Margaret A. Liu (Vice President, Vaccines Research, Chiron Corporation) reviews a wide range of approaches to vaccine design and development. Consideration is given to the particular strengths and weaknesses of protocols ranging from traditional attenuated organisms to the most innovative transgenic plants and DNA-based vaccines, and how well each is likely to meet our future vaccine needs.

Vaccine developments

MARGARET A. LIU

Vaccination as a means of preventing infectious disease has had, arguably, the greatest impact on human health of any medical intervention. Despite the fact that today vaccine development encompasses technologies ranging from the centuries-old approach of modifying pathogens to advanced genetic manipulations of the immune system itself, all vaccines have in common the intention of inducing an immune response designed to prevent infection or limit the effects of infection. Both humoral (antibody-mediated) and cellular arms of the immune system can contribute to the pathogen-specific acquired response that distinguishes vaccine protection from the innate and more general protection afforded by phagocytes, cytokines and physical barriers. Another critical element to vaccines is memory. Because immunization make take place many years before exposure to the pathogen, a long-lived immune response is called for. This article reviews the different approaches taken to produce the wide variety of vaccines that fulfill these requirements and provides a glimpse into the future by considering the scientific rationale for vaccines of the 21st century. (For a more in-depth account, see ref. 1).

Louis Pasteur

Attenuated/related organisms

From an immunological standpoint, perhaps the most obvious vaccine modality is to stimulate protection against a serious illness by prior infection with a weaker or related version or lower inoculum of a pathogen. The ancient Chinese protected against smallpox by the process of variolation, in which small quantities of scabs from a lesion of an infected person were intranasally inoculated[2]. The process was promoted by Lady Mary Montagu, who had observed variolation in Turkey in the early 1700s. In 1796 Edward Jenner used cowpox as a related immunogen against smallpox and, by testing the procedure scientifically, established the scientific precedent for using a related but less dangerous pathogen to engender immune responses that are cross-reactive against the more virulent pathogen[3]. The eradication of smallpox and widespread use of other live virus vaccines, such as those against measles, mumps, rubella, varicella, adenovirus and poliovirus, is testament to the great success of attenuated viruses.

This principle was first applied to a bacterial pathogen by Louis Pasteur in his experiments demonstrating protection of chickens from cholera[4] and of sheep from anthrax[5]. But the most widely known attenuated bacterial vaccine is the BCG (bacille Calmette-Guerin) for protection against tuberculosis, which was first administered in 1921 (ref. 6) and is still widely used today.

A more recent example of a live attenuated virus vaccine is the attenuated or cold-adapted influenza virus vaccine. This vaccine uses strains of influenza virus that optimally replicate at temperatures lower than 37 °C, and thus grow well in the slightly cooler temperatures of the nasal passages, but do not cause disease in the lower respiratory tract[7]. The attenuation thus capitalizes upon a host site restriction while delivering the vaccine to the mucosally desired location and inducing both mucosal and systemic immune responses. These vaccines appear to be efficacious in at least children and may provide an attractive alternative to the traditional influenza vaccine that requires intramuscular (i.m.) delivery and, in children, needs to be administered twice.

Molecular attenuation of pathogens

Efforts are now being made to use the tools of molecular biology to attenuate pathogens. That is, rather than relying on passaging of the organism in culture or in animals with selection of a weaker version, specific mutations can be made, perhaps by mutating or deleting a gene that encodes a protein responsible for a virulence factor or a protein responsible for the tropism of the virus. Recently much attention has been directed towards the potential use of attenuated HIV-1 strains as vaccines for AIDS (see Burton & Moore, page 495 and Heilman & Baltimore, page 532). Using strains of Simian Immunodeficiency Virus (SIV) in which a gene encoding Nef, a protein involved in replication of the virus, was altered or deleted, monkeys were shown to be protected from subsequent challenge with SIV, or SIV and SHIV, respectively (refs 8–10). However, it appears that the level of attenuation of the virus, and its immunogenicity and protective efficacy are inversely correlated[11]. Moreover, studies in neonatal monkeys with an SIV strain with deletions in the *nef* and *vpr* genes, resulted in negative clinical sequelae[12]. And it is possible that the pathogenicity of such a virus could be regained or that integration of such a virus could have other adverse effects, such as acting as a co-factor for the development of tumors. Thus, despite the various successful attenuated virus vaccines in clinical use today, it is currently a topic of debate as to whether a virus such as HIV can be adequately attenuated to make a safe yet effective vaccine.

Edward Jenner

Cross-species reassortant viruses

A new twist to an old technique can be found in rotavirus vaccines currently under development. Reassortant viruses take the best of different viruses and combine them in a new strain.

Examples of such reassortant viruses include strains of influenza virus, made anew each year by taking the existing licensed influenza vaccine, in this case a strain that grows well in eggs (and is therefore useful for the manufacture of the vaccine) as the backbone, and adding genes encoding the surface glycoproteins of each year's circulating strains of virus (replacing the analogous genes in the primary strain).

Rotavirus causes severe diarrhea in children and in developing countries is often fatal. Current efforts to develop a rotavirus vaccine use nonhuman strains of the virus (bovine and monkey strains) as the carrier for reassortment with selected human rotavirus genes. Initial clinical trials with reassortants are showing promise[13,14] (see Sansonetti, page 499).

Viral vectors

Attenuated viruses and bacteria can be modified for use as carriers by inserting genes encoding a protein from a different pathogen into their genome. In this case the carrier virus or bacteria enables the delivery of the antigen-encoding gene to the host, where the antigen is then made. By using a carrier virus or bacteria one can deliver genes from pathogens which themselves might be considered unsafe as an attenuated vaccine (for example, HIV). With *in situ* generation of the protein in the host, one can potentially generate MHC Class I-restricted cytotoxic T lymphocyte (CTL) responses in addition to antibody responses.

Vaccinia and related avipox viruses have been used as such carriers for various genes in preclinical and clinical studies for a variety of diseases. Vaccinia virus vectors in particular have been used extensively, in part because of the experience with the smallpox vaccine, its large capacity for inserted genes[15] and because in some cases it can induce immune responses similar to those seen with infection with the pathogen. Although vaccinia vectors are widely used to generate CTL in the laboratory and have been studied in the clinic, several issues have led vaccinologists to search for vectors other than vaccinia: First, pre-existing immunity in people who received smallpox vaccination may lower the efficacy of subsequent vaccines (although such individuals can develop immune responses against an encoded heterologous protein[16,17]). Second, immune responses against the vector itself can rule out rapid reuse of the vector[18,19]. Third, limited immunogenicity of the encoded antigens has at times been observed. Finally, there exists a potential for disease in immunocompromised people. Recently, in a murine model, a combination of priming with a DNA vaccine (*vide infra*) and boosting with a vaccinia vector was shown to be a more potent regimen than using the vaccinia vector for both the prime and the boost[20].

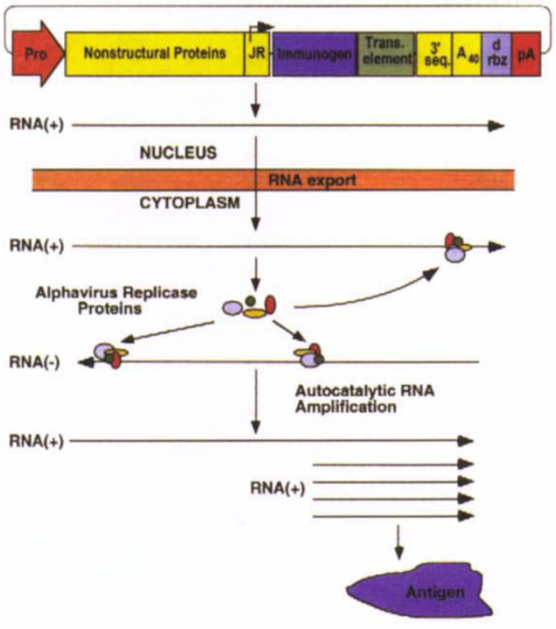


Fig. 1 Alphavirus plasmid DNA-based gene transfer vector.

Replicons

Alpha viruses are RNA viruses that are used as vector systems to deliver the genes of heterologous pathogens. These viruses are attractive because they make many copies of the messenger RNA encoding the structural proteins of the virus. This amplification of the mRNA has the potential to rapidly produce increased quantities of antigen. Replicons can be engineered to consist of a virus coat containing the genome in which the sequences for the structural genes have been replaced by the sequence encoding the antigen. Thus, following infection of the host cell, large quantities of RNA encoding the antigen and hence large quantities of the protein antigen are made, yet the alphavirus itself cannot replicate because it no longer contains the sequences for the necessary structural proteins[21,22] (Fig. 1). One alphavirus, Venezuelan equine encephalitis virus, has tropism for follicular dendritic cells in the lymph node. Dendritic cells are known as professional antigen-presenting cells in light of their ability to present antigen to T cells—an important step in the immune response pathway—and are thus of particular interest as a vaccine vector[23–25].

Bacterial vectors

BCG and salmonella provide examples of bacterial-based vector systems that have been developed because of clinical experience using them as live vaccines. These have been engineered to express heterologous antigen. Shigella[26] and more recently Salmonella[27] have been shown to serve as vectors of a different sort, wherein the bacterium has been used as a delivery system for delivering episomal plasmid DNA into cells that the bacterium invades. One such example is the use of an auxotrophic mutant strain of Shigella that cannot replicate unless a key nutrient is provided. Following invasion of a target cell, replication cannot occur and the bacterium dies, releasing the plasmid DNA encoding the desired antigen into the infected cell. The cell then synthesizes the antigen initiating an immune response. Attenuated vector versions of *Listeria monocytogenes* are also being evaluated[28]. (Attention was directed towards Listeria because of its ability to enter the cytoplasm of infected cells because of production of listeriolysin O[29]).

Subunit vaccines

Subunit vaccines represent technologies ranging from the chemical purification of components of the pathogen grown *in vitro* (the surface glycoproteins hemagglutinin and neuraminidase of influenza or the polysaccharide capsules of *S. pneumoniae*, for example) to the use of recombinant DNA technology to produce a single viral protein (such as hepatitis B surface antigen). Recombinantly made proteins of the envelope of HIV (either the gp120 subunit or full-length gp160) have been evaluated as a means to induce neutralizing antibodies against HIV, but have been disappointing, possibly because of their conformation and the laboratory (rather than primary) isolates from which they were derived[30,31]. Recent clinical studies, however, suggest that recombinant HIV Env may still find utility as a boost in mixed regimens[32].

Clinical trials are underway with virus-like particles (VLPs) made from recombinant L1, the major capsid protein of human papillomavirus. These VLPs have been shown to be capable of inducing antibodies that prevent wart formation in rabbit, bovine and canine models of papillomavirus.

Molecular biology has provided a means to specifically inactivate toxin antigens such as pertussis toxin. This has been accomplished by determining the active site of the toxin, then making a recombinant protein with some of the critical amino acids for the critical residues, thus rendering the toxin enzymatically inactive yet immunologically intact for the generation of protective antibodies. Alternatively, the part of the toxin that binds to the cellular receptor can be disrupted. These detoxified proteins can then be made recombinantly to generate a highly purified, nontoxic, yet antigenically active, vaccine. Examples are detoxified diphtheria, tetanus, cholera and pertussis toxins (reviewed in 1).

Peptides

Perhaps it is a logical progression from whole inactivated viruses or bacteria; to purified components of the pathogen; then to recombinant proteins; and finally to peptide vaccines. In this approach, known B-cell or T-cell epitopes are the immunogen, generally coupled to a carrier molecule designed to increase the immunogenicity of the epitope by providing for the induction of T-cell help. Wheras certain B-cell epitopes may be conserved, for T-cell epitopes, because each individual's MHC molecules are different, a peptide vaccine would likely be limited in the breadth of the population that it would cover. One way around this relative specificity is to combine peptide epitopes covering the major haplotypes. However, even this approach may still provide limited coverage in terms of the number of haplotypes for which epitopes are provided (and hence limit the number of individuals for which the vaccine is effective and the number of epitopes provided per individual), compared to a vaccine based upon a whole protein or organism.

Polysaccharide-protein conjugates

As our understanding of the nature of the contribution of T cells to the generation of certain B cell responses has grown, it has become clear that certain polysaccharide antigens that are T-cell independent are not sufficiently immunogenic for infants. By chemically conjugating these polysaccharides to a carrier protein, the polysaccharide antigen can be transformed into a T-cell-dependent antigen, capable of eliciting protective IgG and memory responses even in the very young. The success of this technology as applied to vaccines against *Haemophilus influenzae* type b, for which several such conjugate vaccines were introduced in the mid-1980s, was startling—the vaccines resulted in not only protection of vaccinated individuals but a decline in the incidence of the disease that exceeded what was expected based on the number of children immunized[33]. Current efforts are directed at developing conjugate vaccines for *Streptococcus pneumoniae* and *Neisseria meningitidis* types A and C. The increasing emergence of strains of *S. pneumoniae* that are resistant to antibiotics[34] is a major impetus to the development of such conjugate vaccines.

Combination vaccines

The successful combination of existing single- or multi-target vaccines presents many challenges. The simple goal is to reduce the number of injections that individuals must receive in order to develop immunity against several diseases, in the hope that fewer immunizations will lead to increased vaccination rates. The formulation of such vaccines can be a problem because it simply may not be possible to take different vaccine modalities (for example, live virus versus recombinant protein on alum) and mix them together. Immunization schedules of the components may also differ and co-injection of certain antigens can result in decreased responses against one or the other of the components.

Adjuvants and delivery systems

Adjuvants are used to increase the immune response to an antigen. Although a number of adjuvants are being evaluated, aluminum salts are currently the only adjuvant in broad usage. Aluminum was thought to work by helping form a depot of antigen but this notion has been challenged by a recent study[35]. Indeed, whereas most adjuvants for prophylactic vaccine use are chemical entities, cytokines are being evaluated for direct usage as adjuvants because they are likely to be the direct proximal mediators of the more 'classical' adjuvants.

Examples of adjuvants being evaluated include monophosphoryl lipid A, which can be formulated with mycobacterial cell walls (termed Ribi adjuvant), MF59 (a microfluidized emulsion of oil and surfactants that is licensed for use as part of an influenza vaccine), SAF-1 (an oil-based emulsion containing muramyl dipeptide and non-ionic block co-polymers), saponin derivatives (such as QS21), and polymers such as polyphosphazene (for recent reviews, see references[36,37]). The challenge is to develop adjuvants that are effective, yet do not elicit too vigorous an immune response either systemically or at the site of injection, leading to sequelae such as granuloma formation.

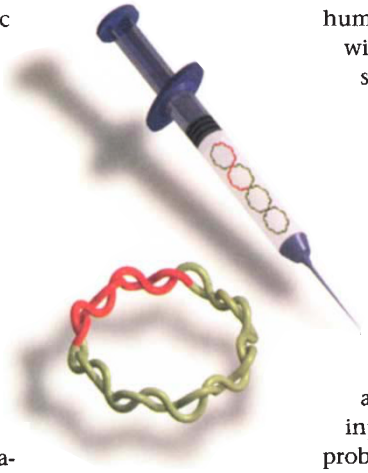
Delivery systems that might lead to prolonged or pulsatile release of an antigen are likewise under evaluation in the hope that they will either reduce the number of immunizations required or boost or prolong immune responses. Such delivery systems include liposomes and microcapsules composed of polymers surrounding the antigen. One polymer used for microcapsules is poly-L-lactide co-glycolide (PLGA), the material used in degradable sutures[38,39]. Particles may be effective for oral administration of antigen, because they can be constructed so as to protect the antigen from degradation in the stomach, and because particles of certain diameters (5–10 μm) are taken up by M (microfold) cells in the intestinal epithelium of Peyer's patches. The route of administration offers not simply convenience but is intended to induce the generation of mucosal immune responses, such as secretory IgA.

Studies on the mechanisms of toxins of enteric bacteria have led to investigation of bacterial toxins as adjuvants for mucosally delivered antigens[40]. These include cholera toxin (CT) and *E. coli* heat-labile toxin (LT). Most recently, when applied as a simple saline mixture on the intact skin of mice, cholera toxin coadministered with bovine serum albumin, diphtheria toxoid or tetanus toxoid resulted in the generation of serum antibodies to the antigen[41]. This remarkable finding may greatly facilitate the administration of future vaccines.

Oral/nasal vaccines

The impressive logistical advantage of orally administered vaccines is perhaps best exemplified by the worldwide polio vaccination days, in which millions of people were immunized in a single day (see Bloom & Widdus, page 480). Such massive efforts are only possible, in terms of both cost and

feasibility, with oral vaccines. Mucosal routes of vaccine administration have the added advantage of mucosal immune responses. Because nearly all infectious diseases enter the host via mucosal surfaces, mucosal responses such as secretory IgA are thought to be particularly effective and important, although serum or systemic responses alone are clearly adequate for many vaccines[42]. For enteric pathogens, oral administration is particularly appealing, whereas for upper respiratory infections, nasal administration is logical. Strategies such as attenuation of particular pathogens enabling vaccination by delivery to the natural site of infection, have been discussed. Efforts are also focused on the delivery of antigens (including formulated or encapsulated antigens) or DNA, intranasally or orally.



Edible plant vaccines

The potential for the global impact of oral vaccines seems even greater when considering the possible use of recombinant foods such as bananas or potatoes. Transgenic plants have been shown capable of producing an antigenic protein whose gene has been incorporated into their host genome. Studies done by feeding animals potato tubers from plants transgenic for an LT fusion protein[43] or a Norwalk virus capsid protein[44] demonstrated that antibodies were made against the antigen. These antibodies were found both in the serum and from mucosal sites. In a more recent report, transgenic potatoes were found to produce the binding subunit of cholera toxin in an appropriate form for immunogenicity even after cooking the potato[45]. Although proteins in cooked transgenic plants have yet to be shown to engender immune responses when fed to animals, this advance may be useful because humans don't consume raw potatoes (although the first human trial of a transgenic potato vaccine called for just that—see Arntzen, page 502). Plants as a production source of vaccine proteins are also being evaluated.

Nucleic acid vaccines

Typically, nucleic acid vaccines are bacterial plasmids carrying genes encoding pathogen or tumor antigens. These plasmids generally use a strong viral promoter to drive the expression of the gene of interest directly in the injected host, rather than *in vitro*. They are usually administered as a simple saline solution by direct intramuscular injection, although use of a 'gene gun' to deliver gold beads onto which DNA has been precipitated is also under evaluation. In a rapidly increasing number of animal models, DNA vaccines have been shown to be effective at generating protective immune responses against a wide variety of diseases (reviewed in[46]). DNA-based vaccines are particularly interesting for several reasons: They are simple; they can generate MHC Class I-restricted CTL responses (without the use of a live vector); antigen is produced with mammalian post-translational modification, conformation and oligomerization; it is relatively simple to combine diverse immunogens into a single preparation (thus decreasing the number of vaccinations required); and the DNA itself seems to act as an adjuvant.

In particular, the generation of CTL responses is thought to be crucial for many disease targets for which no vaccines exist (such as HIV). Furthermore, CTL may provide broader protection against different strains of a virus by targeting epitopes from conserved internal proteins pertinent to many strains of the same virus. The ability to generate CTL by providing intact proteins (rather than peptides) should also allow determinant selection to occur for the broad range of human HLA haplotypes. Because immunization with DNA-encoded proteins results in protein synthesis in the definitive host, such a protein is more likely to be similar or identical to the wild-type protein produced by viral infection, in contrast to many recombinant or chemically modified proteins. However, for bacterial proteins the mammalian post-translational modifications may result in antigens that differ from the bacterial versions, thus resulting in reduced immunogenicity.

Whereas RNA vectors can be used to generate proteins and immune responses by direct injection[47], the inherent instability of RNA is a problem (although the recent demonstration that RNA can directly transfect dendritic cells may provide a better immunologic rationale for such an approach).

The use of DNA to encode replication-defective viral vectors may represent a powerful combination of viral vector technology and DNA vaccines. These vectors are based upon alphaviruses such as Sindbis virus, but utilize a DNA plasmid encoding the antigen and non-structural proteins of the alphavirus. Thus amplification of the antigen-encoding mRNA occurs[48,49]. Such an approach may avoid some of the limitations of traditional vector technology, such as production difficulties and pre-existing immunity, while at the same time providing a means of amplifying the production of protein even when only a few cells are transfected.

Therapeutic vaccines

Whereas this review has been concerned mostly with prophylactic vaccines against infectious agents, it is important to mention recent developments in the area of therapeutic vaccines. Human dendritic cells are now known to be capable of sensitizing target cells to prime CTL *in vitro* when pulsed with RNA-encoded tumor antigen[50]. And in a recent clinical trial, dendritic cells pulsed with tumor cell lysate were shown to be an effective treatment for melanoma[51]. This builds on earlier work demonstrating that dendritic cells could be directly treated with HIV to generate target cells to prime CTL. Such findings open new possibilities for the use of dendritic cells as vaccines even when the key antigen has not been identified.

Gene sequencing and genetic approaches

Complete sequencing of pathogens has led to an increased effort to discover new and more effective protein antigens. The complete sequencing of non-typeable *H. influenzae*, the first pathogen to be sequenced in its entirety[52], provides new possibilities for finding conserved antigens appropriate for developing vaccines for these pathogens. Virulence factors have been sought for *Mycobacterium tuberculosis* and are thought to represent attractive targets for vaccine development. It has, for example, been demonstrated that proteins secreted by *M. tuberculosis* are those that appear to induce cellular immune responses (likely because, when secreted by an organism in an

infected cell, the protein can then enter into the processing pathway by which epitopes are generated and bind MHC Class I molecules, to be presented on the surface of the infected cell and resulting in CTL responses)[53]. Thus, with the full *M. tuberculosis* sequence now available, it will be easier to identify genes with leader sequences indicating that they are secreted, and hence are potential vaccine candidates.

Summary

Increased understanding of the molecular nature of immune responses and advances in the technologies of gene sequencing and molecular biology have resulted in new approaches to vaccine development. And yet, in many ways vaccine development remains somewhat of an empirical art for which the scientific understanding provides not only approaches to and technologies for vaccine development, but also a retrospective explanation for the efficacy of particular vaccines.

Acknowledgments

The author gratefully acknowledges John D. Donnelly and Carol A. Suennen for their critical review of the manuscript.

1. Levine, M.M., Woodrow, G.C., Kaper, J.B. & Cobon, G.S. *New generation vaccines*. Second edn (Marcel Dekker, Inc., New York, 1997).
2. Fenner, F., Henderson, D.A., Arita, L., Jezek, Z. & Ladnyi, I.D. *Smallpox and its eradication* (World Health Organization, Geneva, 1988).
3. Jenner, E. An inquiry into the causes and effects of the variolae vaccinae, a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox. London, 1798, in *Classics of Medicine and Surgery* (ed. Camac, C.N.B.) 213–240 (Dover, New York, 1959).
4. Pasteur, L., Chamberland, C.-E. & Roux, E. Sur la vaccination charbonneuse. *CR Acad Sci,* Paris **92**, 1378–1383 (1881).
5. Pasteur, L. Une statistique au sujet de la vaccination préventive contre le charbon, portant sur quatre-vingt-cinq-mille animaux. *CR Acad Sci, Paris* **95**, 1250–1252 (1882).
6. Calmette, A. La vaccination preventive contre la tuberculose par le BCG (Masson, Paris, 1927).
7. Maassab, H.F. & DeBorde, D.C. Development and characterization of cold-adapted viruses for use as live virus vaccines. *Vaccine* **3**, 355–371 (1985).
8. Daniel, M.D., Kirchhoff, F., Czajak, S.C., Sehgal, P.K. & Desrosiers, R.C. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. *Science* **258**, 1938–1941 (1992).
9. Almond, N. *et al.* Protection by attenuated simian immunodeficiency virus in macaques against challenge with virus-infected cells. *Lancet* **345**, 1342–1344 (1995).
10. Lohman, B.L. *et al.* A partially attenuated simian immunodeficiency virus induces host immunity that correlates with resistance to pathogenic virus challenge. *J. Virol.* **68**, 7021–7091 (1994).
11. Ruprecht, R.M. *et al.* "Attenuated" simian immunodeficiency virus in macaque neonates. *AIDS Res. Hum Retroviruses* **12**, 459–460 (1996).
12. Baba, T.W. *et al.* Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. *Science* **267**, 1820–1825 (1995).
13. Treanor, J.J. *et al.* Evaluation of the protective efficacy of a serotype 1 bovine-human rotavirus reassortant vaccine in infants. *Pediatr. Infect. Dis. J.* **14**, 301–307 (1995).
14. Rennels, M.B. *et al.* Safety and efficacy of high-dose rhesus-human reassortant rotavirus vaccines—report of the National Multicenter Trial. *United States Rotavirus Vaccine Efficacy Group. Pediatrics* **97**, 7–13 (1996).
15. Smith, G.L. & Moss, B. Infectious poxvirus vectors have capacity for at least 25,000 base pairs of foreign DNA. *Gene* **25**, 21–28 (1983).
16. Jones, L., Ristow, S., Yilma, T. & Moss, B. Accidental human vaccination with vaccinia virus expressing nucleoprotein gene [letter]. *Nature* **319**, 543 (1986).
17. Cooney, E.L. *et al.* Safety of and immunological response to a recombinant vaccinia virus vaccine expressing HIV envelope glycoprotein. *Lancet* **337**, 567–572 (1991).
18. Rooney, J.F., Wohlenberg, C., Cremer, K.J., Moss, B. & Notkins, A.L. Immunization with a vaccinia virus recombinant expressing herpes simplex virus type 1 glycoprotein D: long-term protection and effect of revaccination. *J. Virol.* **62**, 1530–1534 (1988).
19. Kundig, T.M., Kalberer, C.P., Hengartner, H. & Zinkernagel, R.M. Vaccination with two different vaccinia recombinant viruses: long-term inhibition of secondary vaccination. *Vaccine* **11**, 1154–1158 (1993).
20. Hanke, T. *et al.* Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine* **16**, 439 (1998).
21. Fries, L.F. *et al.* Human safety and immunogenicity of a canarypox-rabies glycoprotein recombinant vaccine: an alternative poxvirus vector system. *Vaccine* **14**, 428–434 (1996).
22. Frolov, I. *et al.* Alphavirus-based expression vectors: Strategies and applications. *Proc. Natl. Acad. Sci.* U S A **93**, 11371–11377 (1996).
23. Xiong, C. *et al.* Sindbis virus: an efficient, broad host range vector for gene expression in animal cells. *Science* **243**, 1188–1191 (1989).
24. Davis, N.L., Brown, K.W. & Johnston, R.E. A viral vaccine vector that expresses foreign genes in lymph nodes and protects against mucosal challenge. *J. Virol.* **70**, 3781–3787 (1996).
25. Caley, I.J. *et al.* Humoral, mucosal, and cellular immunity in response to a human immunodeficiency virus type 1 immunogen expressed by a Venezuelan equine encephalitis virus vaccine vector. *J. Virol.* **71**, 3031–3038 (1997).
26. Sizemore, D.R., Branstrom, A.A. & Sadoff, J.C. Attenuated Shigella as a DNA delivery vehicle for DNA-mediated immunization. *Science* **270**, 299–302 (1995).
27. Darji, A. *et al.* Oral somatic transgene vaccination using attenuated *S. typhimurium*. *Cell* **91**, 765–775 (1997).
28. Goossens, P.L., Milon, G., Cossart, P. & Saron, M.F. Attenuated *Listeria monocytogenes* as a live vector for induction of $CD8^+$ T cells *in vivo*: a study with the nucleoprotein of the lymphocytic choriomeningitis virus. *Int. Immunol.* **7**, 797–805 (1995).
29. Dietrich, G. *et al.* Delivery of antigen-encoding plasmid DNA into the cytosol of macrophages by attenuated suicide *Listeria monocytogenes*. *Nature Biotechnol.***16**, 181–185 (1998).
30. Hoth, D.F., Bolognesi, D.P., Corey, L. & Vermund, S.H. HIV vaccine development: a progress report. *Ann Intern. Med.* **8**, 603–611 (1994).
31. Clements-Mann, M.L. *et al.* HIV-1 immune responses induced by canarypox (ALVAC) gp-160 MN, SF2 rgp120, or both vaccines in seronegative adults. *J. of Infect. Dis.* (In press).
32. Graham, B.S. & Wright, P.F. Candidate AIDS vaccines. *N. Engl. J. Med.* **333**, 1331–1339 (1995).
33. Murphy, T.V. *et al.* Declining incidence of *Haemophilus influenzae* type b disease since introduction of vaccination. *JAMA* **269**, 246–248 (1993).
34. Hofmann, J. *et al.* The prevalence of drug-resistant *Streptococcus pneumoniae* in Atlanta. *N. Engl. J. Med.* **333**, 481–486 (1995).
35. Flarend, R.E. *et al. In vivo* absorption of aluminium-containing vaccine adjuvants using ^{26}Al. *Vaccine* **15**,1314–1318 (1997).
36. Gupta, R.K. & Siber, G.R. Adjuvants for human vaccines—current status, problems and future prospects. *Vaccine* **13**, 1263–1276 (1995).
37. Cox, J.C. & Coulter, A.R. Adjuvants—a classification and review of their modes of action. *Vaccine* **15**, 248–256 (1997)
38. Gupta, R.K., Chang, A.C., Griffin, P., Rivera, R. & Siber, G.R. *In vivo* distribution of radioactivity in mice after injection of biodegradable polymer microspheres containing 14C-labeled tetanus toxoid. *Vaccine* **14**, 1412–1416 (1996).
39. Anderson, J.M. & Shive, M.S. Biodegradation and biocompatibility of PLA and PLGA microspheres. *Advanced Drug Delivery Reviews* **28**, 5–24 (1997).
40. Tamura, S. *et al.* Synergistic action of cholera toxin B subunit (and *Escherichia coli* heat- labile toxin B subunit) and a trace amount of cholera whole toxin as an adjuvant for nasal influenza vaccine. *Vaccine* **12**, 419–426 (1994).
41. Glenn, G.M., Rao, M., Matyas, G.R. & Alving, C.R. Skin immunization made possible by cholera toxin. *Nature* **391**, 851 (1998).
42. Robbins, J.B., Schneerson, R. & Szu, S.C. Hypothesis: how licensed vaccines confer protective immunity. *Adv. Exp. Med. Biol.* **397**, 169–182 (1996).
43. Haq, T.A., Mason, H.S., Clements, J.D. & Arntzen, C.J. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. *Science* **268**, 714–716 (1995).
44. Mason, H.S. *et al.* Expression of Norwalk virus capsid protein in transgenic tobacco and potato and its oral immunogenicity in mice. *Proc. Natl. Acad. Sci. USA* **93**, 5335–5340 (1996).
45. Arakawa, T., Chong, D.K.X. & Langridge, W.H.R. Efficacy of a food plant-based oral cholera toxin B subunit vaccine. *Nature Biotechnol.* **16**, 292–298 (1998).
46. Donnelly, J.J., Ulmer, J.B., Shiver, J.W. & Liu, M.A. DNA vaccines. *Annu. Rev. Immunol.* **15**, 617–648 (1997).
47. Martinon, F. *et al.* Induction of virus-specific cytotoxic T lymphocytes *in vivo* by liposome-entrapped mRNA. *Eur. J. Immunol.* **23**, 1719–1722 (1993).
48. Ward, G., Reider, E. & Mason, P.W. Plasmid DNA encoding replicating foot and mouth disease virus genomes induces antiviral immune responses in swine. *Journal of Virology* **71**, 7442–7447 (1997).
49. Hariharan, M.J. *et al.* DNA immunization against herpes simplex virus: enhanced efficacy using a sindbis virus-based vector. *J. Virol.* **72**, 950–958 (1998).
50. Macatonia, S.E., Patterson, S. & Knight, S.C. Primary proliferative and cytotoxic T-cell responses to HIV induced in vitro by human dendritic cells. *Immunology* **74**, 399–406 (1991).
51. Nestle, F.O. *et al.* Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nature Med.* **4**, 328–332 (1998).
52. Fleischmann, R.D. *et al.* Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512 (1995).
53. Orme, I.M. Prospects for new vaccines against tuberculosis. *Trends In Microbiology* **3**, 401–404 (1995).

Chiron Vaccines Research
4560 Horton Street, Q3-101
Meryville, California 94608, USA