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REVIEW

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Strategies for enhancing DNA vaccine potency by targeting antigen-presenting cells

Abstract The potency of DNA vaccines to stimulate the immune response, especially the T-cell immune response against viral infections and tumors, depends mainly on the ability of antigen-presenting cells to process and present DNA-encoded antigens. Targeting the specific antigen to antigen-presenting cells is believed to be a crucial step for eliciting the T-cell response. Many strategies for enhancing DNA vaccine potency by targeting antigen-presenting cells have been developed. In this article, we generally introduce a T cell immune system and review some strategies which have been recently developed for enhancing DNA vaccine potency.

Keywords DNA vaccine, antigen presenting cells, targeting, immune response

1 Introduction

DNA vaccination has been proven to be an effective method of immunization against some pathogenic processes in animal models, including HIV, influenza, malaria, rabies (Ulmer et al., 1993; Sedegah et al., 1994; Xiang et al., 1995; Boyer et al., 1997), Ebola, tuberculosis, and neoplasms (Lowrie et al., 1997; King et al., 1998; Xu et al., 1998). DNA-based immunization is also an attractive adjunct for cancer immunotherapy (Freda et al., 2004) and may have potential for the prevention or treatment of autoimmune diseases (Waisman et al., 1996; Ramshaw et al., 1997) or allergy (Delphine et al., 1997). More recently, several DNA vaccination trials in human and animals have been evaluated (Wang et al., 1998; Dadara et al., 2008).

The idea of DNA vaccines or genetic vaccines came from the observation that injection of naked plasmid DNA resulted in the transfection of muscle cells and expression of plasmid-encoded protein β-galactosidase (Wolff et al., 1990). The DNA-encoding nucleoprotein of influenza A virus, when delivered into the quadriceps of BALB/c mice, induced the nucleoprotein-specific CTL response and protection from a subsequent challenge with a heterologous strain of influenza A virus (Ulmer et al., 1993). The DNA was delivered into the skin by using a 'gene gun' delivery system to elicit strong antibody responses against the encoded protein (Tang et al., 1992). Those findings led to the development of a powerful and simple technology of DNA vaccination.

DNA vaccination has a potential advantage over the traditional protein vaccination due to the cellular immune response induced in addition to humoral immune response (Raz et al., 1996) particularly in small experimental animals. In general, however, DNA vaccination seems to be less immunogenic in larger animals (including human beings) than in smaller ones (Wang et al., 1998). This low immunogenicity is the greatest obstacle to its extensive use in clinical or veterinary practice. As a result, the enhancement of DNA vaccine immunogenicity has become the central goal of much current research.

In this article, we review the attempts that have been made to improve the potency of DNA vaccine by targeting the plasmid-encoded proteins to the antigen-processing pathways. We first introduce the T-cell immune response and antigen presentation pathways and then review the strategies for enhancing DNA vaccine potency, especially enhancing the T-cell response.

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2 Induction of T-cell immune response

T cells can be divided into two types based on cell-surface proteins: CD4 and CD8. T cells can recognize foreign proteins in the form of short (9-24aa) fragments (epitopes) that are presented on the major histocompatibility complex (MHC) on cell surfaces. There are two main classes of MHC proteins: MHC class I and MHC class II. $CD8^+$ T cells recognize epitopes presented by MHC class I, whereas $CD4^+$ T cells recognize epitopes presented by MHC class II.

MHC class I molecules are expressed on most somatic cells. In contrast, MHC class II molecules are expressed by a relatively limited number of cell types, most of which are specialized antigen-presenting cells (APCs).

APCs are specialized cells whose function is to phagocytose, process, and present antigen peptides to T cells. In the lymphoid system, the main types of APCs are dendritic cells, macrophages, and B cells. On the majority of cells, MHC class I presents epitopes only from endogenous proteins, i.e., proteins synthesized within the cells. In general, $CD8^+$ T cells will recognize any cell that expresses an unknown (e.g. viral or tumor antigen) protein. In contrast, MHC class II, the expression of which is largely limited to APCs, in most cases, presents the peptides that come from exogenous proteins, phagocytosed from the extracellular milieu. However, APCs differ from most somatic cells. Some APCs seem capable of accepting proteins from the extracellular milieu and presenting them by the MHC class I pathway (cross-priming or cross-presentation) (Rodriguez et al., 1999). Conversely, some endogenously synthesized in APCs can introduce them to the MHC class II pathway (Bonifaz et al., 1999). Naïve T cells can be activated only by the MHC-epitope complex on APC cells, but not triggered by MHC-epitope complex on other somatic cells because they do not express the necessary intracellular adhesion molecules (ICAM-1, or ICAM-3) and costimulatory molecules (B7).

2.1 MHC class I antigen presentation delivered as DNA vaccine

Injected plasmid DNA-encoding antigen protein enters the cells, and passes into the nuclei, where mRNA is transcribed and subsequently translated in the cytosol. A certain proportion of the translated protein molecules are incorrectly folded and preferentially tagged by the covalent attachment of poly-ubiquitination, which acts as a signal for the protein to be taken to the proteasome, where the polyubiquitin is hydrolyzed and the misfolded proteins are degraded into peptides, which are transferred through the TAP transporters into the endoplasmic reticulum (ER). Within the ER, the peptides encounter empty MHC class I molecules to which the peptides are bound, forming the peptide/MHC complexes, which are transported to the Golgi apparatus and hence to the cell surface, where they are available for $CD8^+$ T-cell interaction.

2.2 MHC class II antigen presentation delivered as DNA vaccine

Antigens destined for the MHC class II presentation pathway are usually derived from soluble proteins shed by non-lymphoid cells. These proteins are phagocytosed (usually non-specifically) by APCs and incorporated into a low-pH endosomal compartment, where they are hydrolyzed into short peptides by acid proteases. Meanwhile, MHC class II molecules have been translated and entered the ER, where their epitope-binding site is blocked by a protein named invariant chain (Ii) and then fused with the endosomes, where Ii is removed, with the empty MHC-binding site exposed to the peptides. Those peptides bound most avidly are presented at the cell membranes, where they interact with $CD4^+$ T cells specific for epitopes that present in the original soluble protein.

Plasmids encoding antigens are injected into animal muscular tissues. Most of the antigen genes are expressed in non-lymphoid cells, where the expressed antigen proteins are secreted outside of the cells and taken up by APCs. Some of the plasmids are directly taken up by APCs.

Dendritic cells are professional APCs capable of activating naïve T cells, which are believed to be the major APCs involved in primary immune responses because they induce T-cell proliferation more effectively than any other APCs. Dendritic cells phagocytose and present antigens mainly by micropinocytosis and MHC class II, respectively; at the same time the antigens are also presented by MHC class I (cross-presentation or cross-priming) in some dendritic cell subsets (Pooley et al., 2001; Iyoda et al., 2002). In addition to micropinocytosis, dendritic cells also take up certain antigens with different receptors on dendritic cell membranes. Many strategies for targeting antigens to dendritic cells are based on specific receptor-mediated antigen internalization (Boyle et al., 1998).

3 Strategies for enhancing the potency of DNA vaccines

Several strategies have been developed to enhance the potency of DNA vaccines, including targeting antigen DNA to APCs using bacteria-derived plasmids, directing antigens to APCs by fusion to ligands for APC receptors, targeting antigens for intracellular degradation, and co-administrating antigens with cytokine and co-stimulatory molecules.

3.1 Targeting antigen DNA to APCs using bacteria-derived CpG-rich plasmids

The effects of bacteria DNA on the immune system were first studied in the 1980s (Shimada et al., 1985; Yamamoto et al., 1998). It was observed that the exposure of macrophage to bacteria DNA stimulated the inhibition of interferons (INF) α and β in induction of IL12 production. This in turn led to natural killer cell activation and INF-γ production (Tokunaga et al., 1992; Yi et al., 1996). It was also observed that higher immune responses were induced in mice after being injected with the plasmid recombinant for the ampicillin resistant gene with CpG motifs, than those in mice vaccinated with an identical plasmid recombinant for the kanamycin resistant gene in lack of CpG motifs (Sato et al., 1996). How plasmids entered the target cells remains unclear. It is likely related to scavenger receptors on APCs. These receptors bind a range of polyanions, including sequences of bacteria DNA (Wlochc et al., 1998). The exposure of immune dendritic cells to CpG oligodeoxynucleiotides induces their maturation, and up-regulates MHC class II, CD86 expression, and antigen presenting function (Jakod et al., 1996; Sparwasser et al., 1998). Co-administration of plasmid vector DNA containing CpG motifs acts as an adjuvant when co-administrated with proteins and peptide vaccines or antigen-encoding plasmid DNA. DNA containing CpG motifs, injected with soluble protein antigen, significantly boosts specific humoral and cell-mediated immune responses (Roman et al., 1997; McCluskie and Vavis, 1998).

3.2 Targeting antigens to APCs by fusion to ligands for APC receptors

There are many different receptors expressed on APCs that can be recognized and interacted with special ligands. Several ligands for APC receptors have been chosen for generating fusion proteins with specific antigens to enhance immune responses, such as immunoglobulin Fc fragments (FcγR, FcεR), chemokines (chemokine receptors), C3d component of complement (CD21 and C3d receptors), CTL antigen-4 (CD80/CD86), β-defensin-2 (Toll-like receptor 4), calreticulin (scavenger receptor-A), heat shock proteins (HSPs; CD91 and α2-macroglobulin receptors), RGD motifs of extracellular matrix proteins (integrin), and fragment C of tetanus toxin (FrC; 15 kDa putative receptor).

3.2.1 Fc fragment

The most commonly used ligand is the immunoglobulin Fc fragment that binds Fc receptors expressed on APC membranes. Fc receptors mediate internalization of antigen-Ig complex or antigen-Fc fusion proteins and promote efficient MHC class II-restricted antigen presentation, 1000- to 10000-fold more efficiently than fluid phase pinocytosis (Regnault et al., 1999). FcγR-mediated endocytosis can also cross-present the internalized antigen to MHC class I (Kovacsovics-Bankowski and Rock, 1995). In addition, the interaction of Fc with its FcγRs activates dendritic cells by up-regulating surface molecules and cytokine involved in antigen presentation (Regnault et al., 1999; Pooley et al., 2001; Iyoda et al., 2002). Therefore, Fc receptors represent a privileged antigen internalization route for efficient MHC class I- and II-restricted antigen presentation by APCs.

Another APC receptor ligand, monocyte chemotactic protein3 (MCP-3), is a chemokine, having potent chemoattractant for monocytes and dendritic cells, T cells, basophils, and eosinophils (Luster et al., 1998). Most APCs, particularly dendritic cells, express CCR1, CCR2, and CCR3 chemokine receptors, which bind MCP-3 chemokine (Craig and Barrett, 2001). Antigen proteins fused with MCP-3 can facilitate antigen taken by APCs (Arya et al., 1999).

3.2.2 C3d

The component of complement is also used as a ligand fusion to target APCs (B cells and follicular dendritic cells) and enhance antibody responses (Dempsey et al., 1996). The enhancement of humoral responses by C3d is related to stimulating and expanding the antigen-specific B cells. C3d receptor CD21 is expressed on B cells and follicular dendritic cells (FDC) and associated with the co-stimulatory molecule CD19. The fusion of an antigen to C3d should allow for cross-linking of antigen-specific B-cell receptor and CD21, thereby delivering stranger signals for B-cell activation. CD21 is also expressed on FDCs in germinal centers. It is possible that C3d may facilitate antigen binding to FDCs so that the trapped antigen can select a high-affinity antibody binding site in maturing B cells. It is also reported that the humoral responses against hemagglutinin (HA) of influenza could be enhanced by fusion of C3d (Ross et al., 2000). Therefore, enhancing B-cell signaling by the C3d fusion strategy may be a useful approach for developing DNA vaccine against cancer and other infectious diseases.

3.2.3 CTL-antigen-4 (CTLA-4)

CTLA-4 that has also been exploited to target antigen delivery to dendritic cells is normally expressed by activated T cells and bound with high affinity to CD80/CD86 co-stimulatory molecules expressed by APCs. The antigen fused with CTLA-4 can be easily taken up by APCs through the CD80/CD86 receptors. In a mouse model, vaccination with a DNA construct encoding a human IgG (hIgG) fused with the extra-cellular domain of

CTLA-4 fusion protein was shown to elicit a faster and higher anti-hIgG antibody response than DNA vaccines encoding hIgG alone (Boyle et al., 1998). The CTLA-4 fusion could also enhance the T-cell proliferation.

3.2.4 β-defensin

β-Defensin is a family of cationic peptides expressed by many tissues, especially mucosal tissue and skin, which are involved in host defense against microbial infection. Human β-defensin has been proved to be chemotactic for immature dendritic cells and memory T cells (Yang et al., 1999; Biragyn et al., 2001). Murine β-defensin-2 acts directly on immature dendritic cells via TLR4 (Biragyn et al., 2002), including up-regulation of co-stimulatory molecules and dendritic cell maturation. Thus, defensins play a role in linking innate immunity and adaptive immunity. DNA fusion vaccines of antigen and β-defensin can induce protective and therapeutic immunities (Biragyn et al., 2001).

3.2.5 Heat shock protein (HSP)

Heat shock proteins (HSPs) or heat stress proteins extensively exist in all eukaryotic and prokaryotic species, including plants. HSPs are categorized into several families based on their approximate molecular weight (e.g., the 60 kDa, HSP60 family). In general, HSPs are located in various intracellular compartments and function as intracellular chaperones for peptides. Several HSPs, such as HSP70 and HSP90 (mainly located in cytoplasm), calreticulin (CRT), and HSP96 (mainly located in ER) are peptide-binding HSPs. Immunization with HSP-complexes isolated from tumor or virus-infected cells has proved its ability to elicit antigen-specific cellular immunity to tumor antigens (Ishii et al., 1999). The HSP-based DNA vaccines administrated by fusing antigen gene to HSP genes have been evaluated (Chen et al., 2000; Cheng et al., 2001). The expressed HSP-peptide fusion proteins from non-APCs in immunized mice with HSP-peptide DNA vaccine are phagocytosed by APCs via CD91 and α_2-macroglobulin receptors with peptides processed and re-presented on the surface of the APC by the MHC I molecules of APCs, which then stimulate antigen-specific $CD8^+$ lymphocytes (Udono et al., 1994; Suto and Srivastava, 1995).

CRT has been proved to be associated with peptides delivered into the ER by transporters associated with antigen processing (TAP-1and TAP-2) and also with MHC I-β2 microglobulin molecules to aid in antigen presentation (Sadasivan et al., 1996). L57BL/6 mice vaccinated intradermally with CRT/HPV-16 E7 antigen DNA showed a dramatic increase in E7-specific $CD8^+$ T-cell precursors and an impressive anti-tumor effect against E7-expressing tumors compared with the mice vaccinated with wildtype E7 DNA or CRT DNA.

3.2.6 RGD

Highly conserved Arg-Gly-Asp (RGD) sequence has been found in some extracellular matrix (ECM) proteins, such as fibronectin, fibrinogen, vitronectin, and other adhesive proteins (Ruoslahti and Reed, 1999). RGD sequences in ECM proteins can be recognized by the cell surface receptors, integrins (αvβ3 and αvβ5) on APCs and other cells, which play a central role in cell adhesion biology. Therefore, peptides containing the RGD sequences have been used extensively in mimic proteins to study the interactions between cells and their environment (Massia and Stark, 2001). Penton proteins of adenovirus (Ad) type5 and type2 also contain RGD sequences, which were considered to be associated with coxsackievirus-Ad receptors (CAR; Roelvink et al., 1998) as well as integrins on host cells for Ad infections. Therefore, an Ad-mediated DNA vaccine can effectively deliver antigen to the APCs. Peptide-based vaccines fused with RGD or RGD-like sequences have shown that they can enhance immune responses to the specific antigens (Akira et al., 2003). However, the plasmid-based DNA vaccines fused with RGD sequences have not been reported.

3.2.7 Fragment C (FrC) of tetanus toxin

As a pathogen-derived gene, FrC was commonly used to construct chimeric DNA vaccine with specific antigens. FrC can be recognized by a 15 kDa putative receptor expressed on the membrane of many kinds of cells, including APCs. Compared with parental tetanus toxin, FrC still maintains highly immunogenic but non-toxic characteristics. FrC fused to the single chain Fv (scFv) encoding Id determinants (of B-cell malignancies) can turn the weak scFv DNA vaccine into a powerful scFv-FrC fusion vaccine that can activate high levels of anti-Id antibody and suppress tumors in murine models (Catherine et al., 1998). To increase $CD8^+$ CTL responses, some sequences, which can generate MHC-class I-binding peptides with antigen-derived peptides, were removed from the C–terminus of FrC (Rice et al., 2001). The modified FrC can significantly increase the antigen specific $CD8^+$ CTLs responses.

3.3 Targeting antigen for rapid intracellular degradation to enhance antigen processing and presentation

MHC class I and MHC class II antigen presentation pathways are different in processing and presenting antigens. Accordingly, the strategies for targeting antigen for rapid intracellular degradation to enhance antigen

presentation can be divided into two categories: targeting MHC class I pathway and targeting MHC class II pathway.

3.3.1 Targeting MHC class I pathway

There are several gates to be potentially used to direct antigens into the MHC class I presenting pathway, but most have focused on improving the delivery of antigen peptides (antigen epitopes) to the ER.

3.3.1.1 Using ubiquitin to target antigen protein to proteasome for degradation

Normally ubiquitin can bind misfolded proteins in polyubiquitin form to facilitate proteins into proteasome. Antigen proteins tagged with ubiquitin can be significantly taken up by proteasome for degradation. HIV-1 net protein fused to ubiquitin has been shown to enhance proteasomal degradation of antigen and stimulation of murine and human CTLs responses (Timothy and Robert, 1997).

3.3.1.2 Using antigen epitopes to directly target the ER

Small DNA fragments encoding antigen epitopes, called minigenes (Whitton and Oldstone, 1998), can be introduced into APCs by viral vectors, where they can be translated into very short translation products (often 9 to 11 amino acids), actually endogenous peptides, which can be transported by TAP into the ER for presentation via MHC class I pathway.

3.3.1.3 Generating misfolded antigen proteins for ubiquitinization

Misfolded proteins in cytoplasm are preferentially ubiquitinated and targeted to the proteasome. A destabilizing sequence is introduced in an antigen protein, leading to its apparent misfolding and ubiquitinization (Anton et al., 1999), and therefore enhancing antigen processing and presentations.

3.3.2 Targeting MHC class II pathway

Some studies show that secreted proteins are much more effectively incorporated into the MHC class II pathway, but different antigen proteins show different results in the Th cell responses induced (Haddad et al., 1997; Inchauspe et al., 1997; Torres et al., 1999).

Lysosome-associated membrane protein-1 (LAMP-1) can be used to link antigen proteins to target lysosome/endosome compartments, where MHC class II-restricted peptides are generated (Thomson et al., 1998). DNA fusion vaccines consisting of Hpv E and LAMP-1 show superior protection in a mouse tumor model (Ji et al., 1999; Smahel et al., 2001) and increase $CD4^+$ and $CD8^+$ T-cell responses (Ji et al., 1999).

Another lysosome membrane–related protein, called lysosomal integral membrane protein-II (LIMP-II) (Rodriguez et al., 2001), can be also used to construct fusion DNA vaccines to target lysosome/endososome compartments to induce $CD4^+$ T-cell responses. Minigenes encoding Th epitopes from lymphocyte choriomeningitis virus (LCMV) are linked to the 20-amino acid C-terminal tail of LIMP-II, and the $CD4^+$ T-cell response to the epitope of LCMV can be greatly enhanced, as assessed by INF-γ production.

3.4 Co-administrating antigens with cytokines or co-stimulatory molecules

A number of cytokines and co-stimulatory molecules have been tested to determine whether they can act in amplifying immune responses induced by DNA vaccines. The cytokines, such as GM-CSF (Tao and Levy, 1993), IL1β, IL2 (Dong et al., 1995), IL4 (Chen et al., 1994), IL6, IL7, IL12, and INF-γ (Xiang and Ertl, 1995) have proved their abilities to enhance immune responses induced by specific DNA vaccines.

Co-administrations of antigen and cytokines or co-stimulatory molecules include: (a) co-injecting antigen DNA (plasmids) and cytokines, and (b) co-injecting antigen DNA and cytokine DNA either in the same plasmid or separated plasmids.

Co-expression of IL-2 and hepatitis B virus (HBV) envelope proteins within the same plasmid vector results in a dramatic increase in their abilities to induce humoral and cellular immune responses to HBsAg (Chow et al., 1997). Mice injected with the plasmid encoding HBsAg together with plasmids encoding IL-2 or INF-γ can promote Th1 differentiation and suppress Th2 differentiation. Conversely, co-injection of a plasmid expressing IL-4 can promote Th2 differentiation and suppressed Th1 differentiation. Co-administration of the *IL-2* or *GM-CSF* gene mainly enhances Th1 cell differentiation, leaving Th2 cell development unaffected (Chow et al., 1998).

Co-stimulatory molecules CD80 (B7-1) and CD86 (B7-2) have been observed to provide potent immune signals expressed only in professional APCs, interacting with their receptors CD28 and CTLA-4 present on T cells. As noted previously, Naïve T cells can be activated only by the MHC-epitope complex on APC cells but not on other somatic cells because they are absent of co-stimulatory molecules (B7) or necessary intracellular adhesion molecules (ICAM-1 or ICAM-3). Intramuscular immunization of plasmid DNA antigen is expressed in muscular cells, which do not express the co-stimulatory molecules required for an efficient antigen presentation (Goebels et al., 1992; Hohlfeld et al., 1994). Co-stimulatory molecules as part of DNA vaccines would make transfected somatic cells possess abilities to present

antigen to T cells, therefore they would enhance cellular immune responses. Some studies show that co-immunization with DNA-encoding HIV-1 env antigen and CD86 plasmid rather than CD80 plasmids can result in significant increase of antigen-specific CTL activities (Iwasaki et al., 1997; Tsuji et al., 1997).

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