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MINIREVIEW

On the Use of DNA Vaccines for the Prophylaxis of Mycobacterial Diseases

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The principle of genetic vaccination using naked bacterial plasmid DNA is surprisingly simple, yet the first reports on this technology were only made about a decade ago. In their seminal paper of 1990, Wolff et al. reported that injection of naked plasmid DNA encoding the bacterial enzyme β-galactosidase into muscle cells could lead to direct gene transfer to the muscle cells, transcription in the nuclei, and subsequent synthesis of the enzyme (77). In 1992, Tang et al. demonstrated that plasmid injection could be used to elicit an immune response (60), and the next year, Ulmer et al. were the first to report on the protective efficacy of DNA vaccination against an infectious disease, i.e., influenza A (71). Since then, DNA vaccines have been reported to induce protective immunity in numerous animal models of parasitic, viral, and bacterial diseases (14, 25). DNA vaccines have a number of potential advantages, including ease of preparation, stability, relatively low cost, and safety for immunocompromised patients. A number of clinical trials are actually being performed in the fields of cancer, human immunodeficiency virus (HIV), and malaria.

In a DNA vaccine, the gene for an antigen is inserted into a bacterial plasmid vector, plasmid DNA is amplified in transformed bacteria, and the purified plasmid DNA encoding the immunogen is injected into an immunocompetent host. Cellular targets are, first of all, muscle cells (intramuscular immunization) and keratinocytes (epidermal gene gun immunization), but bacterial DNA also gets into professional antigen-presenting cells (APCs) present in and attracted to the injection site. Once inside the cell, the plasmid translocates to the nucleus in a way that is not really understood. Transcription of the coding information is driven by a strong viral or eucaryotic promoter, very frequently the promoter of IE1, the first immediate-early antigen of cytomegalovirus, followed by its first intron (intron A). The gene of interest is followed by a eucaryotic transcription-termination polyadenylation site, which enables efficient translation and termination of the protein by the ribosomes. An origin of replication (OriC) and an antibiotic (kanamycin or ampicillin) resistance marker are used for selective amplification of the plasmid in *Escherichia coli*.

The methylation pattern of injected plasmid DNA remains of a bacterial nature (methylated adenosines) up to 19 months after injection, indicating that, indeed, no replication occurs in the eucaryotic host (76). Furthermore, sequence homology with eucaryotic sequences is minimal and as a result of these two factors, the risk for homologous recombination and mutagenic or potentially carcinogenic integration is very low. Random integration may take place but at chances of <1 copy in 150,000 nuclei (48).

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STIMULATION OF THE IMMUNE SYSTEM

DNA vaccination is an easy method by which to generate strong humoral and cellular immune responses. The priming of the immune response involves professional APCs (dendritic cells, Langerhans cells in the skin) that endocytose DNA into acidic vesicles for subsequent transport to the nucleus (68). Bacterial DNA has inherent adjuvant properties and triggers production of costimulatory cytokines by these APCs through its interaction with a specific Toll-like receptor, i.e., TLR9 (27). The particular motif in bacterial DNA that interacts with mouse TLR9 is a six-base DNA consisting of an unmethylated CpG dinucleotide flanked by two 5′ purines and two 3′ pyrimidines: GACGTT (31). The major costimulatory cytokines induced are interleukin-12 (IL-12) (which stimulates natural killer cells to produce gamma interferon [IFN-γ] and polarizes the development of a Th1-type T helper subset), IFN-α, tumor necrosis factor alpha (TNF-α), and IL-6 (31). Bacterial DNA is also a potent mitogen for B cells. DNA vaccines stimulate both the exogenous (major histocompatibility complex [MHC] class II-restricted) and endogenous (MHC class I-restricted) antigen presentation pathways. It is particularly this class I-restricted presentation, resulting in strong $CD8^+$-mediated immune responses, that is a hallmark of DNA vaccines and that makes them particularly attractive as subunit vaccine formulations against intracellular viruses, parasites, and bacteria. Following DNA vaccination, antigenic material is generated from within the host cell and endogenous processing can take place in much the same way as following infection with intracellular pathogens. Besides being directly transfected by plasmid DNA, dendritic cells can also take up antigen-loaded apoptotic bodies from transfected myocytes and present processed peptides to $CD4^+$and $CD8^+$ T cells (the so-called cross-priming phenomenon). Vectors coexpressing influenza virus hemagglutinin or nucleoprotein genes and attenuated, mutated caspase genes have been reported to increase T-cell and, to a lesser extent, B-cell responses and to augment protective efficacy against a lethal intranasal viral challenge (55). By virtue of their induction of $CD8^+$ T-cell responses, DNA vaccines strongly mimic

TABLE 1. Tentative summary of DNA vaccine candidates tested so far for protective efficacy

Antigen	Vector	Challenge	Protection[a]	Reference(s)
6-kDa ESAT-6	pCMV	*M. tuberculosis*	+	38
6-kDa ESAT-6	pJW4303	*M. tuberculosis*	±	35
8-kDa mtb8.4	pJA4303	*M. tuberculosis*	+	8
17-kDa MPT63	pJW4303	*M. tuberculosis*	−	35, 39
19-kDa lipoprotein	nCMVintBL	*M. tuberculosis*	−	19
19-kDa lipoprotein	V1J.ns-tPA	*M. tuberculosis*	−	78
22-kDa Lppx lipoprotein	V1J.ns-tPA	*M. tuberculosis*	−	34
23-kDa MPB83	pCMV4	*M. bovis*	±	6, 7, 73
24-kDa alkyl hydroperoxide reductase C	nCMVintBL	*M. tuberculosis*	−	19
26-kDa MPT64 antigen	pJW4303	*M. tuberculosis*	−	29
26-kDa MPT64	pJW4303	*M. tuberculosis*	±	35
30-kDa mycolyl transferase Ag85B	pJW4303	*M. tuberculosis*	+	29
30-kDa mycolyl transferase Ag85B (BCG)	pJW4303	*M. leprae*	+	54
30-kDa mycolyl transferase Ag85B (*M. avium*)	p85B-EGFP	*M. avium*	−	72
32-kDa mycolyl transferase Ag85A	V1J.ns-tPA	*M. tuberculosis*	+	1, 28
32-kDa mycolyl transferase Ag85A (BCG)	V1J.ns-tPA	*M. ulcerans*	+	61
32-kDa mycolyl transferase Ag85A (BCG)	VR1020	*M. bovis*	−	7
32-kDa mycolyl transferase Ag85A (BCG)	p85A-EGFP	*M. avium*	+	72
35-kDa antigen (M. avium)	pJW4303	*M. avium*	+	40
35-kDa antigen (M. leprae)	pJW4303	*M. leprae*	+	41
36-kDa proline-rich antigen PRA	pCMV/pHMG	*M. tuberculosis*	±	65
38-kDa lipoprotein PstS-1	pCDNA.3	*M. tuberculosis*	+	79
38-kDa lipoprotein PstS-1	V1J.ns-tPA	*M. tuberculosis*	−	64
38-kDa lipoprotein PstS-2	V1J.ns-tPA	*M. tuberculosis*	−	64
40-kDa lipoprotein PstS-3	V1J.ns-tPA	*M. tuberculosis*	+	64
43 kDa 1818^{PE_PGRS} protein	pJW4303	*M. tuberculosis*	+	9
39 kDa MTB39 protein from PPE family	pJA4303	*M. tuberculosis*	+	13
65 kDa heat shock protein (*M. leprae*)	pHMG/pCMV	*M. tuberculosis*	++	65
65 kDa heat shock protein (*M. tuberculosis*)	V1J.ns-tPA	*M. tuberculosis*	−	69
65 kDa heat shock protein (*M. avium*)	p65K-EGFP	*M. avium*	±	72
70 kDa heat shock protein	pCMV	*M. tuberculosis*	+	37
84 kDa KatG	pJW4303	*M. tuberculosis*	−	35

[a] ++, very strong protection; +, strong protection; weak protection; −, no protection.

infection with live pathogens, in contrast to vaccines based on protein antigens or killed pathogens that are preferentially processed through the exogenous presentation pathway that generates MHC class II-restricted $CD4^+$ responses.

DNA VACCINATION AGAINST MYCOBACTERIAL DISEASES

Cell-mediated immunity is essential for the control of mycobacterial infections. $CD4^+$ T cells are the key players in the control of *Mycobacterium tuberculosis*, but $CD8^+$ T cells also play an important role, and combination of the two T-cell subsets is necessary to obtain optimal protection (50). Because of the strong cell-mediated immunity they can induce, DNA vaccines were rapidly considered for use against mycobacterial infections and a considerable number of preclinical studies on the subject have been published in recent years. So far, DNA vaccines have been tested in experimental animal models for human and bovine tuberculosis (TB), leprosy, Buruli ulcer, and some nontuberculous mycobacterial infections (Table 1).

M. TUBERCULOSIS

In 1996, Tascon et al. and Huygen et al. were the first to report on the value of naked DNA vaccination against TB by using DNA encoding a 65-kDa heat shock protein from *M. leprae* and the 32-kDa mycolyl transferase or antigen 85A (Ag85A) from *M. tuberculosis*, respectively (28, 65). Subsequently, various degrees of immunogenicity and prophylactic efficacy against TB have been reported (mostly in C57BL/6 mice) with DNA vaccines encoding a vast range of antigens (Table 1). Whereas most plasmids encode mycobacterial proteins that are secreted in mycobacterial culture filtrate or surface exposed on the bacterial cell wall, some plasmids encode cytoplasmic proteins, such as the 65-kDa heat shock protein (65) and the MTB39 protein (13) (belonging to the PPE family), that are found predominantly in bacterial cytosol. Strong humoral, cytotoxic T-lymphocyte (CTL), and Th1-biased cellular immune responses, with nanogram levels of antigen-specific IFN-γ, but little IL-4 or IL-5 can be induced with some of these DNA vaccine candidates, particularly when they are administered by the intramuscular route (63). Interestingly, the epitopic repertoire for both $CD4^+$ and $CD8^+$ T cells is broadened by DNA vaccination compared to infection with live *M. tuberculosis* or vaccination with BCG (12, 17, 62). In $H\text{-}2^b$ haplotype mice, we have no indications for DNA-induced Ag85A- or Ag85B-specific $CD8^+$ T-cell responses (15), and broadening of the repertoire is the result not so much of the appearance of new epitopes but of the induction of stronger responses to initially subdominant epitopes. Antigenic processing and presentation may be different in DNA-vaccinated mice from that in BCG-vaccinated or TB-infected mice in the sense that only complete and correctly folded Ag85 proteins would be available during live infection, whereas truncated or differently folded molecules could be generated in the DNA-trans-

fected cells. In favor of this hypothesis is the fact that mice immunized with purified protein recognize the same epitopes as mice vaccinated with BCG or infected with TB. This also indicates that competition with other antigens in BCG or *M. tuberculosis* is probably not the reason for the narrower repertoire in infected animals. The relevance of these DNA-induced T-cell responses against subdominant epitopes is not clear, but it is tempting to speculate that they could be used to overcome problems in low-responder or nonresponder individuals.

In general, strong cellular immune responses are accompanied by similarly strong humoral responses, although some reports have described antibody production in the absence of T-cell responses (34). An interesting exception to this rule was described for a member of the PE_PGRS family, the surface-exposed 1818^{PE_PGRS} protein encoded by the *Rv1818c* gene. Mice vaccinated with DNA encoding the NH_2-terminal 1818^{PE} domain produced significant (albeit not very high) levels of IFN-γ following antigenic stimulation in the absence of any antibody response. In contrast, mice vaccinated with the complete 1818^{PE_PGRS} gene showed no IFN-γ response but significant antibody levels. Protection against a TB aerosol challenge was only found in 1818^{PE}-vaccinated animals, suggesting that the Gly-Ala-rich PGRS domain could have an inhibitory function (9). Using DNA vaccination, Banu et al. recently demonstrated B-cell immunogenicity for five additional PE_PGRS proteins (3).

A number of these DNA vaccines confer significant protection against TB in mice (Table 1), although it must be admitted that, so far, none of them has worked better than the existing *M. bovis* BCG vaccine, which remains the "gold standard" in all animal experiments. It is difficult to give a clear-cut answer as to why some vaccines work and others do not. Besides the obvious differences in content of immunogenic peptides, a number of variables may be involved: (i) the resting time between the last immunization and a TB challenge, which in some studies was only a couple of weeks, when animals are still bathing in the cytokine milieu induced by the boost; (ii) the CpG content of the plasmid backbone (the ampicillin resistance marker has a higher content of immunostimulating CpG motifs than the kanamycin resistance marker [56], and too many or neutralizing CpG motifs may reduce immunogenicity [32]); (iii) the physical stability and percentage of supercoil of the plasmid (for efficient transcription and translation, one could assume that the plasmid needs to be intact but for the stimulation of TLR-9, linearized oligonucleotides appear to work better); (iv) the route of DNA immunization and the dose used; (v) the route of *M. tuberculosis* challenge (aerosol probably being the most relevant); and (vi) the virulence of the TB isolate. Although most studies have worked with the H37Rv or Erdman strain, the culture conditions have not been standardized. (Concerning this last point, a luminescent *M. tuberculosis* H37Rv strain was developed by Snewin et al. [59] that offers a rapid and cheap alternative to fastidious counting of CFU on Middlebrook agar. Stocks of these bacteria are available at the Pasteur Institute of Brussels and can be distributed to anyone who is interested).

Not all studies have analyzed the immune responses in parallel with protection, but high, antigen-specific prechallenge spleen cell IFN-γ levels often (although not always) correlate with the best protection. Whether a particular antigenic epitope is expressed on infected cells and recognizable by immune T cells is a big unknown, and analysis of cytokine secretion in lung tissue early after a challenge may be indicative but is technically more difficult to achieve.

The use of DNA vaccines for the immunotherapy of TB is a subject of increasing controversy. D. Lowrie and his colleagues reported that four doses of plasmid DNA encoding hsp65 of *M. leprae* administered to BALB/c mice resulted in a rapid and spectacular decline in live *M. tuberculosis* (administered intravenously) in the spleen and lungs up to 5 months later (38). On the other hand, administration of another DNA vaccine with known prophylactic efficacy, i.e., a DNA vaccine encoding Ag85A, failed to protect mice when given in an immunotherapeutic model to mice infected by aerosol with *M. tuberculosis* (69). The discrepancy between the two studies cannot be explained by differences between the mouse strains or plasmid DNA but is perhaps due to differences in virulence between the *M. tuberculosis* isolates and the infection routes (66). Moreover, aerosol TB-infected mice vaccinated immunotherapeutically with DNA vaccines develop classical Koch reactions, characterized by multifocal, discrete regions of cellular necrosis throughout the lung granuloma (66). This adverse reaction is not unique to immunotherapy with DNA vaccines but can also be elicited by administration of whole live or heat-killed mycobacteria, and secretion of TNF-α seems to be the key molecule in the exacerbated lung pathology (47).

hsp65 DNA vaccination at the end of an 8-week chemotherapy course completely prevented the reactivation of bacterial growth after immunosuppressive corticosteroid injection in the Cornell model of reactivation (38). These findings have also been contradicted, this time by Repique et al. who, by using a 10-component DNA vaccine cocktail (very powerful as a prophylactic vaccine [11]), were unable to prevent recrudescence of latent infection after injection of dexamethasone (53).

All of the preclinical TB DNA vaccine studies published so far have been done with mice, with the exception of two that were done with guinea pigs. Baldwin et al. reported that vaccination with DNA encoding Ag85A prevented the onset of caseating disease, which is the hallmark of the aerogenic-infection model in this species (1). Survival was prolonged in Ag85A DNA-vaccinated guinea pigs but shorter than in BCG-vaccinated animals. Garapin et al. reported on DNA immunization with *apa* and *pro*, two genes encoding proline-rich glycosylated proteins from *M. tuberculosis* (23). Positive delayed-type hypersensitivity (DTH) reactions and antibody production were measured in a proportion of outbred Hartley guinea pigs, particularly after intradermal injection of DNA into the flank. An *apa* gene-bearing plasmid conferred some protection against intravenous *M. bovis* BCG in mice, but unfortunately, no data on protection against *M. tuberculosis* in guinea pigs were reported in that study (23).

In cynomolgus monkeys, we have shown that a DNA prime-recombinant protein boost regimen was weakly immunogenic before an intratracheal *M. tuberculosis* challenge but that immune responses could be boosted by and rapidly increased after an intratracheal *M. tuberculosis* infection (J. Langermans, personal communication). Optimization of plasmid vectors for use in primates and humans, with particular attention to their content in immunostimulatory and neutralizing CpG motifs (32), is essential for further studies.

M. BOVIS

The current tuberculin test and slaughter strategy for the control of bovine TB in cattle has proven to be ineffective in countries with important wildlife reservoirs for *M. bovis* such as the badger in Great Britain and the opossum in New Zealand. A recent independent scientific review has concluded that the development of a cattle vaccine against *M. bovis* is the best long-term prospect for TB control in British herds, certainly if this vaccine does not interfere with the current tuberculin skin test (73). Mice vaccinated with DNA encoding the secreted 23-kDa MPB83 protein from *M. bovis* could be protected from an intravenous challenge with virulent *M. bovis* to an extent similar to that of animals vaccinated with the BCG vaccine (6). Vaccination of guinea pigs with the same MPB83 DNA reduced the severity of pulmonary lesions after a low-dose *M. bovis* aerosol challenge but did not protect the animals from hematogenous spread of bacilli to the spleen (7). Vaccination with Ag85A, a promising DNA vaccine for human TB, exerted no measurable protective effect against infection with *M. bovis* in this study (7). Vordermeier et al. have reported that calves vaccinated with DNA encoding MPB83 and MPB70 from *M. bovis* demonstrated potent cellular immune responses, characterized by IFN-γ-producing $CD4^+$ T cells, as well as humoral responses, biased toward immunoglobulin G1 isotypes. Interestingly, none of the MPB83-vaccinated calves showed a positive skin test reaction to bovine or avian tuberculin. Absent or very weak DTH reactions to tuberculin have also been described in mice and guinea pigs immunized with DNA encoding Ag85A (1, 2). However, DTH responses have been reported after DNA vaccination with chlamydial and HIV antigens, indicating that the observed lack of a DTH reaction to tuberculin following vaccination with Ag85A or MPB83 DNA is probably related to the nature of purified protein derivative, which is a mixture of countless cytosolic and culture filtrate proteins. The protective efficacy of these DNA vaccines against *M. bovis* in cattle remains to be determined.

M. AVIUM

M. avium is the most common bacterial infection in patients with AIDS in the United States, leading to substantial morbidity and mortality. C57BL/6 mice were immunized with DNA encoding the *M. avium* 65-kDa heat shock protein, *M. bovis* BCG Ag85A, and *M. avium* Ag85B, all three expressed as fusion proteins with green fluorescent protein. Mice were challenged by the intraperitoneal route with 5×10^6 CFU of MAC101 3 weeks after the third DNA injection. The best protection, resulting in a four- to eightfold reduction in spleen CFU counts, was observed in mice vaccinated with DNA encoding Ag85A from *M. bovis* BCG (72). This protection was comparable to that observed against BCG, whereas the 65-kDa DNA protected to a lesser extent and the *M. avium* Ag85B DNA did not protect at all, which is surprising since *M. avium* strains express Ag85A at lower levels than A85B (49). Protection against *M. avium* serotype 8 was also described in C57BL/6 mice vaccinated with DNA encoding the 35-kDa antigen from *M. avium*. This protein was first identified as an immunodominant antigen in the human response to *M. leprae*. The leprosy and *M. avium* molecules are 95% identical at the amino acid level but are absent in members of the *M. tuberculosis* complex. Mice were challenged by the intravenous route 6 weeks after the third intramuscular 35-kDa DNA injection and showed highly significant and sustained protection in the spleen and liver, equivalent to that conferred by BCG immunization and significantly greater than that observed in a previously treated infection with *M. avium* (40).

Johne's disease is a chronic granulomatous infection of the intestine of ruminants caused by *M. avium* subsp. *paratuberculosis*. Current vaccines (based on live or mostly heat-killed whole bacilli) do not prevent infection but can delay the onset of clinical disease. Unfortunately, these vaccines interfere with the bovine purified protein derivative skin test and farmers are reluctant to use them. Homologs of the Ag85 complex are present in culture filtrates from *M. paratuberculosis* ATCC 19698 and elicit strong Th1 cytokine responses in experimentally infected mice. C57BL/6 mice vaccinated with DNA encoding Ag85A and Ag85B from *M. paratuberculosis* produced strong Ag85-specific antibody responses in their serum and elevated levels of IFN-γ and IL-2 in spleen cell cultures (V. Rosseels, V. Scanlan, A. Vanonckelen, F. Jurion, K. Palfliet, S. Marché, J. Godfroid, K. Walravens, and K. Huygen, Seventh Int. Colloq. Paratuberc., abstr. S2.P21[137], 2002). Vaccination with Ag85B DNA also showed some protective efficacy (V. Rosseels, personal communication).

M. LEPRAE

Britton and his colleagues have reported on the use of DNA vaccines against experimental *M. leprae* infection in mice. Plasmid DNA encoding the immunodominant 35-kDa protein shared by *M. leprae* and *M. avium* (see the previous section) was used to vaccinate outbred Swiss albino mice. DNA immunization stimulated specific T-cell activation and IFN-γ production and significant levels of protection against *M. leprae* footpad infection, comparable to that produced by BCG (41). Protection, albeit somewhat less than that obtained with BCG, was also observed following vaccination with DNA encoding cross-reactive Ag85B from *M. tuberculosis* (54).

M. ULCERANS

Buruli ulcer, caused by *Mycobacterium ulcerans*, is an emerging mycobacteriosis in tropical and subtropical developing countries that is characterized by deep and necrotizing skin lesions caused by a polyketide-like exotoxin. No specific vaccine is available for Buruli ulcer, but evidence from the literature suggests a cross-reactive protective role of the *M. bovis* BCG vaccine, particularly against *M. ulcerans* osteomyelitis in children (52). Vaccination with DNA encoding Ag85A from *M. bovis* BCG was capable of reducing the bacterial load in the footpads of *M. ulcerans*-infected C57BL/6 mice more than 10-fold, as determined by Ziehl-Neelsen staining and actual counting of CFU on Middlebrook agar (61). Sequence comparison of the Ag85A protein from *M. ulcerans* and that of *M. bovis* BCG showed 84.1% identical and 91% conserved amino acid residues (61). Vaccinations with DNAs encoding the Ag85B gene from *M. tuberculosis* and the Ag85A gene from *M. ulcerans* exert similar protective effects (Tanghe et al., personal communication).

MECHANISM OF ACTION AND MODULATION OF EFFICACY OF DNA VACCINES

DNA vaccines induce strong $CD4^+$- and $CD8^+$-mediated immune responses in mice, but the two compartments may differ in relative importance in the protection conferred by the vaccine against TB according to the antigen, the genetic background, and the phase of infection (active infection or latency). It is impossible to determine the role of $CD8^+$ T cells in Ag85A DNA-vaccinated C57BL/6 mice, because the Ag85A molecule does not possess K^b- or D^b-restricted epitopes (15), and therefore, Ag85A-specific cellular immune responses in this mouse strain are exclusively mediated by $CD4^+$ T cells. D'Souza et al. demonstrated that vaccination with Ag85A DNA failed to protect *H-2*^{b} CD4 knockout mice but significantly decreased bacterial replication in the lungs and prolonged the survival of *M. tuberculosis*-infected *H-2*^{b} β2-microglobulin knockout mice, which lack functional $CD8^+$ T cells (15, 18). On the other hand, neither intramuscular nor epidermal gene gun immunization with this Ag85A DNA vaccine conferred protection on BALB/c mice although both immunization routes induced strong $CD8^+$-mediated CTL responses in this mouse strain (63). However, it must be mentioned that the amount of IFN-γ induced in BALB/c mice by this Ag85A DNA vaccine is less than in C57BL/6 mice.

On the other hand, studies with hsp65 DNA-vaccinated BALB/c mice suggest that the most potent protective cells induced by this vaccine are $CD8^+$ $CD44^{hi}$ cytolytic T cells that produce elevated levels of IFN-γ (4, 36). Therefore, one may conclude that DNA vaccines mediate their protection through IFN-γ (and TNF-α?)-secreting $CD4^+$ and $CD8^+$ T cells but that, depending on the nature and localization of the antigen (confined to the phagosome early in infection or released into the cytosol at later time points) and the genetic background (Th1 versus Th2 biased, *H-2* restriction, etc.), either the $CD4^+$ or the $CD8^+$ population will be the most important.

Furthermore, extrapolation to the human situation must be made with great caution. Thus, we have never been able to detect any Ag85-specific CTL responses in BCG-vaccinated or *M. tuberculosis*-infected mice, although such responses do exist in BCG-vaccinated human subjects or healthy given primary infection human subjects (24, 58).

DNA vaccines offer the advantage that they can be formulated to target specific cell compartments for antigenic processing. Plasmids encoding a secreted form of the protein by fusing it to the signal sequence of human tissue plasminogen activator (tPA) are generally more immunogenic, for both B and T cells, than plasmids encoding a mature form (2, 35, 46). Transient-transfection experiments in vitro have shown that plasmids encoding a secreted form show higher expression levels than plasmids encoding nonsecreted proteins, and this is probably the most important factor involved in the increased immunogenicity (35). Better availability of the released antigen to professional APCs may also be of importance. The effect of the tPA leader sequence is most pronounced for weakly immunogenic plasmids or when DNA is used at suboptimal doses or at a low dose number (2). This may explain why some authors have failed to observe differences between DNAs encoding secreted and mature forms of a protein (40). Secreted forms are targeted to the endoplasmic reticulum of the cell, where they are N glycosylated on their asparagine residues, but this glycosylation does not seem to influence their immunogenicity (Montgomery and Huygen, unpublished results).

A specific way to increase MHC class I presentation is by tagging the antigen with ubiquitin, which results in proteins that are targeted preferentially to the proteasome system. The mycobacterial culture filtrate proteins MPT64 and ESAT-6 were expressed as chimeric proteins fused to one of three variants of the ubiquitin protein (UbG, UbA, or UbGR). Immunization with plasmids expressing the UbA and UbGR fusions shifted the host response toward a stronger Th1 type of immunity, which was characterized by absent or low specific antibody levels, a lower number of IL-4-producing cells, and a higher number of IFN-γ-producing T cells. However, despite the higher IFN-γ levels produced in vitro, these ubiquitinated constructs did not provide a better protective response in vivo than the nonubiquitinated tPA-fused antigens (10).

As already mentioned, DNA vaccines are also strong inducers of humoral immune responses. Isotype analysis of these antibodies can give an indirect indication of a preferential activation of the Th1 type of T helper subset following intramuscular DNA injections (63), but the induction of antibodies is less relevant for protection against TB than it is for protection against pathogens that also have an extracellular stage in their replication cycle, such as those that cause HIV and malaria. Use of T-cell epitope-based plasmid DNA vaccines can circumvent the induction of antibodies (21), but so far, no protection data have been published and in view of the extensive HLA polymorphism of human populations, such a peptide-based approach may not be very realistic, albeit that some antigenic epitopes may indeed be recognized in a promiscuous manner by a variety of HLA haplotypes (33).

STRATEGIES FOR INCREASING TB DNA VACCINE POTENCY

A limiting factor for DNA vaccines is their transfection efficacy and the amount of actual protein synthesized. It has been estimated that injection of microgram doses of DNA results in production of only nanogram doses of protein (25). Complexation of DNA in cationic lipids (16) or intramuscular immunization combined with muscle electroporation (67) can increase targeting to APCs and increase muscle transfection, respectively. Other possible ways of increasing immunogenicity would be the use of cationic poly-L-lactide coglycolide microspheres (57, 70), vectors coexpressing mutated caspases (55), and alphavirus-based replicons (26), but for TB genes, these approaches remain to be tested. Coimmunization with a plasmid expressing granulocyte-macrophage colony-stimulating factor can enhance the T-cell immunity of DNA vaccines encoding Ag85B or MPT64 about twofold, but this is not sufficient to improve their protective efficacy at the peak of infection after an aerosol challenge with *M. tuberculosis* (30). Britton and his colleagues have also shown that coimmunization with a self-splicing vector encoding IL-12 and DNA encoding Ag85B or MPT64 increases specific lymphoproliferation and $CD4^+$ and $CD8^+$ IFN-γ secretion, but again, the effect on protective efficacy was very modest (51).

Multisubunit vaccination by coimmunization with different DNA vectors that are not very effective as single vaccines may

result in a greater degree of protection, as indicated by reduced CFU counts (29), and prolonged survival time following a high-dose aerosol challenge compared to that of mice vaccinated with vector DNA only (11). Simple mixing of highly immunogenic plasmids may lead to competition, and construction of hybrid genes may circumvent this problem. The manufacture of hybrid genes (and proteins, for that matter) is also more interesting from an economic point of view. S. Reed and his colleagues at Corixa Corp. have shown very convincing results for an Mtb72F DNA encoding a fusion of the Mtb39 PPE protein and a 32-kDa serine protease (Y. Skeiky et al., personal communication). Another hybrid DNA encoding the 40-kDa phosphate-binding protein PstS-3 and the mycolyl transferase Ag85A also demonstrates increased immunogenicity and efficacy (M. Romano, personal communication).

Finally, by virtue of their intrinsic capacity to prime for polarized Th1-type $CD4^+$ and $CD8^+$ immune responses, DNA vaccines open a very promising avenue for prime-boost immunization strategies. Treatment of Ag85 DNA-primed mice with purified Ag85 protein in adjuvant can increase spleen cell IL-2 and IFN-γ responses two- to fourfold. Intracellular cytokine analysis by flow cytometry showed that these IFN-γ responses were more sustained in time. Finally, protein boosting increased the protective efficacy against an intravenous *M. tuberculosis* challenge, underlining the important role of MHC class II-restricted Th1-type $CD4^+$ helper T cells in mediating protection (62). Recombinant poxviruses can also be used for this type of boosting, but in contrast to the exogenous protein boost, MHC class I-restricted $CD8^+$ T cells are the first target of the viral boost regimen (42). Vaccinia virus has been widely used and has a well-defined safety profile in humans. Compared to naked DNA vaccines, which encode only one particular antigen, recombinant viruses (and recombinant bacteria for that matter) have the disadvantage of being complex vectors with some intrinsic viral immunogenicity and, hence, limited potential for homologous boosting. For TB, heterologous boosting of a DNA-primed immune response with recombinant modified vaccinia virus Ankara (MVA) expressing ESAT-6 and MPT63 was reported to increase the number of both $CD4^+$ and $CD8^+$ IFN-γ-producing cells (44). More recently, heterologous boosting with MVA encoding Ag85A was reported to confer protection against *M. tuberculosis* infection in BALB/c mice equivalent to that obtained with BCG (42). Although very promising, these results have to be taken with some caution as a TB challenge was performed very shortly after the final immunization in these experiments. A phase I clinical study using MVA Ag85A in healthy, skin test-negative volunteers has recently been initiated in the United Kingdom, and similar trials in Gambia and Sénégal are to follow soon (42).

DNA priming, followed by recombinant *S. enterica* serovar Typhimurium secreting ESAT-6, did not enhance protection significantly compared to recombinant *S. enterica* serovar Typhimurium alone (45), but in that study, DNA was administered by intradermal particle bombardment with a gene gun, which may have resulted in priming with only a low number of Th1-type $CD4^+$ T cells (63).

It is a fact that most of the immune analyses done so far have focused on central memory assessed by spleen cell cytokine secretion and little attention has been paid to the induction of effector memory T cells in lung tissue. Intranasal immunization with DNA complexed in cationic lipids is not effective enough at inducing these local pulmonary responses, but we have recently found that a combined intramuscular-intranasal immunization protocol can indeed induce such immune responses in the lung (16).

COMBINATIONS OF PLASMID DNA WITH BCG

Sequential immunization by Ag85B DNA, followed by *M. bovis* BCG, was found to be more effective than BCG immunization alone in protecting C57BL/6 mice against an aerosol *M. tuberculosis* infection (20). Unfortunately, the study did not have a proper control group, which should have been given the empty vector prior to BCG. Also, the conclusion that $CD8^+$ T cells would partially mediate this improved efficacy should be considered with caution because, again, proper controls were lacking, the effect was only seen in the spleen, and moreover, results would be difficult to understand in view of the fact that the Ag85B antigen does not contain K^b- or D^b-restricted epitopes (15). Coimmunization with the self-splicing IL-12 vector during the Ag85B DNA priming step does not increase the protective effect of this DNA-BCG combination vaccination (51). In the bovine model of *M. bovis* infection, priming with hsp65, hsp70, and *apa* DNAs has also been demonstrated to increase the protective efficacy of the BCG vaccine (Hewinson et al., personal communication).

BALB/c mice immunized with BCG adjuvanted in immunostimulatory CpG oligodeoxynucleotides (ODN) demonstrated increased spleen cell IFN-γ production and improved efficacy against an aerosol challenge with *M. tuberculosis* (Erdman), compared to immunization of mice with BCG only (22). Granulomas in BCG-vaccinated mice were smaller and more lymphocyte rich than those from unvaccinated mice, but there were no differences between the BCG-only and the BCG-plus-CpG ODN groups (22). Increased production of IL-12 in the CpG ODN adjuvant-treated group may have been the key factor, as BCG efficacy was similarly increased by intraperitoneal coinjection with 500 ng of recombinant IL-12 protein.

PROSPECTS FOR TB DNA VACCINES

The strong, polarized Th1-type $CD4^+$ and $CD8^+$ responses that they induce, coupled to their inherent adjuvant activity, make DNA vaccines a promising new approach for the immunoprophylaxis of mycobacterial diseases. DNA vaccines are easy to produce and cheap (in theory), they do not require sophisticated purification schemes, nor do they need a cold chain for storage, all important factors for eventual application in developing countries (25). Moreover, these DNA vaccines can be used for repeated boosting because—in contrast to viral or bacterial delivery systems—the plasmid vectors are not intrinsically immunogenic and only elicit immune responses to the heterologous antigens.

Although DNA vaccines have failed so far to induce robust and broad-based immune responses in primates, including humans, phase 1 clinical trials with malaria and HIV DNA vaccines have shown that genuine CTL and $CD4^+$ T-cell responses can be induced (5, 74, 75). For TB DNA vaccines, no phase 1 trials are scheduled at the moment and it is unlikely

that the existing *M. bovis* BCG vaccine will be replaced soon by a DNA-based vaccine, particularly because of the proven efficacy of the BCG vaccine against childhood TB. On the other hand, DNA vaccines may be valuable in combination with BCG by polarizing and focusing $CD4^+$ and $CD8^+$ responses to major protective antigens (DNA prior to BCG) and by boosting waning immunity (DNA post BCG), similar to what is attempted in the first phase 1 clinical trial of a new TB vaccine candidate, i.e., MVA Ag85A (43). Improved vectors and optimal prime-boost immunization protocols will be developed and analyzed in nonhuman primates in the near future. Finally, DNA vaccination is a potent and easy-to-use tool for the screening of protective antigens for mycobacterial diseases and for the characterization of immunodominant epitopes. They can also be helpful in the selection of new candidates for protein subunit and recombinant viral or bacterial expression vectors. We are confident that the National Institutes of Health- and European Economic Community-funded FP6 TB Vaccine Integrated Project will give the scientific community the financial means and opportunities to delineate further the value of DNA vaccines for future immunoprophylaxis of TB and mycobacterial diseases in general.

For more details, visit the following websites: www.genweb.com/dnavax.html and www.fda.gov/cber/points.html (points to consider, safety issues).

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