

IgE Responses in Mice Fed Moderate Protein Deficient and High Protein Diets

Kenji YOSHINO^{1,*}, Kentaro SAKAI², Hiroko OKADA¹, Tohru SAKAI¹ and Shigeru YAMAMOTO¹

¹Department of Nutrition, University of Tokushima School of Medicine, Tokushima 770–8503, Japan

²Department of Environmental Science, Faculty of Human Development, University of Hiroshima Jyogakuin, Hiroshima, 732–0063, Japan

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Summary While severe protein energy malnutrition (PEM) has been known to depress several immune functions, allergies are suppressed by decreasing IgE and impairing vascular permeability and mast cell functions. To address the effect of moderate protein malnutrition without growth arrest and protein hypernutrition on type I allergy, we examined the effect of various levels of protein nutrition on allergy at humoral immunity and the regulation of Th cell function levels. Mice fed 100 g/kg (moderate protein malnutrition; MPM), 200 g/kg (normal protein nutrition; PN) and 400 g/kg (protein hypernutrition; PH) protein diets were intraperitoneally sensitized to ovalbumin (OVA) in aluminum hydroxide. Higher elevations of OVA-specific IgE and total IgE in the serum were observed in the PH group as compared to the PN group. However, OVA-specific IgE in the MPM group was not significantly different from that in the PN group, although the former appeared higher than the latter. While CD3, CD4, CD8 and B220 expressions in the splenic lymphocytes were decreased in the MPM group, B220 expressions were increased in the PH group. Splenic lymphocyte proliferative responses to OVA were augmented in the PH group and depressed in the MPM group. IFN- γ production from splenic lymphocytes was significantly decreased; however, IL-4 production was not affected significantly in the MPM group, and increased in the PH group. These findings suggest that immune functions to specific antigens in the MPM state are depressed at the cytokine level but not in terms of IgE responses. They also suggest that immune functions become Th2-predominant in the PH state, resulting in an increased risk of type I allergy.

Key Words Protein hypernutrition, moderate protein malnutrition, IgE, IL-4, ovalbumin

Allergies have been known to be associated with both genetic and environmental factors, and have increased greatly since World War II. Dietary habits and nutritional state have also changed since then, a fact that could be linked to the development of allergies (1, 2).

In nutritional science over the last two decades, intensive studies on allergies have been focused on lipid sources. It is well known that qualitative differences in fatty acids affect susceptibility to allergies in rodents and human. Specifically, the prevalence of allergies is higher in those who consume more n-6 fatty acids than n-3 line rich diets (3–5). n-6 line fatty acids are sources of arachidonate, and prostaglandin (PG) and leukotriene (LT), act as chemical mediators related to clinical symptoms in allergic patients and immune cell functions. The mediators were biosynthesized from the cascade metabolites by the actions of cyclooxygenase and lipoxygenase, respectively. The E₂ series of PG (PGE₂) enhances Th2 type and depresses Th1 type cytokine production and selectively enhances IgE production (6–8).

Protein-energy malnutrition (PEM) is a serious health problem in people suffering from starvation in

developing countries and the elderly ill. Inversely, the prevalence of allergies is higher in industrialized countries than in developing countries (9). Some investigators have inferred a causality and studied the effect of PEM on the potential for type I allergies. They have found that the level of high affinity IgE receptor I (Fc ϵ RI) expression in the mast cell surface is reduced but intracellular histamine contents are not affected. At the passive cutaneous anaphylaxis level, vascular permeability and antigen-specific IgE are lower than in the normal protein nutrition state (10–13). While PEM has been shown to have preventive effects against allergies, deficiencies of other various immune functions also occur, including atrophy of the thymus, lymph nodes, impairment of phagocytosis and reduction in the number and proliferative responses of circulating T cells, all of which increase mortality and the susceptibility to infections (14–16).

The protein content in deficient diets used in experimental animal studies varies but generally falls into a very low-level range (0 to 50 g/kg protein), which results in severe growth arrest. The relationship between allergies and moderate protein malnutrition with normal growth has not been investigated. Here, we report the effect of moderate protein malnutrition

E-mail: yoshiken0217@ybb.ne.jp

on allergies at the level of humoral immunity and T lymphocyte functions. Although it is noteworthy that excessive protein intake as well as excessive lipid intake and a high level of n-6 fatty acids have recently stood out in industrialized countries, there are few available studies on the effect of protein hypernutrition on immune functions and other biological systems. We hypothesize that protein hypernutrition skews the immune function to cause hypersensitivity in hosts, thus resulting in allergies. We also report the effect of protein hypernutrition on allergies.

MATERIALS AND METHODS

Materials. Albumin B test Wako was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Fetal bovine serum (FBS) and Antibiotic-Antimycotic were from GIBCO Life Technologies (Grand Island, NY, USA). ELISA and cell culture plates were from Greiner GmbH (Frickhausen, Germany). Glass fiber filters, cell harvester, ELISA plate reader and β -scintillator counter were obtained from Packard (Bandhagen, Sweden). Recombinant mouse IFN- γ , Streptavidin-AP, PE-conjugated anti-mouse CD3 and CD4 monoclonal antibodies (mAb), anti-mouse IFN- γ mAb, and mAbs used for determination of OVA-specific IgG, IgG₁, IgG_{2a} and IgE were from Pharmingen (San Diego, CA, USA). Anti-mouse CD3 mAb were from R&D Systems (Minneapolis, MN, USA). IL-4 ELISA kits, FITC-conjugated anti-mouse CD8, and B220 mAbs were from eBioscience (San Diego, CA, USA). Total IgE ELISA kits were from Bethyl Laboratories (Montgomery, TX, USA). TMB solution kits were from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA). ³H-thymidine was from Amersham Pharmacia Biotech (Piscataway, NJ, USA). FACSscan analyzer was from Becton Dickinson (San Jose, CA, USA). Other materials were from Sigma (St. Louis, MO, USA).

Experimental design, diets, and immunization protocol. Three-week-old female BALB/c mice purchased from Japan SLC (Shizuoka, Japan) were housed under specific pathogen-free conditions. The mice were pre-fed normal chow for 1 wk (wk -1 to wk 0), after which they were separated into three groups and fed moderate protein malnutrition (MPM; 100 g/kg), normal protein (PN; 200 g/kg), and protein hypernutrition (PH; 400 g/kg) diets *ad libitum* for 7 wk (wk 0 to wk 7). The ingredients of the experimental diets are detailed in Table 1. Diets were replaced and food intake was measured every 2 d. Body weight was measured once a week. Mice were intraperitoneally immunized with 100 μ g of OVA absorbed in aluminum hydroxide gel as an adjuvant on wk 4 and boosted with the same immunization treatment on wk 6. On wk 7, mice were sacrificed and blood was collected from the jugular vein under diethyl-ether anesthesia and spleens were resected. This experiment received approval from the Animal Research Ethics Committee at The University of Tokushima, Japan.

Determination of serum albumin concentration. Serum albumin concentration was determined by Albumin B Test Wako, based on the bromocresol green method.

Assessment of OVA specific antibody titer in serum by Enzyme Linked Immunosorbent Assay (ELISA). Ninety-six-well microplates were coated with OVA (5 μ g/mL) resolved in 50 mM carbonate buffer (pH 9.6) and were incubated overnight at 4°C. Plate wells were washed four times with PBS containing 0.05% Tween 20 (PBS-T) and were blocked with a 30 g/L bovine serum albumin (BSA) solution for 2 h at room temperature (RT). After washing four times with PBS-T, serum diluted appropriately in 1% BSA resolved in PBS-T (1% BSA/PBS-T) was added to plate wells for 2 h at RT. The serum was diluted 50,000 times for determination of specific IgG and IgG₁, and 20 times for specific IgG_{2a} and IgE. Addition of biotin anti-mouse IgG, biotin anti-mouse IgG₁, IgG_{2a}, or IgE (all: 1 μ g/mL) mAbs to plate wells for 2 h at RT was followed by washing four times with PBS-T. Streptavidin-AP, diluted 1/1000 in 1% BSA/PBS-T, was added for 1 h at RT. Plate wells were washed five times with PBS-T. Phosphatase substrate p-nitrophenyl phosphate tablets (PNPP) resolved in 10% diethanol amine buffer (pH 9.8) were added and incubated

Table 1. Composition of test diets.¹

Ingredient	Groups ²		
	MPM	PN	PH
	g/kg diet		
Crude Casein ³	119.0	238.0	475.0
Cornstarch ⁴	461.0	381.0	223.0
Sucrose ⁴	230.0	191.0	112.0
Soya bean oil	100.0	100.0	100.0
Vitamin mixture ⁵	10.0	10.0	10.0
Mineral mixture ⁶	50.0	50.0	50.0
Cellulose	20.0	20.0	20.0
	% total energy		
Protein	9.84	19.68	39.37
Carbohydrate	68.01	58.17	38.48
Lipid	22.15	22.15	22.15

¹ All were obtained from Oriental Yeast Co. Ltd. (Tokyo, Japan).

² MPM, moderate protein malnutrition; PN, normal protein nutrition; PH, hyperprotein nutrition. Respective protein contents in MPM, PN and PH diets were 100, 200, 400 g/kg diets. All diets isocaloric.

³ Crude protein was 84.3%.

⁴ Cornstarch/Sucrose rate 2 : 1.

⁵ Vitamin mixture was composed of (mg or IU/kg diet): retinol acetate, 5000 IU; ergocalciferol, 1000 IU; tocopherol acetate, 50 mg; menadione, 52 mg; thiamine chloride, 12 mg; riboflavin, 40 mg; pyridoxine-HCl, 8 mg; vitamin B₁₂, 0.005 mg; ascorbic acid, 300 mg; D-biotin, 0.2 mg; folic acid, 2 mg; calcium pantothenate, 50 mg; p-amino benzoic acid, 50 mg; niacin, 60 mg; inositol, 60 mg; choline chloride, 2000 mg.

⁶ Mineral mixture was composed of (mg/kg diet): CaHPO₄/2H₂O, 7280; KH₂PO₄, 12860; NaH₂PO₄, 4680; NaCl, 2330; Ca-lactate, 17750; Fe-citrate, 1590; MgSO₄, 3590; ZnCO₃, 55; MnSO₄/4-5 H₂O, 60; CuSO₄/5H₂O, 15; KI, 5.

until optimal color development at RT. The resulting color development was stopped with 150 mM EDTA and absorbance was read at 405 nm using an ELISA plate reader.

Assessment of Total IgE concentration in serum by ELISA. The total IgE concentration in the serum was determined using commercial kits in accordance with the recommended procedures. Serum was diluted 20 times with 1% BSA/PBS-T. Color development was initiated using a TMB-Solution kit and was stopped by 1N H₃PO₄. Absorbance was read at 450 nm.

Splenic lymphocytes proliferation assay. To obtain single splenic cells, removed spleens were mashed and filtrated through a nylon mesh. The cells were suspended in RPMI 1640 containing L-glutamate and sodium bicarbonate. The suspensions were centrifuged for 5 min at 2,000 rpm. After removing the supernatant, 157 mM NH₄Cl was added for hemolysis. After washing the cells twice using RPMI 1640, they were resuspended in RPMI 1640 supplemented with 10% FBS, 1% Antibiotic-Antimycotic and 50 μ M 2-mercaptoethanol (10% FBS/RPMI). Cell viability was confirmed by microscopic observation following 0.04% trypan blue staining. Cells (3×10^5) were added to 96-well flat-bottom microplates and stimulated with the vehicle, anti-mouse CD3mAb (2 μ g/ml) or OVA (1 mg/ml). The plates were incubated for 72 h including the time pulsed with ³H-thymidine (1 μ Ci) for the last 24 h at 37°C in humidified 5% CO₂. Splenic cells were harvested on glass-fiber filters and dried overnight. ³H-thymidine uptake was measured for 3 min using a liquid scintillation counter. Counts for proliferation response were confirmed by triplicate samples.

Cell culture and ELISA for IL-4 and IFN- γ assessment.

Single splenic cell suspensions were prepared in the same manner as described above. Cells (3×10^6) suspended in 10% FBS/RPMI were added to 24-well flat-bottomed microplates and stimulated with OVA (1 mg/ml) or vehicle (10% FBS/RPMI) for 72 h at 37°C in humidified 5%CO₂, and culture supernatants were collected afterwards.

IL-4 production was assessed using commercial ELISA kits in accordance with the recommended procedures. IFN- γ production was assessed using standard ELISA systems. Briefly, 96-well ELISA plate were coated

with anti-mouse IFN- γ mAb (2 μ g/mL) resolved in 50 mM carbonate buffer overnight at 4°C. After washing four times with PBS-T, plate wells were blocked with 3% BSA solution for 2 h at RT. After washing four times with PBS-T, supernatants from the splenocytes culture and recombinant mouse IFN- γ as the quantitative standard were added and let stand overnight. After washing four times with PBS-T, biotin anti-mouse IFN- γ mAb (1 μ g/mL) was added for 2 h. After washing 4 times with PBS-T, 1,000 times-diluted streptavidin-AP in 10% BSA/PBS-T was added for 2 h and then plate wells were washed five times with PBS-T. Color development was assessed in the same manner as OVA-specific immunoglobulin titers. Absorbance was read at 405 nm.

FACS analysis. Spleen cells (1×10^6) were doubly stained with either a combination of PE-conjugated anti-mouse CD3 and FITC-conjugated anti-mouse B220 mAbs or a combination of PE-conjugated anti-mouse CD4 and FITC-conjugated anti-mouse CD8 mAbs (all: 1 μ g/mL) for 30 min at 4°C in the shade. After washing cells with 1% BSA/PBS, fluorescent intensities on the surfaces of 1×10^4 cells were analyzed by FACSscan.

Statistical analysis. Each result was expressed as the mean $\pm$ SD. Statistical analysis was performed with a computer program, Statview ver 5.0 (Brain Power, Inc., Calabasas, CA, USA). All data were first analyzed by one-way ANOVA and then by Tukey-Kramer test for multiple comparisons among groups. A difference of $p < 0.05$ was considered significant.

RESULTS

Although total food intake and body weight gain did not differ in any of the groups, spleen weight was significantly less in the MPM group as compared to the PN and PH groups. Serum albumin concentration was significantly higher in the PH group as compared to the MPM and PN groups (Table 2).

OVA-specific IgG was significantly lower in the PH group as compared to the PN group but not different from the MPM group. IgG₁ and IgG_{2a} also did not differ among the groups. OVA-specific IgE was higher in the PH group as compared to the PN group (Fig. 1). Only the PH group showed a significantly higher total IgE

Table 2. Body weight, food consumption, serum albumin and spleen weight.¹

	Diet groups		
	MPM	PN	PH
Initial body weight (g)	17.1 $\pm$ 1.2	17.0 $\pm$ 0.5	16.3 $\pm$ 0.8
Final body weight (g)	22.7 $\pm$ 2.4	23.1 $\pm$ 1.2	22.8 $\pm$ 1.0
Weight gain (g)	5.7 $\pm$ 2.8	6.1 $\pm$ 1.4	6.6 $\pm$ 1.3
Total Food intake (g/7wk/mice)	112.0	103.6	100.8
Serum albumin (mmol/L)	5.6 $\pm$ 0.4 ^b	5.8 $\pm$ 0.2 ^b	6.5 $\pm$ 0.4 ^a
Spleen weight (mg)	208.5 $\pm$ 27.0 ^b	242.5 $\pm$ 16.3 ^a	252.6 $\pm$ 16.0 ^a

¹ Values are means $\pm$ SD, $n = 7$. Values in a row with different letters represent a significant difference ($p < 0.05$).

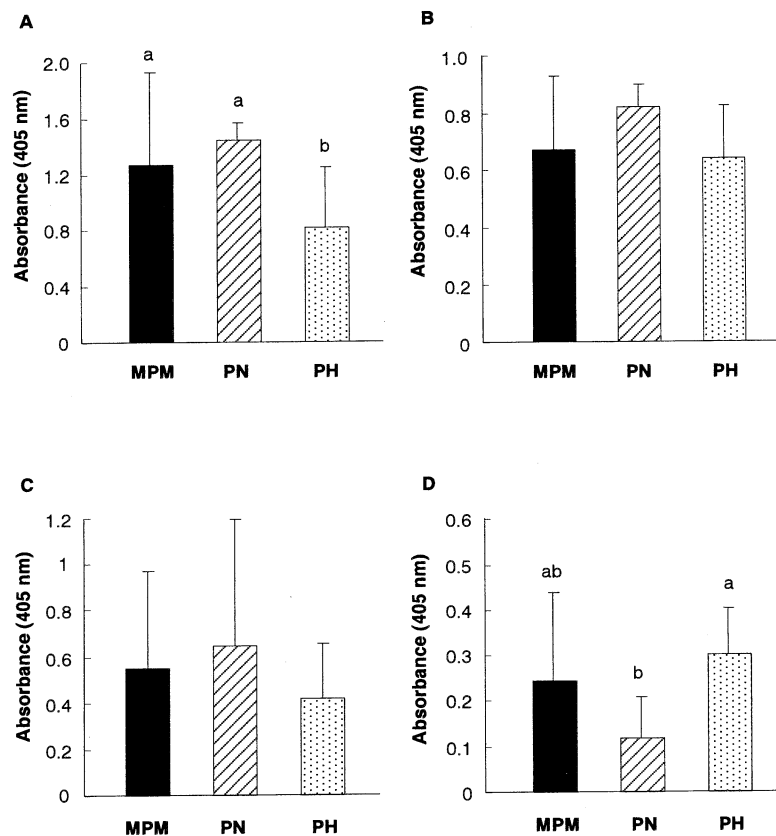


Fig. 1. Ovalbumin (OVA)-specific immunoglobulin titers in serum from OVA-sensitized mice. Mice fed individual diets for 7 wk were intraperitoneally immunized OVA (100 μ g) absorbed into aluminum hydroxide on wks 4 and 6. OVA-specific IgG (A), IgG₁ (B), IgG_{2a} (C), and IgE (D) in serum were assessed by ELISA. Data are mean $\pm$ SD, $n=7$. Different letters represent significant difference ($p<0.05$).

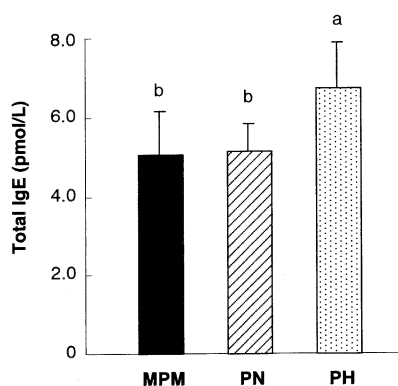


Fig. 2. Total IgE concentration in serum from OVA-sensitized mice. Total IgE concentration was determined by ELISA using the same serum as that mentioned for Fig. 1. Data are mean $\pm$ SD, $n=7$. Different letters represent significant difference ($p<0.05$).

concentration (Fig. 2).

A significant reduction of all subsets expressed in the splenic lymphocytes was observed in the MPM group as compared to the PN group. In the PH group, the B220-expressing lymphocyte population significantly increased; others increased also, but not significantly (Table 3).

The responses to OVA were augmented in the PH

Table 3. CD3, CD4, CD8, B220 expression spleens cell surfaces in individual dietary groups.¹

Expression marker	Dietary groups		
	MPM	PN	PH
	%		
CD3	18.8 $\pm$ 3.5 ^b	31.9 $\pm$ 5.2 ^a	35.4 $\pm$ 5.9 ^a
CD4	10.1 $\pm$ 2.1 ^b	17.4 $\pm$ 2.7 ^a	19.9 $\pm$ 3.4 ^a
CD8	6.2 $\pm$ 1.2 ^b	10.1 $\pm$ 1.6 ^a	8.6 $\pm$ 1.4 ^a
B220	24.2 $\pm$ 7.5 ^c	34.3 $\pm$ 5.6 ^b	43.4 $\pm$ 4.1 ^a

¹ Values are means $\pm$ SD, $n=7$. Values in a row with different letters represent a significant difference ($p<0.05$).

group but were depressed in MPM group as compared to the PN group (Fig. 3A). The response to anti-CD3 mAb was also significantly depressed in the MPM group as compared to the PN group, which was comparable to the PH group in this case (Fig. 3B). There were no significant differences in the proliferative responses among the groups when splenic lymphocytes were stimulated with the vehicle alone.

The MPM group tended to show a reduction of IL-4 production as compared to the PN group. On the contrary, the PH group showed a significant elevation in IL-4 production (Fig. 4A). IFN- γ production in the PH

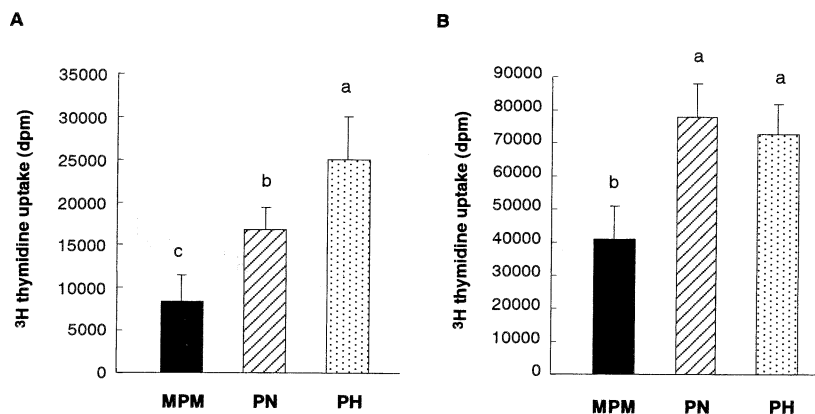


Fig. 3. In vitro splenic lymphocyte proliferative responses in OVA-sensitized mice. Splenocytes in 96-well plates were stimulated with OVA (A; 1 mg/mL) or Anti-CD3 mAb (B; 2 μ g/mL) for 48 h and further incubation for 24 h after the addition of ^{3} H thymidine (1 μ Ci). Cells were harvested on glass-fiber filters and radioactivity was counted using a β -scintillation counter. Indicated values are subtracted from background values. Count unit is dpm. Data are mean $\pm$ SD, $n=7$. Different letters represent significant difference ($p<0.05$).

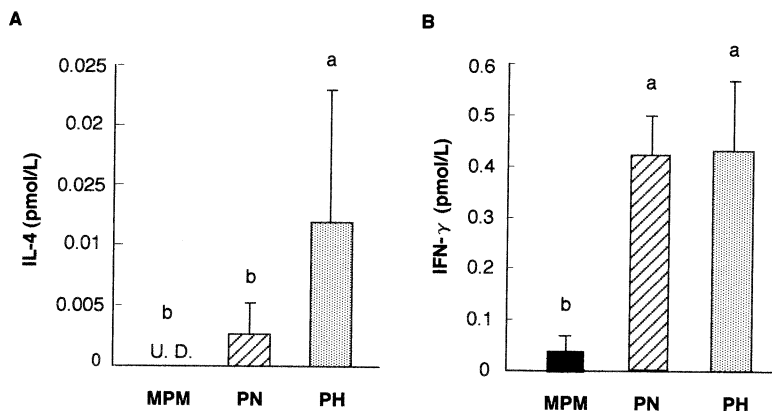


Fig. 4. IL-4 and IFN- γ production from spleen lymphocytes in OVA-sensitized mice. Spleen lymphocytes in 24-well plates were stimulated with OVA (A; 1 mg/mL) for 72 h. IL-4 and IFN- γ concentrations in the collected supernatant were measured by ELISA. Data are mean $\pm$ SD, $n=7$. Different letters represent significant difference ($p<0.05$). U.D., undetectable.

group was similar to that in the PN group, but markedly reduced in the MPM group (Fig. 4B). Spontaneous IL-4 and IFN-gamma secretion were both undetectable (data not shown).

DISCUSSION

It has been reported that mouse helper T lymphocytes could be divided into two subpopulations, Th1 lymphocytes secreting IFN-gamma and Th2 lymphocytes secreting IL-4 and IL-10 (17–19). IL-4 is a typical cytokine to regulate switching from IgM to IgE and IgG₁. IgE bound to Fc ϵ RI expresses on the surface of target cells including mast cells and basophils (20). Chemical mediators, including histamine, serotonin, PG, and LT, are released from the cell populations via signaling cascades (21, 22). On the contrary, IFN- γ suppresses Th2 lymphocyte functions and negatively regulates IgE production (23). IFN- γ and IL-4 greatly reflect on Th1 and Th2 lymphocyte functions, respectively. IgG_{2a} and IgG₁ responses are regulated by Th1 and Th2 cytokines, respectively and also reflect on their functions (18). It has been reported that Th1/Th2 lymphocyte responses shift to predominantly Th2 lymphocyte responses in allergic diseases (24).

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In the PH group, we found significant augmentation in OVA-specific IgE and total IgE but a reduction in IgG as compared to the PN group; IFN-gamma was comparable. In our findings, enhanced specific-IgE responses were accompanied by elevated IL-4 production, while IgG₁ was decreased. Switching from IgM to IgG₁ and IgE is affected by IL-4 concentration; the switch to IgE requires a higher concentration (25). It has been known that switching to IgE occurs sequentially via IgG₁ (26). It may be speculated that increased IL-4 in the PH group directs class switching to IgE rather than IgG₁. In a surface marker analysis on spleen lymphocytes, slight increases in CD3 and CD4 and significant increase in B220 were found. CD3, CD4, CD8, and B220 would be expressed on large populations of T lymphocytes, Th lymphocytes, cytotoxic T lymphocytes and B lymphocytes, respectively. While splenic lymphocyte proliferative responses to OVA were augmented, responses to anti-CD3 were not affected in the PH group as compared to the PN group. Anti-CD3 would

stimulate T lymphocytes specifically. Therefore, T lymphocyte functions themselves are retained in the PH group and it may be speculated that T lymphocyte immune functions to specific antigens were more enhanced in the PH group than in the PN group. Possibly, enhanced IgE production may be responsible for not only enhanced IL-4 production but also some functional augmentation of B lymphocytes in the PH group. Our data suggest that the PH diet predominantly induces Th2 immune responses to specific antigens, thereby enhancing IgE production. However, to determine whether the PH state affects allergic appearance or not, the latter phases of importance in onset of allergies such as mast cell functions and clinical symptoms should be clarified.

Consistent with previous reports, cytokine production from Th lymphocytes was depressed in the MPM group (27). We also evaluated OVA-specific antibody responses and spleen lymphocytes proliferative responses to specific antigens, and lymphocyte subset expressions in the MPM state. As with the severe protein malnutrition state, spleen weight, splenic lymphocytes proliferative responses to OVA, and anti-CD3 mAb were depressed; unlike severe PEM, serum albumin, and body weight (28). All surface marker expressions on splenic lymphocytes tested here were also reduced. IL-4 and IFN- γ production were also reduced. Unexpectedly, OVA-specific IgE was equivalent to or higher than the PN group. It was previously demonstrated that antigen-specific IgE was lower in severe PEM than the normal PN state (29). However, antigen-specific IgE responses in the PEM state were dependent on doses of antigen. The responses in the PEM state are comparable to that in the PN state when administered high doses of antigen, but lower when administered low doses of antigen (13). Although the former observation is consistent with our findings, intact IgE responses in the MPM state are questionable because of reduced IL-4 production. One possible explanation is that an altered Th1/Th2 functional balance that would be reflected from IFN- γ /IL-4 production changed slightly in the MPM group as compared to the PN group (30). Other possible hypotheses to the contradiction are mentioned below. PGE₂ has been known to augment IgE production synergistically with IL-4 (6, 31). It could be biosynthesized from arachidonate, a metabolite of n-6 fatty acid, which the soya bean oil used in our study contains in abundance. Dietary n-6 fatty acids enhance plasma and cutaneous PGE₂ level (32), which is also raised in kwashiorkor (33, 34). In the present study, food intake (energy intake) in the MPM group was comparable to the PN group, and the lipid contents of the diets are greater than that of diets used in other studies. Lipid nutrition may have played a key role in regulating IgE responses in the MPM group, probably in the severe PEM state. Severe protein malnutrition experiments using experimental animals are frequently associated with energy deprivation due to a considerable reduction in food intake when they feed freely. Energy restriction reduces IL-4 production and suppresses anaphylaxis and histamine

release from mast cells in allergic rodent models (35, 36), and also reduces serum IgE in humans (37). However, there is little information on the effect of protein deprivation alone on IgE production and allergies. Some discrepancies remain concerning whether lipid, protein nutrition, or energy intake has more effect on IgE responses. In the present study, although immune depressions involved in lymphocytes certainly occurred in the MPM state at the cytokine level, in splenic lymphocyte proliferative responses to specific antigens and anti-CD3 mAb, and in all subset marker expressions tested on the splenic lymphocytes here, the effects did not reach humoral immune responses, especially IgE.

In conclusion, our study presents the first findings of shifting immune functions toward Th2-predominant and enhanced IgE production in the PH state. This is a new nutritional clue to understanding the high prevalence of allergies as relation to modern food life in industrialized countries. An upper limit of protein nutrition should be determined to prevent immunological alteration and onset of allergies, taking sex, age, physiological activity, etc. into consideration. Our data also imply that an MPM state without energy deprivation does not always suppress the onset of allergies.

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