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Alterations of Pulmonary Defense Mechanisms by Protein Depletion Diet

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Pulmonary defense mechanisms were quantitated in mice that were fed a protein-free diet (PFD) for periods of 2 and 3 weeks. Despite the severe weight loss and emaciation induced by the diet, the bactericidal mechanisms in their lungs were preserved against aerogenic challenges with *Staphylococcus aureus*, *Proteus mirabilis*, and *Listeria monocytogenes*. Phagocytic assays of alveolar macrophages that were retrieved by pulmonary lavage from PFD-fed animals showed a decrease in Fc receptor-mediated binding activity but no alteration in the ingestion of sensitized erythrocytes. In contrast, the PFD induced defects in both the attachment phase and the engulfment phase of the phagocytic process when the challenge organism was *Candida krusei*. The PFD suppressed the pulmonary inflammatory response after mice were infected with influenza virus strain PR8; such mice also failed to eliminate infectious virus from their lungs. Virus infection in control mice suppressed pulmonary antibacterial defenses against challenges with *S. aureus* and *P. mirabilis*, a defect that was ameliorated in the lungs of PFD-fed mice with viral pneumonia. The data demonstrated that pulmonary defense mechanisms were modulated by a PFD but that the observed effect was dependent on the agent used to test host defenses.

Nutritional changes can alter host defenses and, as a consequence, profoundly influence resistance to infection (12, 14, 33, 38). In clinical and experimental studies on the mechanisms by which malnutrition alters nonspecific (phagocytic) and specific (immunological) defenses, workers have focused primarily on the systemic responses. From such studies, it appears that malnutrition has a particularly important influence on thymus-dependent (T-cell) cellular immunity, but that the humoral (B-cell) response, phagocytosis, and the complement system are all affected by nutrition as well (14).

Despite these advances in our knowledge concerning the mechanisms involved with the various nutritional deficiency states that blunt systemic responses to infections, relatively little is known concerning nutritional influences on the defense mechanisms of lungs. In this study we investigated the modulating role of a severe protein deficiency on pulmonary defense mechanisms; specifically, we studied the responses of lungs to aerogenic challenges with *Staphylococcus aureus*, *Proteus mirabilis*, *Listeria monocytogenes*, and influenza virus PR8.

MATERIALS AND METHODS

Animals. Female Swiss outbred mice (MA Bioproducts) weighing 20 to 23 g were used in these experiments. Experimental mice were fed a protein-free diet (PFD) composed of 70% cornstarch, 15% alphacel, 10% vegetable oil, 4% salt mixture, 1% cod liver oil, and a vitamin supplement (ICN Nutritional Biochemicals); these mice were designated PFD mice. Control mice were fed regular rat-mouse-hamster formula (Charles River Breeding Laboratories) containing 27% protein. Under these conditions the control mice showed a mean increase in body weight of 18% after 3 weeks, whereas the PFD mice lost a mean of 40% of their body weight during the same period. PFD mice began to die during week 4 on the diet. Unless otherwise noted, host defense parameters were determined for mice that had been fed the PFD for 3 weeks and were given this diet during the observation period.

Viral infection and titration. The PR8 strain of influenza virus A (adapted in mice) was harvested from the allantoic fluids of 13-day-old chicken embryos after incubation at 37°C for 2 days, titrated in cultures of rhesus monkey tissue, and stored in small portions at −70°C. The titer of the stock culture of virus was 10^8 50% tissue culture infective doses per ml. At 2 weeks after initiation of the PFD, mice were exposed to a 1:100 dilution of this virus stock culture for 30 min in an infectious exposure chamber, as described previously (32). This exposure produced moderate to severe pneumonitis, from which none of the control mice died.

At 1, 3, 5, 7, 9, 10, and 12 days after infection, five control mice and five PFD mice were sacrificed, and their lungs were removed aseptically and scored for gross lung consolidation by the method of Horsfall

(15). Then the lungs were homogenized in citrate-phosphate-buffered Hanks solution (pH 7.0). After centrifugation at 500 × *g* for 5 min, the supernatant fluids from the lung homogenates were pooled. Pulmonary virus titers were determined by allantoic cavity inoculation of 10-day-old embryonic chicken eggs; a 0.1-ml inoculum of a 10-fold dilution of the pooled homogenate supernatant fluid in citrate-phosphate-buffered Hanks solution was inoculated into each of four eggs. The eggs were then incubated at 35°C for 3 days, and the allantoic fluid was harvested and examined for hemagglutinin activity for guinea pig erythrocytes. The 50% egg infectious dose endpoint was calculated by the Karber method (24).

Bacterial challenge. *S. aureus* (coagulase-positive strain 209P, phage type 42D) and a laboratory strain of *P. mirabilis* were labeled with ^{32}P by previously described methods (13). Briefly, the bacteria were each incubated in 125 ml of phosphorus-free culture medium containing 1.0 mCi of ^{32}P. After 18 h of growth at 37°C in a rotating shaker water bath, the labeled cells were centrifuged, washed twice with phosphate-buffered saline (PBS) to remove any unattached label, and suspended in 8 ml of Trypticase soy broth. Uninfected mice which had been fed the PFD for 3 weeks and influenza virus-infected animals which had been fed the PFD for 2 weeks were challenged for 30 min by aerosol inhalation of the radiolabeled bacterial suspension.

L. monocytogenes ATCC 7644 was grown overnight in 125 ml of Trypticase soy broth and concentrated to 8 ml in Trypticase soy broth. Animals which had been fed the PFD for 2 weeks were aerosol challenged for 30 min as described above.

Bacteriological and radioassay procedures. Immediately after bacterial challenge (zero time) with *S. aureus* or *P. mirabilis* and at 4 and 24 h thereafter groups of six animals were sacrificed. The lungs were removed aseptically and homogenized in 3 ml of Trypticase soy broth. Then 1 ml of the homogenate was diluted in PBS and cultured quantitatively in quadruplicate on either Trypticase soy agar containing 5% NaCl (*S. aureus*) or bismuth sulfite agar (*P. mirabilis*). A quantitative measurement of the ^{32}P activity in the lungs was performed with another 1-ml sample of each lung homogenate by liquid scintillation counting techniques, as described elsewhere (19a).

Animals challenged with *L. monocytogenes* were sacrificed at zero time and at 1, 3, 5, 7, 9, and 11 days thereafter. The lungs were handled as described above, except that the viable bacteria in the lung homogenates were quantitated on Trypticase soy agar. In addition, the spleens of the animals were also removed and homogenized in 3 ml of Trypticase soy broth, and the number of viable *Listeria* in them was determined.

Bactericidal activity. The pulmonary bactericidal activity in each animal was calculated by a modification (31) of the radioactive ratio method, as follows: percentage of bacteria remaining (*S. aureus* and *P. mirabilis*) = [(bacterial count/^{32}P count at time *t*)/(mean bacterial count/^{32}P count at zero time)] × 100, where the mean bacterial count/^{32}P count at zero time was calculated by averaging the ratios of bacteria to tracer for the zero time animals. Bactericidal values for control mice, PFD mice, virus-infected mice, and virus-infected PFD mice at time *t* were calculated from similarly treated groups sacrificed at zero time. By using this method we calculated the bactericidal activity of the lungs as a function that was independent of the number of inhaled organisms. Since *L. monocytogenes* was not radiolabeled, the actual numbers of viable bacteria recovered from the lungs and spleens were determined. All experiments with each of the bacteria were performed in duplicate, and the results of the different runs were pooled.

Calculation of physical transport activity. The physical transport of ^{32}P-labeled bacteria from the lungs of control and PFD mice was determined by following the decline in radioactive tracer activity. The ^{32}P counts at 24 h are expressed as percentages of the mean radioactive tracer counts obtained from the animals sacrificed at zero time. As ^{32}P has a relatively short half-life (14.2 days), all samples from each experiment were assayed on the same day. This calculation provided a quantitative measurement of tracer excretion that was independent of the bactericidal activity of the lungs and included the excretion of both viable and nonviable organisms.

Collection of pulmonary cells. Lavages were performed on the lungs of mice that were fed the PFD for 3 weeks. These groups consisted of animals that were infected with virus 7 days before collection or uninfected mice. Mice were sacrificed and exanguinated by cardiac puncture. Pulmonary cells were collected by inserting a Pasteur pipette into the tracheae of surgically removed lungs and introducing and withdrawing 1.5 ml of lavage solution (0.85% NaCl, 0.1% glucose, 0.1% ethylenediaminetetraacetate, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid]) three times; a total of 4.5 ml of lavage solution was used for each lung. After collection, the total number of cells recovered from each individual animal was determined. The suspensions were then centrifuged at 400 × *g* for 10 min, the supernatant fluids were discarded, and the cells were resuspended. Duplicate monolayers of pulmonary cells were prepared for determinations of differential cell counts by cytocentrifugation. One set of cell monolayers was processed with Wright-Giemsa stain, and the second set was processed with α-naphthyl butyrate as described by Li et al. (25) for the cytochemical detection of nonspecific esterases.

Quantitation of alveolar macrophage receptor activity. Murine pulmonary cells were harvested from animals that were fed the PFD for 3 weeks, as described above. More than 97% of the free lung cells were macrophages, and the viability of these cells was >98%. The macrophages were suspended in tissue culture medium 199 supplemented with 10% heat-inactivated fetal calf serum and buffered to pH 7.2 with HEPES (TCM 199). Monolayers of murine macrophages were prepared by incubating 5×10^4 cells on glass cover slips (22 by 22 mm) in small petri dishes for 45 min.

Macrophage immunological (Fc) receptor activity was measured by previously described methods (43). Briefly, immunoglobulin G-coated sheep erythrocytes (EA) were prepared by incubating 7S anti-erythrocyte immunoglobulin and erythrocytes obtained from Cordis Laboratories, Miami, Fla. To determine the per-

centage of macrophages that had a functional Fc receptor capable of binding EA, the fluid covering the monolayers was removed and replaced with 0.5% (vol/vol) EA suspensions in TCM 199 for 1 h at 22°C. Then the monolayers were washed five times with a 0.1% gelatin saline solution to remove the free EA, rapidly air dried, fixed with absolute methanol, and processed with Wright stain. Macrophage-EA rosettes were identified by direct microscopic examination at ×1,000. A minimum of 200 macrophages in each monolayer were selected randomly to determine the percentage of macrophages with attached erythrocytes and the mean number of EA attached per macrophage.

With the following modifications, this technique was also used to measure Fc receptor-mediated phagocytosis. The macrophages were challenged with EA for 1 h at 37°C and rinsed once with gelatin saline solution. To remove extracellular erythrocytes by hypotonic lysis, the EA-challenged monolayers were exposed to distilled water for 20 s, followed by two rinses with gelatin saline solution. Wright-stained preparations were examined microscopically as described above to determine the percentage of phagocytic cells and the mean number of erythrocytes ingested per phagocytic cell.

Macrophage non-immunological (*Candida*) receptor activity (42, 43) was measured as follows. *Candida krusei* was grown in Trypticase soy broth with constant shaking for 18 h at 37°C. The yeast cells were concentrated 10-fold and washed two times in PBS. Then the *Candida* cells were killed by autoclaving, washed two times in PBS, and suspended in TCM 199 at a concentration of 10^7 cells per ml.

To determine the percentage of macrophages that had functional *Candida* receptors capable of binding the yeast cells, the fluid covering the monolayers was removed, a *Candida* suspension (20 yeast cells per macrophage) was added, and the mixture was incubated for 1 h at 22°C. The cultures were then washed three times with PBS to remove the nonadherent *Candida* cells, stained for 5 min with a 0.4% trypan blue–0.2% eosin Y solution in PBS, and washed with PBS to remove the excess stain. A coverslip was then placed over each monolayer, and the wet preparation was examined immediately by phase-contrast microscopy. In this assay, bound *Candida* cells stain pink, whereas the few ingested yeast cells remain unstained (30).

To quantitate non-immunological receptor-mediated phagocytic ingestion, monolayers were incubated with the *Candida* suspension for 1 h at 37°C. Then the cell cultures were washed three times with warm PBS and, to assure the removal of the bound *Candida* cells, the monolayers were incubated with a 0.04% trypsin solution at 37°C for 10 min. After the monolayers were washed with PBS, they were rapidly air dried, fixed with absolute methanol, and processed with Wright stain. Phase-contrast microscopy was used to determine the percentage of phagocytic macrophages and the mean number of *Candida* cells ingested per phagocytic cell.

Histopathology. Histological studies on the lungs of virus-infected test animals were not possible since entire lungs were used for the quantitation experiments. Separate groups of mice were used to study pulmonary histopathology. At 7 days after virus infection, the lungs of these mice were fixed in buffered 10% Formalin, cut into 6-μm sections, and stained with hematoxylin and eosin.

RESULTS

Mice that were fed the PFD progressively lost weight; after 2 weeks they had lost 25% of their initial body weight, whereas control mice had gained 13%. After 3 weeks the weight loss in PFD mice was 40%; this was in contrast to the control mice, which gained 18%.

With time, the PFD mice became increasingly lethargic and emaciated, assuming a hump-backed posture toward the end of week 2 on the diet. At this time, the fur of the animals also became ruffled, and some hair loss occurred around the facial area.

On gross inspection, the lungs of the PFD mice looked normal in that they retained the characteristic salmon pink color. However, because of the severe weight loss the lungs of the PFD mice appeared small compared with the lungs of the control mice. In contrast, severe atrophy of the spleen was observed in the PFD mice; the spleens of the PFD mice appeared to be approximately one-quarter the size of the control mouse spleens.

Table 1 shows the numbers of viable *S. aureus* and *P. mirabilis* cells recovered from the lungs of animals sacrificed immediately after aerogenic challenge. Pulmonary deposition of bacteria was approximately the same in the control and PFD groups.

Figure 1 shows the pulmonary bactericidal activity against inhalation challenges with radiolabeled organisms. The number of viable bacteria declined rapidly in the lungs of control mice, until at 24 h approximately 1% of the initially deposited bacteria were viable. As Fig. 1 shows, the PFD did not significantly ($P > 0.05$, Student's t test) alter the intrapulmonary killing of either bacterial challenge, nor did this diet affect the decline in the amount of radioactive tracer activity in the lungs.

Table 2 shows the cellular composition of the lung lavage fluids. The total count from control animals was $11.4 \times 10^5 \pm 0.7 \times 10^5$ pulmonary cells per lavage, of which more than 98% were alveolar macrophages. Neither the total cell counts nor the differential counts were altered significantly in animals that were fed the PFD.

Table 3 shows the results of the alveolar macrophage membrane receptor-mediated phagocytosis experiments. The mean number of EA attached per positive macrophage and the number of EA attached per 100 positive macrophages (total binding index) were both suppressed significantly by the PFD. On the other hand, the

TABLE 1. *Number of viable bacteria recovered from the lungs of mice immediately after aerosol inhalation challenge with S. aureus or P. mirabilis*

Mice challenged with:	Virus infection	Expt	No. of bacteria in:	
			Control mice	PFD mice
S. aureus	No	1	$2.0 \times 10^5 \pm 0.1 \times 10^5$[a]	$1.4 \times 10^5 \pm 0.2 \times 10^5$
	No	2	$4.6 \times 10^5 \pm 0.6 \times 10^5$	$4.4 \times 10^5 \pm 0.7 \times 10^5$
P. mirabilis	No	1	$6.1 \times 10^5 \pm 1.1 \times 10^5$	$6.6 \times 10^5 \pm 0.9 \times 10^5$
	No	2	$3.6 \times 10^5 \pm 0.7 \times 10^5$	$3.2 \times 10^5 \pm 0.6 \times 10^5$
S. aureus	Yes	1	$8.3 \times 10^4 \pm 1.6 \times 10^4$	$10.5 \times 10^4 \pm 2.5 \times 10^4$
	Yes	2	$21.9 \times 10^4 \pm 4.8 \times 10^4$	$29.4 \times 10^4 \pm 3.2 \times 10^4$
P. mirabilis	Yes	1	$8.7 \times 10^4 \pm 2.4 \times 10^4$	$6.3 \times 10^4 \pm 1.8 \times 10^4$
	Yes	2	$7.5 \times 10^4 \pm 0.9 \times 10^4$	$9.6 \times 10^4 \pm 2.7 \times 10^4$

[a] Each value represents the mean ± standard error of six individual determinations.

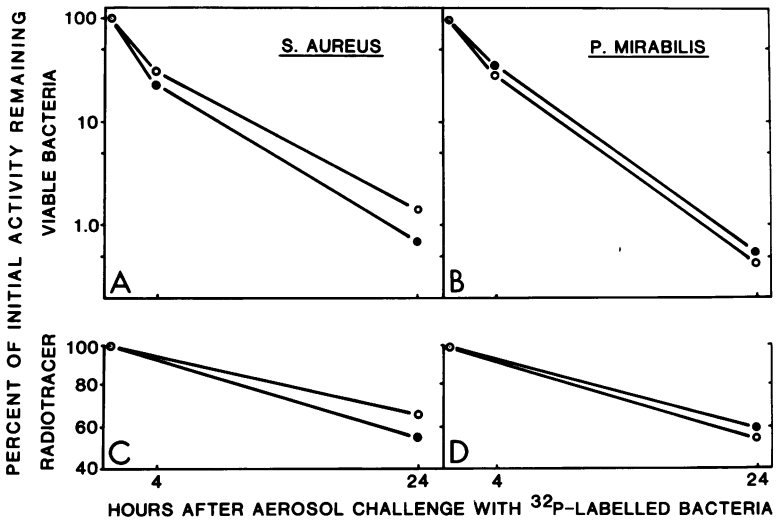

FIG. 1. *(A and B) Comparison of pulmonary bactericidal activities against ^{32}P-labeled S. aureus and P. mirabilis in control mice (○) and mice fed a PFD for 3 weeks (●). (C and D) Comparison of the decline in ^{32}P activity in the lungs of the mice. Each value represents the mean ± standard error of 12 individual determinations.*

TABLE 2. *Cellular response to influenza virus infection on day 7 in murine lungs after 3 weeks of feeding with the PFD*

Mice	Virus infection	Total no. of cells per bronchial lavage ($\times 10^5$)	% of macrophages	% of polymorphonuclear leukocytes	% of lymphocytes
Control	No	11.4 ± 0.7[a]	98.7 ± 0.5	0.5 ± 0.2	0.8 ± 0.5
	Yes	22.5 ± 1.1	57.3 ± 4.9	16.4 ± 5.2	26.2 ± 3.8
PFD	No	10.1 ± 0.7	98.0 ± 0.5	0.2 ± 0.2	1.8 ± 0.4
	Yes	13.1 ± 1.7	73.3 ± 5.8	20.3 ± 5.8	6.4 ± 0.7

[a] Each value represents the mean ± standard error of eight individual determinations.

mean number of EA ingested per phagocytic macrophage and the number of EA ingested per 100 macrophages (total ingestion index) were not altered by the PFD. In contrast to Fc receptor-mediated phagocytic activity, where the PFD inhibited the binding but not the engulfment of particles, both binding and ingestion were suppressed significantly when the challenge organism was *C. krusei*.

Figure 2 shows the courses of pulmonary and systemic infections with *L. monocytogenes*. In contrast to *S. aureus* and *P. mirabilis*, which were killed rapidly in the lungs, *L. monocytogenes* established a long-term infection, which

TABLE 3. *Comparison of alveolar macrophage membrane Fc receptor activity and Candida receptor phagocytic activity in control mice and mice fed a PFD for 3 weeks*[a]

Activity	% of macrophages positive		Mean no. of cells per macrophage		Total activity index	
	Control mice	PFD mice	Control mice	PFD mice	Control mice	PFD mice
Fc receptor binding	85.3 ± 1.3	71.4 ± 0.8 (83.7)[b]	5.90 ± 0.23	4.85 ± 0.26 (82.2)[c]	506 ± 26	355 ± 13 (70.1)[b]
Fc receptor ingestion	75.9 ± 1.5	69.6 ± 2.3 (91.7)	4.94 ± 0.16	4.78 ± 0.14 (96.8)	377 ± 16	332 ± 15 (88.1)
Candida receptor binding	98.1 ± 0.4	89.3 ± 2.7 (90.6)[c]	7.1 ± 0.4	4.7 ± 0.5 (66.2)[c]	701 ± 40	429 ± 61 (61.2)[c]
Candida receptor ingestion	99.1 ±0.2	91.0 ± 2.3 (91.8)[c]	6.4 ± 0.3	4.8 ± 0.5 (75.0)[d]	637 ± 28	444 ± 52 (69.7)[d]

[a] Each value represents the mean ± standard error of eight individual determinations. The values in parentheses are percentages of the control values.
[b] $P < 0.001$, as determined by Student's t test.
[c] $P < 0.01$, as determined by Student's t test.
[d] $P < 0.05$, as determined by Student's t test.

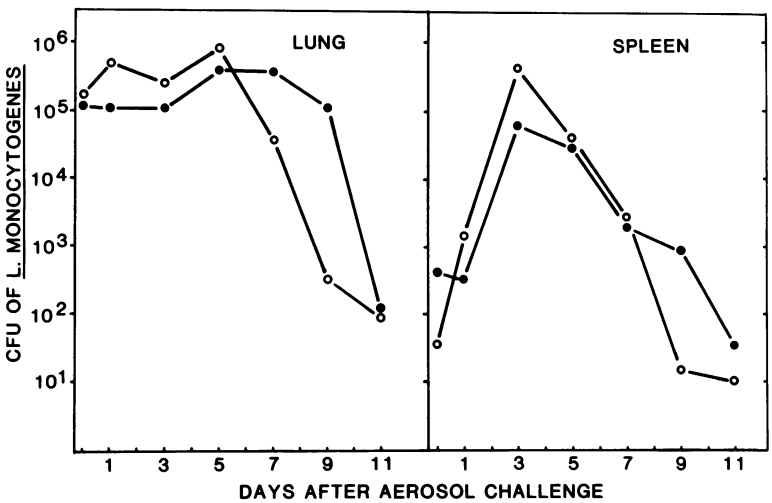

FIG. 2. *Comparison of lung and spleen listericidal activities in control mice* (○) *and mice fed a PFD for 2 weeks* (●). *The animals were maintained on their respective diets during the assay period. Each value represents the mean ± standard error of 12 individual determinations. CFU, Colony-forming units.*

lasted approximately 1 week, after which the organism was eliminated slowly. Similar results were observed in PFD mice, with the exception that the intrapulmonary decline in the number of viable *Listeria* on days 7 and 9 was slower than the decline observed in controls. However, by day 11 this difference no longer existed.

The course of *Listeria* infections in the spleens of mice is also shown in Fig. 2. Within minutes after the cessation of the 30-min aerosol infection procedure, viable *Listeria* were recovered from the spleens. Increasing numbers of organisms were observed until day 3, after which the number of viable *Listeria* declined. The PFD did not alter the infectious process of *L. monocytogenes* in spleens.

Figure 3 shows the courses of pulmonary influenza virus PR8 infections in control and PFD mice. In both groups the virus proliferated extensively in the lungs of the animals until day 5 of infection. Thereafter, the virus was eliminated rapidly from the lungs of the control mice. In contrast, peak pulmonary titers were maintained in the lungs of the PFD mice throughout the 12-day observation period. Also in the PFD group, seven mice died on day 7 of infection and three died on day 12. There were no deaths in the control group.

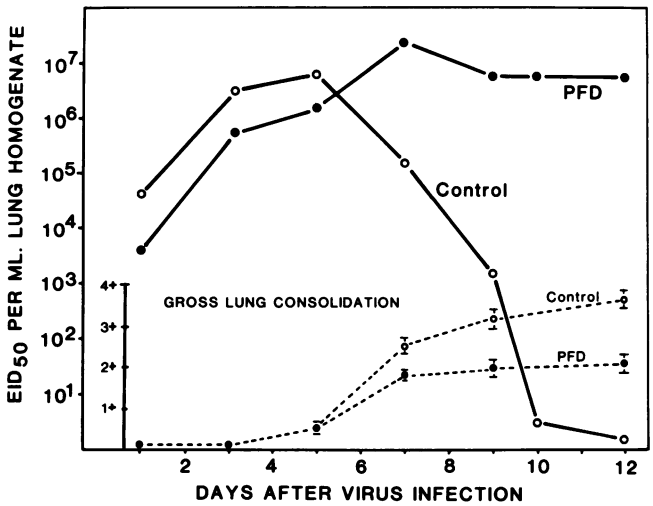

FIG. 3. *Comparison of pulmonary influenza virus titers in the lungs of control mice and mice fed a PFD for 2 weeks. The animals were maintained on their respective diets during the assay period. The inset shows the gross surface consolidation on the surfaces of the lungs, as determined by the method of Horsfall (15): 1+, 0 to 25% consolidation; 2+, 25 to 50% consolidation; 3+, 50 to 75% consolidation; 4+, 75 to 100% consolidation. EID_{50}, 50% Egg infectious doses.*

The extent of gross consolidation on the surfaces of the lungs of virus-infected mice is also shown in Fig. 3. Through day 3 no gross lung consolidation was observed. Thereafter, increasing degrees of surface consolidation were observed in both groups; the amounts of consolidation observed on days 9 and 12 in control lungs were significantly ($P < 0.05$, Student's t test) greater than the amounts observed in the lungs of PFD mice.

Virus-bacterium interactions in the lungs of control and PFD mice are shown in Fig. 4 and 5. In contrast to the rapid intrapulmonary killing of *S. aureus* in the lungs of noninfected mice (Fig. 1), virus infection suppressed pulmonary antibacterial defenses, resulting in a proliferation of *S. aureus* in the lungs (Fig. 4). In contrast, the number of viable staphylococci declined in the virus-infected lungs of PFD mice. The difference between the bactericidal values was significant ($P < 0.05$, Student's t test) at 24 h.

Figure 5 shows the pulmonary bactericidal values against *P. mirabilis* in virus-infected lungs. A greater range of bactericidal values was observed with *P. mirabilis* than with *S. aureus*. For this reason we present the data in the form of individual values for both experiments. At 4 h after aerosol challenge, the numbers of viable bacteria declined in the lungs of both control and PFD mice. However, by 24 h, whereas the number of viable bacteria continued to decline in some animals, extensive intrapulmonary proliferation was observed in others. In general, the

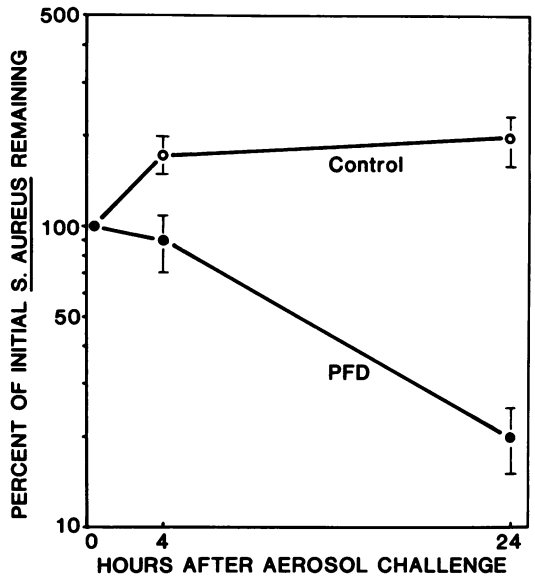

FIG. 4. *Bactericidal activity against S. aureus in the lungs of animals with influenza virus PR8 pneumonia. At 2 weeks after the PFD was begun, the mice were infected with the virus; then the PFD and control mice were maintained on their respective diets for 7 days and challenged with bacteria. Each value represents the mean ± standard error of 12 individual determinations.*

lungs of virus-infected control mice contained larger numbers of bacteria than the lungs of their PFD counterparts. The differences be-

tween the control and PFD groups at 24 h were significant ($P < 0.05$, Mann-Whitney rank test).

Table 2 shows the cellular composition of the lavage fluids from the lungs of virus-infected mice on day 7. We retrieved $22.5 \times 10^5 \pm 1.1 \times 10^5$ cells per lavage from the lungs of control mice, compared with $13.1 \times 10^5 \pm 1.7 \times 10^5$ cells from the lungs of PFD mice ($P < 0.01$, Student's t test). The differential counts revealed that the percentage of inflammatory polymorphonuclear leukocytes was not significantly altered by the PFD. In contrast, the percentage of lymphocytes recovered from the lungs of PFD mice was significantly reduced ($P < 0.01$, Student's t test), and there was a concomitant increase in the alveolar macrophage population.

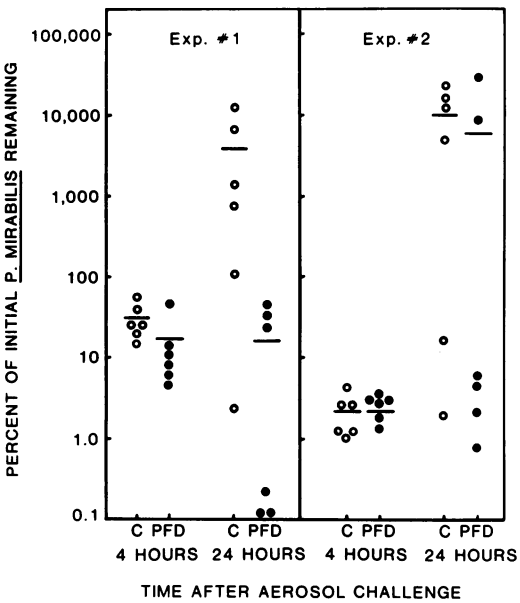

FIG. 5. *Bactericidal activity against P. mirabilis in the lungs of animals with influenza virus PR8 pneumonia. At 2 weeks after the PFD was begun, the mice were infected with the virus; then the PFD and control (C) mice were maintained on their respective diets for 7 days and challenged with bacteria.*

Figure 6 shows a histopathological comparison between day 7 control and PFD virus-infected lungs. Although in each virus-infected lung there were areas that appeared morphologically normal, there were noticeable differences in the affected areas. In control mice, the affected areas were characterized by massive consolidation (Fig. 6A) compared with the PFD lungs, which appeared to be more aerated (Fig. 6B). Upon closer examination of the affected areas, the differences between the control and PFD virus-infected lungs became more apparent, specifically in the bronchial and peribronchial area. In control mice the bronchial abnormalities consisted of intense peribronchial cuffing of inflammatory cells and epithelial cell desquamation. Ciliated epithelial cells, goblet cells, and bronchial mucous gland cells were destroyed. Only the bronchial basal layer remained intact. Beneath the basal layer the basement membrane was swollen, congested, and diffusely infiltrated with predominantly mononuclear cells, the majority of which appeared to be lymphocytes (Fig. 7). In contrast, the bronchial epithelia of the lungs of PFD mice were preserved (Fig. 8) de-

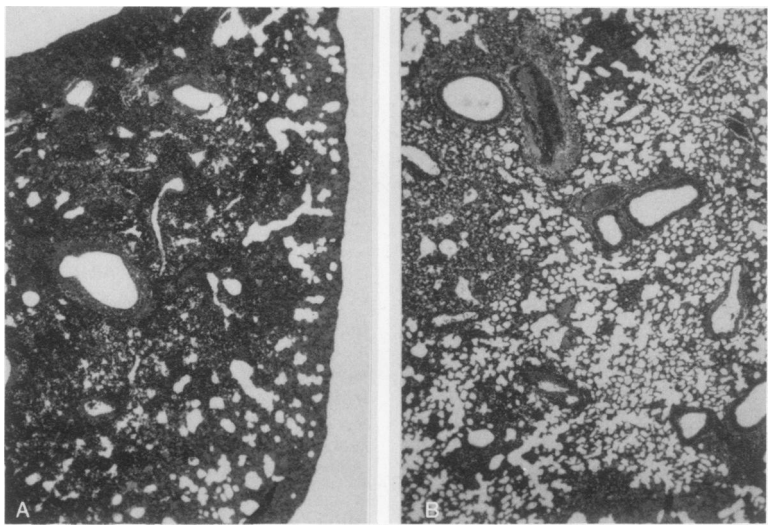

FIG. 6. *Photomicrographs comparing histopathological sections of 7-day influenza virus PR8-infected lungs in control mice (A) and mice fed the PFD (B). Hematoxylin and eosin stain.*

spite the extensive proliferation of the virus and evidence of viral damage. In the virus-infected PFD mice the bronchial epithelia were disorganized, showing ragged inner margins with granularity and vacuolation of the cytoplasm. These degenerative changes were similar to the changes observed in control mice during the early phase of the infection, when the virus was still proliferating in the lungs. In addition to the presence of the bronchial epithelia in the lungs of virus-infected PFD mice, the peribronchial areas were not as densely infiltrated with lymphocytes as the affected peribronchial regions of the lungs of control mice.

DISCUSSION

The role of nutrition in modulating the functional capacity of host defenses has been well established. Although most evidence indicates that malnutrition lowers mechanisms of resistance against infectious agents, conflicting evidence has been found frequently. Recently,

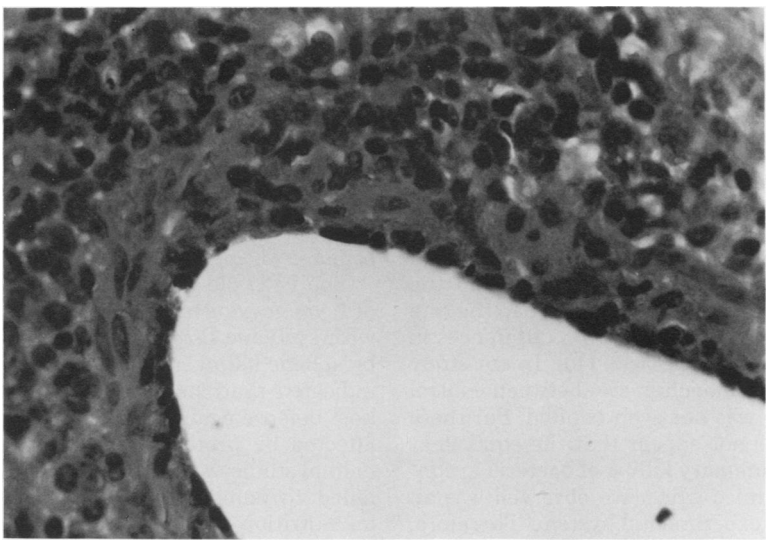

FIG. 7. *Photomicrograph of a medium-sized bronchus in the lung of a mouse infected with influenza virus PR8 7 days previously. An unoccluded bronchus was chosen to show the total epithelial desquamation and the intense inflammatory infiltration in the peribronchial area. Stained with hematoxylin and eosin.*

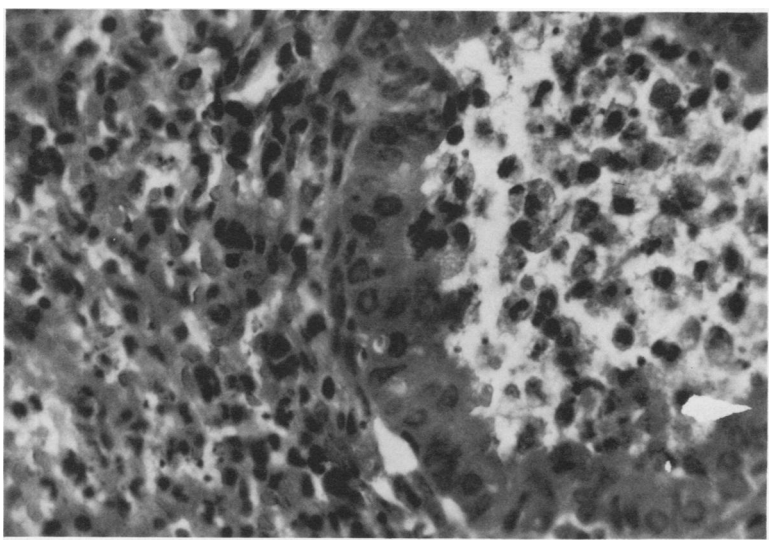

FIG. 8. *Photomicrograph of a medium-sized bronchus in the lung of a virus-infected PFD mouse. Note the presence of the bronchial epithelium. Stained with hematoxylin and eosin.*

Gross and Newberne (14) reviewed the role of nutrition in immunological and phagocytic functions and concluded that in many cases contradictory findings have resulted from the different test systems used. In this study we used several testing techniques to determine the effect of a severe protein deficiency on the pulmonary mechanisms of defense against infectious agents. We believe that this approach was necessary since very little information is available on the effect of malnutrition on lung defenses.

Despite the severe emaciation of the animals that were fed PFD, the bactericidal activity of the lungs against *S. aureus* and *P. mirabilis* remained intact. One possible explanation for this is that fewer bacteria were deposited in the lungs of the PFD mice and therefore the organisms were killed more rapidly (40). However, this did not appear to be the case since approximately the same number of bacteria were recovered from the lungs of both groups immediately after bacterial challenges. Furthermore, in studies in which it was demonstrated that the number of bacteria deposited controlled the rate of intrapulmonary killing, 10-fold differences in inoculum size were compared (40). In our study the difference in inoculum size between control and PFD mice was not even twofold. For these reasons, it does not appear that the equivalent rate of intrapulmonary killing of bacteria in control and PFD mice which we observed was an artifact of our experimental system. Therefore, our results indicate that the antibacterial functions of lungs against *S. aureus* and *P. mirabilis* are not altered by experimental protein depletion malnutrition. This conclusion is supported by the observation that the bactericidal activity of peritoneal macrophages from rats with experimental protein calorie malnutrition also remained intact (21, 22).

In contrast to the observations at the organ level, at the cellular (alveolar macrophage) level the PFD inhibited some phagocytic functions of specific membrane receptors measured in vitro. However, the PFD-induced alveolar macrophage dysfunction depended on the organism used for phagocytic challenge. Both Fc receptor-mediated binding activity and *Candida* receptor-mediated binding activity were suppressed by the PFD, but when the engulfment phase of the phagocytic process was examined, only ingestion of the yeast was affected by the PFD.

The dichotomy between in vivo and in vitro observations serves as a reminder that a precise correlation between the in vitro assessment of phagocytic function and the susceptibility to infectious diseases has not been established firmly. Therefore, care must be taken when attempts are made to correlate in vitro assays with in situ host defenses. Phagocytic challenges of alveolar macrophages with bacteria were not performed in this study because of the divergence in the in vivo and in vitro findings. For example, *S. aureus* is rapidly phagocytized and inactivated by alveolar macrophages in lungs (11); however, this organism is barely ingested and almost not killed when phagocytes are challenged in vitro (23).

We used *L. monocytogenes* in this study since there are major differences in host defenses between pyogenic and intracellular organisms. The former have a marked susceptibility to phagocytic intracellular killing (8), whereas the latter are relatively resistant to such mechanisms (20). Furthermore, the humoral immune system augments phagocytic defenses against pyogenic organisms, whereas protection against *L. monocytogenes* depends primarily on cell-mediated immunity, with the effector cell being the macrophage (3, 26).

The PFD did not alter the infectious process of *L. monocytogenes* in lungs, nor did it interfere with systemic listericidal activity, as measured by splenic killing of the bacterium. Our findings indicated that the series of events involved in host defense against *L. monocytogenes* was not affected by protein deprivation. Other experimental studies with *L. monocytogenes* have also failed to demonstrate a detrimental effect of malnutrition on host defenses. Indeed, acute starvation increased the resistance of mice against hematogenous challenges (46), and peritoneal macrophages from mice subjected to moderate protein deprivation ingested significantly more bacteria than did macrophages from control animals (7). Furthermore, when another facultative intracellular bacterium (*Salmonella typhimurium*) was used, no differences were observed in the intracellular bactericidal activities of peritoneal macrophages from control rats and rats with severe protein calorie malnutrition (23).

Our in vivo studies and the supporting data strongly suggest that malnutrition does not necessarily result in decreased pulmonary resistance to bacterial infections. In contrast, resistance to influenza virus infection was suppressed severely in the lungs of PFD mice, corroborating the results of previous mortality studies with swine influenza virus in mice subjected to dietary protein depletion (36).

There have been relatively few studies on the mechanisms of increased susceptibility to viral infection caused by malnutrition. A loss of host resistance to infection with Wesselbron virus was observed in mice after they were placed on

a protein depletion diet (29). Olson et al. concluded that this increased susceptibility was due at least in part to an impaired antiviral function of macrophages from the observation that protein-deficient peritoneal macrophages in culture supported the growth of Wesselbron virus, whereas macrophages from normal mice did not. Undernutrition (marasmus) increased the susceptibility of mice to coxsackievirus B_3 infection, as shown by an increased incidence of mortality, severe lesions, and elevated and persistent viral titers in the target organs (48). Adoptive transfer of immune lymphoid cells increased the resistance to viral infection. Since the lymphoid tissues were severely atrophic in marasmic mice, the data indicated that this deficiency contributed significantly to the impaired ability of these hosts to recover from viral disease. In a companion study (47), Woodruff showed that marasmic mice had a lower interferon response to viral infection than control animals. Although most evidence indicates that malnutrition decreases host resistance to viral infection, an apparent increase in resistance to pseudorabies virus has also been documented (7).

In this study the PFD induced an increase and a prolongation of virus titers in the lungs, which was accompanied by a decrease in virus-induced pulmonary consolidation. Although the mechanisms by which the PFD interfered with viral elimination and reduced pathological changes were not investigated, clues to potential explanations are available. Resistance to influenza virus infections results from an interaction between interferon and the specific immune response which includes antiviral antibody and specifically sensitized cytotoxic T-lymphocytes (41). Furthermore, it is becoming increasingly clear that whereas the antiviral immune response is instrumental in viral elimination, this response also contributes to the pathogenesis of the disease. This latter conclusion comes from numerous studies in which workers have demonstrated that virus-induced pathological changes correlate with intact antiviral immune responses (4, 16, 34, 37, 39, 49). If in this study the lower percentage of lymphocytes in lung lavages from PFD virus-infected mice can be considered to reflect the immune response, then it follows that the PFD dampened the specific antiviral resistance mechanisms in virus-infected animals. This hypothesis is supported by the following observations: (i) immunosuppressive treatment of mice with cyclophosphamide (19a) and anti-lymphocyte serum (Jakab and Warr, manuscript in preparation) during the course of pulmonary viral infection prolonged the elimination of infectious virus from the lungs; (ii) the treatment with cyclophosphamide and anti-lymphocyte serum reduced the overall inflammatory response to viral infection, with a concomitant shift to a lower percentage of lymphocytes; and (iii) gross lung consolidation was also reduced in the virus-infected mice treated with cyclophosphamide and anti-lymphocyte serum. The almost identical results of the immunosuppressive studies with cyclophosphamide and anti-lymphocyte serum and the PFD studies reported here argue that the increased susceptibility to influenza virus infection may be due in part to a lessening of the antiviral immune response in the PFD mice. This argument is strengthened by other studies which have shown that protein depletion diets are immunosuppressive (14, 17, 38).

We do not know why the PFD had different effects on pulmonary resistance to bacteria and influenza virus. The alveolar macrophage phagocytic system is considered to be the primary defense mechanism of lungs against bacterial infections (11). Our results with *S. aureus* and *P. mirabilis* strongly suggest that the innate bactericidal capacities of alveolar macrophages remained intact in the lungs of PFD animals. On the other hand, resistance to *L. monocytogenes* in normal mice depends on specifically sensitized T-lymphocytes which activate macrophages, thereby giving them increased microbicidal potential to inactivate the bacteria (3). If this is indeed the sequence of events that leads to resistance against *L. monocytogenes*, then the almost identical courses of infection in the lungs and spleens suggest that the entire cascade involved in cell-mediated immunity response was not affected by the PFD. However, T-lymphocyte-deficient nude mice are resistant to *Listeria* infection (5, 6, 10, 28), and recent studies have indicated that the lymphocyte loop of the cell-mediated immunity response can be bypassed in that the macrophages can be activated directly (10). Indeed, acute starvation apparently activates macrophages (10), which may have contributed to the increased resistance of mice against *L. monocytogenes* (46). Our in vivo results do not lend themselves to a mechanistic interpretation; they only demonstrate that the bactericidal defenses of lungs, whether innate or activated, remain preserved in PFD mice. In contrast to bacterial infections, alveolar macrophages do not appear to have a pivotal role in pulmonary resistance against influenza virus infections. Instead, the specific antiviral immune response (with emphasis on the cytotoxic T-cells) is considered to be the primary defense of lungs against influenza virus (41, 45). Since malnutrition appears to have a particularly impor-

tant effect on cell-mediated immunity and a smaller effect on the humoral response (14), a possible explanation for the different effects that the PFD had on pulmonary resistance against virus and bacteria may be that different resistance mechanisms are involved, one affected by the PFD and one not affected.

Viral pneumonias are known to predispose animals to bacterial superinfections by suppressing pulmonary antibacterial defenses (17, 18). *S. aureus* consistently proliferated in the lungs of virus-infected control animals. In contrast, the virus-induced defect was ameliorated in the lungs of the mice that were fed the PFD. In general, the same trend was observed with *P. mirabilis*, although there were considerable differences among the mice within experimental groups. Individual animals differed markedly in resistance, and although mice of the same age and weight were used, the following factors could have contributed to the variability of the data: (i) treatment, the variability being compounded by multiple treatments; (ii) the time interval between the bacterial challenge and the assessment of the intrapulmonary bactericidal activity, when the mechanisms were suppressed; and (iii) the bacterial species used for challenge (gram-positive organisms consistently showed less variability than gram-negative bacteria). These possibilities have been confirmed without exception in many other experiments (data not shown). Thus, despite the variability, it is clear that the virus-induced defect in pulmonary bactericidal mechanisms was mitigated by PFD, as measured with *P. mirabilis*; at 24 h after bacterial challenge, more control mice than PFD mice had high numbers of bacteria in their lungs.

No explanation for the amelioration of the virus-induced bactericidal defect in PFD mice was readily apparent. However, one explanation may explain the data in part. Leukocyte infiltration of the lungs is known to occur during influenza virus infections (50). However, thymus-deficient nude mice develop much less pulmonary infiltration (37) and pathology (45) than immunocompetent furred littermates. Furthermore, nude mice tend to have late deaths and extrapulmonary dissemination of the virus, rather than the early pneumonia deaths observed in immunocompetent animals (37). Delayed deaths also result as a consequence of respiratory virus infections in mice treated with anti-lymphocyte serum (39), cyclophosphamide (16, 32, 35), and X irradiation (1); such immunosuppressive treatment also decreases cellular infiltration into the lungs of virus-infected animals. As stated above, the correlation between an intact antiviral immune response and increased pathological alterations strongly suggests that in the case of influenza virus infections the immunological responses of the host contribute to the pathogenesis of the viral pneumonia. If this is so, then the antiviral immune responses may also be involved in a virus-induced suppression of pulmonary antibacterial defenses. Several lines of evidence from our laboratory suggest that this may be the case. Immunosuppressive treatment of mice with cyclophosphamide and anti-lymphocyte serum (Jakab and Warr, manuscript in preparation) during the course of viral pneumonia: (i) does not interfere with viral replication, (ii) reduces histopathological alterations and leukocyte infiltration into the lungs, and (iii) ameliorates the virus-induced bactericidal defect. It is noteworthy that almost identical results were obtained with the PFD. These observations lend further support to the hypothesis that lowered resistance in PFD mice to virus infection may be mediated through a lesion of the specific (immunological) antiviral mechanisms.

To our knowledge, this is the first study that has examined the effect of malnutrition on pulmonary mechanisms of defense against a wide variety of infectious agents. A severe state of malnutrition was chosen because we wanted to use a maximal stress situation in these initial studies, since different malnutrition states may modulate host defenses against a single organism to varying degrees. The contrasting results which we obtained with the various agents is not surprising. Individual infectious agents have distinctive pathogenic schemes against which hosts have developed primary and perhaps alternate defense strategies. Our data suggest that the PFD affected the acquired immunological defenses of the lungs but did not alter the innate phagocytic defenses.

Finally, it should be noted that the phase of a particular malnutrition state may also affect host resistance against an infectious agent. When the effect of long-term dietary protein depletion on viral susceptibility was investigated in several host-virus systems (35, 36), the relationship was shown to be cyclic in character, involving phases of increased viral susceptibility and decreased viral susceptibility. Thus, the relative resistance of a host appears to be a function of the amount of time on an incomplete diet and, consequently, a function of the state of depletion of the host.

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