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Review

Genetic Vaccines: Strategies for Optimization

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Vaccination with attenuated or killed microbes, purified or recombinant subunit proteins and synthetic peptides is often hampered by toxicity, the presence of infectious agents, weak immune responses and prohibiting costs, especially in the developing world. Such problems may be circumvented by genetic immunization which has recently emerged as an attractive alternative to conventional vaccines. Numerous studies have already shown that immunization of experimental animals with plasmid DNA encoding antigens from a wide spectrum of bacteria, viruses, protozoa and cancers leads to protective humoural and cell-mediated immunity. This review deals with the background and progress made so far with DNA vaccines and their theoretical and practical advantages as well as potential risks, discusses proposed mechanisms of DNA transfection of cells and induction of immune responses to the produced vaccine antigen, and evaluates strategies for the control and optimization of such responses.

KEY WORDS: nucleic acids; genetic immunization; DNA vaccines; gene-gun; cationic lipids; liposomes.

INTRODUCTION

Immunization against microbial infections and cancer is an attractive alternative to chemotherapy. Thus, widespread use of the smallpox vaccine has eradicated the pathogen globally and other vaccines such as those against tetanus, diphtheria, whooping cough, polio and measles prevent millions of deaths each year. However, vaccines consisting of attenuated organisms, although efficacious in producing diverse and persistent immune responses by mimicking natural infections usually without the disease, can be potentially unsafe. For instance, there is a risk of reversion during replication of live viruses or even mutation to a more pathogenic state, and with immunocompromised individuals some of the attenuated viruses may still provoke disease. On the other hand, the extracellular localization of killed virus vaccines and their subsequent phagocytosis by professional antigen presenting cells (APC) or antigen-specific B cells, lead to MHC-II class restricted presentation and to T helper cell and humoural immunity but not to significant cytotoxic T cell (CTL) responses. Moreover, subunit vaccines produced from biological fluids may not be entirely free of infectious agents.

Advances in recombinant DNA and monoclonal antibody technology and the understanding of the immunological structure of proteins and factors regulating immune responses witnessed in the last two decades, have led to a new generation of recombinant subunit and synthetic peptide vaccines (1) that mimic small regions of microbial proteins. These are defined at the molecular level, can elicit specific immune responses and are therefore considered safe. Unfortunately, subunit and peptide vaccines are weak immunogens and are often unable to induce appropriate immune responses. A great variety of experimental immunological adjuvants (1,2) now available go a long way in rendering such vaccines stronger and more efficient. However, seventy odd years after the introduction of aluminium salts as an adjuvant, only one other adjuvant, liposomes (3), has been approved for use in humans (3,4). Thus, inspite of considerable progress, the road to the ideal vaccine appears as elusive as ever, that is until recently.

A novel and exciting concept now developed, namely de novo production of the required vaccine antigen by the host's cells in vivo, promises to revolutionize vaccination, especially

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ABBREVIATIONS: DNA, deoxyribonucleic acid; mRNA, messanger ribonucleic acid; CMV, cytomegalovirus; RSV, Rous sarcoma virus; ISS, immunostimulatory DNA sequences; CpG, unmethylated cytosine adjacent to guanine; IL-2, interleukin 2; IL-4, interleukin 4; IL-5, interleukin 5; IL-6, interleukin 6; IL- 12, interleukin 12; IFN-α, interferon α; IFN-γ, interferon γ; TNF-α, tumour necrosis factor ∝; CTL response, cytotoxic T lymphocyte response; APC, antigen presenting cells; IgG_1, immunoglobulin G_1; IgG_{2a}, immunoglobulin G_{2a}; IgG_{2b}, immunoglobulin G_{2b}; HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency virus; LCMV, lymphocyte choriomeningitis virus; HBV, hepatitis B virus; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; HBsAg, hepatitis B surface antigen; pGL2, luciferase-encoding DNA; pRc/CMV HBS, hepatitis B surface antigen (S region)-encoding DNA; pRSVGH, human growth hormone-encoding DNA; pCMV 4.65, mycobacterium leprosy protein-encoding DNA; pCMV 4.EGFP, fluorescent green protein-encoding DNA; VR 1020, Schistosome protein-encoding DNA; IM, intramuscularly; SC, subcutaneously; IV, intravenously; IP, intraperitoneally; IN, intranasally; IE, intraepidermally; PC, egg phosphatidylcholine; DOPE, dioleoyl phosphatidylethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; SA, stearylamine; BisHOP, 1,2-bis (hexadecylcycloxy)-3-trimethylaminopropane; DOTMA, N[1-(2,3-dioleoyloxy) propyl]-N,N,N, triethylammonium; DC-Chol, 3β-(N,N-dimethylaminoethane) carbonyl cholesterol; DOTAP, 1,2-dioleoyl-3(trimethylammonium)-propane; DODAP, 1,2-dioleoyl-3-dimethylammonium propane.

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where vaccines are either ineffective or unavailable. The concept entails the direct injection of antigen-encoding plasmid DNA which, following its uptake by cells, finds its way to the nucleus where it transfects the cells episomally. Produced antigen, recognized as foreign by the host, is then subjected to pathways similar to those undergone by the antigens of internalized viruses (but without their disadvantages) leading to protective humoural and cell mediated immunity (5–10). The present review will discuss the background and progress made so far with DNA immunization, evaluate mechanisms proposed for the induction of immunity and introduce approaches for its control and optimization.

NAKED DNA IMMUNIZATION

Observations heralding a role of gene delivery in therapy were made originally in the 50's when injection of crude preparations of DNA from tumours led to tumour formation in experimental animals (11,12). These findings were confirmed with the use of purified (13) and recombinant (14) viral DNA. In subsequent work, plasmid DNAs encoding hepatitis B proteins (15), insulin (16) and reporter molecules (17) were able to transfect cells on injection with the gene products exerting their corresponding functions. It was only recently however that the significance of gene expression on direct injection of DNA, in terms of genetic immunization (or vaccination) (18), was realized. Thus, a succession of publications from 1992 onwards established first the ability of plasmid DNA to induce an immune (antibody) response to the encoded foreign protein (human growth hormone) (18) and then, in experiments with DNA encoding influenza nucleoprotein, that immunity was both humoural and cell-mediated and also protective in mice challenged with the virus (19,20). This was the first demonstration of an experimental DNA vaccine. At about the same time, humoural and cell-mediated immunity against HIV-1 using plasmids encoding the HIV *rev* and *env* proteins was reported (21) and similar results were obtained a little later with a gene for the hepatitis B surface antigen (HBsAg) (22). It also appeared that DNA immunization could be applied in cancer immunotherapy: injection of plasmids encoding tumour antigens resulted in the induction of immune responses (23,24) which were protective in an animal model (24). Following these pioneering studies, the concept of DNA immunization has now been adopted by vaccinologists worldwide using an ever increasing number of plasmids encoding immunogens from bacterial, viral and parasitic pathogens and a variety of tumours (Table 1). In many of these studies genetic immunization led to the protection of animals from infection (e.g., refs. 19,24,26,41,51,55–57) or allergic reactions (e.g., ref. 59). Significantly, clinical trials for the therapy of or prophylaxis against HIV, herpes, influenza, carcinomas and hepatitis B are already in progress (9).

The Plasmid Vaccine

A successful DNA vaccine must apparently (7) be supercoiled. Its components consist of the gene encoding the antigen of interest (normally the section of the target pathogen which elicits protective immunity), a promoter sequence (usually derived from cytomegalovirus (CMV) or Rous sarcoma virus (RSV)) to drive the transcription of the antigen gene insert, an mRNA stability polyadenylation region at the 3′ end of the insert to ensure translation, the plasminogen activator gene which controls the secretion of the recombinant product, and ancillary signals. There are in addition an origin of replication for the amplification of the plasmid in bacteria and a gene for antibiotic resistance to select the transformed bacteria. As only one or two representative viral genes are selected for insertion in the DNA plasmid and not the full length viral genetic information, there is no danger of genetic recombination with superinfection by natural viral isolates.

It had been originally assumed that the extent of immune response elicited by the immunized host would be proportional to the amount of antigens produced by the plasmid (8). As a result, appropriate expression-enhancing promoters were attached to the antigen-encoding DNA. It was observed (61) however that plasmid vectors expressing large quantities of the encoded protein did not necessarily promote immune responses against the protein and that, instead, short immunostimulatory DNA sequences (ISS) are required on the plasmid. Thus, the most effective promoters were those that included a six-residue sequence incorporating an ummethylated cytosine motif adjacent to a guanine residue (CpG). It is of interest that, in this respect, bacterial DNA is much richer in unmethylated CpG sequences than is mammalian DNA, hence the immunostimulatory properties of the former (61). Such properties are reflected on the ability of the CpG motif to stimulate (by a hitherto unknown mechanism) B cells to proliferate, secrete interleukins 6(IL-6) and 12 (IL-12) and produce antibodies. CpG also activates macrophages to secrete IL-12 and interferons α (IFN-α) and γ (IFN-γ) which, by stimulating T and natural killer cells, augment the immune response and direct it into the Th1 mode (61,62).

A variety of ancillary signals appended to DNA contributing further to a more effective vaccine include sequences encoding cytokines that could drive immune responses to the antigen towards the desired mode. In recent work (36) for instance, it was shown that HBsAg DNA vaccines co-expressing interleukin 2 (IL-2) not only were much more effective than identical vaccines lacking the IL-2 gene, they were also able to overcome MHC-linked nonresponsiveness to HBsAg. Other appended gene sequences could be those expressing costimulatory molecules apparently needed (64) for the promotion of Th-1 (B7-1) and Th-2 (B7-2 molecule) response, or sequences that could selectively promote uptake of the DNA by such target cells as myoblasts, B lymphocytes and Langerhans or microglial cells in the brain (8). In the case of B cells, targeting was accomplished by using a plasmid DNA encoding an immunoglobulin heavy chain gene under the control of immunoglobulin promoter (63).

From the practical standpoint, DNA vaccines are easy and inexpensive to produce as plasmid vectors can be constructed and tested rapidly. Moreover, plasmids are more temperature stable than live vaccines and their storage in a lyophilized form is straightforward. Recent developments in genetic vaccination technology have led to a common set of operational procedures (64). In brief, after its construction, the plasmid is amplified in *Escherichia coli.* A large set of different *E. coli* host strains have been studied to identify those producing large amounts of DNA per cell of the highest quality. The produced plasmid is then purified from the lysed cells by gel chromatography or density gradient centrifugation, followed by phenol extraction. It is essential that plasmids made for in vivo use are highly

Table 1. Plasmid DNA Vaccines Under Investigation

Target/pathogen	Plasmid DNA-encoded immunogen	Route of injection	Animal model	Reference
Influenza	Nucleoprotein	IM	Mouse	19, 26
Influenza	Haemagglutinin	IM	Mouse	20
HIV-1	gp160, *rev-tax*		Mouse, macaque	21, 27
HIV-1	gp120	IM	Rhesus monkey	28
HIV-1	Nef protein	IM	Mouse	29
HIV-1	*env*	IM	Rhesus monkey	30
SIV	*env* and *gag*	IM	Rhesus monkey	31
LCMV	Nucleoprotein glycoprotein	IM	Mouse	32
HBV	HBsAg	IM	Mouse, chimpanzee	5, 22, 33
HBV	HBsAg (S region)	IM, SC, IV, IP	Mouse	34, 35
HBV	HBsAg (S, Pre-S2 plus region)	IM	Mouse	36
HCV	Envelope glyco-protein E2	IM	Mouse	37
HCV	Core proteins	IM	Mouse	38
HSV-1	Glycoprotein B	IM, IN	Mouse	5, 39
HSV-2	Glycoprotein D	IM	Mouse, guinea pig	40
Measles virus	Nucleocapsid protein	IM	Mouse	41
Measles virus	Haemagglutinin, nucleoprotein	IM	Mouse, neonatal mouse	42, 43
Pseudorabies	gD Glycoprotein	IM	Pig, neonatal pig	44
Sendai virus	Nucleoprotein	IM	Mouse, neonatal mouse	43
Haematopoietic necrosis virus	Nucleoprotein, glycoprotein	IM	Rainbow trout	45
Rotavirus	VP4, VP6 and VP7 proteins	IE	Mouse	46
Newcastle disease virus	F protein	IM	Chicken	47
Cytomegalovirus	Phosphoprotein 89	IM	Mouse	48
Rabies	Glycoprotein	IM	Mouse	49
Tobacco mosaic virus	Coat protein	IM	Rabbit	50
Potato virus	P1 Protein	IM	Rabbit	50
Tetanus	Toxin C fragment	IM	Mouse, neonatal mouse	43
Borrelia burgdorferi (Lyme's disease)	OspA	IM	Mouse	51
Mycobacterium tuberculosis	Antigen 85	IM	Mouse	52
Mycobacterium tuberculosis	Heat shock protein 65	IM	Mouse	53
Plasmodium berghei	Circumsporozoite protein	IM	Mouse	54
Plasmodium yoelii	Circumsporozoite protein	IM	Mouse	55
Leishmania major	gp63	IM	Mouse	56
Mycoplasma pulmonaris	A7-1, A8-1 antigens	IM	Mouse	57
Tumour	Carcinoembryonic antigen			23
Simian virus 40	Tumour antigen			24
Non-Hodgkin's lymphoma	Idiotype of Ig	IM	Mouse	25
B16 tumour	MAGE1, MAGE3	IM	Mouse	58
Dust-mite	Der p5 allergen		Rat	59
Prion diseases	Human prion proteins	IM	Mouse (prion protein deficient PrP^{*})	60

Abbreviations are: HIV-1, human immunodeficiency virus type 1; SIV, simian immunodeficiency virus; LCMV, lymphocyte choriomeningitis virus; HBV, hepatitis B virus; HSV-1, herpes simplex virus type 1; HSV-2, herpes simplex virus type 2; HBsAg, hepatitis B surface antigen; IM, intramuscularly; SC, subcutaneously; IV, intravenously; IP, intraperitoneally; IN, intranasally; IE, intraepidermally.

homogeneous, sterile and free of all contamination, particularly endotoxins, RNA, protein and genomic DNA. To that end, the QIAGEN procedure has been approved by European countries and the USA (65).

Modes of Immunization

Most of the immunization procedures carried out so far have opted (Table 1) for the intramuscular and, to a lesser extent, the intraepidermal route. Other routes such as the oral, nasal, vaginal, intravenous, intraperitoneal and subcutaneous have also been used (64) (Table 1). Plasmid vaccines are given in a variety of diluents including distilled water, saline and sucrose (9). For intramuscular injections, it is not uncommon to pretreat the tissue with cardiotoxin and other drugs that cause muscle damage followed by regeneration (5), or the anaesthetic bupivacaine which dilates local vessels thus enhancing DNA uptake by myocytes (21). The use of bupivacaine has proved successful in improving responses to DNA vaccines (21) although some reports (7) suggest little or no benefit. There are a number of other variables in the way vaccines are administered that need full evaluation before consensus is reached as to their significance in terms of optimal immune response (64). They include the amount of plasmid per dose, the number of doses and time intervals in between and the number of sites over which one dose should be distributed. For instance, with most of the experiments shown in Table 1, the plasmid dose range was 1–200 μg, often given into more than one site, with

injections repeated up to 5 or more times. It appears (64) that in most situations where immunization with plasmid DNA does work, protocols of three injections with about three week intervals between injections have been adopted. It is also clear (64) that booster injections following primary genetic immunization may use the immunogens themselves rather than the plasmids, without reduction of effect.

Mechanisms of Genetic Immunization

Genetic immunization has provided a means to effect greater influence on the immune system, namely to drive the system towards cellular or humoural immunity or both. These two arms of immunity are respectively under the control of Th1 and Th2 cells of the $CD4^+$ cell lineage with the former involving the formation of cytotoxic T-cells (CTL response). Cytokines associated with the Th1 response are IL-2, IL-12, IFN-γ and tumour necrosis factor α (TNF-α) and antibodies that may be formed are of the IgG_{2a} subclass. On the other hand, the Th2 response is characterized by the secretion of interleukins 4 (IL-4) and 5 (IL-5) and the production of IgG_1 antibodies (66). It is now apparent that genetic immunization does not provide an all or nothing choice of Th1 or Th2 immunity but rather a dominant response profile that depends on the formulations, co-adjuvants used, the route of injection and other variables. So how do genetic vaccines work?

To answer the question, two separate sets of events must be elucidated. The first refers to the way naked plasmid DNA enters the cells and ends up in the nucleus. There is evidence that bacterial DNA enters cells by receptor-mediated endocytosis (67) following which, one would expect, DNA is degraded in the secondary lysosomes. Yet, because there is transfection this cannot be completely true. Somehow, at least some of the DNA escapes the lysosomotropic pathway to enter the nucleus, possibly via internal receptors. The mechanism of such pathway is unknown and, inspite of its central importance in genetic vaccination, it has not been widely discussed. A simplistic view would be that some of the endosomes carrying the DNA break up, either spontaneously (it is hardly credible that all endosomes remain intact during intracellular trafficking) or because of DNA presence, and release their contents in the cytosol. Another possibility is that, as with endocytosed protein antigens, there exists a pathway in cells to shunt DNA (bacterial DNA treated as an antigen?) from phagosomes to the cytosol.

The second set of events refers to the cells involved in DNA uptake and transfection. It was initially observed (19,20) and subsequently confirmed (5–10) that intramuscular injection of DNA vaccines leads to such types of immunity as CTL. This was surprising because antigen presentation requires the function of professional APC (66). However, myocytes which were shown (5) to take up the plasmid, albeit only to a small extent and with only a fraction of cells participating in the uptake, are not professional APCs. Although myocytes carry MHC class I molecules and can present endogenously produced viral peptides to the $CD8^+$ cells to induce CTLs, they do so inefficiently (64) as they lack vital costimulatory molecules such as the B7-1 molecule. It has thus been difficult to accept that antigen presentation leading to a CTL response occurs via myocytes. Instead, it was reported (64) that CTL responses are, at least in part, the result of transfer of antigenic material between the muscle cells and professional APC. It is also likely that plasmid is taken up as such directly by APC infiltrating the injected site. Such cells would include dendritic cells which will express and present peptides to $CD8^+$ cells following transport to the lymph nodes or spleen. Indeed, there are several known examples where exogenous antigens enter presentation pathways relevant to $CD8^+$ T cell induction (7). On the other hand, $CD4^+$ cells may be activated by APCs via MHC class II presentation of antigen secreted by the myocytes (or released from them after their destruction via a Tc response) and captured by the cells. Such events would lead to both cellular (Th 1) and humoural (Th 2) immunity. According to a recent report (7), dendritic cells are the essential APC involved in immune responses elicited by intramuscularly given DNA vaccines. This would explain the high efficiency of DNA vaccination achieved by injection into the dendritic cell-rich epidermis (eg. ref 37).

CARRIER-MEDIATED DNA VACCINATION

Vaccination with naked DNA by the intramuscular route relies on the ability of myocytes to engulf the plasmid. As already mentioned, some of the DNA may also be endocytosed by APC infiltrating the site of injection or in the lymph nodes following its migration to the lymphatics. The extent of DNA degradation by extracellular deoxyribonucleases is unknown but, depending on the time of its residence interstitially, degradation could be considerable. In the gut milieu, naked DNA has failed to elicit an immune response, probably because of its anticipated complete degradation (7). It follows that approaches to protect DNA from the extracellular biological milieu, introduce it into cells more efficiently or target it to immunologically relevant cells should contribute to optimal DNA vaccine design.

Microbes

A novel method for DNA delivery and expression in mucosal surfaces has been recently reported (68). It employs attenuated Shigella bacteria which are known to enter the mucosa via M cells, to spread laterally between mucosal cells, and on entry into cells to escape the phagosomes. Administration of highlty attenuated Shigella previously transformed with a plasmid DNA vaccine incorporating a eucaryotic promoter, was found to release the contained DNA into the cytoplasm of mucosa cells and to induce mucosal immune responses as well as systemic immunity (68). The approach could be also applied with bacteria genetically modified with Shigella invasion genes or other bacteria such as *Listeria* that are able to invade cells and break out from the phagosomes into the cytosol.

Gene Gun

The use of gene gun in DNA vaccination was first introduced in 1993 (20) to deliver epidermally submicrogram quantities of plasmids encoding influenza genes in mice. This led to the successful induction of protective humoural and cell-mediated immunity and the adoption of the approach by other workers either for gene vaccination or cancer gene therapy (5–10). The technology employs an adjustable electric discharge to generate a shock wave which accelerates DNA-coated gold particles directly into the cytosol of target cells. The resulting transgene expression levels are at least as high as those obtained with much greater amounts of naked DNA. It is thought that

during its transit in the tissue, DNA detaches itself from the gold particles to end up intracellularly. It is not known however, whether the DNA enters the cells as such or as a complex with the cationic lipid used to link the DNA with the surface of the gold particles. In contrast to intramuscular immunization with naked DNA, gene gun vaccines appear (69) to favour the development of antibodies of the IgG_1 isotype.

Cochleates

Originally developed in 1975 (70), cochleates are rigid, calcium induced structures consisting of spiral bilayers of anionic phospholipids. Cochleates have a unique structure and are different from liposomes in that they are made up of a large continuous solid bilayer sheet rolled up in a spiral form with no internal aqueous phase and with calcium ions bridging the successive layers. Recently, a new type of protein antigen formulation has been developed by encasing antigens within the cochleates during their formation (71). Antigen-cochleate structures have been shown to promote strong and lasting humoural and cell-mediated immunity when given orally, intramuscularly or intranasally (71). It is thought that, in vivo, divalent cations stabilize cochleates in their rolled up structure. Orally administered, cochleates appear to survive the stomach milieu and reach the Peyer's patches (71). The system has now been applied in the delivery of DNA vaccines. In recent communications (R. Mannino and S. Gould-Fogerite; IBC Conferences on Genetic Vaccines, 23–24 October 1996, Washington and 16–18 November 1997, Orlando) it was reported that DNA cochleates formulated with a small amount (3–5 μg) of a plasmid encoding HIV-1, influenza and parainfluenza antigens were able to promote strong and lasting CTL responses after parenteral or oral administration. It was hypothesized that on contact with the cell membrane, the calcium-rich cochleates induce its perturbation, reordering, and a fusion event between the membrane and the outer layer of the cochleate. This apparently results in the delivery of some of the DNA content into the cytosol.

Vesicle-DNA Complexes

Small unilamellar vesicles (SUV), usually made of dioleyl phosphatidylethanolamine (DOPE) and a cationic lipid, are known for their ability to complex DNA on mixing (often leading to the formation of aggregates of vesicle-DNA complexes), and to transfect cells in vitro (72). The pathway to transfection is thought to commence with the binding of the positively charged vesicle-DNA complex entities to the negatively charged cell membranes and endocytosis. This is followed by destabilization of the endosomal membrane whereupon, through lateral diffusion of anionic lipids from the cytoplasm-facing endosomal monolayer, DNA is displaced from the complex and released into the cytosol (73). Intravenous application of the complexes has also been attempted but, in terms of the level of tissue transfection, only with modest success (e.g., ref. 73). Contributing causes include the formation of large aggregates between complexes and anionic plasma proteins leading to the neutralization of the cationic charge and rapid removal of the aggregates in the reticuloendothelial system and hence, reduction or even abolition of transfection activity. So far, results from a few reported studies on genetic immunization with SUV-plasmid DNA complexes have been rather disappointing in that immune responses were either similar (74) to or modestly higher (75) than those achieved with naked DNA. However, improvement of the transfection activity of cationic SUV-DNA complexes may come from a new insight into their structure. It has been reported (76) that mixing of cationic SUV with plasmid DNA leads to the formation of optically birefringent liquid-crystalline condensed globules with a multilamellar structure of alternating lipid bilayers and DNA monolayers. It is suggested by the authors (76) that the observed quantitative control over the structural nature of DNA in the complexes may identify important parameters that are relevant to the system's transfection efficiency.

Liposomes

It was recently proposed (34) that, as APC are a preferred alternative to muscle cells as targets for DNA vaccine uptake and expression, liposomes would be a suitable means of delivery of entrapped DNA to such cells. Locally injected liposomes are known (3) to be taken up avidly by APC infiltrating the site of injection or in the lymphatics, an event that has been implicated (3) in their immunoadjuvant activity. Liposomes would also protect (77) their DNA content from deoxyribonuclease attack. Because of the structural versatility (78) of the system, its tranfection efficiency could be further improved by the judicial choice of vesicle surface charge, size and lipid composition or by the co-entrapment of cytokine genes and other adjuvants (e.g., immunostimulatory sequences), together with the plasmid vaccine. Moreover, as a number of injectable liposome-based drug formulations including a vaccine against hepatitis A has been already licenced in the USA and Europe for clinical use (78), acceptance of the system clinically would be less problematic.

Work from this laboratory (34,35,77) has shown that a variety of plasmid DNAs can be quantitatively entrapped into the aqueous phase of multilamellar liposomes by a mild dehydration-rehydration procedure (77). This consists of mixing preformed SUV with a solution of the DNA destined for entrapment, freeze-drying of the mixture and controlled rehydration of the formed powder followed by centrifugation to remove non-entrapped material. Incorporation values shown in Table 2 were, as expected, higher (57–90% of the amount used) when a cationic lipid was present in the bilayers. No apparent relationship was observed between amount of DNA used (10–500 μg) and values of incorporation for the compositions and lipid mass shown. The possibility that DNA was not entrapped within the bilayers of cationic liposomes but was rather complexed with their surface (as suggested by the similarly high "incorporation" values obtained on mixing; Table 2) was examined by exposing liposome-entrapped and liposome-complexed DNA to deoxyribonuclease: substantially more entrapped DNA remained intact than when it was complexed (77) presumably because of the inability of the enzyme to reach its substrate. The significant resistance of complexed DNA to the enzyme (despite its accessibility) could be attributed to its condensed state (72).

Plasmid-containing liposomes, previously (77) found to transfect cells in vitro regardless of the vesicle surface charge, were tested in immunization experiments (34) using a plasmid (pRc/CMV HBS) encoding the S region of the hepatitis B surface antigen (HBsAg; subtype ayw). Fig. 1 and legend show

Table 2. Incorporation of Plasmid DNA into Liposomes by the Dehydration-rehydration Method

Liposomes	Incorporated plasmid DNA (% of used)					
	pGL2	pRc/cmv HBS	pRSVGH	pCMV4.65	pCMV4. EGFP	VR1020
PC, DOPE[a]	44.2	55.4	45.6	28.6		
PC, DOPE[b]	12.1		11.3			
PC, DOPE, PS[a]	57.3					
PC, DOPE, PS[b]	12.6					
PC, DOPE, PG[a]			53.5			
PC, DOPE, PG[b]			10.2			
PC, DOPE, SA[a]	74.8					
PC, DOPE, SA[b]	48.3					
PC, DOPE, BisHOP[a]	69.3					
PC, DOPE, DOTMA[a]	86.8					
PC, DOPE, DC-Chol[a]		87.1	76.9			
PC, DOPE, DC-Chol[b]			77.2			
PC, DOPE, DOTAP[a]		80.1	79.8	52.7	71.9	89.6
PC, DOPE, DOTAP[b]		88.6	80.6	67.7		81.6
PC, DOPE, DODAP[a]			57.4			
PC, DOPE, DODAP[b]			64.8			

Note: ^{35}S-labelled plasmid DNA (10-500μg) was incorporated ([a]) into or mixed ([b]) with neutral (PC, DOPE), anionic (PC, DOPE, PS or PG) or cationic (PC, DOPE, SA, BisHOP, DOTMA, DC-Chol, DOTAP or DODAP) dehydration-rehydration vesicles (DRV). Incorporation values for the different amounts of DNA used for each of the liposomal formulations did not differ significantly and were therefore pooled (values shown are means of values obtained from 3–5 experiments). PC (16μmoles) was used in molar ratios of 1:0.5 (neutral) and 1:0.5:0:25 anionic and cationic liposomes). PC, egg phosphatidylcholine; DOPE, dioleoyl phosphatidyl ethanolamine; PS, phosphatidylserine; PG, phosphatidylglycerol; SA, stearylamine; BisHOP, 1,2-bis (hexadecylcycloxy)-3-trimethylaminopropane; DOTMA, N[1-(2,3-dioleoyloxy)propyl]-N,N,N, triethylammonium; DC-Chol, 3β-(N,N-dimethylaminoethane) carbonyl cholesterol; DOTAP, 1,2-dioleoyl-3(trimethylammonium) propane; DODAP, 1,2-dioleoyl-3-dimethylammonium propane. Plasmid DNAs used encoded luciferase (pGL2), hepatitis B surface antigen (S region) (pRc/CMV HBS), human growth hormone (pRSVGH), mycobacterium leprosy protein (pCMV 4.65), "fluorescent green protein" (pCMV 4.EGFP) and Schistosome protein (VR1020).

that mice (Balb/c) injected repeatedly by the intramuscular route with 5 or 10 μg plasmid entrapped in cationic liposomes, elicited at all times tested much greater (up to 100-fold) antibody (IgG_1) responses against the encoded antigen than animals immunized with the naked DNA. Responses for other subclasses (IgG_{2a} and IgG_{2b}) for the liposomal DNA were also greater albeit to a lesser extent (up to 10-fold) (Fig. 1). Significantly, IgG_1 responses for the liposome-entrapped DNA were also higher (up to 80-fold) than those obtained for DNA complexed with similar cationic liposomes (34). This was also true for IFN-γ and IL-4 levels in the spleens of immunized mice (Fig. 2) (34). In a more recent study, the role of the route of injection of the pRc/CMV HBS plasmid was examined for both humoural and cell-mediated immunity, using Balb/c mice and an outbred strain (T.O.) of the same species. Results (Fig. 3) comparing responses for liposome-entrapped and naked DNA indicate greater antibody (IgG_1) responses for the former not only by the intramuscular route but also the subcutaneous and the intravenous routes. Interestingly, there was not much difference in the titers between the two strains (Fig. 3) suggesting that immunization with liposomal pRc/CMV HBS is not MHC restricted. A similar patern of results was obtained with IFN-γ and IL-4 in the spleen (unpublished observations).

The way by which liposomal plasmid promotes immunity to the encoded antigen is not likely to involve muscle cells. Although cationic liposomes could in theory bind to the negatively charged myocytes and be taken up by them, protein in the interstitial fluid would neutralize (78) the liposomal surface and thus be expected to interfere with such binding. Moreover, vesicle size (about 800 nm average diameter; ref. 77) would render access to the cells difficult if not impossible. It is then more likely that cationic liposomes are endocytosed by APC, possibly including dendritic cells. As discussed elsewhere (34), it appears that the key ingredient of the DNA liposomal formulations as used in Figs. 1–3 in enhancing immune responses is the cationic lipid. It is conceivable that some of the endocytosed DNA escapes the endocytic vacuoles prior to their fussion with lysosomes (by a mechanism similar to that discussed already for vesicle-DNA complexes) to enter the cytosol for eventual episomal transfection and presentation of the encoded antigen. It is perhaps at this stage of intracellular trafficking of DNA, spanning its putative escape from endosomes and access to their nucleus, that the cationic lipid, (possibly together within the fusogenic phosphatidylethanolamine component), plays a significant but yet unravelled role. However, the question as to why liposome-entrapped DNA is more efficient in inducing immunity to the encoded antigen than complexed DNA (34), still remains.

ADVANTAGES AND DANGERS OF DNA VACCINES

In contrast to the need of an elevated (therapeutic levels), prolonged and often tissue-specific expression of plasmid DNA in gene therapy, genetic vaccination requires only low efficiency, localized gene transduction and transient production of antigen, and probably no more than a few injections, to induce

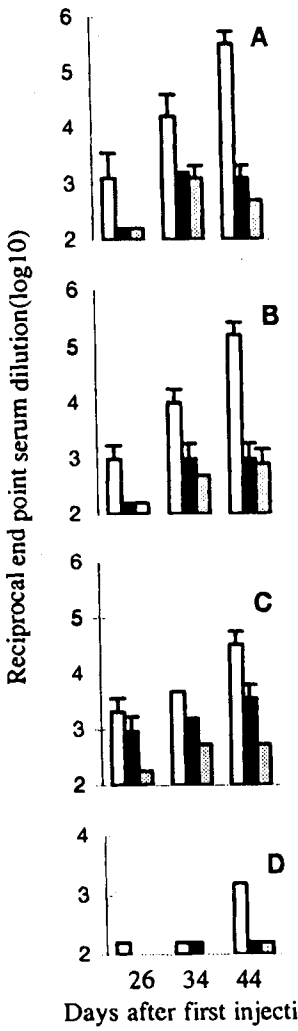

Fig. 1. Immune responses in mice injected with naked, or liposome-entrapped pRc/CMV HBS. Balb/c mice were injected intramuscularly on days 0, 10, 20, 27, and 37 with 5 μg of DNA entrapped in cationic liposomes composed of PC, DOPE and DOTAP (A), DC-Chol (B) or SA(C) (molar ratios 1:0.5:0.25), or in the naked form (D). Animals were bled 7, 15, 26, 34, and 44 days after the first injection and sera tested by ELISA for IgG_1 (white bars), IgG_{2a} (black bars) or IgG_{2b} (dotted bars) responses against the encoded hepatitis B surface antigen (HBsAg; S region, ayw subtype). Values are means ± SD of $\log_{10}$ of reciprocal end point serum dilutions required for OD to reach readings of about 0.2. Similar values (all groups) were obtained in mice injected as above with 10 μg DNA in a separate experiment (results not shown). Sera from untreated mice gave $\log_{10}$ values of less than 2.0. IgG_1 responses were mounted by all mice injected with liposomal DNA but became measurable only at 26 days. Differences in $\log_{10}$ values (all IgG subclasses at all time intervals) in mice immunized with liposomal DNA and mice immunized with naked DNA were statistically significant ($P < 0.0001$–0.002). (Reproduced with permission from ref 34).

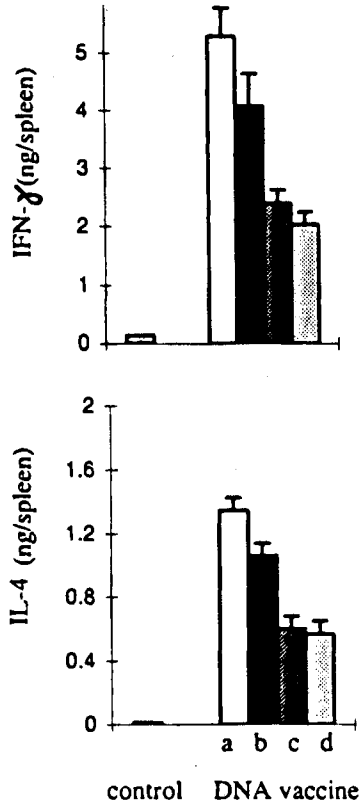

Fig. 2. Cytokine levels in the spleens of mice injected with naked, entrapped or complexed pRc/CMV HBS. Balb/c mice were immunized as in Fig. 1 with DNA entrapped into either cationic (a) or uncharged liposomes (b), mixed with cationic (DOTAP) liposomes (c), or in the naked form (d). "Control" denotes cytokine levels in normal unimmunized mice. Three weeks after the final injection, mice were killed and their spleens subjected to IL-4 and IFN-γ analysis. Each bar represents the mean ± SE of a group of 4 mice. Cytokine values in mice immunized with cationic liposomes were significantly higher than those in the other groups ($p < 0.001$–0.05). (Reproduced with permission from ref 34).

long term protective immunity. This, in conjuction with technical attributes discussed ealier and certain operational or behavioural advantages vis a vis conventional vaccines, places genetic vaccines as the first potentially successful large-scale application of gene therapeutics. Such advantages include simple in vivo gene transfer in the absence of an infectious vector, induction of both humoural and cell-mediated immunity, long-term persistence of antigen expression (which may mean an enduring effector T cell response as well as the establishment of traditional immunological memory), and the possibility of constructing vectors encoding several antigens that can be delivered in a single dose. There is, however, a potential downside of genetic vaccines. For instance, there may be an anti-DNA response which could be autoreactive. Moreover, there is a theoretical possibility of DNA integration into the host's chromosome. This can be minimised if the long terminal repeats of retroviruses, which enable integration and may led to malignancy especially after a long period, are deleted. However high resolution techniques (e.g., polymerase chain reaction) have shown that the chance of a DNA vaccine causing malignancy is less than 1 in 10^6 vaccinees per life span. This is compared with 1 to 1000 for spontaneous tumour incidence. Indeed, it has been estimated that 100 kg of DNA must be injected for the occurrence of one tumorigenic event (10)! It is on this basis that healthy individuals have been allowed to enter into clinical trials with experimental DNA vaccines encoding, for instance, HIV *env* and influenza nucleoprotein (10). In the short period of a few years, research on genetic immunization has mush-

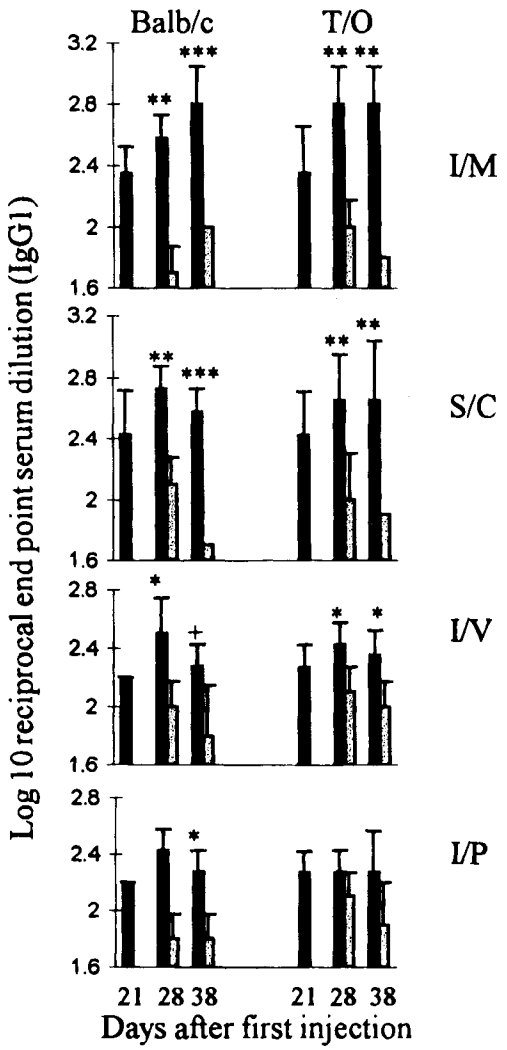

Fig. 3. The effect of route of immunization with pRc/CMV HBS on immune responses in inbred (Balb/c) and outbred (T.O.) mice. Mice in groups of four were injected intramuscularly (I/M), subcutaneously (S/C), intravenously (I/V), or intraperitoneally (I/P) twice on days 1 and 21 with 10 μg of liposome-entrapped (black bars) or naked (dotted bars) pRc/CMV HBS and bled on days 21, 28, and 38. Liposomes were composed of PC, DOPE and DOTAP (1:0.5::0.25 molar ratio). P values are +, <0.05;*, <0.01;**, <0.005;***, $P < 0.008$.

roomed and is now permeating both the practical aspects of vaccinology and basic immunology. Present indications are that this approach to vaccination is likely to improve the performance of current vaccines and also lead to vaccines for diseases which have not yielded to preventive measures. Further optimization of naked DNA vaccines will be undoubtedly achieved by a multitude of means including plasmid DNA targeting to the nucleus via signal peptides (79), tailoring of plasmids with inserts of immunostimulatory sequences or a variety of adjuvant cytokines, and the delivery of such constructs to immunocompetent cells via the use of systems as diverse as liposomes and the gun gun. As stated elsewhere (64), genetic vaccination is now approaching a situation where the capability of the body to respond equally effectively to a multicomponent DNA vaccine may turn out to be the principle limitation of the approach.

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