In addition, injectable microsphere formulations containing the target antigen(s) create a controlled-release mechanism allowing the possibility of providing disease protection after a single inoculation.

Towards the rational design of adjuvants and formulations

In the absence of a detailed understanding of their modes of action, the development of adjuvants and formulations has, in the past, been largely empirical. Recent advances in our understanding of the physiology of immune responses, however, promises to pave the way to a more 'rational' design of adjuvants and formulations, most particularly with the aim of eliciting Th1 immune responses^{1,2,6}. Notwithstanding a potential direct effect on T lymphocytes, the central target for Th1 adjuvants/formulations is the APC. Theoretically, Th1 adjuvants and formulations of the future should have the following properties:

- 1 Attract APCs, for example by providing molecular cues mimicking the natural 'danger' signals that seem to be a feature of bacteria and viruses. A range of molecular stimuli providing 'danger signals' to the immune system has recently been identified. These include, double stranded (ds) RNA, LPS, and unmethylated CpG dinucleotides flanked by two 5' purines and two 3' pyrimidines, either from bacteria or of synthetic origin^{1,6}. Most of these molecules appear to function as ligands for Toll-like receptors (TLR3, TLR4 and TLR9, respectively), and can rapidly stimulate immune cells (T-cells, B-cells, NK-cells and macrophages) to produce pro-inflammatory cytokines, including IL-1, IL-6, IL-12, IL-18, TNF- α and IFN- γ .
- 2 Target antigen presenting cells. Formulating antigens to better target antigen presenting cells might be facilitated by the recent identification of a variety of surface receptors expressed preferentially by APCs¹. These include the high affinity receptor for IgGs (Fc γ RI, CD64), mannose/fucose receptors, certain chemokine receptors, scavenger receptors, molecules capable of binding and capturing heat shock proteins, apoptotic bodies and apoptotic cells (*e.g.* CD14, α V β 5, CD36) or endocytic receptors such as the C-type lectin termed langerin. There is now clear evidence that targeting the antigen to such surface receptors allows antigen internalization, and presentation to T-cells in an MHC class I restricted manner (cross-priming)¹².

- 3 Induce dendritic cell maturation. Maturation or 'conditioning' of APCs can be achieved by cross-linking CD40 molecules with CD40L or anti-CD40 antibodies. This leads to enhancement of antigen-presenting functions presumably by mimicking signals associated with T-cell help¹.

Collectively, the recent insights into pro-inflammatory signals have opened the way to a more rational design of immuno-adjuvants, most particularly of Th1 adjuvants^{1,2,6}. Improved knowledge on the biology of dendritic cells and antigen trafficking and processing also provide clues for designing new formulations. Considering the complex and orchestrated series of events leading to an antigen-specific activation of the immune system, it is very unlikely that a single molecule or component will suffice as a Th1 adjuvant. Rather, it appears important to combine various molecules in order to achieve both recruitment, targeting, and activation/conditioning of APCs in the presence of the desired antigen¹.

Vectors

Viral vectors

Based on the observation that viral infection results in the presentation of virus-specific peptides in association with both MHC class I and MHC class II on the surface of infected cells, strategies have been designed to use viruses as immunization vehicles to elicit antigen-specific immune responses. In such approaches, cDNAs encoding one or several antigens, which may be whole or truncated, are inserted into the viral vector. The resulting recombinant viruses are used to infect the vaccinee, with the aim of causing the expression of the selected antigen(s) *de novo* and their subsequent presentation to the immune system.

For vaccination purposes, the ideal viral vector should be safe with respect to disease-causing potential, transmissibility and long-term persistence in the host. It should enable efficient presentation of expressed antigens to the immune system while preferably exhibiting low intrinsic immunogenicity so that it can be administered repeatedly to boost relevant specific immune responses. Indeed, the strong immunogenicity of adenoviral vectors has been a limiting factor for their use in gene therapy of cancer as well as in vaccination protocols requiring repeated administrations of the immunogen. The vector system must also meet criteria that enable its large scale industrialization. These include; efficient growth on a cell substrate acceptable to regulatory authorities; total genetic stability with respect to attenuation and presence of the foreign gene(s), scalability to tens of millions of doses; easy purification of the vector virus away from cellular debris, and stability in the final formulation¹⁵.

Table 2 Main characteristics (indented text) of viral vectors (bold type) used in (or considered for) human studies¹⁵⁻¹⁷**Retroviruses (diploid positive-strand RNA viruses that replicate through a DNA intermediate)**

Only infect proliferating cells, and transfer their genetic information into the genome of the target cell, leading to a risk of insertional mutagenesis. Thus, their utilization as vectors in humans (e.g. lentiviral vectors derived from HIV-1) appears to be limited. Engineered to be replication defective and thus their production involves packaging cell lines expressing viral proteins necessary for their propagation. Prototypic oncoretroviruses such as the Moloney murine leukaemia virus, have been used in humans as gene delivery vectors not only for gene therapy, but also to genetically modify autologous cancer cells in order to create cellular cancer vaccines. Amphotropic retroviruses bind to a ubiquitous phosphate transporter expressed on most dividing mammalian cells. Cell specificity can be modified using ligand-directed targeting of viruses with a modified *env* gene¹⁵

Poxviruses: vaccinia, MVA, NYVAC, ALVAC, fowlpox (large, enveloped viruses containing a linear double-stranded DNA genome)

Only DNA-containing viruses known to replicate within the cytoplasm of infected cells. Can accommodate large size inserts (30 kb), allowing for the engineering of recombinant viruses expressing multiple foreign gene products. The most frequently used poxvirus vectors, have been attenuated strains such as the Wyeth or Copenhagen vaccinia strains, MVA (modified vaccinia Ankara strain), NYVAC (derived from the Copenhagen strain by further deletion of 18 open reading frames encoding molecules implicated in pathogenicity and host-range regulatory functions). These vectors have been shown to be safe in immunocompromised macaques and in human phase I/II clinical trials. They were also able to elicit both humoral and cellular (lymphoproliferative and CTL) responses against antigens such as HIV *env* or *gag*, *P. falciparum* antigens, HPV16 E6/E7, tumour associated antigens (CEA, Muc1, gp100), etc.

Avipox viruses, such as the canarypox vector ALVAC (derived from the Kanapox strain) or the fowlpox virus, do not replicate in human cells. They can, nevertheless, be produced in fairly high yields using primary chicken embryo fibroblasts. These vectors have an excellent safety profile. Also, they elicit less anti-vector immunity than attenuated vaccinia strains and thus can be used for boosting several times¹⁵. No high-affinity receptor for poxviruses has been identified yet, even if the myxoma virus, a poxvirus that induces a lethal systemic disease in rabbits, is using chemokine receptors (*i.e.* CCR1, CCR5, CXCR4) to infect leukocytes

Adenovirus (medium size, icosahedral viruses containing a double-stranded linear DNA genome)

Replicate in the nucleus of infected cells without integration of viral DNA into the host genome. The majority of adult people (*i.e.* ≥ 85%) have strong (or pre-existing) immune responses, both humoral and cellular, against the most common adenovirus serotypes thereby precluding multiple administrations. First-generation adenovirus-based vectors, based on deletion of the E1A and E1B genes and more recently vectors carrying a double E1 + E4 deletion, have been developed. These replication-defective adenoviral vectors can accommodate up to 7.5 kb of foreign genes and can be generated and amplified to high titres (10¹² infectious units/ml) in 293 cells. Adenoviruses bind efficiently to both replicating and non-replicating epithelial cells following attachment of the viral fibre knob to a common receptor for coxsackie B viruses and adenovirus 2 and 5 (CAR)¹⁵

Adeno-associated viral vector (single-stranded DNA virus)

AAV is not associated with any known human disease. It needs a helper virus (e.g. adenovirus or herpes simplex virus) to replicate, has a limited insert capacity (*i.e.* 4.5 kb) and is difficult to produce in large quantities¹⁵. It can stably integrate its genome in infected cells as double-stranded DNA in a site-specific manner in a locus on human chromosome 19. AAV can infect a wide range of cell types including non-dividing cells. Membrane-associated heparan sulphate proteoglycan has been shown to be a receptor for AAV2

Herpes simplex virus (enveloped virus containing a double-stranded DNA)

Replication-incompetent viruses were obtained following deletion of nine HSV-1 immediate early genes (including ICP4), allowing reduced vector cytotoxicity while allowing for the expression of multiple transgenes. In another approach, disabled infectious single-cycle herpes simplex (DISC-HSV) lacking the gene for the essential glycoprotein H (gH) vectors have been engineered, and tested in humans, both as a vaccine against HSV disease and as vehicles for cancer immunotherapy¹⁵

(continued on next page)

Table 2 (continued) Main characteristics (indented text) of viral vectors (bold type) used in (or considered for) human studies¹⁵⁻¹⁷

Alphaviruses: Semliki Forest virus, Venezuelan equine encephalitis virus, (capped and polyadenylated single-stranded positive sense RNA of approximately 12 kb, surrounded by an icosahedral capsid protein shell)

Virus particles carrying replicons based on the viral replicase. This replicase copies the genome into negative strands which are templates for new positive-strand genomes and utilizes a highly active internal promoter to transcribe a sub-genomic mRNA encoding a foreign gene product. The vector replicons lack the virus structural protein genes and, therefore, are incapable of generating virus particles and causing a productive infection. Replicon-based vaccines produced in the form of either RNA, DNA, or infectious particles have been successfully used to elicit broad immune responses in animals, including primates. Alphaviruses are interesting vectors because of their high level of replication and gene expression and their ability to infect a variety of cell types. Furthermore, these suicidal vectors induce cell death and the release of apoptotic bodies that are efficiently taken up by antigen-presenting cells, thus enhancing immune cross-priming¹⁵

Virus-like particles, from human papilloma viruses, rotaviruses, parvoviruses (self-assembling non-replicative capsid proteins)

VLPs based on L1 from HPV16 and HPV11 have been tested in humans as candidate vaccines against papilloma viruses associated with cervical cancer, or genital warts, respectively. Non-infectious papilloma virus-like particles bind and activate dendritic cells, leading to the induction of Th1 immune responses against inserted T-cell epitopes derived from the E7 antigen¹⁶. VLPs based on the VP2/VP6/VP7 capsid proteins (used alone or in combination) from rotavirus can be produced in SF9 insect cells. Such VLPs can be used as a vaccine against human or bovine rotaviruses, but also as a vehicle to express heterologous polypeptides (up to 600 amino acids long). The determination of the 3-D structure of VP6 had led to the identification of external loops which can be manipulated for insertion or substitution by heterologous polypeptides. VLPs from porcine parvoviruses can present heterologous T-cell epitopes and induce CTLs in mouse models. Following uptake by dendritic cells, VLPs induce DC maturation¹⁷

A large number of RNA and DNA viruses have been developed experimentally as vectors although their flexibility and utility varies greatly (Table 2). Several have been based on attenuated virus strains that have themselves been used as vaccines (*e.g.* vaccinia virus, polio virus, yellow fever virus), whereas others have been specifically manipulated to minimize disease-causing potential while maximizing immunogenic potential¹⁵. Some viral vector systems take advantage of natural host restriction. For example, avipox virus-derived vectors which replicate in avian cells are unable to complete a full round of replication in human cells and are, therefore, apathogenic and unable to transmit person-to-person. They are, however, capable of infecting human cells and express the incorporated antigen gene. On the same concept, other viral vectors such as those based on alphaviruses, adeno-associated viruses and some herpes simplex viruses, have been engineered to remove critical genes required for generating completely infectious particles in humans cells¹⁵. These vectors require helper systems for their propagation in the laboratory. It may be, for vaccination purposes, an advantage for viral vectors to be able to infect human APCs, such as dendritic cells or macrophages. Cytopathic viral

vectors can still induce immune responses following infection of APCs, suggesting that these cells retain their capacity to initiate immune responses, at least during the early phase of the infectious cycle. It may be that apoptotic bodies containing antigens and produced as a result of viral infection can be captured by uninfected APCs.

Recently, a category of new potential vectors has emerged, based on viral-like particles (VLPs)^{16,17}. These vectors consist of capsid protein(s) capable of self-assembly into non-infectious viral particles. Heterologous genes can be inserted, usually as fusion protein with capsid proteins. VLPs based on capsid proteins from human papilloma viruses (HPV), parvovirus or rotavirus, have been produced and tested successfully in animal models (Table 2)^{16,17}. Another potentially useful viral vector for vaccination purposes, which can accommodate a large nucleic acid insert, is based on coronavirus. This virus is responsible for respiratory (*i.e.* common cold) and also enteric diseases in humans and can be used specifically as a vector to elicit immune responses at mucosal surfaces. Given, however, that virtually all humans have developed antibodies to these viruses in prior exposure, the interest of coronavirus as a vector in humans remains to be demonstrated.

Bacterial vectors

In addition to viral vectors, live bacteria are also being tested as carrier systems for DNA vaccines. In this approach, attenuated or mutant strains of both Gram-positive or Gram-negative intracellular bacteria can be used to administer DNA vaccines via mucosal surfaces, or as a direct delivery systems to target APCs¹⁸. In this regard, BCG, *Listeria monocytogenes*, *Salmonella typhi*, *S. typhimurium*, or *Shigella flexnerii* can be considered as vectors. After being phagocytosed by APCs, such bacteria can survive inside the cell, by either preventing the fusion of the phagolysosome with lysosomes, or by exiting from the phagosome into the cytosol where they can release the DNA¹⁸. Such DNA can subsequently enter the nucleus and express the encoded antigen, which can be presented by the APC in association with both MHC class I and class II molecules. Importantly, live, but not heat-inactivated, intracellular bacteria also exhibit a capacity to induce a potent maturation of dendritic cells, thereby optimizing the presentation of heterologous antigens¹⁹. When used as vectors *in vivo* to immunize mice, live intracellular bacteria have been shown to elicit both humoral and cellular responses against heterologous bacterial, viral and tumoural antigens, leading to protection against infectious or tumour challenge. Several Gram-negative bacteria were also found to deliver plasmid DNA to human dendritic cells *in vitro*¹⁸. As an alternative to using whole bacteria, attempts are being made to use bacterial proteins and lipoproteins as carriers for T-cell epitopes²⁰: with this

aim, outer membrane proteins (e.g. OmpA from *Klebsiella pneumoniae*, or Opr1 from *Pseudomonas aeruginosa*) and bacterial toxins (e.g. the adenylate cyclase toxin from *Bordetella pertussis*) have been successfully engineered to accommodate peptides representing heterologous T-cell epitopes^{20,21}. These bacterial proteins have the capacity to target the antigens to dendritic cells and to elicit, at least in murine models, strong CTL responses. The utilization of bacterial proteins as carriers for capsular polysaccharides as antigens has also been very successful (see article by Finn elsewhere in this issue).

Other vectors

Plasmid DNA

Vaccines based on plasmid DNA elicit strong antibody and T-cell responses in animal models, including mice and non-human primates. In contrast, when used in humans to immunize against HIV1 or *P. falciparum* antigens, these vaccines failed to elicit antibodies, even if cellular immune responses (CTLs) were detected when using milligram quantities of the vaccine^{22,23}. Currently, attempts to further enhance immune responses elicited by DNA vaccines are focusing on codon optimization in order to enhance expression in eukaryotic cells, formulation with cationic lipids to improve targeting of APCs and cell entry, and design of DNA vaccines co-expressing the antigen with an immunostimulatory cytokine gene². These approaches, which have given some encouraging results in terms of improvement of immunogenicity in animal models, are still unproven in humans. In another approach, microscopic particles have been coated with a plasmid encoding the hepatitis B surface antigen, and administered to seronegative human volunteers through the skin using the Powderject XR1 particle accelerator. The vaccine was well tolerated, but it failed to induce primary antibody responses²⁴. Another trend today is to associate DNA with other vectors, as part of mixed (prime-boost) immunization regimens: associations for example between DNA and poxviruses, including vaccinia or the canarypox ALVAC, appear to be promising in order to induce both antibody and cellular responses in animals, and are now being tested in humans².

Plant-based edible vaccines

New developments in molecular plant virology, including for example *Agrobacterium tumefaciens*-mediated gene transfer, have helped to generate plant-based systems as a means to produce vaccine antigens or even as an immunization vehicle^{25,26}. Antigens such as the hepatitis B surface antigen, the *E. coli* heat-labile enterotoxin, or the rabies virus glycoprotein have been produced in such plant-based systems and shown

to elicit antibodies (including in some systems mucosal IgAs) when fed orally to mice. In humans, feeding of transgenic lettuce expressing HbsAg or of transgenic potatoes expressing *E. coli* LT or the Newcastle virus capsid protein also elicited significant levels of antigen-specific antibodies²⁵. Plants represent cost-effective expression systems to produce large amounts of recombinant proteins. Such expression systems, however, might not be suitable when the vaccine antigen is a glycoprotein. In addition, vaccination through the oral route usually does not elicit strong systemic immune responses in humans.

Dendritic cells and exosomes

Dendritic cells (DCs) are currently being used as an antigen presentation platform for vaccination in cancer patients²⁷. In this approach, DCs are traditionally expanded *in vitro* from monocyte-derived progenitors, and subsequently loaded with tumour-associated antigens in the form of peptides, proteins, recombinant viruses, plasmid DNA, RNA formulated with cationic lipids, or tumour lysates. DC-based cellular vaccines have been tested thus far in humans against the following cancers (and target antigens): B-cell lymphoma (Ig idiotypes), melanoma (MAGE1, MAGE3, MART1, tyrosinase, tumour lysates), bladder cancer (MAGE3), colorectal cancer (CEA), and prostate cancer (PSM-P1, PSM-P2, PSA, PAP)²⁷. Collectively, these vaccines were very well tolerated, and elicited some level of antitumour CTL responses. Partial remissions and disease stabilization were observed in at least a fraction (usually in the range of 10–30%) of the vaccinees. Despite such encouraging results, procedures to prepare and load DCs with antigens remain expensive and cumbersome, making it difficult to apply on a large scale to current clinical practice. One alternative being explored in humans consists in isolating exosomes (subcellular organelles containing both MHC class I and II and T-cell co-stimulatory molecules) from DCs as a basis for a cell-free vaccine²⁸. In animal models, exosomes isolated from tumour peptide-pulsed DCs could prime efficiently *in vivo* cytotoxic T lymphocytes capable of eradicating or suppressing growth of established tumours.

Devices

A number of new needle-free or modified needle devices, which carry potentially a number of advantages over conventional needle injection, are being developed for vaccine administration^{29–31}. Such advantages include increased safety, acceptability and, therefore, treatment compliance, as well as potentially increased efficacy linked to a broader, or modified, tissue distribution of the antigen, ease of use leading to self-administration, administration of smaller doses of the antigen and adjuvants, as well as

Table 3 New devices for vaccine administration²⁹⁻³⁶

Device	Antigen(s)	Comments
Minineedles (e.g. Macroflux microprojection array)	Various antigens	In this system, a titanium microprojection array with an adhesive patch backing is used. The antigen is adsorbed as a powder onto the minineedles and injected subcutaneously (at a depth of about 100 µm) by patch application to the skin
Needle-less injection		
Spring powered (Advantajet, Injex, Vitajet 3, Medi-Jector)	Hepatitis A, flu antigens, hepatitis B	Spring-powered needle-free devices have been initially designed and used in humans for the administration of insulin or growth hormones. Volumes of 20–500 µl can be administered subcutaneously. Changing the orifice size modulates the administration pressure, in relation to differences in the thickness of skin between patients
Gas powered (Biojector 2000, Penjet, J-Tip, Powderject system)	Many DNA plasmids	Gas-powered systems includes nitrogen, CO ₂ or helium gas powered systems, allowing i.m., subcutaneous or intradermal administration. Such systems allow the administration of volumes of up to 1 ml. Although most systems have been designed to administer antigens as a liquid, one system (Powderject) relies on a pre-filled helium-powered system in which dry-powder formulations, stable at room temperature (e.g. plasmid DNA) are precipitated onto small (3 µm diameter) gold particles for administration through the skin
Patches for transcutaneous immunization	<i>Shigella</i> or <i>Salmonella</i> antigens, CS6 (<i>E. coli</i>), LT	The antigen in combination with an adjuvant (e.g. CT or LT) are administered onto hydrated skin. In various animal models, and more recently in humans, this approach was found to elicit antibodies (both IgGs and IgAs), as well as a strong lymphoproliferative response, in the absence of any adverse event ³⁵
Aerosol for delivery of powder vaccines	Measles	In order to produce fine particles without damaging activity of the virus, a live attenuated measles virus is micronized by jet milling to generate particles with the appropriate size for pulmonary delivery (1–5 µm). Particles are blended with an inert carrier to improve aerosol dispersion with a nebulizer ³⁶

delivery via either the mucosal (nasal or oral), subcutaneous or intradermal route^{29,30}.

Table 3 summarizes devices which have been (or are being tested in humans). The Macroflux microneedle system allows administration of the antigen dry-coated onto microneedles. When pressed onto the skin, the microprojections create mechanical pathways through the superficial skin, allowing intracutaneous delivery of the antigen to an average depth of 100 µm. The antigen dose administered can be controlled by the formulation, wearing time, and system size. The largest experience in the field of needle-free delivery to humans has been gained with a variety of jet-injectors able to deliver vaccines by the subcutaneous route^{32,33}. These devices use forces derived from two sources of power, either a spring or compressed gas, to propel the vaccine through the skin. Needle-free injection was found to increase immune responses to both conventional and DNA-based vaccines: for example, seroconversion rates as well as antibody titres elicited in humans by a hepatitis A vaccine or a trivalent influenza vaccine were found to be increased by at least 10% when using needle-free injections, as opposed to needle and

syringe administration³⁴. Further work is required, however, to control the consistency of the pressure of injection to ensure proper delivery of vaccine to various types of skin. Also, the greater depth of administration, the greater discomfort. An advantage of spring-powered devices is that they are usually lighter and smaller than gas-powered devices. They are also more durable and inexpensive. Coiled springs, however, provide only a limited pressure. In contrast, gas-operated devices are more powerful. As such, they allow the administration of larger volumes through both the subcutaneous and intramuscular route. The gas cartridge needs, however, to be replaced regularly, making these usually large devices more costly than systems operated with a coiled spring. With both systems, the antigen can be administered either in a liquid form, or as a powder (*e.g.* adsorbed onto a microscopic gold particle, as in the Powderject system). Both single-dose injectors, but also high-speed multidose injector systems (allowing mass immunization) are being developed.

Recently, transcutaneous immunization strategies have been introduced as an alternative non-invasive administration route³⁵. In this approach, the antigen is topically applied to intact skin, thereby targeting the antigen to Langerhans cells, which will subsequently migrate through the skin into draining lymph nodes to initiate the immune response. Adjuvants usually associated with the antigen include the CT and LT toxins derived from *V. cholera* and *E. coli*, respectively³⁵. When applied to the rehydrated skin of human volunteers using a patch, such vaccines were shown to be well tolerated, and to elicit strong antibody and lymphoproliferative responses against the antigen, such as LT or the CS6 antigen from enterotoxigenic *E. coli*.

Lastly, attempts are being made to develop aerosol delivery of powder vaccine formulations, using a nebulizer³⁶. Advantages would include ease of use, increased safety, dry powder formulation (which would reduce refrigeration requirements), and potential enhanced mucosal immunity.

Key points for clinical practice

1. New delivery systems for human vaccines are being developed to enhance cellular and mucosal immunity, as well as ease of use
2. There is as of today no Th1 adjuvant efficient in humans. Such adjuvants are needed to develop powerful therapeutic vaccines against cancer or chronic infectious diseases
3. Needle-less injection systems being developed include spring or gas-powered devices, transdermal patches, as well as aerosols for delivery of powder vaccines

References

- 1 Moingeon P, Haensler J, Lindberg A. Towards the rational design of Th1 adjuvants. *Vaccine* 2001; **19**: 4363–72
- 2 Moingeon P. Strategies for designing vaccines eliciting Th1 responses in humans. *J Biotech* 2002; In press
- 3 Czerkinsky C, Anjuere F, McGhee J *et al.* Mucosal immunity and tolerance: relevance to vaccine development. *Immunol Rev* 1999; **170**: 197–222
- 4 Cox J, Coulter AR. Adjuvants, a classification and review of their modes of action. *Vaccine* 1997; **15**: 248–56
- 5 Aguado T, Engers H, Pang T, Pink R. Novel adjuvants currently in clinical testing. *Vaccine* 1999; **17**: 2321–8
- 6 Schijns V. Immunological concepts of vaccine adjuvant activity. *Curr Opin Immunol* 2000; **12**: 456–63
- 7 Lalvani A, Moris P, Voss G *et al.* Potent induction of focused Th1-type cellular and humoral immune responses by RTS,S/SBAS2, a recombinant *Plasmodium falciparum* malaria vaccine. *J Infect Dis* 1999; **180**: 1656–64
- 8 Saul A, Lawrence G, Smillie A *et al.* Human phase I vaccine trials of 3 recombinant asexual stage malaria antigens with Montanide ISA720 adjuvant. *Vaccine* 1999; **17**: 3145–59
- 9 Hoffman SL, Edelman R, Bryan JP *et al.* Immunogenicity, and efficacy of a malaria sporozoite vaccine administered with monophosphoryl lipid A, cell wall skeleton of mycobacteria, and squalane as adjuvant. *Am J Trop Med Hyg* 1994; **51**: 603–12
- 10 Ambrosch F, Wiedermann G, Jonas S *et al.* Immunogenicity and protectivity of a new liposomal hepatitis A vaccine. *Vaccine* 1997; **15**: 1209–15
- 11 Ennis FA, Cruz J, Jameson J, Klein M, Burt D, Tiphawong J. Augmentation of human influenza A virus-specific cytotoxic T lymphocyte memory by influenza vaccine and adjuvanted carriers (ISCOMS). *Virology* 1999; **259**: 256–61
- 12 Raychaudhuri S, Rock KL. Fully mobilizing host defense: building better vaccines. *Nat Biotech* 1998; **16**: 1025–31
- 13 Kunisawa J, Nakagawa S, Mayumi T. Pharmacotherapy by intracellular delivery of drugs using fusogenic liposomes: application to vaccine development. *Adv Drug Deliv Rev* 2001; **52**: 177–86
- 14 Patel GB, Sprott GD. Archeobacterial ether lipid liposomes (archeosomes) as novel vaccine and drug delivery systems. *Crit Rev Biotech* 1999; **19**: 317–57
- 15 Bonnet MC, Tartaglia J, Verdier F *et al.* Recombinant viruses as a tool for therapeutic vaccination against human cancers. *Immunol Lett* 2000; **74**: 11–25
- 16 Rudolf M, Fausch S, Da Silva D, Kast M. Human dendritic cells are activated by chimeric human papillomavirus type 16 virus-like particles and induce epitope-specific human T cell responses *in vitro*. *J Immunol* 2001; **166**: 5917–24
- 17 Sedlik C, Saron M, Sarraseca J, Casal I, Leclerc C. Recombinant parvovirus-like particles as an antigen carrier: a novel nonreplicative exogenous antigen to elicit protective antiviral cytotoxic T cells. *Proc Natl Acad Sci USA* 1997; **94**: 7503–8
- 18 Dietrich G, Kolb-Maurer A, Spreng S, Schartl M, Goebal W, Gentschev I. Gram-positive and Gram-negative bacteria as carrier systems for DNA vaccines. *Vaccine* 2001; **19**: 2506–12
- 19 Rescigno M, Granucci F, Citterio S, Foti M, Ricciardi-Castagnoli P. Coordinated events during bacteria-induced DC maturation. *Immunol Today* 1999; **20**: 200–3
- 20 Miconnet I, Coste I, Beerman F *et al.* Cancer vaccine design: a novel bacterial adjuvant for peptide-specific CTL induction. *J Immunol* 2001; **166**: 4612–9
- 21 Guernonprez P, Ladant D, Karimova G, Ullmann A, Leclerc C. Direct delivery of the *Bordetella pertussis* adenylate cyclase toxin to the MHC class I antigen presentation pathway. *J Immunol* 1999; **162**: 1910–6
- 22 Calarota S, Bratt G, Nordlund S, Hinkula J, Leandersson A-C, Wahren B. Cellular cytotoxic responses induced by DNA vaccination in HIV1 infected patients. *Lancet* 1998; **351**: 1320–5
- 23 Wang R, Doolan D, Le Thong P *et al.* Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine. *Science* 1998; **282**: 476–80

- 24 Tacket C, Roy M, Widera G, Swain W, Broome S, Edelman R. Phase I safety and immune response studies of a DNA vaccine encoding hepatitis B surface antigen delivered by a gene delivery device. *Vaccine* 1999; **17**: 2826–9
- 25 Arntzen C. High-tech herbal medicine: plant-based vaccines. *Nat Biotech* 1997; **15**: 221–2
- 26 Koprowski H, Yusibov V. The green revolution: plants as heterologous expression vectors. *Vaccine* 2001; **19**: 2735–41
- 27 Burdin N, Moingeon P. Cancer vaccines based on dendritic cells loaded with tumor-associated antigens. *Cell Biol Toxicol* 2001; **17**: 67–75
- 28 Zitvogel L, Regnault A, Lozier A *et al*. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* 1998; **4**: 594–600
- 29 Cryz S, Davis SS, Gregoriadis G *et al*. European Commission COST/STD Initiative. Report of the expert panel: vaccine delivery systems. *Vaccine* 1996; **14**: 665–90
- 30 Nugent J, Po AL, Scott EM. Design and delivery of non parenteral vaccines. *J Clin Pharm Ther* 1998; **23**: 257–85
- 31 Sheikh NA, al-Shamisi M, Morrow WJ. Delivery systems for molecular vaccination. *Curr Opin Mol Ther* 2000; **2**: 37–54
- 32 Parent du Chatelet I, Lang J, Schlumberger M *et al*, Imule Investigators Group. Clinical immunogenicity and tolerance studies of liquid vaccine delivered by jet-injector and a new single use cartridge (Imule): comparison with standard syringe injection. *Vaccine* 1997; **15**: 449–58
- 33 Jackson LA, Austin G, Chen R *et al*. Vaccine Safety Datalink Study Group. *Vaccine* 2001; **19**: 4703–9
- 34 Williams J, Fox-Leyva L, Christensen C. Hepatitis A vaccine administration: comparison between jet-injector and needle injection. *Vaccine* 2000; **18**: 1939–43
- 35 Glenn G, Taylor D, Li X *et al*. Transcutaneous immunization: a human vaccine delivery strategy using a patch. *Nat Med* 2000; **6**: 1043–8
- 36 Licalsi C, Maniaci M, Christensen T *et al*. A powder formulation of measles vaccine for aerosol delivery. *Vaccine* 2001; **19**: 2629–36