Immunity, Vol. 3, 165–169, August, 1995, Copyright © 1995 by Cell Press

Exposing the Immunology of Naked DNA Vaccines

Minireview

Drew M. Pardoll and Anne Marie Beckerleg
Department of Oncology
Johns Hopkins University School of Medicine
Baltimore, Maryland 21205

Vaccines represent the most commonly employed immunologic intervention in medicine today. Indeed, they are currently one of the few antigen-specific approaches with clearly documented clinical success. Current estimates by the Centers for Disease Control indicate that greater than 5,000,000 doses of vaccine against some infectious organism are administered yearly in the United States, making vaccines the most commonly administered immunotherapeutic. Current vaccines target only a tiny fraction of infectious diseases, since prophylaxis against some of the most common and deadly infections in the third world is limited by expense and ease of distribution. In addition to the public health concerns of expense and distribution, other features of current vaccines limit their efficacy. While most current vaccines typically elicit reasonable antibody responses, cellular responses (in particular, major histocompatibility complex [MHC] class I–restricted cytotoxic T cells) are generally absent or weak. For many infectious diseases, such as tuberculosis and malaria, humoral responses have been shown to be of little protective value against infection. Another limitation of most current vaccines relates to the limited duration of immunologic memory. Ideal vaccines would provide lifelong prophylaxis, a goal generally not achieved by current formulations.

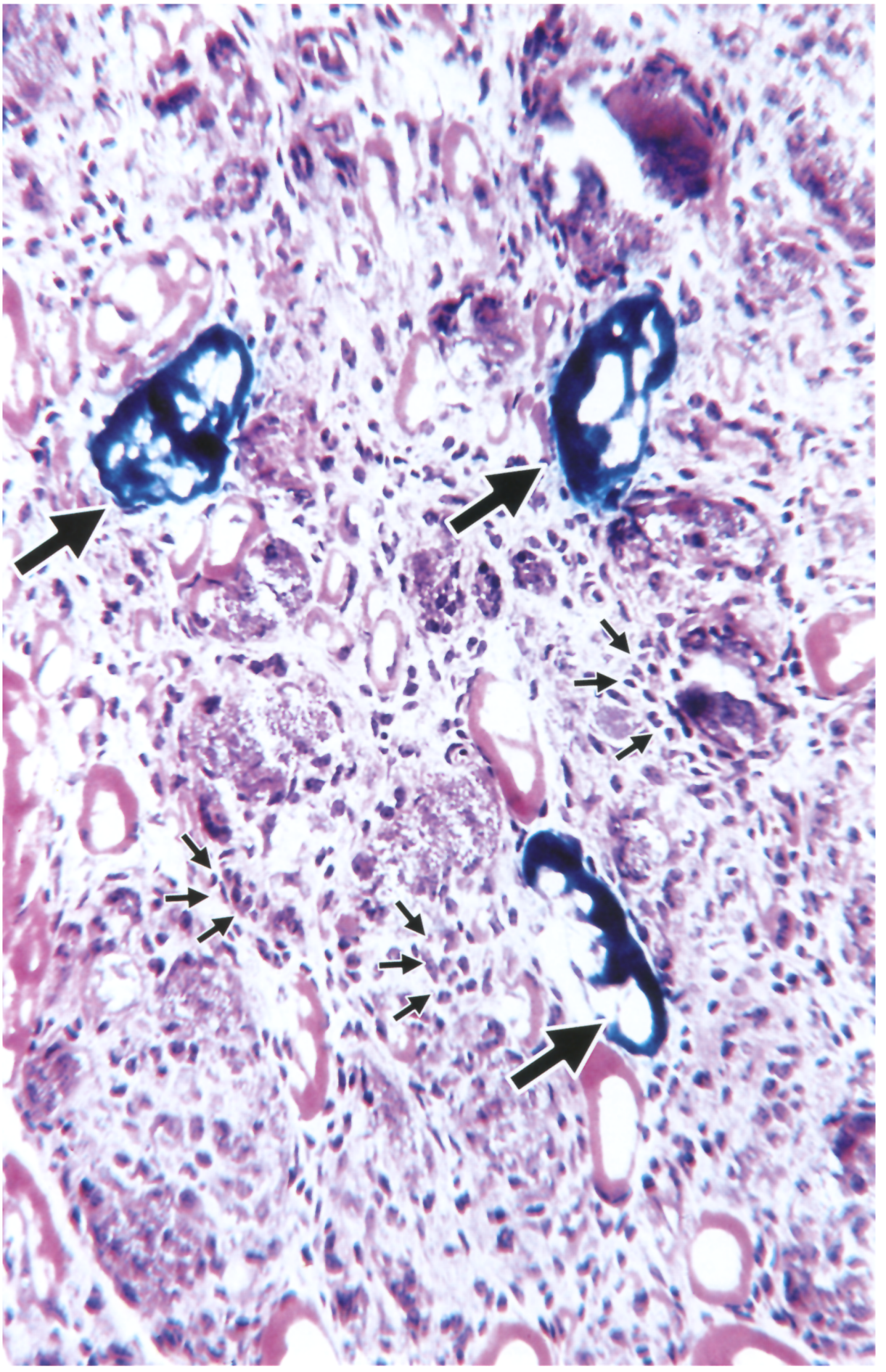
In the last three years, DNA vaccines have burst onto the scene as a radically new approach to infectious disease prophylaxis. One of the most surprising and important features of DNA immunization is that purified "naked" DNA appears to be taken up and expressed by cells in vivo with much greater efficiency than would have been predicted by the experience with DNA transfection in tissue culture. This finding provides the basis for a critical pharmaceutical advantage of DNA vaccines: namely, simplicity of preparation. In addition, naked DNA can be produced in large scale with tremendous purity, allowing for freedom from contamination with potentially dangerous agents. The final pharmaceutical advantage of DNA is its tremendous stability relative to proteins and other biologic polymers, a feature likely to be more relevant for the production of vaccines than the recreation of dinosaurs.

From an immunologic perspective, the unique ability of DNA to either integrate stably into the genome or be maintained long-term in an episomal form provides the potential for long-lived antigen expression. This feature thus has implications for the duration of immunologic memory achievable with nucleic acid vaccines. Despite the flurry of reports documenting the ability of naked DNA vaccines to induce both immunologic and protective responses in animal models, the mechanism by which DNA injections activate the immune system against the encoded antigens remains somewhat mysterious. Nonetheless, given what is now understood about pathways of antigen processing and the requirements for T cell activation, exposing the mechanisms of immune activation by naked DNA may reveal some provocative clues to how the immune system deals with different forms of antigen.

Methods of In Vivo Transduction with Injected DNA

As is so often the case with active areas of investigation, evidence for in vivo transduction by injection of purified DNA was observed many decades ago, but remained largely unnoticed until its more recent resurrection. As early as 1960, Ito (1960) demonstrated the induction of papillomas in rabbit skin by injecting phenol-extracted nucleic acids from the Shope rabbit papilloma virus. Then, in 1990, Wolff et al. demonstrated direct gene transfer into mouse muscle in vivo with reporter constructs (Wolff et al., 1990). Using either chloramphenicol acetyl transferase, luciferase, or β-galactosidase, they demonstrated that injection of either purified RNA or DNA could result in expression of the appropriate enzyme activity within the skeletal muscle. When 100 μg of purified DNA consisting of the reporter gene linked to a Rous Sarcoma Virus LTR (RSVL) promoter was injected, episomal plasmid DNA could be detected by Southern blot 30 days later and enzyme activity persisted for at least 60 days after injection. The stability of this episomal form of DNA is presumably due to the low proliferative state of myocytes in vivo. Numerous subsequent studies have evaluated different parameters of in vivo gene transfer, including injection vehicle, promoter, DNA structure, and route of injection (Davis et al., 1993a; Eisenbraun et al., 1993; Fynan et al., 1993b; Jiao et al., 1992; Manthorpe et al., 1993; Prigozy et al., 1993; Wolff et al., 1991).

Surprisingly, most investigators have found that simple saline solutions appear to be quite reasonable carriers, often resulting in transfection of between 1%–5% of myofibrils in the vicinity of the injection site in the case of intramuscular administration. DNA preparations that are typically used to transfect cells in vitro, such as calcium phosphate precipitates or liposomal preparations, do not appear to enhance the efficiency of in vivo transfer (Manthorpe et al., 1993; Wolff et al., 1990). In fact, most agents tested that might theoretically enhance in vivo transfection efficiency appear to interfere with gene transfer, at least into skeletal muscle. Nonetheless, there are a few reports that coinjections of toxic agents intended to cause muscle necrosis and repair either prior to or concurrently with injection of DNA, can increase gene transfer and expression (Davis et al., 1993a, 1993b; Vitadello et al., 1994; Wang et al., 1993a, 1993b). These include local anesthetics, such as bupivicaine, and myotoxins, such as cardiotoxin. Different promoters that have been compared for efficiency of gene expression for in vivo DNA transfer include CMV-IE, RSV, SV40, SRa, actin, MCK, α-globin, adenovirus, and dihydrofolate reductase. While relatively few direct comparisons have been performed, viral promoters



with broad cell type specificity such as the CMV and RSV promoters appear to generate the most consistently high levels of expression of reporter constructs.

With regard to site of injection, the largest experience is with injection into skeletal muscle. Other tissue types have also been shown to express gene products after DNA injection, including cardiac muscle, liver, and dermis (Acsadi et al., 1991; Malone et al., 1994; Raz et al., 1994). One of the new technologies that has engendered particular interest in intradermal DNA injections is the gene gun. The gene gun, an instrument currently not covered under the assault weapons ban, takes advantage of the ability of ballistically accelerated microscopic gold particles to penetrate cell membranes without killing the cell. By mixing these gold particles with purified DNA in the presence of a polycations such as spermadine, the nucleic acid becomes coated onto the gold particles. These DNA-coated gold particles are loaded into the gene gun and the end is abutted to a shaved area of skin. Discharge of the gene gun results in penetration of the dermis between 0.1–5 mm, depending on the chosen projectile force. This form of DNA injection has been shown to transduce cells in both the dermis and epidermis (Eisenbraun et al., 1993).

Generation of Immune Responses by Naked DNA Vaccines

It is now well established that injection of naked DNA through any of a number of routes reproducibly induces both humoral and cellular immune responses against the encoded antigens. The initial report that genetic immunization could elicit immune responses measured the induction of antibodies against human growth hormone (hGH) subsequent to ballistic injection of DNA-coated gold particles with a gene gun (Tang et al., 1992). hGH gene constructs under transcription control of either the human β-actin promoter or CMV promoter induced specific anti-hGH antibody responses. The titer of antibodies was somewhat variable and strain dependent. In addition, clear-cut booster effects of subsequent DNA immunizations were observed, akin to what is typically seen with recombinant protein immunizations.

Subsequently, Liu and colleagues demonstrated that antigen-specific CTL responses could be induced by intramuscular injection of naked DNA (Montgomery et al., 1993; Ulmer et al., 1993). They utilized an influenza A model to emphasize the advantage of a vaccine strategy that could induce CTL responses, as humoral responses to influenza A tend to be strain specific and poorly cross protective. This is because the major antibody responses are directed against the hemaglutinin (HA) antigen, which varies significantly among different influenza strains. In contrast, epitopes of the influenza nucleoprotein (NP) antigen, a major target for CTL responses, demonstrate significantly less interstrain variability. Using a plasmid containing the NP gene driven by either an RSV or CMV promoter, they demonstrated specific CTL responses against the NP 147–155 epitope presented by the H-2K^d MHC class I molecule. Importantly, animals immunized intramuscularly with NP DNA were protected from intranasal challenge with 102.5 TCID 50 of an influenza isolate, A/HK/68, which arose 34 years after the strain from which the vaccinating NP gene was isolated (A/PR/A/34).

Importantly, CTL responses against NP were found to persist at least 13 months subsequent to the intramuscular DNA injection (Yankauckas et al., 1993). These studies, as well as analogous findings in other animal models of infectious disease, suggested that naked DNA immunization could produce long-term humoral and cellular immune responses qualitatively similar to live attenuated vaccines but without the safety hazards of inoculation of live virus. Other infectious disease models in which successful immunization and at least partial protection against viral challenge have been observed include HIV (using the gp120 or gp160 genes), bovine herpes virus (GIV gene), rabies virus (surface glycoprotein gene), and hepatitis B virus (hepatitis B surface antigen) (Cox et al., 1993; Davis et al., 1993b; Wang et al., 1993a, 1993b; Xiang et al., 1994). In addition to the nucleoprotein gene, DNA vaccinations with other influenza genes including HA and matrix protein have likewise demonstrated protective responses (Fynan et al., 1993a; Montgomery et al., 1993; Robinson et al., 1993). In essentially all cases, significant titers of neutralizing antibody are induced and, in the case of rabies virus and HIV, CTL responses were also documented.

Despite the rapidly expanding volume of reports documenting successful immunization with naked DNA vaccines, there has yet to be a direct systematic comparison of the relative potency of DNA vaccines versus other live attenuated viral or recombinant protein plus adjuvant vaccines in standardized animal models. One report did compare the relative efficacies of naked DNA injections as a function of route of inoculation. Using either the HA subtype 1 (H1) protein in a mouse model of adapted influenza virus or the HA subtype 7 (H7) gene in a chicken model of influenza, six routes of inoculation (intravenous, intraperitoneal, intranasal, intramuscular, intradermal, subcutaneous) were compared in their ability to induce both antibody responses as well as protective immunity (Fynan et al., 1993b). While intramuscular injection of DNA appeared to generate the best response, intravenous, intranasal, intradermal, and subcutaneous immunizations also induced significant protection. When ballistic inoculation of DNA-coated gold particles was evaluated, equivalent levels of protection were achieved using 2–3 logs lower total DNA dose (0.4 μg) than all of the other forms of inoculation. In contrast with what might be expected using a recombinant protein vaccine, intranasal (mucosal) DNA inoculation did not result in enhanced immunglobulin A

Figure 1. Biopsy of Muscle after Injection with Purified DNA Encoding β-Galactosidase

Purified DNA (100 μg) encoding β-galactosidase under transcriptional control of the CMV-IE promoter was injected into the quadriceps muscle of a mouse. The muscle was biopsied 5 days later and stained with bluogal and hematoxylin and eosin. Note the expression of β-galactosidase in large proportion of myofibrils (large arrows) as well as the significant inflammatory infiltrate (small arrows).

(IgA) titers but rather produced IgG responses similar to intramucosal and intravenous injections. Clearly, much additional evaluation needs to be done in developing general principles for the quantitative and qualitative aspects of DNA immunization.

Mechanisms of Initiation of Immune Responses by DNA Vaccines

As eluded to above, many of the advantages of DNA vaccines have been proposed to stem from either stable integration or extrachromosomal maintenance of the DNA within cells of the injected organ. In the case of intramuscular injections, reporter constructs appear to mark myocytes specifically as the targets of integration and gene expression (Figure 1). The previously proposed mechanisms for priming of humoral and cellular immune responses by DNA vaccines have reflected the distinct pathways of MHC class I and class II antigen processing as defined in cell culture. Thus, antibody responses have been proposed to occur when antigens encoded by the transduced myocyte are released into the circulation either via secretion or via cell death. These antigens are then taken up by macrophages and B cells, thereby initiating a T helper–dependent antibody response. Alternatively, CTL priming has been proposed to occur via endogenous proteosome-dependent processing and presentation of antigens within the transfected myocyte followed by TAP-dependent presentation on the myocyte MHC class I molecule (Figure 2A). The stability of the integrated DNA sequences would produce an essentially continuous supply of antigen to drive immune responses indefinitely, thus accounting for the long-term persistence of immune responses against antigens encoded by the injected DNA.

If antigen receptor occupancy by peptide–MHC complexes were the whole story to T cell activation and immunologic priming, these proposed mechanisms would seem the most reasonable. However, they do not account for the critical role of costimulatory signals in initiating immune responses. Indeed, an increasing body of evidence in the self-tolerance field suggests that antigen recognition in the absence of appropriate costimulatory signals results in immune tolerance (by either ignorance, anergy, or deletion) rather than activation. Thus, simple release of antigens by natural processes of cell death do not result in induction of immunity. For example, the myocyte cell death that invariably occurs after running a marathon does not produce autoimmune myocytis. By analogy, it is probably oversimplictic to propose that immune responses against antigens encoded by integrated DNA activate and propagate immune responses when transfected myocytes simply die by natural processes. Similarly, the mounting evidence for costimulatory requirement in CD8 T cell activation implies that direct presentation of endogenously synthesized antigens by MHC class I molecules of transfected myocytes might not be expected to prime CTL precursors.

A critical element to the priming of both humoral and cellular immune responses that is usually left out of the picture in discussions of naked DNA vaccines is the bone marrow–derived antigen-presenting cell (APC). Indeed, although it has never been systematically studied, sites of injected muscle clearly become infiltrated with inflammatory cells (see Figure 1). As with all other circumstances of immunologic activation, it is hard to imagine that bone marrow–derived APCs within these inflammatory infiltrates do not play an important role in the vaccine effect of naked DNA immunizations. In the case of MHC class II–restricted $CD4^+$ T cell priming, these APCs would have the opportunity to pick up locally released antigens in the interstitial spaces and carry them to draining lymph nodes where they could be presented to both B cells and T cells.

With regard to the priming of MHC class I–restricted CD8 T cells, it may also be worth considering these infiltrating APCs as prime suspects in CTL activation (Figure 2B). Despite the earlier cross-priming experiments of Bevan in the 1970s, the notion that host-derived APCs could efficiently ingest released exogenous antigens for processing and presentation on MHC class I molecules in vivo had been considered improbable, because the defined cellular pathways of MHC class I antigen presentation require that the antigen be expressed endogenously (Bevan, 1976). However, recent experiments designed to determine which cell type presents tumor-specific antigens to MHC class I–restricted CD8 T cells may be quite analogous to the CTL priming that occurs with an in vivo transfected myocyte. In both cases, the antigen is initially expressed exclusively by a nonprofessional APC. In the case of tumor vaccines, Huang et al. (1994) used a model system in which a specific antigen (influenza NP) with known MHC class I epitopes is expressed by a tumor. Parent F1 chimeras, in which the MHC haplotypes of the bone marrow–derived cells either did or did not match the MHC haplotype of the tumor cell were vaccinated with NP-expressing tumors. In all cases, the NP-specific CTL generated were specific exclusively for epitopes presented by MHC alleles expressed by the bone marrow–derived cells, not the tumor cell. Thus, the priming of MHC class I–restricted responses involved the transfer of that antigen to a host bone marrow–derived cell before its presentation to $CD8^+$ T cells. Using subsets of macrophages and defined conditions in vitro, exogenous antigens have recently been shown to enter the MHC class I processing pathway (Kovacsovics-Bankowski and Rock, 1995).

The most direct evidence implicating bone marrow–derived APCs in the priming events of naked DNA vaccines comes from a recent study by Ertl and colleagues. Using the murine rabies model, they demonstrated that coinjection of naked DNA encoding murine granulocyte–macrophage colony-stimulating factor (GM-CSF) with the gene encoding rabies glycoprotein enhanced both antibody and cellular responses to the rabies glycoprotein antigen (Xiang and Ertl, 1995). The enhanced immunologic responses correlated with enhanced protection against challenge with the rabies virus in vaccinated animals. These results appear analogous to findings that tumor vaccines genetically modified to express GM-CSF provide enhanced systemic immunity against challenge with lethal doses of unmodified tumor cells injected distant to the vaccine site. The proposed mechanism by which paracrine GM-CSF elaboration enhances antigen-specific immune response relates to the ability of this cytokine to induce the differentiation of hematopoetic progenitors into

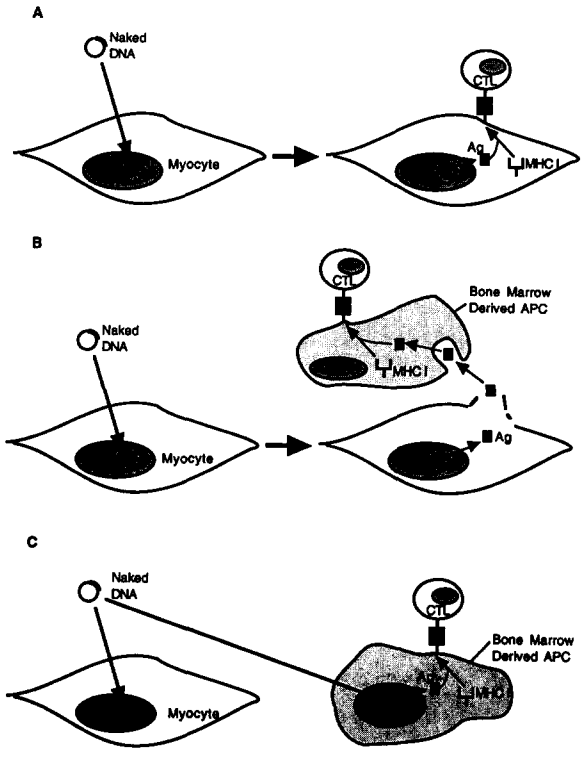


Figure 2. Three Possible Models for Priming of MHC Class I–Restricted CTL Responses by Intramuscular DNA Injections

(A) Direct presentation by transduced muscle cells.

(B) Transfer of protein antigens to bone marrow–derived APCs with crossover into the MHC class I–processing pathway.

(C) Uptake of DNA directly into bone marrow–derived APCs infiltrating the muscle followed by classical MHC class I processing.

"professional" APCs. In addition to macrophages, GM-CSF has been shown to induce the differentiation and maintenance of dendritic cells. This cell type, which is 2–3 three logs more potent on a per cell basis than macrophages in activating naive T cells in vitro, has been proposed to be the critical APC in initiating immune responses in vivo. Qualitative and quantitative differences in APC composition or traffic into different tissue types may, in fact, account for the lower amount of DNA necessary for intradermal vaccination. In particular, the presence of Langerhans cells in the epidermis provides a ready source of APCs at the injection site.

Another model for immunologic priming by naked DNA vaccines bears consideration since it has neither been ruled in or ruled out. While Sutton's law dictates that attention be focused on the myocyte in the case of intramuscular DNA vaccines, it is certainly possible that small numbers of infiltrating bone marrow–derived APCs are themselves directly transfected (Figure 2C). Because these bone marrow–derived cells are motile, while myocytes are stationary, they may have fled the scene by the time the biopsy is taken, thus giving the appearance that myocytes are the only transfected cell. While small in number, such transfected bone marrow–derived APCs may ultimately be the critical players in priming immune responses in draining lymph nodes.

Now that naked DNA has become established as a clear player in the vaccine field, it will be important to dissect the mechanisms by which it activates immune responses. It is only through these studies that intelligent modifications can be introduced to maximize both qualitatively and quantitatively its ultimate potency.

References

Acsadi, G., Jiao, S. S., Jani, A., Duke, D., Williams, P., Chong, W., and Wolff, J. A. (1991). New Biol. *3*, 71–81.

Bevan, M. J. (1976). J. Exp. Med. *143*, 1283–1288.

Cox, G. J., Zamb, T. J., and Babiuk, L. A. (1993). J. Virol. *67*, 5664–5667.

Davis, H. L., Michel, M. L., and Whalen, R. G. (1993a). Hum. Mol. Genet. *2*, 1847.

Davis, H. L., Whalen, R. G., and Demeneix, B. A. (1993b). Hum. Gene Therap. *4*, 151–159.

Eisenbraun, M. D., Fuller, D. H., and Haynes, J. R. (1993). DNA Cell Biol. *12*, 791–797.

Fynan, E. F., Robinson, H. L., and Webster, R. G. (1993a). DNA Cell Biol. *12*, 785–789.

Fynan, E. F., Webster, R. G., Fuller, D. H., Haynes, J. R., Santoro, J. C., and Robinson, H. L. (1993b). Proc. Natl. Acad. Sci. USA *90*, 11478–11482.

Huang, A. Y., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D., and Levitsky, H. (1994). Science *264*, 961–965.

Ito, Y. (1960). Virology *12*, 596.

Jiao, S., Williams, P., Berg, R. K., Hodgeman, B. A., Liu, L., Repetto, G., and Wolff, J. A. (1992). Hum. Gene Therap. *3*, 21–33.

Kovacsovics-Bankowski, M., and Rock, K. L. (1995). Science *267*, 243–246.

Malone, R. W., Hickman, M. A., Lehmann-Bruinsma, K., Sih, T. R., Walzem, R., Carlson, D. M., and Powell, J. S. (1994). J. Biol. Chem. *269*, 29903–29907.

Manthorpe, M., Cornefert-Jensen, F., Hartikka, J., Felgner, J., Rundell, A., Margalith, M., and Dwarki, V. (1993). Hum. Gene Therap. *4*, 419–431.

Montgomery, D. L., Shiver, J. W., Leander, K. R., Perry, H. C., Friedman, A., Martinez, D., Ulmer, J. B., Donnelly, J. J., and Liu, M. A. (1993). DNA Cell Biol. *12*, 777–783.

Prigozy, T., Dalrymple, K., Kedes, L., and Shuler, C. (1993). Som. Cell Mol. Genet. *19*, 111–122.

Raz, E., Carson, D. A., Parker, S. E., Parr, T. B., Abai, A. M., Aichinger, G., Gromkowski, S. H., Singh, M., Lew, D., and Yankauckas, M. A. (1994). Proc. Natl. Acad. Sci. USA *91*, 9519–9523.

Robinson, H. L., Hunt, L. A., and Webster, R. G. (1993). Vaccine *11*, 957–960.

Tang, D., DeVit, M., and Jonston, S. A. (1992). Nature *356*, 152–154.

Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowski, S. H., Deck, R. R., DeWitt, C. M., and Friedman, A. (1993). Science *259*, 1745–1749.

Vitadello, M., Schiaffino, M. V., Picard, A., Scarpa, M. and Schiaffino, S. (1994). Hum. Gene Therap. *5*, 11.

Wang, B., Boyer, J., Srikantan, V., Coney, L., Carrano, R., Phan, C., Merva, M., Dang, K., Agadjanan, M., and Gilbert, L. (1993a). DNA Cell Biol. *12*, 799–805.

Wang, B., Ugen, K. E., Srikantan, V., Agadjanyan, M. G., Dang, K., Refaeli, Y., Sato, A. I., Boyer, J., Williams, W. V., and Weiner, D. B. (1993b). Proc. Natl. Acad. Sci. USA *90*, 4156–4160.

Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990). Science *247*, 1465–1468.

Wolff, J. A., Williams, P., Acsadi, G., Jiao, S., Jani, A., and Chong, W. (1991). Biotechniques *11*, 474–485.

Xiang, Z., and Ertl, H. C. J. (1995). Immunity *2*, 129–135.

Xiang, Z. Q., Spitalnik, S., Tran, M., Wunner, W. H., Cheng, J., and Ertl, H. C. (1994). Virology *199*, 132–140.

Yankauckas, M. A., Morrow, J. E., Parker, S. E., Abai, A., Rhodes, G. H., Dwarki, V. J., and Gromkowski, S. H. (1993). DNA Cell Biol *12*, 771–776.