Impact of Protein Malnutrition on Exogenous Reinfection with Mycobacterium tuberculosis

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Malnutrition may be a predisposing host factor in the development of exogenous-reinfection tuberculosis. Outbred Hartley guinea pigs were given isocaloric diets containing either 30% ovalbumin (control animals) or 10% ovalbumin (low-protein-fed [LP] animals). Equal numbers of control and LP animals were assigned to one of three infection groups: (i) primary pulmonary infection with a low-virulence, streptomycin-resistant (LVsr) isolate of Mycobacterium tuberculosis and then reinfection 6 weeks later by the same route with a high-virulence (HV) isolate; (ii) only the primary infection (LVsr isolate); and (iii) only the secondary infection (HV isolate). Each infection resulted in the development of 4 to 12 pulmonary tubercles. Guinea pigs were skin tested with purified protein derivative and killed 6 weeks after the second infection. Protein deprivation suppressed the dermal responses to purified protein derivative in all infection groups. Primary infection of well-nourished animals with the LVsr isolate induced significant protection against infection with the HV isolate in the reinfected group, based upon the numbers of viable mycobacteria in the lung and spleen. Protein malnutrition did not exacerbate disease in the reinfected group beyond that observed in malnourished animals infected with the HV isolate only, but neither did the infection with the LVsr isolate protect the LP animals against reinfection with the HV isolate. We conclude that malnutrition interferes with the protection normally afforded by primary infection but does not result in more severe disease in reinfected individuals than would be observed in singly infected subjects.

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Postprimary tuberculosis may develop in a previously infected individual as a result of either endogenous reactivation or exogenous reinfection (2, 14). Exogenous reinfection may be defined as the inhalation, retention, and multiplication of virulent tubercle bacilli in an individual who has experienced a primary infection with mycobacteria at some time in the past. Whether clinical tuberculosis develops after exogenous reinfection will depend, in part, upon the virulence of the Mycobacterium tuberculosis strain involved and the levels of innate and residual acquired resistance expressed by the host (2, 14). Previous studies have documented evidence for exogenous reinfection (16), but the relative importance of exogenous as opposed to endogenous tubercle bacilli is still controversial. The distinction may be an important determinant of the control strategies employed in high-prevalence areas (17). Although exogenous reinfection is not believed to be a common cause of disease in the United States (14), Nardell and co-workers (10) described four cases in an outbreak of tuberculosis in a shelter for the homeless in Boston, Mass. The authors reported more severe disease in these putatively reininfected individuals, including extensive lung cavitation and high bacillary loads in sputum. They postulated that preexisting hypersensitivity to mycobacterial antigens may have exacerbated the pulmonary pathology in reinfected patients. The authors further suggested that the debilitated condition, including chronic malnutrition, of these destitute patients may have predisposed them to more severe disease upon reinfection than would be seen after primary infection (10).

There is a discrepancy in the recent literature with regard to the outcome after exogenous reinfection in tuberculosis. Unlike the clinical investigation cited above (10), published studies with experimental animals suggest that previous infection does not exacerbate disease but rather protects against exogenous reinfection (12, 19). However, it has been argued that healthy experimental animals maintained on adequate diets under optimal conditions may not mimic the debilitated human patient in this regard (10).

We tested this hypothesis, namely, that chronic malnutrition would result in more severe tuberculosis in exogenously reinfeched individuals than would occur in malnourished individuals experiencing a primary infection, in a well-established guinea pig model of respiratory tuberculosis (13) applied by us over the past 8 years to the study of protein malnutrition and its effects on Mycobacterium bovis BCG vaccine efficacy. In previous work, we have demonstrated that chronic, moderate protein deprivation results in reversible loss of T-lymphocyte reactivity to tuberculin in vivo and in vitro (7, 8) and, more importantly, that BCG vaccine does not protect protein-deprived guinea pigs against virulent, low-dose pulmonary challenge with M. tuberculosis (1, 5).

(Material and Methods) Experimental animals. Specific-pathogen-free, female Hartley guinea pigs, weighing 150 to 250 g, were obtained from a commercial supplier [Hartley-COBS, Crl: (HA)BR; Charles River Breeding Laboratories, Inc., Wilmington, Mass.]. The animals were housed individually in stainless-steel cages with stainless-steel grid floors and feeders and were given food and tap water ad libitum. Each animal was randomly assigned to a diet and challenge treatment. Body weights were recorded weekly during the experiment.

Experimental diets. The purified experimental diets were obtained commercially (Dyets, Inc., Bethlehem, Pa.) and

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were prepared according to our previously published formulation (9). The control (C) diet was designed to meet recommended nutritional requirements for guinea pigs (11) and contained 30% ovalbumin as the sole protein source. The low-protein (LP) diet was isocaloric and identical to the control diet in every nutrient except protein (10% ovalbumin). Before initiation of the experimental diets, animals were weaned from commercial chow by feeding a mixture of ground chow and powdered C diet which varied gradually from 50% chow to no chow over a 2-week period. Thereafter, guinea pigs were given their assigned powdered diet, with fresh food and tap water provided daily.

**Challenge inocula.** Two strains of *M. tuberculosis* were used. Both were kindly provided by Donald W. Smith of the University of Wisconsin and are the strains used in his previous study of exogenous reinfection (19). One was a high-virulence (HV) isolate from a patient residing in the Chingleput District in India. The other was a low-virulence isolate, from a patient in the same geographic area, which had been made streptomycin resistant (LVsr isolate) by being plated on agar containing streptomycin. The two strains were stored as single-cell suspensions at −70°C (3).

**Respiratory infection.** Guinea pigs were infected via the respiratory route by using an aerosol chamber described previously (18). The infecting inoculum of *M. tuberculosis* introduced into the nebulizer was adjusted empirically to result in the inhalation and retention of approximately 5 to 10 viable organisms per animal. The infection was performed in a biohazard facility designed for use with class 3 human microbial pathogens. Exposure of groups of guinea pigs, selected randomly from the diet treatments, resulted in uniform, reproducible infection of all animals with mycobacteria. There were three infection groups. One group (LVsr/HV group) was infected with the LVsr isolate at the initiation of the experimental diets (week 0) and exogenously reinfected 6 weeks later with the HV isolate. A second group (LVsr group) received only the LVsr isolate at week 0, and a third group (HV group) was infected only with the HV isolate at week 6.

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

**Necropsy procedure.** Six weeks after the second pulmonary challenge (week 12 of the study), groups of five to nine guinea pigs from each treatment group were skin tested, weighed, and killed by the intraperitoneal injection of 1 to 3 ml of sodium pentobarbital (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa). The thoracic and abdominal cavities were opened aseptically, and the entire lung was removed as single lobes and placed in sterile petri dish. The number of primary tubercles was counted by examination under a dissecting microscope. Recovery of viable *M. tuberculosis* was accomplished by homogenizing the right lower lobe of the lung and the spleen in sterile saline with separate Teflon-glass homogenizers. Homogenates were diluted 10-fold in sterile saline, and appropriate dilutions were streaked in duplicate onto Middlebrook 7H10 agar plates. Samples from the LVsr/HV group were also plated on Middlebrook 7H10 agar plates containing 15 µg of streptomycin per ml. After 2 to 3 weeks of incubation at 37°C, the number of colonies was counted, and the data were expressed as the mean log10 viable *M. tuberculosis* organisms per tissue.

**Statistical analysis.** Analysis of variance was utilized to test the effects of diet and challenge paradigm on the dependent variables measured (skin test diameter and number of viable mycobacteria in tissues). When significant treatment effects were indicated, differences between means were assessed by using Duncan's new multiple-range test (15). A 95% confidence level was set for all tests.

**RESULTS**

**Influence of diet on growth and infection level.** Chronic dietary protein deficiency was manifested in all three challenge groups by a cumulative weight loss over the 12 weeks of the study (Table 1). The mean change in body weight was somewhat ameliorated in the protein-deprived guinea pigs infected only with the HV isolate at 6 weeks. This group experienced only about half of the weight loss seen in the other two challenge groups, but the effect of diet was still significant (*P* < 0.05) in all three groups. Animals fed the C diet, however, gained weight steadily regardless of their infection status. There was no significant difference between mean weight gains for C animals from the three challenge groups. Also shown in Table 1 is the infection level attained in each treatment group. The LP diet exerted no detectable effect on the number of primary tubercles visible in the lung at the termination of the study. The infection level was slightly higher for the HV group than for the LVsr group. This difference may be more apparent than real, however, since the tubercles in the LVsr group were smaller and some may have gone undetected on gross examination of the lungs. The number of lung lesions observed in the exogenously infected (LVsr/HV) group was roughly the sum of the two single infections, as expected.

**PPD-induced immunity in vivo.** Table 1 also documents the impact of challenge group and diet on antigen (PPD)-specific T-cell function in tuberculous guinea pigs. Protein deprivation was accompanied by significant impairment (*P* < 0.05) of delayed hypersensitivity to PPD in all three challenge groups. The size of the tuberculin reaction of the LVsr group was significantly (*P* < 0.05) reduced on both diets as compared with that of the HV group or the LVsr/HV animals.

**Effect of diet on exogenous reinfection.** Figure 1 illustrates the ability of the guinea pigs in the three challenge groups to control the accumulation of viable mycobacteria in the lungs. (Data for the LVsr/HV group in Fig. 1 and 2 represent only HV organisms.) The number of residual LVsr organisms in reinfected animals was negligible (1%) of the total

<table>
<thead>
<tr>
<th>Challenge group</th>
<th>Diet</th>
<th>Change in body wt (g)</th>
<th>No. of primary tubercles</th>
<th>PPD skin test diam (mm)</th>
</tr>
</thead>
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<tr>
<td>LVsr</td>
<td>C</td>
<td>+8.4 ± 18.2</td>
<td>7.3 ± 1.0</td>
<td>11.2 ± 0.9</td>
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<td>+62.7 ± 8.7</td>
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<td>15.4 ± 1.3</td>
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<tr>
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<td>−6.3 ± 19.3</td>
<td>5.1 ± 1.0</td>
<td>2.1 ± 1.4</td>
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<tr>
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<td>−27.8 ± 25.0</td>
<td>9.0 ± 0.6</td>
<td>8.2 ± 1.2</td>
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<tr>
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<td>C</td>
<td>−67.6 ± 15.0</td>
<td>14.9 ± 1.0</td>
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<tr>
<td></td>
<td>C</td>
<td>−56.6 ± 12.6</td>
<td>13.4 ± 1.3</td>
<td>15.4 ± 1.3</td>
</tr>
</tbody>
</table>

* All values are means ± standard errors of the mean for four to nine animals per treatment group.  
* Measured from the initiation of experimental diets to sacrifice.
mycobacterial load. In preliminary experiments to choose an infection level, we determined that guinea pigs infected with LVsr organisms had developed significant tuberculin reactivity and had begun to reduce mycobacterial loads in the tissues by 6 weeks postchallenge. In the present study, by 12 weeks after challenge, the LVsr group harbored only low levels of that strain of M. tuberculosis, while significantly higher levels of the virulent strain were recovered from the HV group, which had been infected 6 weeks previously. Diet did not influence either single infection significantly. However, in the LVsr/HV group, protein deficiency severely impaired the ability of guinea pigs to control the second, HV infection. While C animals were protected, as evidenced by significantly (P < 0.05) reduced lung loads of HV mycobacteria in the LVsr/HV group, malnourished animals were incapable of responding to the exogenous reinfection with HV organisms any better than the singly infected HV animals were. It is important to note, however, that the mycobacterial load in the LVsr/HV animals fed the LP diet (LP animals) was not greater than that observed in the HV LP animals.

Similar results were obtained with the spleen (Fig. 2), in which diet did not affect the number of viable M. tuberculosis organisms in either singly infected group but did markedly impair the protection afforded the well-nourished guinea pigs by previous infection with the LVsr strain. Protein-deprived animals controlled the second infection in the spleen somewhat better than that in the lung, but the protection observed in exogenously reinfected LP animals was significantly less (P < 0.05) than that seen in C guinea pigs. Again, reinfection of LP animals did not result in any more disease than that observed in singly infected LP animals.

**DISCUSSION**

The results of this study do not support the hypothesis that chronic malnutrition predisposes tuberculosis patients to more severe disease after exogenous reinfection than would be produced in malnourished individuals after primary infection (10). Protein-deprived guinea pigs challenged exogenously with the HV isolate 6 weeks after primary pulmonary infection with the LVsr isolate of M. tuberculosis harbored no more viable mycobacteria in their tissues (Fig. 1 and 2) and exhibited no increase in the number of visible tubercles in the lungs (Table 1) than did singly infected (HV) animals consuming the same diet. Thus, the contention that in a population of malnourished individuals, exogenously reinfected patients would suffer more extensive tuberculosis than singly infected counterparts is not supported by these results. However, exogenously reinoculated, protein-deprived guinea pigs were not protected against the HV challenge by primary infection with LVsr organisms, as evidenced by the lack of reduction in the numbers of viable HV mycobacteria in the lung and spleen. C animals, on the other hand, exhibited excellent protection against the HV isolate induced by previous infection with the LVsr isolate. The latter observation supports the contention that the initial encounter with low-virulence mycobacteria provides an immunizing stimulus in normal individuals which ameliorates the infection resulting from exogenous reexposure at a later time (19).

Both the loss of protection against infection with the HV isolate observed in the exogenously reinoculated LP animals and the absence of detectable dietary effect on the primary infection with either LVsr or HV organisms (Fig. 1 and 2) extend our previous findings with BCG-vaccinated guinea pigs (1, 5, 7). These results suggest that dietary insult preferentially influences the anamnestic response to a second encounter with the pathogen but has little impact on the primary immune response even to highly virulent mycobacteria like the HV strain used in this study. Data from a recent study of Fc receptor-bearing T cells in this model (D. N. McMurray, R. A. Bartow, and C. L. Mintzer, submitted for publication) suggest that alterations in these putative regulatory T cells which occur in protein-deficient animals may be involved in loss of protection.

The impairment of dermal hypersensitivity reactions to
PPD which we observed in LP animals, even after exogenous reinfection, is consistent with the profound effect which protein deficiency is known to exert on T-cell-mediated immune responses (4). Tuberculin hypersensitivity and acquired resistance to mycobacteria are uncoupled in protein deficiency (5, 7), in the sense that impaired tuberculin reactions are not necessarily accompanied by loss of control of the infection, as is obvious in the HV group in this study. LP animals had the same numbers of viable M. tuberculosis organisms in both lung and spleen as C animals did, but they developed markedly smaller skin tests to 100 U of PPD (Table 1).

An important observation, which we document here for the first time, is that protein malnutrition did not alter the number of pulmonary tubercles which developed after respiratory reinfection (Table 1) but did impair the ability of the deprived animal to control the accumulation of viable mycobacteria within each lesion (Fig. 1), as well as the hematogenous dissemination of tubercle bacilli to extrapulmonary sites like the spleen (Fig. 2). This is consistent with our previous findings that alveolar macrophages lavaged from protein-deficient, tuberculous guinea pigs maintained normal phagocytic function (6). Taken together, these results suggest that the initial interaction between host and mycobacteria in the lung is not affected significantly by chronic, moderate protein deprivation.

In summary, exogenous reinfection does not appear to produce more severe tuberculosis than is observed after primary infection of malnourished individuals, contrary to a hypothesis suggested previously (10). However, protein deprivation does impair the protection afforded well-nourished hosts by previous infection with M. tuberculosis and renders the PPD skin test practically useless as a predictor of cellular reactivity. A number of host factors in addition to nutritional status have been implicated in the development of postprimary tuberculosis (2, 10, 14). Further work will elucidate their roles in exogenous-reinfection tuberculosis.

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LITERATURE CITED