We investigated the effects of high protein intake on host resistance to *Paracoccidioides brasiliensis*. Two-d fasted mice were infected with *P. brasiliensis* and fed on diets with three different levels (54%, 20%, and 5%) of casein. The mice refed the 54% casein diet showed reduced antifungal activity in the spleen and liver as compared with the mice refed the 5% or the 20% casein diet. After infection, increases in spleen and liver mRNA levels of myeloperoxidase, cathespin-G, and elastase-2 were more profound in the mice refed the 54% casein diet as compared with the mice refed the 5% or the 20% casein diet. Infected mice refed the 54% casein diet exhibited greater interferon (IFN)-γ production in the spleen and liver and higher levels of thiobarbituric acid reactive substances (TBARSs) in the liver as compared with those refed the 5% casein diet. These results indicate that high protein intake impairs host resistance to *P. brasiliensis*.

**Key words:** antifungal activity; antimicrobial peptide; high-protein diet; infection; interferon (IFN)-γ

Because supplementation with high protein is often used to improve physical condition in a variety of clinical and social settings, it is important to determine the physiological effects of high protein intake. Previous studies in sheep infected with *Trypanosoma congolense* reported that high protein intake ameliorates the adverse effects arising from infection, as assessed by the severity of anemia and weight change. It has been reported that diet supplementation with high protein can improve resilience and resistance to natural infection by endoparasites in young hair sheep. Conversely, it has been reported that intake of a high-protein (51% casein) diet by aged rats for a short period (15 d) decreased the viability, mitogen-induced proliferating activity, and cytotoxic ability of the lymphocytes as compared with a normal protein (20% casein) diet. Affatoxin B1-induced preneoplastic lesions are greater in rats fed a high-protein (30% casein) diet as compared with rats fed a 12% casein diet. In addition to these findings, it was recently reported that dietary protein restriction without malnutrition can have beneficial effects on host defenses against invading pathogens and cancer. Our previous study indicated that feeding a low-protein (1.5% casein) diet for 7 d enhances host resistance to *Paracoccidioides brasiliensis* as compared with a 20% casein diet. Based on these data, we hypothesized that in certain cases high protein intake can suppress rather than enhance host resistance. Therefore, the present study was conducted to better understand the relationship between high protein intake and reduced host resistance by determining whether *P. brasiliensis*-infected mice receiving a high-protein diet would show decreased antifungal activity.

**Materials and Methods**

**Diet:** Casein, α-corn starch, sucrose, cellulose powder, AIN-76 mineral mixture, AIN-76 vitamin mixture, and choline bitartrate were purchased from Oriental Yeast (Tokyo). dl-methionine was from Wako Pure Chemical (Osaka, Japan). Soybean oil was from NOF (Tokyo). Using food-grade ingredients, purified rodent powder diets were prepared in our laboratory. The compositions of the test diets are shown in Table 1. The three dietary groups examined in our analyses were differentiated by casein weight percentage: 5%, 20%, or 54%. Casein was exchanged isoenergetically with α-corn starch.

**Fungi:** *P. brasiliensis* isolate Pb-18, isolated from a Brazilian patient with paracoccidioidomycosis, was used to infect the mice. The fungal cells used in the experiments were newly derived from the mycelial form of this microbe and were subcultured twice at 35°C at 4-d intervals on 1% glucose-supplemented brain heart infusion (BHI; Difco Laboratories, Detroit, MI) agar slants. Fresh growth (4 d) of the fungus was collected in 0.9% sterile saline with mesh to eliminate cell clumps. After they were washed once, the fungal cells were counted using a hemacytometer. More than 97% of the fungal cells prepared in this manner were viable. They were resuspended at the indicated densities in saline before infection of the mice.

**Animals and experimental design.** Specific-pathogen-free, 5-week-old female BALB/c mice were obtained from Charles River Japan (Atsugi, Japan). The animals were maintained on commercial laboratory chow (Oriental Yeast) and water ad libitum. Approximately...
Table 1. Compositions of Test Diets

<table>
<thead>
<tr>
<th>Composition (g/kg)</th>
<th>Casein in diet</th>
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<tbody>
<tr>
<td></td>
<td>5%</td>
</tr>
<tr>
<td>Casein</td>
<td>50</td>
</tr>
<tr>
<td>cysteine-Methionine</td>
<td>0.75</td>
</tr>
<tr>
<td>α-Corn starch</td>
<td>702.25</td>
</tr>
<tr>
<td>Sucrose</td>
<td>100</td>
</tr>
<tr>
<td>Soybean oil*</td>
<td>50</td>
</tr>
<tr>
<td>Mineral mixture†</td>
<td>35</td>
</tr>
<tr>
<td>Vitamin mixture†</td>
<td>10</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>2</td>
</tr>
<tr>
<td>Cellulose</td>
<td>50</td>
</tr>
</tbody>
</table>

*Fatty acid composition (g per 100 g of fatty acid): 16:0, 17.4; 16:1(ω-9), 0.1; 18:0, 5.7; 18:1(ω-9), 22.3; 18:1(ω-7), 1.2; 18:2(ω-6), 46.9; 18:3(ω-3), 6.1; 20:1(ω-9), 0.2; 22:5(ω-3), 0.1.
†AIN-76 mineral and vitamin mixtures.9

100 g of the non-purified diet contained 23.6 g of protein, 5.3 g of fat, 6.1 g of ash, 2.9 g of fiber, and 54.4 g of nitrogen-free extracts. After an acclimatization period (5d), the mice were fed the test diet containing 20% casein and water ad libitum. One week after commencing the 20% casein diet, the mice were deprived of food for a period of 48 h. During this period of food deprivation, they were allowed free access to water. After the 48-h food-deprivation period, the mice were infected using a single intravenous injection of a 200-μl fungal cell suspension (0.4 × 10^6 cells/ml) through the lateral tail vein. After infection, the mice were immediately assigned to a test diet containing 5%, 20%, or 54% casein. These diets were consumed ad libitum for 7 d. The infected mice consuming the test diets were sacrificed by decapitation on days 0–7 after infection and their blood, spleens, and livers were harvested. All animal housing, handling, and sample collection procedures conformed to the policies and recommendations of the Laboratory Animal Care Advisory Committee of Chiba University (Chiba, Japan).

Measurement of serum urea nitrogen, total protein, and albumin levels. Blood was collected and allowed to clot for 1 h at room temperature. Serum was then separated by centrifugation at 1,500 × g for 20 min at 4 °C. Serum urea nitrogen, total protein, and albumin levels were measured using a urea nitrogen B-test (Wako Pure Chemical), a BCA Protein Assay Kit (Pierce, Rockford, IL), and an A/G B-test (Wako Pure Chemical), respectively. All assays were performed in duplicate and the data averages were statistically analyzed.

Counts of viable fungal cells from organs. Spleens and livers were aseptically removed from the subject mice and homogenized in a glass homogenizer with 4 ml and 19 ml of saline respectively. The samples were diluted with saline. One ml of each diluted homogenate was then plated on BHI agar supplemented with 50 μmol/l of EDTA, 20 ml of horse serum (Gibco Laboratories, Grand Island, NY), and 150 μmol/l of chloramphenicol (Wako Pure Chemical). The agar plates were incubated in a humidified atmosphere at 35 °C, and the colony-forming units (CFUs) of P. brasiliensis were counted after 21 d of incubation.

Measurement of IFN-γ and IL-1β levels. Mouse spleens and livers were homogenized in cold PBS (pH 7.4) with a glass homogenizer and centrifuged at 6,000 × g for 30 min at 4 °C to pellet the debris. IFN-γ and IL-1β levels were determined in sample supernatants using ELISA kits purchased from Endogen (Rockford, IL). All assays were performed following the manufacturer’s instructions.

RNA extraction and quantitative real-time RT-PCR. Total RNA was isolated from mouse spleens and livers with an RNeasy extraction kit following the manufacturer’s instructions (Qiagen, Santa Clara, CA). Complementary DNAs were synthesized from 2 μg of individual total RNA extracts using oligo-dT primers and a Reverse Transcription System (Promega, Madison, WI) following the manufacturer’s instructions. Real-time quantitative RT-PCR analyses were performed in a final volume of 20 μl using a SYBR Green PCR master mix (Applied Biosystems, Foster City, CA) containing 300 nM primers and 10 ng of reverse transcribed total RNA. The resulting amplified products were analyzed in real time using an ABI PRISM 7300 Sequence Detection System. The primer sequences used were as follows (product size and GenBank accession numbers are indicated in parentheses): myeloperoxidase (145 bp, BC053912) forward, 5’-CCGCTCTGAA-CAATCAATGACC-3’; reverse, 5’-ATTACGTTTGGCTGAGTGGG-3’; cathespin-G (186 bp, NM_007800) forward, 5’-AGGACCCAAGAATC-CATCAC-3’; reverse, 5’-AGGACACCTGTCACCAAATCC-3’; elastase-2 (151 bp, NM_015779) forward, 5’-TGCATTGCGTGTTCCTG-3’; reverse, 5’-ACGAAGTTCCTGCAAGTGAAG-3’; inducible nitric oxide synthase (iNOS) (144 bp, NM_010927) forward, 5’-CCCCGTAC-TACTCCATCAGC-3’; reverse, 5’-GCTTCAGCTGCTTACATGCAA-3’; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (177 bp, M32599) forward, 5’-TGACCACCAAATCCTAG-3’; reverse, 5’-GATGTCAGGATGATGTTC-3’. The relative expression levels of the target gene products were calculated by the comparative threshold cycle (Ct) method using GAPDH as the normalization control.

Measurement of lipid peroxidation. The extent of lipid peroxidation in the mouse liver tissue was assayed by measuring one of the end-products of the process, the thiobarbituric acid-reactive substances (TBARSs). Livers were homogenized in a 0.1 M phosphate buffer (pH 7.4) to yield a 10% w/v homogenate. A thiobarbituric acid reaction was carried out by mixing 0.2 ml of sodium dodecylsulfate solution (8.1%, w/v), 1.5 ml of acetic acid buffer (20%, v/v, pH 3.5), 1.5 ml of thiobarbituric acid (0.8%, v/v), and 0.6 ml water with 0.2 ml of tissue homogenate. The resulting reaction mixture was incubated at 95 °C for 60 min and then cooled by mixing it with 1 ml of water and 5 ml of n-butyl alcohol and pyridine (15:1 by volume). The mixture was then centrifuged (4°C, 1,000 × g) for 10 min, and the supernatant was measured spectrophotometrically at 535 nm. Tetraethoxypropane was used as a standard to estimate TBARS formation, which was measured in nanomoles of malondialdehyde equivalents per gram of tissue.

Statistical analysis. All data were expressed as mean plus standard deviation (SD) for n observations. Data were analyzed by Tukey’s test after one-way ANOVA with SPSS software (SPSS, Tokyo). Differences were considered significant at p < 0.05.

Results

Food intake and total body, spleen, and liver weights. The food intake of the mice refed the 54% casein diet was less than that of the mice refed the 5% casein diet throughout the experimental period (Fig. 1A). Two d of starvation resulted in decreases in body weight from 20.6 ± 0.9 g (mean ± SD, before starvation) to 18.0 ± 0.9 g (p < 0.05, n = 10/group). From days 1 to 4 after infection, the mean body weight of the mice refed the 54% casein diet was slightly but significantly lower than that of the mice refed the 5% or the 20% casein diet (Fig. 1B). Two-d of starvation reduced the spleen weight (prestarved versus starved, 101 ± 15 versus 74 ± 10 g, p < 0.05, n = 5/group). The mice refed the 54% casein diet showed greater increases in spleen weight at days 5 and 7 after infection than the mice refed the 5% or the 20% casein diet (Fig. 1C). Two d of starvation also decreased the liver weight (prestarved versus starved, 1.15 ± 0.04 versus 0.93 ± 0.06 g, p < 0.05, n = 5/group). In all groups, the liver weight gradually increased to 1.44–1.59 g by day 7 after infection. Among the dietary groups, the liver weight was not significantly different throughout the experimental period (data not shown).

Serum urea nitrogen, total protein, and albumin levels. Two d of starvation had no significant effect on serum urea nitrogen, total protein, or albumin levels (data not shown). In the mice refed the 54% casein diet, serum
urea nitrogen levels increased to 46.8 ± 0.9 mg/dl by 3 h after infection, and stayed at higher levels until day 7 (Fig. 2A). The serum urea nitrogen levels in the mice refed the 20% casein diet also increased, to 32.6 ± 0.9 mg/dl by 3 h after infection, and then returned to the normal level (day 0 in Fig. 2A) by day 5. In contrast, in the mice refed the 5%, the 20%, and the 54% casein diet, serum total protein and albumin levels were not significantly changed from normal levels from 3 h to day 7 after infection (Fig. 2B, C).

Antifungal resistance
The CFU counts obtained from the spleens were not significantly different among the mice refed the three levels (5%, 20%, and 54%) of casein at day 0 after infection (Table 2). However, at day 3, the mean spleen CFU counts from the mice refed a diet containing 54% casein were 3.6-fold higher than those from the mice refed the 5% casein diet. At day 5, the mice refed the 54% casein diet showed 2.6- and 1.8-fold higher spleen CFU counts than the mice refed the 5% and 20% casein diets respectively. At day 7 after infection, the spleen CFU counts from the mice refed the 54% casein diet were 4.9-fold higher than those obtained from the mice refed the 5% casein diet.

The liver CFU counts also were not significantly different among the dietary groups at day 0 after infection (Table 3). The liver CFU counts from the mice refed the 54% casein diet were 1.7-fold higher than those from the mice refed the 5% casein diet at day 3.
after infection. At day 7, the mice refed the 54% casein diet showed 4.1- and 1.9-fold higher liver CFU counts than those of the mice refed the 5% and 20% casein diets respectively.

**IFN-γ and IL-1β levels in spleen and liver**

Cellular immunity has been shown to be decisive in host resistance to *P. brasiliensis* in the murine system, and IFN-γ and pro-inflammatory cytokines have been suggested to play a pivotal defensive role in this response. Two d of starvation had no significant effect on spleen IFN-γ production (data not shown). The mice refed the 20% or the 54% casein diet showed greater IFN-γ production in the spleen than the mice refed the 5% casein diet at days 5 and 7 after infection (Fig. 3A). Two d of starvation resulted in a reduction in spleen IL-1β production (prestarved versus starved, 7.90 ± 0.40 versus 6.02 ± 0.83 ng/g of tissue, p < 0.01, n = 5/group). In all groups, the spleen IL-1β levels returned to the pre-fasting level by day 3 after infection and stayed at a normal level until day 7, no significant differences being observed among the groups (data not shown). Two d of starvation also had no significant effects on IFN-γ or IL-1β production in the liver (data not shown). The mice refed the 20% or the 54% casein diet exhibited a higher liver IFN-γ level at day 5 after infection than the mice refed the 5% casein diet (Fig. 3B). Liver IL-1β levels were higher in the mice refed the 54% casein diet at day 5 than in the mice refed the 5% casein diet (Fig. 3C). When mice were not infected after 2 d of starvation and were refed the three levels (54%, 20%, and 5%) of casein, the IFN-γ and IL-1β levels in the spleen and liver stayed at the pre-fasting level at days 3, 5, and 7 after refeeding, and were not affected by dietary protein levels (data not shown).

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### Table 2. Recovery of *P. brasiliensis* from Spleens of Mice Refed Diets with Three Levels (5%, 20%, and 54%) of Casein

<table>
<thead>
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<th>Casein in diet</th>
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<tbody>
<tr>
<td></td>
<td>0&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>5%</td>
<td>Mean&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>1,792</td>
<td>144</td>
</tr>
<tr>
<td>20%</td>
<td>1,732</td>
</tr>
<tr>
<td>54%</td>
<td>1,711</td>
</tr>
</tbody>
</table>

<sup>1</sup>Time post-infection (d). Day 0, 3 h after infection/refeeding.

<sup>2</sup>Each value shows mean ± SD (n = 5 per group on each day). The experimental data were assessed by ANOVA following Tukey’s B post hoc test. Mean values with different superscripts (a–c) for the same day are significantly different (p < 0.05).

### Table 3. Recovery of *P. brasiliensis* from Livers of Mice Refed Three Levels (5%, 20%, and 54%) of Casein Diet

<table>
<thead>
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<th>Casein in diet</th>
<th>Colony forming units</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>5%</td>
<td>Mean&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>15,095</td>
<td>2,776</td>
</tr>
<tr>
<td>20%</td>
<td>16,251</td>
</tr>
</tbody>
</table>

<sup>1</sup>Time post-infection (d). Day 0, 3 h after infection/refeeding.

<sup>2</sup>Each value shows mean ± SD (n = 5 per group on each day). The experimental data were assessed by ANOVA following Tukey’s B post hoc test. Mean values with different superscripts (a, b) for the same day are significantly different (p < 0.05).

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**Fig. 3. Levels of IFN-γ in the Spleen (A), IFN-γ in the Liver (B), and IL-1β in the Liver (C) in *P. brasiliensis*-Infected Mice Refed Diets with Three Levels (5%, 20%, or 54%) of Casein.**

Results are expressed as mean ± SD (n = 5/test group at each day). Day 0, before infection/refeeding. Values highlighted with different superscripts (a, b) for the same day are significantly different (p < 0.05).

**The expression of specific genes in the spleen**

Two d of starvation had no significant effect on spleen mRNA levels of myeloperoxidase, cathepsin-G, or elastase-2 (data not shown). In the mice refed the 5% or the 20% casein diet, spleen mRNA levels of myeloperoxidase, cathepsin-G, and elastase-2 stayed at basal levels (day 0 in Fig. 4A–C) throughout the experimental period. In contrast, in the mice refed the 54% casein diet, the spleen mRNA levels of these antimicrobial peptides increased markedly by day 7 after infection. At day 7, the spleen mRNA expression of myeloperoxidase, cathepsin-G, and elastase-2 of the mice refed the 54% casein diet were 5.0-, 4.5-, and 5.0-fold higher than those from mice refed the 5% casein diet respectively. When mice were not infected after 2 d of starvation and were refed the 5%, the 20%, or the...
higher respectively in the mice refed the 54% casein diet in the present study, a high-protein (54% casein) diet reduced host resistance to *P. brasiliensis* as compared with the diets containing 5% or 20% casein. Pal and Poddar have reported that feeding a high-protein diet to aged rats for a long term (60 d) decreased the proliferating activity and cytotoxic ability of the lymphocytes as compared with a normal protein diet. It has also been reported that septic guinea pigs that received a high protein diet had more bacterial translocation in the mesenteric lymph nodes, liver, lung, and blood than animals fed a low protein diet. Taking together, it is conceivable that in certain cases supplementation with high protein has adverse effects on immune functions and thereby suppresses rather than enhances host resistance.

In the present study, IFN-γ production in the spleen and liver and IL-1β production in the liver were greater (Fig. 3A–C), whereas fungal clearance in the spleen and liver was delayed (Tables 2, 3) in infected mice refed the 54% casein diet as compared with those refed the 5% casein diet. Recent studies using animal models have reported that delayed clearance of invading pathogens (*Listeria monocytogenes*, *Candida albicans*, and influenza virus) from target organs leads to greater production of IFN-γ and pro-inflammatory cytokines in these organs, and suggested that the increased response of these cytokines then contributes to clearance of the pathogens. The 54% casein diet-induced increase in IFN-γ and IL-1β production might have resulted from delayed clearance of the fungus.

Antimicrobial peptides constitute part of the innate immune system and play an essential role in defense against infection. It has been reported that neutro-
phils lacking elastase fail to kill phagocytosed gram-

negative bacteria, and that mice deficient in elastase

and/or cathepsin-G have increased mortality after

infection with *Aspergillus fumigatus*. In our previous

study, by contrast, spleen and liver mRNA expression of

myeloperoxidase, cathepsin-G, and elastase-2 increased

less profoundly, while fungal clearance in the spleen and

liver was enhanced, after infection with *P. brasiliensis*
in mice refed the 1.5% casein diet as compared with mice refed the 20% casein diet. In addition, in this study, the mice refed the high-protein (54% casein) diet showed higher mRNA expression of these antimicrobial peptides in the spleen and liver (Figs. 4A–C, 5A–C) and delayed fungal clearance in these organs as compared with the mice refed the 5% or the 20% casein diet. Thus there was no positive correlation between antifungal activity and the expression of any one of genes encoding these three antimicrobial peptides. Further studies are needed to account for these findings. On the other hand, it has been reported that highly reactive halide-derived oxidants produced by myeloperoxidase can cause oxidative damage to host tissues and thus contribute to the pathogenesis of diseases such as atherosclerosis, renal injury, and carcinogenesis. 22) It has also been reported that excess neutrophil serine proteases (e.g., elastase and cathepsin-G) in the lungs play a central role in the pathology of inflammatory pulmonary disease. 23,24) The higher production of these antimicrobial peptides in mice refed the 54% casein diet might have been more detrimental to the host than the lower production of these peptides in the mice refed the 5% or the 20% casein diet.

Neutrophil-derived reactive oxygen species are required for optimum microbicidal activity. It has been reported that a deficiency in the production of reactive

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**Fig. 5.** Liver mRNA Levels of Myeloperoxidase (A), Cathepsin-G (B), Elastase-2 (C), and iNOS (D) in *P. brasiliensis*-Infected Mice Refed Diets with Three Levels (5%, 20%, or 54%) of Casein. The transcript levels for each gene are expressed as relative mRNA levels (GAPDH mRNA). Results are expressed as mean ± SD (n = 5/test group at each day). Day 0, before infection/refeeding. Values highlighted with different superscripts (a–c) at the same days are significantly different (*p* < 0.05).

**Fig. 6.** Liver TBARS Levels in *P. brasiliensis*-Infected Mice Refed Three Levels (5%, 20%, and 54%) of Casein Diet. Results are expressed as mean ± SD (n = 5/test group at each day). Values highlighted with different superscripts (a, b) for the same days are significantly different (*p* < 0.05).
oxygen species leads to increased susceptibility to *P. brasiliensis* infection.\textsuperscript{25} However, it is now well known that infection-induced overproduction of reactive oxygen species, including lipid hydroperoxide, rather contribute to host tissue damage.\textsuperscript{26,27}\textsuperscript{25} For example, influenza virus infection accelerates lipid hydroperoxide production, and treatment with anti-oxidant enzymes and vitamin E reduces lipid hydroperoxide formation and decreases the pathogenicity of the influenza virus.\textsuperscript{28,29}\textsuperscript{25} In the present study, the mice refed the 54% or the 20% casein diet showed greater lipid hydroperoxide production and higher iNOS mRNA expression in the liver than the mice refed the 5% casein diet (Figs. 5D, 6), suggesting that infection-induced oxidative damage in hepatocytes might be increased by refeeding the 54% or 20% casein diet as compared with the 5% casein diet.

There is increasing evidence that when the energy content of the diet is equal, diets with higher levels of protein and reduced levels of carbohydrates promote weight loss.\textsuperscript{30}\textsuperscript{29} The high-protein (54% casein) diet decreased food intake and attenuated body weight gain until day 4 after infection as compared with the diets containing 5% or 20% casein (Fig. 1A, B). The slower recovery of body weight might have contributed to reduced antifungal activity in the mice refed the 54% casein diet. In the present study, the mice in all groups were fed a normal protein (20% casein) diet for 1 week and were then fasted for 2 d before infection. The effect of feeding a high-protein diet both before and after infection on host resistance to *P. brasiliensis* remains to be further elucidated.

**Acknowledgment**

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**References**