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Effects of Diet and Genetics on *Mycobacterium bovis* BCG Vaccine Efficacy in Inbred Guinea Pigs

MICHAEL K. COHEN, REBECCA A. BARTOW, CAROLE L. MINTZER, AND DAVID N. McMURRAY*

Department of Medical Microbiology and Immunology, College of Medicine, Texas A&M University, College Station, Texas 77843

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Strain 2 and strain 13 guinea pigs were vaccinated with *Mycobacterium bovis* BCG and placed on low-protein or protein-adequate diets. Five weeks later all animals were infected by the respiratory route with virulent *Mycobacterium tuberculosis* H37Rv organisms. Four weeks postchallenge, guinea pigs were skin tested with purified protein derivative and sacrificed. Protein deficiency resulted in significant reductions in body weight and thymus weight and in an impairment in the ability to control the *M. bovis* BCG vaccine organisms and to mount delayed hypersensitivity reactions. Protein deficiency also adversely affected the efficacy of the BCG vaccine as demonstrated by the numbers of virulent organisms recovered in spleens and lungs. Strain differences were observed in the number of leukocytes, thymus weight, and the responsiveness of blood lymphocytes to purified protein derivative stimulation. In general, strain 13 guinea pigs responded more dramatically to dietary insult than did their strain 2 counterparts. Protein deprivation completely abolished BCG vaccine protection in the lungs and spleens of strain 13 animals and significantly reduced the protection afforded to strain 2 animals. In both strains, the BCG vaccine protected normally nourished guinea pigs. There was no significant difference between strains with respect to susceptibility to pulmonary infection with virulent mycobacteria. Thus, diet and genetic pedigree each had a significant influence on BCG vaccine efficacy.

The efficacy of the bacillus Calmette-Guerin (*Mycobacterium bovis* BCG) vaccine in the worldwide effort to control tuberculosis has varied significantly depending on undetermined factors specific for a particular population or region (2, 3). One factor which may be associated with a lack of protection by BCG vaccine is malnutrition, prevalent in the same Third World populations in which tuberculosis is common (3, 9, 18).

Specific dietary deficiencies in humans and experimental animals have significant deleterious effects on cell-mediated immune responses to infection (7). Protein deficiency is accompanied by the loss of tuberculin hypersensitivity to purified protein derivative (PPD) (8, 10, 22). Experiments have shown increased numbers of virulent mycobacteria in spleens and lungs of previously BCG-vaccinated, malnourished animals challenged by the respiratory route with *M. tuberculosis* (9, 11). In addition, numbers of lymphocytes and lymphocyte proliferation after stimulation by a mitogen in vitro were both reduced in protein-deprived animals (12).

Studies involving inbred strains of rabbits and mice have focused previously on genetic aspects of the immune response to mycobacterial infections (1, 4, 17). Differences have been observed in the immune responses of the respective resistant and susceptible inbred strains of these animals. Inbred mice have been used most extensively in this particular area (14). These studies have involved high-dose, intravenous injections of virulent *Mycobacterium tuberculosis* or *M. bovis* BCG organisms, whereas most human tuberculosis is transmitted by droplet nuclei via the respiratory route (4, 14). Also, all of the previous work relating protein deficiency and BCG vaccine efficacy has been done with outbred (Hartley strain) guinea pigs administered low-protein diets and challenged by the respiratory route with virulent mycobacteria (9, 11). Two inbred strains of guinea pigs, strains 2 and 13, have been used in studies of basic immune responses and resistance to other infectious diseases (15, 16). Although these strains are generally accepted to differ with respect to susceptibility to mycobacterial infection, the literature contains no direct comparison of these two strains. An experimental advantage of using inbred animals is that one can hold other factors constant while separating two distinct genetic groups within the same species. In addition, the desirability of determining interactions between genetic and nutritional components of the immune response to BCG makes the use of inbred guinea pigs particularly relevant.

In this study, we have observed the effects of chronic, moderate dietary protein deficiency on protection against virulent *M. tuberculosis* H37Rv in BCG-vaccinated strain 2 and strain 13 guinea pigs after pulmonary challenge.

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MATERIALS AND METHODS

Experimental animals. Male and female pathogen-free strain 2 and strain 13 inbred guinea pigs weighing 150 to 250 g were obtained commercially (strain 2-2/CrlBR; Charles River Breeding Laboratories, Wilmington, Mass.; strain 13; Crest Caviary, Raywood, Calif.). The animals were housed individually in polycarbonate cages on stainless steel grid floors with stainless steel feeders. They were each provided with water and food ad libitum. The guinea pigs of each strain were randomly assigned to an experimental diet and to either a vaccinated or nonvaccinated treatment. Body weights were recorded weekly during the experiment.

Experimental diets. The experimental diets were both designed to meet recommended nutritional requirements for

* Corresponding author.

guinea pigs (13). One purified diet was protein deficient (10% ovalbumin), the purified control diet had an adequate amount of protein (30% ovalbumin), and the third diet was commercial guinea pig chow (Ralston Purina, St. Louis, Mo.). The purified diets were maintained isocaloric by inversely varying the cornstarch and ovalbumin levels. The purified diets were obtained commercially (Dyets, Inc., Bethlehem, Pa.) and were prepared according to a previously published formulation (12). Before initiation of the experimental diets, animals were weaned from their commercial chow by first mixing 50% ground chow with 50% control diet and subsequently reducing the proportion of ground chow in the mixture over a period of 2 to 3 weeks. Once the experimental diets were initiated, the animals on low-protein diets and the control animals were fed daily with the respective powder diets. The chow-fed guinea pigs were also fed daily, but the commercial chow was given as pellets. Sixteen guinea pigs were assigned to each of the three diets. Food intake was monitored periodically during the study.

BCG vaccination. Half of the guinea pigs in each diet group and strain were each vaccinated with 0.15 ml containing approximately 10^6 viable *M. bovis* BCG vaccine organisms (Copenhagen 1331, Statens Seruminstitut, Copenhagen, Denmark) reconstituted with sterile physiologic saline. The animals were vaccinated subcutaneously in the left inguinal area 3 weeks before the start of experimental diets.

Respiratory challenge. All animals in the study were challenged by the respiratory route with virulent *M. tuberculosis* H37Rv organisms (ATCC 27294; American Type Culture Collection, Rockville, Md.) 5 weeks postvaccination and 2 weeks after the initiation of the experimental diets. The challenge culture was a dilution of frozen inoculum (−70°C) in sterile saline. According to a published procedure (9, 21), the animals were placed in an aerosol exposure chamber and infected. The concentration of *M. tuberculosis* H37Rv was adjusted to result in the inhalation and retention of about 10^2 viable organisms per guinea pig. Randomly selected groups of 18 animals were exposed simultaneously. This procedure has been shown to result in uniform, reproducible infection in all animals (21).

PPD skin tests. All animals were shaved on the right side and injected intradermally with 0.1 ml of PPD (PPD-RT23; Statens Seruminstitut) containing 100 tuberculin units. These injections were done 24 h before sacrifice, and the mean diameter of induration was measured at sacrifice.

Necropsy procedure. Four weeks after respiratory challenge (6 weeks after the start of experimental diets), all guinea pigs were weighed, and their tuberculin reactions were recorded. Then 1.5 to 2.0 ml of sodium pentobarbital (Sleepaway; Fort Dodge Laboratories, Fort Dodge, Iowa) was injected intramuscularly into each animal. The anesthetized animals were exsanguinated by cardiac puncture with heparinized 10-ml syringes. The thoracic and abdominal cavities of each animal were opened aseptically, and the thymus, spleen, right lower lobe of the lung, and subcutaneous BCG vaccination nodule were aseptically removed. All of the organs were placed in sterile dishes and weighed. The lungs, spleens, and vaccination nodules were each homogenized separately in 2.0 ml of sterile saline in Teflon-coated glass homogenizers. Appropriate 10-fold dilutions were made and inoculated onto duplicate M7H10 (Difco Laboratories, Detroit, Mich.) agar plates, which were incubated at 37°C for 4 to 5 weeks. The plated organisms were counted as colonies and expressed as the mean $\log_{10}$ viable *M. tuberculosis* H37Rv (lung and spleen) or *M. bovis* BCG (vaccination nodule) organisms per gram of tissue. Previous experience has shown that the lungs and spleens of BCG-vaccinated animals no longer harbor detectable levels of viable BCG 9 weeks postvaccination.

By standard clinical procedures, total and differential leukocyte counts were determined from blood samples from each animal. Total serum protein concentrations were measured by the method of Lowry et al. (6) with a bovine serum albumin standard.

Lymphocyte blastogenesis. Lymphocytes from blood were separated by density gradient centrifugation and cultured with tuberculin in vitro. The isolated lymphocytes were suspended in RPMI 1640 medium containing 10% fetal bovine serum–penicillin (100 U/ml)–streptomycin (100 μg/ml)–2-mercaptoethanol (10 μM)–L-glutamine (2 mM) and placed into wells (2×10^5 cells per well) of microtiter plates (Falcon Microtest II; Becton Dickinson Labware, Oxnard, Calif.). Triplicate cultures were stimulated with PPD (Statens Seruminstitut) at final concentrations of 50, 25, and 12.5 μg/ml. Control cultures without antigen were also established. The cells were then incubated for 4 days at 37°C in a 5% CO_2 atmosphere. On the final day of incubation, the cells were labeled for 6 h with 0.8 μCi of tritiated thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.). The cultures were then harvested onto fiber glass filter disks and counted in a liquid scintillation counter (LS8000; Beckman Instruments, Inc., Fullerton, Calif.). The data are expressed as net uptake of tritiated thymidine (counts per minute) of cells stimulated by the dilution of PPD which gave maximum response minus counts per minute in nonstimulated cells from the same animal.

Statistical analysis. The analysis of variance was used to test for the effects of the treatment variables (diet, strain, and vaccination status) on the dependent variables. Mean differences between treatment groups were tested for statistical significance with Duncan's new multiple-range test (20) only when the F test indicated reliable treatment effects. The accepted level of probability was set at 95% for all tests.

RESULTS

Dietary influence on growth and lymphoid tissues. The inbred guinea pigs responded to the diets in a fashion consistent with previous results obtained in outbred (Hartley) guinea pigs. Protein-deprived animals of both strains exhibited significant reductions in total serum protein levels (data not shown). In both strain 2 and strain 13 animals, the chronic administration of the protein-deficient diet resulted in losses or very modest increases in body weight at the time of sacrifice (Table 1). Vaccinated animals of both inbred strains exhibited significantly greater weight gains than their nonvaccinated counterparts in all three diet groups. The only exception was the strain 13 animals fed the low-protein diet. Also, strain differences were observed with respect to body weight changes, in that the strain 2 guinea pigs appeared to adapt better to the low protein diet than did the strain 13 animals.

The thymus weights of nonvaccinated, protein-deprived guinea pigs of both strains were significantly reduced compared with control animals which, in turn, exhibited smaller thymuses than chow-fed guinea pigs. Among vaccinated animals, diet exerted an effect upon the thymus weights of strain 13 but not strain 2 guinea pigs. In general strain 13 guinea pigs given chow had larger thymuses than their strain 2 counterparts, although there was no significant effect of strain alone on this parameter.

A significant strain difference was observed with respect to the number of leukocytes counted (Table 1). In each

TABLE 1. Effect of guinea pig strain, diet, and BCG vaccination on growth, thymus weight, and leukocyte counts in animals challenged with virulent *M. tuberculosis* H37Rv by the respiratory route[a]

Guinea pig strain and dietary treatment[b]	Body wt change (g)		Thymus wt (g)		Total WBC/mm^3 (10^3)	
	Vacc	NV	Vacc	NV	Vacc	NV
2						
Low protein	+18 ± 19*	−46 ± 12*	0.43 ± 0.07*	0.29 ± 0.03*	5.25 ± 2.00*	3.31 ± 0.76*
Control	+75 ± 24†	+13 ± 24†	0.56 ± 0.05*	0.45 ± 0.06†	7.68 ± 0.03†	5.77 ± 1.90*†
Chow	+210 ± 52‡	+174 ± 36‡	0.43 ± 0.04*	0.60 ± 0.05‡	5.18 ± 1.76*	5.46 ± 0.83†
13						
Low protein	−130 ± 8*	−66 ± 12*	0.31 ± 0.03*	0.28 ± 0.05*	6.75 ± 0.21*	6.60 ± 2.20*
Control	+69 ± 13†	−53 ± 11*	0.55 ± 0.08†	0.46 ± 0.04†	9.25 ± 1.03†	8.78 ± 2.16*
Chow	+282 ± 69‡	+208 ± 29†	0.64 ± 0.02†	0.76 ± 0.05‡	12.95 ± 1.50‡	12.12 ± 5.63*

[a] Results are the means ± standard errors of the mean of three or four guinea pigs per treatment. Means denoted by the same superscript (*, †, or ‡) within each vaccination treatment and strain are not significantly different ($P > 0.05$) according to Duncan's new multiple-range test. Vacc, Vaccinated guinea pigs; NV, nonvaccinated guinea pigs; WBC, leukocytes.

[b] Animals were maintained on the experimental diets for a total of 6 weeks before sacrifice.

diet-vaccination treatment group, a significantly greater number of leukocytes was seen in the strain 13 guinea pigs. In general, leukocyte counts tended to be higher in vaccinated animals than in nonvaccinated animals, although the vaccine term was not statistically significant.

Persistence of viable BCG in vaccination site. Figure 1 illustrates the influence of diet and guinea pig strain on the number of viable BCG organisms recovered from the subcutaneous vaccination nodule. Protein-malnourished animals of both strains harbored significantly more viable vaccine organisms than chow-fed animals, with the control-purified group falling in between. When the data were summed across diet groups within each strain, there was an overall significant difference between the number of viable BCG organisms recovered from the two strains of guinea pigs ($P < 0.05$), with strain 2 animals exhibiting higher viable counts.

Effect of diet and inbred strain on tuberculin responses in vivo and in vitro. Results of the tuberculin skin hypersensitivity tests are illustrated in Fig. 2. In both strains, the guinea pigs on the protein-deficient diets had very small or nonexistent dermal reactions to the 100-tuberculin-unit dose of PPD. The effect of guinea pig strain on tuberculin reactions was not significant. However, when the data were broken out by strain and vaccination, strain 2 vaccinated animals had greater average skin test reactions (12.8 ± 2.8 mm) than the nonvaccinated guinea pigs (8.0 ± 2.3 mm), whereas no clear pattern was observed in the strain 13 guinea pigs (vaccinated, 11.8 + 2.1 mm; nonvaccinated, 12.2 ± 1.9 mm). In neither case were the differences statistically significant.

Results from the lymphocyte blastogenesis procedure, measuring proliferative cell responses to tuberculin in vitro, showed a strain difference for blood stimulated with PPD (Fig. 3). The strain term was statistically significant ($P < 0.05$) when the results were summed across diet and vaccination. Background counts were uniformly low (50 to 75 cpm) and were not different between treatments. Counts in PPD-stimulated cultures were at least five times greater than

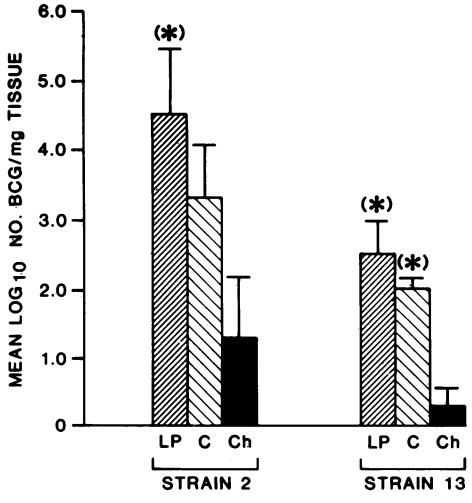

FIG. 1. Numbers of viable *M. bovis* BCG organisms recovered from the subcutaneous vaccination nodules of strain 2 and strain 13 guinea pigs maintained on low-protein (LP), control (C), or chow (Ch) diets; means ± standard errors of the mean of three or four animals per treatment; asterisks indicate significant difference from the chow-fed group ($P < 0.05$).

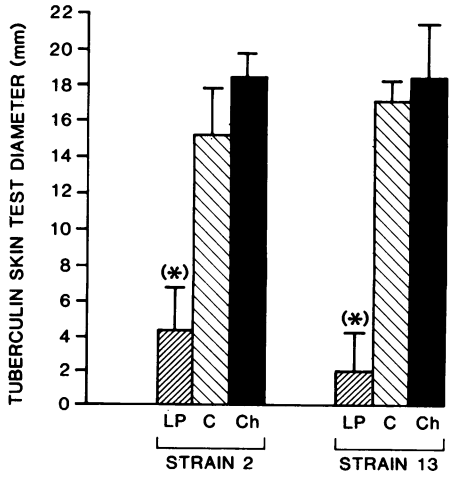

FIG. 2. Delayed hypersensitivity reactions to 100 tuberculin units of PPD in tuberculous strain 2 and strain 13 guinea pigs fed low-protein (LP), control (C), or chow (Ch) diets; means ± standard errors of the mean for six to eight animals per treatment; asterisks indicate significant difference from the control or chow-fed group ($P < 0.05$).

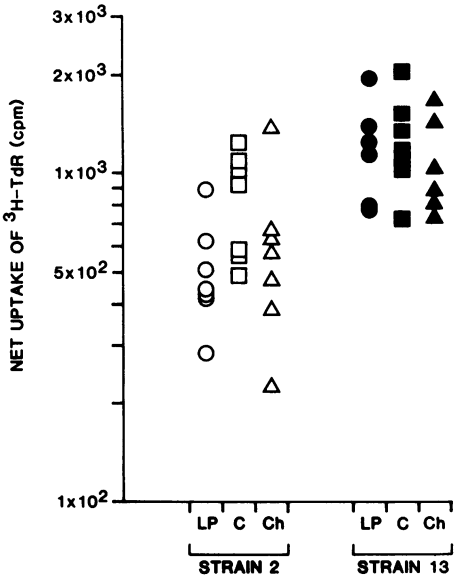

FIG. 3. PPD-induced proliferation in vitro of blood lymphocytes taken from tuberculous strain 2 and strain 13 guinea pigs fed low-protein (LP), control (C), or chow (Ch) diets; results are expressed as the net uptake of [^{3}H]thymidine per 10^6 viable lymphocytes cultured.

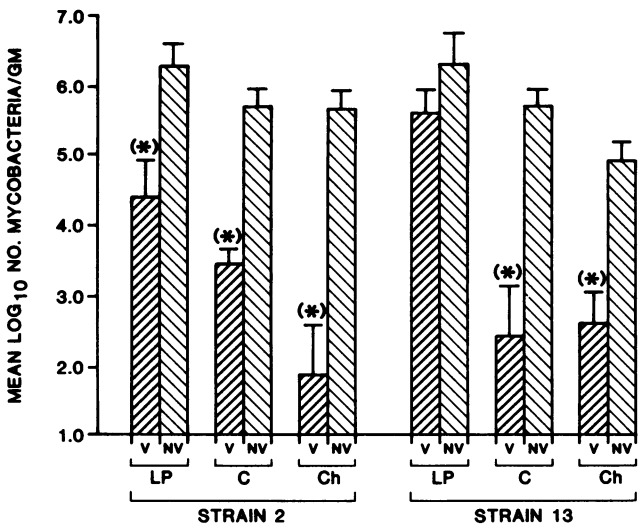

FIG. 4. Numbers of viable *M. tuberculosis* H37Rv organisms recovered from the spleens of BCG-vaccinated (V) and nonvaccinated (NV) strain 2 and strain 13 guinea pigs maintained on low-protein (LP), control (C), or chow (Ch) diets and challenged by the respiratory route; means ± standard errors of the mean of three or four animals per treatment; asterisks indicate significant differences due to vaccination ($P < 0.05$).

in unstimulated cultures. In each diet-vaccination group, lymphocytes from the strain 13 guinea pigs exhibited greater maximum stimulation, although no significant effect of diet was observed.

The data for vaccinated and nonvaccinated animals were pooled for these two variables because the vaccine term was not statistically significant.

Impact of diet and genetic background on BCG efficacy. The protective efficacy of BCG vaccine was determined by the number of virulent *M. tuberculosis* H37Rv organisms quantitatively recovered from the spleen and lung 4 weeks postchallenge. In the spleen (Fig. 4), diet and vaccination each had a significant influence on the number of organisms recovered. In both strains, the BCG-vaccinated animals on the low-protein diets had significantly greater numbers of organisms in the spleen than their control or chow-fed counterparts, suggesting that malnutrition negatively affected the efficacy of the BCG vaccine. A significantly greater number of organisms was recovered from the nonvaccinated control and chow-fed guinea pigs compared with the vaccinated animals, indicating a positive effect of the BCG vaccine in those two groups. In the lungs (Fig. 5), BCG appeared to be effective in that significantly fewer virulent mycobacteria were recovered from the lungs of the vaccinated animals in most strain-diet groups. One notable exception was the low-protein strain 13 group, in which no significant difference was observed between the vaccinated and the nonvaccinated animals in either tissue, indicating no protection afforded by BCG vaccination. As in the spleen, more organisms were recovered from the lungs of the BCG-vaccinated guinea pigs on the protein-deficient diets than from the animals on the protein-adequate diets, indicating a definite nutritional effect on vaccine efficacy. The overall effect of strain on the numbers of virulent mycobacteria recovered from lungs and spleens was not statistically significant.

DISCUSSION

In this study we have demonstrated that the *M. bovis* BCG vaccine protects normally nourished strain 2 and strain 13 inbred guinea pigs against virulent respiratory challenge by *M. tuberculosis* H37Rv. In both the spleen and the lungs, significantly smaller numbers of organisms were recovered from the vaccinated animals (Fig. 4 and 5). This indicates

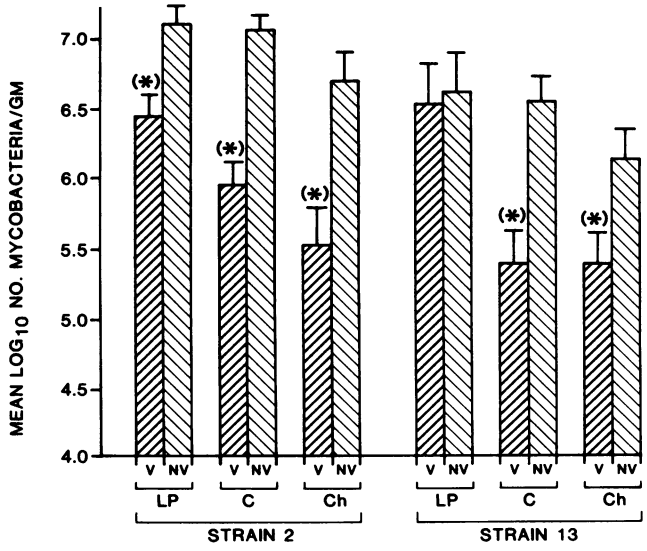

FIG. 5. Numbers of viable *M. tuberculosis* H37Rv organisms recovered from the lungs of BCG-vaccinated (V) and nonvaccinated (NV) strain 2 and strain 13 guinea pigs maintained on low-protein (LP), control (C), or chow (Ch) diets and challenged by the respiratory route; means ± standard errors of the mean of three or four animals per treatment; asterisks indicate significant differences due to vaccination ($P < 0.05$).

protection by the vaccine at both the site of primary implantation (the lungs), as well as in the spleen, which is seeded with mycobacteria by hematogenous dissemination from the lung during development of the infection (5).

We observed no significant differences between strain 2 and strain 13 guinea pigs in susceptibility of the normally nourished animals to the virulent aerosol challenge. The two strains responded to protein deprivation similarly in several ways, including weight loss or very small weight increases (Table 1) and weak reactions to tuberculin (Fig. 2). The latter indicates a definite impairment of the cell-mediated component of the immune system in these malnourished animals (9, 11, 12). This relative tuberculin anergy in protein-deficient guinea pigs was even more dramatic when contrasted with the increased number of viable BCG organisms recovered from the vaccination site (Fig. 1). In spite of the prolonged antigenic stimulation provided by vaccine organisms, protein-deprived guinea pigs could not mount or express a significant delayed cutaneous reaction to PPD. The efficacy of the BCG vaccine appeared to be adequate in the normally nourished animals regardless of the strain as evidenced by the significant differences in the numbers of virulent organisms recovered from the spleens and lungs between the vaccinated and the nonvaccinated animals.

However, several distinct differences were observed between the strain 2 and strain 13 guinea pigs. The strain 13 animals appeared to be more severely affected by the low-protein diet as indicated by more drastic weight loss. It may be that the strain 13 guinea pigs are less adaptable metabolically to these diets, or that their cumulative food consumption was reduced over the course of the experiment. Periodic measurement of daily intake demonstrated no significant difference between strains. The thymuses of strain 13 animals appeared to be very sensitive to dietary insult, as even the modest caloric deficiency noted in the control group was sufficient to cause reductions in thymus weight in that strain. The total number of leukocytes counted in strain 13 animals greatly outnumbered that in their strain 2 counterparts (Table 1). The proliferative responses of blood lymphocytes stimulated by PPD in vitro were also greater in the strain 13 guinea pigs (Fig. 3). Finally, the protein-deficient diet reduced or abolished the protective efficacy of the BCG vaccine in both strains (Fig. 4 and 5). Differences in the numbers of virulent *M. tuberculosis* organisms recovered between the vaccinated and the nonvaccinated animals were substantially diminished in the animals on the low-protein diets. Loss of BCG vaccine efficacy was more pronounced in the malnourished strain 13 guinea pigs, but was similar to that observed previously in outbred (Hartley) guinea pigs studied under identical conditions (9, 11).

Other findings of this study are also consistent with previous work in outbred (Hartley) guinea pigs; e.g., the inbred animals were also adequately protected by the BCG vaccine when properly nourished (9, 11), but exhibited significant weight losses and tuberculin anergy when maintained on the protein-deficient diet (8, 9, 11).

Although it is commonly thought that these two inbred strains of guinea pigs differ in susceptibility to tuberculosis, as do some strains of inbred mice (4, 14), we have not found sufficient evidence to support this contention. In addition, one must be careful in comparing this study to previous work done with inbred strains of rabbits or mice. We have attempted to simulate human tuberculosis as closely as possible, whereas in much of the previous work with other species the experimental protocol has been relatively artificial. The animals in this study were infected via the respiratory route with *M. tuberculosis* organisms, consistent with transmission of the disease in human populations. In contrast, the murine and rabbit studies have involved intravenous or aerosol injections of relatively high doses of BCG or *M. tuberculosis* organisms, respectively (1, 17, 19). These factors should be taken into account when comparing the results of the present study to previous investigations of the role of genetics in resistance to mycobacteria.

Since well-nourished strain 2 and strain 13 guinea pigs responded similarly with respect to BCG vaccine efficacy and PPD hypersensitivity reactions, the decision as to which strain to use in future experiments of this kind may be based upon other criteria such as availability, cost, adaptability to the diets, etc. Inbred guinea pigs will be quite useful because, like the Hartley strain, they have been used successfully to reproduce the conditions relevant to the interaction between tuberculosis and malnutrition in humans. The inbred strains will be particularly advantageous in syngeneic cell transfer and coculture experiments (15). Inbred strains will be helpful in differentiating hereditary and environmental factors which have an impact on the influence of diet on the immune response to tuberculosis. These studies may eventually contribute to the control of tuberculosis in malnourished human populations.

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