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MINIREVIEW

A Striking Property of Recombinant Poxviruses: Efficient Inducers of *in Vivo* Expansion of Primed CD8$^+$ T Cells

Fidel Zavala,* Mauricio Rodrigues,† Dolores Rodriguez,‡ Juan Ramón Rodriguez,‡ Ruth S. Nussenzweig,* and Mariano Esteban‡,[1]

*Department of Medical and Molecular Parasitology, NYU School of Medicine, New York, New York 10010; †Escola Paulista de Medicina, São Paulo, SP, Brazil; and ‡Centro Nacional de Biotecnología, CSIC, Campus Universidad Autónoma, 28049, Madrid, Spain

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INTRODUCTION

Over 200 years ago, in 1796, Jenner introduced vaccination, a name coined by Pasteur, as an effective means to control one of the most devastating diseases of mankind, smallpox. Since then, a large number of vaccines have been developed against many microbial pathogens based on the use of live attenuated, heat-killed, or otherwise inactivated microorganisms or their toxins (Plotkin, 1993). The main protective mechanisms elicited by these vaccines are neutralizing antibodies. After multiple doses of the same immunogen, the titers of antibodies as well as their avidity increase, conferring maximal protection. This strategy is used to induce large antibody responses to intracellular pathogens such as viruses, certain bacteria, fungi, and protozoans. Currently available vaccines, particularly those based on live attenuated carriers, like poxvirus recombinants, can also induce cell-mediated immunity which protects against certain viral diseases (Moss, 1996). However, the evaluation of the magnitude, duration, and efficacy of the CD8$^+$ T cell immune responses induced by these immunogens has been difficult to ascertain in humans due to the limited availability of appropriate, quantitative, antigen-specific methods.

Recent advances in biotechnology have made it feasible to identify and produce recombinant proteins expressing key microbial antigens/epitopes. This has been particularly important in the case of pathogens that have proven to be difficult if not impossible to grow *in vitro*. The corresponding subunit vaccine candidates are being produced as recombinant or synthetic polypeptides, DNA, as well as recombinant bacterial or viral vectors capable of inducing antibodies as well as T cell responses (Liljeqvist and Sthal, 1999). Unfortunately, however, relatively little is known regarding the most efficient immunization strategy for activation and expansion of effector and particularly memory T cells. Moreover, the exact level of activated T cells required for full protection against most infectious organisms remains to be determined. Resolving these questions will be important for the development of new effective vaccines against a variety of pathogens. These include vaccines for newly emerging infections (HIV, Ebola, and hepatitis C), several intracellular parasitic diseases such as malaria, leishmaniasis, and trypanosomiasis, as well as certain malignancies.

Here we review the evidence that the effective booster of T cell-mediated responses requires a different form of antigen/epitope carrier than that used for priming and that recombinant (re) poxviruses are excellent vectors providing a most effective booster.

PRIMING OF SPECIFIC CD8$^+$ T CELL RESPONSES BY SUBUNIT VACCINES

The recent realization that CD8$^+$ and also certain CD4$^+$ T cells play an important role in cell-mediated protective immunity has stimulated intensive research to identify the mechanisms involved in their activation, an essential requisite to advance the rational development of subunit vaccines. This finding has also given impetus to the development of new methodologies to characterize and quantify the T cell responses to different immunogens.

Many delivery systems have been investigated, such as recombinant bacteria, viruses, naked DNA, RNA, as well as various coated particles and synthetic peptides. Overall, the relative ease with which *primary* CD8$^+$ T cell responses can be elicited by immunization with each of these immunogens has been established. However, a

[1] To whom correspondence and reprint requests should be addressed. Fax: 34-91-585-4506. E-mail: mesteban@cnb.uam.es.

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significant *in vivo* expansion of primary $CD8^+$ T cell responses is difficult to achieve. Indeed, as shown in different systems, the magnitude of $CD8^+$ T cell responses induced by a single immunizing dose of these immunogens peaks shortly after immunization and falls thereafter, remaining detectable for several weeks and sometimes even for months (Matloubian *et al.,* 1994; Zimmerman *et al.,* 1996; Murata *et al.,* 1996). Once the $CD8^+$ T cell response is established, the number of antigen-specific T cells remains relatively stable; however, the number of antigen-specific T cells cannot be increased in spite of repeated administration of the same immunogen (Murata *et al.,* 1996).

The inability of the specific $CD8^+$ T cells induced in the primary immune response to increase in number could be due to the antagonistic effects exerted by other simultaneously elicited immune responses, such as neutralizing antibodies and/or certain T cell subsets. These might inhibit the infectivity of the vector and/or accelerate its clearance by the reticuloendothelial system. However, failure to induce a significant secondary $CD8^+$ T cell response also occurs after repeated immunization with MHC class I-restricted nonamer peptides (Miyahira *et al.,* 1998) and plasmid DNA (Schneider *et al.,* 1998). In these cases, it appears unlikely that these vectors could have stimulated additional immune responses that could affect their capacity to induce secondary responses. An intriguing alternative explanation to this phenomenon could be found in an intrinsic inability of primed $CD8^+$ T cells to divide during a prolonged period of time or to a high mortality rate by apoptosis. Regardless of the molecular mechanism(s) which inhibits the expansion of *in vivo* induced primary $CD8^+$ T cell responses, it has been shown that this lack of expansion can be overcome by using an immunization strategy which combines different re vectors expressing the same antigen or epitope (Li *et al.,* 1993; Rodrigues *et al.,* 1994; Murata *et al.,* 1996; Schneider *et al.,* 1998; Sedegah *et al.,* 1998; Robinson *et al.,* 1999; Ramshaw and Ramsay, 2000).

HIGHLY EFFICIENT SECONDARY T CELL RESPONSE ELICITED BY RECOMBINANT POXVIRUSES

The initial striking observation of the potent booster effect of recombinant vaccinia viruses was made by us in 1993 (Li *et al.,* 1993). The key finding was that priming mice with recombinant influenza (reFlu) expressing a $CD8^+$ T cell epitope of the *Plasmodium yoelii* CS protein followed by a booster with recombinant vaccinia viruses (reVV) expressing the same epitope enhanced greatly the specific $CD8^+$ T cell response. Mice immunized according to this protocol displayed not only a strong secondary CS-specific $CD8^+$ T cell response—a 10- to 20-fold increase in magnitude—but also, most importantly, a considerable degree of protection against malaria infection (Li *et al.,* 1993; Rodrigues *et al.,* 1994; Murata *et al.,* 1996). In sharp contrast, the reverse protocol, namely priming with reVV followed by a booster with reFlu, failed to elicit a strong secondary T cell response or protection against malaria challenge. Similar results were obtained in mice immunized with irradiated *P. yoelii* or *Plasmodium falciparum* sporozoites in which the $CD8^+$ T cell response could not be enhanced by repeated exposure to these parasites. Moreover, it was found that parasite-induced CS-specific $CD8^+$ T cell responses could be increased 10- to 20-fold after booster with a reVV expressing the CS epitope (Miyahira *et al.,* 1998). These results clearly demonstrate that reVV is essential for the induction of the large booster effect.

In recent studies using the *P. yoelii* malaria model, it was shown that priming with recombinant virus-like particles (VLPs) derived from a yeast retrotransposon (TyVLPs), carrying the *P. yoelii* CS CTL epitope, followed by boosting with a reVV expressing the CS protein, induced strong secondary $CD8^+$ T cell responses that protected 62% of mice against sporozoite challenge (Oliveira-Ferreira *et al.,* 2000). This is important since recombinant VLPs containing HIV antigens have already been tested in clinical trials in humans with no adverse reactions (Weber *et al.,* 1995; Peter *et al.,* 1997).

In studies in which the immunogenicity of different viral vectors expressing the $CD8^+$ T cell epitope of the *P. yoelii* CS protein was compared, it was found that each of these viral vectors, i.e., Sindbis, Adeno, influenza, and vaccinia viruses, induced a considerable CS-specific $CD8^+$ primary response (Murata *et al.,* 1996; Rodrigues *et al.,* 1997; Tsuji *et al.,* 1998). However, the *in vivo* expansion of an established $CD8^+$ T cell response was difficult to achieve unless a specific combination of immunogens were used as primers and boosters. The fact that other recombinant viruses (Murata *et al.,* 1996), DNA (Schneider *et al.,* 1998), synthetic peptides (Miyahira *et al.,* 1998), and virus-like particles (Oliveira-Ferreira *et al.,* 2000) are incapable or rather inefficient at inducing large secondary $CD8^+$ T cell responses suggests the existence of severe constraints for the *in vivo* expansion of memory T cells. Importantly, these studies indicated that recombinant vaccinia virus is the vector that most efficiently boosts memory $CD8^+$ T cell responses.

The enhancement provided by re vaccinia virus is not restricted to malaria antigens since enhancement also occurred in mice primed with influenza virus and boosted with a reVV expressing the influenza nucleoprotein (NP). The combined immunization also resulted in a greatly enhanced secondary anti-NP-specific $CD8^+$ T cell response (Murata *et al.,* 1996). An equally enhanced T cell response was observed upon immunization with the V3 CTL epitope of the envelope (env) protein of the HIV-1 virus, expressed by reFlu, followed by a booster with reVV expressing the entire env protein (Gonzalo *et al.,* 1999).

BOOSTER EFFECT OF ATTENUATED AND REPLICATION-DEFICIENT POXVIRUSES

In areas where there is a high prevalence of HIV-induced immunosuppression, vaccine safety is particularly important. Therefore, there has been particular emphasis on developing nonpathogenic antigen delivery systems capable of inducing effective $CD8^+$ T cell responses. Synthetic peptides modified by the addition of lipid moieties and recombinant VLPs, and possibly also naked DNA, fulfill these requirements as safe vaccines for human use. Although the T cell response to priming with each of these immunogens is relatively low, it can be greatly enhanced when reVV expressing the corresponding antigen was used for booster.

Importantly, we and others observed that the booster effect of reVV is not dependent on its infectivity, since strong secondary T cell responses and protection against malaria can be elicited by attenuated reVV. In these studies, priming of mice with reFlu virus followed by boosting with the replication-deficient vaccinia virus mutant M7.PYCS (Rodrigues *et al.,* 1994) or priming with a plasmid DNA followed by boosting with the attenuated modified vaccinia virus Ankara strain (Schneider *et al.* 1998) induced higher numbers of specific $CD8^+$-specific T cells and a higher degree of protection than repeated immunization with the same vector.

A most promising attenuated vaccinia strain is the modified vaccinia virus Ankara (MVA), a deletion mutant which resulted from serial passages of the parental VV Ankara strain in chick embryo fibroblast CEF cells, a process during which the virus lost the capability to replicate in mammalian hosts (Mayr *et al.,* 1978; Meyer *et al.,* 1991; Sutter and Moss, 1992). Since MVA has been tested in humans during the smallpox vaccination campaign and proved to be safe, this viral strain has become the vector of choice for booster of vaccine candidates. Specific characteristics of the MVA vector, such as lack of expression of several immunomodulatory proteins and induction of type I IFN (Blanchard *et al.,* 1998), may explain the greater immunogenicity of recombinant viruses based on MVA. This has also been noticed in other studies with MVA recombinants expressing simian immunodeficiency virus (SIV) and influenza antigens (Sutter *et al.,* 1994; Moss *et al.,* 1996).

The work by Degano *et al.* (1999) has shown that in the prime/boost regime, large $CD8^+$ T cell responses and levels of protection were achieved when using DNA delivered by intramuscular injection or a gene gun followed days after by iv administration of reMVA. Furthermore, the simultaneous enhancement of the $CD8^+$ T cell response against two epitopes from different pathogens, HIV and Plasmodium, has been achieved in mice by the DNA/reMVA prime/boost regime, using DNA and MVA vectors, both expressing a multiepitope polypeptide (Hanke *et al.,* 1998).

In another study, it was shown that recombinant VLPs derived from a yeast retrotransposon (TyVLPs), containing the CTL epitope of the *Plasmodium berghei* CS protein, was found to induce specific antimalaria CTLs (Allsop *et al.,* 1996). Combined immunization with TyVLPs followed by the administration of reMVA expressing the *P. berghei* CS protein CTL epitope provided 95% protection against malaria in mice (Gilbert *et al.,* 1997; Plebanski *et al.,* 1998; Schneider *et al.,* 1999).

An important finding was that the efficiency of the combined prime/boost approach to induce high levels of $CD8^+$ cells is not restricted to murine models. The prime/boost regime using a nonreplicating poxvirus to boost the immune response elicited by a different vector has also been successfully applied in macaques. Immunization of macaques (*Macaca nemestrina*) by priming with DNA and boosting with a recombinant fowlpox (reFPV), both encoding HIV-1 env, gag, and pol antigens, was found to generate a marked enhancement of HIV-1-specific CTL and Th responses and protection against a nonpathogenic HIV-1 challenge (Kent *et al.,* 1998). Neither of these two vectors was by itself able to generate a consistent CTL response. In another study in rhesus monkeys, using several immunization protocols, a T cell-mediated protective immune response against chimeras between simian and human immunodeficiency viruses was only obtained by priming with DNA followed by boost with reFPV (Robinson *et al.,* 1999). Further demonstration of the efficacy of the heterologous prime/boost approach in macaques was provided by Hanke *et al.* (1999), demonstrating that high levels of $CD8^+$ T cells specific for the gag protein from SIV were induced in rhesus macaques by sequential immunization with plasmid DNA and a reMVA.

The immunological mechanisms of the booster effect of reVV on the expansion of $CD8^+$ T cells are not yet known. It is currently accepted that during priming with a variety of vectors (i.e., DNA, VLPs, live virus), there is a moderate activation of $CD8^+$ T cells triggered by a few epitopes and that after booster with reVV expressing the same antigen there is expansion of the specific $CD8^+$ T cell population in addition to the priming effect on naive immune T cells. The prime antigen-specific memory cells can react faster and expand more rapidly. The potent $CD8^+$ T cell expansion effect of reVV might also be related to virus-induced activation of immunomodulators. Indeed, VV infection of animals can trigger the induction of some cytokines (Gherardi *et al.,* 1999). Considering that poxviruses encode a large number of proteins that control the host immune responses such as complement, interferons, other cytokines, and chemokines (Alcami and Koszinowski, 2000), it is likely that many of these soluble inhibitors influence the extent of immune responses. Significantly, MVA does not encode many of the soluble cytokine receptors, like IFN-α/β, IFN-γ, TNF, or the virus chemokine binding protein. Future studies

should aim at modulating the poxvirus vector immunogenicity by the selective inactivation of viral inhibitors on immune functions and/or by the incorporation in the vector genome of selected cytokines. In this way, we might be able to further enhance and modulate the specific $CD8^+$ T cell responses to selected antigens induced by recombinant poxvirus vectors.

In conclusion, these studies demonstrate that there are relatively few restrictions for the induction of primary specific $CD8^+$ T cell responses by immunization with different vectors. However, a significant expansion of these cells during secondary T cell responses only occurs under very restricted conditions of immunization. Such an expansion is most efficiently accomplished by using re poxviruses expressing the same foreign antigen/epitope. The promise of the prime/boost immunization approach is highlighted by several human vaccine trials against malaria, HIV, and melanoma, currently approved or in the planning stage, all based on prime/boost immunization using recombinant poxvirus vectors. We hope that this or a related approach, and the corresponding findings, will provide the basis for the design of new, safe, and effective vaccines against various pathogens which still cause severe human diseases.

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