

QUANTITATIVE EFFECTS OF NUTRITIONAL ESSENTIAL  
AMINO ACID DEFICIENCY UPON IMMUNE  
RESPONSES TO TUMORS IN MICE\*

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Protein-calorie malnutrition may produce profound and sometimes paradoxical changes in the immune defense mechanisms against infection and malignancy.<sup>1</sup> Depression of host resistance to pyogenic and intracellular bacterial infection (1) and increased resistance to some viral infections (2, 3) and malignant tumors<sup>1</sup> (4, 5) have been reported in nutritionally deprived animals. Our previous studies have demonstrated that animals fed limiting amounts of a casein diet showed intact cytotoxic cell-mediated immune responses to tumor antigens at a protein intake that resulted in profound depression of specific humoral antibody responses, including serum "blocking antibody" (6).<sup>1</sup> These findings suggested that specific cell-mediated cytotoxic immunity may operate more effectively against tumor cells in the moderately protein-deficient animal, because of the absence of serum inhibiting factors. Further reduction in the level of protein in the diets of tumor-bearing animals resulted in depression of both humoral and cellular responses. In addition, a persistent defect in cytotoxic cell-mediated function was found in animals after nutritional protein deprivation at a young age.<sup>2</sup> Thus the animal's immune resistance could be either increased or depressed, depending on the timing and the severity of the nutritional deprivation.

Similar inhibitory effects upon the incidence and growth of malignant tumors have been reported in animals fed diets imbalanced or deficient in the essential amino acids (7-11). Ten amino acids have been found essential for normal growth of mice (9, 12, 13). The mouse does not require arginine and can synthesize cystine from methionine (9). The requirement for tyrosine is influenced by the phenylalanine intake. At least tryptophane, phenylalanine, and methionine have been found essential for specific antibody responses (14, 15), but the amino acid requirements for complete humoral and cellular immune responses in the mouse have not been fully defined.

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<sup>2</sup> Jose, D. G., and R. A. Good. 1972. Manuscript in preparation.

Limitation of casein in the diet inhibits first the humoral antibody response and, at a lower dietary level, the cytotoxic cell-mediated response.<sup>1</sup> Casein diets become limiting first in the amino acids cystine and methionine and, on further reduction of the intake, in tryptophane. Thus the effects on the immune response observed in earlier studies after quantitative reduction of casein in the diet might be a manifestation of limiting intakes of one or more essential amino acids.<sup>1</sup>

The purpose of the present study was to measure the effect of different degrees of deficiency of essential and semiessential amino acids upon specific immune responses to allogenic tumor cells in mice. Synthetic amino acid diets were prepared from purified amino acids mixed in concentrations approximately equivalent to a casein diet containing 15% protein-calories (9). The effect of deficiency of a single amino acid was tested by feeding a series of diets in which the selected amino acid was reduced to 50, 25, or 10% of its concentration in the standard amino acid diet. Primary humoral and cellular immune responses to allogenic tumor cells were assessed, by in vitro assay, in groups of mice fed the various diets.

#### *Materials and Methods*

*Animals.*—Male C3Hf/Umc low pathogen mice were obtained from the University of Minnesota colony at 5 wk of age. A spontaneous DBA/2 mammary adenocarcinoma was maintained in serial passage in syngenic male animals by subcutaneous inoculation and was used as test material on the sixth and seventh passages.

*Diets.*—Purified amino acids (Nutritional Biochemicals Corp., Cleveland, Ohio) were mixed in the approximate composition of a 15% casein ( $N = 1.8\%$ ) diet (9). The detailed constituents of this standard amino acid diet are shown on the following page. Additional diets were prepared according to the same formula, but with each essential amino acid selectively reduced in concentration to approximately 50, 25, or 10% of the standard amino acid diet. Groups of C3H mice were fed diets containing four different levels of each essential amino acid in turn and were compared with groups of mice fed casein diets containing 28, 15, or 6% protein-calories. Animals were placed on the various diets ad lib. at 5 wk of age; they were inoculated with allogenic tumor at between 50 and 60 days of age, and their immune responses were tested 11 days later. The quantities of each diet actually consumed were measured regularly.

*Tumor Inoculation.*—DBA/2 mammary adenocarcinoma cells were established in monolayer culture in R.P.M.I.-1640 tissue-culture medium with 20% fetal calf serum. Portions of the same tumor were inoculated, by intraperitoneal injection of  $20 \times 10^6$  viable tumor cells in a single cell suspension, into C3Hf/Umc mice in various dietary groups. Spleens and sera were harvested from these mice, and from uninoculated control mice, 11 days later.

*Assay of Cellular Immunity.*—Tumor monolayers were labeled on the 4th day by the addition of tritiated thymidine, specific activity 26 Ci/mmmole, 10  $\mu$ Ci/ml final concentration (Amersham Searle Corp., Arlington Heights, Ill.). Cytotoxic cell-mediated immunity of spleen cell suspensions from inoculated mice in various dietary groups was assessed by the thymidine-washout technique as previously described.<sup>1</sup> Results were expressed as the percentage lysis of target cells by comparing the percentage decrease in radioactivity in cultures of target cells with lymphocytes at a 1:100 ratio with the radioactivity in target cells alone.

*Antibody Determinations.*—Blocking antibody activity was measured by the percent inhibition of cell-mediated lysis by sensitized lymphoid cells from normally fed mice in the presence of inactivated serum, at a 1:10 dilution, from mice in different dietary groups.

*Standard Amino Acid Diets*

	<i>g/100 g</i>
L-Phenylalanine	0.60
L-Tyrosine	0.30
L-Threonine	0.50
L-Valine	0.70
L-Tryptophane	0.15
L-Methionine	0.40
L-Cystine	0.20
L-Isoleucine	0.50
L-Leucine	0.80
L-Arginine·HCl	0.24
L-Lysine·HCl	1.12
L-Histidine·HCl	0.40
L-Alanine	0.53
L-Aspartic acid	1.23
Glycine	0.23
L-Proline	1.96
L-Serine	1.04
Monosodium L-glutamate	4.80
Corn oil	7.00
Cornstarch	35.00
Dextrose	35.00
Salt mix (USP XIV)	4.00
Vitamin mix	2.00
Inert	1.30

Hemagglutinating antibody was determined by the polyvinylpyrrolidone method (16) using DBA/2 mouse erythrocytes as indicator cells.

*Statistics.*—Statistical analysis was performed using Student's *t* test.

## RESULTS

Mice fed the full standard amino acid diet, when tested 11 days after primary inoculation with allogenic tumor cells (Table I), showed no significant differences from animals on 28% or 15% casein diets in their capacity to develop cytotoxic cell-mediated immunity, serum blocking of cellular cytotoxicity, or hemagglutinating antibody. Reduction of each essential amino acid group to approximately half its concentration in the standard amino acid diet (*A* series of diets) resulted in a 50% reduction in cellular lysis in animals fed leucine *A* diets and slight ( $P < 0.01$ ) decreases in cellular lysis in animals fed tryptophane *A* and lysine *A*. No significant changes were found in serum blocking activity or hemagglutination titer in the *A* diet animals (Table II).

In animals fed the *B* diets, only those groups deficient in leucine and tryptophane showed further decreases in cytotoxic cellular immunity. Profound depression of serum blocking activity and hemagglutinating antibody titers

TABLE I  
*Cytotoxic Cell-Mediated Immunity, Serum Blocking Activity for Cell-Mediated Lysis, and Hemagglutination Titers of Immunized C3H Mice on Various Diets*

Diets		Cell-mediated lysis		Serum	
Protein content	Total calories per mouse per day	cpm $\pm$ sd* remaining in cultures	Mean inhibition of culture	Blocking $\ddagger$	Mean log H.A. titer
			%	%	
28% casein	20	2386 $\pm$ 193	76	92	5.2
15% casein	21	1914 $\pm$ 187	81	96	4.7
6% casein	18	2574 $\pm$ 213	74	5	1.5
Full amino acid	19	2133 $\pm$ 186	79	98	5.1
Nonimmune	—	9342 $\pm$ 1036	7	0	0

\* Mean radioactivity (counts per minute) remaining in DBA/2 tumor target cell microcultures after incubation with sensitized spleen cells at a 1:100 ratio.

$\ddagger$  Mean percentage inhibition of normal immune spleen cell cytotoxicity for tumor target cells by serum from mice on various diets at a 1:10 dilution.

TABLE II  
*Cytotoxic Cell-Mediated Immunity, Serum Blocking Activity, and Mean Log Hemagglutination Titers of Immunized C3H Mice on Diets with Selective Reduction of One Amino Acid*

Diets		Cell-mediated lysis		Serum	
Limiting amino acids	Content	cpm $\pm$ sd* remaining in cultures	Mean inhibition of culture	Block-ing*	Mean log H.A. titer
	g/100 g		%	%	
Full amino acid diet		2133 $\pm$ 186	79	98	5.5
L-Phenylalanine	Ph. A (0.6%, P.4)	1822 $\pm$ 134	82	96	5.4
L-Tyrosine	T.2)				
	Ph. B (0.3%, P.2)	2991 $\pm$ 256	70	15 $\ddagger$	3.6
	T.1)				
	Ph. C (0.15%, P.1)	8531 $\pm$ 794	15 $\ddagger$	0 $\ddagger$	2.1
	T0.05)				
L-Threonine	Thr. A (0.25%)	2841 $\pm$ 271	72	87	5.1
	Thr. B (0.15%)	3011 $\pm$ 293	70	10 $\ddagger$	3.8
	Thr. C (0.05%)	9022 $\pm$ 846	10 $\ddagger$	0 $\ddagger$	2.7
L-Valine	Val. A (0.4%)	2015 $\pm$ 176	80	88	5.2
	Val. B (0.2%)	3046 $\pm$ 236	70	0 $\ddagger$	3.3
	Val. C (0.1%)	9002 $\pm$ 785	10 $\ddagger$	0 $\ddagger$	1.8
L-Tryptophane	Tryp. A (0.1%)	3184 $\pm$ 273	68	99	4.4
	Tryp. B (0.05%)	4429 $\pm$ 392	56 $\ddagger$	0 $\ddagger$	3.1
	Tryp. C (0.02%)	7176 $\pm$ 784	28 $\ddagger$	0 $\ddagger$	1.5

\* See footnote Table I.

$\ddagger$  Significantly different from standard amino acid diet ( $P < 0.01$ ).

were found in these dietary groups, the greatest depression occurring in serum from animals fed methionine-cystine B, valine B, tryptophane B, threonine B, and phenylalanine-tyrosine B diets. Except for the tryptophane diet, the pattern of immune response in animals fed these diets was similar to the pattern observed previously with limiting casein diets. Thus a dietary level was defined at which cytotoxic cellular responses remained intact while blocking antibody was depressed. Blocking activity in serum was reduced by 50% in animals fed leucine B and isoleucine B, but was not significantly changed in animals fed arginine B, lysine B, or histidine B.

Further reductions in amino acids resulted in marked depression of all three immune responses in mice fed diets deficient in phenylalanine-tyrosine, threonine, valine, tryptophane, leucine, isoleucine, and methionine-cystine. These data indicate the threshold nutritional amino acid requirements for maintaining normal cellular and humoral responses.

The C level diets of arginine, lysine, and histidine failed to produce more than partial depression of immune responses, suggesting that the minimum requirements of the mice were less than the amounts of these amino acids in the C diets or else that they are nonessential for the immune response in this strain.

The growth of animals on the A diets did not differ from that of animals fed 15% casein or standard amino acid diets. The mean body weight was reduced by 20% or more in animals fed methionine B, phenylalanine B, threonine B, valine B, and tryptophane B, or in those fed lysine C. There were only slight decreases in body weight in mice fed arginine C and histidine C. Thus diets that resulted in a depression of blocking antibody activity in serum also produced some retardation of growth.

#### DISCUSSION

Protein deficiencies in laboratory animals have consistently been shown to impair immunoglobulin synthesis (17).<sup>1</sup> Depression of specific antibody responses to heterologous red blood cells has been found in rats and mice restricted in protein (18, 19), tryptophane, phenylalanine, or methionine (14). Tryptophane-deficient rats have also been observed to have poor responses to diphtheria antigen (15). Conflicting results have been obtained after the administration of the synthetic antigen Glu<sup>52</sup>, Lys<sup>33</sup>, Tyr<sup>15</sup> to *Cebus* monkeys on methionine-deficient, methionine-excess, or methionine-supplemented diets (20).

We have previously reported evidence of profound depression of hemagglutinating, cytotoxic, and blocking antibody to allogenic and syngenic tumors in C57BL6/J and C3Hf/Umc male mice fed diets containing less than 10% casein.<sup>1</sup> Animals fed 6% casein failed to develop primary blocking antibody responses, although cytotoxic cell-mediated immunity remained intact; and these mice showed increased resistance to autochthonous mammary tumors.

A similar split inhibition of immune responses to allogenic tumor cells has been demonstrated, in this study, in mice fed diets limiting in the essential amino acids methionine-cystine, valine, threonine, phenylalanine-tyrosine, tryptophane, and isoleucine (Tables II, III). Only slight depression of immune responses was found in mice restricted in arginine, lysine, or histidine. Leucine restriction resulted in a paradoxical depression of cytotoxic cell-

TABLE III  
*Cytotoxic Cell-Mediated Immunity, Serum Blocking Activity, and Mean Log Hemagglutination Titers of Immunized C3H Mice on Diets with Selective Reduction of One Amino Acid*

Diets		Cell-mediated lysis		Serum	
Limiting amino acids	Content	cpm $\pm$ sd* remain- ing in cultures	Mean in- hibition of culture	Block- ing*	Mean log H.A. titer
	<i>g/100 g</i>		<i>%</i>	<i>%</i>	
L-Methionine	Me. A (0.3%, M.2)	2510 $\pm$ 233	75	85	4.3
L-Cystine	C.1				
	Me. B (0.2%, M.2)	2976 $\pm$ 214	70	0†	2.7
	Me. C (0.1%, M.1)	6813 $\pm$ 603	32†	0†	1.7
L-Isoleucine	Isol. A (0.3%)	2831 $\pm$ 213	72	95	4.3
	Isol. B (0.2%)	3243 $\pm$ 284	68	45†	4.2
	Isol. C (0.1%)	9964 $\pm$ 763	0†	0†	2.4
L-Leucine	Leu. A (0.30%)	6493 $\pm$ 664	35†	92	4.3
	Leu. B (0.20%)	7231 $\pm$ 602	28†	45†	3.5
	Leu. C (0.10%)	7795 $\pm$ 683	22†	0†	1.8
L-Arginine	Arg. A (0.2%)	2811 $\pm$ 177	72	95	4.2
	Arg. B (0.1%)	3168 $\pm$ 239	68	92	3.9
	Arg. C (0.05%)	3808 $\pm$ 246	62	35†	3.1
L-Lysine	Lys. A (0.8%)	3909 $\pm$ 302	61	92	5.2
	Lys. B (0.2%)	3598 $\pm$ 273	64	85	4.2
	Lys. C (0.1%)	5441 $\pm$ 384	46†	10†	3.0
L-Histidine	Hist. A (0.2%)	2491 $\pm$ 186	75	83	5.1
	Hist. B (0.1%)	3342 $\pm$ 239	67	96	4.2
	Hist. C (0.05%)	2937 $\pm$ 215	71	84	3.9

\* See footnote Table I.

† Statistically significantly different from standard amino acid diet ( $P < 0.01$ ).

mediated responses at a dietary level that left blocking antibody unaffected. Thus the immune response to allogenic tumors may be profoundly altered, both in magnitude and in the nature of the response, by nutritional deficiencies.

Limitation of the intake of essential amino acids in mice has been reported to exert different effects on the growth of malignant tumors and of the tumor-bearing host. Restriction of dietary tyrosine and phenylalanine has resulted in significantly greater growth inhibition of S91 melanoma than of the tumor-free host (11). Such growth inhibition might reflect the unique substrate re-

quirements for melanin-pigment synthesis in this particular tumor, but a similar tumor growth inhibition by phenylalanine-tyrosine-restricted diets has also occurred in mice with sarcoma 180 (10), hepatoma (8, 21) and various adenocarcinomata (7, 9). In studies (without controls) of the effect of phenylalanine-tyrosine restriction in the treatment of human patients with malignant melanoma (22, 23) and other malignant tumors (22), regression of primary tumor growth, cessation of the metastatic spread, and improvement in hematological and subjective parameters have been reported. The relative roles of limitation of essential substrates for tumor growth and of increased immune resistance to the tumor through depression of serum blocking activity, suggested in these studies, are unknown.

Similar selective inhibition of tumor growth has been shown to occur in mice fed diets deficient in valine and isoleucine (9, 10, 24), cystine (25, 26), methionine (9, 24), and threonine (9, 10). Dietary deficiencies of lysine, leucine, histidine, and arginine produced little diminution of tumor growth without profoundly depressing the host's body weight (9, 10). Hence limitation of the amino acids that selectively inhibit tumor growth with least effect on the host's body weight has been shown, in this study, to correlate closely with the amino acids that result in the characteristic splitting of the immune responses to tumors. In addition, those amino acids whose restriction depresses both tumor growth and body weight either have no demonstrable effect upon immune responses in this study or depress both cellular and humoral responses at a similar level of deprivation.

Leucine has proven the exception in these studies: its restriction has resulted in depression of cell-mediated cytotoxic immunity while serum blocking activity has remained intact (Table III). Other studies have also reported unusual effects in leucine deficiency, or in leucine loading. The growth of C57BL mouse adenocarcinoma showed a 19% increase on a moderately restricted leucine diet (9). The amino acid imbalance induced in rats by leucine loading has been corrected by isoleucine and valine (27); and in human infants leucine loads may depress plasma isoleucine and valine concentrations (28). These findings suggest that diets deficient in leucine but adequate in other amino acids may depress host immune resistance to tumors, and that diets containing excess leucine (29) may increase host resistance to tumors because of the resulting isoleucine-valine deficiency. A possible beneficial effect has been reported in human leukemia in which amino acid imbalance was induced by loading the diet with excess phenylalanine and tyrosine (30). The beneficial effect of the antileukemic drug L-asparaginase may be achieved by a similar mechanism. Specific metabolic inhibitors of other essential amino acids may allow some degree of manipulation of the immune response in man.

#### SUMMARY

Mice were fed diets deficient in a single essential amino acid, and the primary immune responses to inoculation of allogenic tumor cells was measured

by *in vitro* assay of cellular immunity. Moderate reduction of the amino acids phenylalanine-tyrosine, valine, threonine, methionine-cystine, isoleucine, and tryptophane in the diet produced profound depression of hemagglutinating and blocking antibody responses, although cytotoxic cell-mediated immunity remained intact. These diets had previously been shown to result in a selective depression of tumor growth in mice. Limitation of the amino acids arginine, histidine, and lysine in the diets gave rise to only slight depression of the immune responses. These diets had previously been shown to produce a proportional decrease in both tumor growth and host body weight. Moderate leucine restriction resulted in a paradoxical depression of cytotoxic cell-mediated immunity with little effect on serum blocking activity. Slight increases had previously been noted in the weight of tumors in mice fed leucine-restricted diets. Deficiency or imbalance of essential amino acids in the diet may produce profound depression of immune responses and apparent, marked changes in the immune resistance of the host animal to tumors.

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