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Effect of Dietary Essential Amino Acid Limitations upon the Susceptibility to *Salmonella typhimurium* and the Effect Upon Humoral and Cellular Immune Responses in Mice

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We investigated the effects of dietary essential amino acid limitations on the susceptibility of mice to *Salmonella typhimurium* infections and on humoral and cellular immune (cell-mediated immune) responses of mice. Mice fed synthetic diets limited (significantly less than optimum concentration) in a single essential amino acid (leucine, isoleucine, valine, or lysine) for 3 weeks after they were weaned exhibited significantly enhanced susceptibility to *S. typhimurium* infection, as evidenced by the higher levels of mortality and spread of the bacterial cells in their livers and spleens compared with mice fed the control diet. Compared with mice fed the control diet, mice fed the diet limited in leucine had a lower ability to clear *S. typhimurium* cells from the peritoneal cavity 5 min after intraperitoneal injection, whereas mice fed the diet limited in lysine had a greater ability. The in vivo phagocytosis and in vitro bactericidal kinetics against *S. typhimurium* cells by peritoneal macrophages were not significantly different in the control group and the groups of mice fed experimental diets. Certain experimental groups exhibited significantly lower resistance and antibody response against *S. typhimurium* SL3770 on day 5 after immunization with heat-killed *S. typhimurium* SL3770. On day 8 after immunization, the levels of serum antibody against *S. typhimurium* in the mice fed the experimental diets were comparable to the levels in mice fed the control diet. However, the levels of serum transferrin and complement C3 were significantly lower in mice fed certain experimental diets. The cellular immune capacities of mice fed any of the experimental diets were not impaired compared with the capacities of mice fed the control diet, as measured by spleen cell responsiveness to phytohemagglutinin and the ability to clear infecting *Listeria monocytogenes* cells from livers and spleens.

Clinical studies have shown that children who lack adequate protein in their diets (protein malnutrition) are more susceptible to infection, disease, and death (22). Similarly, dietary protein deficiencies have rendered experimental animals more susceptible to infection with *Staphylococcus aureus* (5), *Mycobacterium tuberculosis* (23), *Streptococcus pneumoniae* (27, 28), *Trichinella spiralis* (24), *Escherichia coli* (28), and *Salmonella typhimurium* (18). The increases in susceptibility to *M. tuberculosis* and *S. aureus* infections were reversed when the deficient diets were supplemented by a mixture of essential amino acids (5). Dietary limitations of essential amino acids also render hosts susceptible to infection (24). Dietary deficiencies of specific essential amino acids may result from consumption of low-quality proteins, such as gluten or soya α-protein, which are deficient in one or more essential amino acids (5), or consumption of certain proteins in limited quantities, such as 8% casein (11).

Infection itself may also result in or augment amino acid malnutrition (17). Wannemacher has hypothesized that low levels of serum branched-chain amino acids during some infections are due to increased catabolism of these amino acids by muscle tissue as sources of energy (26). Shigella enterotoxin (1) and diarrhea (7) have been implicated in the inhibition of transport and malabsorption of amino acids by intestines, as well as in the creation of deficiencies of minerals and vitamins. Therefore, a host may become deficient in essential amino acids due to diet or infection.

The resistance of a host to infections, including *S. typhimurium* infections, is dependent upon the humoral (8, 9) and cell-mediated immune systems (2, 25), as well as several nonspecific factors, such as complement (12), transfer-

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rin (15), lysozyme, and the phagocytic abilities of blood and tissue macrophages (6, 8). Therefore, any increased susceptibility of mice fed diets limited in proteins or essential amino acids may be due to an impairment in one or more of these host defense systems. Recently, we reported that female mice fed from weaning semisynthetic diets that were limited in leucine, isoleucine, valine or lysine had normal levels of serum immunoglobulin G but higher than normal levels of serum immunoglobulin A and immunoglobulin M (19). However, the specific humoral immune response against *S. typhimurium* and the cell-mediated immune system were not evaluated in mice fed the experimental diets.

Until now, most investigators have used protein-deficient diets which were not defined with respect to the total nitrogen balance or the optimum concentration of essential amino acids for a specific host or both. Also, most experimental studies have used adult hosts without taking into account the levels of resistance (native or acquired) that already existed in such hosts. The purpose of the present investigation was to determine the effects of individual dietary essential amino acid limitations upon the susceptibility of mice to experimental infections with *S. typhimurium* and the effects of these limitations upon the specific humoral immune and cell-mediated immune systems in mice.

MATERIALS AND METHODS

Mice and bacterial strain. Female CF1 and CD1 weanling mice (10 to 12 g) were obtained from the Charles River Breeding Laboratories, Inc., Wilmington, Mass. Female Swiss Webster weanling mice were obtained from Cox Farm, Indianapolis, Ind. Unless stated otherwise, CF1 mice were used in most experiments.

S. typhimurium SR11 (courtesy of S. W. Rockwood) and *S. typhimurium* SL3770 (courtesy of R. W. Treick) were maintained on nutrient agar medium and grown overnight in nutrient broth before each experiment. *Listeria monocytogenes* (courtesy of A. A. Blazkovec) was cultured initially in Trypticase soy broth. Cells from stock suspensions were grown for 12 h in broth at 37°C before each injection. The cells were harvested by centrifugation at 27,000 × *g*, washed twice, suspended in sterile saline, adjusted spectrophotometrically (optical density at 550 nm) against a McFarland number 1 $BaCl_2$ standard to a concentration of approximately 3×10^8 cells per ml, and diluted to the appropriate concentrations needed for injections. The exact number of viable cells was determined by counting the number of colony-forming units (CFU) on an appropriate agar medium after incubation at 37°C for 24 h.

Diets. Each group of weanling mice was fed ad libitum one of five semisynthetic diets (limited in leucine, isoleucine, valine, or lysine or not limited [control]) for 3 weeks (19). These mice were used in the experiments described below. The control diet contained all of the essential amino acids at the levels needed to achieve maximum growth, as described by John and Bell (11). The growth response of the mice to each diet was determined by weighing the animals at 4-day intervals for 3 weeks; the growth response was similar to the weight gain previously described regardless of the strain used (19).

Experimental infection and immunization. After the mice in each dietary group were fed the specific diets for 3 weeks, each mouse was injected intraperitoneally (i.p.) with approximately 300 cells of *S. typhimurium* SR11 or 10^4 cells of *S. typhimurium* SL3770. In a pilot study, we determined that a dose of 300 *S. typhimurium* cells killed 50% of CF1 mice fed the control diet in 10 days after i.p. injection. A total of 10^8 washed and heat-killed (1 h at 100°C) *S. typhimurium* SL3770 cells were used for immunization of each mouse. On days 5 and 8 after immunization, at least 5 mice from each dietary group were bled via the branchial plexus, and their sera were collected, and at least 15 mice from each dietary group were each challenged i.p. with approximately 1.3×10^5 viable *S. typhimurium* SL3770 cells (10 times the 50% lethal dose) at that time. Mortality was monitored for 14 days. The days for collection of sera and challenge of mice after vaccination were based on a pilot experiment that determined the time of peak antibody response in control mice.

Enumeration of bacterial cells in spleens and livers. Groups of 15 to 20 mice were each fed one of the diets for 3 weeks. Every mouse in each dietary group was injected i.p. with approximately 300 *S. typhimurium* SR11 cells or 10^4 *L. monocytogenes* cells. The number of *S. typhimurium* or *L. monocytogenes* cells present in the livers and spleens of the mice in each dietary group was determined by a procedure described previously (2). At least five injected mice per dietary group (barring mortalities) were selected randomly on days 1, 3, and 5 after injection with *S. typhimurium* and on days 3, 5, and 7 after injection with *L. monocytogenes* and were sacrificed. The spleens and livers were individually removed, weighed, and homogenized in saline with a motorized Teflon pestle tissue homogenizer (Thomas). The tissue homogenates were diluted in saline, and 0.2 ml of each dilution was spread in duplicate onto an appropriate agar medium. The numbers of *S. typhimurium* and *L. monocytogenes* cells per milligram of organ were determined on the basis of the numbers of CFU on EMB and Trypticase soy agar, respectively, after 24 to 48 h at 37°C.

Phagocytic and bactericidal activities of peritoneal macrophages. The bactericidal activity of the peritoneal macrophages from each dietary group of mice was determined by a previously described procedure (2), with slight modifications. Five mice from each dietary group were each injected i.p. with approximately 10^6 *S. typhimurium* SR11 cells. After 5 min, the mice were sacrified, and each peritoneal cavity was flushed twice with 2 ml of Hanks balanced salt solution that contained 10% calf serum, 5 U of heparin, and 6 μg of chloramphenicol per ml to inhibit the intracellular multiplication of *S. typhimurium* during the in vitro bactericidal assay. The peritoneal

washes of five mice from each dietary group were pooled in a siliconized tube and kept at 0°C. The suspension was washed twice, the initial supernatant fraction was diluted in saline, and 0.2 ml of each dilution was spread onto EMB agar to determine the number of nonphagocytized CFU.

The pellet of peritoneal exudate cells (PEC), which contained the phagocytized *S. typhimurium* cells, was suspended in 4 ml of Hanks balanced salt solution and divided equally among three siliconized test tubes, which were rotated at 9 rpm at 37°C. After 0, 30, or 60 min, the macrophages were lysed by freezing and thawing the suspension three times, using liquid nitrogen and a 37°C water bath, respectively. This suspension was diluted in saline, and 0.2 ml of each dilution was spread onto EMB agar to determine the number of CFU that remained inside the PEC after 0, 30, and 60 min. The bactericidal activity of the PEC was determined for only the 60-min preparation since Blanden et al. (2) demonstrated that maximum bactericidal activity of PEC occurs by 60 min and suggested that observations after 60 min might not be valid because of multiplication of surviving *S. typhimurium* cells inside the PEC.

Serum bacterial agglutination. At 5 and 8 days after immunization with 10^8 heat-killed *S. typhimurium* SL3770 cells, the sera of mice from each dietary group were examined for the relative concentration of agglutinating antibody specific for SL3770. Serial dilutions of the sera were reacted with a constant concentration (7.5×10^7 cells per ml) of heat-killed *S. typhimurium* SL3770 for 10 h at 37°C. Serial twofold dilutions (25 μl) were made by using a microtiter dilution system (Dynatech Laboratories, Inc., Alexandria, Va.). The reciprocal of the highest dilution of a serum to agglutinate the *S. typhimurium* cells was considered to be the titer.

Evaluation of serum complement C3 and transferrin levels. On days 5 and 8 after immunization with 10^8 heat-killed *S. typhimurium* SL3770 cells, the sera of mice from each dietary group were examined for the relative levels of complement C3 and transferrin. We used the radial immunodiffusion technique of Mancini et al. (16), as previously described (19), to determine the relative levels of these serum proteins.

Transformation of splenic T lymphocytes by PHA. The ability of phytohemagglutinin (PHA) to stimulate the spleen cells of mice was assayed by a previously described procedure (10), with minor modifications. After the 21-day feeding regimen, spleens were removed from at least five mice per dietary group and placed into pH 7.2 RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) which contained 100 U of penicillin per ml and 100 μg of streptomycin per ml to inhibit bacterial multiplication. Cells were washed twice, and the erythrocytes were lysed with tris(hydroxymethyl)aminomethane–0.16 M ammonium chloride (pH 7.2) buffer at 37°C for 2 min and suspended in RPMI 1640 medium containing 10% fetal calf serum. More than 95% viability was obtained, as determined by the trypan blue exclusion procedure. Each final suspension was adjusted to a concentration of 2.0×10^6 cells per ml in 2 ml of RPMI 1640 medium containing 10% fetal calf serum. This concentration of spleen lymphocytes and 10 mg of PHA-P (Difco Laboratories, Detroit, Mich.) yielded a maximum response. Control suspensions were not exposed to any PHA-P. AFter 72 h of incubation at 37°C in a 5% CO_2 atmosphere, 1 μCi of [*methyl*-^{3}H]thymidine (20 Ci/mmol; lot 1258.136) was added to the PHA-P-stimulated and control cultures. After 24 h, the reactions were stopped by plunging each culture tube into a cold ice bath, and cells were filtered onto membrane filter disks (pore size, 0.4 μm; Nuclepore Corp., Pleasanton, Calif.) and washed with 5 ml of 5% cold trichloroacetic acid to lyse the cells and precipitate the deoxyribonucleic acid onto the filter disks. The disks were rinsed in absolute ethanol, and the amount of radioactive [^{3}H]thymidine incorporated into deoxyribonucleic acid was determined by using a scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.) and toluene-ethanol scintillation fluid.

Statistical evaluation of the data. We used the chi-square test to determine any significant differences in the mortality experiment and the Student *t* test to determine any significant differences between the control values and experimental values in the other experiments. *P* values of ≤ 0.05 were considered to be significant.

RESULTS

Susceptibility of mice to experimental infection with *S. typhimurium*. Mice are susceptible to infection with most strains of *S. typhimurium*, and the death rates after infection are related to the number of bacterial cells in the livers and spleens (2). The number of mortalities for the CF1 mice fed a diet limited in isoleucine, leucine, or valine was significantly higher after injection with *S. typhimurium* SR11 than the number of mortalities in the control group (Table 1). The mortality in the lysine-limited group was higher but not significantly so. Except for the CD1 mice fed the leucine-limited diet, the Swiss Webster and CD1 mice fed the experimental diets exhibited higher mortalities after *S. typhimurium* injections. However, the differences in mortality between the CD1 control and experimental groups were not significant. With the exception of the leucine-limited group, the experimental groups of CF1 mice also exhibited significantly higher mortalities after infection with *S. typhimurium* SL3770, a less virulent strain than SR11 (Table 1). Each dead mouse had 10^8 to 10^9 *S. typhimurium* cells per liver and spleen, indicating that the higher mortalities in the experimental groups were due to increased susceptibilities to *S. typhimurium*.

Enumeration of CFU in the livers and spleens. Another measure of susceptibility to *S. typhimurium* infection is the degree of bacterial proliferation in livers and spleens (2). On day 1 after injection, all groups of mice fed experimental diets had significantly more *S. typhimurium* cells in their livers and spleens (Table 2). On day

TABLE 1. *Effects of dietary essential amino acid limitations on the susceptibility of mice to S. typhimurium*[a]

Dietary limitation	CF1 mice injected with 300 SR11 cells		CF1 mice injected with 10^4 SL3770 cells		Swiss Webster mice injected with 200 SR11 cells		CD1 mice injected with 250 SR11 cells	
	No. injected	% Mortality	No. injected	% Mortality	No. injected	% Mortality	No. injected	% Mortality
None	21	57	23	4	10	0	12	42
Leucine	20	95[b]	22	14	10	20	12	33
Isoleucine	18	100[b]	19	21[b]	9	66[b]	13	62
Valine	19	84[b]	17	71[b]	6	33[b]	13	69
Lysine	22	77	22	45[b]	9	66[b]	13	54

[a] After 21 days on specific diets, female CF1, Swiss Webster, and CD1 mice were injected i.p. with the indicated number of *S. typhimurium* cells, and mortalities were monitored for 14 days.
[b] Significantly different than mice fed the control diet ($P \leq 0.05$).

TABLE 2. *Effects of dietary essential amino acid limitations on the proliferation of S. typhimurium SR11 in the livers and spleens of CF1 mice*[a]

Dietary limitation	No. of mice injected	No. of *S. typhimurium* CFU per liver and spleen ($\log_{10}$) on:			No. of mice dead by day 5
		Day 1 after injection	Day 3 after injection	Day 5 after injection	
None	18	3.28 ± 0.46[b]	5.54 ± 0.91	5.12 ± 0.70	0
Leucine	19	4.84 ± 0.52[c]	6.29 ± 0.94	4.56 ± 2.05	2
Isoleucine	20	5.19 ± 1.32[c]	6.54 ± 0.92[c]	6.28 ± 1.68	3
Valine	19	5.00 ± 0.63[c]	6.50 ± 0.85[c]	6.59 ± 0.98[c]	4
Lysine	18	4.31 ± 0.58[c]	6.14 ± 0.99	4.90 ± 1.76	1

[a] Female CF1 mice were injected i.p. with 300 live *S. typhimurium* SR11 cells. The livers and spleens of six mice were examined for CFU on days 1 and 3 postinjection, and the numbers of CFU on day 5 postinjection were determined only for surviving mice.
[b] Mean ± standard deviation.
[c] Significantly different than mice fed the control diet ($P \leq 0.05$).

3 after injection, mice fed isoleucine- or valine-limited diets had significantly more CFU in their livers and spleens than mice fed the control diet. On day 5 after injection, all experimental groups experienced one or more deaths, indicating increased numbers of CFU in the livers and spleens of all experimental groups. Of the survivors, only mice fed the valine-limited diet exhibited higher levels of CFU in their livers and spleens than mice fed the control diet. The day 5 CFU values for each experimental group of mice would be much higher if the data from dead mice were included (10^8 to 10^9 CFU per liver and spleen of each dead mouse).

Macrophage phagocytic and bactericidal activities. Native macrophage phagocytic and bactericidal activities are important in native resistance of mice against *S. typhimurium* infection. Mice fed the leucine-limited diet exhibited significantly higher levels of CFU (90%), whereas mice fed the lysine-limited diet demonstrated significantly lower levels (39%) of *S. typhimurium* CFU in their peritoneal fluids, compared with the CFU levels in the mice fed the control diet (Table 3). The number of CFU remaining

TABLE 3. *Effects of dietary essential amino acid limitations on the ability of mice to clear S. typhimurium SR11 from the peritoneal cavity*

Dietary limitation	No. of *S. typhimurium* cells injected per mouse	*S. typhimurium* remaining (% of cells injected) in:[a]	
		Intracellular fluid	Extracellular fluid
None	1.01×10^6	3	69 ± 10
Leucine	0.94×10^6	4	90 ± 10[b]
Isoleucine	1.20×10^6	4	59 ± 32
Valine	1.01×10^6	3	54 ± 16
Lysine	1.31×10^6	4	39 ± 5[b]

[a] A minimum of five mice per dietary group were injected i.p. with 10^6 *S. typhimurium* cells. After 5 min each peritoneal cavity was washed with Hanks balanced salt solution, and the number of *S. typhimurium* cells associated with peritoneal cells was determined by lysing the peritoneal cells with water and rapid freezing and thawing. The numbers of CFU in the extracellular fluids were determined by a quantitative plate count technique.
[b] Significantly different than mice fed a control diet ($P \leq 0.05$).

in the peritoneal cavities of mice fed the valine- or isoleucine-limited diet was not significantly different than the number of CFU in the control group. It appears that a clearance mechanism present in the peritoneal cavity was enhanced in the mice fed the lysine-limited diet and was impaired in the mice fed the leucine-limited diet.

The number of *S. typhimurium* cells phagocytized after 5 min by the PEC from mice fed any of the experimental diets was relatively small (3 to 4%) and was not significantly different than the number of cells found in the PEC from mice fed the control diet (Table 3). Similar values were obtained when the PEC were disrupted with a tissue homogenizer. This suggests that the freeze-thaw technique for disruption of PEC was not responsible for the observed small percentage of *S. typhimurium* cells in the PEC. Between 70 and 90% of the phagocytized *S. typhimurium* cells were killed in 60 min by the PEC from the control and experimental diet groups of mice (Fig. 1). The kinetics of the in vitro bactericidal activities exhibited by the PEC from mice fed the experimental diets were not significantly different than the kinetics of the activities exhibited by the PEC from mice fed the control diet.

Humoral immune responses against *S. typhimurium*. On days 5 and 8 after vaccination, all groups of mice fed experimental diets had significantly higher resistance to SL3770 than mice that were fed the control diet and were not vaccinated (Table 4). On day 5 after vaccination, this resistance (as expressed by percent mortality) was significantly lower in the mice fed the leucine-, isoleucine-, or valine-limited diet than in vaccinated mice fed the control diet. However, experimental groups of mice challenged on day 8 after vaccination did not exhibit significantly different levels of resistance than the vaccinated control mice.

On day 5 after vaccination, mice fed the valine- or lysine-limited diet exhibited significantly lower levels of serum antibody against SL3770 than vaccinated control mice (Table 5). The lower resistance of the valine-limited group (Table 4) was accompanied by a lower level of serum antibody against SL3770. On the other hand, mice fed the lysine-limited diet exhibited normal resistance (Table 4) and expressed significantly lower levels of serum antibody than control mice. The reverse was true for mice fed the leucine-limited diet. The serum antibody levels on day 8 compared with day 5 after vaccination were higher, but the levels in the experimental groups on day 8 were not significantly different than the levels in the control mice.

The serum antibody levels against *S. typhimurium* SL3770 in the vaccinated experimental and control mice which survived the SL3770 challenge dose (Table 4) were examined on day 16 and 23 after challenge. The serum antibody levels against *S. typhimurium* in these vaccinated and challenged mice were significantly higher than the levels in the vaccinated mice. Mice fed experimental diets had levels of antibody which were not significantly different than the levels in mice fed the control diet (Table 6). These data indicate that antibody synthesis was impaired only in the early phase and not in the later phase of humoral immune response in the mice fed the experimental diets.

It was of interest to determine whether the synthesis of antibody was at the expense of the synthesis of other resistance factors, such as transferrin and complement C3, in mice fed the experimental diets. Therefore, the serum levels of transferrin and complement C3 were meas-

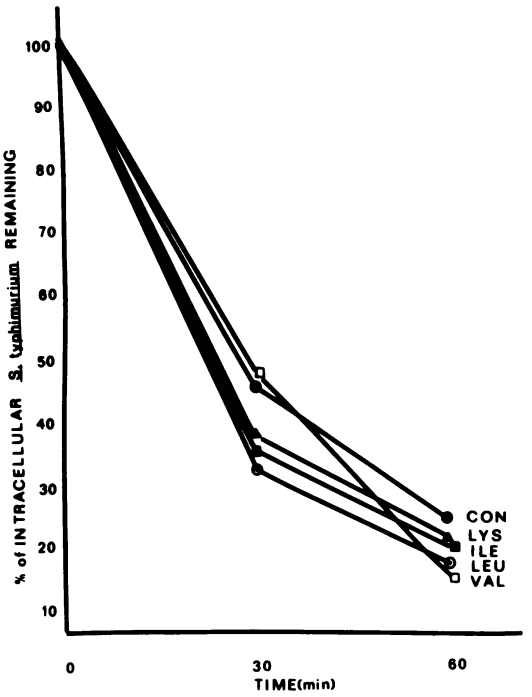

FIG. 1. *Effects of dietary amino acid limitations on the in vitro killing of i.p. injected S. typhimurium cells by murine peritoneal cells. Mice from control (CON) and experimental groups (leucine limited [LEU], isoleucine limited [ILE], valine limited [VAL], and lysine limited [LYS]) were injected i.p. with 10^6 cells of S. typhimurium; after 5 min their peritoneal cavities were flushed with Hanks balanced salt solution, the peritoneal cells were separated, and the in vitro killing of peritoneal cell-associated S. typhimurium cells was determined at 0, 30, and 60 min by the procedure described in the text.*

TABLE 4. *Effects of dietary essential amino acid limitations on the resistance of mice to a challenge with live S. typhimurium SL3770 on days 5 and 8 after vaccination with heat-killed SL3770*

Dietary limitation	Day 5 after vaccination[a]		Day 8 after vaccination[a]	
	No. challenged	% Mortality	No. challenged	% Mortality
None	17	0	14	0
Leucine	17	23[b]	15	0
Isoleucine	15	33[b]	17	18
Valine	15	27[b]	15	20
Lysine	17	0	16	13
Control[c]	10	100[b]		

[a] After 21 days on a dietary regimen, female CF1 mice were injected i.p. with 10^8 heat-killed *S. typhimurium* SL3770 cells; mice were challenged 5 or 8 days later with 10 times the 50% lethal dose of live SL3770, and mortalities were monitored for 14 days.

[b] Significantly different than vaccinated mice fed the control diet ($P \leq 0.05$).

[c] Mice were fed the control diet and injected without vaccination with 10 times the 50% lethal dose of live SL3770; all mortalities occurred by day 6.

TABLE 5. *Effects of dietary essential amino acid limitations on the humoral immune response against S. typhimurium SL3770*

Dietary limitation	Agglutination titer (log_2) at:[a]	
	5 Days after vaccination	8 Days after vaccination
None	3.09 ± 0.94[b]	3.88 ± 0.92
Leucine	3.00 ± 1.26	3.60 ± 1.17
Isoleucine	2.20 ± 1.48	3.85 ± 1.34
Valine	2.00 ± 1.32[c]	4.00 ± 1.06
Lysine	1.71 ± 1.25[c]	4.00 ± 1.69

[a] After 21 days on a dietary regimen, female CF1 mice were injected i.p. with 10^8 heat-killed *S. typhimurium* SL3770 cells; sera were collected 5 and 8 days later, and antibody against SL3770 was assayed by bacterial agglutination.

[b] Mean ± standard deviation.

[c] Significantly different than mice fed the control diet ($P \leq 0.05$).

ured 5 and 8 days after vaccination with 10^8 heat-killed SL3770 cells. On day 5 after vaccination, the serum levels of transferrin and complement C3 were not significantly different in mice fed the experimental diets compared with mice fed the control diet (Table 7). On day 8 after vaccination, the levels of serum transferrin increased slightly in the mice fed the control diet or the leucine- or isoleucine-limited diet compared with the levels observed on day 5. However, mice fed the valine- or lysine-limited diets had significantly lower levels of serum transferrin on day 8 after vaccination than mice fed the control diet. Similarly, mice fed the leucine-, valine-, or lysine-limited diet exhibited significantly lower levels of serum complement C3 on day 8 after vaccination than mice fed the control diet. Only mice fed the isoleucine-limited diet expressed a normal level of complement C3 on day 8 after vaccination.

Cellular immunity. The T lymphocytes are the central lymphoid cells of cellular immune resistance. The total number of spleen cells found in the mice fed the isoleucine-, valine-, or lysine-limited diet was significantly lower than the number of spleen cells in the mice fed the control diet (Table 8). However, when the concentration of the spleen cell suspension from each dietary group was adjusted to 2×10^6 cells per ml, no impairment of PHA stimulation was observed in any of the experimental groups (Table 9). The spleen cells from mice fed the isoleucine- or lysine-limited diet had significantly higher responsiveness to PHA than the spleen cells from mice fed the control diet.

The cellular immunity of the host was also determined in vivo by evaluating the clearance of *L. monocytogenes* from livers and spleens (3). On day 3 after injection mice fed the isoleucine-limited diet and on day 5 after injection mice fed the valine-limited diet demonstrated a decrease in liver and spleen *L. monocytogenes* CFU levels (Table 10). No experimental group of mice demonstrated significantly higher levels of CFU than the mice fed the control diet. On day 7 after injection, all experimental groups of mice experienced some mortality. Dead mice had 10^8 to 10^9 CFU per liver and spleen at the time of death. The CFU values from dead mice were not included in the day 7 data. The surviving mice fed experimental and control diets experi-

TABLE 6. *Effects of dietary essential amino acid limitations on the murine antibody response against S. typhimurium SL3770*

Dietary limitation	Agglutination titer (log_2) at:[a]	
	16 Days after secondary challenge	23 Days after secondary challenge
None	4.20 ± 1.09[b]	6.00 ± 1.00
Leucine	3.60 ± 0.89	5.40 ± 1.14
Isoleucine	4.00 ± 0.81	6.20 ± 1.09
Valine	4.60 ± 0.89	6.50 ± 1.57
Lysine	5.50 ± 1.00	6.20 ± 1.48

[a] After 21 days on a dietary regimen, CF1 mice from each dietary group were vaccinated with 10^8 heat-killed SL3770 cells; 8 days later they were challenged with 10 times the 50% lethal dose of live SL3770, and the survivors were bled for sera on days 16 and 23 and assayed for bacterial agglutination against SL3770.

[b] Mean ± standard deviation.

TABLE 7. *Effects of dietary essential amino acid limitations on the serum levels of transferrin and complement C3 after vaccination with heat-killed S. typhimurium SL3770*

Dietary limitation	Area of RID precipitation pattern (mm^2)[a]			
	Transferrin		Complement C3	
	5 Days after vaccination	8 Days after vaccination	5 Days after vaccination	8 Days after vaccination
None	29.3 ± 2.9[b]	38.1 ± 15.4	85.7 ± 10.1	93.3 ± 7.7
Leucine	31.8 ± 7.7	36.3 ± 8.1	95.6 ± 16.9	75.5 ± 14.3[c]
Isoleucine	33.7 ± 3.0	46.6 ± 7.2	92.0 ± 16.1	91.4 ± 19.0
Valine	28.5 ± 7.7	25.6 ± 1.1[c]	75.6 ± 20.0	73.5 ± 12.8[c]
Lysine	24.0 ± 7.5	21.3 ± 8.3[c]	72.1 ± 17.5	49.2 ± 10.9[c]

[a] After 21 days on a dietary regimen, mice were injected with 10^8 heat-killed *S. typhimurium* SL3770 cells; sera were collected on days 5 and 8, and transferrin and complement C3 levels were measured by the radial immunodiffusion (RID) technique.

[b] Mean ± standard deviation.

[c] Significantly different than mice fed the control diet ($P \leq 0.05$).

TABLE 8. *Effects of dietary essential amino acid limitations on the number of murine spleen cells*

Dietary limitation	No. of spleen cells per mouse ($\times 10^7$)[a]
None	9.47 ± 1.60[b]
Leucine	8.42 ± 1.37
Isoleucine	3.13 ± 1.85[c]
Valine	3.59 ± 0.97[c]
Lysine	2.84 ± 0.74[c]

[a] Mice were fed a dietary regimen for 21 days, spleens were removed and forced individually through a 60-gauge screen, and numbers of cells were enumerated with a hemacytometer.

[b] Mean ± standard deviation.

[c] Significantly different than mice fed the control diet ($P \leq 0.05$).

TABLE 9. *Effects of dietary essential amino acid limitations on the responsiveness of murine spleen cells to PHA*

Dietary limitation	[^{3}H]thymidine incorporation (cpm, $\times 10^3$)[a]	
	With PHA	Without PHA
None	22.2 ± 17.6[b]	1.00 ± 0.33
Leucine	27.3 ± 26.4	2.20 ± 1.31
Isoleucine	44.4 ± 21.6[c]	2.99 ± 2.23
Valine	44.3 ± 28.8	3.36 ± 1.04
Lysine	46.5 ± 16.4[c]	2.93 ± 1.46

[a] The spleens of mice from each dietary group were homogenized to a cell suspension and adjusted to 2×10^6 cells per ml in RPMI 1640 medium containing 10% fetal calf serum and 10 μg of PHA-P per ml. The culture was incubated for 4 days, and the incorporation of [^{3}H]thymidine into deoxyribonucleic acid was measured for the last 24 h.

[b] Mean ± standard deviation.

[c] Significantly different than mice fed the control diet ($P \leq 0.05$).

enced decreases in the number of CFU per liver and spleen. This clearance was probably due to the development of cell-mediated immunity against *L. monocytogenes*, which has been shown to develop at about day 5 after injection (3). The surviving mice fed the leucine-limited diet had significantly lower numbers of CFU per liver and spleen than the mice fed the control diet.

DISCUSSION

On the basis of the data described above, we can state that CF1 female mice fed diets limited in single essential amino acids are more susceptible to infection with *S. typhimurium* SR11 and SL3770, as determined by the levels of mortality and the numbers of *S. typhimurium* cells in livers and spleens. Increases in susceptibility were also observed in the experimental groups of Swiss Webster mice.

Not much is known regarding the nature of preimmune (native or nonspecific) defense mechanisms in early resistance to *S. typhimurium*. However, acquired resistance to *S. typhimurium* infection in mice has been associated with macrophage activation, a cellular immune function (2), and specific antibodies (a humoral immune function) (13). Therefore, the observed increases in the susceptibilities of the mice fed the diets limited in essential amino acids may have been the result of an impairment in the development of preimmune defense mechanisms or the result of an impairment in the development of specific defense mechanisms of the immune systems against *S. typhimurium* or both.

The PEC of all dietary groups in this investigation exhibit low degrees of in vivo phagocytic activity against *S. typhimurium*, as would be expected in nonimmunized mice (2). However, there is a clearance phenomenon in the perito-

TABLE 10. *Effects of dietary essential amino acid limitations on the proliferation and clearance of L. monocytogenes in the livers and spleens of mice*[a]

Dietary limitation	No. of mice injected	No. of *L. monocytogenes* CFU per liver and spleen (log_{10}) on:			No. of mice dead by day 7
		Day 3 after injection	Day 5 after injection	Day 7 after injection	
None	15	5.29 ± 0.77[b]	5.38 ± 0.94	4.24 ± 0.98	0
Leucine	15	4.77 ± 1.48	5.32 ± 1.60	2.49 ± 0.55[c]	3
Isoleucine	14	3.72 ± 0.86[c]	5.02 ± 0.78	3.73 ± 0.58	1
Valine	15	5.02 ± 1.14	4.34 ± 0.53[c]	3.70 ± 0.17	1
Lysine	15	5.66 ± 0.84	6.03 ± 1.02	4.96 ± 1.87	3

[a] Female CF1 mice from each dietary group were injected i.p. with 10,000 live *L. monocytogenes* cells, the livers and spleens of four to five mice were examined for CFU on days 3 and 5 postinjection, and the numbers of CFU on day 7 postinjection were determined only in surviving mice (three to five mice per group).

[b] Mean ± standard deviation.

[c] Significantly different than mice fed the control diet ($P \leq 0.05$).

neal cavity which accounts for the loss of nonphagocytized *S. typhimurium* cells from the supernatant fraction of the peritoneal exudate. This clearance phenomenon is significantly enhanced and impaired in mice fed lysine- and leucine-limited diets, respectively. The exact mechanism of this peritoneal cavity clearance is not apparent. Dietary amino acid limitations do not affect the in vitro bactericidal activity of the PEC.

This investigation clearly demonstrates that significant humoral resistance to *S. typhimurium* infection could be acquired in mice fed the control diet, as well as the experimental diets, when the mice were vaccinated with heat-killed *S. typhimurium*, compared with mice that were fed the control diet and were not vaccinated. It is well known that vaccination with heat-killed *S. typhimurium* elicits a strong humoral immune response that plays a vital role in resistance against *S. typhimurium* infection (9). Significantly lower resistance in the leucine-, isoleucine-, and valine-limited groups on day 5 but not on day 8 after vaccination suggests that the early immune response is impaired in these dietary groups. Only the lower resistance of the valine-limited group can be explained by a lower antibody level. The lower resistance of the leucine- and isoleucine-limited groups may be explained by the impairment of other host defense functions, such as inability to clear *S. typhimurium* cells from the peritoneal cavity and an impairment of the quality of antibody in vivo. Indeed, Reinhardt and Steward (20) have shown that protein malnutrition adversely affects the affinity of antibody for antigen. Significantly depressed levels of serum transferrin and complement C3 in certain experimental groups by day 8 after immunization with heat-killed *S. typhimurium* suggested that these dietary groups may have used the limited amino acid preferentially for antibody synthesis, resulting in an impairment of the synthesis of transferrin and complement. Except for the valine- and lysine-limited groups on day 5, antibody synthesis was not affected in the experimental groups, as well as in the control group, after vaccination and challenge.

As reported by other investigators (4), the parameters of cell-mediated immunity measured in this study indicate that this host immune response is not affected adversely by dietary limitations involving essential amino acid. Although the total numbers of spleen cells may in fact have been lower in three experimental groups, the percentages of T lymphocytes present in the spleen cell populations or the T lymphocyte responsiveness was greater in mice from two of these dietary groups (isoleucine- and lysine-limited mice). Khorshidi and Mohagheghpour (14) have shown that an increased PHA responsiveness of spleen cells from protein-malnourished mice was due to a decrease in the number of suppressor lymphocytes in the spleen cell population.

The resistance to *L. monocytogenes* is a cell-mediated immune function. The ability of a host to clear *L. monocytogenes* starts by day 3 and is fully developed by day 5 after infection (3). The preimmune (before day 3 after infection) macrophage phagocytic and bactericidal activities have been associated with the early resistance of mice to *L. monocytogenes* infections (29). No experimental group exhibited higher *L. monocytogenes* levels in livers and spleens on days 3 and 5 after injection than the control group, indicating that the cell-mediated immune function was not impaired. On day 7 after injection, although death occurred in experimental groups due to acute infection, the surviving mice from the experimental and control groups demonstrated clearance of *L. monocytogenes*, except

the leucine-limited group, which exhibited a significantly higher clearance. However, the deaths on day 7 in the experimental groups indicate that the cell-mediated immune responses in mice fed the experimental diets might not have reached as high a level as the level in the control group.

The increased susceptibilities of experimental groups of mice to *S. typhimurium* infection were not due to the impairment of one specific host defense mechanism resulting from the dietary limitations. The levels of transferrin, C3 in serum after immunization, early humoral immune response, and peritoneal clearance were affected adversely by certain dietary limitations. As measured by (i) spleen cell responsiveness to PHA and (ii) ability to clear infecting *L. monocytogenes* cells from livers and spleens, cell-mediated immunity was not impaired in the mice fed the experimental diets.

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LITERATURE CITED

1. **Binder, H. J., and D. S. Whiting.** 1977. Inhibition of small intestinal sugar and amino acid transport by the enterotoxin of *Shigella dysenteriae*. Infect. Immun. **16**: 510–512.
2. **Blanden, R. V., G. B. Mackaness, and F. M. Collins.** 1966. Mechanism of acquired resistance in mouse typhoid. J. Exp. Med. **124**:585–600.
3. **Cheers, C., I. F. C. McKenzie, H. Pavlov, C. Ward, and J. York.** 1978. Resistance and susceptibility of mice to bacterial infection: course of listeriosis in resistant or susceptible mice. Infect. Immun. **19**:763–770.
4. **Cooper, W. C., R. A. Good, and T. Mariani.** 1974. Effects of protein insufficiency on immune responsiveness. Am. J. Clin. Nutr. **27**:647–664.
5. **Dubos, R. J., and R. W. Schaedler.** 1958. Effect of dietary proteins and amino acids on the susceptibility of mice to bacterial infections. J. Exp. Med. **108**:69–81.
6. **Friedman, R. L., and R. J. Moon.** 1977. Hepatic clearance of *Salmonella typhimurium* in silica-treated mice. Infect. Immun. **16**:1006–1012.
7. **Ghadimi, H., S. Kamar, and T. Abaci.** 1973. Endogenous amino acid loss and its significance in infantile diarrhea. Pediatr. Res. **7**:161–165.
8. **Herzberg, M., P. Nash, and S. Hiho.** 1972. Degree of immunity induced by killed vaccines to experimental salmonellosis in mice. Infect. Immun. **5**:83–90.
9. **Hochadel, J. F., and K. F. Keller.** 1977. Protective effects of passively transferred immune T or B lymphocytes in mice infected with *Salmonella typhimurium*. J. Infect. Dis. **135**:813–823.
10. **Janossy, G., and M. F. Greaves.** 1971. Lymphocyte activation. I. Response of T and B lymphocytes to phytomitogens. Clin. Exp. Immunol. **9**:483–488.
11. **John, A., and J. M. Bell.** 1976. Amino acid requirements of the growing mouse. J. Nutr. **106**:1361–1367.
12. **Johnston, R. B.** 1977. Biology of the complement system with particular reference to host defense against infection—a review, p. 295–307. *In* R. M. Siskind (ed), Malnutrition and the immune response. Raven Press, New York.
13. **Kenny, K., and M. Herzberg.** 1968. Antibody response and protection induced by immunization with smooth and rough strains in experimental salmonellosis. J. Bacteriol. **95**:406–411.
14. **Khorshidi, M., and N. Mohagheghpour.** 1979. Effects of protein deficiency on suppressor cells. Infect. Immun. **24**:770–773.
15. **Kvach, J. T., T. I. Wiles, M. W. Mellencamp, and I. Kochan.** 1977. Use of transferrin-iron-enterobactin complexes as the source of iron by serum exposed bacteria. Infect. Immun. **18**:439–445.
16. **Mancini, G., A. O. Carbonara, and J. F. Heremans.** 1965. Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry **2**:235–240.
17. **Moore, R. N., B. A. Johnson, and L. J. Berry.** 1977. Nutritional effects of salmonellosis in mice. Am. J. Clin. Nutr. **30**:1289–1293.
18. **Newberne, P. M., C. E. Hunt, and V. R. Young.** 1968. The role of diet and the reticuloendothelial system in the response of rats to *Salmonella typhimurium* infection. Br. J. Exp. Pathol. **49**:448–457.
19. **Petro, T. M., and J. K. Bhattacharjee.** 1980. Effect of dietary essential amino acid limitations upon native levels of murine serum immunoglobulins, transferrin, and complement. Infect. Immun. **27**:513–518.
20. **Reinhardt, M. C., and M. W. Steward.** 1979. Antibody affinity and clearance function studies in high and low antibody affinity mice. The effect of protein deficiency. Immunology **38**:735–739.
21. **Robertson, E. C., and M. E. Doyle.** 1936. Higher resistance of rats fed casein than those fed vegetable proteins. Proc. Soc. Exp. Biol. Med. **35**:374–376.
22. **Scrimshaw, N. S., C. E. Taylor, and J. E. Gordon.** 1968. Interactions of nutrition and infection. W.H.O. Monogr. Ser. **57**:3–329.
23. **Sriramachari, S., and C. Goplan.** 1958. Nutrition and tuberculosis. III. Effect of some nutritional factors on resistance to tuberculosis. Indian J. Med. Res. **46**:105–110.
24. **Taliaferro, W. H., R. L. Woolridge, and E. P. Berditt.** 1949. Dietary protein and resistance to *Trichinella spiralis* infection. Science **109**:443.
25. **Venneman, M. R., and L. J. Berry.** 1971. Cell-mediated resistance induced with immunogenic preparations of *Salmonella typhimurium*. Infect. Immun. **4**:381–387.
26. **Wannemacher, R. W.** 1980. Key role of various individual amino acids in host response to infection. Am. J. Clin. Nutr. **30**:1268–1280.
27. **Watson, M.** 1937. Studies on influence of diet on resistance to infection. II. The effect of various diets on the resistance of mice to bacterial infection. J. Hyg. **37**:420–425.
28. **Wissler, R. W.** 1947. The effect of protein depletion and subsequent immunization upon the response of animals to pneumococcal infection. II. Experiments with male albino rats. J. Infect. Dis. **80**:264–277.
29. **Zinkernagel, R. M., and R. V. Blanden.** 1975. Macrophage activation in mice lacking thymus derived (T) cells. Experentia **31**:591–593.