Effects of Dexamethasone and Transient Malnutrition on Rabbits Infected with Aerosolized Mycobacterium tuberculosis CDC1551

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Malnutrition is common in the developing world, where tuberculosis is often endemic. Rabbits infected with aerosolized Mycobacterium tuberculosis that subsequently became inadvertently and transiently malnourished had compromised cell-mediated immunity comparable to that of the rabbits immunosuppressed with dexamethasone. They had significant leukopenia and reduced delayed-type hypersensitivity responses. Malnutrition dampened cell-mediated immunity and would interfere with diagnostic tests that rely on intact CD4 T-cell responses.

Tuberculosis is a major global infectious disease accounting for more than 2 million deaths annually. The majority of cases occur in the developing world where calorie malnutrition is common. Cegielski and McMurray recently reviewed the effect of micronutrient and protein calorie malnutrition on the risk of developing active tuberculosis (2). Although the evidence in human populations is mostly indirect and observational, these data, when combined with results from animal models, suggest that malnutrition increases the risk of developing active tuberculosis (6, 11, 12, 27, 30).

Animal models have shown that protein and calorie malnutrition can impair macrophage activation (28), alter T-cell function and cytokine production, and decrease the number of circulating lymphocytes (13, 21). In both mice and guinea pigs, malnutrition increases the organ burden of disease, resulting in poor outcomes (3, 23), and compromises the ability of the BCG vaccine to protect animals against pulmonary infection with virulent Mycobacterium tuberculosis (1, 5, 25, 26).

Here, we report the effects of transient, inadvertent malnutrition in rabbits. Rabbits were housed according to established and approved protocols at the George Washington University Medical Center, Johns Hopkins University, and the United States Army Research Institute of Infectious Diseases. Rabbits were infected with aerosolized M. tuberculosis CDC1551 at the United States Army Research Institute of Infectious Diseases by previously published methods (22). Animals were then transported back to George Washington University Medical Center in HEPA-filtered containers and then housed under biosafety level 3 conditions. Four animals were necropsied on the first day after exposure to aerosolized M. tuberculosis. Lungs were homogenized in phosphate-buffered saline. CFU were enumerated by serial dilution on Middlebrook 7H10 agar, supplemented with oleic acid-dextrose-catalase (Becton Dickinson, Inc., Sparks, MD) and four antibiotics (trimethoprim, 20 μg/ml; carbenicillin, 50 μg/ml; cycloheximide, 50 μg/ml; and polymyxin, 200 U/ml). The right upper lobe was homogenized and plated to enumerate the number of viable bacilli. The average number of bacilli in the right upper lobe of the lung ± standard deviation was 406 ± 102 CFU, and the number was 3,741 ± 921 CFU when extrapolated to the total lung. Impinger samples were taken at the time of infection with aerosolized M. tuberculosis and plated. Impinger concentrations of the organism, combined with each rabbit’s plethysmography, were used to calculate the number of bacilli inhaled. The mean number ± standard deviation was 15,000 ± 1,500 CFU. The difference between the dose cultured from rabbits on the day after aerosolized M. tuberculosis exposure is the same as that described by the original aerosol experiments conducted by Lurie et al. in 1950 (17, 19). Only about 30% of inhaled particles are retained in the alveolar passages, presumably due to clearing of the upper air passages by the ciliary motion of the bronchial epithelium.

Two days after aerosolized M. tuberculosis infection, the drinking water of eight rabbits was switched to bottled water containing 2.5% aminoguanidine, eight rabbits were treated with intramuscular dexamethasone 0.1 mg/kg of body weight/day after one loading dose of 10 mg/kg/day, and the third group of eight rabbits was left untreated. Because the aminoguanidine was so unpalatable and water bottles rather than the automatic water-providing system were used, the rabbits in this group refused to drink. Observation of the water levels in the bottles showed that the rabbits ingested none of the aminoguanidine-treated water, and therefore a toxic effect of the ami-
noguanidine was unlikely. In addition, the rabbits also stopped eating the food pellets because they were not drinking water. As a result, 3 days later, these animals required subcutaneous fluids and were placed back on automatic water. In spite of this, the animals continued to have poor appetites and weight loss for the next 3 weeks, although they had no other overt sign of illness. The exact food intake was not measured. After the fourth week, the rabbits began eating and rapidly regained their pretreatment weights (Fig. 1A). The underlying reason for their persistent inanition and then very rapid recovery is enigmatic. Interestingly, the effect within each group was uniform, as can be seen by the small mean standard errors associated with the weekly weights. All animals in the experiment survived to the 5-week necropsy endpoint. The eight untreated, infected controls and the eight infected rabbits treated with intramuscular dexamethasone maintained their weights for the duration of the experiment.

Four weeks after aerosolized Mycobacterium tuberculosis infection, all animals were skin tested with old tuberculin (Wyeth Lederle, Pearl River, NY). Delayed-type hypersensitivity (DTH) reactions were read at 48 h by previously published methods (22). Dexamethasone-treated animals had statistically significantly depressed skin responses (mean $\pm$ standard error) to old tuberculin compared to untreated controls (193 $\pm$ 1130 cells/mm$^3$, $P < 0.01$ [Table 1]). However, the malnourished rabbits had a rise in their white-blood-cell counts by 5 weeks concomitant with the recovery of weights to baseline (6,230 $\pm$ 910 cells/mm$^3$). (Fig. 1 and Table 1)

Five weeks after aerosolized M. tuberculosis infection, the rabbits were euthanized with an intravenous pentobarbital preparation (Euthasol; National Logistic Services Animal Health, Baltimore, MD). The number of grossly visible pulmonary tubercles was counted, and their diameters were measured (10, 18). Spleen, hilar lymph nodes, and right upper lung lobes were homogenized, and aliquots were plated on Middlebrook 7H10 agar supplemented with oleic acid-dextrose-catalase. Steroid-treated rabbits had a trend toward significantly increased CFU (mean $\pm$ standard deviation, 1,294 $\pm$ 727 CFU; $P = 0.12$) in their lungs when compared to untreated controls (285 $\pm$ 185 CFU) (see histology below). Despite this higher bacillary burden of disease in steroid-treated animals, the number of grossly visible tubercles was significantly lower (Table 1), apparently because of lymphocyte depletion and the relative paucity of inflammatory cells. This lack of inflammation is corroborated by the steroid-treated rabbits’ lower total lung weights (means $\pm$ standard deviations of the steroid-treated-rabbit group versus the control group, 9.1 $\pm$ 0.05 g versus 10.56 $\pm$ 0.33 g; $P < 0.01$) and spleen weights (means $\pm$
standard deviations, 0.9 ± 0.09 g versus 1.37 ± 0.09 g; P < 0.01) (Table 1). Although the mean number of CFU of the malnourished rabbits was higher than that of the controls, it was not statistically significant because of rabbit-to-rabbit variability. None of the rabbits had culturable CFU in the spleen. The relative differences between groups in the burden of bacilli were also blunted because of the relatively low virulence in rabbits of the strain used (CDC1551). Control rabbits contained infection with this strain readily with little evidence of caseous necrosis 5 weeks after aerosolized M. tuberculosis infection (22).

The proportions of rabbits with culture-positive lymph nodes in the untreated control group and the malnourished group were similar. The dexamethasone-treated rabbits had marked lymphoid depletion, significantly smaller spleens, and marked adrenal atrophy. Their lymph nodes were small and difficult to find. Therefore, the reduced hilar lymph node CFU may not be reliable in this group of rabbits. However, the reduced CFU listed in Table 1 is consistent with the findings of Lurie et al. with cortisone-treated rabbits (17, 20).

A portion of the lung and spleen of three animals from each group was weighed and then cut into small pieces that were treated with liberase, an enzyme used to break down connective tissue, and incubated at 37°C for 1 h. Lung pieces were then sieved through sterile strainers to obtain single-cell suspensions. The suspensions were centrifuged, washed, fixed with 4% paraformaldehyde and incubated with fluorescence-conjugated antibodies to CD4, CD8, CD11b, or immunoglobulin M (Serotec, Raleigh, NC) to compare the relative proportions of different cell populations by flow cytometry. Lymphoid and myeloid cells were gated by size based on forward and side scatter. CD4, CD8, and immunoglobulin cell marker-positive cells were represented as a proportion of the total number of gated lymphocytes. The dexamethasone-treated rabbits had significantly lower percentages of CD4+ and CD8+ T cells and B cells in the spleen. The malnourished rabbits and untreated controls had statistically equivalent percentages of CD4+, CD8+ T cells, and B cells in spleen. (Fig. 1B). Similar results were obtained when cell types were calculated as absolute numbers of the splenocytes in the entire spleen. The total numbers of CD4+ T cells and CD8+ T cells in lymph nodes were significantly higher in the malnourished animals (log_{10} cell counts, 7.33 ± 0.13 and 6.81 ± 0.32, respectively) than in either the dexamethasone-treated animals (log_{10} cell counts, 6.51 ± 0.26 and 5.66 ± 0.35, respectively) or control infected animals (log_{10} cell counts, 6.71 ± 0.56 and 6.29 ± 0.51, respectively). This may be due to immune reconstitution, as these rabbits had regained baseline weights by the time of necropsy. Interestingly, the malnourished rabbits had a significant decrease in the percentages of CD4+ T cells in their lungs, despite recovery of their peripheral-white-blood-cell counts. The steroid-treated rabbits also had a significant decline in the lung CD4+ T-cell populations. Although the results are enigmatic, the percentages of B cells and CD11b+ cells were significantly higher than those of either of the other two groups (Fig. 1C). The absolute numbers of cells were also calculated from the weight of the piece of lung from which single-cell suspensions were obtained, and similar trends were observed. Because the number of tubercles within each lung piece was not determined, the standard deviation from the mean was much larger, since cell counts could not be normalized to the number of tuberculous granulomas in each lung piece.

Histologically, in the dexamethasone-treated rabbits, alveolar plugs of partly disintegrating epithelioid cells could be found in a few of the microscopic tuberculous lesions. These plugs were similar to those described by Lurie et al. (18, 20). Acid-fast staining demonstrated tubercle bacilli in some of these plugs. Few, if any, acid-fast bacilli could be found histologically in lesions of either the control or malnourished rabbit groups. The dexamethasone-treated rabbits seemed to have about the same number of microscopic lesions as the controls. Many of these microscopic lesions were not grossly visible at the time of necropsy.

**Discussion.** In guinea pigs, diminished skin test responses to tuberculin with protein calorie malnutrition have been noted (5, 24). In addition, malnourished guinea pigs have impaired
lymphocyte proliferation to tuberculin antigens in both peripheral blood and draining lymph node lymphocytes. This impairment cannot be rescued by augmentation of the acquired immune response with prior vaccination with BCG (21, 24). Macrophages from guinea pigs with protein calorie malnutrition and tuberculosis infection produced less tumor necrosis factor alpha and more tumor growth factor beta (7). In malnourished tuberculous mice, lung-specific decreases in gamma interferon, tumor necrosis factor alpha, and inducible nitric oxide synthase have been reported by Chan et al. (3). These cytokine changes can be rescued with subsequent protein supplementation.

In humans with protein malnutrition, peripheral lymphoid tissues (spleen, lymph node, tonsils, Peyer’s patches, and appendix) become atrophied with depletion of lymphocytes (8, 14). They had profound declines in the absolute and relative numbers of mature circulating T cells (especially CD4+). The relatively small decline in the number of CD8+ T cells leads to a reduction in the CD4 T-cell-to-CD8 T-cell ratio. Interestingly, B cells are not as affected by calorie malnutrition as the results seen in our rabbits (4). Because malnutrition affects both the generation and the maturation of functional T lymphocytes, the impaired cell-mediated immunity to mycobacterial infection is not surprising.

Although our data for rabbits with malnutrition were generated with only a small number of animals, the peripheral blood leukopenia and blunted DTH skin test responses were statistically significant. If the animals had been necropsied at 4 weeks or if the malnutrition had been more prolonged, higher bacillary burdens of disease might have been observed. Because of the paucity of immunologic reagents in rabbits, other studies on the antigen-specific functions of the T cells in the malnourished animals could not be obtained. To repeat this exact experiment would be unethical. Nonetheless, our data are consistent with studies on malnourished human beings: malnourished, BCG-vaccinated persons are more likely to be DTH skin test negative (15, 16, 29). Also, in malnourished individuals, other diagnostic tests for tuberculosis that depend on cellular immune responses, such as enzyme-linked immunospot assay or QuantiFERON-TB, may also have reduced sensitivity.

The findings reported herein showed that compared to the tuberculous controls, both tuberculous, dexamethasone-treated rabbits and the inadvertently malnourished, tuberculous rabbits showed significantly decreased white-blood-cell counts (at 4 weeks) and decreased dermal tuberculin sensitivity (Table 1). Dexamethasone-treated rabbits also had (i) decreased numbers of visible pulmonary tubercles, (ii) decreased lung and spleen weights, (iii) smaller hilar lymph nodes, and (iv) smaller adrenals (Table 1). In the spleens and lungs of dexamethasone-treated rabbits, the percentages of CD4+ T cells were significantly decreased. Although the rabbits in the malnourished group regained their baseline weights during the last week of infection, a persistent decrease in the proportion of CD4+ T lymphocytes remained in the lungs but not in the spleens. Cell-mediated immune responses after tuberculosis infection in rabbits and in humans are dampened by both steroid treatment and malnutrition.

Further studies of the rabbit are warranted because all stages of tuberculosis can be modeled (9, 17) and the forthcoming genome sequence will add to the immunologic reagent armamentarium.

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REFERENCES


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