INFECTION AND IMMUNITY, May 1999, p. 2624–2626 Vol. 67, No. 5
0019-9567/99/$04.00+0

Vaccination of Guinea Pigs with Nutritionally Impaired Avirulent Mutants of *Mycobacterium bovis* Protects against Tuberculosis

GEOFFREY W. DE LISLE,* THERESA WILSON, DESMOND M. COLLINS, AND BRYCE M. BUDDLE

AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand

Received 14 October 1998/Returned for modification 1 December 1998/Accepted 27 January 1999

Four nutritionally impaired strains of *Mycobacterium bovis* produced by illegitimate recombination were tested for their ability to protect guinea pigs against intratracheal challenge with virulent *M. bovis*. All four strains and *M. bovis* BCG induced significant levels of protection as measured by the reduced spread of infection to the spleen and liver. In animals vaccinated with BCG or two of the other strains, the bacterial counts from the lungs were significantly lower than those of the nonvaccinated animals.

Mycobacterium bovis, a member of the *Mycobacterium tuberculosis* complex, is the principal cause of tuberculosis in domestic animals and can produce human disease which is indistinguishable from that caused by *M. tuberculosis*. *M. bovis* and *M. tuberculosis* are genetically very similar (15), and the widely used human tuberculosis vaccine, BCG, is an *M. bovis* strain that became attenuated for unknown reasons during extended subculturing. In many industrialized countries, *M. bovis* has been eradicated from cattle or reduced to very low levels through the implementation of control programs based on test-and-slaughter principles. Bovine tuberculosis continues to be a major problem in countries which cannot afford such programs or where these programs have been only partially effective due to wildlife reservoirs of infection (12). Important reservoirs of *M. bovis* infection include the badger in the United Kingdom and the brushtail possum in New Zealand. In these countries, vaccination is being investigated in order to achieve more effective control of the disease (1, 4, 11). The variable effectiveness of the currently available vaccine reduces its usefulness for controlling bovine tuberculosis in cattle or wildlife (5, 12) and necessitates the development of a better vaccine. A better tuberculosis vaccine for animals will likely also have application for use in humans.

Four nutritionally impaired strains of *M. bovis* were selected, from a group of 440 *M. bovis* mutants produced by illegitimate recombination, on the basis of their inability to grow in minimal medium (16). The strains were shown to be avirulent for guinea pigs following the subcutaneous inoculation of 10^6 CFU. In the present study, we examined the potential of these strains to protect guinea pigs against subsequent intratracheal challenge with a virulent *M. bovis* strain (WAg201) originally isolated from a cow and previously used in this laboratory (17). Groups of four to eight Duncan-Hartley guinea pigs weighing 700 to 900 g were vaccinated by subcutaneous injection in the right flank with 10^5 CFU of either one of the nutritionally impaired strains or BCG. A control group of 10 animals was not vaccinated. Eight weeks postvaccination, all animals were each anesthetised by an intramuscular injection of 3 mg of ketamine hydrochloride (Parnell Laboratories, Auckland, New Zealand) per kg of body weight and 2.5 mg of xylazine hydrochloride (Rompun; Bayer, Auckland, New Zealand) per kg. Then they were challenged with 10^2 CFU (0.1 ml) of virulent *M. bovis* WAg201 introduced through the mouth into the trachea via a cannula. As an additional control, 10^5 CFU of each nutritionally impaired strain was inoculated subcutaneously into three guinea pigs that were not challenged.

All animals were sacrificed 13 weeks after vaccination. The following parameters of infection were measured: the number and distribution of macroscopic and microscopic lesions, the weights of the spleens, and the number of *M. bovis* CFU present in the lungs, spleen, and liver. *M. bovis* CFU were determined by a plate count procedure. Delayed-type hypersensitivity to tuberculin was measured immediately before vaccination and prior to sacrifice. Bovine purified protein derivative (4 U; Ministry of Agriculture and Forestry, Central Animal Health Laboratory, Auckland, New Zealand) was injected intradermally, and the diameter of the erythema was measured 24 h later. No delayed-type hypersensitivity reactions were observed when the animals were tested prior to being inoculated with mycobacteria. There were no statistical differences as determined by an analysis of variance (ANOVA) among the skin test responses of the groups of challenged animals, including the nonvaccinated group, when they were examined immediately prior to being sacrificed. The responses ranged from 12.9 to 20 mm in diameter.

Macroscopic lesions consistent with tuberculosis were ob-

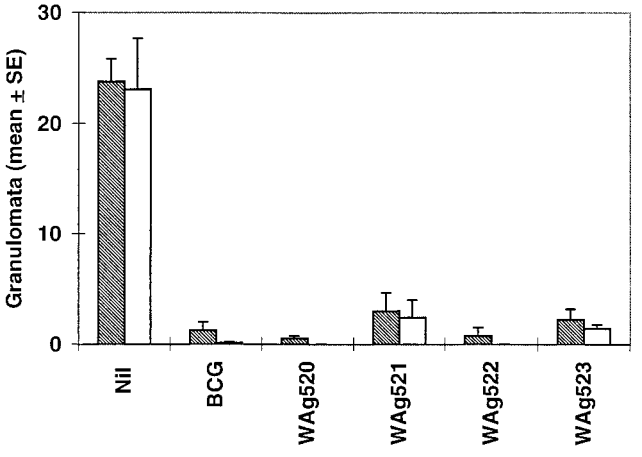

FIG. 1. Microscopic granuloma in spleen (▨) and liver (▭) for vaccinated and control guinea pigs 5 weeks after challenge with virulent *M. bovis* strains. A granuloma was defined as a discrete accumulation of cells consisting predominantly of macrophages.

* Corresponding author. Mailing address: AgResearch, Wallaceville Animal Research Centre, P.O. Box 40-063, Upper Hutt, New Zealand. Phone: 64 4 5286 089. Fax: 64 4 5281 380. E-mail: delisleg@agresearch.cri.nz.

TABLE 1. Vaccine-induced protection of guinea pigs as measured by *M. bovis* CFU[a]

Immunization group (no. of animals)	Lung		Spleen		Liver	
	Log_{10} CFU (mean ± SE)	Log_{10} protection	Log_{10} CFU (mean ± SE)	Log_{10} protection	Log_{10} CFU (mean ± SE)	Log_{10} protection
Nonvaccinated (10)	5.27 ± 0.1	0 A	5.04 ± 0.19	0 A	3.17 ± 0.17	0 A
BCG (8)	4.33 ± 0.21	0.94 B	1.76 ± 0.38	3.28 BC	0.8 ± 0.09	2.38 C
WAg520 (6)	4.35 ± 0.32	0.92 B	1.21 ± 0.36	3.83 C	0.7 ± 0	2.47 C
WAg521 (5)	4.34 ± 0.28	0.92 AB	3.3 ± 0.09	1.74 B	1.84 ± 0.26	1.33 B
WAg522 (4)	3.98 ± 0.12	1.28 B	1.87 ± 0.61	3.17 BC	0.7 ± 0	2.48 C
WAg523 (5)	4.39 ± 0.22	0.88 AB	2.35 ± 0.55	2.69 BC	0.95 ± 0.14	2.23 C

[a] Values within columns followed by the same letter are not significantly different ($P > 0.05$). The minimum detectable level was 0.7 log_{10}.

served in the lungs of the guinea pigs challenged with virulent *M. bovis*, and affected portions were cultured. Tissue samples for histology and bacteriology were taken from the left and right lobes of the liver, and a transverse section through the middle of the spleen was taken for histological examination. Bacteriological results for the lungs and liver are expressed as CFU/gram of tissue, and those for the spleens are expressed as CFU/spleen. The results of the vaccination experiment are summarized in Tables 1 and 2 and Fig. 1. The four nutritionally impaired strains and BCG all gave significant levels of protection ($P < 0.05$) as measured by spleen weights (data not shown), number of macroscopic and microscopic spleen and liver lesions, and number of *M. bovis* CFU isolated from the spleen and liver. Analyses of spleen weights and numbers of macroscopic and microscopic lesions were undertaken with ANOVA of raw data, while analyses of bacterial counts were conducted with ANOVA of log_{10}-transformed data. Significant differences in lung CFU were observed between the nonvaccinated group and the groups vaccinated with either BCG or one of the nutritionally impaired strains WAg520 and WAg522.

No macroscopic or microscopic lesions were observed in the spleens, livers, or lungs of the control animals that were vaccinated with the nutritionally impaired strains but were not challenged with virulent *M. bovis*. No *M. bovis* organisms were isolated from the spleens of these animals. These results are comparable to those of our earlier study of virulence, in which a 10-fold-higher dose was inoculated (16).

The results of this study support the use of nutritionally impaired strains as vaccines for tuberculosis. All four nutritionally impaired strains and BCG gave significant levels of protection, as demonstrated by the reduced spread of infection to the spleen and liver. A significant reduction in the bacterial lung counts, compared with those in the unvaccinated animals, was also observed in the guinea pigs vaccinated with two of the nutritionally impaired strains and BCG. The recent study of Baldwin et al. (3) demonstrated that certain vaccine strategies against *M. tuberculosis* may reduce the bacterial burden but exacerbate pulmonary pathology. A limitation of our study was that material was not available to determine whether the nutritionally impaired mutants influenced the development of necrotizing lesions in the lungs.

TABLE 2. Vaccine-induced protection in guinea pigs as measured by macroscopic lesions[a]

Immunization group (no. of animals)	No. (mean ± SE) of:	
	Spleen lesions	Liver lesions
Nonvaccinated (10)	118 ± 8.92 A	263.5 ± 123.6 A
BCG (8)	1.75 ± 0.88 B	0.38 ± 0.38 B
WAg520 (6)	0.33 ± 0.21 B	0.0 ± 0.0 B
WAg521 (5)	4.20 ± 1.59 B	0.0 ± 0.0 B
WAg522 (4)	0.25 ± 0.25 B	0.0 ± 0.0 B
WAg523 (5)	8.20 ± 5.69 B	3.0 ± 3.0 B

[a] Values within columns followed by the same letter are not significantly different ($P > 0.05$).

Despite its drawbacks, BCG remains the standard against which all new vaccines will ultimately be measured. Currently, no subunit or DNA vaccine has been shown in animal experiments to induce greater levels of protection against tuberculosis than BCG, and in most cases they provide less protection than BCG (2, 6, 13). These nonliving vaccines also have the disadvantage of requiring multiple vaccinations, in contrast to BCG (3, 8, 9) and the nutritionally impaired strains examined in this study. A concern about live tuberculosis vaccines is that the organisms may multiply unchecked in some immunocompromised animals. A recent study by Guleria et al. (7) has shown that nutritionally impaired *leuD* mutants of BCG have the potential to be safe even in very immunocompromised animals, such as SCID mice.

A notable feature of three of the nutritionally impaired strains and BCG is that compared to *M. tuberculosis*, they have major DNA deletions of between 10 and 65 kb (10, 14, 16). These large deletions do not all occur in the same place in the chromosome, indicating that there are many genes which are not required for in vitro growth but are essential for the maintenance of virulence. The remaining nutritionally impaired strain, WAg520, has only a 2-bp deletion in a possible undecaprenol kinase gene, and this strain was one of two nutritionally impaired strains which produced significant protection in the lungs. In contrast to BCG, the nutritionally impaired strains have genetic changes that have been found by identifying alterations to the parent strain. Although some major deletions of DNA in BCG have been identified (10, 14), the possibility of smaller genetic changes has not been excluded. In the absence of the parent strain of BCG or the complete genome sequence of BCG, the identification of all such changes will be difficult. New live vaccines against tuberculosis are likely to be acceptable for routine use only if their genetic changes have been precisely defined. While the major challenge remains identifying the portions of the chromosome which can be inactivated to produce the safest and most efficacious tuberculosis vaccine, the results of this study demonstrate the potential of nutritionally impaired strains of members of the *M. tuberculosis* complex to be effective and safe vaccines against tuberculosis.

We thank Lillian Morrison for statistical analysis.

This study was supported by grants from the Foundation of Research Science and Technology and MAF Policy, New Zealand.

REFERENCES

1. **Aldwell, F. E., A. Pfeffer, G. W. de Lisle, G. Jowett, J. Heslop, D. Keen, A. Thompson, and B. M. Buddle.** 1995. Effectiveness of BCG vaccination in protecting possums against bovine tuberculosis. Res. Vet. Sci. **58:**90–95.
2. **Andersen, P.** 1994. Effective vaccination of mice against *Mycobacterium tuberculosis* infection with a soluble mixture of secreted mycobacterial proteins. Infect. Immun. **62:**2536–2544.
3. **Baldwin, S. L., C. D'Souza, A. D. Roberts, B. P. Kelly, A. A. Frank, M. A. Lui, J. B. Ulmer, K. Huygen, D. M. McMurray, and I. M. Orme.** 1998. Evaluation of new vaccines in the mouse and guinea pig model of tuberculosis. Infect. Immun. **66:**2951–2959.
4. **Buddle, B. M., G. W. de Lisle, A. Pfeffer, and F. E. Aldwell.** 1995. Immunological responses and protection against *Mycobacterium bovis* in calves vaccinated with a low dose of BCG. Vaccine **13:**1123–1130.
5. **Buddle, B. M., F. E. Aldwell, and D. N. Wedlock.** 1995. Vaccination of cattle and possums against bovine tuberculosis, p. 111–115. *In* F. Griffin and G. de Lisle (ed.), Tuberculosis in wildlife and domestic animals. Otago Conference Series 3. Otago University Press, Dunedin, New Zealand.
6. **Ellner, J. J.** 1997. The immune response in human tuberculosis—implications for tuberculosis control. J. Infect. Dis. **176:**1351–1359. (Review.)
7. **Guleria, I., R. A. McAdam, G. Kaplan, W. R. Jacobs, Jr., and B. R. Bloom.** 1996. Auxotrophic vaccines for tuberculosis. Nat. Med. **2:**334–337.
8. **Horwitz, M. A., B.-W. E. Lee, B. J. Dillon, and G. Harth.** 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of *Mycobacterium tuberculosis*. Proc. Natl. Acad. Sci. USA **92:** 1530–1534.
9. **Huygen, K., J. Content, O. Denis, D. L. Montgomery, A. M. Yawman, R. R. Deck, C. deWitt, I. M. Orme, S. Baldwin, C. D'Souza, A. Drowart, E. Lozes, P. Vandenbussche, J.-P. van Vooren, M. A. Lui, and J. B. Ulmer.** 1996. Immunogenicity and protective efficacy of a tuberculosis DNA vaccine. Nat. Med. **2:**893–898.
10. **Mahairas, G. G., P. J. Sabo, M. J. Hickey, D. C. Singh, and C. K. Stover.** 1996. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. J. Bacteriol. **178:**1274–1282.
11. **Newell, D. G., and R. G. Hewinson.** 1995. Control of bovine tuberculosis by vaccination. Vet. Rec. **136:**459–463.
12. **O'Reilly, L. M., and C. J. Daborn.** 1995. The epidemiology of *Mycobacterium bovis* infection in animals and man: a review. Tubercle Lung Dis. **76**(Suppl. 1)**:**1–46.
13. **Orme, I. M.** 1997. Progress in the development of new vaccines against tuberculosis. Int. J. Tuberc. Lung Dis. **1:**95–100.
14. **Philipp, W. J., S. Nair, G. Guglielmi, M. Lagranderie, B. Gicquel, and S. T. Cole.** 1996. Physical mapping of *Mycobacterium bovis* BCG Pasteur reveals differences from the genome map of *Mycobacterium tuberculosis* H37Rv and from *M. bovis*. Microbiology **142:**3135–3145.
15. **Sreevatsan, S., X. Pan, and J. M. Musser.** 1997. Restricted structural gene polymorphism in the *Mycobacterium tuberculosis* complex indicates evolutionarily recent global dissemination. Proc. Natl. Acad. Sci. USA **94:**9869–9874.
16. **Wilson, T., B. J. Wards, S. J. White, B. Skou, G. W. de Lisle, and D. M. Collins.** 1997. Production of avirulent *Mycobacterium bovis* strains by illegitimate recombination with deoxyribonucleic acid fragments containing an interrupted *ahpC* gene. Tubercle Lung Dis. **78:**229–235.
17. **Wilson, T. M., G. W. de Lisle, and D. M. Collins.** 1995. Effect of *inhA* and *katG* on isoniazid resistance and virulence of *Mycobacterium bovis*. Mol. Microbiol. **15:**1009–1015.

Editor: S. H. E. Kaufmann