Elsevier
ELSEVIER

PII: S0264-410X(96)00274-5

Vaccine, Vol. 15, No. 8, pp. 830–833, 1997
© 1997 Elsevier Science Ltd. All rights reserved
Printed in Great Britain
0264-410X/97 $17+0.00

Immunogenicity and efficacy of a tuberculosis DNA vaccine encoding the components of the secreted antigen 85 complex

Evelyne Lozes*, Kris Huygen*§, Jean Content*, Olivier Denis*, Donna L. Montgomery†, Anne M. Yawman†, Paul Vandenbussche*, Jean-Paul Van Vooren‡, Annie Drowart‡, Jeffrey B. Ulmer† and Margaret A. Liu†

BALB/c and C57BL/6 mice were injected intramuscularly with plasmid DNA encoding the three components of the immunodominant 30–32 kDa antigen 85 complex (Ag85A, Ag85B, and Ag85C) from Mycobacterium tuberculosis *culture filtrate, in order to investigate the utility of nucleic acid vaccination for induction of immune responses against mycobacterial antigens. Ag85A and Ag85B encoding plasmids induced a robust Th1-like response towards native Ag85, characterized by elevated levels of interleukin (IL)-2, interferon-γ, and TNF-α. Levels of IL-4, IL-6, and IL-10 were low or undetectable. Plasmid encoding Ag85C was not effective. Cytotoxic T cell activity was also generated in* in vitro *restimulated splenocyte cultures from Ag85A and Ag85B DNA vaccinated mice. Finally, Ag85A and Ag85B DNA vaccination conferred significant protection against mycobacterial replication in lungs from B6 mice, subsequently challenged. Therefore, this technique may be useful for the definition of protective antigens of* M. tuberculosis *and the development of a more effective tuberculosis vaccine. © 1997 Elsevier Science Ltd.*

Keywords: tuberculosis; DNA vaccine; antigen 85

Tuberculosis remains a major health problem, affecting millions of people worldwide. It is estimated that each year, 8 million new cases develop and that 3 million people die of the disease[1]. The attenuated *Mycobacterium bovis* BCG vaccine continues to be widely administered, but its efficacy remains controversial[2]. It is clear that a more efficient vaccine would be the most definitive way of controlling this disease. Experimental vaccination with mycobacterial culture filtrates (CF) has demonstrated that secreted, extracellular proteins are essential for protection[3], but the precise CF antigens are not completely defined at present. The three components of the Ag85 complex, a 30–32 kDa family of proteins (Ag85A, Ag85B, and Ag85C) constitute a major portion of the secreted proteins in such CF[4]. We have previously shown that the Ag85 complex induces strong T cell proliferation and interferon-γ (IFN-γ) secretion in PBMC cultures from healthy individuals with a primary infection with *Mycobacterium tuberculosis/Mycobacterium leprae*[5] and in spleen cell cultures from mice vaccinated with live *M. bovis* BCG[6]. Sensitization against Ag85 occurs very early in mycobacterial infection. Hence, recognition of this antigen may be an effective strategy of the host to control initial bacterial replication, thereby making the proteins of the Ag85 complex reasonable vaccine candidates. Here we show that vaccination of mice with Ag85A DNA and Ag85B DNA induces a strong T helper 1 type and cytotoxic T cell response as measured after *in vitro* restimulation, and confers protection against subsequent mycobacterial challenge.

*Pasteur Institute of Brussels, Mycobacterial Immunology, Department Virology, 642 Engelandstraat, 1180, Brussels, Belgium. †Merck Research Laboratories, West Point, PA 19486, USA. ‡Hôpital Erasme ULB, 808 Route de Lennick, 1070, Brussels, Belgium. §To whom correspondence should be addressed.

MATERIALS AND METHODS

Plasmids

Genes encoding the mature Ag85A, Ag85B, and Ag85C from *M. tuberculosis* were PCR amplified, BglII digested and inserted in either the V1Jns.tPA vector (in which the mycobacterial genes are preceeded by the signal sequence for human tissue plasminogen activator, resulting in a secreted form), or in the V1Jns vector (without tPA signal, resulting in a mature form)[7].

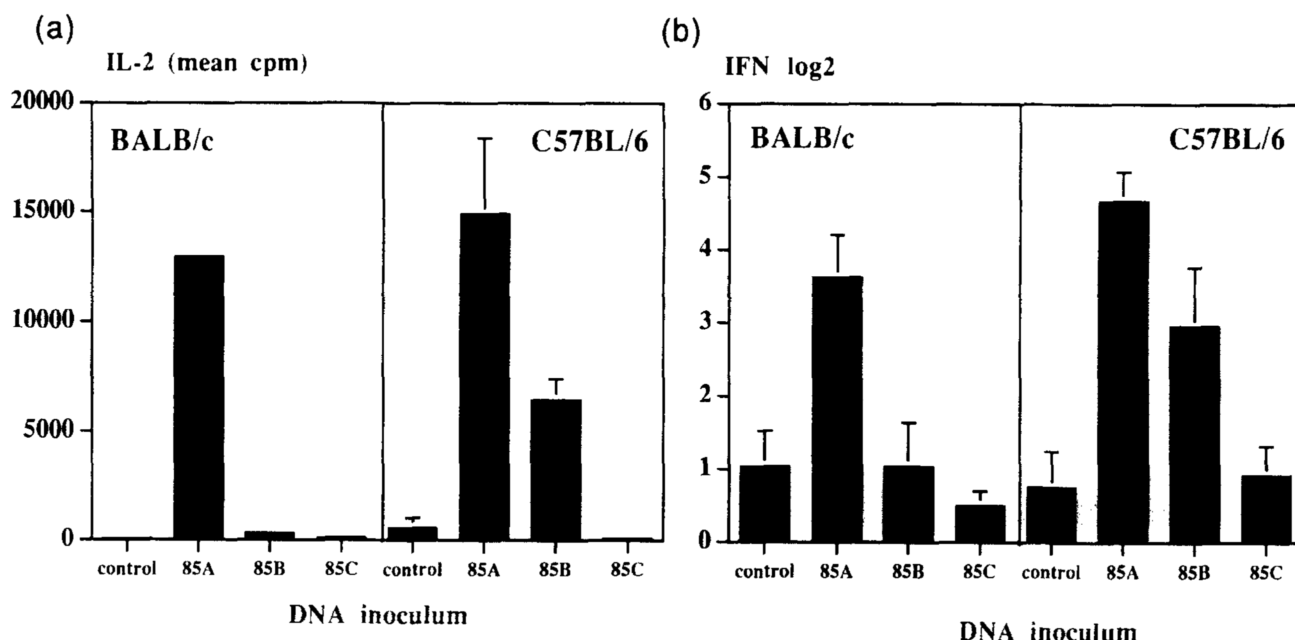

Figure 1 Mean IL-2 (a) and IFN-γ (b) levels in 24 h and 72 h culture supernatants of spleen cells from DNA vaccinated BALB/c and B6 mice, stimulated *in vitro* with purified Ag85 (mean±S.D. of three mice in each group, tested separately)

DNA vaccination

BALB/c and C57BL/6 (B6) mice were vaccinated intramuscularly (i.m.) three times at 3 week intervals with 100 μg of cesium chloride/ethidium bromide purified plasmid DNA. As a control for BCG-vaccination, a separate group of mice was infected intravenously (i.v.) with 2×10^6 c.f.u. of surface-grown pellicle *M. bovis* BCG, simultaneously with the first DNA vaccination.

Antigens

Native Ag85 (30–32 kDa) was purified from *M. bovis* BCG CF by sequential chromatography on Phenyl-Sepharose, DEAE-Sephacel and Sephadex G75[8].

Cytokine production and analysis

Three weeks after the third DNA injection, mice were killed and spleen cells (4×10^6 ml^{-1}) were stimulated *in vitro* with purified Ag85 (5 μg ml^{-1}). Three mice were analyzed separately in each group. Culture supernatants were harvested after 24 h [interleukin (IL)-2 and TNF-α] and 72 h (IFN-γ, IL-4, IL-6, IL-10). Cytokines were measured using ELISA or bio-assay as reported before[9]. Briefly, IL-2 activity was meaured using a thymidine uptake of IL-2 dependent CTLL-2 cells (detection limit 10 pg ml^{-1}, 10000 c.p.m. equals about 1000 pg ml^{-1}) and IFN-γ activity was measured using a cytopathic effect reduction assay of vesicular stomatitis virus on mouse L929 cells (detection limit 150 pg ml^{-1}, 1 log2 equals 220 pg).

CTL activity

Spleen cells from Ag85 DNA vaccinated B6 mice were amplified with BCG CF for 7 days and CTL activity was determined using a neutral red dye uptake of thioglycollate-induced syngeneic peritoneal exudate cells pulsed *in vitro* with purified Ag85 and cultured in the presence of amplified effector cells at ratios up to 10:1 for 16 h. Neutral red was released by acetic acid/ethanol and the O.D. was measured in a microELISA autoreader at 540 nm.

Table 1 Cytokine secretion following vaccination with AG85 DNA

	IL-4	IL-6	IL-10	TNF-α
BALB/c				
Control DNA	<15[a]	47	<100	23
85A DNA	<15	101	<100	162
85B DNA	<15	20	<100	32
85C DNA	<15	<5	<100	<5
C57BL/6				
Control	<15	19	<100	6
85A DNA	<15	10	<100	370
85B DNA	<15	5	<100	203
85C DNA	<15	9	<100	<5

[a]Mean cytokine levels (pg ml^{-1}) in spleen cell culture supernatant from DNA vaccinated mice stimulated *in vitro* with purified Ag85 (5 μg ml^{-1}) (mean of three mice analyzed in each group)

BCG challenge

DNA vaccinated B6 mice were challenged iv. with 2×10^6c.f.u. of BCG. The number of CFU in lungs was determined by plating appropriate dilutions of cell suspensions on Middlebrook 7H11 Bacto Agar.

RESULTS

Cytokine production by splenocytes from Ag85 DNA vaccinated mice

As shown in *Figure 1*, elevated levels of IL-2 (1000 pg ml^{-1}) and IFN-γ (4000 pg ml^{-1}) could be measured in spleen cell culture supernatants from BALB/c and C57BL/6 mice vaccinated with plasmid encoding a secreted form of Ag85A and restimulated *in vitro* with native Ag85 purifed from *M. bovis* BCG CF. Ag85B encoding plasmid was also effective in B6

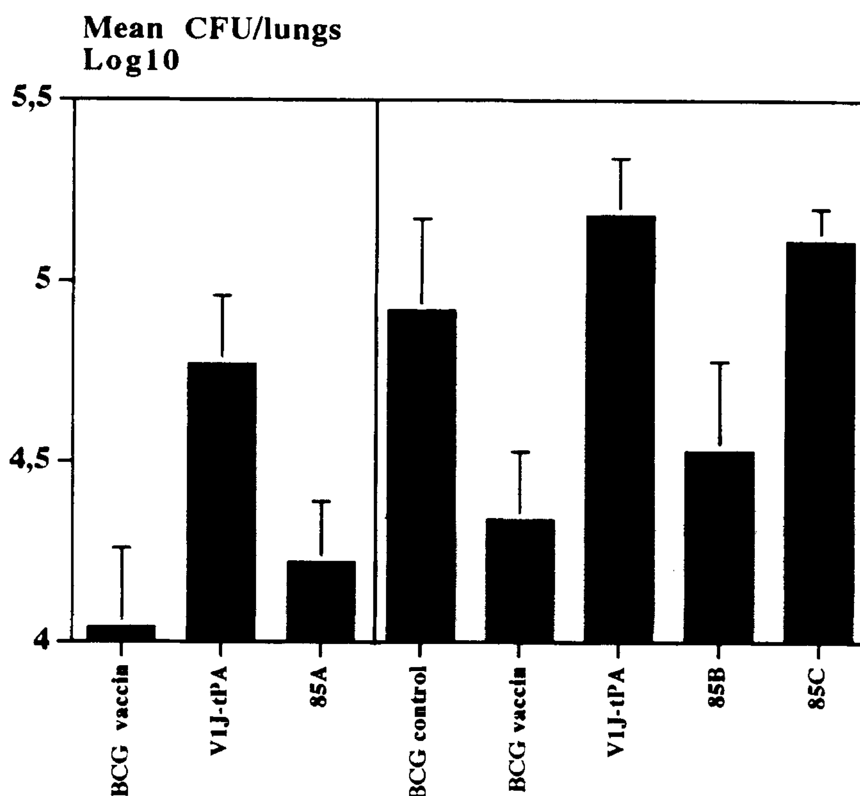

Figure 2 Number of CFU in lungs from B6 mice, vaccinated with Ag85A DNA (A) or Ag85B and Ag85C DNA (B). Mice were challenged i.v. at day -9 with 2×10^6 c.f.u. of *M. bovis* BCG. c.f.u. values are compared to mice vaccinated with control V1J-tPA DNA, mice vaccinated with BCG and untreated mice (mean log10±S.D. of at least five mice in each group, tested separately)

mice but not in BALB/c, whereas plasmid DNA encoding the Ag85C component was not active in either strain. Significant Th1-type cytokine production by splenocytes was also found in response to stimulation with PPD and CF but not with whole BCG bacilli or BCG cytoplasmic extract (data not shown). IL-4 and IL-10 levels were undetectable in these Ag85 stimulated cultures. IL-6 production was also very low, although some activity was measured in splenocytes from BALB/c mice vaccinated with Ag85A DNA. Production of TNF-α finally, was significantly increased in spleen cells from mice vaccinated with Ag85A DNA (BALB/c and B6) and Ag85B DNA (B6) (*Table 1*).

Generation of cytotoxic T lymphocytes by splenocytes from Ag85 DNA vaccinated mice

B6 mice vaccinated with plasmids encoding a mature form of Ag85A or Ag85B demonstrated significant CTL activity against Ag85 pulsed peritoneal exudate cells, whereas mice vaccinated with plasmid encoding mature Ag85C did not (*Table 2*). Significant CTL activity was also induced in restimulated splenocytes from BALB/c mice vaccinated with DNA encoding the mature form of Ag85A (data not shown).

Vaccination with Ag85 encoding plasmids protects against mycobacterial replication

As shown in *Figure 2*, vaccination with DNA encoding a secreted form of Ag85A (*Figure 2*A) or Ag85B, but not of Ag85C (*Figure 2*B), conferred significant protection against mycobacterial replication in the lungs from B6 mice following a subsequent challenge, with a reduction level comparable to the one observed with the BCG vaccine.

Table 2 CTL activity following vaccination with AG85 DNA

Effector:target	2.5:1	5:1	10:1
Control DNA	0[a]	0	16.6±4.5
85A DNA	0	29.4±9.1	80.2±9.8
85B DNA	0	48.1±12.6	101.4±1.9
85C DNA	0	0	39.4±6.3

[a]% specific lysis (mean±S.D. of three mice analyzed separately) as measured in a neutral red dye uptake of H-2[b] peritoneal exudate cells pulsed with Ag85 and using CF amplified spleen cells from DNA vaccinated B6 mice as effectors

DISCUSSION

Protective immunity against mycobacterial infection is mediated by interactions between specifically sensitized CD4+ and CD8+ T lymphocytes and activated macrophage effector cells harboring the intracellular pathogen. IFN-γ, a potent activator of macrophages which is produced by both CD4+ and CD8+ T cells plays a pivotal role in anti-mycobacterial protection, as demonstrated in knock-out mice, genetically deleted for IFN-γ or IFN-γ receptor[10]. Here we have shown that DNA vaccination with plasmids encoding mycobacterial extracellular proteins is a potent method for the generation of specific helper responses with a Th1-like phenotype, characterized by splenocytes which upon antigen-specific stimulation secrete elevated levels of

IL-2 and IFN-γ and minimal levels of IL-4. Furthermore, significant CD8-mediated CTL responses were also induced by Ag85 DNA vaccination and most importantly, this vaccination generated a protective immune response, as demonstrated by reduced mycobacterial replication in the lungs of Ag85 DNA vaccinated mice comparable to reductions observed in BCG-vaccinated mice following BCG challenge.

Plasmid encoding the Ag85A component was consistently found to be the most effective plasmid, whereas DNA encoding Ag85C was not active, when considering Th1 cytokine production, CTL activity or protection. On the other hand, all Ag85 constructs were capable of inducing substantial humoral immune responses (Ulmer *et al.*[11]), suggesting that each plasmid was expressed *in vivo* but that induction of T-cell responses against Ag85C was impaired.

Protective antigens for tuberculosis (and leprosy) are ill-defined at present, because effective vaccination methods and specific adjuvants for the generation of strong cellular immunity are not readily available, and also because classical purification of mycobacterial proteins is time-consuming and difficult. DNA vaccination may provide a simple way to overcome some of these problems and may prove to be a powerful tool for screening as yet unidentified mycobacterial antigens for protective efficacy. This technology may open new horizons for effective vaccination against tuberculosis, because strong cell-mediated immune responses, including CTL and Th1-type cytokines can be induced.

ACKNOWLEDGEMENTS

The authors would like to express their gratitude to K. Palfliet, F. Jurion, J. Ooms, L. De Wit, A. Vanonckelen, M. De Cock, C. DeWitt and M. Snyder for excellent technical assistance.

REFERENCES

1 Snider, D.E., Raviglione, M. and Kochi A. Global burden of tuberculosis. In: *Tuberculosis: Pathogenesis, Protection and Control* (Ed. Bloom, B.R.). ASM Publications, Washington, 1994, pp. 3–11

2 Bloom, B.R. and Fine P.E.M. The BCG experience: Implications for future vaccines against tuberculosis. In: *Tuberculosis: Pathogenesis, Protection and Control* (Ed. Bloom, B.R.). ASM Publications, Washington, 1994, pp. 531–557

3 Orme, I.M. Prospects for new vaccines against tuberculosis. *Trends Microbiol.* 1995, **3**, 401–404

4 Wiker, H.G. and Harboe, M. The antigen 85 complex: a major secreted product of *M. tuberculosis. Microbiol. Rev.* 1992, **56**, 648–661

5 Launois, P., DeLeys, R. and Niang, M.N. *et al.* T cell epitope mapping of the major secreted mycobacterial antigen Ag85A in tuberculosis and leprosy. *Infect. Immun.* 1994, **62**, 3679–3687

6 Huygen, K., Lozes, E. and Gilles, B. *et al.* Mapping of TH1 helper T cell epitopes on major secreted mycobacterial antigen 85A in mice infected with live BCG. *Infect. Immun.* 1994, **62**, 363–370

7 Shiver, J.W., Perry, H.C. and Davies, M.E. *et al.* Immune responses to HIV gp120 elicited by DNA vaccination. In: *Vaccines 95* (Eds Chanock R.M., Brown, F. and Ginsberg, H.S. *et al.*). Cold Spring Harbor Laboratory Press, New York, 1996, pp. 95–98

8 De Bruyn, J., Huygen, K. and Bosmans, R. *et al.* Purification, characterization and identification of a 32 kD protein antigen of *Mycobacterium bovis* BCG. *Microbiol. Pathogen.* 1987, **2**, 351–366

9 Huygen, K., Abramowicz, D. and Vandenbussche, P. *et al.* Spleen cell cytokine secretion in *Mycobacterium bovis* BCG infected mice. *Infect. Immun.* 1992, **60**, 2880–2886

10 Dalton, D.K., Pitts-Meek, S. and Keshav, S. *et al.* Multiple defects of immune cell function in mice with disrupted interferon-γ genes. *Science* 1993, **259**, 1739–1742

11 Ulmer, J.B., Liu, M.A. and Montgomery, D.L. *et al.* Expression and immunogenicity of *M. tuberculosis* antigen 85 by DNA vaccination. *Vaccine* 1997, **15**, 792–794