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# Cell-Mediated Immunity in Malnourished Guinea Pigs After *Mycobacterium bovis* BCG Vaccination

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Specific-pathogen-free guinea pigs were vaccinated with viable *Mycobacterium bovis* BCG and maintained on purified, isocaloric diets containing either 30% or 7.5% casein, or commercial chow. At intervals of 4, 5, 6, and 8 weeks postvaccination, groups of guinea pigs from each diet treatment were skin tested with purified protein derivative and killed. Protein-deficient animals exhibited progressive reductions in total serum proteins and albumin. Significantly greater numbers of viable *M. bovis* BCG were recovered from the vaccination site and inguinal lymph nodes of protein-deficient guinea pigs at all intervals. In contrast, the development of delayed hypersensitivity was markedly retarded in the 7.5% casein group and was also reduced somewhat in the 30% casein group as compared to chow control. Peripheral blood lymphocytes from protein-deficient animals did not respond normally in vitro to a polyclonal T cell mitogen, phytohemagglutinin. These results demonstrate that protein-calorie malnutrition in this model impairs the development of cell-mediated immunity as evidenced by skin test anergy, lymphocyte hyporesponsiveness, and failure to control levels of viable *M. bovis* BCG after vaccination.

Immune dysfunction secondary to malnutrition is probably the most common form of "acquired" immunological deficiency in man. Numerous studies have demonstrated that both severe and moderate nutritional deficiencies are accompanied by significant immune impairment, particularly cell-mediated immunity in vivo and in vitro (5, 10, 23, 34). Experimental animal models have contributed to our appreciation of the role of single nutrient deficiencies (2, 6) while illustrating the differential effect of moderate protein-calorie malnutrition on humoral and cellular immune mechanisms (9, 18). Few would dispute the association between malnutrition and measurable perturbations of immune response; however, the biological significance of nutrition-mediated immune dysfunction, in man or animals, has not been established. There appears to be a relationship between protein malnutrition and increased susceptibility to infectious disease in humans and animals (21, 30), but few experimental studies have included response to infection along with other measures of immunological status.

*Mycobacterium bovis* BCG vaccine is used world-wide to prevent tuberculosis and in immunotherapy of some forms of cancer. Since nutritional deficiencies occur in the majority of preschool children in many developing countries (8, 23), and are common in patients with malignancy (28), it is critical to evaluate the impact of malnutrition on the response to *M. bovis* BCG. Several studies done in experimental animals and human subjects suggest that protein-calorie malnutrition impairs immunity to *M. bovis* BCG (3, 34), whereas other results appear to show no effect (25, 29). Previously, we have demonstrated that malnourished, *M. bovis* BCG-vaccinated guinea pigs exhibit impaired delayed hypersensitivity to purified protein derivative (PPD) and lymphocyte hyporesponsiveness to mitogenic stimulation in vitro (22, 24a). In contrast, we have observed enhanced macrophage activity under similar conditions (24b), suggesting that acquired cellular resistance may not be affected.

In the present study, we examined the influence of a protein-deficient diet on the time-course of *M. bovis* BCG infection, the development of delayed hypersensitivity, and the blastogenic activity of peripheral blood lymphocytes in *M. bovis* BCG-vaccinated guinea pigs.

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## MATERIALS AND METHODS

**Experimental animals.** Outbred, albino, specific-pathogen-free female guinea pigs, weighing 250 to 300 g, were obtained from a commercial source (Hartley-COBS, Charles River Breeding Laboratories, Inc., Wilmington, Mass.). They were housed individually in polycarbonate cages on stainless-steel wire mesh floors and provided with tap water and food ad libitum. Each animal was randomly assigned to an experimental treatment. Body weights were recorded weekly during the experiment.

**Experimental diets.** The experimental diets were based upon the current recommended nutritional requirements for guinea pigs (26). Two purified diets containing 30 or 7.5% casein were used. These diets were isocaloric, with the proportion of casein and cornstarch varying inversely to provide the desired protein content. The formulations of the basic diet, mineral mix, and vitamin mix were published previously (24a). The casein was supplemented with arginine and methionine in the dietary group receiving 30% casein. The diets were given as a powder or mixed with tap water to form a paste. Fresh diet was provided every other day. An additional group was maintained on a commercial stock diet (Guinea Pig Chow, Ralston Purina, St. Louis, Mo.) containing 18% protein.

***M. bovis* BCG vaccination.** On the same day the experimental diets were started, guinea pigs were vaccinated with viable *M. bovis* BCG vaccine (Copenhagen 1331, Statens Seruminstitut, Copenhagen, Denmark). Each animal received 0.1 ml of saline containing about $10^3$ viable bacilli subcutaneously in the left inguinal area. The viability of the vaccine was determined by plating appropriate dilutions on oleic acid albumin agar (Difco Laboratories, Detroit, Mich.).

**PPD skin tests.** Delayed hypersensitivity was elicited in *M. bovis* BCG-vaccinated guinea pigs two days before sacrifice by the intradermal injection of 0.1 ml of PPD (PPD-RT-23; Statens Seruminstitut). One dose of PPD, 100 tuberculin units, was injected on a shaved area of the side. The reactions were measured with a transparent plastic ruler 48 h later, and the mean diameter of induration was recorded in millimeters.

**Autopsy procedure.** Four, five, six, and eight weeks after vaccination and initiation of the experimental diets, groups of 5 to 7 guinea pigs from each group were killed by cervical dislocation. The animals were exsanguinated by cardiac puncture with a heparinized 10-ml syringe. The abdominal cavity was opened aseptically, and the subcutaneous vaccination site and inguinal lymph nodes draining the site were removed to sterile petri dishes and weighed. These organs were then homogenized in separate Teflon-glass homogenizers in 2 ml of sterile 2% albumin solution, and appropriate dilutions were plated on oleic acid albumin agar to recover viable *M. bovis* BCG. The plates were incubated for 3 to 4 weeks at 37°C, and the number of *M. bovis* BCG colonies were counted. Viable counts are expressed as mean $\log_{10}$ viable *M. bovis* BCG per milligram (wet weight) of tissue.

Total protein content of the serum was measured using the Lowry method (20). Protein electrophoresis was carried out on cellulose acetate strips, which were stained and quantified in a scanning densitometer (DCD-16, Gelman Sciences, Inc., Ann Arbor, Mich.). The concentration of serum albumin was calculated using these data.

**Lymphocyte blastogenesis.** The response of peripheral blood lymphocytes to the polyclonal T cell mitogen phytohemagglutinin (Difco) was measured by in vitro culture (11). Whole, heparinized blood was diluted 1:6 with tissue culture medium (RPMI 1640, Microbiological Associates, Bethesda, Md.) containing 10% fetal bovine serum. Aliquots of 250 μl were placed in the wells of a microtiter plate (Falcon Plastics, Oxnard, Calif.). Triplicate cultures were stimulated with four dilutions of phytohemagglutinin (1:10, 1:50, 1:100, 1:200) in 10 μl of tissue culture medium and incubated at 37°C in a 5% $CO_2$ atmosphere for 72 h. Each well was then pulsed with 10 μl of tissue culture medium containing 0.8 μCi of tritiated thymidine (6.7 mCi/mmol; New England Nuclear Corp., Boston, Mass.). Twenty-four hours later, the cultures were harvested onto fiber-glass filter disks with a multiple automated sample harvestor (Otto Hiller, Inc., Madison, Wisc.). The disks were bleached with 5% hydrogen peroxide, placed in liquid scintillation vials with cocktail (Omnifluor, New England Nuclear), and counted in a liquid scintillation counter (Beckman LS 8000, Beckman Instruments, Inc., Fullerton, Calif.). Counts were corrected for background and quenching and adjusted to represent the response of $10^6$ lymphocytes. The phytohemagglutinin dilution giving the optimal response was selected for each animal and used to calculate the blastogenic index, which was defined as the ratio of counts per minute in mitogen-stimulated versus non-stimulated cultures of the same animal's cells.

**Statistical analysis.** The analysis of variance was used to test for the effect of dietary treatment on the dependent variables measured. Where appropriate, Student's $t$ test was employed to assess the significance of differences between group means. The confidence level was set at $P < 0.05$.

## RESULTS

**Effect of diet on growth and protein status.** There was no difference between the initial mean body weights of the 7.5% casein group (283 ± 18 g), the 30% casein group (267 ± 20 g), and the chow group (275 ± 18 g). The effect of diet on the body weights of guinea pigs killed at the four intervals is illustrated in Fig. 1. The chow control animals were significantly heavier than those consuming the purified diets at all intervals. Food intake was estimated periodically, and both groups consuming purified diets were taking in about one-fifth less food per day than the chow controls. There was no difference in food consumption between the 7.5 and 30% casein groups. Between 5 and 8 weeks, the 30% casein group gained somewhat, whereas the 7.5% casein group lost weight gradually. The protein-deficient animals were significantly lighter than those on the 30% casein diet at 6 and 8 weeks postvaccination.

Between 4 and 8 weeks after initiation of experimental diets and *M. bovis* BCG vaccination, protein deficiency was observed to exert a progressive effect on serum total protein con-

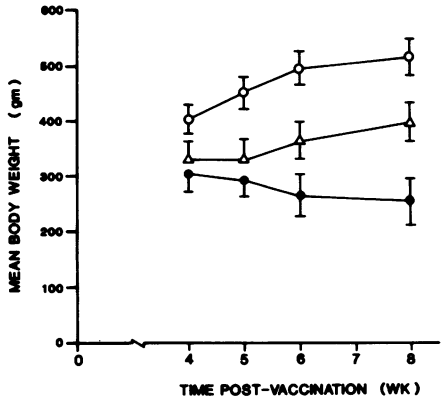


FIG. 1. Growth of *M. bovis* BCG-vaccinated guinea pigs maintained on 7.5% casein (●), 30% casein (△), or commercial chow (○); vertical bars indicate standard error of the mean.

centrations (Fig. 2A). In the group consuming the 7.5% casein diet, total protein levels were already significantly reduced compared to chow controls at 4 weeks, and declined markedly to less than 5 g/dl by 8 weeks. In the 30% casein group, a slight reduction was seen at 4 weeks, but there were no significant differences from the chow control thereafter. A similar pattern was observed in the response of serum albumin (Fig. 2B), which was subnormal at 4 weeks in the 7.5% casein group and declined further to less than 2.5 g/dl by 6 weeks. There were no differences in the mean serum albumin concentrations of the 30% casein and chow groups at any interval.

**Time course of *M. bovis* BCG infection.** Figure 3 illustrates the influence of diet on the recovery of viable *M. bovis* BCG from the inguinal lymph nodes draining the vaccination site (Fig. 3A) and subcutaneous vaccination site itself (Fig. 3B). In all animals, the number of viable *M. bovis* BCG increased between 4 and 6 weeks and then declined by week 8 postvaccination. Total numbers of viable mycobacteria were about the same in both tissues within each treatment group. The weights of the inguinal lymph nodes and vaccination site were reduced about one-third in the 7.5% casein group at 5, 6, and 8 weeks (0.08 g versus 0.12 g for lymph nodes; 0.24 g versus 0.32 g for the vaccination site). Significantly more *M. bovis* BCG per milligram were recovered from both tissues taken from protein-deficient (7.5% casein) guinea pigs than from chow controls, especially at the 4-, 5-, and 6-week intervals. Animals consuming the 30% casein diet also had more viable *M. bovis* BCG per milligram in both tissues at 4 weeks and in the inguinal nodes at 5 weeks. There was no significant difference between bacillary populations of the 30% casein and chow groups at the later intervals.

**Development of delayed hypersensitivity.** Figure 4 documents the influence of dietary treatment and time postvaccination on the size of delayed hypersensitivity reactions after intradermal tests with 100 tuberculin units of PPD. Reactions were minimal in all diet groups 4 weeks after vaccination, but increased rapidly in the chow controls to reach a plateau of 18 to 20 mm of induration by 6 and 8 weeks. A modest increase in responsiveness was observed in the two groups consuming purified diets at 5 weeks, after which no further change was observed in the protein-deficient (7.5% casein) guinea pigs. In contrast, PPD reactivity continued to increase in the 30% casein group, although the skin tests were never as large on the average as those of the chow controls. At 6 and 8 weeks, all of the chow and 30% casein animals had measurable reactions to PPD, whereas some of the 7.5% casein guinea pigs were completely anergic and those which responded had much smaller areas of induration distributed over a range of reaction sizes from 0 to 10 mm of induration.

**Lymphocyte blastogenesis.** The effect of diet on the mitogenic responses of peripheral blood lymphocytes to a polyclonal T cell mitogen,

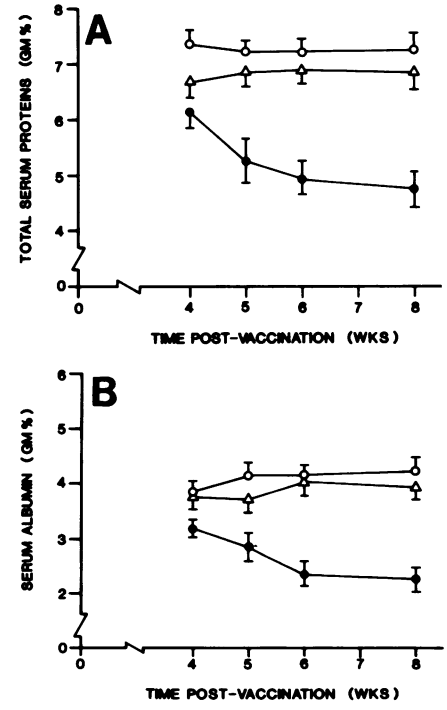


FIG. 2. Effect of duration of diet on total serum proteins (A) and serum albumin (B) of *M. bovis* BCG-vaccinated guinea pigs maintained on 7.5% casein (●), 30% casein (△), or commercial chow (○); vertical bars indicate standard error of the mean.

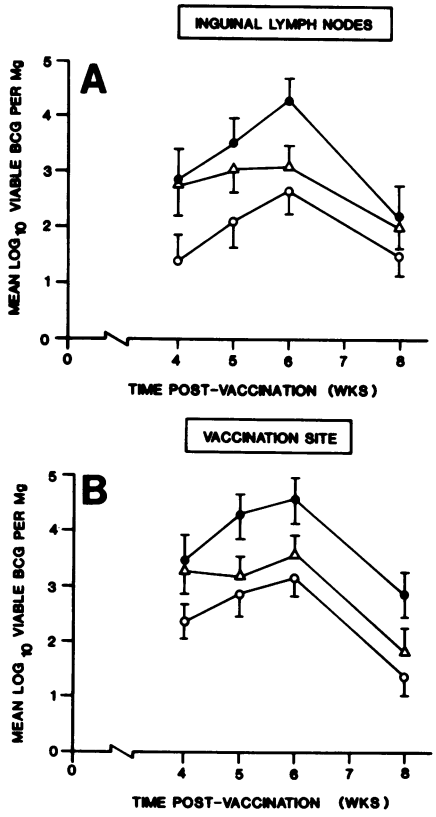


FIG. 3. Time course of *M. bovis* BCG infection in the inguinal lymph nodes (A) and vaccination site (B) of guinea pigs maintained on 7.5% casein (●), 30% casein (△), or commercial chow (○); vertical bars indicate standard error of the mean.

phytohemagglutinin, is summarized in Table 1. The optimum dilution of phytohemagglutinin varied slightly from animal to animal, but there was no detectable effect of diet on the dose response. Although the blastogenic responses of lymphocytes from guinea pigs fed the 30% casein diet were routinely lower than the control responses, the differences were not statistically significant at any interval. In contrast, significant impairment of blastogenesis was observed at all intervals in lymphocytes taken from protein-deficient (7.5% casein) guinea pigs. Moreover, there was a progressive deterioration of lymphocyte responses in vitro with increased duration of dietary deficiency. By 8 weeks, the response of the 7.5% casein group was only one-tenth that of control.

## DISCUSSION

Malnutrition impairs the ability of guinea pigs to respond immunologically to infection with viable *M. bovis* BCG. Animals fed a protein-deficient diet (7.5% casein) beginning on the day of vaccination did not control the accumulation of viable *M. bovis* BCG in the vaccination site and draining lymph nodes as successfully as the protein-adequate (30% casein) or chow control groups (Fig. 3). In spite of this increased antigenic stimulation, the delayed hypersensitivity responses to PPD in the 7.5% casein group were weak or absent, implying that the development of cell-mediated immunity to mycobacterial antigens was impaired. A possible mechanism for this impairment is suggested by the hyporesponsiveness of peripheral blood thymus-dependent (T) lymphocytes from protein-deficient guinea pigs to stimulation in vitro with a polyclonal mitogen (Table 1).

Our previous experience with guinea pigs suggested that slow adaptation to the purified diets resulted in growth retardation even in the protein-adequate (30% casein) group (24a). In the present study, the 30% casein group demonstrated some of the hallmarks of marasmus in children, with low body weight but no significant reduction in total serum proteins or serum albumin. This marasmus-like condition had a measurable effect on delayed hypersensitivity reactions to PPD, even at 8 weeks when the growth rates and serum protein and albumin levels of the 30% casein and chow control groups were comparable (see Fig. 4). Our results are similar to published reports of reduced tuberculin reactions in moderately underweight *M. bovis* BCG-vaccinated children (12, 13, 23, 34). Some workers have reported little effect of clinical marasmus on delayed hypersensitivity after vaccination with *M. bovis* BCG (25, 29, 31), whereas others have demonstrated significant impairment of PPD reactions in such children (1, 24). Heresi and colleagues have found impaired T lymphocyte blastogenesis in spleen cells of severely calorie-restricted rats (14), but demonstrated no impairment of production of a lym-

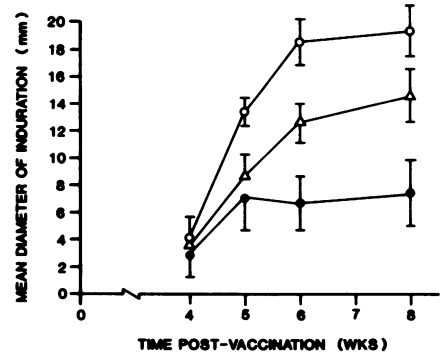


FIG. 4. Development of delayed hypersensitivity reactions to 100 tuberculin units of PPD in *M. bovis* BCG-vaccinated guinea pigs maintained on 7.5% casein (●), 30% casein (△), or commercial chow (○); vertical bars indicate standard error of the mean.

phokine, leukocyte inhibitory factor, by the cells of marasmic children (15). Our results suggest that a history of calorie restriction in the guinea pig, as expressed by low body weight, is accompanied by a measurable delay in the development of delayed hypersensitivity, but is not reflected in the other measures of immune response (that is, numbers of viable *M. bovis* BCG, lymphocyte blastogenesis), which are adversely affected by protein malnutrition. The timing of the immunization with respect to the nutritional insult is probably critical, since Koster et al. have observed retarded development of delayed hypersensitivity in children immunized before nutritional rehabilitation was initiated (17).

As in previous studies, restricted protein intake (7.5% casein) for several weeks produced a nutritional state in guinea pigs similar to protein-calorie malnutrition in children, with low body weight accompanied by significant reductions in total serum proteins and albumin. We have already demonstrated that serum albumin levels in guinea pigs are correlated positively with some immunological functions, such as delayed hypersensitivity and lymphocyte blastogenic responses in vitro (24b). Bhuyan and co-workers have reported previously that protein-malnourished guinea pigs and rabbits responded poorly to PPD and did not develop a normal granulomatous response after *M. bovis* BCG vaccination (3, 4). The biological validity of our model system is evident from the similarity between our results and studies of cell-mediated immunity in humans with protein-calorie malnutrition. Several groups have reported reduced skin reactivity and T lymphocyte hyporesponsiveness after *M. bovis* BCG vaccination of children with protein-calorie malnutrition (1, 24, 28, 31).

Although the number of viable *M. bovis* BCG recovered from the injection site and draining nodes was significantly greater in the protein-deficient (7.5% casein) group, the population declined markedly between 6 and 8 weeks post-vaccination (see Fig. 3). This indicates that the development of effective acquired cellular resistance was delayed, but not abolished, by malnutrition. It is important to note that apparent control of *M. bovis* BCG accumulation occurred in the 7.5% casein group without concomitant conversion to PPD skin test reactivity (see Fig. 4). This suggests that delayed hypersensitivity may not be a useful indicator of host response to *M. bovis* BCG vaccination. Protein-deficient guinea pigs demonstrated enhanced macrophage enzymatic activity in previous studies, suggesting a mechanism by which *M. bovis* BCG levels might be controlled even in the absence of a strong T cell response (24b). Alternatively, the decline observed in viable *M. bovis* BCG in the malnourished animals may not be due to specific immunity but rather to deteriorating tissue conditions which are unable to support growth of the organisms. Other workers have observed increased mycobacterial populations in histological sections of tissues taken from protein-malnourished guinea pigs, but the viability of these organisms was not determined (3).

Collins and Auclair reported essentially no growth of viable *M. bovis* BCG at the vaccination site and peak numbers in draining nodes much earlier in a study of normal guinea pigs (7). The discrepancies between their results and our observations in chow control animals are likely due to differences in *M. bovis* BCG vaccine strain and dose. Collins et al. injected more than $10^6$ viable *M. bovis* BCG per guinea pig, 1,000-fold more than in the present study. The advantage of employing minimal effective doses of *M. bovis* BCG to increase the discriminating power between levels of acquired resistance has already been demonstrated (19).

Delayed hypersensitivity to PPD is widely used as an indicator of host defense status and *M. bovis* BCG vaccine efficacy in humans. Unfortunately, skin test reactions are impaired by both severe and moderate malnutrition in the very populations which would benefit most from protection against tuberculosis. Our results indicate that the PPD skin test is sensitive both to protein deprivation and a past history of reduced food intake, whereas only protein deprivation is accompanied by deranged lymphocyte function in vitro and temporary impairment of acquired

TABLE 1. Influence of dietary treatment on phytohemagglutinin-induced blastogenesis of peripheral blood lymphocytes

| Diet group | Blastogenic index[a] | | | |
|---|---|---|---|---|
| | 4 wk | 5 wk | 6 wk | 8 wk |
| Chow | 33.4 ± 7.5[b] (5)[c] | 35.2 ± 9.0 (5) | 46.1 ± 12.4 (5) | 29.0 ± 6.5 (5) |
| 30% Casein | 21.2 ± 4.8 (7) | 22.9 ± 7.4 (7) | 34.3 ± 7.6 (10) | 20.3 ± 4.9 (6) |
| 7.5% Casein | 10.4 ± 3.9 (7)[d] | 5.3 ± 2.9 (7)[d] | 3.3 ± 1.9 (5)[d] | 2.8 ± 0.6 (5)[d] |

[a] Ratio of counts per minute in stimulated versus unstimulated cultures.
[b] Mean ± standard error of the mean.
[c] Number of observations contributing to the mean.
[d] Denotes values significantly different from chow control ($P < 0.05$).

cellular resistance in vivo. The implications of these observations for the use of *M. bovis* BCG in preventing clinical tuberculosis in malnourished populations are emphasized by the recent *M. bovis* BCG field trial failure in India (33). An experimental approach utilizing low-level respiratory infection of malnourished *M. bovis* BCG-vaccinated guinea pigs with *M. tuberculosis* should help to clarify this question (16).

## ACKNOWLEDGMENTS

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