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DNA vaccines and apoptosis: to kill or not to kill?

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The apoptotic machinery has become the latest target of vaccinologists attempting to improve the efficacy of DNA vaccines. While workers have previously sought to induce apoptotic death in transfected DCs as a means to activate immunity, a new approach (see related article on pages 109–117) instead seeks to delay apoptosis in host DCs after DNA vaccination.

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The honeymoon period that vaccinologists had with the new technology of DNA immunization is over. It ended with the realization that DNA vaccines were not as effective as hoped against the most serious threats such as HIV or cancer. Disappointing results from ongoing preclinical work and from clinical trials have put a serious damper on the enthusiasm that characterized the early days of DNA vaccines. It nevertheless seems to us that an overwhelming set of theoretical and practical advantages justify a redoubling of effort to get DNA vaccines to work effectively in humans. This is particularly the case when the menace of bioterrorism looms ever larger, and threats of new epidemics caused by emerging infectious diseases, such as Severe Acute Respiratory Syndrome, seem to be materializing. It is, needless to say, critically important to have vaccine vectors that can rapidly be engineered and administered to large numbers of people using a pathogen's genetic information. Nucleic acid vaccines represent such a vaccine vector — the requisite cultivation and expansion of new pathogens for the creation of a live attenuated or killed vaccine is of course not necessary when all one needs for construction of a vaccine is the bug's genetic identity.

For this reason, research on DNA vaccines has moved to its second phase with the emphasis now on improving immunogenicity and efficacy (reviewed in ref. 1). This includes: (i) improved DNA plasmids used as vectors in an attempt to enhance antigen expression and focus antigen targeting; (ii) better delivery systems for more efficient transfection of cells in vivo; and (iii) the development of molecular adjuvants to enhance immune responses to the inoculum, including the codelivery of cytokine (2) or other adjuvant molecules (3).

The drive to improve DNA vaccine function is fueled by the consensus that DNA vaccines may be immunologically benign, that is to say, they are simply not carrying enough of the signals necessary to trigger a strong innate immune response. While immunostimulatory DNA sequences (CpG motifs) are believed to be primarily responsible for the adjuvant properties of prokaryotic DNA (4), the adjuvant capacity of CpG that naturally occur on plasmids may not be sufficient for many applications. This is especially true when dealing with weakly immunogenic antigens or self-antigens, as is the case with cancer. The issue of immunostimulatory DNA is further complicated by the identification of species-specific requirements for these motifs. Thus, there is an urgent need for more robust and universally applicable adjuvant strategies.

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Induction of apoptosis enhances DNA vaccine immunogenicity

The immunostimulatory properties of apoptotic death have been debated intensively in recent years (5–9). It appears that the controversy over whether apoptosis or necrosis are either immunostimulatory or immunosuppressive were — at least in part — due to the misguided view that apoptotic death came in a single variety. Based on early descriptions, apoptosis was defined as a particular kind of cell death occurring in the absence of inflammation with predictable and invariable lack of immune stimulation. More recent studies have made it clear that apoptotic death can be triggered by a wide variety of mechanisms, which depending on the trigger can be accompanied by the production and release of various factors that help the immune system make a decision about the handling of the dead cells (10). Thus, apoptosis has been redefined as a particular set of defined molecular events with myriad variations.

Various reports have shown the immunogenicity of antigenic material associated with dead or dying cells (7, 11) and several studies have applied these findings in their effort to enhance DNA vaccine efficacy. Workers have codelivered genes for proapoptotic molecules with DNA vaccines to specifically induce apoptosis in transfected cells. For example, $CD4^+$ and $CD8^+$ T cell responses were improved when the genes for mutated caspases 2 or 3 were coinjected with the antigen-carrying

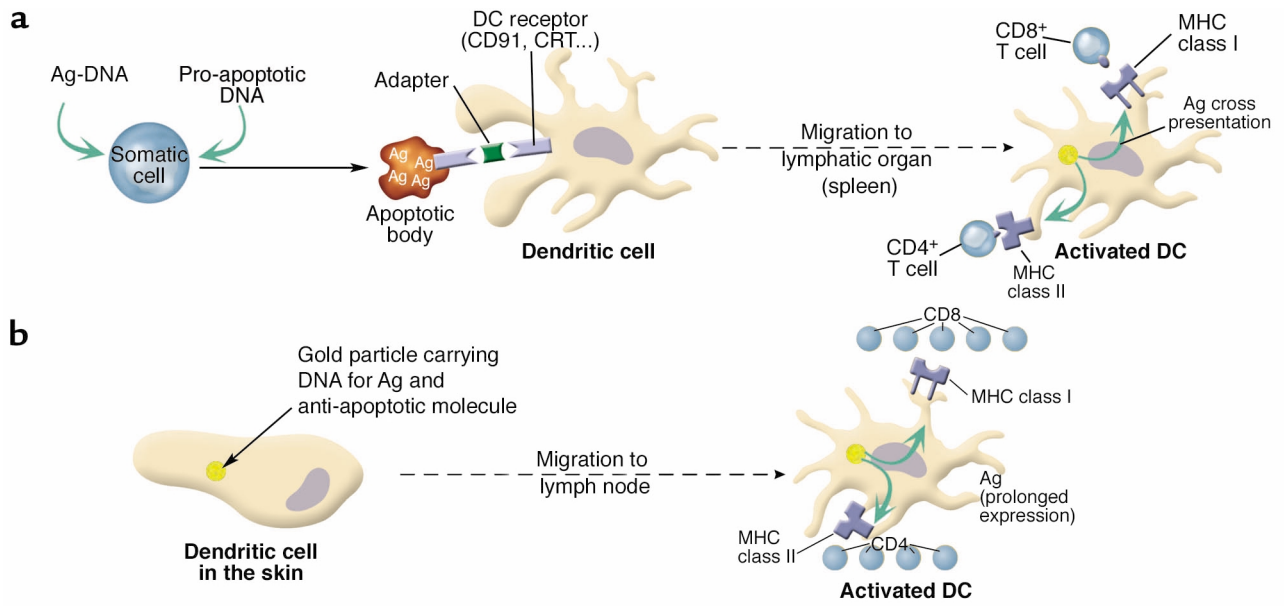

Figure 1
The enhancement of DNA-vaccine induced immune responses by the co-delivery of either pro- and antiapoptotic genes may be explained by the following model: (**a**) Mechanism for enhancement of DNA vaccines by induction of apoptosis. Proapoptotic genes are delivered together with the genes for the antigen of interest into somatic cells where both are expressed. The resulting apoptotic cells are recognized by professional APCs through an array of molecules found on the surfaces of dying cells through receptors such as CD36, ABC-1, CD14 CD19, or class A scavenger receptor. After ingestion and degradation of the antigen-loaded apoptotic cells the antigen presenting cell migrates to a lymphatic organ and presents the antigen of interest to $CD4^+$ and $CD8^+$ T cells. (**b**) Mechanism for enhancement of DNA vaccines by prevention of apoptosis. Antiapoptotic genes together with the genetic sequence for an antigen of interest are delivered by gene gun into the skin. DCs in the skin (Langerhans cells) are directly transfected and activated by the immunization (25). These activated APCs migrate to the local lymph node where they present antigen to T cells. This antigen may have been produced from the DNA plasmid by the DC itself or may have been produced by another transfected cell in the skin and then ingested by the DC. Due to the co-expression of antiapoptotic molecules, the life span of the DC is increased thus allowing the prolonged presentation to T cells, resulting in enhanced T cell priming. Alternatively, the expression of antiapoptotic genes may protect the antigen-presenting cell from direct killing by activated T cells. CRT, calreticulin.

plasmid (12, 13), demonstrating that apoptosis can provide an adjuvant effect (14). Similarly, the codelivery of the *fas* gene induced apoptosis of the transfected host cells resulting in enhanced CTL activity (15). Using a completely different approach to reach the same goal, we have employed apoptosis-inducing alphavirus replicase-based RNA and DNA constructs to deliver antigens of interest. These naked nucleic acid vaccines owe their enhanced immunogenicity not to increased antigen production, but to the requisite production of double-stranded RNA species, which results in the quantitative induction of apoptosis and innate immunity (16, 17).

It was recently shown that cells transfected with such replicase-based nucleic acid vaccines appear to interpret the transfection as a viral infection, thereby triggering the activation of antiviral pathways, which eventually lead to apoptotic cell death (18). Apoptosis is a consequence of infection with various viruses. Apoptosis of virally infected cells directly limits viral spread, but components of the enzymatic machinery of apoptosis, namely caspases, are also involved in the cleavage of IL-1β and IL-18 to their active forms (10). Apoptosis also can facilitate the initiation of a T cell response through improved uptake of the apoptotic material by DCs and presentation to $CD8^+$ T cells through cross-priming (19). In our own experience, interfering with apoptotic death induced by replicase-based DNA vaccines in vivo through codelivering an antiapoptotic gene significantly reduces vaccine efficacy (W.W. Leitner and N.P. Restifo, unpublished observations).

Improved function of DNA vaccine by inhibition of apoptosis

Considering studies that demonstrate that the induction of apoptosis enhances immune responses induced by a DNA vaccine, it is quite surprising that the reverse strategy, i.e., a reduction of in vivo apoptosis, was found to strongly enhance the immunogenicity of a DNA vaccine. In their study in the current issue of the *JCI*, Kim et al. (20) codelivered plasmids containing antiapoptotic genes with an antigen-containing plasmid to enhance vaccine efficacy. Interestingly, a plasmid containing the gene for the Bcl-2 family member Bcl-X_L yielded the strongest enhancement of antigen-specific T cell responses and resulted in efficient tumor rejection.

The codelivery of proapoptotic molecules most likely leads to the apoptotic death of transfected somatic cells, which would become attractive targets for infiltrating antigen-presenting cells. In contrast, Kim et al. argue that the target of the antiapoptotic molecules they codelivered by gene gun bombardment of the skin are directly transfected antigen-presenting cells (particularly DCs). The antiapoptotic molecules are hypothesized to prolong the lifespan of these critically important DCs thus allowing longer expression and presentation of the antigen with which they were

cotransfected. An alternative explanation stems from the observation that antigen-presenting DCs are susceptible to killing by the very same T cells they helped activate (21). Protection of antigen-presenting DCs from presentation-related death might allow these DCs more time to activate T cells.

A model for the differential impact of apoptosis in DNA vaccines

How can the immunogenicity of nucleic acid vaccines be enhanced both by the induction of apoptosis and its prevention? Kim et al. (20) reconcile these apparently contradictory findings by focusing on the differences in antigen and route of delivery. While Kim et al. deliver plasmids to the skin by gene gun, workers exploiting inducers of apoptosis have generally delivered the genetic immunization by an intramuscular route. This explanation may not encompass all of the data, as gene gun delivery was used by Sasaki et al. to deliver antigen plus proapoptotic modified caspases to enhance cellular and humoral immunity (22).

An alternative model may explain the conflicting observations: in this model, the effect of the pro- or antiapoptotic molecules that are codelivered with the DNA vaccine are on different types of cells. While various types of cells are transfected at the injection site, proapoptotic molecules selectively kill somatic cells. Reports have suggested that DCs may be much less susceptible to proapoptotic stimuli (23). For example, DCs reportedly become activated by proapoptotic signals delivered by Sindbis virus replicons (24). Thus, dying, antigen-loaded somatic cells would become attractive targets for the local DCs. When coimmunizing with antiapoptotic genes, the DCs would express both antigen of interest and a gene product that increases their lifespan. Once these DCs arrive at the local lymphatic organ, they present antigen for a prolonged period of time and in addition may be better protected from the fas-mediated attack by freshly primed T cells. Thus, this type of molecular adjuvant would postpone the scheduled elimination of DCs, which is part of the normal downregulation of an immune response (21).

Kim et al. (20) tested the immunogenicity of various molecules that would be able to interfere with apoptosis. In addition to the truly antiapoptotic molecules (Bcl-2, Bcl-X_L, and the X-linked inhibitor of apoptosis protein), they also employed dominant-negative mutants of caspases that are involved in the activation phase of apoptosis. Each of these inhibitors is expected to interfere with apoptosis at a different step in the apoptotic cascade. The finding that each of the inhibitors used here provided some level of adjuvant effect offers the opportunity to study the codelivery of multiple inhibitors with the goal to further improve DNA vaccine efficacy. Should the model be correct, one might envision an approach that takes advantage of both pathways (Figure 1). For such an approach, tissue-specific promoters could trigger apoptosis in somatic cells (such as keratinocytes or fibroblasts) while DC-specific promoters would limit expression of antiapoptotic genes to DCs. Both types of cells would be allowed to express the antigen of interest. The observations reported so far certainly opens up exciting new possibilities for innovative researchers trying to develop better DNA vaccines. It also provides an interesting springboard for immunologists trying to understand the underlying mechanisms of T cell activation.

Molecular adjuvants and the future of DNA vaccines

Limited immunogenicity may be the price that DNA vaccines pay for their safety and lack of serious side effects. The development of effective molecular adjuvants and improved delivery methods based on a deeper understanding of apoptosis, APC function, and immune cell activation may be the breakthrough that DNA vaccines urgently need. Significant improvements in our scientific understanding could make nucleic acid vaccines useful in the fight against infectious diseases or allergies or perhaps some day even cancer. If we are ever to begin to realize these goals, there is a critical need for the evaluation of these new strategies in human clinical trials.

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