ficient to prime naive T cells, a fact which is thought to prevent the inappropriate activation of naive T cells that may encounter self antigens on MHC molecules of normal tissues in the body.

The second, non-antigen specific signal must be delivered to the T cell for full activation to ensue. The best characterized source of "second signal" to date is engagement of CD28 on T cells by its ligands CD80 (B7-1) or CD86 (B7-2), which are exclusively expressed on activated APCs. This paradigm predicts that muscle cells would normally be ill-equipped to initiate T-cell responses, because they fail to express the T cell costimulatory molecules B7-1 or B7-2, and because other, less well-defined signals provided by the lymphoid microenvironment are lacking at the intramuscular injection site.

The identity of the cell type responsible for initiating T-cell priming is especially problematic in the case of MHC class I restricted CD8^{+} CTLs. Antigenic peptides presented on MHC class I molecules are thought to be derived largely from endogenous proteins, i.e., they result from the degradation of proteins synthesized by the cells that present them. If this is true, however, then how are CTL responses generated against antigens exclusively expressed by nonhematopoietic tissues that cannot provide T-cell costimulation? This paradox recently became evident in an analysis of immune responses generated by tumor cell vaccines.

A number of strategies have explored the injection of irradiated cancer cells as a means to prime antitumor immune responses. Many of these responses are critically dependent on the generation of tumor-specific MHC class I restricted CTLs. If tumor antigens can only access MHC class I molecules as endogenous proteins synthesized by the cancer cell, then tumor specific CTLs would have to be directly primed by the tumor cells, which lack expression of costimulatory molecules. The resolution of this paradox came through a series of experiments in which bone marrow chimeras were created that had APCs (which are derived from the bone marrow) expressing MHC molecules of a distinct haplotype from those on the immunizing tumor cells[6]. When these chimeras were immunized with irradiated tumor cells, the CTLs were found to always be restricted to the MHC haplotype of the APCs. This result strongly supported an alternate pathway of MHC class I antigen processing, known as "cross-priming"[7]. Specifically, exogenous antigens (produced by the tumor cells) are taken up by bone marrow derived APCs and are then processed and presented on the APCs' MHC class I molecules for presentation to CTLs.

When this same experimental approach was applied to the analysis of CTLs primed by DNA vaccination, the identical result was found, i.e., even though transfected muscle cells were the predominant source of antigen production, CTLs raised to the engineered protein are primed by APCs[8,9]. One difference between the tumor vaccine system and DNA vaccination, however, is that in the latter case, presentation of antigen by APCs could arise either from uptake of exogenous antigen (perhaps released by muscle cells), or by direct transfection of the APCs themselves. The latter possibility is hard to rule out, although PCR based assays have usually failed to detect plasmid in the draining lymph nodes of mice immunized with DNA. In any event, the chimera experiments appeared to rule out muscle cells as the dominant APC in CTL priming.

All this seemed to be falling nicely into place until the present demonstration by David Weiner and colleagues that coinjection of plasmids encoding B7-2 (but not B7-1) dramatically enhances CTL priming. Does the provision of B7-2 endow "non-professional" APCs (muscle) with all that is needed to prime CTL? In the absence of inflammation, muscle cells express very low levels of MHC class I antigens and are virtually devoid of MHC class II expression. Nevertheless, these molecules can be upregulated–in the presence of inflammatory cytokines, along with adhesion molecules involved in T-cell/APC interactions. It is interesting that T-cell proliferative responses to antigen (probably mediated by CD4^{+} T cells) are also enhanced by the coinjection of plasmid encoding B7-2. If myocytes are the dominant APC in this vaccine approach, then good MHC class II expression on these cells would be obligatory.

Fortunately, a fairly straightforward experiment similar to those described above should soon resolve this debate (see Fig. 1). Parent (haplotype "A") into F1 (haplotype "AxB") bone marrow chimeras would have

APCs of a single haplotype, but because the T-cell repertoire would develop in an F1 thymus, T cells would be capable of recognizing antigen presented on either haplotype A or B. Muscle cells of the F1 recipient would, of course, express both haplotypes. Immunization of these chimeras with plasmid DNA encoding an antigen that contains both an "A" restricted and a "B" restricted epitope together with a plasmid encoding B7-2 would give one of two possible results. If only "A" restricted CTL were generated, then priming would still be occurring by host bone marrow derived APCs. On the other hand, if both A and B restricted responses were seen, then muscle indeed would have been made to behave as a professional APC.

Of course, as with any interesting result, either answer will raise even more interesting questions. If muscle has been made to serve as the dominant APC, are the transfected myocytes destroyed by the primed CTL, and if not, why not? Alternatively, if bone marrow derived APCs are the dominant cell in priming CTL, one is still left to ponder why transfection of APCs with B7-2 would make a difference. After all, these cells can already express this molecule. Perhaps the level of B7-2 or the timing of its expression in relation to antigen can effect the nature of the T cell response. Only one thing is certain; the answers to these questions are likely to lead to additional strategies with which to enhance the antigen specific immune response.

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Liposomal gene delivery: A complex package

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Cationic liposomes have become one of the most widely used vehicles to deliver DNA into cells. Following the initial report by Debs's group[1] describing the delivery of lipo-

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some–DNA complexes into mice via intravenous injection, there has been a concerted effort to develop formulations that bring about high-level and reproducible transgene expression in various tissues. After some initial disappointment, several groups are now reporting successful delivery of plasmid DNA in mice by tail vein injection[2-6]. In this issue, Templeton et al.[7] describe a novel liposomal formulation composed of 1,2-diacyl-3-

trimethylammonium propane (DOTAP) and cholesterol that is another step forward in improving gene expression in vivo.

The difficulties involved with the intravenous use of cationic liposome–DNA complexes arise as a result of their underlying transfection mechanism. Cationic liposomes form complexes with the negatively charged DNA via charge interactions. Optimal transfection of the liposome–DNA complex relies on the presence of excess positive charge, which is required for an efficient interaction with the negatively charged cell membrane. Neutralization of excess positive charge in the liposome–DNA complex by negatively charged serum proteins is likely to result in a decreased transfection efficiency. Also, DNA might be released from liposome–DNA complexes by anionic molecules in the serum, rendering DNA more susceptible to enzymatic degradation[8]. Furthermore, serum proteins can induce an aggregation of liposome–DNA complexes, leading to a rapid clearance of liposome–DNA complexes from the blood by the reticuloendothelial system (RES). These problems, together with others, severely limit the intravenous application of cationic liposome/ DNA complexes.

Recently, it has been demonstrated that the serum sensitivity of liposome–DNA complexes can be resolved in several different ways (summarized in Table 1). Several important conclusions can be drawn from these studies. First, excess cationic charge in the complex with a charge ratio (+/-) ranging from about 2 to more than 10 seems to be required for efficient gene transfer[2-7]. This may be due to the fact that excess amounts of lipids can overcome the neutralization effect of the serum proteins. Second, a colloidally stable structure seems to enhance intravenous gene delivery. For example, cholesterol has been shown in several studies to enhance gene expression in vivo, whereas dioleoylphosphatidylethanolamine (DOPE) significantly decreases the transduction efficiency of the liposome– DNA complex[3,4,6,7]. This is in contrast to in vitro

transfection, where DOPE is found to improve gene expression in many studies. The enhancement of transfection in vivo by cholesterol may be due to its ability to stabilize the liposome bilayer. Finally, condensation of DNA is also important for a high level of gene expression in vivo.

In the present study, Templeton et al. use a liposomal formulation with a unique structure in which DNA is condensed in the interior of invaginated liposomes between two lipid bilayers and demonstrate high-level expression of the packaged gene in vivo. This is in agreement with a recent study in which

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the inclusion of protamine sulfate was shown to enhance the in vivo activity of the liposome –DNA complex[5]. In the latter study, plasmid DNA was partially condensed by protamine and the protamine–DNA complex then interacted with liposomes to form small particles with a size about 100–200 nm[5].

Intravenous administration of liposome –DNA complexes can bring about gene expression in many tissues, including those of the heart, lung, liver, spleen, and kidney. Although the level of gene expression varies from study to study, the lung invariably shows the highest expression levels and

endothelial cells are the major cell type transfected. This may be due to a "first-passage effect" because the lung is the first capillary bed the liposome–DNA complex encounters after intravenous administration. Indeed, gene expression in the lung has been shown to decrease 100-fold when the liposome–DNA is injected into the portal vein[5]. Therefore, targeted gene delivery to other organs via intravenous administration of cationic liposome–DNA complexes often proves difficult. In the study by Templeton et al., incorporation of a targeting ligand, asialofetuin, increased gene expression in the liver by sevenfold. Nevertheless, the level of gene expression in this organ was still much lower than that in the lung. Thus, the goal of tissue-specific targeting via intravenous administration of cationic liposome–DNA complexes remains unmet.

A significant advantage of liposomal vectors over viral vectors is their low immunogenicity, which allows repeated injections in order to achieve long-term gene expression. One surprising observation from a recent study is that there is an unresponsive or poorly responsive period of about two weeks between the two injections during which repeated injections are ineffective[6]. At present, it is not known whether this effect is toxicity-related or due to other inactivation mechanisms. One alternative to this problem is the use of a novel plasmid DNA with an extended half-life, as reported by Thierry et al.[2] In their study, gene expression lasted for about 3 months upon a single injection of an episomally replicative DNA plasmid complexed with cationic liposomes.

Progress in the development of liposomal vectors for intravenous gene delivery represents a good beginning, but there is a long way to go before it can be translated into clinical applications. More toxicity studies are required. Also, much remains to be learned about the interactions of liposome –DNA complexes with serum proteins and other components in circulation. On the basis of experience of developing "stealth" liposomes, the design of an effective and target-specific colloidal delivery system for DNA will require detailed study of numerous interactions with blood components.

Table 1. Parameters to be considered when devising a liposome–DNA complex for intravenous administration.

Parameter	Importance	Reference
Cationic lipid	Choice of lipid critical: Not all lipids equal, some better than others	6,7
Helper lipid	Cholesterol more efficient than DOPE	3,4,6,7
Charge	Higher (+/-) ratio enhances delivery	2–7
DNA packaging	Condensation of DNA enhances delivery	2,4,5,7
Toxicity	Only toxic at high doses	5–7
Stability	A few weeks to a few months	4,5,7
Repeated injection	Possible after a recovery period	6
Transgene expression level	Picogram to nanogram gene product per milligram of tissue	2–7
Duration of expression	A few weeks to a few months	2,5,6

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