DESIGNING IMMUNE RESPONSES WITH GENETIC IMMUNIZATION AND IMMUNOSTIMULATORY DNA SEQUENCES

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Genetic immunization or DNA vaccination represents a rapidly developing technology with new perspectives for the prevention and therapy of infectious diseases and it offers new approaches for the treatment of autoimmunity, tumors and even allergy. DNA vaccines are comprised of plasmid DNA which encodes antigen molecules directly in the transfected cells of a target organism. In contrast to protein-induced immune responses, DNA vaccines stimulate both humoral and cell-mediated immune reactions.

In the present review we present a palette of unique features of genetic immunization like the effect of CpG motifs, the influence of mode and site of gene delivery and the modulation of immune responses by co-delivery of cytokines, colony stimulating factors, adhesion molecules and other stimulatory molecules. In addition, modulation of the immune response via translation, processing and presentation will be discussed, which in sum demonstrate the elegant possibilities of genetic immunization to induce tailor-made immune responses.

The first publications in 1990 (WOLFF et al. 1990) and 1992/93 (TANG et al. 1992, ULMER et al. 1993) demonstrating that injection of plasmid DNA (Fig.1) is able to induce potent humoral and cellular immune responses against the encoded genes opened a new era of vaccine research. *In vivo* transfection of epidermal tissue with the gene gun obviously resulted in transcription and translation of genes followed by the proper presentation of gene products to the immune system. Considering the similarity of these events with the endogenous pathways involved in anti-viral responses, it was originally postulated that this new method would primarily be suitable for vaccination against virus infections. However, within several years after the first publications, a large number of reports had demonstrated the enormously broad range of possible applications (AGADJANYAN et al. 1997; ANGUS et al. 1996; BARRY et al. 1995; BOURNE et al. 1996; GEISSLER et al. 1997; GONZALEZ ARMAS et al. 1996; LOWRIE et al. 1994; MANICKAN et al. 1995; MOR et al. 1995; XIANG et al. 1994; XU and LIEW 1995; XU et al. 1998). Genetic immunization proved to induce protective immunity against bacterial and parasitic infections and also seemed to offer new perspectives for the treatment of cancer (CONRY et al. 1995; WANG et al. 1995) and even allergy (HARTL et al. 1999; HSU et al. 1996; RAZ et al. 1996).

1. Principles of DNA vaccination

Although the precise mechanisms involved in the induction of an immune response following DNA immunization have not yet been determined, we have a fairly good understanding of how antigens are processed, presented and recognized by cells of the immune system (Fig.2).

In general, exogenous antigens are presented in the context of major histocompatibility complex (MHC) class II molecules. Internalization of proteins into endocytic compartments from the extracellular

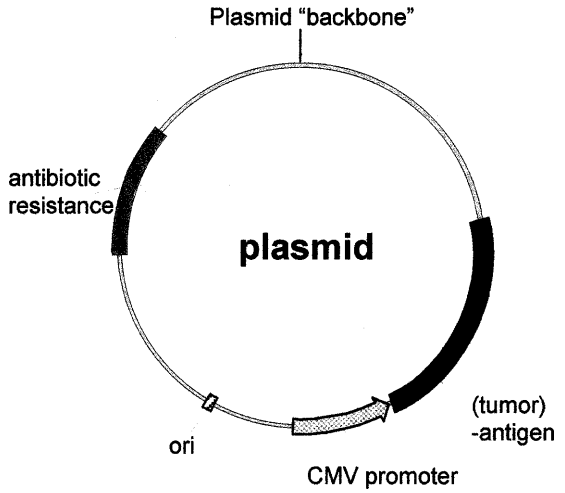

Fig 1
Minimum essential elements of a DNA vaccine. The gene encoding the antigen of interest is under the control of a strong promoter (in most cases a CMV-promoter). The plasmid is replicated in bacteria using a procariotic origin of replication (ori). Bacteria carrying the plasmid are selected based on antibiotic resistance, which is mediated by the gene carrying the resistance marker.

space is followed by denaturation (low pH and proteases) and degradation yielding peptide fragments. A small fraction of suitable, 12-20 amino acid long peptides will bind to MHC class II molecules. The peptide-class II complexes are transported to the cell surface and are recognized by antigen-specific $CD4^+$ T cells. This typical pathway for antigens from the extracellular milieu does not appear to be connected with the processing of endogenous proteins.

In contrast, $CD8^+$ lymphocytes recognize a complex of the MHC class I molecule with an embedded 8-12 amino acid long peptide antigen. The default source of peptides presented on MHC class I molecules are intracellular proteins generated by proteasomal processing. The resulting peptides are delivered into the endoplasmic reticulum (ER) via the transporter associated with antigen presentation (TAP), bound to MHC class I molecules and delivered to the cell surface.

A strict segregation of the processing pathways would exclude any presentation of antigens from the extracellular milieu on MHC class I molecules and explains the poor efficacy of protein-based vaccines for inducing cytotoxic T cell (CTL) responses. However, there is increasing evidence that this barrier between the processing-pathways is bypassed at least in certain antigen presenting cells (APC) such as immature DCs or macrophages (Heath and Carbone 1999; Kumaraguru et al. 2000; Lu et al. 2000; Ridge et al. 1998). These cells are able to take up exogenous antigens for class I-restricted presentation ("cross-priming"). The possibility that the same professional APC may simultaneously process and present both, MHC-I and MHC-II epitopes of a single protein immunogen in the appropriate MHC context may provide a mechanism to improve or facilitate cellular communication between antigen-specific $CD4^+$ and $CD8^+$ T cells. Moreover, at the effector phase, the activation of both T cell subsets may enhance the spectrum and repertoire of anti-pathogen immune mechanisms.

The events involved in the activation of T cells already represent a downstream event in the initiation of an adaptive immune response. T cell priming requires the presentation of antigen by professional APCs. Furthermore, presentation must occur in the context of certain costimulatory signals. The absence of appropriate costimuli results in anergy or activation induced cell death by apoptosis (Boise et al. 1995). A number of studies have demonstrated that DCs are the most effective cell type to sense and respond to various "danger" signals (Cella et al. 1997; Matzinger 1994; Steinman 1991). In general, such signals are the result of tissue and/or cell damage, but also molecules of bacterial origin such as lipopolysaccaride (LPS) and even bacterial DNA itself can act as a danger signals due to the presence of unmethylated CpG motifs as discussed later. The latter seem to reflect the interaction of certain pathogens with the immune system in an evolutionary context. After being activated, DCs are capable of capturing antigens in the periphery and subsequently migrate to T cell areas of lymphoid organs such as lymph node or spleen, where the interaction with and activation of T cells takes place.

One question arising from this widely accepted picture of basic immune mechanisms was how DNA vaccines are able to trigger the different arms of the immune system. With respect to data indicating long-term gene expression and translation of proteins in transfected myocytes it was initially postulated that these cells had been recruited as antigen presenting

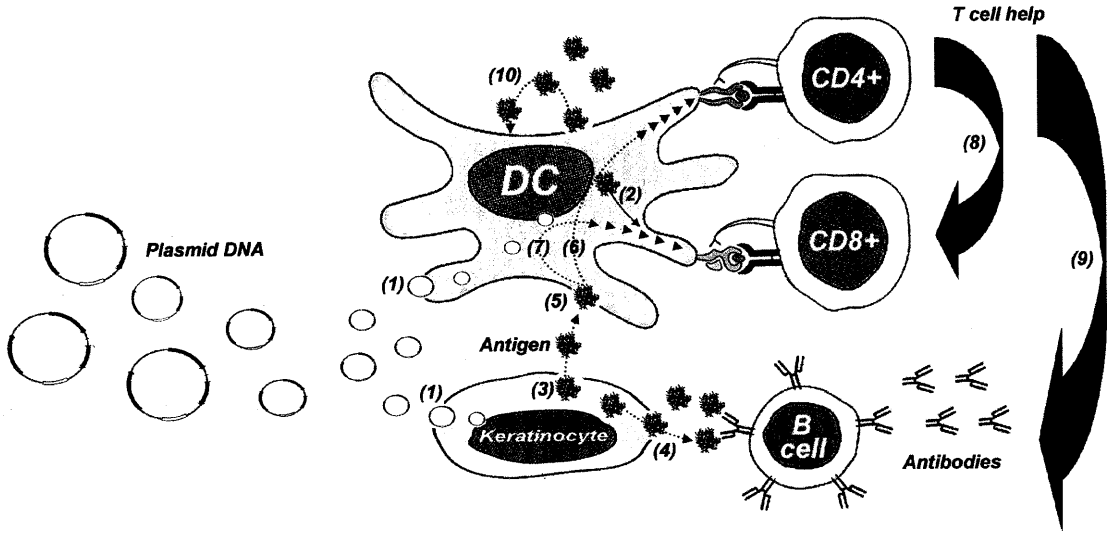

Fig 2

Mechanisms of antigen presentation after DNA immunization: Following tranfection (1) of dendritic cells (DC) or keratinocytes via i.d. injection (or myocytes after i.m. injection) plasmid DNA is translated into antigen by both cell types. In the DC the default pathway for endogenous protein is presentation on MHC class I molecules as indicated (2). Antigens produced by keratinocytes (3) are available for B cells (4) and DCs (5) thus entering the MHC class II pathway (6) via endosomes and the MHC class I pathway via cross-priming (7). T helper cells act as stimulators for both CD8+ cells (8) and B cells (9). In addition, DCs may also take advantage of their own antigen production (10).

B cell processing and presentation on MHC class II molecules as well as MHC class I presentation by keratinocytes have not been considered in this diagram. Both cell classes play no major role in the initiation of the response because neither nad've B cells nor keratinocytes display

cells (Wolff et al. 1992). Cytotoxic response were thought to be induced by the production, processing and presentation of plasmid-derived protein, whereas the release (or secretion) of soluble antigen provided material for T helper cell activation and also for B cells as a prerequisite for specific antibody production. Several publications addressing the induction of CTL responses in bone marrow chimeras following DNA immunization presented the following picture: Intramuscular as well as intradermal injection of plasmid DNA revealed that the initiation of cell-mediated immune responses required antigen presentation by professional APCs (Corr et al. 1996; Doe et al. 1996; Fu et al. 1997; Iwasaki et al. 1997; Ulmer et al. 1996). The acquisition of antigens by APCs is not fully understood, however, the mechanism of cross-priming represents an attractive explanation for how soluble antigens (e.g. translated in muscle cells or keratinocytes) gain access to the MHC class I restricted pathway. Very recent findings even suggest that one mechanism for the immunogenicity of DNA vaccines consists in the presentation of peptides to CD4 T cells by in vivo plasmid-transfected myocytes (Stan et al. 2001).

2. The role of CpG motifs in DNA vaccines

DNA vaccines do not only carry the genetic information for the antigen of interest but also deliver an adjuvant-effect due to the presence of immunostimulatory CpG motifs within the bacterial backbone. Increasing the number of CpG motifs in plasmids used for genetic immunization increases the immunogenicity and the Th1 bias of the DNA-induced immune response (Hartl et al. 1999; Hartl et al. 1999; Krieg et al. 1998). Besides immunostimulatory CpG sequences, neutralizing CpG motifs have been uncovered in adenoviral DNA, which suppress the adjuvant effect of immunostimulatory CpG DNA. Removal of such neutralizing CpG motifs from immunization vectors enhanced both, the IgG2a/IgG1 ratio as well as CTL activity (Krieg et al. 1998).

Therefore, the successful design of a powerful DNA vaccine requires intimate knowledge of the effects that DNA sequences within a DNA plasmid can have on the host's immune system.

2.1. Historical and general background of the action of CpG motifs. The antitumor-effects of microbial material were already recognized in the 19th century. In 1866, W. Busch reported tumor regression following severe infection (Busch 1866). W. Coley was the first to explore the possibilities of treating cancer patients with live or killed bacteria but the success of such treatment was limited due to severe systemic toxicity of these so-called "Coley´s toxins" (Coley 1894). During the following decades, lipopolysaccharide (LPS) of gram-negative bacteria was identified as the component responsible for both, tumor regression as well as toxicity. However, LPS does not exert these effects directly but through activating a variety of immune cells. Among them are most notably monocytes, macrophages and DCs that respond to the stimulation with a complex pattern of physiological changes, such as expression of certain surface molecules and secretion of soluble mediators including tumor necrosis factor alpha (TNF-α) (Beutler and Cerami 1986), (Carswell et al. 1975). TNF-α, although originally described as an anti-tumor factor, has now been well characterized as a pro-inflammatory cytokine. By acting on vascular endothelial cells it causes damage to blood capillaries and, thus, mediates both, some of the clinic symptoms seen in septic shock as well as destruction of tumor tissue by depriving it from nutrients and oxygen (Aderka 1991).

In addition to LPS, a variety of other microbial substances such as lipoteichoic acid and muramyl dipeptide from gram-positive bacteria or mannan from yeast are potent stimulators of the innate immune system. Interestingly, they act in much the same way as LPS. In the 1980s, the immunostimulatory properties of bacterial DNA were discovered (Tokunaga et al. 1984), (Yamamoto et al. 1988). Bacterial DNA differs from that of mammalians in the frequency of CpG dinucleotides, which are avoided ("CpG suppression") in mammals. Furthermore, in contrast to mammalian DNA the cytosine residues in bacterial CpG dinucleotides are generally not methylated (Cardon et al. 1994). Studies with synthetic oligodeoxynucleotides have revealed a hexameric consensus sequence for immunostimulatory CpG motifs consisting of a central CpG dinucleotide flanked by two purines at the 5´end and two pyrimidines at the 3´end (5´-PuPuCGPyPy-3´) (Krieg et al. 1995). However, it turned out that both, base-sequence as well as backbone chemistry influence the immune-stimulatory efficacy of individual CpG motifs (Pisetsky and Reich 1999). Individual CpG motifs also exhibit marked species specificity (Hartmann and Krieg 2000). This is of particular interest with respect to vaccine design since CpG motives that proved optimal in a mouse model may be ineffective in humans.

2.2. Recognition of CpG motifs. The cellular events of macrophage activation by CpG DNA strikingly parallel those achieved with other microbial products. These substances are recognized by the macrophage via so-called "pattern recognition receptors" (PRR) that were developed early in evolution and represent a mechanism of the innate immune system to signal "danger" based on structural surface characteristics that are shared by a variety of infectious agents but are absent on host cells. Examples of PRR are the mannose receptor (Fraser et al. 1998), CD14 (Pugin et al. 1994) and several members of the Toll-like receptor (TLR) family. Based on the genetic difference between LPS sensitive (C3H/HeN) and LPS resistant (C3H/HeJ) mouse strains, Tlr-4 was identified as the receptor for LPS (Poltorak et al. 1998). Tlr-2 is the receptor for bacterial peptidoglycan, lipoteichoic acid and mycobacterial cell wall components (Schwandner et al. 1999; Underhill et al. 1999) and, most recently, Tlr-9 has been found to be responsible for detecting CpG DNA. (Hemmi et al. 2000).

2.3. Signal transduction induced by CpG motifs. CpG mediated intracellular signaling requires endocytosis of CpG DNA/Tlr-4 complexes and acidification of endosomes. Signal transduction involves the myeloid differentiation factor MyD88, IRAK and TRAF6, activation of IkB kinase, mitogen-activated protein kinases, the stress kinases N-terminal c-Jun kinases JNK1/2 and p38 and, finally, results in transcriptional activation of multiple genes involving ATF-2, AP-1 and NF-kB ((Hacker et al. 1998; Hacker et al. 2000; Sparwasser et al. 1997; Yi and Krieg 1998). Many immunoregulatory genes contain NF-kB responsive elements in their promoter regions and

Table 1:

Cell type	Direct effect	Effector molecules	Functions
Macrophages and / or Dendritic cells	yes	IFN α/β	MHC-I up-regulation; AG processing; AG presentation
		IL-1	adhesion molecules on endothelial cells; IL-6 up-regulation
		TNF-α	adhesion molecules on endothelial cells; IL-6 up-regulation; (autocrine) activation of macrophages; (vascular damage)
		IL-6	B-cell differentiation Antibody secretion Class switch
		IL-12	IFN-γ (by NK and Th1 cells); Th1 cell differentiation; Suppression of Th2 cells
		MHC-I ICAM-1 CD40 CD86	AG presentation to $CD8^+$ Tc stabilizes cell-cell contact co-stimulatory signal, IL-12; co-stimulatory signal
Natural killer cells	no	IFN-γ	activation of APC; Th1 development; MHC-I up-regulation
		Cytolytic activity	destruction of tumor cells
Th1 cells	no	IFN-γ	activation of APC; Th1 development; MHC-I up-regulation
B cells	yes	MHC-II CD40 CD86 IL-6	AG presentation to $CD4^+$ Th co-stimulatory signal; co-stimulatory signal; B-cell differentiation Antibody secretion Class switch

ype 1 gag gene [In Process Citation]. J Virol **74,** 2628-2635, 2000

inhibition of NF-kB also abrogates CpG mediated immune cell activation (YI and KRIEG 1998).

2.4. Cellular responses to CpG DNA . So far, a variety of immune cell species have been identified that – directly or indirectly – respond to CpG DNA. These include professional APCs (monocytes, macrophages and DCs) as well as NK, B and T cells (see table 1). The direct action APCs is probably most important for the immunostimulatory effect of CpG DNA. These cells, when encountering CpG DNA, undergo dramatic physiological changes affecting both, the immunoregulatory functions of the APC themselves as well as – via secretion of cytokines – the activation status of other immune cells.

2.4.1 Macrophages. Macrophages are induced by CpG DNA to secrete a panel of cytokines, in particular type-1 interferons (IFNα/β), IL-1β, TNF-α ,

IL-6 and IL-12 (Chace et al. 1997; Krieg 1999; Sparwasser et al. 1997; Stacey et al. 1996; Sun et al. 1998) and – when primed by IFN-γ – to express inducible nitric oxide synthase (iNOS) (Sweet et al. 1998). Interferons up-regulate the surface expression of MHC-I molecules on most cell types, resulting in increased sensitivity to $CD8^+$ CTL-mediated cell lysis as well as enhanced antigen presentation by APC, which is required for T-cell activation (Boehm et al. 1997). Interferons also enhance the cytolytic activity of NK cells and, in humans, facilitate Th1 cell development by promoting the expression of IL-12 receptor on these cells (de Waal Malefyt 1997). IL-1 is a mediator of local inflammation that acts on endothelial cells to elevate the expression of leukocyte adhesion molecules and, hence, helps to recruit immune cells to the site of infection. TNF-α, when produced at high concentrations, e.g. in the case of systemic infection, is a central mediator in septic shock. At lower concentrations TNF-α, similarly to IL-1, acts on endothelial cells to recruit neutrophils and monocytes to the site of infection.

Furthermore, TNF-α activates monocytes and macrophages in an autocrine manner and, both, TNF-α and IL-1 are potent inducers of IL-6. IL-6, in turn, promotes terminal B-cell differentiation, secretion of antibodies from plasma cells and differentiation of myeloid stem cells. IL-12 is a key cytokine for the development of cell-mediated immunity and the development of Th1-type immune reactions in general.

In addition to these effects, CpG DNA increases the expression of several surface molecules on macrophages, such as MHC-I, ICAM-1, CD40 or CD80/86. These molecules are involved in antigen presentation and activation of lymphocytes. The alterations triggered by CpG oligonucleotides have been shown to persist for prolonged time in mice after injection of CpG oligonucleotides and enhance an immune response with a Th1 bias to subsequently injected antigens ((Kobayashi et al. 1999, 2000).

Activated macrophages exhibit direct tumor cytotoxicity that was initially attributed primarily to the production of TNF-α and reactive nitric oxide intermediates. However, many tumor cell lines that are lysed by activated macrophages in a cell-cell contact-dependent manner are not sensitive to recombinant TNF-α or NO (Mateo et al. 1996). Furthermore, a soluble factor that is distinct from TNF-α and NO has been described and partially purified (Harwix et al. 1992; Schwamberger et al. 1992) but the exact mechanism of this kind of macrophage tumor cytotoxicity is not yet fully understood.

2.4.2. Dendritic cells. Dendritic cells are unique in their ability to activate nad'ye T lymphocytes and, therefore, play a key role as APC for the induction of a primary immune response. Similar to macrophages, bone marrow-derived DCs respond directly to CpG DNA by secretion of TNF-α, IL-6 and IL-12. They also express increased levels of CD40, CD86 and MHC-II on their surface (Sparwasser et al. 1998).

2.4.3. Natural killer (NK) cells. Activated NK cells play an important role in the early phase of an immune reaction. These cells provide the majority of IFN-γ before this cytokine is supplied in relevant concentrations by activated T cells. However, purified NK cells appeared not to be activated directly by CpG DNA but are potently stimulated by type-1 interferons, IL-12 and TNF-α, that are secreted by macrophages and DCs in response to CpG DNA (Ballas et al. 1996). IFN-γ is the central cytokine for macrophage activation. Macrophages primed by this cytokine produce nitric oxide and reactive oxygen intermediates and, hence, show enhanced microbicidal activity towards phagocytosed microorganisms. IFN-γ also promotes the expression of MHC-I and -II as well as co-stimulatory molecules, such as CD40 or CD86. In addition, IFN-γ enhances the expression of molecules involved in antigen processing including the "transporter of antigenic peptides" (TAP) or the proteasome subunits LMP-2 and LMP-7. Finally, IFN-γ plays a key role during the differentiation of T lymphocyte and promotes the establishment of a Th1 type environment by stimulating IL-12 production.

Besides providing IFN-γ, NK cells also exhibit increased cytolytic activity when activated as a consequence of the presence of CpG DNA *in vivo*. In fact, NK cell-mediated antitumor activity was the first biological effect of bacterial DNA that had been described (Yamamoto et al. 1988).

In summary, the activation of immune cells by CpG DNA initiates a complex network of cell-cell interactions and cytokine production cascades that result in an overall enhancement of immune functions in an antigen-independent manner. Thus, CpG

DNA has been shown in a multitude of independent studies to be a potent adjuvant when applied prior to or together with protein or DNA vaccines.

2.5. Effects of CpG DNA on the Th1/Th2 cytokine balance. Helper T cells can be divided into at least two functionally different subsets, Th1 and Th2 lymphocytes (Mosmann and Coffman 1989). These $CD4^+$ T cell populations are defined by the distinct sets of cytokines they produce. IFN-γ is the signature cytokine of Th1 cells whereas IL-4, IL-5 and IL-13 are products characteristic for Th2 cells. These cytokines also – directly or indirectly – control and stabilize the Th1/2 balance of an immune response through the following mechanisms: (1) IFN-γ promotes Th1 cells by enhancing IL-12 production by macrophages and expression of IL-12 receptors on $CD4^+$ T cells. IL-12, in turn, mediates the differentiation of Th1 cells by activation of STAT4. Simultaneously, IFN-γ suppresses the development of Th2 cells. (2) Conversely, IL-4 is required for Th2 cell differentiation via activation of STAT6 while IL-4 and IL-13 partially inhibit IL-12 production. This consequently suppresses the development of Th1 cells.

Both helper T cell subsets are associated with distinct effector functions: A Th2 profile leads to immunoglobulin class switching to IgG1 and IgE in mice and IgG4 and IgE in humans and, therefore, mediates IgE and eosinophil/mast cell-dependent immunity. This type of response is important for eliminating parasite infections but is also implicated in allergic disease.

A Th1 profile enhances Th1-antibody production by B cells (IgG2a in mouse); it activates phagocytic cells against microbial infections as well as NK cells and is required for the development of CTL-mediated immunity against viral infections or malignant cells. Hence, with respect to cancer vaccine design, the most attractive aspect of CpG- based adjuvants is the development of a Th1-biased immunity, no matter, whether a given tumor is more sensitive to CTLs or to ADCC involving NK cells.

In addition to the effects describe above, most bacterial adjuvants induce toxic cytokines such as TNF-α and IL-1. These cytokines, when expressed systemically in large amounts may lead to severe symptoms such as septic shock. Hence, for vaccine design, the most challenging aspect is to find adjuvants that promote an optimal Th1 response while minimizing the production of toxic cytokines and the associated side effects.

Although many processes that occur after activation with various microbial products are strikingly similar, CpG DNA may be superior over other adjuvants due to its ability to trigger the release of large amounts of IL-12. When comparing the effect of LPS and CpG oligonucleotides on the macrophage-like cell line RAW264 investigators found that the latter yielded significantly more IL-12 than LPS (Cowdery et al. 1999). IL-12 has already been mentioned previously as the key factor for Th1 development. Thus, comparing an adjuvant´s potential to induce IL-12 versus TNF-α (and IL-1) might provide a means for evaluating its applicability.

3. The potential of DNA immunization for vaccine research

Without a doubt, one of the outstanding features of DNA immunization is the opportunity to co-deliver information together with to the antigen-coding sequence on the plasmid DNA. In recent years several strategies have successfully been used to modulate the immune response after DNA immunization, such as: (i) different modes and sites of gene delivery, (ii) co-delivery of genes or adjuvant molecules with regulatory and/or stimulatory properties and (iii) modification of the vector sequences by inserting or deleting cytosolic or endosomal transport signals.

These possibilities, either as single approaches or in different combinations opened an enormous playground for experimentation and elicited interesting aspects for both vaccine development as well as basic immunological processes.

3.1. The influence of mode and site of gene delivery. Two early studies demonstrating efficient humoral and cellular responses by injection of plasmid DNA used a biolistic technology (gene gun), which was originally developed for improving transfection in plants (Tang et al. 1992, Ulmer et al. 1993). In subsequent publications also intramuscular (Sedegah et al. 1994; Wang et al. 1993; Watanabe et al. 1993; Yankauckas et al. 1993) and intradermal DNA in-

jection (Raz et al. 1994; Sato et al. 1996; Shiver et al. 1995) were used to stimulated antigen-specific humoral and cellular immune responses.

Interestingly, gene gun and intradermal or intramuscular application differed strikingly in terms of the type of immune response induced by the different gene-delivery methods. A predominant Th2 type response was observed after gene gun immunization whereas intramuscular (i.m.) or intradermal (i.d.) injection elicited a more Th1 biased response (Feltquate et al. 1997).

The different efficacies of gun versus needle injection in different pathogen models may reflect the requirement of a Th1 or Th2 biased response type for efficient protection against different pathogens. For both gene gun immunization as well as for needle injection specific and superior protection data have been demonstrated (Bennett et al. 1999; Leitner et al. 1997, Lodmell et al. 1999, 2000; Tanghe et al. 2000; Weiss et al. 2000).

The reason why different modes targeting the same site (such as the delivery of plasmid DNA into the skin by gene gun or by needle) results in such strikingly different types of responses is still not fully understood. In both cases DCs such as Langerhans cells play a crucial role in the primary response triggered by the DNA vaccines. Although DNA immunization results in transfection of a small proportion of DCs only, it leads to a widespread activation of DCs in the draining lymph node – at least after gene gun immunization -, thus providing necessary signals for effective T cell activation (Porgador et al. 1998). One explanation for the difference in the delivery methods is that they provide qualitatively and quantitatively different "danger" signals at the site of injection. With respect to gene gun inoculation a sufficient activation signal for DCs apparently comes from the bombardment of the skin with gold particles itself independent of plasmid DNA (Porgador et al. 1998). Upon i.d. injection of saline DNA, however, the dominant activating signal presumably comes from immunostimulatory sequences (ISS) within the plasmid DNA itself (discussed in detail later). These ISS containing an unmethylated CpG dinucleotide core in a defined base context ("CpG motifs") stimulate Th1 cytokines such as IL-12 and IFNs from DCs, macrophages and NK cells. In addition, CpG motifs enhance the expression of various co-stimulatory ligands such as CD80, CD40, and ICAM-1 on APCs. Approximately 100-times more plasmid DNA is used for i.d. needle injection compared to gene gun delivery, which may be responsible for the predominant Th1 type response (Barry and Johnston 1997).

In some pathogen models gene gun immunization has proven to be superior to either i.d. or i.m. injection of plasmid DNA. Interestingly, this was not restricted to humoral responses (Bennett et al. 1999; Leitner et al. 1997; Lodmell et al. 2000; Weiss et al. 2000), which could have been explained by the more efficient transfection of cells with a biolistic device, but also affected the induction of CTL responses and protective immune responses. Increased CTL responses and more efficient seroconversion were reported after gene gun injection (compared to i.m. needle injection) of plasmid DNA encoding the entire ovalbumin (OVA) or a specific OVA CTL epitope (Yoshida et al. 2000). A comparison of CTL responses after gene gun or i.d. injection of a ras p21 minigene DNA vaccine encoding a nested CTL epitope within a T-helper epitope revealed a similar superiority of the gene gun delivery-method (Bristol et al. 2000). This was true for different combinations of constructs expressing various ras mini-gene sequences (CTL epitope and/or helper epitope) in different forms (with or without an ER-targeting leader sequence) indicating that the enhanced efficiency of the gene gun was independent of the physical form and presentation of the encoded mini-gene (manuscript in preparation).

With respect to the predominant Th2 type responses induced by gene gun, the question arises how and why gene gun delivery is a superior method for the induction of CTL responses – especially for the treatment of tumors – compared to the Th1-biased i.d. or i.m. injection mode. To a certain extent this apparently paradox observation may simply be a consequence of the strict definition of gene gun raised responses as strong Th2 and i.d. or i.m. induced reactions as clear Th1 type. Despite a distinct serological Th2 type profile with high levels of IgG1 and IgE but low levels of IgG2a antibodies, in many experiments gene gun immunization induced high levels of IFN-γ in supernatants of stimulated spleen cells (Fensterle et al. 1999; Vinner et al. 1999; Yoshida et al. 2000).

3.2. Modulation of immune responses by co-delivery of immune stimuli. A unique feature of DNA vaccines is that they make it very easy to deliver additional information to the immune system. This is generally done by: (i) expression of additional information by a separate cassette on one plasmid construct, (ii) co-injection of a separate plasmid which contains the additional information and (iii) adjuvant substances.

3.2.1. Colony stimulating factors as genetic adjuvants. A large number of publications have addressed the co-delivery of immunostimulatory factors in various combinations in different pathogens or tumor models. The granulocyte-macrophage colony-stimulating factor (GM-CSF) is one of the most intensively studied molecules regarding its costimulatory effects for DNA immunization. GM-CSF is important for growth, differentiation and maturation of the most potent professional APCs, the DCs (Banchereau et al. 2000; Fong and Engleman 2000).

Intramuscular injection of plasmid DNA encoding MAGE-1 and MAGE-3 resulted in improved anti-tumor immunity when the plasmid co-expressed GM-CSF (and also CD80) in a B16 melanoma mouse model (Bueler and Mulligan 1996). A similar approach was used by another group that immunized rats with a bicistronic plasmid which independently expressed the antigens E1 or E2 of the hepatitis C virus (HCV) and the GM-CSF gene (Lee et al. 1998). The antibody response as well as lymphoproliferation was significantly increased by co-expression of the GM-CSF gene. Intramuscular DNA immunization with a herpes simplex virus gene encoding the HSV-2 gD protein together with a plasmid coding for GM-CSF enhanced the production of IgG, IgE and IgA antibodies, lymphoproliferation and cytokine secretion when compared to immunization with the HSV-2 gD gene alone (Sin et al. 1998). The drastic increase of the IgG1 antibody subclass indicated a Th2 type bias of this response. Furthermore, co-injection improved protection from lethal challenge with HSV-2. Similar serological results were found in a mouse model of aerosol tuberculosis with plasmid DNA encoding the *M. tuberculosis*-secreted proteins, MPT64 and Ag85B (Kamath et al. 1999). Intramuscular co-injection of a plasmid containing the GM-CSF gene elicited enhanced humoral and cellular immune responses but did not improve the protective efficacy after challenge.

To find the most effective hematopoietic growth factor for genetic vaccination the authors of one study compared the effect of costimulation with GM-CSF, M-CSF and G-CSF (Kim et al. 2000). As with HCV, HSV and tuberculosis, the HIV-1 DNA delivered together with GM-CSF DNA showed increased antibody and proliferative responses. Testing the possible combinations of all three growth factors revealed that M-CSF in any combination provided the best enhancement of $CD8^+$-CTL responses.

3.2.2. Cytokine genes as immunomodulators for genetic immunization. Among the T-cell growth factors, the cytokines IL-2 and IL-7, and the Th1-biasing cytokines IL-12 and IFN-γ were intensively studied in co-expression experiments. In a hepatitis B virus (HBV) mouse model both a fusion construct encoding the middle envelope and IL-2 protein as well as a bicistronic vector separately encoding the middle envelope protein and IL-2 were used (Chow et al. 1997). Coexpressing IL-2 resulted in enhanced humoral and cellular immune responses and a slight Th1 type modulation. In addition, vaccine efficacy was increased by at least 100-fold by the coexpression of IL-2 as measured by the dose of vaccine needed.

The immune response in mice following gene gun inoculation of constructs encoding HIV-1 gp120 or nucleoprotein from influenza shows a Th2-bias (Prayaga et al. 1997). However, the co-delivery of IL-2, IL-7, or IL-12 plasmid DNA blocked this effect by markedly enhancing gp120- specific IFN-γ production, and suppressing IL-4 and IgG1 responses.

In another study, the authors attempted to modulate the immune response against HIV-1 by co-delivery of genes for IL-12 and GM-CSF along with DNA vaccines (Kim et al. 1997). Co-expression of IL-12 reduced the humoral response while GM-CSF increased antibody production. Both cytokines mediated significant antigen-specific T cell stimulation but only co-delivery of IL-12 resulted in an increase in specific CTL responses.

Significant enhancement of Th1 cells (including high titers of IgG2a and a decreased production of IgG1 antibodies) was obtained after co-expression of IL-12 and IFN-γ together with HBV plasmid DNA (Chow et al. 1998). In contrast, co-delivery of IL-4 induced a clear Th2 profile with increased produc-

tion of IgG1 antibodies and suppression of IgG2a antibodies. Co-injection of the IL-2 or the GM-CSF gene enhanced the development of Th1 cells, while the development of Th2 cells was not affected, and the production of IgG1 and IgG2a antibodies were both increased. IL-12 and IFN-γ had the most pronounced effect on CTL responses, whereas IL-4 suppressed this activity. Upon challenge with HBsAg-expressing syngeneic tumors, significant reduction of tumor growth was observed in mice that were co-administered the IL-12 gene but not the IL-4 gene. IL-12 co-expression was also demonstrated to be necessary for effective anti-tumor immunity induced by gene gun immunization with p53 mutant protein tested in the Meth A sarcoma model (Tuting et al. 1999).

INF-α represents another potential candidate of a Th1-biasing cytokine, which is generally associated with viral infections. As with IL-12, IL-18 and IFN-γ, it was shown that co-expression of INF-α shifted the response towards a Th1 phenotype (Tuting et al. 1999).

3.2.3. Co-Injection of DNA vaccines and recombinant cytokines. In addition to the co-delivery as genes, cytokines can be also co-injected in the form of recombinant molecules. This was demonstrated in an experimental murine tumor expressing the model tumor-associated antigen ß-galactosidase (ß-gal) and plasmid DNA expressing ß-gal delivered by gene gun. Significant reduction in the number of established lung metastases was observed when human rIL-2, mouse rIL-6, human rIL-7, or mouse rIL-12 were administered after DNA inoculation; mouse rIL-12 as an adjuvant had the most profound effect (Irvine et al. 1996).

These adjuvant strategies have been extended to primate models with similar results applying co-expression strategies with IL-12, IL-18 and IFN-γ together with HIV vaccines (Kim et al. 1999).

3.2.4. Co-delivery of co-stimulatory and adhesion molecules. Very soon after the establishment of the first DNA immunization protocols, co-stimulatory molecules such as CD80 and CD86, which provide the crucial second signal required for optimal T cell activation, became the target of intensive investigation. The complex immune modulation of CD80, CD86, CD28 and CTLA4 in DNA vaccines were investigated by several groups. For the model antigen ß-galactosidase (Horspool et al. 1998), for HIV-1 antigens (Kim et al. 1997) and for the nucleoprotein of influenza (Iwasaki et al. 1997) the authors demonstrated that CTL induction but not humoral responses were primarily affected by co-expression of CD80 and CD86 with a more pronounced effect of CD86. However, immunization with plasmid DNA encoding a minimal CTL epitope of ovalbumin (OVA) revealed different results (Corr et al. 1997). Co-expression of CD80 was more potent at stimulating CTL response than CD86. Furthermore, in challenge experiments using OVA-transfected tumors co-delivery of CD80, but not of CD86, prolonged survival. In a murine malaria model we noted that antibody responses as well as protection from challenge were strongly affected by co-immunization with a CSP-DNA vaccine and the B7.1 gene. Depending on the interval between the co-immunizations the immune response was either strongly enhanced or suppressed (W.W.Leitner, J.A.Lyon, unpublished observation).

A recent study indicated that adhesion molecules may also serve as potent candidates for immunomodulation by co-injection of the respective genes together with plasmid DNA (Kim et al. 1999). Here, the authors examined the role of intracellular adhesion molecule-1 (ICAM-1), lymphocyte function associated-3 (LFA-3), and vascular cell adhesion molecule-1 (VCAM-1) along with DNA immunogens. Antigen-specific lymphoproliferation and cytotoxic responses were enhanced by co-expression of ICAM-1 and LFA-3. Furthermore, co-injection of both these constructs also increased the level of IFN-γ, macrophage inflammatory protein-1α and ß. The pathways involved in ICAM-1 and LFA-3 costimulatory processes do not seem to be associated with CD86/CD28 pathways and may act synergistically *in vivo*.

Another example for the innovative use of costimulatory or regulatory factors of the immune systems in DNA vaccines was demonstrated by using components of the complement system to enhance immunogenicity (Ross et al. 2000). Upon complement activation, a fragment of the third complement protein (C3d) is released and binds to CD21, which exerts B cell stimulatory properties (Fearon and Carter 1995). A DNA vaccine encoding different forms of hemagglutinin (HA) from influenza virus was

fused to three tandem copies of the murine homologue of C3d. According to the hypothesis the HA moiety of the fusion would bind to anti-HA immunoglobulin receptors on B cells and signal through the B cell receptor, while the C3d moiety of the fusion would bind to CD21 on the B-cell surface and signal through CD19. Indeed, this approach enhanced the avidity maturation of antibodies against HA and accelerated the appearance of HA-inhibition activity. Furthermore, the accelerated antibody responses correlated with a more rapid appearance of protective immunity. Complete protection from challenge could be achieved with significantly lower doses of the vaccine.

3.2.5. Co-delivery of epitopes recruiting T helper cells. T helper cells not only play a key role in the humoral branch of the immune system but also cytotoxic responses depend on – or are improved by – T cell help. This has been demonstrated for antiviral DNA vaccines encoding entire genes (Wild et al. 1999) and single epitopes (Maecker et al. 1998). In the latter study, an eight amino acid-long epitope from the influenza A virus hemagglutinin was inserted into the hepatitis B surface antigen sequence, which – in this context – served as a T helper antigen for the influenza minigene thus indicating that the helper epitope can be of heterologous origin.

In a recent study (Bristol et al. 2000) we investigated a DNA mini-gene coding for a single peptide sequence derived from the ras oncogenes (Abrams et al. 1996) that contained both $CD8^+$ and $CD4^+$ T cell epitopes in a nested configuration (Abrams et al. 1996). This peptide represents ras sequence 4-16, and contains the substitution of Gly to Val at position 12. Gene gun immunization with this construct induced both antigen-specific proliferative and cytotoxic responses. These results suggest that a single protein immunogen containing nested mutant ras-specific $CD4^+$ and $CD8^+$ T cell epitopes can be processed *in vivo* to induce both subset-specific T lymphocyte responses and leads to the generation of a quantitatively enhanced $CD8^+$ CTL response, likely due to the intimate coexistence or cooperation of $CD4^+$ help (Bristol et al. 2000).

In summary, the principle picture rising from this scenario points to the enormous combinatorial possibilities of DNA vaccines for modulating the immune response at different levels. At present, however, it must be pointed out that general conclusions need to be drawn carefully, because differences in the way of presentation, the form of the antigen and the immunization protocol may yield different, sometimes paradox results.

3.3. Modulation of the immune response via translation, processing and presentation. Genetic immunization has successfully produced antibody responses against secreted, membrane-bound and cytoplasmically sequestered antigens. Several proteins such as rabiesG (Xiang et al. 1995) and hepatitis B surface protein (Chow et al. 1997) were equally immunogenic in their membrane-bound and secreted form. For a number of antigens from different sources (including allergens) export from the cytosol does not even seem to be necessary. This raises the question how effectively native antigen is made accessible to APCs and especially to B cells for the induction of a strong antibody response (Chang et al. 1998; Hartl et al. 1999; Hsu et al. 1996; Raz et al. 1996; Velaz-Faircloth et al. 1999). There is also experimental evidence that a given antigen can be highly immunogenic in one form and poorly or non-immunogenic in another. Examples for such antigens are the circumsporozoite protein (CSP) of plasmodia (Leitner et al. 1997; Scheiblhofer et al. 2001) and the outer surface protein C (OspC) of the lyme disease causing spirochete (Weiss et al. 1999). Both antigens need to be secreted using an ER-targeting signal sequence in order to become immunogenic or protective. Genetic immunization with a hepatitis C virus nucleocapsid antigen and with ovalbumin follows similar rules in that a secretion signal drastically increases immunogenicity (Boyle et al. 1997; Inchauspe et al. 1997). The complexity of these problems can be depicted by comparing the results obtained from experiments with the rabies G protein and the malaria CSP: Despite similar humoral and cellular responses against secreted and non-secreted antigens, the secreted rabies G protein provided inferior protection. In contrast, the secreted form of CSP was superior to the non-secreted form in terms of the humoral response as well as protection (Scheiblhofer et al. 2001). Furthermore, increasing secretion influenced the type of response by mediating a Th2 bias. The effect of the physical form of the antigen not only plays a role in the general immuno-

genicity but also in the Th1/Th2 bias which was also indicated by the results of a study using the tick-borne encephalitis virus envelope protein E (Aberle et al. 1999). From currently available data we can conclude that the form of the antigen is crucial for the induction and efficiency of the immune responses. In certain cases, however, the nature of the protein itself may play an additional role.

Yet another strategy for deliberately modulating intracellular events was the co-delivery of ubiquitin in the form of a fusion protein. In contrast to the effects induced by the leader sequences, tagging proteins with this or similar sequences accelerates and increases the proteasomal degradation. While this approach will improve the induction of CTL responses it will also drastically decrease the antibody response (Fu et al. 1998; Rodriguez et al. 1997; Vidalin et al. 1999; Wu and Kipps 1997). This approach holds a significant promise for the design of DNA vaccines against tumors. Most successful anti-tumor responses are based on cytotoxic T cells and not on antibodies. Using the methods described above, the immune response induced by a genetic vaccine can be tailored to achieve optimal efficacy in various disease systems for which qualitatively distinct responses are required.

An important aspect of genetic vaccines is the codon usage in the genes expressed by the vaccine. Manipulating the codon usage allows optimization of antigen expression and/or immunogenicity of various pathogen genes. For example, it enables vaccine designers to replace certain codons with immunostimulatory CpG motifs (discussed below) without changing the amino acid sequence of the antigen. Several reports have demonstrated improved immunogenicity based on such modifications (Andre et al. 1998; Nagata et al. 1999; Uchijima et al. 1998; Vinner et al. 1999; zur Megede et al. 2000). When the gene for the antigen of interest comes from a prokaryotic organism the codon usage can be optimized for expression of this antigen in the eukaryotic host.

In conclusion, these data indicate exciting possibilities for modulating the conditions of antigen presentation at a very early and obviously crucial state of the immune response. Already now, various combinations of the attempts described above enable us to program a specific outcome for the immunization with an epitope, polytope or entire gene encoded on a DNA vaccine. More detailed knowledge about molecules regulating the transport mechanisms between the various compartments within cells will make even more sophisticated approaches for DNA vaccine development possible in the future.

3.4. Mini-gene and polytope-gene vaccines. In addition to using the entire coding region of an antigen on a plasmid DNA construct, genetic immunization has proven to be an excellent method for inducing immune responses against even single T or B cell epitopes of antigens. This so-called mini-gene or minimal-epitope approach takes advantage of the fact that plasmid DNA encoded antigenic peptides can be loaded onto MHC class I molecules via the endogenous antigen-processing pathway. The potential of this concept for the treatment of viral infections and tumors was first demonstrated with a minigene coding for a single epitope of HIV gp120 or mutant p53, respectively (Ciernik et al. 1996). Protective CTL responses were induced in both cases by using epidermal delivery by gene gun. Genetic immunization with both the entire coding region as well as with an immunodominant peptide region of p53 results in the induction of potent cytotoxic activity (Petersen et al. 1999).

Protective anti-tumor responses were also obtained in another surrogate tumor model (P815 cells transfected with a plasmid coding for the influenza nucleoprotein NP 147-155) by intramuscular injection of plasmid DNA encoding the entire NP or the minimal epitope 147-155 (Iwasaki and Barber 1998). Cell depletion experiments confirmed that $CD8^+$ cells were responsible for protection.

Fusing the epitope-coding sequence with an ER-targeting leader sequence can further enhance the immune response to a minimal epitope. For the minigene approach this effect was demonstrated with the p53 epitope after gene gun application (Ciernik et al. 1996), the nucleoprotein epitope of measles virus and the M2 protein epitope of respiratory syncytial virus after intradermal injections (Hsu et al. 1998). The basic mechanism here is to circumvent the proteasome – and thus degradation of the antigen – by direct translation of a signal sequence plus peptide into the ER. The signal sequence is subsequently cleaved by a signal peptidase thus releasing the anti-

genic peptide into the ER. Using this approach, the antigenic peptide – provided that it is of proper length and has the appropriate motif for MHC class I loading – is directly delivered to the compartment where it is associated with MHC molecules. Bypassing the proteasomal complex may be particularly important for typical CTL epitopes (8-10 amino acids long) since any further degradation of these peptides would result in the loss of binding to MHC class I. However, for DNA vaccines encoding entire genes (as discussed above), the same approach i.e., fusion with an ER-targeting leader sequence, mediates improved presentation on MHC class II molecules and T cell help through increasing the amount of secreted native antigen available for APCs.

The mini-gene approach has been expanded by some investigators who linked several epitopes together to create multi-epitopes or polytopes for vaccination. The feasibility of this idea was demonstrated with a mini-gene cassette encoding a mixture of CTL, T helper and B cell epitopes from herpes simplex virus (HSV) (Yu et al. 1998). This polytope plasmid DNA stimulated epitope specific cytotoxic reactions comparable to the response induced with live HSV. T cell proliferation, antibody production and protection were significant however less effective than those induced with the live virus. Analogue approaches showed long-lasting CTL responses generated by multiple contiguous minimal murine CTL epitopes (Thomson et al. 1998) and a polyepitope minimal vector encoding two murine epitopes from human immunodeficiency virus and *Plasmodium falciparum* (Hanke et al. 1998).

4. The influence of DNA immunization and CpG motifs on the retionoic acid receptor status in spleen and on the activity of type I iodothyronine 5'-deiodinase in liver

4.1. Retinoic acid receptor . Retinoids and retinoic acid receptors (RAR) represent well known examples for the interaction of two important networks, the immune system and the endocrine system. In general, retinoids, in particular *all-trans* retinoic acid (RA), are essential for a normal development and homeostasis of vertebrates. All-trans retinoic acid action on the regulation of specific gene expression is mediated by three distinct nuclear retinoic acid receptor subtypes, RARα, β, γ belonging to the steroid/thyroid nuclear receptor family. The RAR receptors act as transcriptional activators by binding to retinoic acid responsive elements (RAREs) near the promoters of target genes. Many of RAREs that consist of a direct repeat of two motifs (AGGTCA) separated by a 5-base pair spacer have been identified in a number of genes that respond to RA in vivo (Gudas 1994).

RA and RARs have been demonstrated to be involved in various aspects of the immune system. Antigen presenting cells (APC) like Langerhans cells up-regulate their ability to present allo-antigens by RA (Meunier et al. 1994) and monocytes are induced to produce increased TGF-β and TNF-α levels (Szabo et al. 1994). Experiments using RARγ – transgenic animals indicated a role of RAR inducing cytotoxic T-lymphocytes in vivo and of skin graft rejection (Pohl et al. 1993). Expression studies in antigen-stimulated T lymphocyte cultures demonstrated an upregulation of RARα mRNA by RA thus pointing to a function of RARα as a ligand-inducible transcriptional enhancer factor (Friedman et al. 1993). Retinoids also act on B cells, RAR-ligands are able to inhibit cell activation and can prevent apoptosis in B lymphocytes (Lomo et al. 1998). Furthermore, retinoic acid-induced gene transcription seems to have strong impacts also on the expression and regulation of adhesion molecules like ICAM-1 (Aoudjit et al. 1994, 1995; Babina et al. 2001; Bassi et al. 1995) and cytokines like interleukin-2 (Ballow et al. 1997, de Grazia et al. 1994) interleukin-4 (Racke et al. 1995) and IFN-γ (Cippitelli et al. 1996). Recently, it has also been reported that RA is capable to inhibit the growth of breast cancer cells by upregulating ICAM-1 expression (Baj et al. 1999). Concerning the role of RA and RAR in the immune system, an increasing number of publications are focused on the regulation of apoptosis, i.e. RA can prevent activation-induced T cell apoptosis by inhibiting the induction of Fas ligand expression (Baj et al. 1999; Szondy et al. 1998; Yang et al. 1994). Human peripheral unstimulated B cells were found to express mRNA of the RA receptor α, β, and γ (Worm et al. 1998), and retinoic acid is able to mediate its growth-inhibitory effect on B lymphocytes via their cognate nuclear receptors (Naderi and Blomhoff 1999).

In a recent study we investigated in vivo effects of DNA-based immunization of mice on binding parameters of all-trans retinoic acid receptors (RARs) in spleen cell nuclei (Brtko et al. 2000). An eucaryotic expression vector encoding the gene for the model enzyme ß-galactosidase of Escherichia coli was used for intradermal injection. Furthermore, immunostimulatory CpG motifs, which stimulate the expression of various cytokines and may serve as a "danger signal" for the mammalian immune system, were coinjected as oligodeoxynucleotides. The results demonstrated that the concentration of RARs was significantly reduced in the late phase of the primary immune response (21 days after injection of plasmid DNA – indicated by high affinity IgG antibodies and IFN-γ expression). Coinjection of CpG motifs did not change the course of the humoral response but enhanced and accelerated the proliferative response and expression of IFN-γ, which correlated with the reduced RAR concentration.

4.2. Type I, iodothyronine 5′-deiodinase. Type I, iodothyronine 5′-deiodinase (5′-DI), an enzyme consisting of two identical 27-kDa subunits with one atom of selenium per molecule, catalyzes the conversion of the main secretory product of the thyroid, the prohormone thyroxine (T_4) to the active 3,5,3′-triiodothyronine (T_3), predominantly in liver, kidney, and thyroid (Kohrle et al. 1995). In liver, it is localized in the cytosolically oriented leaflet of the rough and smooth endoplasmic reticulum (Auf Dem Brinke et al. 1979). The role of the cytokines, TNF, interleukins and interferones on the 5′-DI has been studied, but the results were contradictory depending on the experimental design and animal model used in the study (Kohrle 1994). The study of the direct effects of cytokines upon the 5′-DI activity in phi1 rat liver cells has shown that three cytokines, tumour necrosis factor-α (TNF-α), interleukin-1ß (IL-1ß) and interleukin-6 (IL-6) stimulated the 5′-DI activity. These data do not support the hypothesis that inflammatory cytokines may mediate the euthyroid sick syndrome by causing inhibition of 5′-DI activity (Davies et al. 1997).

In a similar approach like cited above (Brtko et al. 2000) we investigated the effect of intradermal DNA immunization and co-injection of CpG motifs on the expression of 5'DI. (Brtko et al. 1999). From the data we conclude that the activity of 5′-DI in mouse liver when compared to non-immunized animals (100 %) was found to be significantly enhanced by DNA immunization two weeks (175.7 %) or three weeks (192.6 %) after the plasmid injection. In addition, the activity of the 5′-DI in mouse liver was markedly enhanced two weeks (252.4 %) or three weeks (243.3 %) after the injection when CpG motifs were applied together with the plasmid DNA. This is in good agreement with the publication demonstrating the *in vitro* direct effect of three cytokines (TNFα, IL-1ß and IL-6) on stimulation of the 5′-DI activity in phi1 rat liver cells (Davies et al. 1997) and may serve as *in vivo* evidence not supporting the hypothesis that inflammatory cytokines mediate the euthyroid sick syndrome by inhibiting the 5′-DI activity.

In summary, both studies demonstrate that DNA-based immune responses, similar like protein-based responses (Brtko et al. 2000; Friedman et al. 1993; Meunier et al. 1994; Szabo et al. 1994), involve important endocrine pathways. CpG motifs, the DNA vaccine-inherent adjuvant part, seem to additionally enhance or accelerate the interactions between the endocrine and the immune system.

At present, we cannot interpret these results from a functional point of view, because RAR and 5'-DI are involved in various aspects of the immune system in a very complex context. However, the data clearly demonstrate that RAR specific binding characteristics or 5'-DI activity are influenced by in vivo immune responses. Furthermore, we also conclude, that, despite different pathways of processing and presentation of antigen by immunization with protein in comparison with immunization with plasmid DNA, similar basic immune mechansims are induced during a primary response.

5. Safety of DNA vaccines and CpG based adjuvants

5.1. DNA vaccines. Three key areas of concern have been identified by regulatory agencies considering the application of DNA vaccines in humans: (i) the potential of genomic integration of the DNA, (ii) induction of anti-DNA antibodies induced by the injected plasmid DNA and (iii) induction of autoimmunity (or tolerance) as a result of the special fea-

tures of DNA immunization. A number of publications have addressed these aspects. In mice injected i.m. with plasmid DNA encoding influenza NP no integration of the injected plasmid has been detected so far (Nichols et al. 1995). Also low doses of DNA vaccines have not been shown to induce tolerance (Liu et al. 1997) and induction of autoimmunity caused by massive immune destruction can also be excluded (Wolff et al. 1990). Furthermore, animal studies also suggest that the induction of anti-DNA antibodies by DNA vaccines is unlikely (Jiao et al. 1992; Katsumi et al. 1994).

Very recent phase I clinical studies including DNA vaccines against HIV-1 (MacGregor et al. 1998), hepatitis B surface antigen (Roy et al. 2000; Tacket et al. 1999), malaria (Le et al. 2000) and prostate cancer (Mincheff et al. 2000) confirmed the picture emerging from the animal studies. The results demonstrated that DNA vaccines are well tolerated with no or only mild local reactogenicity and systemic symptoms. No anti-DNA antibodies or muscle enzyme elevations could be detected (MacGregor et al. 1998).

5.2. CpG motifs. A unique feature of CpG DNA based adjuvants, even when compared to other bacterial product derived adjuvants, is their strong ability to induce IL-12, the central mediator of Th1 reactions (Cowdery et al. 1999; Sparwasser et al. 1998). This is of special interest with respect to many forms of immunotherapy (e.g. successful anti-tumor immunization relies on the induction of sufficiently strong cellular immunity, in particular induction of tumor specific CTL and these require a Th1 cytokine profile to proliferate and differentiate).

However, the downstream events of CpG DNA and other bacterial products, such as LPS are remarkably similar and multiple studies have shown that CpG DNA induce TNF-α *in vitro* and *in vivo* (Sparwasser et al. 1997a,b; Stacey et al. 1996). Hence, it cannot be ruled out completely that administration of CpG DNA may carry a potential risk. In selected experimental systems CpG DNA induced septic shock symptoms or promoted the development of autoimmunity. In one study, administration of bacterial DNA or CpG oligonucleotides caused a rapid increase in TNF-α serum levels and, in galactosamine sensitized mice, resulted in lethal septic shock (Sparwasser et al. 1997a, b). Furthermore, these authors and others also found that CpG DNA and LPS synergized in the induction of TNF-α (Cowdery et al. 1996). The potential promotion of autoimmunity by CpG DNA was investigated in NZB/W mice, which are prone to systemic lupus erythematosus-like syndrome. Immunization of these mice with *E.coli* DNA but not with calf thymus DNA resulted in elevated levels of anti DNA-antibodies. Nevertheless, treatment of these mice with *E.coli* DNA improved survival and reduced the autoimmune symptoms in these animals despite the increase in anti DNA-antibodies (Gilkeson et al. 1996). In another model, CpG DNA induced experimental autoimmune encephalomyelitis (EAE) by activation of Th1 effector cells specific for myelin basic protein and this effect was dependent on the CpG DNA-mediated induction of IL-12 (Segal et al. 1997). These data clearly indicate the necessity of further investigations to improve our knowledge about the complex in vivo influences of CpG motifs during immune responses.

6. The future of DNA vaccines and DNA-based adjuvants.

Considering the availability of other vaccines, namely recombinant and attenuated viruses and recombinant protein, one cannot help asking the question: Do we really need such a radically new approach ? (Leitner 2001) The simple answer is that all the currently used vaccines have practical problems. For many decades, a variety of attenuated viruses have successfully protected us against their wild-type brethren. However, such viral vaccines always carry the risk of inadvertent infection when the virus is not sufficiently weakened. A recent outbreak of Polio caused by an unsufficiently attenuated vaccine has served as a painful reminder of this problem (Greensfelder 2000). In general, stronger attenuation makes a given vaccine safer at the cost of decreased efficacy. Furthermore, vaccination with an attenuated virus may be unacceptable in the case of highly pathogenic viruses such as HIV or Ebola, regardless of the degree of attenuation.

On the other hand, selecting recombinant proteins from the pathogen as the active compound of vaccines carries absolutely no risk of infection, but displays the shortcomings of stimulating only one arm

of the immune system, antibody production, while failing to effectively activate the cellular branch. Furthermore, recombinant protein vaccines require strong adjuvants and may therefore not be safe for humans.

DNA vaccines, in contrast, are very cheap, easy to construct, and capable of stimulating both cellular and humoral responses. DNA vaccines would also meet the requirements for broad applicability in Third World contries, including easy manufacturing as well as uncomplicated transportation and storage.

CpG adjuvants in combination with DNA vaccines (and also with protein vaccines) expand the range of possible immune modulations. Oligodeoxynucleotides containing CpG motifs directly or indirectly activate various types of cells of the immune system. A key feature with respect to immunostimulation is the activation of APCs, which respond by increased antigen processing and presentation and by secretion of cytokines. Importantly, CpG DNA induces large amounts of IL-12, which is a central mediator of Th1 immune reactions. A strong Th1 environment promotes NK cell activity and the development of CTL. NK and CTL mediated immunity represent important effector mechanisms for the eradication of viruses and tumor cells. It has been suggested by a series of independent studies that CpG based adjuvants may be more attractive for clinical applications than other microbial derivatives because of their comparably lower toxicity. Aside from the undisputed potential for vaccine research against pathogens, DNA vaccines and CpG adjuvants are intensively investigated concerning their usefulness in cancer vaccination and treatment.

Nevertheless, DNA vaccines are far from being perfect replacements for conventional ones. While it should not be their primary purpose to replace currently used vaccines with a good track record, i.e. high efficacy and few side-effects, new approaches are urgently needed for diseases that have so far resisted vaccination attempts. One of the major advantages of DNA vaccines is the rapid realization of novel scientific findings and insights. In contrast to any other way of vaccine development, DNA vaccines enable construction and evaluation of new vaccine concepts and strategies in animal models within months. Furthermore, the enormous potential of specific modulations of immune responses (with or without additional CpG adjuvants) opens the possibilitiy for a tailor-made immune response against any given pathogen.

According to our current knowledge, DNA vaccines represent a clean and safe way to induce an immune response to well-defined antigens and this exciting and promising new technology deserves a fair chance to prove itself.

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