Influence of Dietary Protein Type on the Immune System of Mice[1]

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ABSTRACT The effect of graded amounts of dietary lactalbumin (L), casein (C), soy (S), wheat (W) protein and Purina rodent chow (stock diet) on the immune responsiveness of C3H/HeN mice has been investigated by measuring the specific humoral immune response to sheep red blood cells (SRBC), and horse red blood cells (HRBC) as well as the nonspecific splenic cell responsiveness to phytohemagglutinin (PHA) and concanavalin A (Con A) after stimulation with *Mycobacterium bovis*, strain BCG. The nutritional efficiency of these diets was normal and similar. The immune response of mice fed the L diets, was found to be almost five times higher than that of mice fed the corresponding C diets. The humoral immune response of mice fed C, S, and W diets was substantially lower than that of mice fed stock diet, whereas that of mice fed L diet was higher. The above-described immune effect of all tested proteins was obtained at 20 g/100 g concentration with no further increments with 30- and 40 g/100 g protein in the diet. Mitogen responsiveness to PHA and Con A in L diet–fed mice was only slightly higher than that of C diet–fed mice. Little difference in immune responses was noted among mice fed C, S or W protein diets. The principal factor responsible for the observed immune effect does not appear to be the availability or concentration of single essential amino acids but rather the composite effect of the specific amino acid distribution in the protein. J. Nutr. *113:* 1415–1421, 1983.

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Much has been written about the effects of protein concentration in diet, on host defense mechanisms (1–4), but little is known about the effects of the type of dietary protein on the immune system and its responses. Our interest in the effect of dietary amino acid on immune responsiveness, was prompted by the observation (5) that lowering the concentration of phenylalanine (Phe) in a casein equivalent amino acid mixture, to the level present in lactalbumin, produced a 100% increase in the plaque-forming cells' (PFC) response to sheep red blood cells (SRBC). Moreover, we have shown recently that mice fed a 28 g/100 g lactalbumin hydrolyzate diet exhibit a 4.7-fold increase in PFC response to SRBC in comparison with mice fed a nutritionally equivalent 28 g/100 g casein hydrolyzate diet (6).

Reported here are the results of a comparative study on the effects of stock diet (Purina rodent chow, Ralston Purina Co., St. Louis, MO) and of defined formula diets (DFD) containing four naturally occurring purified proteins at a 20, 30, or 40 g/100 g concentration, on the immune responsiveness of mice. An attempt was made to evaluate the relative role played by certain single es-

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sential amino acids in relation to the difference in immune response noted in mice fed casein as compared to lactalbumin.

MATERIAL AND METHODS

Mice. Male C3H/HeN mice were purchased from Canadian Breeders, Montreal, Canada, at 6 weeks of age.

Dietary treatment. A detailed composition of the defined formula diets (4.3 kcal/g) is given in table 1. The diets contained 20, 30, or 40 g/100 g of lactalbumin (L), casein (C), soy (S) or wheat (W), vitamin-free protein. All diets contained the same amount of fat, minerals and vitamins: the amount of protein varied from 20 to 40 g/100 g and the carbohydrate fraction was adjusted accordingly. These diets will be designated L diet, C diet, S diet or W diet. In some experiments 0.19 g of valine (Val), 0.28 g of Phe, 0.35 g of tyrosine (Tyr), 0.32 g of histidine (His) or 0.16 g of arginine (Arg) per 100 g diet was added to the 20 g/100 g L diet to bring the concentration of each of these amino acids in the L diet to the level present in the corresponding C diet. These diets will be respectively designated L + Val, L + Phe, L + Tyr, L + His and L + Arg. In other experiments 0.16 g of isoleucine (Ile), 0.15 g of leucine (Leu) 0.21 g of cystine (Cys), 0.51 g of threonine (Thr) or 0.15 g of lysine (Lys) per 100 g diet was added to the 20 g/100 g C diet to bring the concentration of each of these amino acids in the C diet to the level present in the corresponding L diet. These diets will be respectively designated C + Ile, C + Leu, C + Cys, C + Thr and C + Lys. In some experiments either 0.25 or 0.50 g of Cys per 100 g diet was added to the 20 g/100 g L diet and 0.50 g of Cys was added to the 20 g/100 g C diet; these diets will be designated high Cys L and high Cys C diets, respectively.

Other animals were fed a commercial laboratory diet (estimated 23% protein). This diet is designated P. In a final experiment 12 g/100 g of either L, C, S or W were added to the stock diet. These diets are designated P + L, P + C, P + S, P + W, respectively.

Diets refrigerated between feeding were given thrice weekly. They were continuously available in powder form in stainless-steel feeders specially designed to avoid spillage and spoilage. Drinking water was allowed ad libitum. The mice, housed in wire-bottomed cages to prevent coprophagy, were placed on the various diets at 6–8 weeks of age, and immunological studies commenced 2 weeks later. Dietary treatment was continued

TABLE 1

Amino acid composition of test diets[1,2]

Amino acid	20 g/100 g lactalbumin	20 g/100 g casein	20 g/100 g soy protein	20 g/100 g wheat protein
	g/100 g diet			
Isoleucine	1.01	0.85	0.80	0.66
Leucine	1.65	1.50	1.33	1.22
Valine	0.88	1.07	0.85	0.78
Methionine	0.35	0.42	0.20	0.27
Cystine	0.26	0.05	0.05	0.15
Phenylalanine	0.54	0.82	0.85	0.88
Tyrosine	0.50	0.85	0.66	0.53
Threonine	1.12	0.61	0.70	0.41
Tryptophan	0.22	0.19	0.24	
Lysine	1.42	1.27	1.12	0.48
Histidine	0.27	0.59	0.46	0.36
Arginine	0.40	0.56	1.27	0.53
Glycine	0.32	0.32	0.75	0.61
Serine	0.85	0.90	0.88	0.65
Alanine	0.74	0.56	0.75	0.49
Proline	0.93	1.65	0.93	2.11
Aspartic acid	1.58	1.38	2.01	0.56
Glutamic acid	3.23	3.50	3.26	5.90

[1] The amino acid content of the 20% protein diets provides approximately 17% amino acid in the diet. All diets contained in addition 18% corn oil, 2.8% salt mixture, 0.33% vitamin mixture and 2% fiber. The 20% protein diets were then made to 100 g by addition of 57% carbohydrate in the form of partially hydrolyzed cornstarch. The 30 and 40% protein diets contained the same proportion of amino acids listed above with the protein content increased to 30 and 40 g/100 g diet, respectively. Carbohydrate was reduced accordingly. The presence of lactose, ash and moisture in lactalbumin (20 g/100 g) and ash and moisture in casein, soy and wheat protein (15 g/100 g) was taken into consideration. [2] The vitamin mixture provided in milligrams per 100 g diet: ascorbic acid, 31.5; niacin, 5.04; riboflavin, 0.38; thiamin, 0.32; folic acid 0.063; vitamin B-6, 0.25; biotin, 0.032; pantothenic acid, 1.9; choline, 53.2 and per 100 g diet: vitamin A, 1007 IU; vitamin D, 253 IU; vitamin E, 6.3 IU; vitamin B-12, 1.26 μg; and phylloquinone, 63 μg. The mineral content of ions or cations (expressed in milligrams per 100 g diet) and the actual chemical compounds fed were: Ca, 378 ($CaHPO_4 \cdot 2H_2O$ and $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$); P, 208 ($K_2HPO_4 \cdot 2H_2O$); Fe, 7.7 ($FeSO_4 \cdot 2H_2O$); Mg, 44 (MgO); Cu, 0.38 ($CuSO_4 \cdot 5H_2O$); Zn, 2.5 ($ZnSO_4 \cdot 7H_2O$); Mn, 0.63 ($MnSO_4$); Cl, 840 ($C_5H_{14}ClNO$); K, 1050 ($K_2HPO_4 \cdot 2H_2O$); Na, 245 (NaCl).

throughout the experiment. Each dietary group comprised 10 mice.

PFC assay. The method used for assaying IgM PFC was essentially the one described by Cunningham and Szenberg (7) with certain minor modifications (5). The mice were injected intravenously (i.v.) with 5×10^6 SRBC and assayed for PFC on day 5 when the response was shown to peak (5). In other experiments mice were injected i.v. with 5×10^8 SRBC or horse red blood cells (HRBC) and assayed for PFC on day 4.

Mitogen responses. The test of mitogenic responses to phytohemagglutinin (PHA) and concanavalin A (Con A) were performed by using the method described by Lapp et al. (8). Several concentrations of the mitogens were used, and the results obtained with the optimum concentrations have been reported here.

BCG treatment. Mice were inoculated intraperitoneally (i.p.) with 6×10^6 of colony forming units (CFU) of living *Mycobacterium bovis*, strain BCG (TMC #1029, Phipps strain, Trudeau Institute, Saranac Lake, NY). The BCG was given 2 weeks after commencement of dietary treatment and 1 week before killing the animals.

Statistical analysis. Statistical evaluation of differences between groups was done by Student's *t*-test and by analysis of variance (*F*-test).

RESULTS

Nutritional data. In table 2, data are presented on the nutritional efficiency of the different diets. Mice fed the 20 and 30% protein diets and stock diet increased in body weight by approximately the same amount (table 2) with similar food consumption ranging from 3.4 to 3.6 g/24 hours. No significant differences were observed between these dietary groups in serum protein values and white cell counts (data not shown). At the 20 and 30% protein level, the spleen weights of all mice fed the L diets were higher than those of mice fed the other defined formula diets. No significant difference was noted in relative spleen weight between C, S and W

TABLE 2

Effect of 3 weeks dietary regimen on body growth and spleen weight of 6-week-old C3H male mice[1,2]

Protein		Initial weight	Final weight[3]	Spleen weight:body weight ratio	
				Unimmunized	Immunized[4]
g/100 g		*g*	*%*		
20	Lactalbumin	18.8 ± 0.4	122.1 ± 1.0	49.0 ± 0.8	59.4 ± 1.8
	Casein	19.4 ± 0.3	122.0 ± 1.4	39.4 ± 3.7	50.0 ± 2.3
	Soy protein	19.1 ± 0.4	123.0 ± 1.8	38.5 ± 1.7	47.6 ± 1.2
	Wheat protein	19.2 ± 0.5	122.0 ± 1.4	40.9 ± 1.4	48.9 ± 1.7
30	Lactalbumin	18.5 ± 0.3	124.0 ± 2.4	50.1 ± 1.8	58.1 ± 1.1
	Casein	19.0 ± 0.4	125.1 ± 2.1	40.0 ± 0.5	45.9 ± 2.3
	Soy protein	18.5 ± 0.3	121.3 ± 2.7	42.6 ± 1.2	48.6 ± 2.2
	Wheat protein	18.9 ± 0.3	124.9 ± 2.0	42.0 ± 2.1	45.1 ± 1.5
40	Lactalbumin	19.1 ± 0.4	113.0 ± 3.0	35.4 ± 4.7	44.9 ± 1.3
	Casein	19.3 ± 0.2	117.6 ± 1.2	34.5 ± 0.8	39.3 ± 2.0
	Soy protein	19.9 ± 0.5	107.4 ± 3.1	44.1 ± 1.6	48.8 ± 3.6
	Wheat protein	20.0 ± 0.5	116.4 ± 2.4	37.2 ± 0.9	45.9 ± 1.9
	Stock diet[5]	19.7 ± 0.6	121.0 ± 1.8	43.9 ± 1.9	55.8 ± 2.2

[1] Means ± SEM; $n = 10$ mice. [2] Spleen weight:body weight ratio of L-fed groups vs. corresponding C-fed groups at the 20 and 30 g/100 g level: $P < 0.025$ or less by Student's *t*-test. Spleen weight:body weight ratio of the 20 g/100 g L and 20 g/100 g C groups vs. the corresponding 40 g/100 g L and 40 g/100 g C groups: $P < 0.025$ or less by Student's *t*-test. [3] Percentage of initial weight. [4] Five days after i.v. immunization of mice with 5×10^6 sheep red blood cells. [5] Ralston Purina Co., St. Louis, MO: estimated 23 g/100 g protein.

diet-fed mice. With all diets at the 40% protein level, growth was slightly impaired and spleen weight of L and C diet-fed mice was lower than that of mice fed the corresponding 20 and 30% diets.

Humoral immune response. The number of PFC per spleen 5 days after i.v. inoculation with 5×10^6 SRBC was in the L diet groups nearly five times greater than the corresponding values for mice fed the C diets. The values for the latter groups were approximately 35 and 50% higher than the corresponding values observed in the S diet and W diet groups (fig. 1). The pattern of immune responses in relation to protein type was strikingly similar at 20, 30 and 40 g/100 g protein level in diet (data not shown). This impressive enhancement of the PFC response cannot be ascribed to presensitization of the L diet–fed group with cross-reacting antigens present in L because only low numbers of PFC per spleen ($0.4–0.6 \times 10^{-3}$) were found in nonimmunized mice and, moreover, these did not differ between the dietary groups. A similar effect of protein type in diet was observed following immunization with 5×10^8 SRBC or 5×10^8 HRBC (fig. 1). The number of PFC per spleen in response to 5×10^6 SRBC in L diet–fed mice was found to be 154% of that noted in their stock diet–fed counterparts. Conversely, the PFC to SRBC produced by mice fed the C, S and W diets were respectively only 33, 21 and 17% of the corresponding value of stock diet–fed mice. The addition of 12 g/100 g of either L, C, S or W to stock diet failed to influence the humoral immune response (fig. 1).

Mitogen responses. In mice not subjected to an immunogenic stimulus, no difference was seen between the various dietary groups in spleen cell mitogen responses. When the mice are stimulated with BCG 1 week prior to death, it is found in the PHA and Con A stimulated groups, that mice fed the L diets exhibit moderately higher mitogen responses than mice fed the corresponding C, S and W diets (fig. 2). The response to PHA is higher in the C diet–fed mice than in the corresponding S diet–fed mice. No substantial difference is seen between the C diet groups

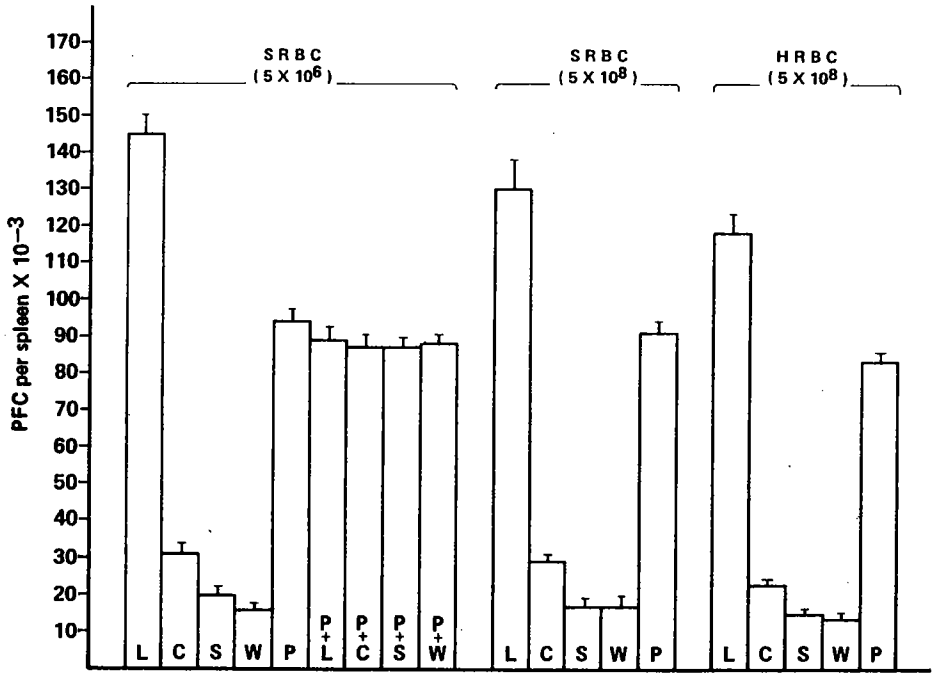

Fig. 1 Number of plaque-forming cells (PFC) per spleen after immunization with sheep red blood cells (SRBC) or horse red blood cells (HRBC); effect of 2 weeks of dietary treatment with 20 g/100 g of either lactalbumin (L), casein (C), soy (S) or wheat (W) protein in diet, or Purina stock diet (P) or P + 12 g/100 g of either L, C, S, W. Each value represents the mean + SEM, $n = 10$ mice. By Student's t-test, the effect of the type of protein is: L vs. C, C vs. S, L vs. P and P vs. C: $P < 0.01$ or less.

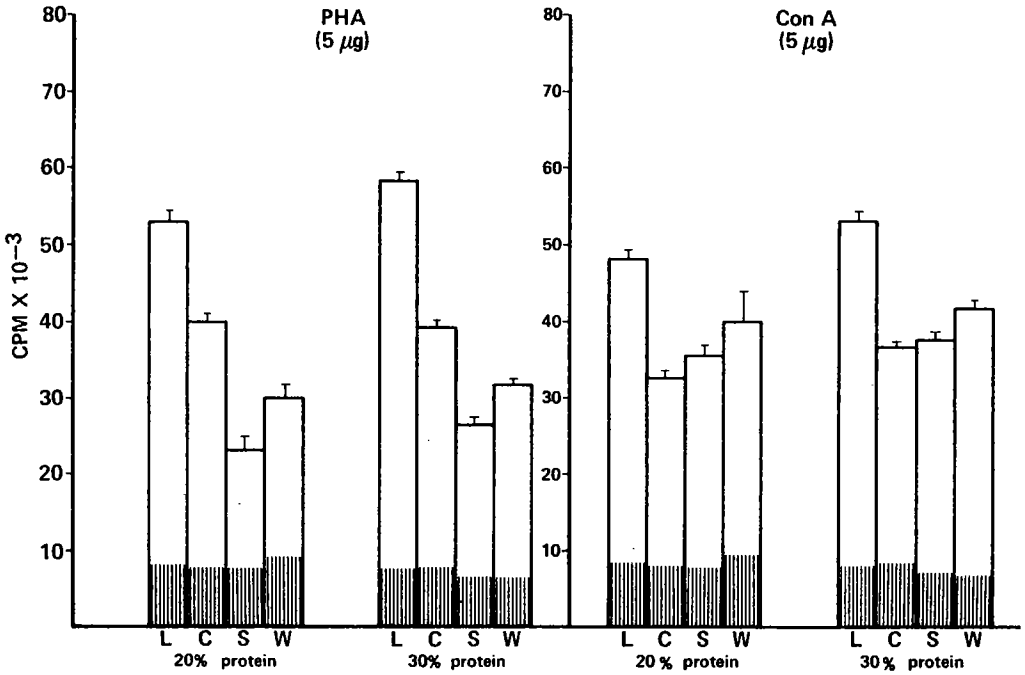

Fig. 2 Effect of 2 weeks of dietary treatment with either lactalbumin (L), casein (C), soy protein (S) or wheat protein (W) diets on the spleen cell mitogen responses of BCG-stimulated mice. Hatched area indicates background (no mitogen) values. Each value represents the mean ± SEM; n = 10 mice. By the two-way analysis of variance (F-test), the effect of the concentration of protein is not significant. The effect of the type of protein is: L vs. C for PHA and Con A responses: $P < 0.005$; C vs. S for PHA responses: $P < 0.005$.

and the S and W diet groups in their responses to Con A. Strikingly similar results were obtained in mice fed 20 and 30 g/100 g protein in diet.

Effect of individual amino acids. Our data (fig. 3) show a 30% drop in the splenic PFC response to SRBC when the Phe level in the L diet is raised to that present in the C diet. The addition of either Val or Tyr or His or Arg to the L diet, on the other hand, failed to influence the immune response. No significant effect on splenic PFC response to SRBC was noted when the level of either Ile, or Leu, or Thr, or Lys in the C diet was raised to that present in the L diet. However, a 25% increase in the PFC response was noted when the Cys level in the C diet was raised to that present in the L diet. Mice fed the two high Cys L diets exhibited almost identical splenic PFC responses to SRBC (145 ± 6.1 and 146 ± 5: both values are similar to the value of 144 ± 6.6 noted in control L diet–fed mice). The response of mice fed the high Cys C diet was also similar to that of mice fed the C + Cys diet (data not shown).

DISCUSSION

Our nutritional studies show that the various 20 g/100 g protein formula diets and stock diet all sustain normal growth of mice and that the 30 g/100 g diets, with higher amino acid content, do not enhance body growth beyond that of the 20 g/100 g diets. On the other hand, the 40 g/100 g protein diets appear to be slightly deficient (table 2).

In mice not challenged with an immunogenic stimulus, diet alone was found to have little or no effect on a variety of parameters examined. Thus, body growth, serum protein, circulating leukocyte number and the spleen cell mitogen responses to Con A, PHA (data not shown) were all within normal limits. The only difference noted was a higher spleen weight: body weight ratio in the L diet–fed mice (table 2).

After challenging mice with an immune stimulus and measuring either the specific humoral immune response to SRBC (fig. 1) or the nonspecific splenic cell responsiveness to mitogens after stimulation with BCG (fig.

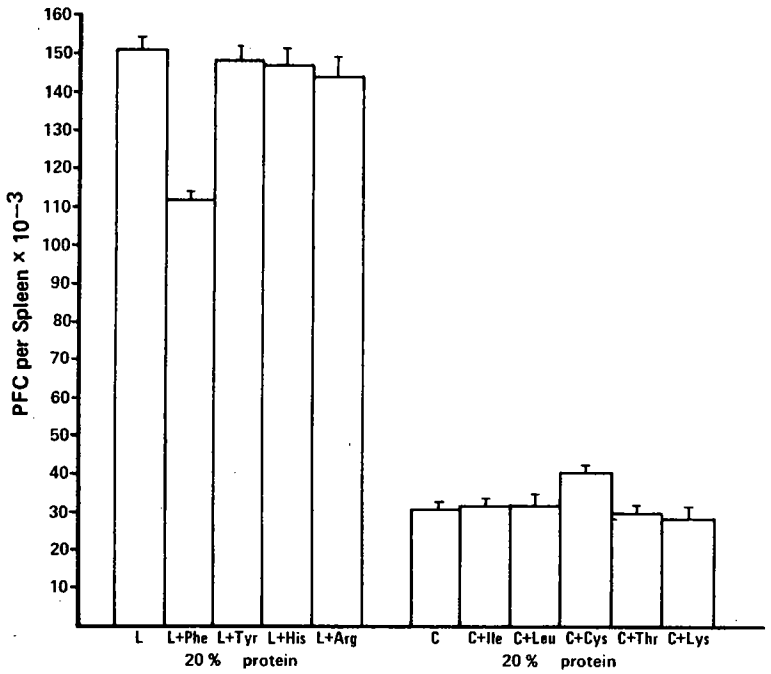

Fig. 3 Effect of 2 weeks of dietary treatment on the number of plaque-forming cells per spleen 5 days after immunization with 5×10^6 sheep red blood cells. Role of individual amino acid. Each value represents the mean ± SEM; $n = 10$ mice. L diet vs. L + Phe diet: $P < 0.005$. C diet vs. C + Cys diet: $P < 0.05$ by Student's t-test.

2), it was observed first of all that the responses of the mice fed the L diets were consistently greater than those of the mice fed the corresponding C, S and W diets. These differences were similar at 20, 30 and 40 g/100 g protein level in diet. No substantial enhancement of humoral immune responses was noted in all dietary groups when the protein level was raised from 20 to 30 and 40 g/100 g. A similar pattern of immune response was noted in mice fed 20 g/100 g protein diets and challenged with a different antigen such as HRBC (fig. 1).

Previous experiments have shown that the spleen cell PFC responses to SRBC were greatly increased when the content of L hydrolyzate in diet was raised from 12 to 28 g/100 g (6). The current experiments indicate that a maximum in the intensity of immune responsiveness of the L diet–fed mice is apparently reached around the 20 g protein per 100 g level in diet. In the C diet–fed groups the maximum immune response is probably reached at or below 12 g/100 g protein in diet (6).

Major differences in immune responsiveness are noted between L diet–fed mice, on the one hand, and mice fed the other types of proteins, with little or no differences between the C, S, and W diet–fed groups. The reason for the divergent immune effect of L and C proteins is a matter of speculation. The relative concentration of some essential amino acids is higher, similar to, or lower in L than in C and vice versa (table 1). Despite a slight drop in immune reactivity, the abolition of the difference in Phe level between L and C diets, fails to lower the immune response of mice fed L + Phe to the level of mice fed C diets (fig. 3). Similarly the addition of Cys to casein produces only a moderate increment in the immune response of the spleen cells. The experiments in which the differences in concentration of individual essential amino acid between L and C diets were abolished, clearly indicate that the specific immune effect of the protein moiety is not related to the concentration or availability of one particular amino acid, but rather to the composite effect of the specific amino acid distribution in protein. This assumption is strengthened by the fact that the pattern of humoral immune responses, with different protein diets, is similar at the 20, 30 and 40

g/100 g protein level and by the lack of any significant effect on humoral immunity with the addition of either L, C, S or W to the stock diet. The substantial drop in humoral immune response noted when mice are switched from stock diet to DFD's containing either C, S or W protein is intriguing.

This effect does not appear to be related to unidentified immune-depressive material present in the purified proteins since the addition of a substantial amount of either C, S or W failed to lower the immune response in mice fed stock diet (fig. 1). It is possible that differential protein-dependent changes in gut-associated microflora may influence the immune responses of mice while not affecting their growth and general nutritional status. Decreases in some specific strains of enteric bacteria have been reported in humans following the use of elemental DFD's (9–11). In view of the immunological interactions between the host and its intestinal microbes. (12–14), it is conceivable that dietary protein type may influence systemic immunity by altering the relative composition of the intestinal microflora.

Although the mechanism underlying the effect of L on immune responsiveness remains totally unknown, the practical importance of these findings is self-evident.

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