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COMMENTARY

Nanotechnology in Vaccine Development: A Step Forward

Adnan Nasir[1]

The study of nanotechnology for epicutaneous delivery of pharmaceuticals and vaccines is burgeoning. Topically applied nanomaterials have been shown to enter tape-stripped skin and reach draining lymph nodes in an inbred strain of mice. Nanomaterials in the form of plasmid DNA, proteins, and virus particles accumulate in hair follicles, diffuse via dendritic cells to draining lymph nodes, and elicit antigen-specific humoral and cell-mediated immunity. Topically immunized mice have also demonstrated resistance to infection with live virus. Advantages of nanotechnology include uniformity, reproducibility, and precision in the synthesis and manufacture of candidate compounds. Combined with novel pharmacokinetics and the possibility of targeted therapy, nanotechnology-based vaccines may prove superior to existing vaccines and have the potential to open therapeutic avenues for treating infectious disease and malignancy.

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The history of infectious disease in humans is a cyclical tug-of-war between host and parasite and host and pathogen (Donelson and Donelson, 2008). The cost to society over the course of history has been staggering, with epidemics ravaging whole populations. Over time, this cost has not diminished, in either its scope or its extent. According to the World Health Organization, of the top 10 causes of death in the world, 5 are the result of infectious disease, nearly surpassing ischemic heart disease and cerebrovascular disease combined (World Health Organization, 2003) (Table 1). Although the impact of infectious disease is universal, its effects are particularly acute in developing nations, where the heaviest burden falls on the poor (Table 2).

Looking beyond these mortality statistics, the five most prevalent diseases worldwide are infectious or parasitic. They strike where literacy rates are low, poverty is epidemic, and resources are scarce. They drain the reserves of affected individuals and families, and rob communities and nations of productive citizens. Beyond basic sanitation and infrastructure, one of the most cost-effective ways to manage infectious disease is vaccination. Traditional vaccines have considerable disadvantages, and novel approaches to make vaccines more versatile and more effective are urgently needed. The ideal vaccine would be easy to manufacture, inexpensive, readily transported, and stable in a broad range of ambient temperatures and levels of humidity. Finally, a vaccine should be easy to administer. Needle-based vaccines require an infrastructure of sterile materials and trained personnel, and they generate hazardous waste (Glenn *et al.*, 2000). Furthermore, they are painful and can meet with psychological and cultural resistance, making needle-free alternatives desirable. One advantage of epicutaneous vaccination is that resident antigen-presenting cells can induce powerful and sustained immune responses; in at least one study, these allowed for antigen dilution to one-fifth, potentially reducing vaccine cost (Nasir, 2008) and increasing vaccine availability (Nel *et al.*, 2006).

Nanoparticles and nanomaterials with biological activity exist in nature (Oberdorster *et al.*, 2005). Viruses are one of the more salient examples. Viruses can induce natural immunity through clinical or subclinical infection via epicutaneous, respiratory, and gastrointestinal routes. The induction of "artificial" immunity using nanomaterials is not a recent phenomenon. Smallpox has existed for millennia and has been well documented in history. Clinical examples of skin lesions can be identified on the mummy of Ramses V (Hornung, 1997). Smallpox descriptions taper off in late Roman and early Christian texts, suggesting that the disease was dormant for centuries before making a resurgence in Medieval Europe and then in Asia.

The earliest description of the process of inoculating a naive individual with (viral) nanoparticles may have been given in 1549 by Wu Quan (Temple, 1986). It is most likely that therapy for smallpox was developed at this time, although some China scholars

Table 1. Top causes of mortality worldwide

Rank	Cause	% of Deaths
1	Ischemic heart disease	12.6
2	Cerebrovascular disease	9.7
3	Lower respiratory infection	6.8
4	HIV/AIDS	4.9
5	Chronic obstructive pulmonary disease	4.8
6	Diarrheal disease	3.2
7	Tuberculosis	2.7
8	Malaria	2.2
9	Cancer of trachea/ bronchus/lung	2.2
10	Road traffic accidents	2.1

From the World Health Organization, 2003.

Table 2. Global burden of disease

Rank	Disease	No. infected (millions)
1	Malaria	800
2	Filariasis	280
3	Leishmaniasis	200
4	Schistosomiasis	200
5	Trypanosomiasis	13

From the World Health Organization, 2003.

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contend that the origins can be traced to the Song Dynasty (960–1279 A.D.) (Needham, 1999). Wu Quan described a powder made from the crust of smallpox lesions that was blown into the nasal passages of the vaccinees. At the time, it was understood that inoculation of live material from patients who had full-blown disease (*Variola major*) led to high mortality, and the procedure was therefore banned. Lesional crust for incorporation into the powder could be harvested only from subjects who had a few crusts by proxy (*Variola minor*). This practice of inoculation became widespread in Central Asia, South Asia, and Asia Minor (Wujastyk, 1995). It was later varied to include scarification of the skin and was introduced to England by Lady Mary Wortley Montagu in 1717, practiced anecdotally by farmers and milkmaids, and subsequently perfected by Edward Jenner in 1796 (Bray and Roy, 2004).

Predecessor vaccination methods were cost-effective. However, as indicated by the mortality due to inappropriate administration of vaccines (e.g., from *Variola major*), these methods were nonspecific and imprecise. The advent of nanotechnology enabled the precise manufacture of materials with desirable properties and the removal of undesirable features (Table 3). This selectivity, combined with the compactness of the nanoscale, allows the development of a wide range of useful agents. Safety issues notwithstanding, the potential for nanotechnology in vaccine development is significant (Castanedo-Tardan *et al.*, 2007; Nasir, 2008; Nasir and Gaspari, 1996; Nel *et al.*, 2006; Oberdorster *et al.*, 2005; Warheit *et al.*, 2007).

The skin has both active and passive barrier defenses that retard the entry of pathogens. To be successful, nanomaterials must bypass these defenses. A variety of studies have demonstrated that nanomaterials can penetrate the skin. Penetration is typically through the follicular route and diminishes with increasing nanomaterial size (Mortensen *et al.*, 2008; Vogt *et al.*, 2006). Particles larger than 100 nm are not likely to penetrate intact skin. However, skin that is damaged, flexed, or otherwise altered may be more permeable to particles. Patients with atopic dermatitis demonstrate slightly greater vulnerability to cutaneous viral infections (for example, by molluscum contagiosum or herpes simplex) (Bork and Brauninger, 1988; Erdmann *et al.*, 2004; Kakourou *et al.*, 2005; Wollenberg *et al.*, 2003). Furthermore, nanomaterials or their vehicles may be modified to gain purchase on the skin or to enter via follicular and interfollicular routes.

In this issue, Mahe *et al.* (2009) report a mouse model for testing nanomaterial penetration and immunogenicity. The authors increase the potential for nanomaterial penetration by three means. First, the skin to which the antigen is to be applied is tape-stripped. Tape-stripping not only may decrease barrier function but also may increase the expression of immunostimulatory cytokines and surface markers on keratinocytes. Second, the mice are anesthetized for 1 hour to allow undisturbed penetration of the applied material. Third, the authors selected the C57BL/6 inbred mouse strain, which has unique immunological (they favor a Th1 response) and behaviorial (they tend to groom and barber as a form of dominance) properties (Sarna *et al.*, 2000; Von Stebut and Udey, 2004).

In a series of elegant experiments, the authors found that nanomaterials can penetrate tape-stripped skin. They used several methods to demonstrate penetration (Figure 1). Fluorescent 40- and 200-nm particles called fluorospheres emit a yellow–green fluorescence at 515 nm. *In vivo* confocal laser scanning microscopy demonstrated the accumulation of these particles in round aggregates at a depth of 80 μm, corresponding to the location of hair follicles. The initial distribution was compact and bright after 5 hours, but

> Nanomaterials can penetrate tape-stripped skin effectively.

gradually diffused over 24 hours, suggesting skin uptake. When epidermal cells were harvested 3 hours after application and sorted for antigen-presenting cells ($CD11c^+$, $CD207^+$), nearly one-third contained fluorospheres. At 4, 24, and 48 hours after application, the authors harvested draining lymph nodes and compared them with nondraining lymph nodes for the presence of fluorospheres. They were able to demonstrate accumulations as early as at 4 hours—and a peak of 40 nm fluorospheres by 24 hours—in draining lymph nodes, and there was an absence of fluorescence in nondraining lymph nodes. Results for 200-nm fluorospheres showed a smaller peak at 4 hours (approximately one-third of the 40-nm peak) and subsequent diminution over 24 and 48 hours. When $CD11c^+$ cells were purified from these draining lymph nodes, they were found to contain fluorospheres. These studies suggest a rapid accumulation of nanoparticles in hair follicles, attachment to and uptake by dendritic cells, and migration of fluorosphere-laden dendritic cells to the ipsilateral lymph node within 4–24 hours. The absence of fluorescence in nondraining nodes indicates a cutaneous–lymphatic route of migration rather than a cutaneous–circulatory route.

Next, the authors tested the ability of nanoparticulate antigens to generate an immune response after topical application. The antigens selected were

Table 3. Sources of nanomaterials

Anthropogenic	Natural	Incidental	Engineered
Frying	Volcanic ash	Forest fires	Carbon nanotubes
Cooking	Ocean spray	Plasma	Quantum dots
Sandblasting	Biomagnetite	Meteorites	Sunscreen
Mining	Ferritin	Mineral composites	Semiconductor wires
Metalwork	Virus		Fullerenes
Diesel exhaust	Clouds		

Adapted from Oberdorster *et al.*, 2005.

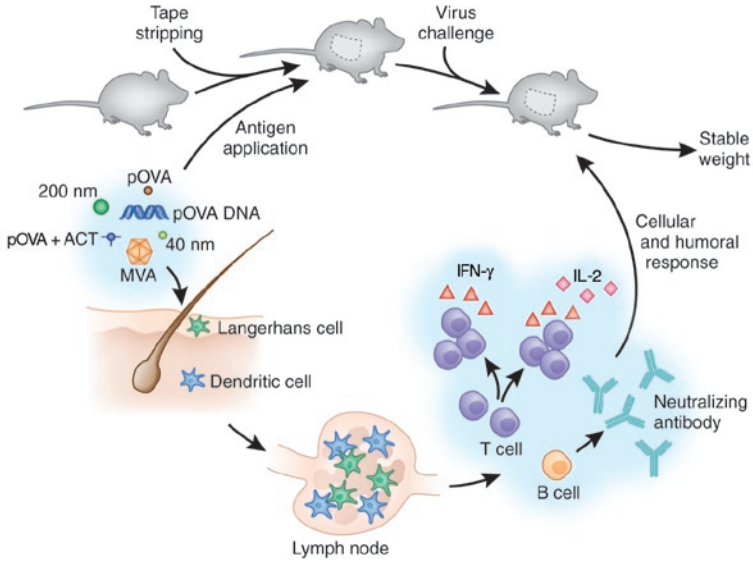

Figure 1. Tape-stripped C57BL/6 mice permit perifollicular penetration of topically applied nanoparticles. Particles tested include 40-nm beads, 200-nm beads, ovalbumin protein (pOVA), pOVA coupled to cholera toxin (pOVACT), plasmid DNA–encoding ovalbumin protein (pOVA DNA), and 290-nm modified vaccinia Ankara virus (MVA). When applied topically, nanoparticles aggregate around hair follicles and disperse into the skin, where they are taken up by epidermal Langerhans cells and dermal dendritic cells. Skin surrounding the upper part of the hair follicle is especially rich in antigen-presenting cells. These cells migrate to ipsilateral lymph nodes and present antigen to T cells, leading to T-cell proliferation and cytokine production as well as neutralizing antibody production. Mice topically immunized with MVA respond to live virus challenge by maintaining stable weight, whereas control mice lose up to 11% body weight within 1 week of live virus challenge.

plasmid DNA encoding ovalbumin (OVA), OVA protein, and OVA conjugated with cholera toxin. Based on my calculations and the current literature, these particles measure 20–70, 5, and 5.5 nm, respectively (Braga and Ricci, 2004). In these experiments, host mice were first intravenously administered 4 million prelabeled naive CD8-OT1 cells from a strain of mice with a specific immune response to peptides 257–264 (SIINFEKL) of OVA. The cells lost the intensity of their label through cytoplasmic dilution as they underwent proliferation and division.

Cell-mediated responses to topical application of antigen compared with PBS controls were measured at day 4 by flow cytometry of fluorescent labeling for $CD8^+$ cells in draining and nondraining lymph nodes. The authors found that CD8-OT1 proliferation was greatest in response to OVA protein (83% proliferating cells in draining lymph nodes vs. 68% in nondraining lymph nodes) as compared with OVA plasmid DNA (21% proliferating cells in draining lymph nodes vs. 15% in nondraining lymph nodes). The efficacy of plasmid DNA was roughly equivalent whether it was applied topically or injected into the tibialis anterior muscle. Humoral responses were assessed by measuring anti-OVA IgG at day 14. Whereas phosphate-buffered saline (PBS) and OVA protein elicited negligible antibody responses, OVA protein + cholera toxin and OVA plasmid DNA elicited vigorous and nearly equivalent responses.

The authors subsequently determined whether a modified vaccinia Ankara (MVA) virus could penetrate skin and elicit immune responses. This virus particle, when modified with green fluorescent protein to allow visualization, is approximately 290 nm in diameter. The authors were able to detect particles *in situ* using confocal fluorescent microscopy and anti-MVA antibodies, and they demonstrated the same intense follicular localization of MVA with diffusion and loss over 24–48 hours. MVA antigen could be seen in $CD207^+$ cells, particularly around perifollicular dendrites. MVA antigen could also be detected in draining lymph nodes within 4 hours of topical application, but not in nondraining lymph nodes. Application of 15×10^6 particle-forming units (pfu) of MVA topically elicited vigorous production of IFN-γ by CD4 and CD8 cells at day 14. Similarly, a vigorous elevation in neutralizing antibody titer was noted at day 28 in immunized animals compared with PBS controls.

Finally, Mahe *et al*. immunized mice topically with 5×10^6 pfu MVA or PBS control. Two months later, the mice were challenged intranasally with 5×10^6 pfu of live vaccinia virus. Animals were measured for weight loss as a percentage of body weight. Topically immunized mice lost a peak of 5% body weight during the study, whereas control mice lost nearly 11%, suggesting modest *in vivo* protection from live virus challenge.

The authors have nicely demonstrated in this model system that nanoparticles in the 40- to 200-nm range can effectively penetrate tape-stripped skin and enter lymph nodes via dendritic cells and Langerhans cells. They have demonstrated that proteins and plasmid DNA can traffic to draining lymph nodes by the same mechanism and elicit cell-mediated and humoral immunity. Further, the authors have shown that a vaccinia virus particle approximately 290 nm in size can penetrate tape-stripped skin and migrate within dendritic cells to draining lymph nodes, where they stimulate T-cell cytokine production and neutralizing antibody production, thereby conferring relative immunity against live virus challenge.

Several interesting questions remain. Do the applied nanomaterials localize solely in hair follicles; if so, why? Are nanomaterials on nonfollicular skin groomed off or rubbed away? Or do they accumulate and aggregate in the follicles through some funnel or drain effect? Are there properties of the infundibulofollicular epithelium (for example, charge, pH, lipids, commensal organisms) permissive to nanoparticle accumulation? Once nanoparticles accumulate in the follicle, do they require a threshold

density or concentration to penetrate into the skin, and is this density lacking in interfollicular skin? Would occlusion enhance epicutaneous penetration, even in interfollicular skin? What form of nanoparticles do dendritic cells prefer for uptake: dispersed, aggregated, bulk? What surface properties of nanoparticles favor dendritic cell uptake? (He *et al.,* 2007; Ramakrishna *et al.,* 2007). For example, in some models, nanoparticles are more readily taken up by cells if they are spherical rather than cylindrical, whereas in other models (Gratton *et al.,* 2008; Lee *et al.,* 2007*;* Zhang *et al.,* 2008*)*, a cylindrical shape is favored. Is there some aspect of tape stripping that favors dendritic cell migration? Do keratinocytes serve as bystanders or costimulators in this process? (Burns *et al.* , 1999; Gaspari *et al.,* 1998a; Gaspari *et al.,* 1998b). Are there other vehicles that can be used to enhance the penetration of nanomaterials or simulate the ancillary effects of tape stripping? What combination of adjuvants would be preferable for topical preparations? (He *et al.,* 2007; Kenney *et al.,* 2004; Martin and Gaspari, 2008; Yeh *et al.,* 1997). What antigen or vehicle variables can be exploited to maximize cell-mediated responses versus humoral responses? Are the responses seen in this animal model unique to the C57BL/6 strain or are they generalizable? Is this methodology suitable for the administration of multiple vaccines? (Glenn *et al.,* 2000). Can multiple vaccines be designed to have synergistic effects?

Pathogens have developed a number of strategies to evade the immune system, from hiding intracellularly to inducing tolerance, to shedding surface markers, to serially switching dominant antigenic epitopes (Berriman *et al.,* 2005; Donelson and Donelson, 2008; El-Sayed *et al.,* 2005; Nasir *et al.,* 1987; Von Stebut and Udey, 2004). Some of these mechanisms are also used by tumors to evade immune surveillance. The road to vaccine design and development is long, tangled, and unpredictable. Countless investigator years and solid supportive preliminary data have led to one of the largest and costliest HIV vaccine clinical trials in history. Yet the result—*increased* infection rates in treated individuals—was both puzzling and profoundly disappointing. The complexity of the population being studied, their prior history of non-HIV viral infections, and difficulty in reaching the necessary strength of humoral and cell-mediated arms of immunity all likely contributed to the failure (Cohen, 2007; Sekaly, 2008). With the advent of nanotechnology, tools are becoming available to develop the next generation of vaccines, featuring easy application, transportability, stability, compact size, specific targeting, and enhanced effectiveness. The results reported by Mahe and co-workers represent a notable step in that direction.

CONFLICT OF INTEREST

The author states no conflict of interest.

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Targeting Apoptosis Defects in Cutaneous T-Cell Lymphoma

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Cutaneous T-cell lymphomas (CTCLs) are non-Hodgkin's lymphomas characterized by the clonal proliferation of skin-invasive mature T lymphocytes. Because of their low proliferative potential, the accumulation of clonal T cells is considered to be potentially due to their inability to undergo Fas-mediated apoptosis, a crucial process involved in T-cell homeostasis. Several reports have indicated a decrease in or absence of Fas expression in tumor cells in significant proportions of CTCL patients, whether or not associated with overexpression of apoptosis inhibitors. In this issue, Wu *et al*. confirm the impaired expression of Fas in CTCL cells. Moreover, they show that CTCL cells genetically engineered to re-express Fas acquire a sensitivity to FasL-induced apoptosis, opening new avenues for therapeutic intervention strategies in CTCL.

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Mycosis fungoides (MF), the most common type of cutaneous T-cell lymphoma (CTCL), and its leukemic variant, Sézary syndrome (SzS), are mature T-cell neoplasms characterized by the clonal proliferation of skin-invasive T lymphocytes that have the phenotype of mature helper T cells. Because of the low replicative potential of CTCL cells, it is proposed that their accumulation may, to some extent, be the result of defective regulation of T-cell apoptosis.

Apoptosis can be triggered by a series of stimuli, among which are signals transmitted by a family of cell-surface receptors—called death receptors—that belong to the tumor necrosis factor–receptor family. Six death receptors are known—Fas (CD95, Apo-1), TRAIL-receptor 1 (DR4), 2 (DR5, Apo-2), tumor necrosis factor–receptor 1, TRAMP (DR3, WSL-1, Apo-3), and DR6—and all contain a cytoplasmic sequence called the "death domain" that couples the death receptor to downstream caspases, the activation of which leads to apoptosis.

The acquisition of resistance to apoptosis as a mechanism involved in tumorigenesis is well documented. In several tumor types, a decrease in or loss of cell-surface Fas expression compared with that observed in nontumor cells of the same lineage has been reported. Likewise, mutations in the DNA sequence of death receptors such as Fas, DR4, and DR5 have been reported in several human cancer types. Furthermore, in both mice and humans, Fas gene mutations leading to defective Fas/FasL signaling have been shown to result in lymphoproliferative disorders as a consequence of lymphocyte accumulation (Rieux-Laucat *et al.*, 1995). Resistance to apoptosis can also occur as a consequence of the aberrant expression of intracellular apoptosis inhibitors in tumor cells, as reported in many cancer types. Among them is the FLICE-inhibitory protein (cFLIP), which is an intracellular protein that is highly homologous to caspases 8 and 10 and is able to block the autoproteolytic cleavage of caspase-8 that is normally induced upon triggering Fas-mediated apoptosis. An overexpression of cFLIP associated with a high expression of Fas has been reported in FasL-resistant Hodgkin's/Reed–Sternberg cells, the malignant cells of Hodgkin's lymphoma (Dutton *et al.*, 2004). Similar observations have since been reported for non-Hodgkin's lymphoma and several other types of cancer. The relevance of cFLIP overexpression, and indirectly that of death receptor-mediated apoptosis in the pathogenesis of lymphoma, is supported by mouse models of lymphoma engineered to overexpress cFLIP in which tumor progression is enhanced (Djerbi *et al.*, 1999).

Appropriate Fas-mediated cell death is essential in the control of lymphocyte homeostasis. Elimination of T cells after encountering a specific antigen or superantigen normally proceeds via activation-induced cell death (AICD), a process that is dependent on activation

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