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Review

Immune responses to DNA vaccines

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Abstract. DNA vaccines, based on plasmid vectors expressing an antigen under the control of a strong promoter, have been shown to induce protective immune responses to a number of pathogens, including viruses, bacteria and parasites. They have also displayed efficacy in treatment or prevention of cancer, allergic diseases and autoimmunity. Immunologically, DNA vaccines induce a full spectrum of immune responses that include cytolytic T cells, T helper cells and antibodies. The immune response to DNA vaccines can be enhanced by genetic engineering of the antigen to facilitate its presentation to B and T cells. Furthermore, the immune response can be modulated by genetic adjuvants in the form of vectors expressing biologically active determinants or by more traditional adjuvants that facilitate uptake of DNA into cells. The ease of genetic manipulation of DNA vaccines invites their use not only as vaccines but also as research tools for immunologists and microbiologists.

Key words. DNA vaccines; B and T cell responses; antigen presentation; genetic adjuvants; antigen targeting; expression vectors.

Introduction

DNA vaccines, also termed genetic vaccines, polynucleotide vaccines or nucleic acid vaccines, are bacterial vectors that carry a pathogen's gene under the control of a strong constitutively active promoter such as the one derived from cytomegalovirus (CMV). Inoculation of DNA vaccines into muscle or skin by a syringe or a propulsion device such as a gene gun results in uptake of the DNA into cells, followed by transcription and translation of the pathogen's gene and, consequently, an immune response composed of antibodies, T helper cells and cytolytic T lymphocytes (CTLs) [1–3]. DNA vaccines have several advantages over traditional vaccines: clinical studies have shown them to be well tolerated, they stimulate a full spectrum of immune responses including CTLs generally not induced by protein vaccines and they generate exceptionally long-lasting immune responses [4, 5]. They provide their own adjuvant in the form of unmethylated bacterial CpG sequences [6–8] that induce an innate immune response which in turn sponsors activation of an antigen-specific immune response.

DNA vaccines, which were first described in 1992 [1], have been shown to induce immune responses to a variety of viral, bacterial and parasitic antigens. In addition, they have shown efficacy in treatment of allergic diseases, autoimmunity and tumor models. In this article we provide a review of DNA vaccines with emphasis on the mechanistic basis of the immune response elicited to plasmid vector-encoded antigen.

Vectors used for DNA vaccines

Vectors employed for the construction of DNA vaccines are bacterial plasmids which are otherwise commonly used for in vitro expression of proteins in

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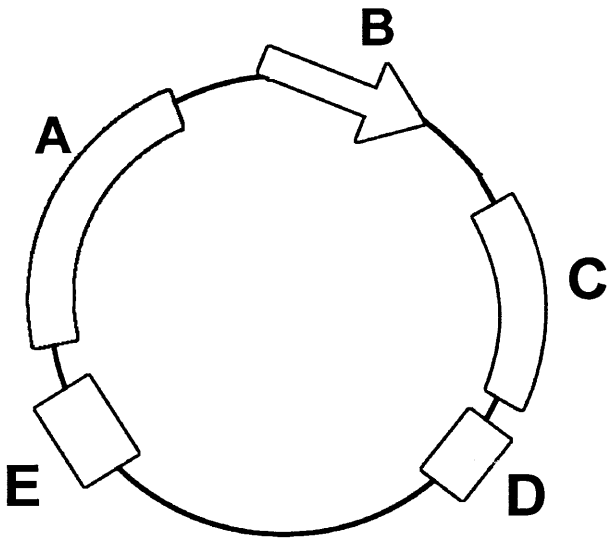

Figure 1. A hypothetical vector map. A, resistance gene (ampicillin or kanamycin); B, promoter; C, transgene; D, termination/polyadenylation sequences; E, origin of replication.

mammalian systems (fig. 1). Most of these vectors carry viral promoters that cause constitutive expression of antigen in a large variety of cell types. These include the human cytomegalovirus immediate/early promoter (CMV/IE), the Rous sarcoma virus (RSV) long terminal repeat (LTR) and the simian virus (SV) 40 early promoter. The SV40 promoter is ~ 40 times weaker than the CMV promoter and only works in some systems, such as the one based on the rabies virus glycoprotein [9], which causes cell death upon overexpression by a more potent promoter. It is also possible to induce a good immune response using the major histocompatibility (MHC) class I promoter [10] that drives expression in most cells. The MHC class II promoter, which causes antigen expression only in specialized cells, mainly of the immune system, such as macrophages, dendritic cells and B cells, was also found to drive expression of vector-encoded antigens to levels sufficient for induction of an immune response. Nevertheless, vectors based on this promoter give significantly lower immune responses when compared with vectors carrying a viral or MHC class I promoter [10]. Most DNA vaccine vectors contain an intron, which is an element that can increase expression of genes. This 'cassette' is followed by the gene encoding the antigen of interest flanked by the SV40 or bovine growth hormone 3′-untranslated region (BGH 3′-UTR) transcript termination/polyadenylation sequences. This part of the vector is often referred to as the transcriptional unit responsible for antigen synthesis. The other part is the plasmid backbone that contains an origin of replication (*ori*) enabling high-yield production in *Escherichia coli* along with an antibiotic resistance gene, such as ampicillin (not approved by federal agencies for use in humans) or kanamycin (a resistance marker suitable for human vaccines), to confer antibiotic-selected growth in bacteria. This part of the plasmid backbone contains unmethylated CpG sequences that possess important immunomodulatory properties and provides an intrinsic adjuvant effect for DNA vaccines [7]. It is thought that the magnitude of the immune response to DNA vaccines directly correlates with the level of antigen expression, measured in vitro upon transient transfection of cells. Other factors that can regulate the promoter's activity in vivo, such as cytokines, affect message stability or stability of the encoded antigen and are likely to influence expression levels in situ in a currently unpredictable fashion, thereby affecting the immune response by lowering the antigenic load. Vectors expressing a single antigen or even a fragment thereof have been used in numerous studies. In some reports, DNA vaccines expressing multiple antigens were tested. These DNA vaccines either expressed different antigens by individual constructs or by dicistronic vectors [11]. Even using a multitude of several hundred vectors encoding different antigenic fragments in an expression library immunization (ELI) approach, protective immunity to a complex bacterial antigen could be induced [12]. Together these data suggest that vectors expressing different antigens do not have a markedly negative effect on the immune response to individual antigens [13]. This is highly promising, as it will eventually allow vaccinologists to use combination DNA vaccines either to enhance protection against a single pathogen or to simultaneously induce protection against a multitude of infectious agents.

Alternative vectors are being developed, such as a construct based on self-replicating Similiki Forest virus RNA that was shown to induce a strong immune response to the influenza virus nucleoprotein in experimental animals [14]. A replication-incompetent, T antigen-deleted SV40 vector was generated as a vaccine carrier for the hepatitis B surface antigen. This construct upon intraperitoneal or subcutaneous inoculation, two routes that are fairly inefficient for immunization with classical DNA vaccines, induced neutralizing antibodies to hepatitis B virus, whereas antibodies to the vaccine carrier, that is antigens of SV40, were not elicited [15].

Applications of DNA vaccines: infectious diseases

So far, vaccination is the most efficacious strategy to prevent morbidity and mortality due to infectious

Table 1A. Vaccine to viruses.*

Virus	Antigen	Route of immunization	Animal model	Reference
Influenza	NP	i.m.	mice	1, 16–18
	NP	i.m., gene gun	mice	19
	NP	i.d.	mice	20
	HA	i.m.	mice	21
	HA	gene gun	chicken	22
	HA	gene gun	pigs	23
	HA		mice	24
	HA	i.m., i.v., i.n.	mice, ferrets	25
	NP, HA, matrix	i.m.	ferrets	26
	HA, NA, M, NP, NS1	gene gun	mice	27
HIV-1	env	i.m.	mice	28
	env		mice	29
	env	i.v., i.m., gene gun	mice	30
	env	i.m.	rhesus monkeys	31
	env	intranasal	mice	32, 33
		intravaginal	mice	34
			mice	35
		gene gun	rhesus monkeys	36
		i.m.	chimpanzees (HIV+)	37
		i.m.	chimpanzees	38
			humans (HIV+)	39
HIV-2	env	i.m.	mice	40
SIV	multiple	i.v., i.m., gene gun	rhesus macaques	41
	env, gag		rhesus macaques	42
FIV	env	i.m.	cats	43†, 44
	multiple	i.m.	cats	45
HTLV-1	env, rex	i.m.	rabbits	46
Hepatitis B	HBsAg	i.m.	mice	47
	HBsAg	i.m.	mice	48, 49
	env		mice	50
	env	i.m.	chimpanzees	51
	HBsAg	i.d., i.m.	Aotus monkeys	52
			neonatal chimpanzees	53
Hepatitis C	C, E2			54
	C		mice	55, 56
Hepatitis E	ORF3	i.m.	mice	57
Rabies	GP	i.m.	mice	3, 9, 58
	GP	i.m.	neonatal mice	59
	GP	gene gun	mice	60
	GP	i.d., gene gun	cynomolgus monkeys	61
Pseudorabies	IE 180	gene gun	mice	62
	IE 180	gene gun	pigs	63
	gD (env)	i.m.	mice	64
	gC or gD			65
Dengue 2	preM+env		mice	66
Measles	HA, NP	gene gun	mice	67
Encephalomyocarditis	VP1	i.m.	mice	68
LCMV	NP	i.m.	mice	69
Rotavirus	VP6	i.m., i.d.	mice	70
	VP4, VP6, VP7	gene gun	mice	71
	VP6	oral	mice	72
Simian virus 40	T-Ag	i.m.	mice	73
Bovine herpesvirus 1	gD	i.m., i.d.	cattle	74, 75
Bovine RSV	gD	i.m., i.d.	cattle	76
Coxsackievirus	VP1 or multiple	i.m.	mice	77
Ebola	NP, GP		guinea pig	78
	NP, GP	gene gun	mice	79

Table 1A. (Continued).

Virus	Antigen	Route of immunization	Animal model	Reference
HSV-1	gB	i.m.	mice	80
	ICP 27			
	ICP 27		guinea pig	81
	gB	ocular	mice	82
HSV-2	gD2			83, 84
Parvovirus	VP1	i.m.	dogs	85
CRPV	LI	i.m.	rabbits	86
RSV	F	i.m., i.d.	mice	87
St. Louis encephalitis				88

HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; FIV, feline immunodeficiency virus; HSV, herpes simplex virus; LCMV, lymphocytic choriomeningitis virus; RSV, respiratory syncitial virus; CRPV, cottontail rabbit papilloma virus; NP, nucleoprotein; HA, hemagglutinin; M, matrix protein; NA, neuraminiase; NS, nonstructural protein; ORF, open reading frame; IE, immediate early; HBsAg, hepatitis B virus surface antigen; C, Core protein; i.m., intramuscular; i.d., intradermal; GP, glycoprotein. † Enhancement of disease upon DNA vaccination. * Animals were vaccinated with DNA plasmids encoding the appropriate antigen(s) and then tested for a response against the protein.

agents. DNA vaccines have been tested in a number of viral, bacterial and parasitic systems as detailed below.

Viral infection

By far the majority of related literature addresses the use of DNA vaccines for prevention or treatment of viral infections using a multitude of different viruses. The DNA was administered by various routes into a number of avian and mammalian species. DNA vaccines even induced immunity in fish. A list of publications dealing with DNA vaccines to viruses is given in table 1A. In most cases, DNA vaccination resulted in an immune response that provided protection to challenge. In some instances protection was mediated by neutralizing antibodies, in others by cell-mediated immunity. In one example, a DNA vaccine failed to induce protective immunity in cats to infection with feline immunodeficiency virus (FIV) but rather exacerbated disease upon subsequent challenge [43]. Two other FIV studies in cats nevertheless yielded more promising results and showed at least some protection against infection [44, 45]. Numerous studies tested vectors expressing individual antigens; others used combinations of antigens expressed by different vectors or dicistronic vectors expressing two antigens within the same plasmid [11]. One study used a chimeric construct expressing a poorly immunogenic antigen, that is the hepatitis C virus core antigen with parts of a highly immunogenic protein such as the hepatitis B virus envelope protein [55]. Incorporating sequences of the immunogenic protein into the vector augmented the immune response to the less immunogenic core protein, presumably by providing additional stimulation of T helper cells. DNA vaccines have mainly been used in young adult animals. They were also shown to be efficacious in neonatal animals [59] and in aged mice [18]. Most DNA vaccines were used prior to challenge. Several studies in monkeys and humans tested DNA vaccines to human immunodeficiency virus (HIV)-1 in infected individuals. In a mouse model of lymphocytic choriomeningitis virus (LCMV), which establishes persistent infections, a DNA vaccine was shown to induce viral clearance from all infected organs [69].

A number of routes of immunization have been investigated, with intramuscular (i.m.) and intradermal (i.d.) gene gun delivery being the most frequently tested. Protection could be induced by either route, although some reports observed a clear advantage of one route over the other. I.m. immunization generally results in a Th1 bias, whereas i.d. immunization favors Th2 responses [19]. Depending on the pathogen, different immune effector mechanisms contribute to protection, which might in part explain the different observations using different routes. Immunity could also be induced upon mucosal administration, a route that in our hands only elicits comparatively weak immunity upon applying DNA in saline, suggesting that mucosal DNA vaccination might require the use of adjuvants such as cholera toxin [89] to optimize the response. One report even described immunization upon ocular application of DNA [82]. A very elegant study achieved induction of immunity including fecal immunoglobulin A (IgA) secretion by oral immunization with encapsulated DNA, the route of choice for mass vaccination. In this study the DNA was incorporated into biodegradable polylactride-coglycolide microspheres. This apparently protected the DNA as it passed through the upper intestinal tract. It might furthermore have facilitated uptake of the DNA into M cells or other antigen-presenting cells of the intestine [72].

Table 1B. Vaccines to bacteria

Bacteria	Antigen	Route of immunization	Animal model	Reference
Mycobacterium tuberculosis	hsp65		mice	91, 92
Mycobacterium tuberculosis	Ag85A, B, C	i.m.	mice	93–95
Mycobacterium tuberculosis	19kDa, AhpC		mice	96
Borrelia burgdorfei	OspA	i.m., i.d.	mice	97, 98
Clostridium tetani	tetanus toxoid		mice	99
Mycoplasma pulmonis	all (ELI)	gene gun	mice	12
Clamydia trachomatis	MOMP, CTP	i.m.	mice	100
Salmonella typhii	OmpC			101

MOMP, major outer membrane protein; CTP, cytosine triphosphate synthetase.

In another report, the wild-type codons of a viral protein, that is the gp120 of HIV-1, was replaced with codons from highly expressed human genes. The vector containing the synthetic gp120 with optimized codon usage gave clearly superior immune responses compared with the original gene, suggesting the potential of this strategy to enhance vaccine efficacy while decreasing the risk of recombination with wild-type virus [90].

Bacterial diseases

Vaccination with DNA plasmids has been shown to induce immune responses against bacteria and to provide protection against infection, as detailed in table 1B. Induction of antibody responses were demonstrated against *Mycobacterium tuberculosis* antigen 85 [93] and hsp65 [91], tetanus toxin C [99] and the OmpC protein of *Salmonella typhi* [101]. The efficacy of DNA vaccines encoding proteins of *Mycoplasma pulmonis* was demonstrated by an ELI approach where several thousand distinct plasmids were used as a cocktail vaccine [12]. Although this approach is unlikely to be practical for mass vaccination, it allows screening of a large number of antigens for their potential ability to induce protective T cell responses. Upon several rounds of selection, the antigens can eventually be pinpointed that should form the basis of a focused vaccine. This technique relies on protection by T cells; neutralizing humoral responses are most likely not induced by ELI, which uses ubiquinated fragments of the pathogen's DNA of ~ 300–600 base pairs in length for incorporation into the DNA vaccine.

Parasitic diseases

DNA vaccination has been shown to confer protection in experimental animals against both uni- and multicellular parasites, as shown in table 1C. Following plasmid DNA inoculation, antibodies have been raised successfully against paramyosin from *Schistosoma japonicum* [113], the major surface glycoprotein gp63 from *Leishmania major* [103, 104] and different antigens, including the hepatocyte erythrocyte protein 17 (HEP17), the circumsporozoite protein (CSP) and the sporozoite surface protein 2 (SSP2), from *Plasmodium yoelii* and *Plasmodium berghei* [107–112]. DNA vaccines to *Leishmania*- and *Plasmodium*-induced protection was based on cell-mediated responses, either on Th1 T helper response [103, 104] or CTLs, IFN-γ and release of nitric oxide [108, 111]. The route of DNA administration was critical in generating an antiparasitic immune response. In mice, the strongest antibody response to SSP2 was observed after i.d. immunization, whereas for CSP and HEP17 the i.m. route of vaccination resulted in superior responses. Upon challenge with the pathogen, both i.d. and i.m. immunization with HEP17 DNA protected mice equally well, whereas plasmids encoding CSP or SSP2 showed higher efficacy upon i.m. injection [109].

Allergic disorders

Allergic reactions are triggered by exposure to allergens which bind to IgE on the cell surface of mast cells and basophils. This binding leads to the release of histamine and inflammatory mediators [114], followed by the infiltration of eosinophils into the site of allergen exposure.

Traditional immunotherapy of allergic disorders is based on repeated injections of gradually increasing doses of allergen; this induces blocking antibodies of the IgG isotype. A serious risk of triggering anaphylactic reactions, variability in efficacy for many allergens and last but not least the cost and time expenditure has limited this mode of treatment, which is being replaced with new and improved drugs. Nevertheless, allergy medications not only have side effects but also require chronic use. DNA immunization with plasmids encoding allergens may have the potential to provide an alternative immunotherapy of allergic disorders. As mentioned previously, i.m. DNA vaccination induces

Table 1C. Vaccines to parasites.*

Parasite	Antigen	Route of immunization	Animal model	Reference
Cowdria ruminantium	MAP1	i.m.	mice	102
Leishmania major	gp63	i.d.	mice	103, 104
Trypanosoma cruzii	trans-sialidase		mice	105
	all (ELI)	i.m.	mice	106
Plasmodium yoelii	CSP	i.m.	mice	107–109
	CSP, PyHEP17, PySSP2	i.m.	mice	110, 111
Plasmodium berghei	CSP	i.m.	mice	112
Schistosoma japonicum				113

MAP1 major antigenic protein 1; CSP-circumsporozoite protein; PyHEP17, 17-kDa hepatocyte erythrocyte protein.
* Animals were vaccinated with DNA plasmids encoding the appropriate antigen(s) and then tested for a response against the protein.

mainly IgG2a antibodies, which may neutralize allergens. DNA vaccines, by their strong bias of inducing Th1 responses, actively inhibit antigen-specific IgE synthesis and activation of eosinophils [115, 116]. Indeed, in vivo experiments performed in animal models have shown that DNA vaccines downregulate antigen-specific IgE antibodies and reduce the late-phase allergic responses, that is eosinophil infiltration, tissue damage and allergic inflammation [115, 117, 118]. Moreover, in contrast to classical immunotherapy, it seems that due to the low level of antigen secretion, DNA immunization is relatively unlikely to cause anaphylactic reactions. Nevertheless, the caveat should be mentioned that in the case of an adverse reaction, the longevity of the in situ antigen expression by DNA vaccines would cause a serious and potentially untreatable threat to afflicted individuals.

Autoimmunity

One study tested the effect of a DNA vaccine on experimental allergic encephalomyelitis (EAE). This disease, which mimics human multiple sclerosis in mice and rats, can be induced by immunization with myelin basic protein in adjuvants. In mice the disease is mediated by pathogenic T cells expressing the variable region Vβ8.2 T cell receptor [TcR]. A DNA vaccine encoding the Vβ8.2 TcR given several times i.m. rendered mice resistant to the development of EAE by causing a shift from a pathological Th1 response to a protective Th2 response, a finding that was surprising, considering that in microbial systems DNA vaccines given by that route generally induce a Th1-type immune response [119].

Tumors

Several laboratories have reported on the use of vector DNA encoding tumor-specific antigens as vaccines to cancer. Several vaccines were based on idiotypes expressed on B cells for treatment of lymphomas [120–122]. One study tested a DNA vaccine expressing the human carcinoembryonic antigen (CEA) for vaccination of dogs, which developed serological and cellular immune responses to this antigen [123, 124]; the result was expected, as a human protein was being expressed in a different species. Additional studies showed that DNA vaccines could induce immunity in homologous systems. In one study, a DNA vaccine expressing mouse gp75/tyrosine related protein-1, which is overexpressed in melanoma cells, induced in mice an immune response that protected against challenge with tumor cells expressing high levels of this protein [125]. Mice also developed autoimmunity, which was characterized by coat depigmentation. Also, in a mouse melanoma system, T and B cell responses could be induced by a DNA vaccine expressing human gp100, a melanocyte-specific antigen. Protection to challenge with tumor cells was mediated by CTLs [126]. Another study showed that immunization of mice with a DNA vaccine expressing a mutated p53 minigene induced protective immunity to challenge with tumor cells expressing the same p53 mutation. The vaccine efficacy could be enhanced by adding the adenovirus E3 leader sequence to the p53 sequence, thus targeting the antigen towards the endoplasmic reticulum (ER) [127].

Induction of immunity to DNA vaccines

The immune system requires three signals for activation of antigen-specific immune responses. Signal 1 is the antigen; in the case of DNA vaccines, this antigen is encoded by the transcriptional unit of the expression vector. Signal 2 is a costimulatory signal provided primarily by determinants of the B7 family, that is B7.1 or B7.2 molecules, which are expressed on mature, activated antigen-presenting cells (APCs) [128]. Certain cytokines such as interleukin (IL)-2 can also provide signal 2 to B cells. Signal 0, also called the danger signal

[129], is a molecularly yet to be identified factor or a multitude of redundant factors that cause(s) activation of professional antigen-presenting cells, mainly dendritic cells, that in their immature (resting) stage are highly efficient at digesting and processing antigen but are fairly inefficient at stimulating T cells. The danger signal supposedly activates resting dendritic cells, which then upregulate expression of MHC determinants and costimulatory molecules. Upon activation dendritic cells also start synthesis of cytokines and chemokines and gain the ability to migrate to lymphatic tissue, where stimulation of T and B cells takes place. Antigen taken up by antigen-presenting cells without a signal 0, such as proteins released from apoptotic cells, is thought to remain immunologically silent or to induce tolerance.

In most DNA vaccinations, the plasmid is inoculated into either muscle or skin; immune responses have also been reported after intravenous or intranasal administration. Following i.m. injection, myocytes appear to be the predominant cell type transfected by the DNA, as was shown using vectors expressing reporter proteins [130]. Following skin inoculations, antigen expression occurs mostly in keratinocytes [131, 132]. Although a potential role of keratinocytes in antigen presentation remains to be evaluated, all available evidence suggests that these nonmigratory cells, that is myocytes or keratinocytes, have little if any contribution to the stimulation of the naive immune system by DNA vaccines. At the beginning of the era of DNA vaccines it was discussed that antigens presented by MHC class I determinants on transfected myocytes or keratinocytes stimulate cytolytic T cells [2]. It was further suggested that cytokines such as IFN-γ induce MHC class II expression on these cells, thus enabling them to present antigenic fragments to $CD4^+$ T helper cells [133]. However, presentation of antigen by nonprofessional APC's, which can provide signal 1 (the antigen) but only in the absence of signal 2 (the costimulatory signal), results in peripheral tolerance rather than in activation of antigen-specific T cells. This has not been observed upon DNA vaccination of adult individuals. One study showed induction of tolerance upon DNA vaccination of neonatal mice [134]. This might reflect the relative lack of APCs in newborn mice. In this scenario an abundance of antigen expressed on cells providing signal 1 in the absence of signal 2 might indeed cause tolerance. Nevertheless, none of the other reports on DNA vaccination of neonatal animals [59, 135, 136] described induction of tolerance, but rather observed stimulation of an immune response that was qualitatively indistinguishable from that of adult mice.

It was subsequently demonstrated experimentally that indeed the immune response to DNA vaccines follows, as expected, the same pathway as the immune response to traditional protein-based antigens. Studies, using bone marrow chimeras, have shown that the T cell response upon i.m. DNA vaccination is directed to the MHC haplotype of the donor cells rather than to that of the host [136, 137], implicating bone marrow derived cells as the source of antigen presentation. Other studies reported direct transfection of dendritic cells upon intradermal DNA vaccination, which could shortly afterwards be demonstrated in the draining lymph nodes [138–140]. While the dermis of the skin is relatively rich in professional APCs, such as Langerhans' cells, dendritic cells and tissue macrophages, professional APCs are sparse within muscle tissue, and their additional recruitment following the injection of DNA might be required.

In addition to providing signal 1 by the antigen and signal 2 by direct (i.e. transfection) or indirect (i.e. reprocessing) antigen expression on dendritic cells, DNA vaccines also seem to provide signal 0 to the immune system. The bacterial part of DNA vaccines is rich in unmethylated CpG sequences, which are either absent or methylated in the mammalian genome [141]. Such palindromic CpG motifs follow the formula 5′-purine-purine-CG-pyrimidine-pyrimidine-3′. CpG sequences carried by oligonucleotides or bacterial DNA were shown to cause activation of B cells and to trigger polyclonal Ig secretion [7]. CpG sequences directly activate monocytes, macrophages and dendritic cells, resulting in the production of various cytokines including interferon (IFN)-α, -β and -γ, IL-18 and IL-12 [142, 143]. CpG sequences upregulate expression of costimulatory molecules on APCs and activate natural killer (NK) cells. Bacterial DNA or CpG containing oligonucleotides provide a strong adjuvant effect to protein-based immunogens and can cause tumor rejection [141]. The ability of such DNA motifs to induce secretion of IL-12 might be the cause of the strong Th1 bias of immune responses to i.m.-injected DNA vaccines [144]. The occasionally observed Th2 response upon i.d. injection of DNA vaccines suggests that CpG sequences might have different effects at different anatomic locations.

The importance of CpG sequences present in the bacterial part of expression vectors in providing signal 0 to the immune system was underscored by the observation that DNA vaccines treated with the SssI enzyme, which methylates CpG sequences, fail to induce an immune response [145]. As a caveat to this report, it should be pointed out that the CMV promoter, which was the regulatory element in the DNA vaccine used in this study, contains a CpG motif; methylation of this site by the SssI enzyme strongly reduces the transcriptional activity of the CMV promoter. These experiments thus remain to be repeated using a promoter which is not affected by methylation to prove beyond doubt that CpG sequences in DNA vaccines indeed provide signal 0 to the immune system.

Induction of humoral immunity by DNA vaccines

Inoculation of plasmid DNA has been reported to be an effective way of generating humoral immune responses for a variety of viral, bacterial and eucaryotic antigens. The first report of induction of an antibody response utilized particle bombardment of gold beads coated with DNA encoding the human growth hormone and the human α-1 antitrypsin [1]. This initial report using a so-called gene gun which propels DNA directly into cells was rapidly confirmed using simple i.m. or i.d. injection for a diverse array of the other antigens listed in tables 1A–C.

In some cases, the DNA vaccine induced antibodies that neutralized the infectious agents and thus provided protection to challenge. Such antibodies have been detected in animals vaccinated with DNA encoding HIV envelope protein [146, 147], herpes simplex glycoproteins [80, 81, 84, 148], rabies virus glycoprotein and influenza hemagglutinin (HA) [16, 21, 149, 150]. In one of our studies we tested the antibody response to the rabies virus nucleoprotein. This antigen, if presented to the immune system as a recombinant protein or within the context of the rabies virus core, induces a potent B cell response that provides protection to peripheral viral challenge [151, 152]. Nevertheless, upon immunization of mice with a number of different vectors expressing the rabies virus nucleoprotein under the control of the CMV or SV40 promoter, only a marginal B cell response could be elicited after several booster immunizations, and no response was detected after a single immunization. B cells respond to extracellular antigen; they are oblivious to protein hidden behind cell membranes. DNA vaccines, as opposed to many viruses including rabies virus, are noncytopathic and thus fail to release antigen by causing cell death; they might therefore be unable to efficiently induce humoral responses to antigens that are maintained within the nucleus or the cytoplasma of transfected cells. Neutralizing antibodies, the main correlate of protection for many infectious diseases, are generally directed against secreted or cell surface-expressed antigens. Nevertheless, such antigens often show substantial variability between different strains or even isolates of a pathogen due to selective pressure from the immune system. Nucleoproteins are more conserved and thus provide useful additions to broaden the spectrum of vaccine efficacy. The failure of DNA vaccines to elicit strong humoral immunity to proteins contained within cells, which was also confirmed using ovalbumin as a test antigen [153], might be circumvented by reconstructing such antigens, for example adding a signal sequence that allows their secretion or display on cell membranes. Although this might be suitable for some proteins, others might not fold properly and consequently will become trapped in the ER. For such antigens, DNA vaccines expressing short secreted sequences encoding linear B cell epitopes might provide an alternative to those encoding a full-length protein.

The structure of proteins, which is recognized by conformation-dependent antibodies, is greatly influenced by posttranslational addition of side chains such as sugar moieties. DNA vaccines add mammalian-type glycosylation patterns to proteins. Viruses use the host cell machinery for posttranslational modification of their antigens, and thus have the same pattern as mammalian cells or DNA vaccines expressing an antigen in mammalian cells. In contrast, higher organisms such as bacteria glycosylate sites that are not utilized by mammalian cells. Bacterial glycoproteins expressed by DNA vaccines are therefore glycosylated differently from those encoded by bacteria, which might reduce the antibody response that cross-reacts with the pathogen. Changing codons that encode mammalian glycosylation sites and reconstructing codons that encode bacterial glycosylation sites could at least in part correct the structure of the protein and prevent masking of B cell epitopes by superfluous sugar residues. Needless to say, incorporating such changes, which must preserve the correct folding of the protein, will be a Herculean task of trial and error.

Inoculation of plasmid vectors into mice can cause life-long immunity, as was demonstrated with influenza nucleoprotein (NP) [154], HA [155], hepatitis B surface antigen [47] and hepatitis C core protein [56]. Although specific antibodies may be detected as early as 1 week after plasmid injection [5, 156], generally genetic vaccines result in slowly rising antibody titers which reach peak levels 8–12 weeks after immunization. These titers then persist at high levels for several months. The unusual kinetics of the B cell response to DNA vaccination is presumably a reflection of the comparatively long-lasting persistence of vector DNA and of vector-encoded proteins in transfected cells. Once a B cell response has been initiated, the longevity of the vector-encoded antigen favors its deposition in the form of immunocomplexes on follicular dendritic cells. Such complexes are released when antibody titers decline and cause further activation of memory B cells.

The precocity and strength of the humoral response depends on the dose of vector DNA. Antibody levels may be further increased by a second DNA injection. Antibody titers can also be increased by using a traditional vaccine such as peptides or viral recombinants for booster immunization [157–160]. Although DNA vaccines induce antibody titers that in many systems suffice to provide protection against challenge with the pathogen, as a rule, titers achieved by DNA vaccines are more persistent but otherwise well below those that can be elicited by a traditional subunit vaccine such as a

recombinant virus. Notwithstanding, DNA vaccines are remarkably well suited to prime the antibody response, which upon subsequent booster immunization with a fairly low dose of a recombinant vaccine reaches titers exceeding those achieved by the vaccine given at optimal doses alone once or even repeatedly.

The isotypes of serum antibodies induced by DNA vaccination are generally IgG (predominantly IgG2a in mice), but serum IgM and IgA have also been detected [21, 155]. Upon i.m. DNA vaccination, antibodies of the IgG2a isotype predominate, indicating stimulation of a Th1 response. This is in contrast to protein vaccines given in alum, one of the few adjuvants suitable for vaccination of humans. Such vaccines induce a Th2 response with IgG1 and IgE antibody production and IL-4, IL-5 and IL-10 secretion [161, 162]. The preferential induction of Th1 responses upon DNA vaccination presumably reflects, as mentioned above, the ability of CpG sequences to induce IL-12, a cytokine known to promote activation of Th1 responses while suppressing induction of Th2-linked responses. The inhibition of Th2-type responses by DNA vaccines was shown to downregulate production of IgE antibodies to allergen, suggesting that DNA vaccination may be effective in the treatment of allergic responses [115].

T cell-mediated immune responses

Induction of CTLs as a rule requires de novo synthesis of antigen. In the cytoplasma some of the antigen is degraded by proteosomes into peptides which are translocated by a peptide transporter system (TAP) into the ER. Here association of peptides with MHC class I determinants takes place, causing stabilization of MHC class I molecules and allowing their transport to the cell surface. Only peptides with appropriate anchoring residues complementary to the pockets of the MHC groove are able to bind. The affinity of these residues to MHC causes a hierarchy of responsiveness in which peptides with high affinity are immunodominant, whereas those with lower affinity are subdominant or even cryptic. Most antigens have one or two immunodominant epitopes, which differ depending on the MHC haplotype of the host, a phenomenon referred to as Ir-gene control [163]. Subdominant epitopes induce at best a low immune response, and cryptic epitopes are immunologically silent [164]. The same hierarchy that governs stimulation of T cells also applies to the induction of tolerance induced to self proteins: immunodominant epitopes of self induce solid unresponsiveness whereas cryptic epitopes are ignored, thus failing to induce tolerance. Subdominant epitopes are in a gray zone; they may or may not induce tolerance.

Induction of CTL activity upon DNA vaccination was initially demonstrated after in vitro restimulation of lymph node lymphocytes or splenocytes isolated from vaccinated mice. This assay system primarily detects memory T cells. Activation of effector CTLs was subsequently confirmed by testing freshly isolated splenocytes for lysis of antigen-expressing target cells. CTLs were detectable upon DNA vaccination of mice and primates to a variety of antigens, including the NP from influenza A virus [2], hepatitis B surface [165] and core antigens [166], HIV envelope (Env) and gp160 [167–171], rabies glycoprotein [3], LCMV NP [69] and HSV ICP 27 [172]. In some experimental systems, specific CTL activity could be detected as early as 3–6 days after DNA injection; their activity reached its maximal level by 12 days and was maintained for several months [173] or even for the lifetime of the animals. Booster immunization with a second dose of DNA or a recombinant vaccine expressing the same antigen enhanced the CTL response [158].

Cytoplasmic degradation of proteins, a prerequisite for TAP-mediated transport into the ER and association of peptides with MHC class I determinants, is enhanced by the addition of ubiquitin, a host cell sequence that provides a 'trash' signal to the cells. Genetic modification of vector-encoded antigen by incorporating a ubiquitin sequence to the 5′ end of the antigen's coding sequence was shown to enhance the CTL response at the expense of the B cell response, which was strongly reduced [174, 175]. Although such modification might not be needed for DNA vaccines encoding a single full-length antigen, library approaches [12] which use a mixture of many sequences from a pathogen, most of which do not contribute to the induction of T cell immunity, require such modifications to reach antigenic threshold levels.

$CD4^+$ T helper cells respond to peptides associated with MHC class II determinants. In most cases these peptides are derived from extracellular antigens that, once taken up by APCs, are degraded into peptides within the lysosomal pathway prior to binding to their restricting element. In some instances, endogenous antigen can also enter this pathway and associate with MHC class II molecules. Similar to $CD8^+$ T cells, $CD4^+$ T cells respond preferentially to immunodominant epitopes which show high binding affinity to the MHC class II groove; they fail to respond to cryptic epitopes.

$CD4^+$ T cells are divided into three functionally distinct subsets according to their cytokine secretion pattern [176]. In mice, Th0 cells are uncommitted T cells that secrete IL-2; Th1 cells produce IFN-γ and support development of cellular immune response, including CTLs and activation of macrophages. They promote activation of B cells secreting IgG2a and inhibit devel-

opment of IgE-secreting B cells [177]. Th2 cells produce IL-4, IL-5, IL-6 and IL-10, activate eosinophils (IL-5) and promote B cell activation and antibody secretion with a predominance of IgG1 (IL-4) [178–180].

As T helper cells respond preferentially to exogenous antigen processed by the lysosomal pathway, modifications that favor secretion of antigen might augment activation of these T cell subsets. Indeed, in some systems the addition of a signal sequence was shown to promote activation of B cell responses which in part might have been attributed to increased T cell help. A further improvement might be achieved by adding a signal sequence to the 5′ end of the coding sequence and an lysosome associated membrane protein (LAMP) sequence to the 3′ end. The LAMP sequence causes immediate uptake of secreted protein into the lysosomal pathway, where association of MHC class II determinants takes place. Although this modification has not yet been tested for DNA vaccines, one study demonstrated that such an addition could dramatically improve the efficacy of a recombinant vaccine by facilitating activation of T cells. In this report, the E7 protein of human papilloma virus (HPV)-16 was expressed either in its wild-type form or upon addition of a signal/LAMP sequence by a vaccinia virus recombinant. Upon vaccination, mice were challenged with a tumor cell line also expressing E7 of HPV-16; mice vaccinated with the wild-type E7 construct developed tumors, those vaccinated with the modified form of E7 remained disease-free [181, 182].

Targeting of a DNA vaccine-encoded antigen to professional APCs by expressing a fusion protein containing either CTLA4 or L-selectin, two molecules that can bind to activated dendritic cells, also improved activation of antigen-specific immune responses [183].

Intriguingly, some DNA vaccines were shown to induce T cell responses not only to immunodominant epitopes, previously defined by protein vaccines, but also to additional epitopes which were presumably subdominant or even cryptic [165]. As a result, DNA vaccines to the hepatitis B virus surface antigen elicited responses in mouse strains that were nonresponsive to protein antigen. Although this is potentially exceedingly useful to overcome genetic nonresponsiveness, especially to cancer vaccines that encode self proteins, it might also pose a potential risk for the development of autoimmune responses to DNA vaccines. To reiterate, the immune system does not induce tolerance to cryptic epitopes [164, 184]. A vaccine that induces a T cell response to a cryptic foreign antigen cross-reactive with a cryptic self epitope might induce a self-destructive immune response. This has not yet been observed after 6 years of intensive studies and thus presumably provides a minor risk. The mechanism of the DNA vaccine-mediated induction of immunity to a broadened epitope profile is currently not understood but might be a reflection of the longevity of antigen expression, allowing interactions between naive T cells and rare epitopes.

Genetic adjuvants

The type and magnitude of immune responses to DNA vaccines can be modulated by the use of so-called genetic adjuvants (table 2). Genetic adjuvants are plasmid vectors encoding a cytokine, a costimulatory molecule or a ligand. Genetic adjuvants are generally given concomitantly with the DNA vaccine, thus allowing expression of the antigen and the immunomodulatory entity in close proximity. The first publication addressing the use of genetic adjuvants reported a strong enhancement of the antigen-specific B and T helper cell response upon immunization of mice with a mixture of a DNA vaccine to rabies virus and a vector encoding granulocyte-macrophage colony-stimulating factor (GM-CSF). The GM-CSF-mediated augmentation of the immune response [190], which was confirmed using additional microbial systems [188, 192], was attributed to the ability of this cytokine to activate and recruit APCs. In the same study, IFN-γ was shown to reduce the efficacy of the immune response to DNA vaccines [10], which might reflect that this cytokine downregulates the activity of most viral promoters [195–197]. Subsequent studies demonstrated that IFN-γ could augment immune responses to the hepatitis B virus surface antigen [188, 189]. IL-4 was shown to enhance B and T cell response to a rabies virus DNA vaccines [190]. This cytokine, which is considered a Th2 cytokine, did not affect the type of the immune response to rabies virus, which remained of the Th1 type. In fact, IL-4 even augmented the cytolytic T cell response to the DNA vaccine. Although the mode of action of IL-4 as a genetic adjuvant is currently not fully understood, this cytokine might act by reducing IFN-γ production in vivo. In other models, IL-4 was found to selectively enhance the Th2 response at the expense of the Th1 response [180, 189]. IL-5 and IL-10, two Th2-related cytokines, as well as IL-18, a cytokine with IFN-γ like biological functions, were shown to augment humoral immune responses to DNA vaccines [185]. IL-12 in one study was shown to enhance cytolytic T cell responses to DNA vaccines while causing a moderate reduction in antibody titers [188]. In another study using a different antigen, IL-12 enhanced the T helper cell and antibody response [185]. TNF-α was shown to enhance T helper cell activity [185]. In several systems, IL-2 enhanced the overall immune response to DNA vaccines [185–187]. TGF-β, a cytokine that in general suppresses immune responses, was shown to reduce antibody responses to a DNA vaccine [189]. Costimulatory molecules such as

Table 2. Modulation of immune responses after DNA vaccination.*

Modulator	Antigen	Effect	Reference
Il-2	HIV gp120	Augments humoral and T-cell responses; however, immune response may be dependent on the temporal relationship between Ag and cytokine delivery Enhances the development of Th1 cells Increases IgG production Moderatelly augments CTL response	185, 186
	HBsAg	Helps to overcome MHC-linked nonresponsiveness to HBsAg vaccination	187, 188
	Transferrin	Augments humoral and T cell responses	189
Il-4	Rabies virus GP	Increases B and T cell responses, including CTL activation	190
	Transferrin	Selectively increases IgG1	189
	HBsAg	Significantly enhances the development of specific Th2 cells Increases production of IgG1 Ab Suppresses Th1 differentiation and IgG2a production Enhances antigen-specific B cell response	188
	HIV gp120	Enhances antigen-specific B cell response	185
Il-5	HIV gp120	Enhances antigen-specific B cell response	185
IL-10	HIV gp120	Enhances antigen-specific B cell response	185
Il-12	HIV gp120	Enhances stimulation of CTLs, decreases B cell responses	186
	HBsAg	Significantly enhances Th1 cells Increases production of anti-HBV IgG2a Ab Inhibits Th2 cells Decreases production of IgG1 Ab Augments CTL activity	188
Il-15	HIV gp120	Augments CTL activity	185
Il-18	HIV gp120	Enhances antigen-specific B cell response	185
IFN-γ	Rabies GP	Decreases the B and Th immune responses	10, 191
	HBsAg	Significantly enhances Th1 cells and increases production of anti-HBV IgG2a Ab Inhibits Th2 cells and decreases production of IgG1 Ab. Augments CTL activity.	188, 189
TNFα	HIV gp120	Increase in antigen-specific Th proliferation Enhances CTL response	185
GM-CSF	Rabies GP	Enhances the development of Th1 and B cells	191
	HBsAg	Moderately augments CTL response	188
	PyCSP	Increases antigen-specific Ab production, Il-2 secretion and Th proliferation	192
TGF-β1	Transferrin	Decreases Ab response	189
CD80	HIV	Slightly increases CTL and T cell responses	193
CD86	HIV	Increases CTL induction and Th cell proliferation No changes in B cell responses	193
CD154 (CD40L)	β-galactosidase	Increases immune responses	194

Ag, antigen; Ab, antibody; Th, T helper.
* Animals were vaccinated with DNA plasmids encoding appropriate antigen(s) and then tested for a response against the protein. Modulators were co-injected in the form of plasmids encoding a modulating agent.

B7.1 (CD80) and B7.2 (CD86) were also shown to enhance the immune response, that is mainly the T cell response, to DNA vaccines [193]. In our system, based on the rabies virus glycoprotein, the effect of either of these molecules was marginal and could only be observed in some experiments using suboptimal doses of the antigen-encoding vector for immunization. The mechanisms by which costimulatory molecules enhance the immune response to DNA vaccines is obscure; it has been suggested that costimulatory molecules might enable muscle cells to efficiently present antigen to naive T cells. This seems highly unlikely considering that immune responses are initiated in lymphatic tissues. Vectors encoding ligands, such as CD40L, which binds to costimulatory molecules and provides an early activation signal to dendritic cells, were also shown to strongly augment the immune response to DNA vaccines [194].

In summary, although a number of cytokines and costimulatory molecules were found to augment the immune response to DNA vaccines, the most consistent and impressive effects were achieved with molecules

Table 3. DNA vaccines in comparison with traditional vaccines.

	Attenuated pathogen	Inactivated pathogen	Live recombinant vector	Protein vaccine	Peptide vaccine	DNA vaccine
Antibody response	yes	yes	yes	yes	yes	yes
Antibody rise	fast	fast	fast	fast	fast	slow
CTL induction	yes	no	yes	no	variable	yes
T helper induction	yes	yes	yes	yes	yes	yes
Complete antigen repertoire	yes	yes	no	no	no	possible
Immune response(s) to the vaccine carrier	no	no	possibly	no	no	no
Duration of response	long	short	long	short	short	long
Number of required vaccine doses	one	multiple	multiple	multiple	mulitple	one or more
Safety (especially for pregnant and immuno-comprised individuals)	no	yes	no	yes	yes	probably
Risk of reversion	yes	no	yes	no	no	no
Impaired efficacy in the presence of maternal antibodies	yes	yes	yes	yes	yes	no
Ease of production	variable	difficult	difficult	difficult	difficult	easy
Cost	variable	expensive	expensive	expensive	expensive	inexpensive

that drive activation of dendritic cells, that is GM-CSF and CD40L, suggesting that dendritic cell activation might be a limiting factor to initiate the immune response to DNA vaccines. The often contradictory results obtained with some of the interleukins and interferons might reflect differences in the amount of biologically active mediators in situ, which is not only controlled by the dose of vector but also by promoter activity, message stability and other factors.

Additional adjuvants

Other substances have been explored for their use in DNA vaccination. Initial reports showed that reagents used for in vitro transfection of cells actively inhibited the immune response to DNA vaccination, presumably by interfering with DNA uptake. Hypertonic solutions such as sucrose solution were shown to slightly increase the in situ transfection rate [198], as did pretreatment of muscle tissue with myotoxic reagents such as cardiotoxin and buvocain. An increase in B and T cell responses to DNA vaccines was also observed by incorporating DNA in cationic liposomes [199, 200] or monophosphoryl A lipids [201]. Interestingly, DNA incorporated into the latter substance showed reduced in situ antigen expression, suggesting that the adjuvant effect of this lipid was not mediated by facilitating DNA uptake. Several reports have attempted to specifically target a DNA vaccine to APCs. Dendritic cells express mannose receptors. DNA encapsulated in mannose-coated liposomes [202] or mannan-coated *N*-butyl-*N'*-tetradecyl-3-tetradecylamino-propionamidine [203] markedly enhanced the T and B cell-mediated immune response to an HIV gp160-encoding DNA vaccine. Alternatively, several reports demonstrated an increase in DNA vaccine efficacy by targeting the antigen, rather than the vector, to APCs [204].

DNA vaccines in comparison with traditional vaccines

As outlined above, DNA vaccines induce, upon de novo synthesis of antigen in transfected cells, a full spectrum of immune responses, including antibodies, T helper cells and cytolytic T cells (table 3). This is also achieved by live attenuated vaccines or viral recombinant vaccines. DNA vaccines lack the inherent risks of attenuated vaccines and, unlike recombinant vaccines, immune responses to the vaccine carrier are not an issue upon vaccination with plasmid DNA. DNA vaccines, unlike viral recombinants, can be used repeatedly for different immunogens. Inactivated vaccines and protein vaccines have minor side effects and induce antibodies and T helper cells, two immune mechanisms that fully suffice for protection to many pathogens. Nevertheless, most inactivated vaccines or protein vaccines require addition of adjuvants which induce a local inflammatory reaction, that provide the danger signal to the immune system. The severity of the inflammatory reaction, which commonly correlates with the efficacy of the adjuvant, limits the use of many adjuvants in humans. DNA vaccines provide their own adjuvant through unmethylated CpG sequences present in the bacterial part of the vector. Inactivated vaccines and proteins commonly induce Th2 responses, which in the case of some infections such a those with respiratory syncitial virus might actually exacerbate disease upon infection. DNA vaccines, at least upon intramuscular immunization, induce Th1 responses. Peptide vaccines induce monospecific B or T cell responses. This might be particularly useful for treatment of cancer, where

known point mutation of a self protein, such as p53, creates a T cell epitope [205]. Peptides require the addition of adjuvants, especially if the induction of $CD8^+$ T cells is sought. Furthermore, peptides are in general poorly immunogenic, which in part reflects their short half-life in serum [206]. DNA vaccines carrying minigenes for expression of single epitopes can readily be constructed to replace peptide vaccines. Many of the traditional vaccines fail to induce immune responses in neonates, whereas DNA vaccines readily stimulate T and B cell responses in newborn animals [207–209]. While the immune response to most traditional vaccines is impaired in the presence of maternally transferred antibodies [210–212], DNA vaccines given to neonates are not or only marginally affected by the dam's immune status [213–215].

Compared with many traditional vaccines, DNA vaccines induce comparatively low immune responses. This might limit their usefulness for pathogens that invade their host at high numbers or that replicate very efficiently. For reasons we currently fail to fully understand, DNA vaccines are exceedingly potent in priming the immune response. Experimental animals inoculated with a DNA vaccine develop very high immune responses upon booster immunization with a low dose of a traditional vaccine expressing the same antigen. This might be linked to the adjuvant effect of CpG sequences that by creating a unique cytokine milieu might favor activation of memory T helper cells. For that reason, even if DNA vaccines on their own are eventually shown to lack efficacy for many of the human pathogens, they might secure their place in vaccinology as priming agents.

DNA vaccines as gene therapy vehicles

The usefulness of expression vectors as vaccines was discovered serendipitously during preclinical gene therapy trials. Similarly, E1-deleted adenoviral recombinants were initially developed for gene therapy of genetic diseases such as cystic fibrosis. Both types of constructs were shown to induce strong B and T cell responses which led to rapid cytolytic T cell-mediated destruction of infected or transfected cells and, in the case of adenoviral recombinants, impaired uptake of the gene therapy vehicle upon subsequent reapplication [216–218]. Vaccinologists embraced both of these constructs and showed them be highly suitable vaccine carriers [219]. Gene therapy aims at the long-term replacement of missing or faulty genes, whereas vaccinology has the objective to induce a potent immune response—two opposing goals that might nevertheless both be achieved by DNA vaccines. While inoculation of plasmid vectors was shown to result in a strong local inflammatory reaction, CpG-methylated vectors fail to induce such a response [220]. By the same token, one report described that methylated DNA vaccines do not stimulate an immune response to the transgene product [145]. This later study was conducted with a DNA vaccine carrying the CMV promoter which loses activity upon CpG methylation; it is thus not yet established beyond doubt that the observed lack of an immune response to the methylated construct was caused by inactivation of the immunostimulatory CpG motif or by the strong reduction of protein expression following methylation of the promoter. Nevertheless, taken together these two sets of data suggest that methylated plasmid vectors might not induce an immune response to the encoded antigen. This could be exploited by gene therapists for long-term replacement of potentially immunogenic proteins.

Advantages and disadvantages of DNA vaccines

One major advantage of DNA vaccines, at least for those involved in basic research, is the ease with which they can be generated, modified and purified. Generating a recombinant vaccine takes several months, whereas production of an attenuated pathogen can take years. Production of a recombinant protein, followed by purification, takes weeks. Generation of a DNA vaccine takes between 2–4 days; amplification and purification take less than 24 h.

The ease of their construction invites their use for 'nonvaccine' purposes. They have been used to generate monoclonal antibodies [221], to help to identify the antigen of a pathogen that induces protective immunity [79] and to gain insight into the in vivo action of immunomodulatory molecules such as cytokines, chemokines or ligands during a primary antigen-specific immune response (table 2). From a more practical standpoint, DNA vaccines are inexpensive to mass-produce, an important consideration for the production of vaccines for use in developing countries or in veterinary medicine. They are extremely stable; the DNA can be boiled, precipitated in ethanol and shipped across the globe at room temperature; they do not require cold chains, which are very difficult to maintain in less-developed countries.

Immunologically, DNA vaccines induce the same spectrum of immunity as a natural infection, they also induce at least in small rodents exceptionally long-lasting immunity, which remains to be confirmed in larger animals.

Thus far DNA vaccines have not shown any severe adverse reactions. There is little evidence for integration of DNA into the host cell genome [222]. Autoimmune reactions such as stimulation of persistent antibodies to

DNA has not been observed. One study reported induction of antibodies to DNA upon DNA vaccination [223]; however, the titers were very low and transient. In our system even multiple immunizations with high doses of DNA failed to elicit detectable antibodies to double or single-stranded DNA [9].

In some systems, DNA vaccines seem to overcome genetic unresponsiveness [65, 111]. DNA vaccines were shown to induce immunity in neonatal animals [59]. Passive transfer of antibodies inhibits the immune response to DNA vaccines in adult mice [213]. In neonatal mice, maternally transmitted antibodies or hyperimmune serum given iatrogenically failed to reduce the B cell response to DNA vaccines [213]. This suggests their potential usefulness for early childhood vaccination, which is currently unfeasible with traditional vaccines to many common infections due to the maternal antibody-mediated inhibition of the infant's own immune response to active immunization.

DNA vaccines have disadvantages. They result in rather slow rising antibody responses, which disallows their use for postexposure vaccination to certain infections such as with rabies virus. The humoral immune response to DNA vaccines is not overly potent, suggesting that DNA vaccines (unless they can be improved) might best serve as priming agents in combination vaccines. DNA vaccines persist for weeks to months at the site of inoculation; antigen is produced for several weeks. Although this is advantageous for enhancing the immune response in the case of an adverse reaction, the continued production of antigen that can only be terminated by surgical intervention will be problematic. The two most serious possible side effects of DNA vaccination are transformation due to integration of DNA into the host cell genome or the development of autoimmunity due to induction of T cells to cryptic epitopes. Neither of these has been observed. Nevertheless, as with every bioactive substance, these adverse events might be sufficiently rare to only become apparent after mass administration. One side effect that has been observed is the development of a mild inflammatory reaction at the site of an i.m. inoculation. This is common to all vaccines and thus poses only a minor concern for humans. For meat-producing livestock such as cattle, scarring of muscle tissue is unwelcome, and an alternative route of DNA vaccination is required. One technical disadvantage of DNA vaccines is that their mode of administration currently involves an invasive procedure, that is either an injection by syringe or the use of a gene gun, which is cumbersome for mass vaccination. Although some investigators reported induction of immunity upon intranasal application of DNA, the response was low. The addition of cholera toxin, which is well tolerated by mice but causes prohibitive diarrheal purges in humans, to achieve good responses was required [89]. The development of optimized delivery vehicles such as microencapsulation or emulsion in cationic lipids will most likely eventually allow administration of DNA per os or by the intranasal route [72, 199–202].

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