Pharmaceutical Research, Vol. 13, No. 12, 1996

Review

Drug Delivery Issues in Vaccine Development

Michael F. Powell[1,2]

Received August 21, 1996; accepted September 8, 1996

Although significant headway has been made in vaccine development, there are several delivery-related issues that must be overcome to advance tomorrow's candidate vaccines. Some of these are in the areas of: single-shot subunit vaccines, therapeutic vaccines for cancer, the use of cytokines as vaccine adjuvants, DNA-based vaccines, and the development of vaccines that provide sterilizing immunity, as might be required for an affective HIV-1 prophylactic vaccine. The hurdles for vaccine advancement in these areas are briefly described.

KEY WORDS: vaccines; targeted drug delivery; therapeutic cancer vaccines; cytokines; DNA vaccines; sterilizing immunity; single-shot vaccines.

INTRODUCTION

Vaccination against smallpox, polio, diphtheria, pertussis, tetanus, measles and other pathogens has reduced mortality more than any other disease intervention (1–4). Despite these successes, vaccine development has significant hurdles, both social and scientific, largely because of the nature of prophylactic vaccines. At the time of vaccine administration, the subject is often an infant or child, with no personal perception of immediate benefit (5). Indeed, the parents of young vaccinees often not only perceive little immediate benefit, but also have little awareness of the risk/benefit consequences of vaccinating (or not vaccinating), including the societal implications of vaccination (herd immunity) (6,7). Because of such social issues, vaccines must be perceived as 'completely' safe, easy to administer resulting in high compliance, cause little pain upon delivery, and be effective against the pathogens of the region. Our current regime of vaccines can hardly be described as such. Few parents perceive vaccines as 'completely' safe, and often show undue concern about whether or not to vaccinate their infants based on the few, highly publicized incidences of breakthrough associated with the vaccine (8,9). Today's current regime of childhood vaccines is also complex, as children require multiple office visits for both primary and booster immunizations which typically take several years for completion. The complexity of a complete childhood vaccination schedule leaves ample room for delayed or missed booster immunizations, possibly resulting in an unwanted, and unknown, lack of vaccine efficacy.

Novel vaccine development has a number of special delivery issues compared to other drugs. Because vaccines are typically injected either subcutaneously or intramuscularly to maximize the immune response, the macroscopic delivery of vaccines is straightforward—most are simply injected. Alternatively, the microscopic delivery and targeting of the vaccine antigen and adjuvant to the desired cell types, as well as the regulation of how the antigen is processed and presented to the host immune system, has several delivery issues, including:

1. single-shot subunit vaccines
2. therapeutic vaccines for cancer
3. cytokines as vaccine adjuvants
4. DNA vaccines
5. sterilizing immunity vaccines (e.g., for HIV-1).

SINGLE-SHOT SUBUNIT VACCINES

Current Status and Unmet Needs

Subunit vaccines, made from one or more proteins of the parent pathogen, often require multiple boosting before maturation of the immune and memory response occurs (10). This need for multiple boosting often results in poor compliance resulting in reduced efficacy. The ultimate goal of an ideal single-shot vaccine is to provide an 'autoboost' of antigen at a defined time(s) with only a single injection. Most single-shot vaccines in development today are designed to mimic boosting using controlled-release delivery systems administered s.c. or i.m., wherein a pulsatile release of antigen from a delivery device or vehicle is released at later prescribed interval(s). Even these few restrictions/guidelines dictate what an optimal single-shot vaccine might look like. Such a vaccine should deliver a bolus of antigen and adjuvant shortly after injection for primary immunization, followed by one or more autoboosts of antigen after a prescribed duration, preferably after several months. The timing of the autoboost(s) should be well defined and optimized for the antigen selected. The non-toxic delivery vehicle should not be subject to catastrophic degradation and accidental release of antigen at early times, and should be fully biodegraded shortly after the final autoboost is completed. Finally, the anti-

[1] Genentech, Inc., 460 Pt. San Bruno Blvd., South San Francisco, California 94080.

[2] To whom correspondence should be addressed.

0724-8741/96/1200-1777$09.50/0

gen that is released should be, in many cases, fully intact and not denatured so as to elicit a maximal neutralizing immune response.

Hurdles and Opportunities

Based on these criteria and today's sustained-release technology, prototype single-shot vaccines have been designed using polymeric microspheres containing antigen, such as the poly-lactide-co-glycolide (PLGA) microspheres (11–18), or those made of other polymer types (19). There are several delivery issues surrounding the use of polymer-based microspheres for use in single-shot vaccines. First, the synthesis of microspheres is complex, where dozens of parameters often require optimization before the desired microspheres are made (20). Each of these parameters, such as polymer choice and molecular weight, polymer end capping, antigen loading, added stabilizers and bulking agents, as well as processing parameters such as primary stir speed, choice of emulsifier(s), reaction temperature, quenching bath type, drying process and the like greatly affect the type of microsphere made, and the antigen release profile. This release profile, in turn, is crucial for the 'correct' delivery of antigen. Although the definition of optimal microspheres using today's technology for microsphere synthesis is technically difficult and requires significant engineering (20,21), there are no technical hurdles that cannot be overcome to make this a reality, at least as a general process for making microspheres that autoboost antigen at prescribed times.

The stability of intact antigen within the polymer microspheres may be an issue, in that certain antigens are fairly robust and may survive the encapsulation process, whereas others are fragile (22) and may denature during the production of the microspheres (20). The prediction of antigen stability towards the encapsulation process is not well understood, and will likely continue to be a subject of interest for years. For example, it has been shown that the subunit protein gp120 can be microencapsulated using a process involving organic solvents and rapid stirring rates without significant denaturation (11–16). On the other hand, numerous research groups have attempted to microencapsulate tetanus toxoid and only recently has progress been made showing that this unstable antigen may eventually be incorporated into a polymeric single-shot vaccine (19,23). Even if the antigen is successfully incorporated into the microspheres without denaturation, the antigen should not degrade within the microspheres after injection in-vivo, nor before release during the autoboost phase. In that most polymer microspheres undergo hydration where they take up water and swell (24,25), the local environment of the microencapsulated antigen after injection is believed to be an aqueous milieu of pH approximately 7.4, and 37°C. Indeed, microsphere hydration is necessary for bulk erosion to occur so that the antigen can be released from the polymer matrix. Further, the degradation of certain polymers such as PLGA results in an increase in the number density of the terminal carboxylic acid groups, and so the local pH within the microsphere often drops, sometimes as low as approximately pH 4 (25). In that proteins show maximum stability at different pHs depending on their primary sequence, structure, and degradation pathway(s) (22), this uncertainty of local pH within the microsphere before bulk erosion occurs introduces another degree of uncertainty regarding antigen stability. The requirement that the antigen remain substantially intact in an aqueous environment at 37°C for several months is a difficult one to overcome with today's polymeric technology, and there is no polymer technology on the horizon that addresses this directly. In-vivo antigen stability within the polymer matrix is likely to remain one of the unsolved problems in single-shot vaccine design for the next generation. Based on these considerations, it is likely that single-shot subunit vaccines will become a reality for some subunit antigens and not others, depending largely on the nature and stability of the antigen.

THERAPEUTIC VACCINES FOR CANCER

Current Status and Unmet Needs

Therapeutic vaccines are categorically different than prophylactic vaccines because they are designed to fight an established pathogen, cancer, or an autoimmune disease state, all of which have already gained a foothold in the host at the time of vaccination. The demand placed on a typical therapeutic vaccine is significantly higher than for a prophylactic vaccine, and suffers from a number of shortcomings including: the lack of knowledge about the optimal choice of antigen (if indeed there is a disease-specific antigen available), the lack of precedence for therapeutic vaccine efficacy, and the need for cellular, rather than humoral, immunity to be induced. This is a tall order for any vaccine, in that the target antigen is often an autologous protein (such as in the targeting of the autologous HER-2 protein that is upregulated in certain breast and ovarian cancers (26)) and by their very nature, autologous proteins make poor immunogens, often requiring the breaking of tolerance before mounting an immune response (27,28). Even if the cancer target antigens are modified autologous antigens, they often are structurally close to autologous antigens such that immune recognition is not trivial (29). This is made even more difficult by a key delivery problem—if the vaccine antigen is not delivered 'correctly' to the appropriate compartment within the antigen presenting cell (APC), then an 'incorrect' immune response will be made, and efficacy may not be achieved. These delivery issues relate to the microscopic delivery of the vaccine antigen, including how antigen is processed and presented, the nature of the antigen itself, and the adjuvant formulation used. In order to understand how these factors affect microscopic delivery of antigen, it is appropriate to define the two major pathways for antigen presentation and the delivery factors which affect each.

When the immune system encounters a foreign protein, it is usually taken up by either of two pathways, either the Class I Major Histocompatibility Complex (MHC) 'endogenous' pathway where a cellular response is induced, or by the Class II MHC pathway resulting in primarily an antibody type of response. The pathway selected by the immune system is dictated primarily by the delivery route of the antigen. Delivery of foreign protein to the cytosol of cells results in predominantly a CD8+ T-lymphocyte, or cellular response. By this route, proteins are proteolytically degraded into peptides (30–32) and then transported to the endoplasmic reticulum (33–36) where the peptides bind selectively to the Class I MHC heavy chain. After a stable complex with β2-microglobulin is formed, this complex is transported to the cell surface by intracellular chaperons (37,38). Peptides presented by Class I MHC molecules to other immune competent cells are generally 8–10 amino

acids in length (39–42), and result in an upregulation of a cellular immune response, believed to be important in tumor reduction and therapeutic vaccine efficacy. Alternatively, when antigen is delivered to the endosomal compartment of APCs, such as macrophages, dendritic cells or B-lymphocytes, the Class II mediated pathway predominates resulting in primarily CD4+ T-lymphocyte-mediated responses and antibody responses. In this pathway, foreign proteins are transported into acidic endosomes of APCs, where they are degraded proteolytically to give small peptides (43–46). The endosomal processing and Class II MHC molecule biosynthetic pathways intersect (47,48) and the peptides bind to the Class II molecule, displacing the invariant chain (49,50) before transport of this peptide-loaded Class II MHC molecular complex to the cell surface. Peptides expressed in association with Class II MHC are typically 12–25 amino acids in length (51–53), and result in an upregulation of a humoral, or antibody, immune response. The delivery of antigen to each pathway results in a different immune response, and represents one of the hurdles in vaccine design. Typically if soluble proteins are simply injected parenterally they are processed primarily by the Class II pathway, resulting in an antibody and CD4+ T-lymphocyte responses. Unfortunately, a humoral response is often believed to be insufficient for tumor reduction or clearance. Getting antigen exclusively into the Class I MHC presentation pathway is significantly more difficult because this requires intracellular delivery of antigen to the cytosol.

Hurdles and Opportunities

Significant headway in this area has been achieved in the last few years, where adjuvants have been developed specifically to induce a CD8+ T-lymphocyte response (54–56). Although it has been shown that humoral immune responses against cancer-associated antigens have been detected in cancer patients (57–59); including a correlation with clinical outcome (60), it is generally believed that therapeutic vaccines should be more effective if they induce tumor antigen-specific cytoxic T-lymphocytes (CTLs), driven by targeting antigen to the Class I MHC pathway. The generation of a cellular response, however, does not guarantee vaccine effectiveness in tumor size reduction or elimination, in that the neoplasm may be resistant to cellular killing, or may be poorly vascularized resulting in ineffectual killing due to inability of CTLs to reach the tumor. In fact, it has been shown that, even though several murine tumors may be successfully treated by therapeutic vaccination, when analogous studies were carried out in primates there was a dramatic reduction in efficacy. Predicting the future efficacy of therapeutic vaccines is made more difficult by the lack of precedence of successful therapeutic vaccines with which to base a prediction (61). There are a number of therapeutic vaccines that have shown efficacy to date, such as metastatic melanomas (62–64), colorectal cancer (65), and a number of viral-associated cancers (66). A striking commonality between most studies of this type is that survival (or life-expectancy) is extended slightly, but few therapeutic vaccines totally clear the neoplasm, resulting in non-declining mortality curves. Although some encouraging findings have been reported, a survey of the present day data suggest that therapeutic cancer vaccines still require significant optimization of the immune response, and that appropriate delivery and processing of antigen may be one of the hurdles in this area (67–70).

CYTOKINES AS VACCINE ADJUVANTS

Current Status and Unmet Needs

There are only a few factors that affect the immune response to subunit vaccines. These are the nature and the dose of antigen (71–73), the route of administration (intradermal, subcutaneous, intramuscular, oral, nasal, pulmonary, and vaginal) (10), the nature of the vaccine (species, haplotype, age, and immune status) (74–76), the immunization schedule and timing of boosters (subunit vaccines usually benefit from spacing out the booster immunizations, presumably due to maturation of the high affinity precursor B-cells) (77), and the adjuvant used (10,78,79). One particular class of adjuvants, the cytokines, have significant delivery hurdles because the cytokine should be delivered to the specific set of lymphocytes requiring upregulation in the antigen processing and presentation pathway, without causing toxicity to other cell types nearby.

Because our immune system is made up of different cell types, each with its own role to play in host defense, and are often regionally distinct from each other, mechanisms have evolved to allow these cells to interact with each other. Some of these interactions occur by cell-to-cell contact, and some are regulated through the use of soluble factors or cytokines, often referred to as interleukins (IL) (80). Cytokines are produced by different T-lymphocytes. Infection with intracellular pathogens or tumors typically induces a cellular response effected by CD8+ T-lymphocytes (81,82). The induction of a humoral response in these types of infections may actually exacerbate disease, probably by down-regulating the Type 1 response (83,84). In that different cytokines upregulate and downregulate these responses, it has long been recognized that cytokines themselves may make powerful and selective adjuvants (85–87). This notion is further supported by the observation that different types of vaccine immunogens and adjuvants induce the production of different cytokines (88).

Hurdles and Opportunities

What are the delivery hurdles for using cytokines as adjuvants? Firstly, cytokines are highly potent molecules. Cytokines are expressed and secreted by T-lymphocytes (and other cells) for sending 'messages' to nearby cells (such as APCs). Even though the local concentration, say between two proximal cells, may be significant, the systemic cytokine level is extremely low and usually below detection levels. When cytokines are administered systemically, usually by parenteral injection, the amount of cytokine required to act as adjuvant often produces systemic toxicity (89). In that little is known about the delivery of large proteins (including cytokines) to lymphocyte subsets after parenteral injection, it is unlikely that this hurdle will be overcome in the near future, thus relegating cytokine delivery to the empirical science that it is. Secondly, the nature of the immune system is such that it is not affected by one cytokine at a time, but by several at once. In fact, it is likely that cytokines not only synergize with each other, but also act as antagonists, for example, in the IL-4 and IL-10 down-regulation of immune responses by suppressing macrophage antigen presentation and

the production of Th1 cytokines such as γ-IFN (90). In that a systematic study of cytokine interactions (as they pertain to adjuvant activity) has not been carried out, it is unlikely that the synergistic/antagonistic effects of cytokines will be unraveled in the near future. Thirdly, cytokines are often species-specific. For example, human γ-IFN will increase neopterin levels in primates (91), but does not do so in rodents (89,92), suggesting that mice and rats do not appropriately recognize human γ-IFN. This represents an enormous hurdle for the pharmaceutical use of cytokines as adjuvants, particularly because of the failure of animal models to accurately predict safety of human molecules (89,92). This is further complicated by the deleterious anti-cytokine immune response induced when testing cytokines derived from one species in another species (e.g., testing human γ-IFN in mice or baboons). Because of this species-specificity, xenogenous cytokines will not target correctly, likely resulting in a lack of adjuvant activity. Fortunately, headway is being made to clone cytokines from different species and then test in autologous systems as an integral part of vaccine adjuvant programs (86,93). Fourth, the development of cytokines as adjuvants is hampered by a significant 'non-delivery' issue that deserves mention here—the lack of knowledge regarding the type and magnitude of immune response required for efficacy. In that subunit vaccines are significantly different from the infectious pathogens they are supposed to protect against, augmentation of vaccine immunogenicity with a particular, but 'incorrect' cytokine may result in vaccine failure. Indeed, the failure to produce certain cytokines has been associated with vaccine nonresponsiveness as in the hepatitis B vaccine (94). These delivery hurdles to the development of cytokines as vaccine adjuvants make this one of the more challenging areas of vaccine research, with few short term successes on the immediate horizon, but because of their specificity, enormous potential in the long term.

DNA VACCINES

Current Status and Unmet Needs

Genetic immunization is carried out by injecting antigen-encoding DNA plasmids directly into muscle or skin, resulting in low level expression of the gene product (the antigen itself), with resultant host immunity against this antigen (95–98). The gene products are often correctly glycosylated, folded and expressed by the host cell. Because DNA translation and transcription occur intracellularly, delivery of the DNA plasmids to the cytosol represents one of the greatest hurdles for novel DNA vaccines. In order to deliver these plasmids to the nucleus of the target cells, several clever approaches have been used (99), including coating gold microparticles with the plasmid and delivering these directly into the skin by a particle bombardment device, such as a "gene gun" (96,100,101) or by viral vector delivery (102), as well as other organisms including attenuated Shigella (103). Genetic vaccination has been applied to several systems since its recent inception in 1992, including immune responses against cancer antigens (104), mycoplasma (105), tuberculosis (106), malaria (107), parasites (108), and many virus infections (109), including influenza (110) and HIV (111).

Hurdles and Opportunities

These successes represent some of the more exciting advances in novel vaccine design, although DNA vaccination has a few hurdles to overcome before this technology can be standardized (112). For example, the delivery of DNA to the target muscle cells (113) shows low delivery yields if simply injected (100), such that sophisticated delivery techniques are mandatory, including the use of 'adjuvants' (114), liposomes (115), gene gun delivery (96), or live vectors (103). Even with specialized delivery, the animal-to-animal variation has been great, and many of these studies have been carried out with small numbers of animals per group. This delivery issue is compounded by the relatively large amount of DNA required for gene product expression, where often hundreds of micrograms of DNA are required. Although there is no immediate solution to the *macroscopic* delivery problem of efficiently delivering DNA intracellularly, these issues are being presently addressed by a number of researchers.

A second delivery issue for genetic vaccination is signaled by the conspicuous dearth of reports in primates, presumably because of the difficulty of inducing a primate immune response (116,117). Most studies to date have focused on rodents, where successful genetic vaccination is well documented. Comparison of luciferase activity in rodents and rhesus monkeys showed that the luciferase expression levels were significantly reduced in monkeys compared to rodents, presumably because of the increased perimysium connective tissue in monkeys compared to rodents (116). Recently, DNA inoculation of cynomologous monkeys using bupivicaine as 'adjuvant' has shown to be effective in the induction of both humoral and cellular responses against HIV, although the number of animals tested was limited and the titers low (118). Interestingly however, the sera from these cyno monkey was effective at HIV-1 neutralization, hinting at the enormous potential of genetic immunization. In general, it is believed that this failure to see good immunogenicity in non-human primates is also due the difficulty in delivering DNA to the muscle cells of higher species, and this delivery problem is currently not well understood.

The third delivery issue for DNA based vaccines relates to the difficulty in boosting the immune response. Most studies thus far have shown only a modest immune response, although protection in several models has been demonstrated (110,119). Typically, when an immune response wanes, a repeat booster injection is given to increase the magnitude and the affinity of the immune response, particularly for non-replicating vaccines such as whole-killed, or subunit vaccines. For most conventional vaccines, this booster response is often magnitudes higher than the primary response. Herein lies one of the delivery challenges for DNA vaccines—can they be administered so that a decent booster response is observed after boosting? Thus far, significant boosters with DNA vaccines have not been dramatic (111), and have caused some concern that boosting is inherently difficult by DNA injection, possibly due to its low and variable delivery.

Mentioned only for completeness-sake, there are also a few non-delivery related hurdles in the development of DNA vaccines including safety concerns such as anti-DNA antibody formation, local reactogenicity and systemic toxicity, genetic and reproductive toxicity (120), DNA stability and purity (such as the removal of lipopolysaccharides) (121), and the device or adjuvant used to increase the delivery of DNA (95). Although these issues are real and time-consuming, they are not the major hurdles for the development of DNA vaccines; the greatest challenge is the targeted delivery of functional DNA to the host.

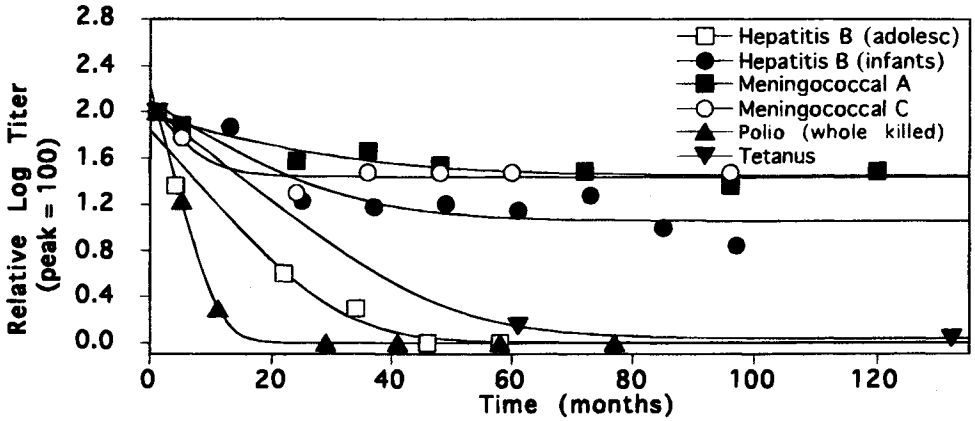

Fig. 1. Duration of the humoral immune response in humans for several antigens (see text). Because the titer values for the different antigens spanned several log values, all were normalized to a 'peak titer' of 100 shortly after final boosting, and the decay of the peak titers displayed in comparison to this reference point of 100 (or log 2.0).

STERILIZING IMMUNITY VACCINES

Current Status and Unmet Needs

Perhaps the greatest demand placed a non-live vaccine is 'sterilizing immunity', or the complete prevention of infection. It may be desirable to have 'sterilizing immunity', that is, the complete absence or prevention of infection, for diseases such as HIV-1, where the pathogenesis is not well understood. The pundits of HIV-1 vaccine design have cited several reasons why a subunit AIDS vaccine cannot be efficacious, including: the difficulty in inducing long-lasting sterilizing immunity, rapid genetic variation of the HIV-1 envelope, the lack of knowledge about the infection process and whether to focus on a parenteral or a mucosal delivery (each which has it's own specific set of delivery problems), and the lack of effectiveness of vaccine sera to neutralize field isolates of primary HIV-1 (122). The proponents of making a subunit HIV-1 vaccine rebut that vaccine effectiveness rarely correlates with laboratory neutralization assays (5), (indeed, for many pathogens these assays simply do not exist), and that the infection rate per contact is low (123), suggesting that any modulation of the immune system prior to infection will alter the infection rate, thus resulting in a partially effective vaccine. Although there is no single preclinical experiment that *proves* HIV-1 vaccine efficacy in humans, vaccine protection of chimpanzees against HIV-1 challenge suggests that sterilizing immunity is possible (124,125) providing the antibody titers are maintained at high levels.

Hurdles and Opportunities

Maintaining an elevated and durable immune response is one of the hurdles in the development of sterilizing vaccines. The duration of the immune response following vaccination is affected largely by two factors, the nature of the immune response itself, and by the sustained release of antigen from the vaccine. The first of these is intrinsic to the species being tested; after subunit vaccination the humoral response generally shows fairly rapid decay of the immune response, followed by low-level, prolonged titers that often last several years (Figure 1) (14,126–131). This plateau phase may be important for an effective HIV-1 vaccine if high antibody levels are required for protection, as were observed in the chimpanzee protection experiments (124,125). Thus, a human HIV-1 vaccine that demonstrates long-lasting, antibody titers comparable to titer levels achieved in protected chimpanzees at the time of HIV-1 challenge may afford sterilizing immunity in humans (132).

Maintaining high antibody titers by the sustained release of antigen from a delivery device (such as polymeric microspheres (11,16,17)) is the key delivery issue crucial to making a sterilizing vaccine. In that most adjuvants are not effective in altering the antibody decay half-lives or persistence, presumably because there is little sustained release of antigen after a few days or weeks (14), researchers have attempted to use sustained release formulations to make a vaccine giving high, long-lasting titers. The use of a polymeric-based vaccine that releases antigen at significantly later times after the primary immunization results in sustained titers, presumably due the continuous stimulation of the immune system by low level amounts of antigen released as the polymer undergoes hydrolysis and bulk erosion. Thus, the combination of soluble antigen and adjuvant for the primary response, and a polymer-encapsulated antigen for the release of antigen at later times is predicted to give a delayed-release formulation capable of maintaining high and long-lasting titers.

There are also other modifications of the vaccine that might alter the immune response decay kinetics, such as using a particulate antigen. It has been shown that particulate Hepatitis B surface antigen shows a slower decay of the baboon immune response than does soluble gp120 (77), and the particulate antigen Fluogen® gives slower decay of the immune response than does soluble ovalbumin in mice (117). Further, the addition of soluble adjuvant to sustained release formulations has also been demonstrated to maintain higher titers for longer periods (12). Although it has been demonstrated that high, long-lasting titers can be made by encapsulating antigen in polymeric microspheres, and these titers are functionally active in that they neutralize virus, there are several hurdles that need to be overcome, including optimizing the polymer type, particle size, antigen loading, release profile, combinations of microspheres, sterilization procedures, adjuvant encapsulation methodology to mention a few.

ACKNOWLEDGMENTS

I would like to thank Mark Newman, Fred Vogel, Tue Nguyen, Tim Gregory, Phil Berman, Don Francis, Mark Reddish, Jeff Cleland, and Larry Lachman for their helpful comments and advice regarding controversies in vaccine development. Administrative support was also provided by Milianne Chin and is gratefully acknowledged.

REFERENCES

1. S. A. Plotkin and E. A. Mortimer. *Vaccines*. Philadelphia: Saunders, (1988).
2. E. Brown, G. Dougan, E. M. Hoey, S. J. Martin, B. K. Rima, and A. Trudgett. *Vaccine Design*. New York: Wiley & Sons, (1993).
3. R. B. Fisher. *Edward Jenner 1749–1823*. St. Edmunds, Suffolk: St. Edmundsbury Press, pp. 361 (1991).
4. S. L. Plotkin and S. A. Plotkin. A short history of vaccination. In: S. A. Plotkin, E. A. Mortimer, (eds.) *Vaccines*. Philadelphia: Saunders, pp. 1–11 (1994).
5. D. P. Francis. Laboratory empiricism, clinical design, and social value. In: M. F. Powell, M. J. Newman, (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*. New York: Plenum Press, pp. 135–139 (1995).
6. P. Aaby. Malnutrition and overcrowding/intensive exposure in severe measles infection: Review of community studies. *Rev. Infect. Dis.* **10**:478–49 (1988).
7. I. M. Longini-Jr. M. E. Halloran, M. Haber, and R. T. Chen. Measuring vaccine efficacy from epidemics of acute infectious agents. *Stat. in Medicine* **12**:249–263 (1993).
8. L. B. Schonberger, J. E. McGowan-Jr., and M. B. Gregg. Vaccine-associated poliomyelitis in the United States. *Am. J. Epidemiology* **104**:202–211 (1976).
9. H. V. Wyatt. Polio immunization: Benefits and risks. *J. Family Practice* **3** (1978).
10. M. J. Newman and M. F. Powell. Immunological and formulation design considerations for subunit vaccines. In: M. F. Powell, M. J. Newman, (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*. Vol. 6. New York: Plenum Press, pp. 1–42 (1995).
11. J. L. Cleland, M. F. Powell, A. Lim, L. Barron, P. W. Berman, D. J. Eastman, J. H. Nunberg, T. Wrin, and J. C. Vennari. Development of a single-shot subunit vaccine for HIV-1. *AIDS Res. Human Retrovir.* **10**:S21–S26 (1994).
12. J. L. Cleland, L. Barrón, P. W. Berman, A. Daugherty, T. Gregory, A. Lim, J. Vennari, T. Wrin, and M. F. Powell. Development of a single-shot subunit vaccine for HIV-1: Part 2. Defining optimal autoboost characteristics to maximize the humoral immune response. *J. Pharm. Sci.* (1996) (in press).
13. J. L. Cleland, L. Barrón, A. Daugherty, D. Eastman, C. Kensil, A. Lim, R. P. Weissburg, T. Wrin, J. Vennari, and M. F. Powell. Development of a single-shot subunit vaccine for HIV-1: Part 3. Effect of adjuvant and immunization schedule on the duration of the humoral immune response to recombinant MN gp120. *J. Pharm. Sci.* (1996) (in press).
14. J. L. Cleland, M. F. Powell, A. Lim, L. Barron, P. W. Berman, J. H. Nunberg, D. J. Eastman, T. Wrin, J. C. Vennari, K. K. Murthy, C. R. Kensil, and J. Y. Wu. Development of a single administration subunit vaccine for HIV-1. *Proceed. Intern. Symp. Control. Rel. Bioact. Mater.* **22**:570–571 (1995).
15. J. L. Cleland, A. Lim, A. Daugherty, L. Barrón, N. Desjardin, E. T. Duenas, D. J. Eastman, J. C. Vennari, T. Wrin, P. Berman, K. K. Murthy, and M. F. Powell. Development of a single-shot subunit vaccine for HIV-1: Part 5. Programmable in vivo auto boost and long lasting neutralizing response. In preparation, 1996.
16. J. L. Cleland, A. Lim, L. Barrón, E. T. Duenas, and M. F. Powell. Development of a single-shot subunit vaccine for HIV-1: Part 4. Optimizing microencapsulation on pulsatile release of MN gp 120 from biodegradable microspheres. *J. Pharm. Sci.*, (1996) (in press).
17. J. H. Eldridge, J. K. Staas, J. A. Meulbroek, J. R. McGhee, T. R. Tice, and R. M. Gilley. Biodegradable microspheres as a vaccine delivery system. *Molecular Immunology* **28**:287–294 (1991).
18. D. T. O'Hagan, D. Rahman, J. P. McGee, H. Jeffery, M. C. Davies, P. Williams, S. S. Davis, and S. J. Challacombe. Biodegradable microparticles as controlled release antigen delivery systems. *Immunology* **73**:239–242 (1991).
19. J. Hanes, M. Chiba, and R. Langer. Polymer microspheres for vaccine delivery. In: M. F. Powell, M. J. Newman, (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*. Vol. 6. New York: Plenum Press, pp. 389–412 (1995).
20. J. L. Cleland. Design and production of single-immunization vaccines using polylactide polyglycolide microsphere systems. In: M. F. Powell, M. J. Newman, (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*. Vol. 6. New York: Plenum Press, pp. 439–462 (1995).
21. W. R. Gombotz, M. S. Healy, and L. R. Brown. Very low temperatures casting of controlled release microspheres. (1991). Enzytech, Inc. 5/28/91 Patent #5019400.
22. M. F. Powell, J. L. Cleland, and S. J. Shire. The development of stable protein formulations: A close look at protein aggregation, deamidation, and oxidation. *Crit. Rev. Thera. Drug Carr. Sys.* **10**:307–377 (1993).
23. B. Gander, C. Thomasin, H. P. Merkle, Y. Men, and G. Corradin. Pulsed tetanus toxoid release from PLGA-microspheres and its relevance for immunogenicity in mice. *Proc. Int. Symp. Control. Rel. Bioact. Mater.* Washington DC: Controlled Release Society, pp. 65–66 (1993).
24. R. A. Miller, J. M. Brady, and D. E. Cutright. Degradation rates of oral resorbable implants (polylactates and polyglycolates): Rate modification with changes in PLA/PGA copolymer ratios. *J. Biomed. Mater. Res.* **11**:711–719 (1977).
25. R. A. Kenley, M. O. Lee, T. R. Mahoney-II, and L. M. Sanders. Poly(lactide-co-glycolide) decomposition kinetics in vivo and in vitro. *Macromolecules* **20**:2398–2403 (1987).
26. W. W. Wong, S. Vijayakumar, and R. R. Weichselbaum. Prognostic indicators in node-negative early stage breast cancer. *Am. J. Med.* **92**:539–548 (1992).
27. G. J. V. Nossal. Molecular and cellular aspects of immunologic tolerance. *Eur. J. Biochem.* **202**:729–737 (1991).
28. S. Adelstein, H. Pritchard-Briscoe, T. A. Anderson, J. Crosbie, G. Gammon, R. H. Loblay, A. Basten, and C. C. Goodnow. Induction of self-tolerance in T cells but not B cells of transgenic mice expressing little self antigen. *Science* **251**:1223–1225 (1991).
29. J. L. Urban, and H. Schreiber. Tumor antigens. *J. Immunology* **10**:617–644 (1992).
30. M. G. Brown, J. Driscoll, and J. J. Monaco. Structural and serological similarity of MHC-linked LMP and proteosome (multicatalytic proteinase) complexes. *Nature* **353**:335–357 (1991).
31. C. K. Martinez, and J. J. Monaco. Homology of proteosome subunits to a major histocompatibility complex-linked LMP gene. *Nature* **353**:664–667 (1991).
32. A. L. Goldberg, and K. L. Rock. Proteolysis, proteosomes and antigen presentation. *Nature* **357**:375–379 (1992).
33. J. J. Monaco, S. Cho, and M. Attaya. Transport proteins in the murine MHC: Possible implications for antigen processing. *Science* **250**:1723–1726 (1990).
34. A. Kelly, S. H. Powis, L. Kerr, I. Mockridge, T. Elliott, J. Bastin, B. Uchanska-Ziegler, A. Ziegler, and A. Townsend. Assembly and function of the two ABC transporter proteins encoded in the human major histocompatibility complex. *Nature* **355**:641–644 (1992).
35. T. Spies, V. Cerundolo, M. Colonna, P. Cresswell, A. Townsend, and R. DeMars. Presentation of viral antigen by class I MHC molecules is dependent on a putative peptide transporter heterodimer. *Nature* **355**:644–646 (1992).
36. W. Suh, M. F. Cohen-Doyle, K. Fruh, K. Wang, P. A. Peterson, and D. B. Williams. Interaction of MHC class I molecules with the transporter associated with antigen processing. *Science* **264**:1322–1326 (1994).
37. E. Degen, and D. B. Williams. Participation of a novel 88 kD protein in the biogenesis of murine class I histocompatibility molecules. *J. Cell. Biol.* **112**:1099–1115 (1991).
38. M. R. Jackson, M. F. Cohen-Doyle, P. A. Peterson, and D. B. Williams. Regulation of MHC class I transport by the molecular chaperon, calnexin (p88, IP90). *Science* **263**:384–387 (1994).
39. K. Falk, O. Rotzschke, and H. Rammensee. Cellular peptide

composition governed by major histocompatibility complex class I molecules. *Nature* **348**:248–251 (1990).
40. K. Falk, O. Rotzschke, S. Stevanovic, G. Jung, and H. G. Rammensee. Allele-specific motifs revealed by sequencing of self-peptides eluted from MHC molecules. *Nature* **351**:290–295 (1991).
41. G. M. van-Bleek, and S. G. Nathenson. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2K^b molecule. *Nature* **348**:213–216 (1990).
42. O. Rotzschke, K. Falk, O. Deres, H. Schild, M. Norda, J. Metzger, G. Jung, and H. Rammensee. Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells. *Nature* **348**:252–253 (1990).
43. D. S. Collins, E. R. Unanue, and C. V. Harding. Reduction of disulfide bonds within lysosomes is a key step in antigen processing. *J. Immunol.* **147**:4054–4059 (1991).
44. J. M. van-Noort, J. Boon, A. C. M. Van-der-Drift, J. P. A. Wagenaar, A. M. H. Boot, and C. J. P. Goog. Antigen processing by endosomal proteases determines which sites on sperm-whale myoglobin are eventually recognized by T cells. *Eur. J. Immunol.* **21**:1989–1996 (1991).
45. L. Vidard, K. L. Rock, and B. Benacerraf. The generation of immunogenic peptides can be selectively increase or decrease by proteolytic enzyme inhibitors. *J. Immunol.* **147**:1786–1791 (1991).
46. B. Bennett, I. J. Check, M. R. Olsen, and R. L. Hunter. A comparison of commercially available adjuvants for use in research. *J. Immunol. Meth.* **153**:31–40 (1992).
47. P. J. Peters, J. J. Neefjes, V. Oorschot, H. L. Ploegh, and H. J. Geuze. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to the lysosomal compartments. *Nature* **349**:669–676 (1991).
48. C. V. Harding, D. S. Collins, J. W. Slot, H. J. Geuze, and E. R. Unanue. Liposome-encapsulated antigens are processed in lysosomes, recycled and presented to T-cells. *Cell* **64**:393–401 (1991).
49. J. J. Neefjes, V. Stollorz, P. J. Peters, H. J. Geuze, and H. L. Ploegh. The biosynthetic pathway of MHC class II but not class I molecules intersects the endocytic route. *Cell* **61**:171–183 (1990).
50. M. S. Anderson, and J. Miler. Invariant chain can function as a chaperon protein for class II major histocompatibility complex molecules. *Proc. Natl. Acad. Sci.* **89**:2282–2286 (1992).
51. A. Sette, S. Buus, C. Colon, C. Miles, and H. M. Grey. I-A^d binding peptides derived from unrelated proteins share a common structural motif. *J. Immunol.* **141**:45–48 (1988).
52. D. F. Hunt, H. Michel, T. A. Dickinson, J. Shabanowitz, A. L. Cox, K. Sakaguchi, E. Appella, H. M. Grey, and A. Sette. Peptides presented to the immune system by the murine class II major histocompatibility complex molecule I-A^d. *Science* **256**:660–662 (1992).
53. A. Sette, J. Sidney, C. Oseroff, M. del-Guerico, S. Southwood, T. Arrhenius, M. F. Powell, S. M. Colon, F. C. A. Gaeta, and H. M. Grey. HLA DR4w4-binding motifs illustrate the biochemical basis of degeneracy and specificity in peptide-DR interactions. *J. Immunology* **151**:3163–3170 (1993).
54. C. H. Kensil, U. Patel, M. Lennick, and D. Marciani. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* molina cortex. *J. Immunol.* **146**:431–437 (1991).
55. J. Wu, B. H. Gardiner, C. I. Murphy, J. R. Seals, C. R. Kensil, J. Recchia, G. A. Beltz, G. W. Newman, and M. J. Newman. Saponin adjuvant enhancement of antigen-specific immune responses to an experimental HIV-1 vaccine. *J. Immunol.* **148**:1519–1525 (1992).
56. M. J. Newman, J. Wu, B. H. Gardner, K. J. Munroe, D. Leombruno, J. Recchia, C. R. Kensil, and R. T. Coughlin. Saponin adjuvant induction of ovalbumin-specific CD8$^+$ cytotoxic T lymphocyte responses. *J. Immunology* **148**:2357–2362 (1992).
57. L. J. Old. Cancer immunology: The search for specificity-GHA Clowes Memorial Lecture. *Cancer Res.* **41**:361–375 (1981).
58. K. S. Furukawa, K. Furukawa, F. X. Real, L. J. Old, and K. O. Loyd. A unique antigenic epitope of human melanoma is carried on the common melanoma glycoprotein gp95/p97. *J. Exp. Med.* **169**:585–590 (1989).
59. A. Ryghetti, V. Turchi, C. A. Ghetti, G. Scambia, P. B. Panici, G. Roncucci, S. Mancuso, L. Frati, and M. Nuti. Human B cell immune response to the polymorphic epithelial mucin. *Cancer Res.* **53**:2457–2459 (1993).
60. P. O. Livingston, E. J. Natoli-Jr., M. J. Calves, E. Stockert, H. F. Oettgen, and L. J. Old. Vaccines containing purified GM2 ganglioside elicit GM2 antibodies in melanoma patients. *Proc. Natl. Acad. Sci.* **84**:2911–2915 (1987).
61. J. Bystryn, S. Ferrone, and P. Livingston. *Specific Immunotherapy of Cancer with Vaccines*. Vol. 690: New York, pp. 411 (1993).
62. D. L. Morton, L. J. Foshag, D. S. B. Hoon, J. A. Nizze, E. Famatiga, L. A. Wanek, C. Chang, D. G. Davtyan, R. K. Gupta, R. Elashoff, and R. F. Irie. Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. *Ann. Surg.* **216**:463–482 (1992).
63. D. Berd, H. C. Maguire-Jr., P. McCue, and M. J. Mastrangelo. Treatment of metastatic melanoma with an autologous tumor-cell vaccine: Clinical and immunologic results in 64 patients. *J. Clin. Oncol.* **8**:1858–1867 (1990).
64. P. Hersey, A. Edwards, A. Coates, H. Shaw, W. H. McCarthy, and G. W. Milton. Evidence that treatment with vaccinia melanoma cell lysates (VMCL) may improve survival of patients with stage II melanoma. *Cancer Immunol. Immunother.* **25**:257–265 (1987).
65. L. G. Durrant, G. W. L. Denton, and R. A. Robins. Immunization with human monoclonal antiidiotypic antibody in colorectal cancer. In: J. Bystryn, S. Ferrone, P. Livingston (eds.) *Specific Immunotherapy of Cancer with Vaccines*. Vol. 690. New York: NY Acad. Sci., pp. 334–342 (1993).
66. M. R. Hilleman. The promise and the reality of viral vaccines against cancer. In: J. Bystryn, S. Ferrone, P. Livingston (eds.) *Specific Immunotherapy of Cancer with Vaccines*. Vol. 690. New York: NY Acad. Sci., pp. 6–18 (1994).
67. A. J. McMichael and W. F. Bodmer. *A New Look at Tumor Immunology*. Cold Spring Harbor, NY: Cold Spring Harbor Lab. Press, (1992).
68. T. Boon. Teaching the immune system to fight cancer. *Sci. Am.* **266**:82–89 (1993).
69. B. M. Longenecker, M. Reddish, R. Koganty, and G. D. MacLean. Immune responses of mice and human breast cancer patients following immunization with synthetic sialyl-Tn conjugated to KLH plus Detox adjuvant. *Ann. N. Y. Acad. Sci.* **690**:276–291 (1993).
70. D. M. Pardoll. Cancer vaccines. *Immunol. Today* **14**:310–316 (1993). See also: D. M. Pardoll, Cancer vaccines: A road map for the next decade. *Curr. Opin. Immunol.* **100**:619–621 (1996).
71. H. N. Eisen, and G. W. Siskind. Variations in affinities of antibodies during the immune response. *Biochemistry* **3**:996 (1964).
72. L. A. Steiner, and H. N. Eisen. The relative affinity of antibodies synthesized in the secondary response. *J. Exp. Med.* **126**:1185–1205 (1967).
73. D. Salk, and J. Salk. Vaccinology of poliomyelitis. *Vaccine* **2**:59–74 (1984).
74. V. A. Fulginiti. Immunizations: Current Controversies. *J. Pediatr.* **101**:487–494 (1982).
75. M. Bruguera, M. Cremades, R. Salinas, J. Costa, M. Grau, and J. Sans. Impaired response to recombinant hepatitis B vaccine in HIV-infected persons. *J. Clin. Gastero.* **14**:27–30 (1992).
76. J. S. MacKenzie. Influenza subunit vaccine: Antibody responses to one and two dose of vaccine and length of response, with reference to the elderly. *Br. Med. J.* **1**:200–202 (1977).
77. K. P. Anderson, C. Lucas, C. V. Hanson, H. F. Londe, A. Izu, T. Gregory, A. Ammann, P. W. Berman, and J. W. Eichberg. Effect of dose and immunization schedule on immune response of baboons to recombinant glycoprotein 120 of HIV-1. *J. Infect. Diseases* **160**:960–969 (1989).
78. M. F. Powell, and M. J. Newman. *Vaccine Design: The Subunit and Adjuvant Approach*. In: R. T. Borchardt, ed. Pharmaceutical Biotechnology. Vol. 6. New York: Plenum Press, pp. 949 (1995).
79. F. R. Vogel, and M. F. Powell. A compendium of vaccine adjuvants and excipients. In: M. F. Powell, M. J. Newman (eds.) *Vaccine Design: The Subunit and Adjuvant Approach*. Vol. 6. New York: Plenum Press, pp. 141–228 (1995).
80. W. E. Paul, and R. A. Seder. Lymphocyte responses and cytokines. *Cell* **76**:241–251 (1994).
81. E. G. Pamer. Cellular immunity to intracellular bacteria. *Curr. Opin. Immunol.* **5**:492–496 (1993).

82. S. H. E. Kaufmann. Immunity to intracellular bacteria. *Annu. Rev. Immunol.* **11**:129–164 (1993).
83. B. S. Graham, G. S. Henderson, Y. Tang, X. Lu, K. M. Neuzil, and D. G. Colley. Priming immunization determines T helper cytokine mRNA expression patterns in lungs of mice challenged with respiratory syncytial virus. *J. Immunol.* **151**:2032–2040 (1993).
84. R. L. Modlin, and T. B. Nutman. Type 2 cytokines and negative immune regulation in human infections. *Curr. Opin. Immunol.* **5**:511–517 (1993).
85. D. D. Ho, M. G. Sarngadharan, M. S. Hirsch, R. T. Schooley, T. R. Rota, R. C. Kennedy, T. C. Chanh, and V. L. Sato. Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. *J. Virol.* **61**:2024–2028 (1987).
86. A. W. Heath. Cytokines as immunological adjuvants. In: M. F. Powell, M. J. Newman, (eds.) *Vaccine Design: The Subunit and Adjuvant Approach.* New York: Plenum Press, pp. 645–658 (1995).
87. L. B. Lachman, L. Shih, X. Rao, S. E. Ullrich, and J. L. Cleland. Cytokine-containing liposomes as adjuvants for subunit vaccines. In: M. F. Powell, M. J. Newman, (eds.) *Vaccine Design: The Subunit and Adjuvant Approach.* New York: Plenum Press, pp. 659–671 (1995).
88. W. H. Alwan, F. M. Record, and P. J. M. Openshaw. Phenotypic and functional characterization of T cell lines specific for individual respiratory syncytial virus proteins. *J. Immunol.* **150**:5211–5218 (1993).
89. T. G. Terrell and J. D. Green. Comparative pathology of recombinant murine interferon-gamma in mice and recombinant human interferon-gamma in Cynomolgus Monkeys. *Intl. Rev. Exp. Pathol.* **34**:73–101 (1993).
90. T. R. Mosmann and R. L. Coffman. TH1 and TH2 cells: Different patterns of lymphokine secretion lead to different functional properties. *Annu. Rev. Immunol.* **7**:145–173 (1989).
91. G. Reibnegger, D. Fuchs, L. C. Fuith, A. Hausen, E. R. Werner, G. Werner-Felmayer, and H. Wachter. Neopterin as a marker for activated cell-mediated immunity: Application in malignant disease. *Cancer Detection & Prevention* **15**:483–490 (1991).
92. J. D. Green and T. G. Terrell. Utilization of homologous proteins to evaluate the safety of recombinant human proteins—case study: Recombinant human interferon-gamma (rhIFN-g). *Toxicology Letters* **64/65**:321–327 (1992).
93. P. Dong, C. Brunn, and R. J. Y. Ho. Cytokines as vaccine adjuvants: Current status and potential applications. In: M. F. Powell, M. J. Newman, (eds.) *Vaccine Design: The Subunit and Adjuvant Approach.* New York: Plenum Press, pp. 625–643 (1995).
94. J. Vingerhoets, G. Vanham, L. Kestens, G. Penne, G. Leroux-Roels, and P. Gigase. Deficient T-cell responses in non-responders to hepatitis B vaccination: Absence of TH1 cytokine production. *Immunol. Lett.* **39**:163–168 (1994).
95. F. R. Vogel, and N. Sarver. Nucleic acid vaccines. *Clin. Microbiol. Rev.* **8**:406–410 (1995).
96. S. A. Johnston, and D. C. Tang. Gene gun transfection of animal cells and genetic immunization. *Methods Cell Biol.* **43**:353–365 (1994).
97. N. R. Rabinovich, P. McInnes, D. L. Klein, and B. F. Hall. Vaccine technologies: View to the future. *Science* **265**:1401–1404 (1994).
98. E. F. Fynan, R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. DNA vaccines: Protective immunizations by parenteral, mucosal, and gene-gun inoculations. *Proc. Natl. Acad. Sci.* **90**:11478–11482 (1993).
99. M. Nakanishi. Gene introduction into animal tissues. *Crit. Rev. Thera. Drug Carr. Sys.* **12**:263–310 (1995).
100. E. F. Fynan, R. G. Webster, D. H. Fuller, J. R. Haynes, J. C. Santoro, and H. L. Robinson. DNA vaccines: A novel approach to immunization. *Intl. J. Immunopharmacol.* **17**:79–83 (1995).
101. M. D. Eisenbraun, D. H. Fuller, and J. R. Haynes. Examination of parameters affecting the elicitation of humoral immune response by particle bombardment-mediated genetic immunization. *DNA & Cell Biol.* **12**:791–797 (1993).
102. A. R. Fooks, E. Schadeck, U. G. Liebert, A. B. Dowsett, B. K. Rima, M. Steward, J. R. Stephenson, and G. W. Wilkinson. High-level expression of the measles virus nucleocapsid protein by using a replication-deficient adenovirus vector: Induction of an MHC-1-restricted CTL response and protection in a murine model. *Virology* **210**:456–465 (1995).
103. D. R. Sizemore, A. A. Branstrom, and J. C. Sadoff. Attenuated Shigella as a DNA delivery vehicle for DNA-mediated immunization. *Science* **270**:299–302 (1995).
104. R. A. Spooner, M. P. Deonarain, and A. A. Epenetos. DNA vaccination for cancer treatment. *Gene Therapy* **2**:173–180 (1995).
105. W. C. Lai, M. Bennett, S. A. Johnston, M. A. Barry, and S. P. Pakes. Protection against Mycoplasma pulmonis infection by genetic vaccination. *DNA & Cell Biol.* **14**:643–651 (1995).
106. D. B. Lowrie, R. E. Tascon, M. J. Colston, and C. L. Silva. Towards a DNA vaccine against tuberculosis. *Vaccine* **12**:1537–1540 (1994).
107. S. L. Hoffman, M. Sedegah, and R. C. Hedstrom. Protection against malaria by immunization with a Plasmodium yoelii circumsporozoite protein nucleic acid vaccine. *Vaccine* **12**:1529–1533 (1994).
108. W. Yang, G. J. Waine, and D. P. McManus. Antibodies to Schistosoma japonicum (Asian bloodfluke) paramyosin induced by nucleic acid vaccination. *Biochem. Biophys. Res. Comm.* **212**:1029–1039 (1995).
109. M. Yokoyama, J. Zhang, and J. L. Whitton. DNA immunization confers protection against lethal lymphocytic choriomeningitis virus infection. *J. Virol.* **69**:2684–2688 (1995).
110. R. G. Webster, E. F. Fynan, J. C. Santoro, and H. Robinson. Protection of ferrets against influenza challenge with a DNA vaccine to the haemagglutinin. *Vaccine* **12**:1495–1498 (1994).
111. D. H. Fuller, and J. R. Haynes. A qualitative progression in HIV type 1 glycoprotein 120-specific cytotoxic cellular and humoral immune responses in mice receiving a DNA-based glycoprotein 120 vaccine. *AIDS Res. Human Retrovir.* **10**:1433–1441 (1994).
112. A. Fomsgaard. Genetic immunization—"the biological equivalent of cold fusion?". *Ugeskrift for Laeger* **157**:4932–4936 (1995).
113. M. Vitadello, M. V. Schiaffino, A. Picard, M. Scarpa, and S. Schiaffino. Gene transfer in regenerating muscle. *Human Gene Therapy* **5**:11–18 (1994).
114. I. Danko, J. D. Fritz, S. Jiao, K. Hogan, J. S. Latendresse, and J. A. Wolff. Pharmacological enhancement of in vivo foreign gene expression in muscle. *Gene Therapy* **1**:1–8 (1993).
115. R. J. Mannino, and S. Gould-Fogerite. Liposome mediated gene transfer. *BioTechniques* **6**:682–690 (1988).
116. S. Jiao, P. Williams, R. K. Berg, B. A. Hodgeman, L. Liu, G. Repetto, and J. A. Wolff. Direct gene transfer into nonhuman primate myofibers in vivo. *Human Gene Therapy* **3**:21–33 (1992).
117. M. J. Newman. Personal communication. (1996).
118. B. Wang, J. Boyer, V. Srikantan, K. Ugen, L. Gilbert, C. Phan, K. Dang, M. Merva, M. G. Agadjanyan, M. Newman, R. Carrano, D. McCallus, L. Coney, W. V. Williams, and D. B. Weiner. Induction of humoral and cellular immune responses to the human immunodeficiency type 1 virus in nonhuman primates by in vivo DNA inoculation. *Virology* **211**:102–112 (1995).
119. C. C. Zarozinski, E. F. Fynan, L. K. Selin, H. L. Robinson, and R. M. Welse. Protective CTL-dependent immunity and enhanced immunopathology in mice immunized by particle bombardment with DNA encoding an internal virion protein. *J. Immunol.* **154**:4010–4017 (1995).
120. J. S. Robertson. Safety considerations for nucleic acid vaccines. *Vaccine* **12**:1526–1528 (1994).
121. H. A. Smith. Regulatory considerations for nucleic acid vaccines. *Vaccine* **12**:1515–1519 (1994).
122. D. P. Bolognesi. The dilemma of developing and testing AIDS vaccines. In: G. M. Cooper, R. G. Temin, B. Sugden, (eds.) *The DNA Provirus: Howard Temin's Scientific Legacy.* Washington DC: Amer. Soc. Microbiol., pp. 301–312 (1995).
123. S. H. Pincus, K. G. Messer, P. L. Nara, W. A. Blattner, G. Colclough, and M. Reitz. Temporal analysis of the antibody response to HIV envelope protein in HIV-infected laboratory workers. *J. Clin. Investigation* **93**:2505–2513 (1994).
124. P. W. Berman, T. Matthews, D. Eastman, G. Nakamura, M. A. Champe, J. P. Porter, F. M. Wurm, R. D. Hershberg, E. K. Cobb, and J. W. Eichberg. Protection of chimpanzees from infection by

HIV-1 after vaccination with recombinant gp120 but not gp160. *Nature* **345**:622–625 (1994).
125. P. W. Berman, K. K. Murthy, T. Wrin, J. Vennari, E. K. Cobb, D. J. Eastman, M. Champe, G. Nakamura, D. Davison, M. F. Powell, J. Bussiere, T. J. Gregory, T. Matthews, and J. F. Obijeski. Protection of MN-rgp120 immunized chimpanzees from heterologous infection with a primary isolate of HIV-1. *J. Infect. Dis.* (1995) (in press).
126. J. E. Salk. How many injections of poliomyelitis vaccine for effective and durable immunity? *J.A.M.A.* **167**:1–7 (1958).
127. D. Salk, A. L. van Wezel, and J. Salk. Induction of long term immunity to paralytic poliomyelitis by use of non-infectious vaccine. *Lancet* **2**:1317–1321 (1984).
128. K. M. Zangwill, R. W. Stout, G. M. Carlone, L. Pais, H. Harekeh, S. Mitchell, W. H. Wolfe, V. Blackwood, B. D. Plikaytis, and J. D. Wenger. Duration of antibody response after meningococcal polysaccharide vaccination in US Air Force personnel. *J. Infect. Dis.* **169**:847–852 (1994).
129. Z. Mintai, L. Kezhou, D. Lieming, and R. A. Smego-Jr. Duration and efficacy of immune response to hepatitis B vaccine in high-risk Chinese adolescents. *Clin. Infect. Dis.* **16**:165–167 (1993).
130. D. Lieming, Z. Mintai, W. Yinfu, Z. Shaochon, K. Weiqin, and R. A. Smego-Jr. A 9-year follow-up study of the immunogenecity and long-term efficacy of plasma-derived hepatitis B vaccine in high-risk Chinese neonates. *Clin. Infect. Dis.* **17**:475–479 (1993).
131. L. G. Payne, S. A. Jenkins, A. Andrianov, and B. E. Roberts. Water-soluble phosphazene polymers for parenteral and mucosal vaccine delivery. In: M. F. Powell, M. J. Newman, (eds.) *Vaccine Design: The Subunit and Adjuvant Approach.* Vol. 6. New York: Plenum Press, pp. 473–493 (1995).
132. D. Francis, P. W. Berman, J. L. Cleland, J. McElrath, T. J. Gregory, A. Lim, T. Wrin, J. Vennari, and M. F. Powell. Decay and persistence of the humoral immune response to recombinant MN gp120 in humans. (1996) *In preparation.*