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DNA Vaccine for the Prevention and Treatment of Tuberculosis

Contents

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Abstract

Traditional systems for developing drugs and vaccines are failing spectacularly to deliver the goods in the fight against tuberculosis (TB). The disease that afflicts the developing world defies the imagination in its scale. One third of the world's population – 2 billion people – is infected with *Mycobacterium tuberculosis*, and 16 million have active TB. Shockingly, TB hit an all-time high in 1999 with 8 million new cases – 95% of them in developing countries – and 2 million deaths. The disease is spreading rapidly throughout the world. The toll is set to rise; AIDS activates the dormant form of the disease, while multidrug resistance is spreading across the planet.

The last new drug for TB was introduced over thirty years ago and industry has been reluctant to invest in discovering new families of drugs because of the financial risks in investing in products destined largely for developing country markets. If global health is left to market forces, historians will remember this era as one in which humanity stood idly by while half the planet languished in sickness. Fortunately some researchers have realized this, and are driving forward new models for TB therapy and vaccine discovery. One of the latest sign of this trend is the development of a DNA vaccine for the prevention and treatment of TB by our research group.

Over the last few years, some of our experiments in which mycobacterial antigens were presented to the immune system, as if they were viral antigens (DNA vaccine), have had a significant impact on our understanding of protective immunity against tuberculosis. They have also markedly enhanced the prospects for new vaccines. We now know that individual mycobacterial-protein antigens expressed from DNA-vaccine constructs can confer protection equal to that from live BCG vaccine in mice. A critical determinant of the outcome of immunization appears to be the degree to which antigen-specific cytotoxic T cells are generated by the immune response. We have demonstrated that DNA vaccination is an effective way of establishing long lasting cytotoxic T-cell memory and protection against tuberculosis. Moreover, our new preclinical work shows that DNA vaccines, initially designed to prevent infection, can also have a dramatic therapeutic action. In infected mice, the immune response can be caused to switch from one that is relatively inefficient and gives bacterial stasis to one that kills the bacteria, eliminating the persistant bacteria simply by giving DNA vaccination. We can speculate that application of such immunotherapy, in conjunction with conventional chemotherapeutic antibacterial drugs, might result in faster or more certain cure of the disease in man. Furthermore, similar vaccines used prophylactically and therapeutically might be able to both prevent establishment of this persistent state and eliminate it if it is already established.

Key words: Tuberculosis; DNA vaccination; Cytokines; Th1/Th2, costimulatory and adhesion recptors, PLGA microspheres.

Background

Tuberculosis kills 3 million people every year (Kochi, 1991; Snyder, 1994). The disease is due to respiratory infection with *Mycobacterium tuberculosis* and the World Health Organization places its hope to bring tuberculosis under control on a combination of vaccination with bacillus Calmette-Guérin (BCG) to boost immunity and antibacterial drug treatment to directly kill the bacteria (Kochi, 1991). Despite these efforts, there are still 10 million new cases and 3 million deaths worldwide every year, mainly in developing countries, and this changes little from year to year (Snyder, 1994). The disease is now increasing again in the more affluent countries that have been so complacent about the situation elsewhere. Increasingly the disease is often caused by multi-drug-resistant varieties of *M. tuberculosis*, and it is then not only highly infectious but also essentially incurable (Fine, 1989; Snyder, 1994). HIV-infected people are exquisitely susceptible and represent an increasing fraction of population everywhere. Moreover, there are few signs of new antituberculosis drugs appearing in the near future; but there is real hope that recombinant DNA techniques will quickly produce major advances in the development of materials for vaccination.

At present, nothing is being done to prevent the emergence of disease in these individuals. Chemoprophylaxis of latent infections can reduce the risk of reactivation, but it is not widely used because it is deemed too costly and too dificult to administer. Elimination of tuberculosis as a public health problem will not be easily achieved without an effective and affordable intervention targeting this vast reservoir of contagion.

Why is tuberculosis so difficult to treat? The drugs themselves are not at fault: the same compounds that are so poorly effective *in vivo* will sterilize a culture of *M. tuberculosis in vitro* within days. The possibility of suboptimal penetration of drugs into tuberculous tissue has also been eliminated by analysis of lung tissue from patients who were injected with a radiolabeled drug prior to surgery. The mysterious physiological state of the persistent organisms may hold the key to the problem, because conventional drugs are ineffective against bacteria that have exited the cell-division cycle and become stationary.

Current anti-tuberculosis regimens require administration of multiple drugs for a minimum of 6-9 months. Without directly observed therapy, patient adherence to such lengthy and complex regimens is poor, resulting in righ rates of treatment failure, relapse, and development of bacterial drug resistance. Directly observed therapy, in which a health care provider or social worker administers each dose of drugs, dramatically improves patient adherence. At present, however, only 15% of patients worldwide receive the directly observed therapy regimen recommended by the World Health Organization. In these circumstances, immunotherapy to boost

the efficiency of the immune system in infected patients could be a valuable adjunct to antibacterial chemotherapy. We have shown in mice that DNA vaccines can switch the immune response from one that is relatively inefficient and gives bacterial stasis to one that kills bacteria. Application of such immunotherapy in conjunction with conventional chemotherapeutic antibacterial drugs might result in faster or more certain cure of the disease in humans.

The BCG vaccine is in widespread use against tuberculosis but has doubtful impact on the global position. It is a live vaccine, derived from *M. bovis* by virulence attenuation during prolonged cultivation of the bacteria in the laboratory. In ten randomized controlled trials of BCG vaccines carried out since 1930, the protective efficacy against tuberculosis has ranged from 0 to 80% in different populations (Fine, 1988, 1989). Thus, BCG is far from being an ideal vaccine against tuberculosis and in several countries it is virtually unused, not only because of doubts about its efficacy, but also because it prevents the subsequent use of skin-sensitivity tests to detect tuberculosis infection (Fine, 1989). Therefore, a new vaccine that contained only a few key protective antigens could be the answer, but how did we identify the protective mycobacterial antigens amongst the hundreds comprising the whole of the microorganisms? The classical approaches of separating bacterial components according to chemical, physical or immunological properties and testing the purified antigens has been disappointing because only modest protective effects have been obtained in non-human models. Initially, our answer was to take the cloned genes encoding some of the most immunologically prominent mycobacterial protein antigens and express them directly in antigen-presenting cells in mice (Silva et al., 1992; Lowrie et al., 1994); we used DNA vaccination or a retroviral vector to transfect antigen-presenting cells to do this.

Immunization With a Retroviral Vector

The initial steps to this realization were taken when we used a retroviral shuttle vector to express the hsp65 antigen of *Mycobacterium leprae* in the monocyte-like tumor cell line J774 (BALB/c origin) (Silva et al., 1992). The cells presented the expressed antigen for specific recognition by T cells of the $CD4^+$, $CD8^+$, and g/d TCR types (Silva et al., 1993). When BALB/c mice were given a series of injections (ip or iv) of the transfected cells (J774-hsp65), they acquired a remarkably high degree of protection against a subsequent ip or iv challenge infection with virulent *Mycobacterium tuberculosis* H37Rv; bacterial numbers declined exponentially in internal organs and were, for example, 100-fold lower in livers after 5 weeks compared to control animals (Silva & Lowrie, 1994; Silva et al., 1994). Injection of the protein with adjuvant was

ineffective. Protection was dependent on tumor-cell viability and was antigen specific; for example, there was no protection against challenge with *Listeria monocytogenes* or when C57BL/6 mice (haplotype d) were used (Lowrie, 1994; Silva et al., 1994). The same retroviral construct was also used to transfect bone-marrow cells *in vitro*; and these cells were also able to generate specific protective immunity when they were used to reconstitute lethally gamma-irradiated mice (Silva et al., 1995). Evidently, features unique to tumor cells were not essential for the protective response. Thus we investigated whether the virus-like endogenous origin of the antigen synthesized by the transfected cell was the key.

Immunization With Plasmid DNA

DNA vaccination is an alternative means of generating endogenous antigen; and we reported the first evidence that this too could be used to generate protective anti-tuberculosis immunity at the first World Health Organization DNA vaccine meeting in 1994 (Silva et al., 1994). We have obtained similar results with two different promoters, those for CMV immediate early gene and murine hydroxymethylglutaryl-CoA-reductase, expressing a range of individual mycobacterial antigens following direct injection of the plasmid DNA into muscle (2 x 50 mg on 3 or 4 occasions at 3-4 week intervals) (Lowrie et al., 1997). Effective genes were those encoding hsp65, hsp70, 36 kDa proline-rich antigen or ESAT-6 and the degree of protection could equal that obtained with live BCG, depending on mouse strain and antigen (Lowrie et al., 1997). Analysis of the immune response to DNA expressing hsp65 showed that it was predominantly of Th1 type (abundant IFN-γ, minimal IL-4) by ELISA and RT-PCR assays of tissues and antigen-stimulated T cells. Cytotoxic $CD8^+$ T cells responded to peptides representing putative MHC class I restricted epitopes of hsp65 (Lowrie et al., 1997; Lowrie et al., 1997[a]; Lowrie et al., 1997[b]).

Therapy of Tuberculosis by DNA Vaccination

Our studies in mice show that DNA vaccines, initially designed to prevent infection, can have a dramatic therapeutic action too. In infected mice, the immune response can be caused to switch from one that is relatively inefficient and gives bacterial stasis to one that kills the bacteria, eliminating the persistent bacteria simply by giving DNA vaccination (Lowrie et al., 1999).

Since the development of the bactericidal cytotoxic component of the immune response appears to be more elicited by DNA vaccination than by mycobacterial infection, we asked whether DNA vaccination would be beneficial against

an established tuberculosis infection. An infection initiated by intravenous injection of virulent *M. tuberculosis* H37Rv was allowed to develop for 8 weeks, during which the number of bacteria in the internal organs slowly increased about 4-fold, reflecting an essentially bacteriostatic immunity. Then the mice were given a series of 4 doses of plasmid DNA intramuscularly at 2-week intervals. The numbers of live bacteria in internal organs were rapidly declining 2 months and 5 months after the first dose of DNA when the plasmid expressed antigen hsp65. Much smaller effects were obtained with plasmid expressing other mycobacterial antigens (70 kDa heat-shock protein or 6 kDa early-secretory antigen; hsp70, ESAT6); and this parallels the lower efficacy of these antigens in prophylactic DNA vaccination (Lowrie et al., 1999). There was virtually no change induced with plasmid that contained no insert, or by a single dose of the standard BCG vaccine, compared to untreated or saline-treated control mice. The therapeutic effect of DNA vaccination was associated with a switch from a predominantly Type 2 to a predominantly Type 1 immune response as judged from frequencies of lymph node T cells producing IFN-γ or IL-4 vaccination (Lowrie et al., 1999). DNA vaccine therapy was equally effective against an established infection with a clinical isolate of *M. tuberculosis* that is resistant to isoniazid, one of the main chemotherapeutic drugs; and an identical change in T cell phenotypes was observed in vaccination (Lowrie et al., 1999).

When modern chemotherapy fails, it most often does so as a result of re-growth of fully drug-sensitive bacteria that have persisted in a non-replicating and physiologically drug-resistant form. We tested whether the addition of DNA vaccination at the end of chemotherapy could reduce this problem. Three intramuscular doses of hsp65 DNA vaccine appeared to eliminate residual bacteria from a proportion of the mice when the DNA was given after chemotherapy. Injections of immunossuppressive corticosteroid were unable to reactivate bacterial growth, indicating a sterilizing effect. The proportion of mice with spleens or lungs that appeared to be sterile increased significantly as the number of DNA doses was increased in vaccination (Lowrie et al., 1999).

It appears that in tuberculosis the killing component of the immune response is inadequate because it is under-evoked. This can be corrected by enhancing the contribution made by antigen-specific cytotoxic T lymphocytes that kill *M. tuberculosis* when they lyse infected macrophages. This correction can be applied by DNA vaccination and is effective even in heavily infected mice, where one might imagine the immune system being strongly committed by the antigen burden towards the established inadequate responses elicited by other vaccines (Lowrie et al., 1999).

Immune Response Elicited by DNA Vaccine

Type 1 Cytokines

A striking difference between the immune response to DNA vaccination and the immune response to either BCG or *M. tuberculosis* infection is that DNA induces almost entirely a protective Th1 response, whereas the mycobacterial infections have a major component of non-cytotoxic T cells that produce a Th2 response (Bonato et al., 1998). During infection or after immunization, CD4 and CD8 hsp65-reactive T cells increased equally in spleens. During infection, the majority of these cells was $CD44^{lo}$ and produced IL-4, whereas after immunization the majority were $CD44^{hi}$ and produced IFN-γ. In adoptive transfer of protection to naive mice, the total CD8-cell population purified from spleens of immunized mice was more protective than that from infected mice. When the cells were separated into CD4 and CD8 types and then into $CD44^{hi}$ and $CD44^{lo}$ types, $CD44^{lo}$ cells were essentially unable to transfer protection. The most protective $CD44^{hi}$ cells were CD8 and those from immunized mice were much more protective than those from infected mice. Thus, whereas the $CD44^{lo}$ IL-4-producing phenotype prevailed during infection, protection was associated with the CD8/$CD44^{hi}$ IFN-g-producing phenotype that predominated after immunization. This conclusion was confirmed and extended by analysis of 16 hsp65-reactive T-cell clones from infected mice and 16 from immunized mice; the most protective clones, in addition, displayed antigen-specific cytotoxicity (Bonato et al., 1998).

Persistence of Memory for Specific Cytotoxicity After DNA Vaccination

In an experiment comparing different means of immunizing with hsp65, we have now established that protocols which generate the antigen endogenously (J774-hsp65 or DNA vaccination), or that deliver the antigen into the cytosol (protein-loaded liposomes), generate protection and this is associated with a strong $CD8^+$ response in which $CD44^{hi}$ memory-associated cells are prominent (Lowrie et al., 1997[a]). The greatest protection was seen 1 week after immunization with J774-hsp65, but declined substantialy by 8 months. Liposomes and DNA vaccination also gave substantial early protection equal to BCG. Whereas protection after liposomes declined like the protection after J774-hsp65, protection after hsp65-DNA was sustained like the BCG-induced protection. DNA vaccination also induced a sustained increase in the proportion of $CD8^+$ splenocytes that were $CD44^{hi}$. The frequencies of cytotoxic hsp65-responsive cells among $CD44^{hi}/CD8^+$ splenocytes at different intervals after the various vaccination protocols show that at 1 week after immunization the highest frequency was observed with J774-hsp65 where the frequency reached 1 in 8, which had substantially declined by 8 months after immunization. Hsp65-liposomes

and hsp65-DNA also resulted in high frequency of $CD44^{hi}$ cells at 1 week (about 1 in 200), which subsequently declined in the case of hsp65-liposomes but increased to 1 in 12 by 8 months in the case of DNA vaccination. This rising frequency of cytotoxic hsp65specific $CD8^+$ memory cells was also seen after BCG vaccination. One hypothesis to account for these findings is that only live BCG and DNA vaccination provides a persistent source of intracellular antigen and IFN-γ which is needed to sustain the cytotoxic T cell memory required for long-lasting protection.

The Importance of Macrophages and a Cytotoxic T Cell Response

Despite many years of research, the effector mechanisms by which *Mycobacterium tuberculosis* is killed, when the immune response mounts its most successful defense, remain contentious. Bacteriostasis is the most prominent feature of immunity, and is essential in the absence of effective bactericidal processes, but probably also contributes to bacterial dormancy and persistence. Stronger bactericidal processes would be preferred in order to minimize the problems posed by reactivation of dormant infection many years later.

Adoptive transfer experiments have established beyond doubt that protection is cell-mediated and not antibody-mediated. However, the evidence that protection requires activation of macrophages by antigen-specific T lymphocytes, so that the macrophages acquire an ability to kill the mycobacteria, remains less than compelling. Our new evidence suggests that cytotoxic T cells may also directly kill the bacteria, depending on their ability to deliver potent bactericidal proteins such as granulysin from their granules. It could be important, therefore, for any new vaccines to boost such killing mechanisms (Silva & Lowrie, 2000).

Although adoptive transfer of protection with T lymphocytes from infected or immunized rats into naive animals established over 20 years ago that acquired immunity is cell-mediated (Kochi, 1991), attempts to define the phenotype and function of the protective T cells have given conflicting results. CD4+, CD8+, and g/d -TCR T cells have all been implicated, as have IFN-γ and cytotoxicity, without establishing how they actually contribute to protection (Fine, 1988, 1989; Snyder, 1994). The accepted paradigm has been that protection is mainly due to antigen-specific CD4+ Th1 cells that produce IFN-γ to activate macrophages that then kill the mycobacteria during phagocytosis. Bacteria that are not killed by this process have their multiplication inhibited inside IFN-γ-activated macrophages. A subsidiary function has been served by cytotoxic CD8+ cells that release intracellular bacteria from infected cells so that they can be killed during phagocytosis by activated macrophages (Silva et al., 1992). Even allowing for some crossover between the activating and cytotoxic functions of CD4+ and CD8+ cells, it is now clear that this is not the whole story.

Involvement of CD8+ T cells in protective immunity against tuberculosis has been recognized for some time (Silva et al., 1992, 1993; Lowrie et al., 1994), although the antigen specificity and function of these cells was not clear. Recently, significant progress has been made from several directions. The discovery that immunization of mice with a single mycobacterial antigen (hsp65) could give substantial protection against tuberculosis challenge (Silva & Lowrie, 1994; Silva et al., 1994) led to analyses in which the diversity of antigens recognized by T cells responding to *M. tuberculosis* was no longer a variable. The key to eliciting protection was in the use of immunization procedures favoring presentation as an endogenous antigen. Thus, *in vivo* expression from retroviral vector-transfected bone-marrow cells, or from a transfected macrophage-like cell line, or DNA vaccination of muscle or skin, or intravenous cationic liposome delivery of the protein were all effective (Silva et al., 1995). Endogenous antigen favored responses from MHC class I-restricted CD8+ T cells. Adoptive transfer of protection with hsp65-specific T cell lines or clones raised from such immunized animals showed that, individually, the most protective were indeed CD8+ cells, although CD4+ and g/d T cells also protected; and marked synergy occurred with all three types transferred together (Lowrie et al., 1995; Silva et al., 1996). Protection partly reflected the ability of the cells to produce IFN-γ; IL-4 producing cells were not protective and protection with IFN-γ producing cells was decreased by administering antibody against IFN-γ. However, the most protective CD4+ and CD8+ clones also displayed antigen specific cytotoxicity *in vitro* and selectively lysed macrophages that were infected with *M. tuberculosis* (Silva et al., 1996; Lowrie et al., 1997). This is consistent with the view (Silva et al., 1992) that cytotoxicity also has a positive role in protection, but what we think that role is depends on how we think the mycobacteria are actually killed.

IFN-γ is now known to be absolutely essential for protection (Lowrie et al., 1997[a]; Lowrie et al., 1997[b]). This could be largely due to its role in activating macrophages for mycobacteriostasis (Silva et al., 1992; Bonato et al., 1998), rather than for killing; but it also impacts many other aspects of immunity besides macrophage activation (Boehm et al., 1998). Similarly, there is compelling evidence for the importance of nitric oxide production by macrophages in protection (Flesch & Kauffman, 1991; Chan et al., 1992, 1995; MacMicking et al., 1997), but this might be more due to a regulatory function of this key intracellular signaling agent (Satriano & Schlondorff, 1994; Arnold et al., 1977; Benbernou et al., 1997) upstream from the actual lethal events, rather than due to direct toxicity of nitric oxide for the bacteria. We can question, in fact, whether active killing by macrophages has a major role in either the initial arrest of bacterial population growth or in the subsequent slow decline in population.

It seems likely that appropriately activated macrophages can indeed sometimes kill virulent *M. tuberculosis* (O'Brien et al., 1991; Chan et al., 1992; Sato et al., 1998), but are not usually sufficient for sterilizing immunity. Effects seen *in vitro* are generally modest and the target is clearly a difficult one for macrophages to kill. Extensive efforts have produced only sporadic claims to demonstrate substantial killing and independent confirmatory evidence is sparse, particularly for human cells (Warwick-Davies et al., 1994). Possibly the right combination and sequence of differentiation and activation signals (Adams & Hamilton, 1992) and a high rate of replacement of expanded macrophages occurs *in vivo*, particularly in the high-turnover granulomas characterizing the earlier stages of the immune response (Papadimitriou & Spector, 1971). This might provide major and sustained bactericidal action but has not yet been reproduced *in vitro*.

Since killing by macrophages seems so inefficient, we should take note of the increasing evidence that some T lymphocytes can directly kill the bacteria. Macrophages may not even be the main source of bactericidal products in protection against TB after all. Perhaps the first indication of this came from evidence that intracellular BCG could be killed when human monocytes containing the bacteria were lysed by specific lymphocytes which induced apoptosis by releasing ATP (Molloy et al., 1994; Lammas et al., 1997). Virulent *M. tuberculosis* has not been reported killed by this mechanism and other inducers of apoptosis or necrosis were ineffective. It has not been established what the toxic factor is that is generated under these conditions. Similarly, Pascal Meylan and associates (Oddo et al., 1998) have reported that cytotoxic CD4+ T lymphocytes from man killed virulent *M. tuberculosis* when they lysed infected macrophages by the Fas-FasL pathway. The bacteria were also killed when apoptosis was induced with TNF-α, suggesting that the lethal product came from the macrophage rather than the lymphocyte.

When we examined 28 CD4+/CD8- and 28 CD4-/CD8+ T cell clones that were specific for hsp65 and were derived from J774-hsp65 or DNA-vaccinated or *M. tuberculosis* infected mice, more than half were cytotoxic for macrophages infected with *M. tuberculosis* while none were cytotoxic for uninfected macrophages (Silva & Lowrie, 2000). Cytotoxicity was measured as release of ^{51}Cr. The cytotoxicity of all except 2 of the 14 cytotoxic CD4+/CD8- clones was inhibited by antibody against either Fas or FasL and not by incubation with the degranulating agent Sr^{2+} prior to cell/cell contact. The remaining 2 clones were inhibited by Sr^{2+} and not by the antibodies. In contrast, 15 of the 18 cytotoxic CD4-/CD8+ clones were inhibited by Sr^{2+} and only 3 were inhibited by anti-Fas or anti-FasL (Silva & Lowrie, 2000). Although the 32 cytotoxic clones all showed similar degrees of cytotoxic activity (^{51}Cr release), they differed markedly in their antibacterial activity during lysis of infected

macrophages. Only clones that used the granule-mediated pathway (Sr^{2+}-inhibitable) caused > 50% decrease in the viability of *M. tuberculosis* when the clones were incubated with infected macrophages for 24 hours (Silva & Lowrie, 2000). The loss of bacterial viability caused by these clones ranged up to 95%. Furthermore, there was a close correlation (r^2 = 0.9218, P<0.0001) between antibacterial activity and the granule content of the clones as measured by the availability of a granule marker enzyme, BLT esterase, for release by Sr^{2+} (Silva & Lowrie, 2000). Loss in bacterial viability was abrogated by prior degranulation of the clones. The magnitude of the decrease in viability during 24 hours and its correlation with cytotoxic granule content suggest that T-cell granule enzymes probably actively kill the bacteria. These are discharged into the infected macrophages during the lytic process and most likely contain a murine homologue of human enzyme granulysin that has been shown to kill virulent *M. tuberculosis* when granular cytotoxic T lymphocytes lyse infected monocytes. This would explain that part of the contribution of these cells to protection which is not inhibited by antibody against IFN-γ (Silva & Lowrie, 2000).

In summary, several distinct bactericidal mechanisms may operate in cellular immunity against tuberculosis. Different mechanisms may predominate at different stages of infection, to kill both actively multiplying and dormant bacteria, and cumulatively decrease the duration of persistence of the infection. It is likely that both cytokines, released in response to specific antigens, and direct cell:cell interactions modulate differentiation of these mechanisms. DNA vaccines provide a new means of exploring these processes and may lead to practical vaccines that bring bactericidal rather than bacteriostatic mechanisms to the fore. The clinical impact of such developments could be substantial.

The Importance of T-Cell Migration and Costimulation in the Activation of Immune Response

For the generation of a T-cell immune response, antigens need to be presented to a T cell receptor (TCR) of the appropriate specificity by antigen presenting cells (APC). However, the functional outcome of TCR ligation varies, depending on the presence or absence of additional costimulatory signals delivered to the T cell via a number of accessory molecules, which serve as receptors for specific ligants expressed on APC. Therefore, antigenic stimulation can lead either to a productive immune response characterized by proliferation, differentiation, clonal expansion and effector function or, in the absence of appropiate costimulation, to a state of long-lasting antigen-specific unresponsiveness termed anergy (Jenkins & Schwartz, 1987; Mueller et al., 1989). One such costimulatory signal critical, most studied for both the productive outcome of the immune response and the prevention of induction of

the anergic state, is provided by the members of B7 family, B7-1 (CD80) (Freeman et al., 1989) and B7.2 (CD86) (Azuma et al., 1993), and their counterreceptor CD28 (Harding et al., 1992).

The receptors B7-1 and B7-2 display a pattern of expression on APC and, depending on type of APC, can be induced by a variety of stimuli (Boussiotis et al., 1994). B7-2 is constitutively expressed on resting human peripherical blood monocytes (Azuma et al., 1993); however, its expression is not constitutive on lung macrophages (Chelen et al., 1995). On blood monocytes, B7-2 is up-regulated following stimulation (Azuma et al., 1993), whereas B7-1 is expressed only after IFN-γ activation (Freedman et al., 1991). In contrast, IL-10 prevents the up-regulation of B7-1 and B7-2 on macrophages (Ding et al., 1993). In several studies, B7-1 and B7-2 have been shown to be constitutively expressed on dendritic cells (Larsen et al., 1994). After B cell activation, B7-2 is more rapidly expressed than B7-1 (Boussiotis et al., 1993) and all other APC display the identical sequence.

The couterreceptors for B7-1 and B7-2 are CD28 and CTLA-4, expressed on T cells. CD28 is constitutively expressed on 95% of resting CD4+ and 50% of resting CD8+ peripherical blood human T cells, and its expression increases following activation (Turka et al., 1990). In the mouse, CD28 is expressed on all T cells (Gross et al., 1992). CD28 is the low affinity but the major costimulatory receptor for B7-1 and B7-2 (Linsley & Ledbetter, 1993). The second receptor on T cells for the B7 receptors is CTLA-4, expressed on activated T cells (Brunet et al., 1987; Lindstein et al., 1993) with 20-fold higher affinity than CD28 by their ligants (Linsley et al., 1991).

Following a TCR-engagment, the costimulatory signal delivered by CD28-B7 interaction induces an increase of IL-2 mRNA transcription, IL-2 secretion, up-regulation of IL-2 receptor, T cell proliferation and interleukin secretion (Gimmi et al., 1991; Gross et al., 1992; Reiser et al., 1992). On the other hand, CTLA-4 expression has been described as a down-modulator of T cell activation (Gribben et al., 1995; Chambers et al., 1997).

Besides costimulatory receptors, other molecules have been found that can regulate the T-cell activation (Damle *et al.*, 1992, Sato et al., 1995). Most of these molecules, which belong to the integrins and immunoglobulins families, were first described such as mediators of migration process (Springer, 1990). Besides, recent evidences have shown that either costimulatory or adhesion receptors are essential to provide the intercellular contact (Wülfing & Davis, 1998; Viola et al., 1999) that induces efficient T-cell activation.

Intracellular pathogens, such as *Leishmania donovani* and *M. tuberculosis*, can survive within host cells. This surveillance is favored by ability of pathogens to escape host cellular immune response. Studies have shown that one of the escape

mechanisms can involve the down-modulation of costimulatory receptors (Kaye et al., 1994; Saha et al., 1994). We have used a model of systemic experimental tuberculosis to evaluate the expression of costimulatory (CD28, CTLA-4, B7-1 and B7-2) and adhesion receptors (ICAM-1, VLA-4 and b2 integrin) in lymphocytes and macrophages from infected and non-infected mice. The expression of these molecules was analyzed in BALB/c and C57BL/6 strains after 7, 15, and 42 days of infection with virulent *M. tuberculosis* by intraperitoneal route.

Our results showed that there was a significant *ex vivo* down-modulation of VLA-4 expression on peritoneal lymphocytes from infected BALB/c mice when compared with the cells from non-infected mice. We also detected a significant up-regulation of CD18 on peritoneal lymphocytes from infected BALB/c mice after 7 days of infection. On the other hand, peritoneal lymphocytes from infected C57BL/6 mice did not present significant down-modulation of VLA-4 expression, whereas the VLA-4 level of expression was very similar between infected and non-infected mice. We also detected a significant up-regulation of CD18 expression on peritoneal lymphocytes from infected C57BL/6 mice even after 42 days of infection and a higher number of peritoneal CD28+ lymphocytes than in BALB/c mice. However, CD86, and CD18 expression were down-modulated on peritoneal machophages from infected BALB/c and C57BL/6 mice. The ICAM-1 and CD80 level of expression between infected and non-infected BALB/c or C57BL/6 mice did not change significantly. We did not detect the *ex vivo* CTLA-4 expression either on peritoneal lymphocytes from infected BALB/c mice or on C57BL/6 mice.

The results obtained by our group showed a differential pattern of expression in VLA-4, CD18 and CD28 receptors among infected BALB/c and C57BL/6 mice. This finding suggest that the type of acquired immune response by susceptible (BALB/c) or resistent strain (C57BL/6) can also be related to the pattern of expression of cell surface receptors. The down-modulation on these receptors can contribute to the decrease of T-lymphocyte influx to the site of infection and for the impairment of intercellular contact. In this way, the levels of VLA-4 and CD18 expression by cells from infected C57BL/6 mice could explain in part the higher influx of T cells to the site of infection in this mice strain than in the BALB/c mice. However, other experiments are necessary for evaluating the exact role of these T cells in the protective response against tuberculosis. Moreover, a better undestanding and characterization of the host-*M. tuberculosis* relationshiop and its influence on the development of an efficient immune response are essential to determine whether these cell-surface receptors can be used as tools in immunotherapy or to improve the DNA vaccine.

New Strategies for DNA Vaccine Administration

DNA vaccination involves the deliberate introduction into tissues of a DNA plasmid carrying an antigen-encoding gene that transfects cells *in vivo* and results in long-lived antigen-specific humoral and cellular immune responses. Over the last few years, some of our experiments in which mycobacterial antigens were presented to the immune system as endogenous antigens (transfected macrophages, liposomes, DNA vaccine) have had a significant impact on our understanding of protective immunity against tuberculosis (Lowrie et al., 1995; Lowrie et al., 1997[b]) and hold promise for the rapid development of an effective new vaccine.

Although the use of naked DNA was able to elicit an effective immune response in our experimental model of tuberculosis, the amount of plasmid required is very high. One of the reasons for the high doses of plasmid is that the extracellular delivery injection results in degradation by nucleases after conventional administration (Levy et al., 1996). Based on these observations, the use of delivery systems capable of protecting plasmid DNA represents an interesting strategy for vaccine administration avoiding vector loss. Thus, to improve the efficacy of our vaccine or therapeutic device against tuberculosis we are testing different ways to deliver the DNA vaccine.

Concerning DNA vaccination, the levels of protein expression after direct injection are transient due to the eventual loss of unintegrated plasmid DNA from transfected nuclei (Wolff et al., 1990; Manthorpe et al., 1993). The gene-gun technique, which consists of adsorbing the plasmid onto gold microparticles to be shot at the host's dermis, has shown excellent results in terms of expression efficiency with very low DNA amounts. However, as in the intramuscular-injection method, boosters are still necessary. Thus, sustained delivery of plasmid DNA at the tissue site may represent a source of continuous gene uptake thereby providing the duration of expression. In addition to the known and efficient delivery systems used to improve the efficacy of DNA vaccines such as gene gun and encapsulation of DNA into liposomes, we are beginning to use encapsulation of DNA into polymeric microspheres that release their content in a controlled manner continuously over time (Jong et al., 1997).

Biodegradable microspheres such as those based on poly-lactic poly-glycolic co-polymers (PLGA) have the potential to act as mediators of DNA transfection targeted to phagocytic cells such as macrophages, and to protect against biological degradation by nucleases (Ando et al., 1999). PLGA is a biocompatible and biodegradable material with an extensive record of safe use in medicine (Brannon-Peppas, 1995). Microsphere uptake by antigen-presenting cells has been extensively studied and is directly related to particle diameter (Eldridge et al., 1991). Microspheres less than 10 mmm in diameter are actively taken up by cells such as macrophages, and those

smaller than 5 mmm in diameter are taken up by the M cells of the Payer's patches of the small intestine, which is of special interest in the design of oral vaccines. The ability of these systems to elicit the immune response at mucosal surfaces constitutes an additional advantage especially against pathogens that gain access trough mucosal surfaces such as the respiratory, gastrointestinal, and urinogenital tracts.

Our results have shown that plasmid DNA can be encapsulated into PLGA microspheres without compromising its functionality. Several strategies have been developed to increase the amount of entrapped DNA and to reduce particle diameter (Ando et al., 1999). We found that the double-emulsion/solvent-evaporation method used to prepare the microspheres resulted in particles with adequate features for our purpose. Particles were smaller than 10 mmm, a fundamental characteristic to target phagocytic cells, and presented good entrapment efficiency. We demonstrated *in vitro* that phagocytic cells take up PLGA microspheres which release the entrapped plasmid, which in turn is able to drive the expression of antigenic protein (Lima et al., submitted). After intramuscular injection, DNA vaccine-loaded PLGA microspheres were able to elicit similar levels of specific antibodies to intramuscular injection of naked DNA, in BALB/c mice serum.

Studies using bone marrow chimeras showed that the antigenic peptide involved in priming a CTL response is presented in the context of the MHC-encoded class I molecule of bone marrow-derived cells and not by injected myocytes (Corr et al., 1996; Doe et al., 1996). Thus, the immune responses are initiated by antigen that is acquired by antigen-presenting cells (APCs). Taken together, these observations support the need of targeting specific cells to successfully elicit an immune response after DNA vaccination. In this respect, the use of PLGA microspheres may be an interesting strategy to target DNA directly to APCs, either by intramuscular or subcutaneous route. This ability may be due to particles ranging from 1 to 10 mmm in diameter, which are too large to be nonspecifically endocytosed, and therefore are taken up by phagocytic cells such as macrophages. In addition, microsphere vaccination results in a depot after subcutaneous or intramuscular injection followed by recruiting cells that can, hypothetically, facilitate APC transfection without ulcerative manifestations at the injection site (Lima & Rodrigues Júnior, 1999). Another advantage of these systems includes administration by pulmonary route, where the particles could target macrophages in lung, which are also the *M. tuberculosis* natural infection site.

Another approach to this system is the possibility of developing a single-shot vaccine since a range of PLGA-derived polymers that present different degradation rates is available. Thus, by combining particles made of different polymers we could

obtain a formulation capable of releasing the DNA at different times and thus mimicking booster. This represents an excellent strategy, since *M. tuberculosis* kills about 3 million people every year, especially in developing countries where compliance with vaccination booster is very low.

In conclusion, our results suggest that PLGA microspheres offer an effective approach to gene delivery *in vivo*, especially to phagocytic cells. The use of this system as carrier for a DNA vaccine against tuberculosis based on the hsp65 gene could contribute to the improvement of the vaccination and therapeutic scheme in a model in which large amounts of naked DNA and boosters are required.

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