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Mycobacterium bovis BCG Vaccine Fails to Protect Protein-Deficient Guinea Pigs against Respiratory Challenge with Virulent *Mycobacterium tuberculosis*

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Specific-pathogen-free Hartley guinea pigs were maintained on isocaloric-purified diets either adequate (30%) or moderately deficient (10%) in protein. Half of each diet group was vaccinated with viable *Mycobacterium bovis* BCG. Six weeks later, all animals were challenged by the respiratory route with virulent *Mycobacterium tuberculosis* H37Rv. At intervals of 1, 2, and 3 weeks postchallenge, guinea pigs from each diet and vaccination group were skin tested with tuberculin and sacrificed. Protein deficiency resulted in loss of tuberculin hypersensitivity. Vaccination with *M. bovis* BCG protected control animals, as determined by significant reductions in the number of *M. tuberculosis* H37Rv organisms recovered from lungs, spleen, and bronchotracheal lymph nodes 2 and 3 weeks postchallenge. Based upon the same criteria, the degree of protection afforded protein-deficient animals by *M. bovis* BCG vaccine ranged from partial (spleen and lymph nodes) to none at all (lungs). Approximately the same numbers of tubercle bacilli were recovered from nonvaccinated guinea pigs in both diet groups. Protein deficiency appears to impair *M. bovis* BCG-induced immunity while not affecting primary pulmonary infection with virulent *M. tuberculosis*.

Tuberculosis is a major cause of morbidity and mortality in the developing world today despite the availability of a vaccine, *Mycobacterium bovis* BCG. While there is very good evidence for the efficacy of *M. bovis* BCG in protecting vaccine recipients against clinical tuberculosis, the recent field trial failure in South India suggests that unknown environmental or host factors may render *M. bovis* BCG less efficacious in certain Third World populations (2, 19).

Malnutrition coexists with tuberculosis in many parts of the world in which vaccination is the only cost-effective means of controlling the disease. Dietary deficiencies of specific nutrients have been associated with alterations in immunological competence in humans and experimental animals (1, 6). Cell-mediated immunity in general and thymus-dependent (T) lymphocyte numbers and functions in particular appear to be predictably and profoundly altered in the malnourished host (8, 9). The extrapolation of these observations to T cell-mediated infectious disease resistance, however, has not been straightforward. Some macrophage-dependent host defense mechanisms are apparently unaffected or even enhanced in nutritionally deprived individuals (13, 20). Given the central role of T lymphocytes and macrophage activation by their products in resistance against tuberculosis, it is likely that nutritional deficiencies would alter mycobacterial immunity (2, 3, 7). Early studies in malnourished, *M. bovis* BCG-vaccinated children demonstrated that such children did not develop normal tuberculin skin reactivity after vaccination and presumably were not as protected against virulent challenge (6, 11, 16). The latter hypothesis has never been tested in humans or experimental animals.

We have adapted a guinea pig model of experimental pulmonary tuberculosis to study the effects of dietary deficiencies on *M. bovis* BCG vaccine efficacy (17). Our previous results revealed specific lesions in the immune responses of protein-deficient guinea pigs vaccinated with *M. bovis* BCG. In general, protein-malnourished guinea pigs failed to mount a significant tuberculin reaction, even to increased doses of tuberculin, although these animals eventually controlled infection with *M. bovis* BCG and other attenuated mycobacteria (12, 10). The experiments we describe here were designed to determine whether protein-malnourished guinea pigs could be protected by *M. bovis* BCG vaccine against respiratory challenge with virulent *Mycobacterium tuberculosis*.

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MATERIALS AND METHODS

Experimental animals. Outbred, albino, specific-pathogen-free female guinea pigs, weighing 150 to 200 g each, were obtained from a commercial source (Hartley-COBS, Crl:(HA)BR; Charles River Breeding Laboratories, Inc., Wilmington, Mass.). They were housed individually in polycarbonate cages on stainless-steel grid floors and provided food in stainless-steel feeders and tap water ad libitum. Each animal was randomly assigned to an experimental diet and vaccination treatment group. Body weights were recorded weekly during the experiment.

Experimental diets. The experimental diets, based upon ovalbumin as the protein source, were designed to meet current recommended nutritional requirements for guinea pigs (14). The biotin content of the diet was increased to counter the effects of avidin, a biotin-binding protein found in ovalbumin. Two diets representing an adequate level of protein (30%) and a protein-deficient level (10%) were obtained from a commercial source (Dyets, Inc., Bethlehem, Pa.). The diets were isocaloric, with the proportion of corn

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TABLE 1. Influence of diet on growth and protein status[a]

Dietary treatment	Vaccination status[b]	Body weight (g) at wk:			Serum albumin (g/dl) at wk:		
		1	2	3	1	2	3
Control	VACC	387 ± 32[c]	391 ± 57[c]	413 ± 17[c]	3.62 ± 0.17[c]	3.35 ± 0.18[c]	2.93 ± 0.16[c]
	NV	368 ± 22[c]	365 ± 20[c]	342 ± 24[d]	3.57 ± 0.30[c]	3.24 ± 0.16[c]	2.88 ± 0.10[c]
Low protein	VACC	287 ± 23[d]	297 ± 14[d]	257 ± 25[e]	2.77 ± 0.09[d]	2.90 ± 0.13[d]	2.74 ± 0.14[c,d]
	NV	262 ± 21[d]	248 ± 18[e]	230 ± 21[e]	3.05 ± 0.07[d]	2.87 ± 0.22[d]	2.61 ± 0.09[d]

[a] Values are mean ± standard error of the mean of four to six observations at indicated week post-respiratory challenge with virulent *M. tuberculosis*.
[b] VACC, Vaccinated guinea pigs; NV, nonvaccinated guinea pigs.
[c,d,e] Means within each interval labeled by the same superscript letter are not statistically different ($P > 0.05$); means labeled with different superscript letters are statistically different.

starch and ovalbumin varying inversely to provide the desired protein content. The formulation of the diets has been published previously (12). All animals were given a mixture of the fully supplemented diet (30% protein) and decreasing proportions of powdered commercial guinea pig stock diet (Ralston Purina Co., St. Louis, Mo.) over a 2-week adaptation period before assignment to one of the experimental diets. The food was given as a powder, and fresh diet was provided every other day. Food intake was monitored periodically throughout the study.

***M. bovis* BCG vaccination.** On the same day the experimental diets were started, half of the guinea pigs in each diet treatment were vaccinated with viable *M. bovis* BCG vaccine (Copenhagen 1331; Statens Seruminstitut, Copenhagen, Denmark). Each animal received 0.1 ml of saline solution containing approximately 10^3 viable bacilli subcutaneously over the left inguinal region. The viability of the vaccine was determined by plating appropriate dilutions on M7H10 agar (Difco Laboratories, Detroit, Mich.).

Respiratory infection. *M. tuberculosis* H37Rv (ATCC 27294) was obtained from the American Type Culture Collection, Rockville, Md. The challenge inoculum was prepared and stored at −70°C by a published procedure (5).

After 6 weeks on the experimental diets (i.e., 6 weeks postvaccination with *M. bovis* BCG), all guinea pigs were infected via the respiratory route in an exposure chamber described previously (10, 20). The infection was carried out in a biohazard facility for use with class 3 human microbial pathogens. The concentration of viable *M. tuberculosis* H37Rv organisms in the nebulizer fluid was adjusted empirically to result in the inhalation and retention of about 10^2 viable mycobacteria per guinea pig. The viable count was determined by plating appropriate dilutions of the challenge culture on M7H10 agar plates (Difco). The actual challenge level was estimated from the number of primary tubercles observed grossly in the lungs of nonvaccinated animals at 3 weeks postinfection. Diet did not influence significantly the number of tubercle bacilli inhaled and retained by this criterion. Groups of guinea pigs, selected randomly from each of the vaccination and dietary treatments, were exposed repeatedly to result in uniform, reproducible infection of all animals with mycobacteria (18, 20).

PPD skin tests. One day before sacrifice, guinea pigs received two intradermal injections of 0.1 ml of solution containing either 5 or 100 tuberculin units (TU) of purified protein derivative (PPD) (PPD-RT23; Statens Seruminstitut, Copenhagen, Denmark) on a shaved area of the side. The reactions were measured with a transparent plastic ruler 24 h later, and the mean diameter of induration was recorded in millimeters.

Necropsy procedure. One, two, and three weeks after respiratory infection with virulent *M. tuberculosis* H37Rv, groups of four to six guinea pigs from each treatment were killed by cervical dislocation. A blood sample (5 to 7 ml) was drawn immediately from each animal by cardiac puncture into a 10-ml syringe. The serum was separated from each sample and stored at −20°C. The right lower lobe of the lung, the spleen, and the bronchotracheal lymph nodes were removed aseptically, placed in sterile petri dishes, and weighed. All infected tissues were handled in a biohazard hood with appropriate precautions for protection of personnel. The tissues were then homogenized separately in 4.5 ml of sterile physiologic saline in Teflon-glass homogenizers. Appropriate dilutions were inoculated onto duplicate M7H10 agar plates which were incubated at 37°C for 3 to 4 weeks. The number of colonies was counted, and the results were expressed as mean $\log_{10}$ viable organisms per gram (wet weight) of tissue to account for differences in organ size due to dietary deficiency and sampling procedures.

Statistical analysis. The analysis of variance was used to test for the effects of the treatment variables (diet and vaccination) on the dependent variables (extent of disease and delayed hypersensitivity). Mean differences between treatment groups were tested for statistical significance by Student's *t* test or Duncan's new multiple range test. The accepted level of probability was set at 95% for all tests.

RESULTS

Dietary influence on nutritional status. The low-protein diet had the expected effects on growth and metabolism of guinea pigs (Table 1). Body weights of protein-deficient animals were significantly lower at all sacrifice intervals. Nonvaccinated animals appeared to lose more weight than vaccinated animals in both diet groups. The significant reduction in serum albumin concentrations in the low-protein group is evidence of the protein-deficient status of those guinea pigs. No differences in food intake were noted between the diet groups at any time during the experiment.

Effect of diet and vaccination on tuberculin hypersensitivity. Dermal reactions in both doses of tuberculin were profoundly impaired in protein-deficient guinea pigs. The same treatment effects were seen in response to both the 5- and 100-TU doses, although the absolute reaction size and the magnitude of the differences between treatments were greater with the larger dose of tuberculin, and therefore only those data are presented (Fig. 1). Vaccinated guinea pigs consuming the control diet exhibited strong positive reactions at all sacrifice intervals, while their nonvaccinated counterparts developed significant dermal reactions to PPD as their disease developed. In contrast, protein-deficient animals were hyporesponsive at all intervals. Neither previous vaccination with *M. bovis* BCG nor extensive replication of virulent *M. tuberculosis* was accompanied by significant

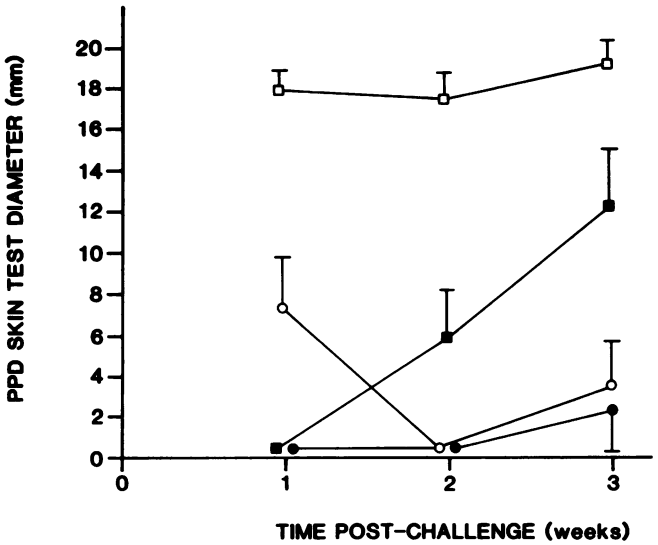

FIG. 1. Influence of dietary protein and *M. bovis* BCG vaccination on delayed hypersensitivity reactions to 100-TU PPD in vaccinated (open symbols) and nonvaccinated (solid symbols) guinea pigs maintained on either normal (squares) or protein-deficient (circles) diets and challenged at zero time with virulent *M. tuberculosis* H37Rv; mean ± standard error of the mean of four to six animals per data point is shown.

delayed hypersensitivity reactions to a 100-TU dose of tuberculin.

Impact of diet on protective efficacy of *M. bovis* BCG vaccine. Vaccine-induced protection was defined as a significant reduction in the number of virulent *M. tuberculosis* organisms recovered quantitatively from the tissues. In the lung (Fig. 2), vaccination with *M. bovis* BCG conferred protection on normally nourished guinea pigs, as evidenced by the 10- to 100-fold reduction in the number of virulent tubercle bacilli, compared with their nonvaccinated counterparts at 2- and 3-week intervals. Protein deficiency, on the other hand, completely eliminated any benefit from prior vaccination. There was no difference between the bacillary loads in the lungs of *M. bovis* BCG-vaccinated, low-protein animals and either nonvaccinated group at any sacrifice interval. In the spleen (Fig. 3), similar results were obtained. Compared with animals consuming the control diet, protein-deprived guinea pigs were significantly less protected by *M. bovis* BCG vaccination, although the loss of protection in the spleen was partial rather than complete. Figure 4 illustrates the effects of dietary protein and vaccination on the number of virulent *M. tuberculosis* organisms in the lymph nodes draining the lungs. Both vaccinated groups had significantly fewer organisms in their lymph nodes 2 weeks postchallenge, while this protection had begun to wane by 3 weeks in the protein-malnourished group. There were no significant differences in the bacillary loads of protein-deprived vaccinated and nonvaccinated guinea pigs at the last interval. In all organs, no dietary influence was observed on the ability of nonvaccinated animals to control a primary, pulmonary infection with virulent tubercle bacilli.

DISCUSSION

We have demonstrated that chronic, moderate dietary protein deficiency impairs the ability of *M. bovis* BCG vaccine to protect guinea pigs against respiratory challenge with virulent *M. tuberculosis* H37Rv. Protein deprivation was characterized by weight loss or reduced growth velocity and modest but statistically significant reductions in the concentration of serum albumin (Table 1). Protein-deprived animals were incapable of mounting significant delayed hypersensitivity reactions even to a large dose of tuberculin (100 TU) and even in the presence of extensive, extrapulmonary tuberculosis (Fig. 1). Although we did not skin

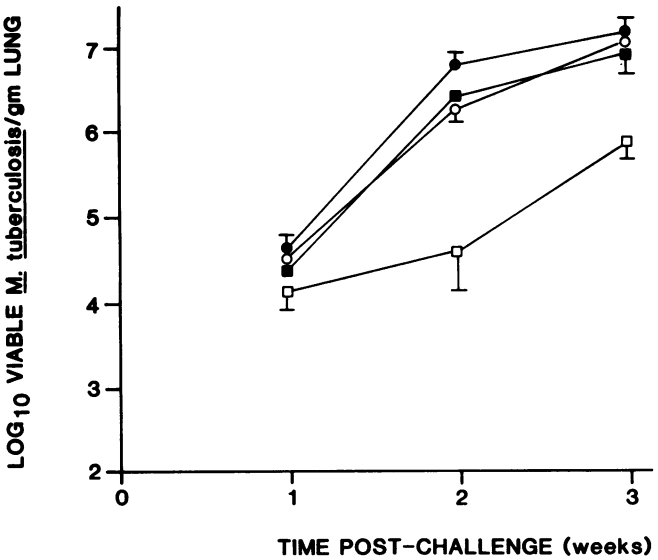

FIG. 2. Effect of dietary protein and *M. bovis* BCG vaccination on the number of viable *M. tuberculosis* H37Rv organisms recovered from the lungs of vaccinated (open symbols) and nonvaccinated (solid symbols) guinea pigs maintained on either normal (squares) or protein-deficient (circles) diets; mean ± standard error of the mean of four to six animals per data point is shown.

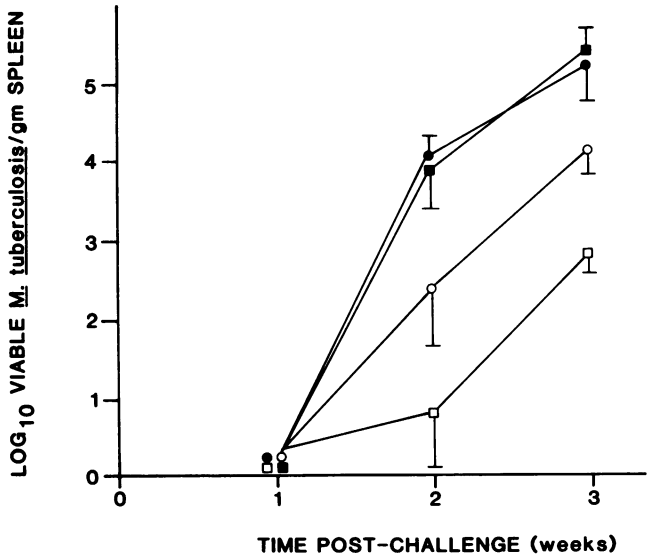

FIG. 3. Effect of dietary protein and *M. bovis* BCG vaccination on the number of viable *M. tuberculosis* H37Rv organisms recovered from the spleens of vaccinated (open symbols) and nonvaccinated (solid symbols) guinea pigs maintained on either normal (squares) or protein-deficient (circles) diets; mean ± standard error of the mean of four to six animals per data point is shown.

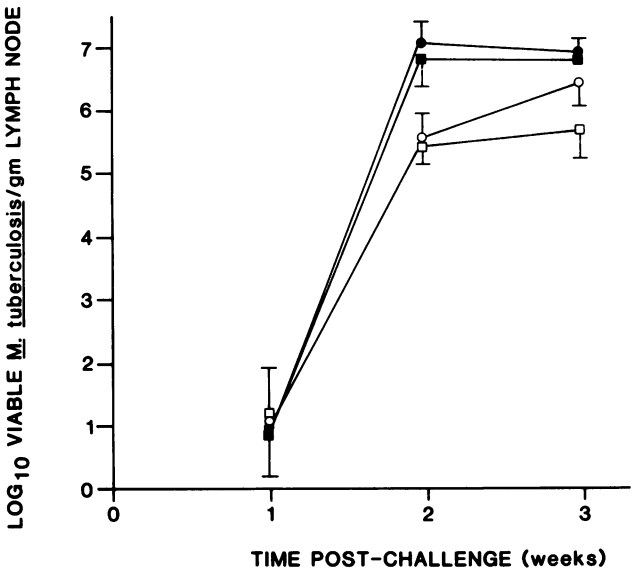

FIG. 4. Effect of dietary protein and *M. bovis* BCG vaccination on the number of viable *M. tuberculosis* H37Rv organisms recovered from the bronchotracheal lymph nodes of vaccinated (open symbols) and nonvaccinated (solid symbols) guinea pigs maintained on either normal (squares) or protein-deficient (circles) diets; mean ± standard error of the mean of four to six animals per data point is shown.

test animals prior to respiratory challenge, previous experience indicates that the protein-malnourished guinea pigs would have been responsive to PPD with reactions similar to those seen at 1 week (12). These observations confirm our previous reports of tuberculin anergy in protein-malnourished guinea pigs infected with *M. bovis* BCG and attenuated *M. tuberculosis* H37Ra (10, 12) and support the contention that malnutrition interferes with the diagnostic usefulness of PPD skin tests in individuals with clinical tuberculosis (D. N. McMurray, Editorial, Chest **77**:4–5, 1980).

Among the most consistent and predictable reports of impaired cell-mediated immune function in malnourished humans have been the observations of failure to develop normal tuberculin skin reactivity after *M. bovis* BCG vaccination (6, 11, 16). While it is tempting to suggest that lack of conversion to PPD postvaccination implies that the vaccine has been unsuccessful in eliciting a protective response in the malnourished vaccine recipient, that hypothesis has never been formally tested either in human field trials or in experimental animals.

Using a guinea pig model similar to the one described here, Smith and his colleagues (4, 18) have demonstrated that successful vaccination with *M. bovis* BCG results in control of mycobacterial accumulation in the primary lung lesions as well as limitation of bacillemic spread of virulent organisms to the spleen and the lymph nodes. A statistically significant inverse correlation has been established between the number of viable *M. tuberculosis* H37Rv organisms recovered from the lungs between 3 and 5 weeks postchallenge and the ultimate survival of vaccinated and nonvaccinated guinea pigs (20). Based upon that rationale, we observed that *M. bovis* BCG did not protect protein-deficient guinea pigs against virulent *M. tuberculosis*. The loss of vaccine-induced protection was complete in the lung and partial but significant in the spleen and the lymph nodes draining the lung. It appears that protein deprivation not only prevents control of mycobacterial accumulation in the pulmonary lesions but interferes with the ability of vaccinated animals to limit extrapulmonary dissemination. Although we believe that these data are predictive of the ultimate outcome of infection, studies of longer challenge-sacrifice intervals may be desirable to confirm the deleterious effect of protein deficiency on vaccine efficacy.

Given the failure of protein-deficient guinea pigs to mount a protective immune response after vaccination with *M. bovis* BCG, it was surprising to observe no effect of diet on the course of primary tuberculosis in nonvaccinated animals. Identical numbers of virulent mycobacteria were recovered from lung, spleen, and lymph nodes of low-protein and control, nonvaccinated groups. We had observed previously that protein-deficient guinea pigs ultimately controlled primary infections with *M. bovis* BCG or attenuated *M. tuberculosis* H37Ra as successfully as normally nourished animals (10, 12). In those experiments, as in the ones reported here, control of mycobacterial populations in nonvaccinated animals was seen in the absence of significant tuberculin hypersensitivity. The development of apparently effective resistance to progressive tuberculosis has been observed occasionally in anergic patients (McMurray, Editorial, Chest, 1980). These observations suggest that dermal reactivity and antimicrobial resistance in tuberculosis may be dissociable events, possibly mediated by different T lymphocyte subpopulations, as reported in mice (15).

The extrapolation of these results to the effects of malnutrition on *M. bovis* BCG vaccine efficacy in human populations must be qualified. Variables such as the severity and chronicity of protein deprivation, the virulence of the prevalent strains of *M. tuberculosis*, and the endpoint used to define protection (quantitative reduction of bacillary loads versus absence of clinical tuberculosis) might affect the conclusions reached if a similar study were carried out in humans. However, the fact that our observations were made in an animal model designed specifically to approximate the relevant human condition, with respect to both diet and infection, suggests that nutritional variables may have contributed to previous failures of *M. bovis* BCG vaccine in human trials.

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