Pulmonary Necrosis Resulting from DNA Vaccination against Tuberculosis

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The use of DNA constructs encoding mycobacterial proteins is a promising new approach to vaccination against tuberculosis. A DNA vaccine encoding the hsp60 molecule of *Mycobacterium leprae* has previously been shown to protect against intravenous infection of mice with *Mycobacterium tuberculosis* in both the prophylactic and immunotherapeutic modes. It is shown here, however, that this vaccine was not effective in a more realistic aerosol infection model or in a model of latent tuberculosis in the lungs. Moreover, when given in an immunotherapeutic model the immunized mice developed classical Koch reactions characterized by multifocal discrete regions of cellular necrosis throughout the lung granulomas. Similar and equally severe reactions were seen in mice given a vaccine with DNA coding for the Ag85 antigen of *M. tuberculosis*. This previously unanticipated safety problem indicates that DNA vaccines should be used with caution in individuals who may have already been exposed to tuberculosis.

Disease caused by *Mycobacterium tuberculosis* is the leading cause of death from an infectious disease. Currently, it is estimated that over 2 million people die from tuberculosis yearly and that 8 million people contract the disease (5). Individuals who have been exposed to the bacillus but may have controlled it in the form of a latent infection, and who therefore are at risk of reactivation disease, may number in the hundreds of millions (6).

There has been a gradual realization that the existing vaccine against tuberculosis, *Mycobacterium bovis* BCG, loses its effectiveness in individuals as they pass the teenage years (3, 21), and in certain clinical trials its overall effectiveness has been zero (14). As a result there is now a concerted effort to try to identify more effective vaccines against tuberculosis, using well-characterized animal models for initial screening (16).

To date, the most effective vaccine used in mouse studies is a DNA vaccine encoding the 65-kDa heat shock protein of *Mycobacterium leprae* (hsp60/lep). Mice immunized with this DNA vaccine have shown 2- to 3-log reductions in bacterial load after intravenous challenge with virulent *M. tuberculosis*, and the vaccine is equally protective if given prophylactically, as an immunotherapeutic vaccine, or in a Cornell type model in which bacteria are eliminated by chemotherapy, leaving only bacilli that are in a latent or dormant form (12).

In the study presented here the hsp60/lep vaccine was tested using the more realistic pulmonary infection model in the mouse, in which the animal is exposed to a small challenge dose by aerosol exposure. In this model the hsp60/lep DNA vaccine was ineffective in both the prophylactic and Cornell modes. Moreover, this vaccine, as well as the previous described highly protective Ag85/TB DNA vaccine (1, 8, 25),

both induced progressively severe pulmonary necrosis in an immunotherapeutic vaccination model, especially when given to a susceptible mouse strain. While this class of DNA vaccines appears to be completely safe in terms of inoculation into naïve individuals, the data presented here suggest that if given unknowingly to individuals already exposed to tuberculosis, there may be a risk of exacerbating disease or triggering reactivation of latent tuberculosis. This previously unanticipated problem thus raises a significant safety issue.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female C57BL/6 and BALB/c mice, 6 to 8 weeks old, were purchased from the Jackson Laboratories, Bar Harbor, Maine. The mice were maintained under barrier conditions in the biosafety level 3 biohazard facility at Colorado State University. They were given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments.

Vaccinations. The DNA vaccine encoding the hsp60 protein antigen of *M. leprae* was constructed using the plasmid vector pcDNA3 (12). Ag85/TB DNA was prepared and used as previously described (1, 8). Mice received injections intramuscularly (i.m.) three times at 3-week intervals with 50 µg of hsp60-DNA per quadriceps muscle using a 30-gauge needle. As negative controls, mice received injections with control plasmid DNA or with saline. As a positive control, mice received subcutaneous injections with BCG at 10⁶ CFU in 200 µl of sterile 1× phosphate-buffered saline (single inoculation).

Animal challenge studies. In all experiments, mice were challenged by low-dose aerosol exposure with *M. tuberculosis* strain H37Rv using a Glas-Col (Terre Haute, Ind.) aerosol generator calibrated to deliver 50 to 100 bacteria into the lungs. Bacterial counts in the lungs were determined by plating serial dilutions of individual whole-organ homogenates on nutrient 7H11 agar and counting the CFU following 3 weeks incubation of at 37°C. In the prophylactic model, mice were challenged 4 weeks after the last DNA injection, and lungs were harvested for bacterial counts and histological analysis 4 and 10 weeks following aerosol infection. In the Cornell model, mice were aerogenically infected with *M. tuberculosis* 4 weeks prior to receiving isoniazid (Sigma) in their drinking water at 100 mg/ml for 60 days. Immediately following drug treatment, the mice received i.m. injections with hsp60-DNA three times as described above. Three weeks after the last DNA injection, mice received intraperitoneal injections with 200 µl of dexamethasone (Sigma) at 6 mg/kg of body weight every 2 days for 8 weeks. Lungs were harvested 18 days later for bacterial counts and histological analysis. In the therapeutic model, mice received the first hsp60-DNA injection 8.5 weeks

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FIG. 1. Prophylactic vaccination in C57BL/6 mice. Animals were given DNA or control vector DNA three times and then rested for 1 month prior to low-dose aerosol challenge with *M. tuberculosis* H37Rv. Controls received injections of saline or a single dose of BCG. Data shown are the mean numbers of bacteria recovered from the lungs 30 days after aerosol exposure ($n = 5$) (error bars, standard errors of the means). Only mice receiving BCG were protected against the challenge infection ($P > 0.01$).

after the aerosol infection. Lungs were harvested for bacterial counts and histological analysis 4 and 7 weeks after the last DNA injection.

Histological analysis. The middle right lung lobe was obtained from each mouse and immediately inflated with and stored in 10% formal saline. Tissues were processed routinely and sectioned for light microscopy such that the maximum surface area of each lobe could be viewed. Sections were then stained with

hematoxylin and eosin. Slides were examined by a veterinary pathologist with no prior knowledge of the experimental groups and were subjectively graded for both quality and quantity of cellular accumulation.

RESULTS

Prophylactic vaccination studies. In a first series of experiments BALB/c were immunized either three or four times with hsp60/lep DNA prior to challenge by aerosol with *M. tuberculosis* strain H37Rv. In these studies no evidence of protection was seen, and so the experiments were repeated using an intrinsically more resistant strain, C57BL/6, in which previously good results have been obtained with other DNA vaccines (1). However, here again no reduction in the bacterial load in the lungs was observed (Fig. 1). Because of previously observed (24) detrimental pathology using a similar vaccine (hsp60/TB DNA) the lungs of these mice were carefully examined. In sections of lungs examined 30 days after challenge infection the overall pathology observed was similar in the vaccinated mice and in saline controls, with both showing widespread granulomatous inflammation (Fig. 2A and B). In sections examined 80 days later the aggregates of lymphocytes and macrophages were large and appeared to be much more extensive in the mice receiving the DNA vaccine, suggesting an increased cellular influx (Fig. 2C and D).

Postchemotherapeutic vaccination studies. A second series of studies were performed in both BALB/c and C57BL/6 mice using the so-called Cornell protocol (19), in which infected mice are given chemotherapy to reduce the bacterial load to below detectable levels, after which steroid immunosuppression is given to try to reactivate residual surviving bacteria. Following the protocol previously described for the hsp60/lep

FIG. 2. Photomicrographs of lungs of C57BL/6 mice harvested 30 days (A and B) or 80 days (C and D) after aerosol exposure to *M. tuberculosis*. Mice received prophylactic vaccination with hsp60/lep (A and C) or saline (B and D). Alveoli contain large numbers of macrophages and lower numbers of lymphocytes, but there is no appreciable neutrophil infiltration or necrosis. At day 80 perivascular accumulations of lymphocytes are seen; these were particularly prominent in the hsp60/lep vaccinated animals.

FIG. 3. Effect of hsp60/lep vaccine in the modified Cornell model. Four weeks after exposure to low-dose aerosol challenge with *M. tuberculosis* H37Rv, the bacterial load was assessed (day 30 counts), and then the remaining mice were treated with isoniazid for 60 days, after which the reduction in bacterial load was assessed (Post-INH). Mice were then given three vaccinations with the hsp60/lep DNA or control injections. Thirty days after the final vaccination the bacterial load was determined, and it was then determined again after 8 weeks of treatment with dexamethasone. In each case the mean value is shown ($n = 5$) (error bars, standard errors of the means).

DNA vaccine (12) inoculation with this vaccine was given prior to steroid reactivation. Despite the large number of mice used, only a small percentage of animals showed evidence of reactivation (range of one to three mice having a detectable bacterial colony in their lungs per group of 15 animals), which did not permit analysis of any potential differences between groups. In view of this, we attempted to address this problem by repeating the experiment using a deliberately less effective chemotherapeutic regimen, isoniazid alone for 60 days, that we knew from previous experience will reduce the bacterial load considerably but would not completely eliminate the bacterial load in the infected mice, at least not to the extent that this could be determined bacteriologically. In this study (Fig. 3) isoniazid treatment reduced the bacterial load by 3 logs in the lungs of the infected mice. Groups of mice were then given hsp60/lep DNA, vector alone, or saline, and the bacterial load was assessed 30 days after the third vaccination. As shown, none of these materials had any effect on the regrowth seen in the bacterial load in the lungs. Moreover, since these values were still relatively low, a second group of treated mice were given 8 weeks of treatment with daily doses of dexamethasone to see if this altered any of the groups. As shown, the steroid therapy exacerbated the bacterial load to a similar degree in all three groups, measured 18 days after cessation of 8 weeks of dexamethasone treatment. These data thus indicate that the DNA vaccine had no effect, even when the bacterial load was very low.

Immunotherapeutic vaccination studies. In a third series of experiments, we examined the ability of the hsp60/lep and the Ag85 DNA vaccines to reduce the bacterial load in the lungs if given after the mouse had already been infected with *M. tuberculosis*. The first set of studies were performed in BALB/c mice that are relatively susceptible to aerosol infection. The protocol was designed to follow the bacterial load starting 1 month after the final DNA vaccine administration, but a few days after this vaccination was completed many of the mice began to die, and others appeared to be in severe distress and

were therefore euthanized in accordance with our animal usage approval protocol.

The lungs from these mice were then examined and animals receiving saline or control DNA had expected discrete granulomas consistently seen in mice 4 to 5 months after aerosol exposure. These lesions contained mixtures of lymphocytes and macrophages, with some of the latter cells appearing foamy and being gradually surrounded by increasing fibrosis (Fig. 4A). In contrast, granulomas in mice receiving hsp60/lep DNA were very big (as much as 10-fold larger than those in controls) and contained multiple areas of destructive necrosis throughout these structures (Fig. 4B). Higher magnification of these areas revealed foci consisting of groups of dead macrophages, often containing large clumps of acid fast staining bacteria (Fig. 4C and D). Similar areas of necrotic foci were seen in mice given the Ag85 DNA vaccine (data not shown).

Again, given these unexpected results, the studies were repeated using C57BL/6 mice, and with the protocol modified to reduce the vaccine frequency given from four to three. In these subsequent experiments no deaths were observed in any of the animals, but no reductions in the bacterial load were seen 30 days after the third inoculation with the DNA vaccine (Fig. 5). Examination of the lungs of these animals at this time point again revealed large areas of degenerative pathology in the DNA vaccinated mice (Fig. 6). Interestingly, a similar pattern was seen in the lungs of mice 100 days after the final vaccination, but in many sections the pathology seemed less pronounced, suggesting that some degree of tissue repair had taken place (Fig. 7).

DISCUSSION

The results of this study show that a plasmid DNA vaccine (hsp60/lep) encoding the hsp60 molecule of *M. leprae* that has been previously shown to be highly effective against intravenous or intraperitoneal inoculation with virulent *M. tuberculo-*

FIG. 4. Representative histologic appearance of lungs from BALB/c mice given immunotherapeutic vaccination with hsp60/lep. (A) In mice given saline only small granulomatous foci were seen in the lungs of these animals 130 days after low-dose aerosol challenge with *M. tuberculosis* H37Rv. Perivascular aggregates of lymphocytes can be seen extending into the adjacent parenchyma, which is variably surrounded by macrophages. Size bar = 100 μ m. (B) In mice given hsp60/lep DNA the lesions are very large (size bar = 100 μ m) and show evidence of extensive tissue destruction. There is a sharp line of demarcation with normal lung parenchyma, and alveoli are filled with infiltrates of mononuclear cells and some neutrophils. There is loss of alveolar wall detail, with multifocal necrosis. (C) At a higher power of magnification (size bar = 10 μ m), these multiple necrotic foci are characterized by fibrillar acidophilic cellular debris with clear clefts that represent extracellular cytoplasmic lipid deposition (arrows). These foci contain a mixture of normal to completely degenerate macrophages. (D) Acid-fast staining revealed multiple bacilli (example indicated with an arrow) visible within the cytoplasm of foamy macrophages as well as in the extracellular space of necrotic foci. Such bacilli were only rarely seen in lesions of control mice at this stage of the infection.

FIG. 5. Immunotherapeutic vaccination in C57BL/6 mice. Animals were given three injections of DNA, vector DNA control, or BCG on days 60, 81, and 102 after exposure to low-dose aerosol challenge with *M. tuberculosis* H37Rv. The data shown are the mean numbers of bacteria recovered from the lungs after a further 30 days ($n = 5$) (error bars, standard errors of the means). No protection was seen in any groups at this time, or after a further seventy days (data not shown).

sis H37Rv failed to protect mice if the infection was given by the more realistic pulmonary route.

The previous observation (12) that the hsp60/lep had such a substantial protective effect in mice given this DNA construct as a prophylactic vaccine against intravenous challenge is of great interest, since it implies that the bacterium produces large amounts of this antigen early during the course of the infection. In contrast, it is very interesting that culture filtrates obtained from bacteria growing progressively in nutrient medium *in vitro* contain only trace amounts of hsp60, and such cultures only begin to produce significant amounts of hsp60 if deprived of nutrients or exposed to low oxygen tensions. Whatever the explanation, the data in the present study suggest that alveolar and/or local resident macrophages in the lungs do not present the hsp60 antigen, or the bacillus does not produce it perhaps because of the very high local oxygen tension. Alternatively, hsp60 antigen may be expressed by infected macrophages in the lungs, but due to the influx of other such (uninfected) cells giving rise to the characteristic epithelioid macrophage granuloma in which the great majority of the cells do not contain live bacteria, immune T cells fail to reach those cells actively infected. This situation may arise because, unlike secreted proteins which are recognized very early on (15), the kinetics of presentation of hsp60 may be slower as a result of bacilli only starting to produce this protein when exposed to host defenses, such as reactive oxygen and nitrogen radicals (4, 7). Of course, if this is the correct explanation, then it implies

FIG. 6. Histologic appearance of lungs of C57BL/6 mice 30 days after the third immunotherapeutic vaccination with hsp60/lep. (A) Alveoli and interalveolar septae are effaced by infiltrates of macrophages, some of which have abundant foamy cytoplasm. There are random foci of necrosis containing karyorrhectic and cellular debris with intralesional aggregates of neutrophils (indicated by an arrow). Mice given saline (B) or DNA vector control (C) have prominent perivascular accumulations of lymphocytes and intra-alveolar macrophages but no necrosis or neutrophilic infiltration.

that the vaccine would almost certainly be ineffective under clinical conditions. Having said that, this does not diminish the importance of trying the DNA class of vaccines under such conditions, especially considering that nearly a century has passed since the BCG vaccine was first developed as a potential

defense against tuberculosis, and the field has failed since then to provide a valid alternative.

Our failure to observe no more than minimal reactivation in our initial study using the Cornell model was almost certainly due to two reasons. Firstly, the combination chemotherapy

FIG. 7. Histologic appearance of lungs one hundred days after immunotherapeutic vaccination with hsp60/lep DNA. Foci of necrosis contain debris (small arrows) and clefts of accumulated cholesterol (large arrows). Alveoli contain macrophages with abundant foamy cytoplasm, and aggregates of lymphocytes are prominent. Overall, the appearances of lungs from vaccinated (A) and saline controls (B) were similar.

protocol is extremely potent in the mouse, and 4 months of treatment will reduce the number of potentially reactivating mice to a low percentage (in this study the highest reactivation observed was 3 out of 15 mice) as well as increasing the number of mice needed per group to preserve statistical power. Secondly, although most if not all mice can be induced to reactivate if sufficient steroid treatment is subsequently given, this treatment generally has to be given for an extended period of time to be fully effective. Whereas only three injections of dexamethasone resulted in full reactivation of all control animals and more than 10^4 bacterial colonies being detected in the lungs of each of these mice in the study described by Lowrie et al. (12), in our own experience (2) at least seven injections of the more potent hydrocortisone only gave rise to partial reactivation of mice and bacterial counts of <100 . In a more recent, very comprehensive analysis of this type of model, a study by Scanga et al. (19) took even longer to induce reactivation in mice given a similar chemotherapeutic regimen, and again the recovery of bacterial colonies was very low (a mean of 70).

To try to address this we used a modified protocol that we knew would not completely reduce the bacterial load to undetectable levels. We reasoned that although it is probable that this protocol would not drive all bacteria into a truly latent state, as some proponents of the Cornell model seem to believe, it would still be a fair test of the capacity of the hsp60/lep DNA vaccine to reduce or eliminate the presence of the remaining bacilli. However, as shown above we were unable to find any evidence that the DNA vaccine could reduce the bacterial load in the lungs.

Administration of both the hsp60/lep and the Ag85/TB DNA as immunotherapeutic vaccines to BALB/c mice already in a state of chronic disease resulted in death of the animals within a few days of receiving the final i.m. inoculation. Autopsy of the few remaining mice in both cases revealed multiple foci of small circular areas of necrotic tissue damage throughout the granulomatous structures, with similar damage seen in mice receiving either vaccine. Moreover, in both cases lesions were noticeably much larger than in controls, suggesting a florid lymphocytic influx induced by the vaccines. A repeat of this study in resistant C57BL/6 mice in which the DNA immunization frequency was reduced did not result in fatalities, but the vaccination had no effect on the bacterial load in the lungs. Some lung damage was observed in the DNA-vaccinated mice, but interestingly this did not appear to have further progressed in animals examined 100 days later, possibly indicating that some degree of tissue repair had taken place.

The results shown here, as well as very similar recent results by others (17) attempting DNA vaccination in an immunotherapeutic or Cornell style model, clearly show that the generation of protection against an active infection using vaccines in an immunotherapeutic manner creates a major challenge. Since the vaccine would be given at a distant site, T cells generated as a result would have to be able to rapidly home to the site of infection and have the necessary molecules to effectively invade the granulomatous tissue (which by that time could be in an advanced state of formation). Moreover, by that time the increasing local fibrosis and micronecrosis that is a primary facet (18) of chronic tuberculosis in the mouse lung would

almost certainly be a further hindrance. Having said that, the data presented here seem to suggest that the hsp60/lep induced a considerable lymphocytic cellular influx, which was seen in both the prophylactic and immunotherapeutic models.

Collectively, these data indicate that the previously described hsp60/lep DNA vaccine, so effective in intravenous infection models (12), lacks the capacity to protect mice against pulmonary tuberculosis when delivered by the more realistic aerogenic route. In addition, a previously unanticipated observation concerned the apparently accelerated development of widespread necrotic lesions in the lungs of such animals, especially if the vaccine was given in an immunotherapeutic mode.

Two findings should be strongly stressed here. The first is that the common finding that both DNA vaccines tested here, as well as our previous experience with an hsp60/TB DNA construct (24), seems to imply that the pathological effects observed appear to be a general phenomenon of DNA vaccines encoding immunogenic mycobacterial antigens and are clearly not limited to vaccines encoding the heat shock chaperonin proteins. The frequency of immunization is also an important factor, since no adverse effects were seen until the mice had received at least three injections. In a previous study in which Ag85 DNA was given just twice we saw no protection, but we also no evidence of untoward pathological effects (23). Additionally, the relative resistance of the host seems to be important, given the lack of mortality seen in the C57BL/6 mice. It is interesting that it has previously been demonstrated (9) that BALB/c mice infected with *M. tuberculosis* react much more strongly to hsp60 protein than mice on the B6 background.

The second finding to be stressed is that all of the DNA vaccines tested to date in this laboratory appear to be completely safe in the context of the inoculation procedure itself. It is only after the animal has been exposed to *M. tuberculosis* infection that these pathological effects become evident, and only then if they are given repetitively. Moreover, since these pulmonary lesions take time to develop this undoubtedly explains why they were not noted in the intravenous challenge model described by Lowrie et al. (12), in which no histological data appear to have been collected. Nevertheless, these findings raise a safety issue and may imply that DNA vaccines given to individuals who unknowingly already have an undetected lesion in their lungs containing chronic or latent bacilli run the risk of inducing necrotic reactivation. In this regard, there are several DNA vaccines under test that have given good results to date (16) including ESAT-6, MPT64, and others, and it is unknown if such potential vaccines would also induce these problems.

The potential use of hsp60, at least in the context of a protein vaccine, has had a checkered history. It is clearly highly immunogenic, especially if bacteria are heat killed as a result of which their last physiological response is to make large amounts of this material (10). Inoculated in this manner there is a potent TH2 type response, resulting in large amounts of antibody production, but also evidence of a strong cytotoxic T lymphocyte response (28), an observation also seen in terms of the response to the DNA vaccine. However, their promise as a vaccine was initially discounted by studies over 15 years ago by Wiegshauss and Smith, who failed to protect guinea pigs

against aerosol infection with virulent *M. tuberculosis* using a recombinant vaccine (27). As a result, the use of this highly conserved heat shock protein, with its attendant autoimmune potential counteracting any potential benefits as an antimicrobial vaccine (26), remains debatable.

In this regard, the multiple pockets of necrosis seen in the lungs of the DNA-vaccinated mice in the immunotherapeutic studies are reminiscent of the famous Koch reaction in which Koch found that immunization of mice with a suspension of mycobacterial antigens triggered necrosis in preexisting lesions in the lungs of these animals (11). A potential explanation for these observations is the generation of potent cytotoxic T lymphocyte CD8 responses induced by the hsp60/lep DNA vaccine, as Silva and colleagues have demonstrated (20).

It is also potentially possible that BCG vaccination can itself induce such reactions. In mice given BCG in the immunotherapeutic mode we previously observed evidence for a pyogenic response in the lungs, suggesting lung damage (23). It is therefore interesting to speculate that in the south India BCG vaccine trial (22), administration of BCG to individuals already latently infected with tuberculosis may have induced such Koch reactions, thus explaining the higher incidence of eventual tuberculosis in the vaccinated group compared to the unvaccinated controls. Even today the basis of Koch's observation is not known precisely (13), and so we have begun immunotherapeutic vaccination experiments using various models with mice with gene disruptions to try to identify if it is indeed CD8 T cells, or perhaps others, that underlie such potentially fatal responses, and which thus act adversely against an otherwise useful vaccination strategy.

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