Effects of protein calorie malnutrition on tuberculosis in mice

(Interferon γ/tumor necrosis factor α/nitric oxide synthase/granuloma/reactive nitrogen intermediates)

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ABSTRACT Infectious diseases and malnutrition represent major burdens afflicting millions of people in developing countries. Both conditions affect individuals in industrialized nations, particularly the aged, the HIV-infected, and people with chronic diseases. While malnutrition is known to induce a state of immunodeficiency, the mechanisms responsible for compromised antimicrobial resistance in malnourished hosts remain obscure. In the present study, mice fed a 2% protein diet and developing protein calorie malnutrition, in contrast to well-nourished controls receiving a 20% protein diet, rapidly succumbed to infection with Mycobacterium tuberculosis. Malnourished mice exhibited a tissue-specific diminution in the expression of interferon γ, tumor necrosis factor α, and the inducible form of nitric oxide synthase in the lungs, but not the liver. The expression of these molecules critical to the production of mycobactericidal nitrogen oxides was depressed in malnourished animals in the lungs specifically at early times (<14 days) after infection. At later times, levels of expression became comparable to those in well-nourished controls, although the bacillary burden in the malnourished animals continued to rise. Nevertheless, urinary and serum nitrate contents, an index of total nitric oxide (NO) production in vivo, were not detectably diminished in malnourished, mycobacteria-infected mice. In contrast to the selective and early reduction of lymphokines and the inducible form of nitric oxide synthase in the lungs, a marked diminution of the granulomatous reaction was observed in malnourished mice throughout the entire course of infection in all tissues examined (lungs, liver, and spleen). Remarkably, the progressively fatal course of tuberculosis observed in the malnourished mice could be reversed by restoring a full protein (20%) diet. The results indicate that protein calorie malnutrition selectively compromises several components of the cellular immune response that are important for containing and restricting tuberculosis infection, and suggest that malnutrition-induced susceptibility to some infectious diseases can be reversed or ameliorated by nutritional intervention.

Tuberculosis is a disease historically known to be particularly influenced by malnutrition (ref. 1; for review, see ref. 2), and is a major cause of morbidity and mortality in developing countries where protein calorie malnutrition (PCM) is also prevalent (3–6). Substantial experimental evidence suggests that malnutrition can lead to secondary immunodeficiency that increases the host’s susceptibility to infections (1, 3, 4). Although it is clear that protection against Mycobacterium tuberculosis is dependent upon cellular immunity (7), which is known to be diminished by PCM (reviewed in refs. 4 and 5), the specific cell-mediated microbicidal mechanisms compromised in dietary protein deficiency that result in increased susceptibility to tuberculosis remain unclear. In previous studies (8–11), we established that the principal mechanism by which murine mononuclear phagocytes kill or inhibit the growth of M. tuberculosis is the arginine-dependent production of NO and related reactive nitrogen intermediates (RNI). This suggested the possibility that PCM might lead to diminution in the expression of inducible NO synthase (iNOS) or the lymphokines responsible for its induction, thus compromising the host’s protective immune response to M. tuberculosis. We have tested this hypothesis in a murine tuberculosis model designed to elucidate relevant components of cell-mediated immunity and antimycobacterial mechanisms whose functions are specifically attenuated by PCM.

MATERIALS AND METHODS

Animals and Diets. Eight- to 10-week-old female CD1 and C57BL/6 mice were used in this study. Animals were kept in a biosafety level 3 facility and monitored for common murine pathogens as described previously (9). Ingredients of the regular casein-based mouse diet ( Bioserv, Frenchtown, NJ) were as described (12, 13). Protein-deficient diets, in solid or liquid forms, were made isocaloric to the basic diet by carbohydrate supplementation (12, 13). Post-manufacture analyses of dietary protein, carbohydrate, and fat content of the various diets used were routinely performed for quality control.

Mycobacteria. The virulent M. tuberculosis Erdman and the attenuated bacillus Calmette–Guérin (BCG) Pasteur were passaged in Middlebrook 7H9 broth supplemented with oleic acid-albumin-dextrose complex (OADC) (Difco). Details in passaging cultures of these mycobacteria and preparation of the organism for infection have been described previously (9–11). Mice were infected intravenously (i.v.) with 1 × 106 colony-forming units (cfus) of M. tuberculosis via the lateral tail vein using a 26-G needle (9–11). In certain experiments, an infection dose of 103 cfu was used. For studies performed to determine the activity of the RNI-generating pathway in response to mycobacterial infection, mice were inoculated with 103 cfu of BCG Pasteur intraperitoneally.

Measurement of Urinary Nitrate Content. Activity of the L-arginine-dependent, RNI-generating pathway in mice fed diets containing various amounts of protein after BCG infection was assessed by quantitating urinary nitrate content. Animals were kept in metabolic cages for daily 24-hr urine collection. Liquid diets, administered in graduated feeding cylinders, were used in these studies for easy monitoring of coloric intake by various groups of mice. Urinary nitrate was first reduced to nitrite using the enzyme nitrate reductase of Pseudomonas oleovorans as described (14). Nitrite content was determined by the Griess reaction (8).

Abbreviations: BCG, bacillus Calmette–Guérin; cfu, colony-forming unit; IFN-γ, interferon γ; iNOS, inducible nitric oxide synthase; PCM, protein calorie malnutrition; RT–PCT, reverse transcription–PCR; RNI, reactive nitrogen intermediate; TNF-α, tumor necrosis factor α.

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Assessment of Disease Progression and Preparation of Tissues for Analyses. At days 1, 3, 5, 7, 10, 14, 25, and 30 after infection, animals were sacrificed as described previously (9–11). The susceptibility to *M. tuberculosis* of mice from various experimental groups were assessed by mortality; quantitation of viable cfus in livers, lungs, and spleens; and by histopathological examination of these organs (9–11). Mice exhibiting severe disease or appearing moribund were sacrificed to avoid suffering and scored as succumbing to *M. tuberculosis* infection.

Quantitation of Viable Bacilli in Infected Tissues. For cfu quantitation, aseptically removed tissues were homogenized in phosphate-buffered saline (PBS) with 0.05% Tween 80, and the number of viable organisms per organ was determined by plating onto 7H10 agar plates in serial dilutions. Because each organ was partitioned for various studies, tissue portions were harvested for a particular study based on their anatomical location (10). For cfu quantitation in the lungs, the left upper lobe was used; in the liver, the right lobe was used; and in the spleen, the caudate quarter was used.

Histopathological Studies. Samples for histopathological studies were prepared as described previously (9–11). Briefly, tissues were fixed in 10% buffered-formalin before embedding in paraffin. Hematoxylin/eosin (H&E) and Ziehl-Neelsen acid fast staining of sections from paraffin blocks were used in histological examination to assess pathological changes and bacillary load, respectively.

Immunohistochemical Staining. For immunohistochemical studies, tissues were frozen in O.C.T. (Miles) in liquid nitrogen and stored at −80°C until used. Preliminary studies had shown that tissues so preserved yielded the best results for staining by a rabbit, affinity-purified polyclonal antibody against murine macrophage iNOS (11). O.C.T.-embedded fresh tissues were sectioned at 5–6 μm. Anti-iNOS antibodies were applied at a dilution of 1:100 for 3–4 h. A conventional avidin-biotin complex-based method was performed using the ABC Vectastain Kit (Vector Laboratories). Peroxidase-diaminobenzidine (DAB) method was used for colorization. The sections were then counterstained with hematoxylin.

Reverse Transcription–PCR (RT-PCR). Tissues used for assessing message levels of iNOS, interferon γ (IFN-γ), and tumor necrosis factor α (TNF-α) by RT-PCR were stored at −80°C after initial freezing in liquid nitrogen. RNA extraction from tissues were carried out using the TRIZOL reagent (Life Technologies, Grand Island, NY). RT-PCR was performed using standard protocols (15). The relative levels of expression of the various target mRNA’s were compared after normalization to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) messages. PCR products were resolved by agarose gel electrophoresis. Negatives of photographs taken on ethidium bromide-stained gels (Kodak 677 films) were processed as described by the manufacturer, and signals of the various PCR products were then subjected to densitometric analysis. The primer pairs used in these experiments were as follows: iNOS, 5′-tctcagggcttcgacaag (upstream) and 5′-ccagccctcaccatattc (downstream), or 5′-ggaggaacctgctcagc (upstream) and 5′-ggcgcgctgttggtcttg (downstream); IFN-γ, 5′-aatcagctactatagggtaagccagctggtct (upstream) and 5′-ttgcttctgctgctgctgcc (downstream); TNF-α, 5′-gttccagttggcatgtgctgcg (upstream) and 5′-ctcagcctgccagggcag (downstream); GAPDH, 5′-ctctttctctctctctctcc (upstream) and 5′-ctctttctctctctctctctcc (downstream), or 5′-cctcttctctctctctctcc (upstream) and 5′-cctcttctctctctctctcc (downstream).

Electron Microscopic Studies. Tissue sections prepared from paraffin blocks were deparaffinized in three changes of xylene at 50°C for 3 h. Tissues were then rehydrated sequentially in 100%, 95%, 80%, 70%, and 50% ethanol. Rehydrated samples were washed in 0.1 M cacodylate buffer containing 10 mM sucrose and 3 mM CaCl$_2$ for 2 h. Samples were then treated sequentially with 4% formaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer, postfixed for 1 h in 1% osmium, followed by dehydration in 50%, 70%, 80%, 95%, and 100% ethanol, and finally treatment with two changes of acetone and infiltration using a mixture of araldite–Epon resin. After polymerization, 1 μm sections were cut and stained with toluidine blue for evaluation by light microscopy. For ultrastructural studies, thin sections were stained with uranyl acetate and lead citrate before examination by a Siemens (Iselin, NJ) model 102 electron microscope.

**RESULTS AND DISCUSSION**

The principal aim of this study has been to clarify the mechanisms by which malnutrition enhances susceptibility to *M. tuberculosis*. In particular, we sought to identify components of cell-mediated immunity and known antimycobacterial mechanisms compromised by PCM during infection with *M. tuberculosis*. Because we had previously demonstrated that the L-arginine-dependent generation of RNI is the principal mycobactericidal mechanism (8–11), we explored particularly whether PCM enhanced susceptibility to the tubercle bacillus by adversely affecting this macrophage microbicidal pathway.

Susceptibility of Malnourished Mice to *M. tuberculosis* Infection. We established a murine tuberculosis model suitable for examining the effect of PCM on tuberculosis in mice, based on previous studies that demonstrated increased bacterial growth and dissemination in malnourished animals infected with the attenuated BCG vaccine strain (13). Preliminary studies were carried out to determine optimal experimental parameters, including the infectious challenge dose of *M. tuberculosis*, the tolerance of mice to dietary protein restriction, and the appropriate time for infection after introduction of protein-deficient diets. In addition, the general characteristics of the clinical course of disease in this PCM system were established. In this model system, mice were infected with 1 × 10$^6$ cfu i.v. of the virulent Erdman strain of *M. tuberculosis* 10 to 14 days after initiation of a 2% protein diet. Results in Fig. 1 indicate that, after challenge with the virulent Erdman strain, mice given the low protein diet experienced fulminant and rapidly fatal tuberculosis infection. While all of the animals receiving a full protein (20%) diet survived the infection (uninfected mice with PCM have been observed to survive for at least 6 months), 100% of infected mice with PCM succumbed by day 66 postinfection, with a mean survival time of 43.3 ± 13.7 days. Acid-fast stain of tissues obtained from the malnourished mice demonstrated a marked increase in bacterial burden compared with control animals (Fig. 2).

![Fig. 1](image-url) (Left) CD1 mice with PCM (2% protein diet) infected with 10$^5$ (□ n = 12 mice) or 10$^6$ (● n = 7 mice) virulent Erdman strain of *M. tuberculosis* experienced an exacerbated course of tuberculous infection compared with controls fed a full protein diet (■ n = 9 mice) and ○ (n = 7 mice) represent control animals infected with 10$^5$ and 10$^6$ bacilli, respectively. (Right) Reinstating a full protein diet to *M. tuberculosis*-infected CD1 mice with PCM reverses the fulminating course of tuberculosis associated with malnutrition. Fourteen days after initiation of the diet, mice fed a protein-deficient diet (2% protein; □) or a regular diet (20% protein; □) were infected with 10$^6$ virulent Erdman strain of *M. tuberculosis*. At 18 days postinfection, the diets were switched between the two groups. Seven mice per group were studied.
Increased susceptibility of malnourished mice to BCG (13), the cytotoxic mechanism of macrophages may contribute to the observation, with the number of bacilli at day 28 postinfection in the lungs of the infected malnourished animals exceeding that in mice receiving a full protein diet by 2–3 logs (Fig. 3).

Effect of PCM on Granuloma Formation. Histopathological studies of tissues obtained from animals at various time intervals postinfection revealed that mice with PCM formed poorly structured granulomas compared with control animals receiving a normal diet (Fig. 2). Electron microscopic studies of these tissues showed that the cellular components of the granuloma of mice with PCM were loosely organized, whereas those of animals fed a full protein diet were tightly adjoined to one another (Fig. 4). Interestingly, tissue necrosis, a major characteristic of human disease not typically seen in murine tuberculosis, was observed in the lungs of animals with PCM. This pathological tissue reaction was, however, not seen in the liver or spleen of these animals, or in any of the organs of mice receiving a normal protein diet. Similar results were obtained when a 100-fold lower inoculum (1 × 10^3 cfu) of the virulent M. tuberculosis was used to challenge mice with PCM, although the kinetics of the mortality curve showed a less rapid course (Fig. 1) with a mean survival time of 69.3 ± 7 days.

Taken together, these results indicate that the antimycobacterial defense mechanisms of mice receiving a protein-deficient diet are severely compromised, as manifested by a rapidly progressive course of infection associated with a markedly increased bacterial burden and a strikingly diminished granulomatous reaction. Significantly, the progressively fatal course of tuberculosis in protein-deficient mice could be reversed by reinstating a full protein diet (Fig. 1) at 18 days after challenge with the tubercle bacillus (10^6 cfu), a time in the course of infection when increased tissue bacterial load is grossly apparent. Finally, when C57BL/6 mice, an inbred strain found to be relatively resistant to M. tuberculosis, were given a 2% protein diet, they too developed fulminant tuberculosis infection with severity similar to that observed in CD1 mice (data not shown).

Failure to Detect a Diminution in Urinary Nitrates in Malnourished Mice. Because our laboratory had previously demonstrated that deficiency of the L-arginine-dependent cytotoxic mechanism of macrophages may contribute to the increased susceptibility of malnourished mice to BCG (13), the effect of PCM on this RNI-generating pathway was examined. Our initial experiments explored the possibility that protein calorie malnourished mice might be deficient in the amino acid L-arginine, the substrate required for the generation of NO by macrophage iNOS. Urinary nitrate levels, which reflect the level of NO produced in vivo from the substrate L-arginine (14), were determined to assess whether the enhanced susceptibility of malnourished animals to mycobacteria was simply the consequence of an L-arginine insufficiency. Results of these studies indicated that the urinary nitrate contents of BCG-infected mice with PCM were not diminished compared with those of well-nourished controls (Fig. 5), indicating that sufficient amounts of arginine were available for the generation of RNI after mycobacterial infection. BCG was used for these experiments for biosafety reasons, because comparable analysis of in vivo NO production by quantitating daily 24-h urinary nitrates cannot be performed with mice infected with virulent mycobacteria. Nevertheless, nitrate contents in random serum samples of malnourished mice infected with the virulent M. tuberculosis Erdman (10^6 i.v.) were comparable to those of well-nourished controls (data not shown). Together, these results indicate that L-arginine availability is unlikely to be the factor limiting the mycobacteriocidal RNI-generating pathway in malnourished mice infected with M. tuberculosis.

Fig. 2. Immunohistopathological studies of tissues of M. tuberculosis-infected CD1 mice fed a 20% (Left) or 2% (Right) protein diet. Kinyoun’s acid fast stain of lung tissues (Upper) reveals markedly increased bacillary loads in mice with PCM (25 days postinfection) compared with those of controls. Difference in tissue bacillary loads is observed as early as 14 days postinfection in the lungs. Studies of hepatic tissues demonstrated similar findings. Immunocytochemical staining of lung tissues (2.5×) using anti-iNOS antibodies (Lower) demonstrates deficient expression of macrophage iNOS in the lungs of M. tuberculosis-infected mice with PCM. Sections represent tissues obtained from mice 10 days postinfection.
The data reflect, however, the total L-arginine utilization by the various isoforms of NO synthase from a wide variety of cell types, and therefore, may not be revealing of the activity level of the mycobacteriocidal RNI-generating pathway in infected tissue macrophages within the granuloma. This led us to explore the possibility that the expression of iNOS, the key enzyme required for the generation of NO by macrophages, may be decreased in malnourished, \textit{M. tuberculosis}-infected mice.

**Effect of PCM on the Expression of iNOS, IFN-\(\gamma\), and TNF-\(\alpha\).** The relative abundance of iNOS mRNA in mice with PCM and well-nourished controls during tuberculous infection was assessed by RT-PCR, using constitutive GAPDH mRNA as an internal standard. The results of these experiments (Fig. 6) revealed a tissue-specific dysregulation of iNOS expression in dietary protein-deficient mice during tuberculous infection. While iNOS mRNA levels were down-regulated in the lungs of \textit{M. tuberculosis}-infected mice with PCM compared with infected animals fed a normal diet, its expression in the liver was comparable between the two groups. This diminution in pulmonary iNOS expression was most apparent at the early phase of infection (before day 10 postinfection). By 2 weeks after infection, iNOS mRNA levels in the lungs of the two groups were indistinguishable. Expression of the enzyme itself in the lungs was evaluated using an affinity purified polyclonal anti-iNOS antibody (11). In keeping with the diminished expression of iNOS mRNA, immunohistochemically reactive iNOS in pulmonary tissues obtained from \textit{M. tuberculosis}-infected mice with PCM appeared to be decreased compared with controls in the same period (Fig. 2). This observation must be interpreted with caution, however, because of the paucity of pulmonary granulomas per tissue section observed as a result of the expected low number of bacilli (\(\approx 1\%\) of the inoculum) delivered to the lungs via the intravenous route (16). Similar to the results obtained using RT-PCR to evaluate iNOS mRNA levels, immunohistochemical studies on lung tissues from the two groups of mice at later time points revealed similar expression of this enzyme at the protein level (data not shown).

Two lines of evidence suggest that dysregulation of IFN-\(\gamma\) and TNF-\(\alpha\) may play a role in the diminution of iNOS expression in mice with PCM during \textit{M. tuberculosis} infection. First, these cytokines are the key molecules that synergistically activate the production of mycobacteriocidal RNI by macrophage iNOS (17–19). Second, diminished production of several cytokines has been associated with malnutrition (refs. 20–22; reviewed in ref. 23). We therefore examined the expression of IFN-\(\gamma\) and TNF-\(\alpha\) in protein-malnourished, \textit{M. tuberculosis}-infected mice. Results from these experiments (Fig. 6) again revealed a tissue-specific diminution in IFN-\(\gamma\)
and TNF-α mRNA levels in dietary protein-deficient mice during tuberculous infection. Expression of both IFN-γ and TNF-α mRNAs was down-regulated in the lungs of infected PCM mice compared with mice fed a normal diet, while the relative mRNA levels of these two cytokines in the liver were comparable. As in the case of iNOS, this diminution in the expression of IFN-γ and TNF-α was apparent early in infection, beginning on day 5; by 2 weeks after infection, mRNA levels of these two cytokines were comparable.

Comment. Results of this study provide evidence that PCM compromises the ability of mice to mount protective cell-mediated immune responses to tuberculous infection at multiple levels. (i) The three critical elements involved in the activation of the mycobactericidal L-arginine-dependent cytotoxic pathway of macrophages (8), IFN-γ, TNF-α, and iNOS, are all diminished in a tissue-specific fashion in the lungs during the early phase of infection when the antimicrobial function may be most effective (24–26). In contrast, the overall L-arginine levels, assessed by quantitating urinary and serum nitrates, are not decreased in malnourished animals during mycobacterial infection. The tissue-specific effect of PCM on iNOS and lymphokine expression in malnourished mice indicates that PCM does not engender a generalized or systemic defect in protein synthesis, and that determination of overall NO production by measurement of urinary nitrates may not be sensitive enough to allow detection of a selective deficit of the RNI-dependent cytotoxic pathway in a particular organ. Consistent with the significance of RNI in host defense against M. tuberculosis (8–11, 27), the increased tissue bacillary burden in malnourished mice is most striking in the lungs, where dysregulation of the RNI-generating pathway is most pronounced, and where the decisive confrontation between the bacillus and host response is played out. (ii) The granulomatous response, a major tissue reaction believed to be critical for protection by containing M. tuberculosis (13, 28), is dramatically reduced in all tissues examined throughout the course of infection. This may explain the failure of malnourished animals to control the infection even after lymphokine and iNOS levels rise to the those of well-nourished mice.

It is noteworthy that while the initial reduced expression of IFN-γ, TNF-α, and iNOS in the lungs of M. tuberculosis-infected, protein-malnourished mice attains a level comparable to that of controls given a normal diet by 5–10 days postinfection, the granulomatous reaction remains inadequate in all tissues examined throughout the entire course of the disease. This differential effect of PCM on the kinetics of tissue cytokine expression (abnormal only in the early phase of infection) and granuloma formation (deficient throughout the entire disease course) indicates that malnutrition affects the cell-mediated immune response in a remarkably specific manner. This specificity is further emphasized by our findings that IFN-γ, TNF-α, and iNOS are selectively deficient in the lungs. Indeed, studies on the effects of PCM on other immunological parameters such as murine neutrophil mobilization during staphylococcal infection (29), ingestion of IgG-coated sheep erythrocytes by mouse peritoneal macrophages (30), serum non-C3 complement levels (31), have also demonstrated specificity, indicating that the immunosuppression in the malnourished hosts affects selective components of the immune system.

The normalization of the deficient expression of the RNI-generating pathway by 2 weeks postinfection in malnourished M. tuberculosis-infected mice indicates that some T-cell and macrophage functions may not be irreversibly defective in PCM. This is supported by the finding that the the fat course of tuberculosis in malnourished animals could be reversed upon “re-nutrition” with a 20% protein diet. This finding suggests the possible existence of NO-independent antimycobacterial mechanism(s) whose optimal expression during tuberculous infection is dependent on dietary protein content. Finally, the reversibility of the fat course of tuberculosis in mice with PCM by protein supplementation suggests that dietary intervention may impact the outcome of infectious diseases in malnourished individuals. It is hoped that a better understanding of the mechanisms underlying the deficiencies in cell-mediated immunity in PCM will eventuate in new and inexpensive nutritional or therapeutic strategies that will enable malnourished hosts to be protected against tuberculosis and other infectious diseases.

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