Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 10496–10498, September 1997

Commentary

The immunologist's grail: Vaccines that generate cellular immunity

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Many of the past efforts to develop vaccines have focused upon the generation of antibody responses. In a number of current efforts to develop both new vaccines against many infectious diseases and effective immunotherapies for cancer, the target has been the generation of cytotoxic T lymphocyte (CTL) responses. Unlike B cells (and antibodies) that can recognize chemically diverse antigens (e.g., proteins, polysaccharides) in a variety of contexts (cell- or pathogen-associated or soluble), T cells generally recognize only a bimolecular complex consisting of an antigenic peptide bound to certain cell membrane glycoproteins, the major histocompatibility complex (MHC) class I and class II molecules. These MHC molecules provide a genetic identity for each individual since each parentally derived haplotype is codominantly expressed.

Thus T cells have a dual specificity in that, with the exception of instances of organ transplantation when T cells of one individual recognize nonself "foreign" MHC molecules, their receptors are specific for both their own MHC molecules and for the relevant antigenic peptide that has bound to those MHC molecules. The awarding of the 1996 Nobel prize in physiology or medicine to Peter Doherty and Rolf Zinkernagel denotes their important contribution to the field in their demonstration of this dual specificity, that CTL must recognize two determinants on a virally infected cell: one specific for the virus and one specific for the MHC of the host.

One subset of T cells, which express a cell surface glycoprotein CD8, recognize peptide antigens associated with MHC class I molecules. These $CD8^+$ T cells can also secrete cytokines in response to engagement of their T cell receptors, but their name reflects their ability to lyse the cell which present the antigen. Because in contrast to MHC class II proteins whose expression is limited largely to professional antigen-presenting cells (APCs), the MHC class I molecules are present on the surface of most nucleated cells, $CD8^+$ T cells can kill a variety of cell types, thus eliminating cells that are infected with virus (and hence producing more virus) or tumor cells, etc. Peptides associated with MHC class I molecules are usually derived from proteins present in the cytoplasm of cells, a routine occurrence with viral infection or for any other protein endogenously synthesized by the host cell, but not for exogenous proteins (such as an injected protein, which are generally taken up into endosomes for degradation by the endolysosomal pathway). The challenge for the induction of these so-called "MHC class I-restricted cytotoxic T cells" or CTLs is how to artificially introduce antigens into the MHC class I pathway, via the cytoplasm.

With the elucidation of the mechanisms of antigen processing and presentation for the generation of MHC class I-restricted CTL came efforts to generate such responses either by the use of viral vectors encoding heterologous antigens or of nonviral systems. Four articles recently published in the *Proceedings* (1–4) describe various approaches to generating MHC class I-restricted CTLs. It is of interest to describe these papers for the strategies and observations which they present and also to compare the approaches with one another and with other immunization modalities currently being studied in the laboratory and in early clinical trials.

The study by Saron *et al.* (1) capitalizes upon the ability of a toxin (from *Bordatella pertussis*) to translocate the toxin domain of the molecule into the cytoplasm of a cell. By inserting an MHC class I-restricted peptide epitope from a virus into this enzymatic toxin domain, this peptide was thus translocated directly into the cytoplasm of cells as part of the toxin domain and effectively immunized mice for protection against an otherwise lethal challenge with the virus. The study by Bronte *et al.* (4) provides additional insights into a strategy pioneered in the Moss laboratory, which has entered into clinical evaluation as well as being a widely utilized laboratory tool: recombinant vaccina viruses which express heterologous protein antigens. The two papers by Buschle *et al.* (2) and Schmidt *et al.* (3) provide a new twist on the well-known approach of peptide immunization by delivering the peptides mixed with polycations such as polylysine and polyarginine, with the result being protection in two tumor systems. Each study is important for demonstrating not only potential utility for immune activation capable of mediating protection *in vivo* against viral or tumor challenge (or for immunotherapy), but for further elucidating our understanding of key issues of delivery of antigen for optimal activation of protective/therapeutic immunity.

In their efforts to deliver epitopes to the cytosol of APCs for eventual association with nascent MHC class I molecules, Saron *et al.* (1) made a fusion protein by inserting the peptide epitope of interest derived from the nucleoprotein of lymphocytic choriomeningitis (LCMV) into the adenylate cyclase toxin of *B. pertussis*. The toxin has the ability to invade eukaryotic cells and deliver its N-terminal portion (the catalytic domain) directly through the plasma membrane into the cytosol. Peptides can be inserted into certain locations of the N-terminal catalytic region and thus be transported directly into the cytoplasm where they may be available for processing and presentation on MHC class I molecules. While others, including our laboratory, have utilized other fusion proteins to transport peptides or proteins into the cytoplasm of cells (5, 6), this paper provides a demonstration of the effectiveness *in vivo* of such an approach for generating CTL responses. Moreover, the CTL responses were sufficient to protect mice from an otherwise lethal intracerebral infection with LCMV. As an additional and important demonstration, the authors also showed that a fusion protein, made with the same LCMV epitope inserted into a genetically detoxified toxin, also induced MHC class I-restricted CTL and protective immunity.

The use of pertussis toxin, which directly translocates the enzymatic domain into the cytoplasm may be critical for the ability of this particular toxin–fusion protein to direct the epitope into the correct intracellular compartment for *in vivo* priming of CTL. For example, *Pseudomonas aeruginosa* exotoxin A (PE toxin) apparently utilizes a more complex retrograde pathway from endosomes to the Golgi complex and

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endoplasmic reticulum for translocation to the cytoplasm (7, 8). And although fusion proteins made with the binding and translocating domains of PE toxin and peptides of influenza A matrix protein and nucleoprotein were able to sensitize target cells for lysis by CTL (5), they were never shown to be capable of inducing CTL by *in vivo* administration.

Different MHC molecules generally bind different peptides; thus a vaccine consisting of a single CTL epitope would be effective only for a segment of an outbred population. Given the MHC haplotypic diversity of the human population, it would be preferable to make a toxin–fusion protein vaccine that provided a complete protein antigen rather than haplotype-specific epitopes to permit determinant selection to provide for an increased breadth of coverage for the population by providing a number of different peptide epitopes for any given antigen. Pastan and colleagues (9) demonstrated that a fusion protein composed of the binding and translocating domains of PE toxin and the enzyme barnase, was able to deliver functional barnase protein to the cytoplasm of cells. Their ability to substitute an intact protein for the toxin moiety of the PE toxin and the demonstration in the paper by Saron et al. (1) that the catalase activity of the toxin could be inactivated without destroying the ability of the toxin to deliver the epitopes, raise the question of whether the pertussis toxin can be made to deliver an entire protein, rather than just an epitope, by substituting a protein for, or inserting a protein into, the N terminus of the toxin. For PE fusion proteins, the proper refolding of the recombinant proteins presented a challenge; it is not known whether inclusion of an entire protein rather than a peptide into the pertussis toxin would introduce difficulties for the refolding of such a fusion protein into a functional delivery molecule. It is of interest that the authors were able to immunize the animals repeatedly because one potential drawback of this approach might be the generation of an immune response against the toxin protein itself (which was not evaluated in this paper).

The paper by Bronte *et al.* (4) utilized a poxvirus (vaccinia) vector strategy that has been employed both as a vaccine/immunotherapeutic strategy and as a tool for the study of CTL responses and mechanisms. They have further added to the understanding of factors that are important for optimizing the effectiveness of this approach (and potentially other recombinant viral vector technologies) by demonstrating an additional mechanism whereby expression of an antigen by a recombinant vaccinia virus is more effective from an immunologic standpoint if the expression is driven by an early promoter (i.e., a promoter that is active immediately after the virus infects a cell, prior to replication of the viral DNA) rather than by a late promoter (active after viral DNA replication), despite the latter being considered a stronger promoter. Although a number of cell types express the most antigen when the recombinant vaccinia virus utilizes a late promoter, dendritic cells expressed the model tumor antigen and activated antigen-specific CTL only when the antigen expression was controlled by an early promoter. The paper explored the possibility that APCs may be somewhat nonpermissive compared with other cells for viral infection; hence a vaccinia construct employing a late promoter would result in less expression of the encoded antigen, and hence poorer immunogenicity.

However, as the authors point out, other factors can account for differences in immunogenicity of vaccinia constructs employing early vs. late promoters. Earlier studies by Coupar *et al.* (10) demonstrated that utilization of late promoters for vaccinia expression of influenza hemagglutinin (HA) resulted in inhibition of the presentation of HA epitopes to HA-specific CTL. Townsend *et al.* (11) extended these observations utilizing vaccinia vectors encoding different forms of HA or influenza nucleoprotein (NP). They demonstrated that inhibition also occurred to a lesser extent during the early stages of infection, and that the inhibition was epitope-dependent and could be overcome if the antigen were constructed to be more rapidly degraded. They postulated that as vaccinia infection proceeds, an antigen processing defect develops that may be due to the production of a vaccinia-encoded protease inhibitor; thus an antigen that is targeted for faster degradation, rather than one that is more stable, would effectively rescue the defective antigen processing occurring in the vaccinia-infected cell. The inhibition of host cell protein synthesis by vaccinia may also decrease the synthesis of MHC class I molecules, thus providing another etiology of the decreased effectiveness of antigen presentation for constructs using a late promoter.

Nevertheless, the findings are important because, if the expression of protein by APCs vs. expression in other cell types determines the effectiveness of recombinant vaccinia as vaccines, the vectors must be constructed to maximize expression in those cells specifically. Highlighting the importance of expression of protein (antigen, in the case of a vaccine) specifically in APCs demonstrates the need to evaluate expression vectors in the target cells of interest. Yet a requirement for expression of protein by professional APCs, which is an underlying assumption of this approach, perhaps should not be taken for granted. For example, in some systems, transfer of antigen (12) or "cross-priming" (13) is thought to be responsible for delivering antigen from the cell synthesizing the antigen to the APC, which then presents it to CTL. Thus the demonstration that the use of early promoters was more effective for generating the desired immune responses because of their greater activity (compared with late promoters) in APCs refocuses the efforts for vector construction at least for poxviruses, specifically upon expression in APC rather than general levels of expression in a broad range of cell types.

Some groups have eschewed efforts to deliver protein antigens to the cytosol for entry into the MHC class I processing pathway in favor of peptides that have the potential to bind to MHC molecules at various points of the pathway, including extracellularly. The Birnstiel group (2) describes the ability of polycations to deliver peptides to APCs *in vitro.* They show that both negatively charged and neutral peptides can be delivered. The mode of introduction of the peptides into the cells appears to be different for different compounds, with polylysine apparently transiently permeabilizing cell membranes, whereas polyarginine acts via endocytic mechanisms. In their companion paper (3), a percentage of mice immunized with a peptide derived from a tumor (P815 mastocytoma) mixed with polylysine were protected from tumor challenge. Utilizing a melanoma system, they showed that subcutaneous immunization of mice with a combination of four peptides with certain polycations provided a degree of protection when given prophylactically or therapeutically. In both systems the protection provided by irradiated tumor cells (engineered to secrete cytokines) provided equivalent or better protection. Although the partial protection seen in these early studies is interesting, the mechanism of the protection is unclear. There is no demonstration of CTL nor of T helper cell responses (i.e., cytokine production) following immunization alone. (Splenocytes from animals that were first inoculated with tumor cells then therapeutically received one of the peptide-cationic mixes secreted γ-interferon in response to coculture with the melanoma cells, but inoculation with the tumor would have primed the animals.) Because none of the experiments contained control groups immunized with the polycations alone and no antigen-specific responses were demonstrated for animals receiving vaccine only, it is not clear if the protection seen was specific for the tumor antigens or was a nonspecific effect. Although in some species poly-L-lysine (PLL) can act as a classical T-dependent antigen presented in the context of MHC class II molecules, and in fact was used for the initial demonstrations of Ir gene control of antibody responses in guinea pigs (14, 15), in most strains of mice haptens conjugated

to PLL behave as T-independent antigens. Indeed, to engender protective MHC class I-restricted T cells by CTL, the administration of MHC class I-restricted peptides alone in the absence of provision of T cell help, would likely be suboptimal. Thus while the protection seen may indicate utility for this approach, it would be important to elucidate the mechanism of protection to determine how specific the immunity is and how widely applicable such an approach would be for other antigens and systems.

Vaccine approaches as described by the Leclerc and coworkers (1) and the Birnstiel group (2, 3) are based upon the delivery of specific peptides rather than proteins as the antigen. While the studies both demonstrate protective and/or therapeutic efficacy in mice for their respective infectious disease and cancer models, the peptide approach raises three issues: (*i*) peptides provide a limited rather than a broad base of CTL (or helper) epitopes for a population that has the haplotypic diversity that humans do; (*ii*) no B cell epitopes are provided, should antibody responses be needed for the full immune complement of both antibody and cellular responses; and (*iii*) helper T cell epitopes, which are important for optimal generation of CTL responses, are not provided. Although the first issue can be approached by providing a mixture of peptide epitopes to cover the majority of haplotypes, provision of an entire protein or several proteins, as mentioned above, would be expected to give broader coverage. Such provision may be important to avoid the generation of monospecific CTL responses that might lead to antigenic escape variants of the virus or tumor antigen. Indeed, administration of large numbers of autologous CTL that were monospecific for a single epitope from the nef protein of HIV to an individual resulted in the development of a viral strain missing that epitope (16). Regarding generation of T cell help, in the case of the toxin–antigen fusion protein, the toxin presumably is responsible for the provision of helper T cell epitopes for the generation of the primary response, although obviously the helper responses would be based upon a protein not subsequently present at the time of infectious challenge. For the peptide–polycation approach, it is not clear whether specific cellular responses of either the helper or cytolytic phenotype were generated by immunization itself.

Thus these three approaches all provide tantalizing demonstrations of the ability of various strategies to generate protective immunity *in vivo*. Although the poxvirus (4) and the toxin–peptide fusion protein (1) both clearly demonstrated the ability to generate CTL, the mechanism for the protection of the peptide–polycations (2, 3) is less clear. A number of other technologies that generate CTL responses (and in general good antibody responses as well) are being studied preclinically and clinically including various adjuvants, DNA vaccines (17), and other vector delivery systems (e.g., other recombinant viruses, retroviruses, and bacteria). The simplest approach which circumvents the peptide issues (limitation of epitopes for populations with diverse MHC haplotypes and the need to provide diverse T cell help for generation of CTL) and the potential limitation caused by immune responses directed against the delivery "vehicle" or vector (toxin delivery moiety or virus vector, for example), is the DNA vaccine approach (17) wherein a bacterial plasmid encoding a protein antigen under the control of a viral promoter results in the *in situ* generation of the antigen with subsequent development of antibody and cellular immune responses. This approach was first demonstrated to be effective for generating protective MHC class I-restricted CTL (18). Importantly, the protection was cross-strain effective; that is, animals were protected against a lethal challenge with a different strain of influenza virus than the one from which the gene encoding the antigen was derived. In contrast, antibody-mediated protection for influenza virus is acutely specific for a given strain of virus. This technology has been demonstrated to provide protective immunity against challenge in a number of viral, bacterial, and other preclinical disease models (cancer, allergy, and autoimmune diseases) (17) by the generation of CTL and/or antibody responses. It is a relatively generic technology with no capability of causing infectious disease itself (as may occur with viral or bacterial vectors) and has the ability to provide entire protein antigens or mixtures of antigens (via their genes), thus circumventing one of the major limitations of the peptide-based vaccine approaches (epitopic restriction). No significant immune responses have been seen against the vector itself, thus permitting the vector to be reused for booster doses or for different vaccines; this issue may be a limitation for the utility of fusion proteins or vaccinia vectors. These DNA vaccines can be easily manipulated to target the encoded protein to different cellular locations should that prove to be important for enhancing particular arms of the immune response (antibodies, T cell help, CTL).

These various technologies need to be directly compared for particular applications to evaluate the relevant immune responses in the context of the specific pathophysiology of the disease. While the preclinical efficacy may not be predictive of clinical utility, each of the approaches described in these papers has increased our armamentarium for developing vaccines and immunotherapeutics, and they provide additional tools for the continued quest to further understand antigen processing and mechanisms of protective immunity.

I wish to thank Drs. Herman Eisen, John Donnelly, Jeff Ulmer, Tong Fu, and John Shiver for their critical reading of this manuscript.

1. Saron, M. F., Fayolle, C., Sebo, P., Ladant D., Ullmann, A. & Leclerc, C. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3314–3319.
2. Buschle, M., Schmidt, W., Zauner, W., Mechtler, K., Trska, B., Kirlappos, H. & Birnstiel, M. L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3256–3261.
3. Schmidt, W., Buschle, M., Zauner, W., Kirlappos, H., Mechtler, K., Trska, B. & Birnstiel, M. L. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3262–3267.
4. Bronte, V., Carroll, M. W., Goletz, J. J., Wang, M., Overwijk, W. W., Marincola, R., Rosenberg, S. A., Moss, B. & Restifo, N. P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 3183–3188
5. Donnelly, J. J., Ulmer, J. B., Hawe, L. A., Friedman, A., Shi, Z. P., Leander, K. R., Shiver, J. W., Oliff, A. I., Martinez, D., Montgomery, D. M. & Liu, M. A. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 3530–3534.
6. Milne, J. C., Blanke, S. R., Hanna, P. C. & Collier, R. J. (1995) *Mol. Microbiol.* **15,** 661–666.
7. Chaudhary, V. K., Jinno, Y., FitzGerald, D. & Pastan, I. (1990) *Proc. Natl. Acad. Sci USA* **87,** 308–312.
8. Pelham, H. R. B., Roberts, L. M. & Lord, J. M. (1992) *Trends Cell Biol.* **2,** 183–185.
9. Prior, T. I., FitzGerald, D. J. & Pastan, I. (1991) *Cell* **64,** 1017–1023.
10. Coupar, B. E., Andrew, M. E., Both, G. W. & Boyle, D. B. (1986) *Eur. J. Immunol.* **16,** 1479–1487.
11. Townsend, A., Bastin, J., Gould, K., Brownlee, G., Andrew, M., Coupar, B., Boyle, D., Chan, S. & Smith, G. (1988) *J. Exp. Med.* **168,** 1211–1224.
12. Huang, A. Y. C., Golumbek, P., Ahmadzadeh, M., Jaffee, E., Pardoll, D. M. & Levistsky, H. (1994) *Science* **264,** 961–965.
13. Bevan, M. J. (1976) *J. Immunol.* **117,** 2233–2238.
14. Benacerraf, B., Green, I. & Paul, W. E. (1967) *Cold Spring Harbor Symp. Quant. Biol.* **32,** 569–575.
15. Bluestein, H. G., Green, I. & Benacerraf, B. (1971) *J. Exp. Med.* **134,** 471–481.
16. Koenig, S., Conley, A. J., Brewah, Y. A., Jones, G. M., Leath, S., Boots, L. J., Davey, V., Pantaleo, G., Demarest, J. F., Carter, C., Wannebo, C., Yannelli, J. R., Rosenberg, S. A. & Lane, H. C. (1995) *Nat. Med.* **1,** 330–336.
17. Donnelly, J. J., Ulmer, J. B., Shiver, J. W. & Liu, M. A. (1997) *Annu. Rev. Immunol.* **15,** 617–648.
18. 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., Friedman, A., Hawe, L. A., Leander, K. R., Martinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. L. & Liu, M. A. (1993) *Science* **259,** 1745–1749.