Serum Levels of Th2-Type Immunoglobulins are Increased in Weanling Mice Subjected to Acute Wasting Protein-Energy Malnutrition

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ABSTRACT

The bulk of our knowledge on immunosuppression in malnutrition comes from the experiments done on cell-mediated immunity. However, malnutrition-induced modifications of humoral immunity have been less understood. The objective of this study was to determine the effects of acute protein-energy malnutrition on serum levels of immunoglobulins and their subclasses in murine models. Male and female C57BL/6J mice were allocated to one of the four groups: (1) zero-time control (ZC) (19 days of age); (2) *ad libitum* intake of a complete purified diet (control group [CG]); (3) restricted intake of the complete diet (restricted group [RG]); and *ad libitum* intake of an isocaloric low-protein diet (low-protein group [LP]). The three groups other than the zero-time control were maintained on their respective regimens for 14 days, i.e. from 19 through 33 days of age. The restricted intake protocol produced weight loss through energy deficiency (marasmic-type malnutrition), whereas the low-protein diet caused wasting through inadequate protein nitrogen and induced a condition mimicking incipient kwashiorkor. Though serum levels of IgG1 and IgE (Th2-type immunoglobulins) in RG and LP mice were significantly higher than those in CG mice, serum levels of IgG2a and IgG3 (Th1-type immunoglobulins) did not show any significant difference between those three groups. Interestingly, serum levels of IgG2b (another Th2-type immunoglobulin) in LP mice were significantly higher than those in CG and RG mice. In ZC mice serum levels of total IgG, IgG1, IgG2b and IgG3 were significantly higher than those in the other three groups. We concluded that during acute malnutrition, Th1/Th2 balance is apparently shifted towards Th2 arm. This deviation seems to be more prominent during acute protein deficiency. The increased serum levels of immunoglobulins in ZC mice were probably due to the intestinal uptake of those proteins from maternal milk.

Keywords: Mice, Protein-energy malnutrition, Serum immunoglobulins

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INTRODUCTION

Immunocompetence and nutritional status are two of the most important determinants of mortality and morbidity. They are not independent indices and the

nature of their relation is central to many health problems. This fact is widely appreciated, but the knowledge of the nature of the relation is incomplete and possibly misunderstood, with consequences for international health, particularly for developing but also for developed countries.[1] The bulk of evidence on immunosuppression in protein-energy malnutrition (PEM) comes from works done on cellular immunity.[2-4] It has been said that blood immunoglobulin levels are normal and even elevated in PEM but much of the blood antibody is a polyreactive type not characteristic of the mature, high-specificity antibody response.[5] Malnutrition mostly reduces T cell numbers than B cells[6] and hence causes an elevation in T/B ratio. A depression in a thymus-dependent antibody response in an experimental animal model has been reported.[7] Cytokine changes in malnutrition have recently been the focus of concern. The production of such inflammatory cytokines as interleukin (IL)-1, tumor necrosis factor (TNF)- α and IL-6 is depressed in PEM.[5] Some human studies have reported Th-2 type cytokine activation during energy restriction in autoimmune diseases, in which Th-1 cytokines are over-produced.[8] The effect of cytokine changes on B cells and immunoglobulin subclasses and isotypes is not fully known. The aim of this study was to elucidate the effects of severe protein-energy malnutrition on immunoglobulin isotypes and subclasses in murine models.

MATERIALS AND METHODS

Briefly, male and female weanling C57BL/6J mice were randomly allocated to one of the four experimental groups, i.e. energy-restricted, protein-restricted *ad libitum*, and *ad libitum* control. The fourth group of mice were killed and analyzed just after weaning to get the baseline data (zero-control). After 2 weeks experimental period, all mice were bled and killed under CO2 anesthesia. Serum immunoglobulins were determined using double-sandwich enzyme-linked immunosorbent assay and carcasses were analyzed using chemical methods.

Animals, Feeding Protocols and Blood Sampling

Mice from in-house breeding colonies (Department of Nutritional Sciences, University of Guelph) of C57BL/6J were used. The stock from which these colonies were derived was purchased several years earlier from the Jackson Laboratory (Bar Harbor, ME). The mice were weaned at 18 days of age, acclimated for 1 day to a complete purified diet and, subsequently, allocated to experimental groups. Following acclimation the mice were allocated to one of the four groups: (1) zero-time control (ZC) (19 days of age); (2) *ad libitum* intake of a complete purified diet (control group (CG)); (3) restricted intake of the complete diet (restricted group [RG]) so that daily weight loss of about 2% of initial body weight was produced; and (4) *ad libitum* intake of an isocaloric low-protein diet (low-protein group [LP]). The three groups other than the zero-time control were maintained on their respective regimens for 14 days, i.e. from 19 through 33 days of age. Mice were housed individually in plastic cages with stainless steel tops. The animal facility was a windowless room maintained at 25-27°C and at a relative humidity of 60-70%. The room was supplied with fluorescent light daily between 0800 and 2200 h. All animals had unimpeded access to clean tap water at all times. At the end of the experimental period, retro-orbital plexus blood was sampled under CO_2 anesthesia and then mice were killed by cervical dislocation. Blood samples were incubated for 30-45 min and then centrifuged at 3500 g at room temperature. Sera were separated and transferred into fresh tubes. Because of low quantity of each single sample recovered, every 2-4 samples in a same group were pooled and considered as one sample. Serum samples and carcasses were kept at – 80°C until the day of examination. The experiments and procedures were approved by the Animal Care Committee of the University of Guelph, and were performed in accordance with the recommendations of the Canadian Council on Animal Care as specified in the *Guide to the Care and Use of Experimental Animals*.

Diets

The complete purified diet has been described in detail elsewhere.[9] Briefly, it was formulated with spray-dried egg white (80% crude protein; U.S. Biochemical, Cleveland, OH), cornstarch (St. Lawrence Starch, Port Credit, ON, Canada), glucose (cerelose 2001; CPC International, Englewood Cliffs, NJ), cellulose (celufil non-nutritive bulk; U.S. Biochemical), corn oil (Mazola, no additives or preservatives; Best Foods Canada, Etobicoke, ON, Canada) and a vitamin-mineral supplement that provided, most notably, 4 mg biotin/kg diet. A typical proximate analysis for this diet is 92.3% dry matter, 18.8% crude protein, 8.1% ether extract, 2.6% ash, 3.1% crude fiber and 17.0 kJ/g gross energy.[10] The low protein diet was formulated by replacement of all but 5 g of the egg white with an equal weight of cornstarch. By this means, a similar proximate analysis to that of complete diet is maintained with the exception of crude protein, which measures 0.6%.[10,11] The low protein diet used in the present investigation produces edema when fed to weanling mice for a period exceeding 3 wk.[12] Coprophagy was permitted both in the present investigation and in the aforementioned studies pertaining to the development of edema.

Immunoglobulin Assays

Reagent

Purified normal mouse IgG, IgG1, IgG2a, IgG2b and IgG3 and rabbit anti-mouse IgG, IgG1, IgG2a, IgG2b or IgG3 (gamma chain specific) and goat anti-mouse Fab-specific IgG conjugated with horseradish peroxidase (HRP) were obtained from Sigma-Aldrich. Purified normal mouse IgE, anti-mouse IgE capture monoclonal antibody, biotynilated rat anti-mouse IgE and avidin-HRP were all purchased from PharMingen. The substrates *O*-phenylenediamine (OPD) and tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich and PharMingen, respectively.

Procedures

The serum immunoglobulin levels were determined using enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well micro-plates were coated with properly diluted rabbit anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 gamma chain specific IgG or anti-mouse IgE capture monoclonal antibody in a 100 µL volume for 1hour at 37°C. After washing with phosphate-buffered saline with Tween-20 (PBST), the wells were blocked with 1% solution of bovine serum albumin (BSA) in PBS for 30 min at room temperature. Wells were washed with PBST again and then 100 µL of the pre-diluted serum samples, mouse IgG, IgG1, IgG2a, IgG2b, IgG3 or IgE standards were transferred to the appropriate wells. After one hour incubation at room temperature and washing with PBST, 100 µL of the properly diluted goat anti-mouse Fab-specific IgG/HRP or biotynilated rat anti-mouse IgE and then avidin-HRP were added to the wells. For IgG and its subclasses, OPD and for IgE, TMB was used as a substrate. The plates were read at 490 nm (IgG and its subclasses) or at 450 nm and then corrected at 570 nm (IgE). The serum levels of each immunoglobulin were determined using standard curves.

Carcass Analyses

Dry matter content of carcasses was determined by freeze-drying followed by oven-drying under vacuum for 16 h at 90°C. Crude protein and total lipid concentrations were measured, using samples of dried carcasses, as described elsewhere.[10]

Statistical Analyses

Results were subjected to two-tailed Student's *t* test or to ANOVA followed, if justified, by Tukey's Studentized range test or Duncan's New Multiple Range test.[13] Several data sets were subjected to logarithmic transformation to meet the requirement that a normal distribution be achieved. The predetermined upper limit of probability for significance throughout this investigation was $p<0.05$. All statistical analyses were done using Windows 2000/SPSS 10 package.

RESULTS

Growth Indices

Weight loss within the malnourished group averaged 27% of initial body weight, whereas the well-nourished control animals gained an average of 140% of their initial weight during the 14-day experimental period. Carcass compositional analysis was conducted only on pooled samples thereby precluding statistical analysis of the data (Table 1). The results were similar

Table 1. Feeding characteristics and carcass analysis data of the mice studied.

	Zero-Time Control (n=6)	Age-Matched Control (n=8)	Low-Protein (n=8)	Energy-Restricted (n=7)	Pooled SEM
IBW (g/mouse)[1]	8.4	8.6	8.7	8.8	0.013
FBW (g/mouse)	——	19.2[a]	6.8[b]	6.6[b]	0.414
FI (g/mouse/14d)[1]	——	65.9[a]	21.7[b]	12.6[c]	0.019
Carcass composition:					
dry matter (% wet wt)[1]	31.7[a]	30.5[a]	27.8[b]	27.1[b]	0.029
protein (% wet wt)[2]	16.8[b]	15.8[b]	16.0[b]	18.2[a]	12.510
lipid (% wet wt)	9.6[a]	10.5[a]	4.3[b]	1.8[c]	0.575

[1]Means are antilogs of ln-transformed means.

[2]Means are square roots of square-transformed means.

All statistical comparisons by Duncan's New Multiple Range test. Numbers in a row not sharing a common superscript are significantly different.

Abbreviatives: FBW: Final Body Weight; FI: Food Intake; IBW: Initial Body Weight; SEM: Standard Error of Mean.

Table 2. Serum immunoglobulin levels in the mice studied.

Immunoglobulin	*p* value	Zero-Time Control (n=6)	Age-Matched Control (n=8)	Low-Protein (n=8)	Energy-Restricted (n=7)
Total IgG (µg/mL)	<0.0001	569.3 ± 45.6[a]	217.9 ± 31.3*c*	394.8 ± 42.8[b]	289.4 ± 23.0[bc]
IgG1 (µg/mL)	<0.0001	115.8 ± 10.5[a]	46.6 ± 6.8[b]	82.0 ± 10.1[c]	89.4 ± 7.3[c]
IgG2a (µg/mL)	0.1919	1.7 ± 0.07	1.6 ± 0.1	2.0 ± 0.2	1.6 ± 0.1
IgG2b (µg/mL)	<0.0001	290.5 ± 24.3[a]	120.8 ± 17.1[b]	216.0 ± 25.4[c]	131.0 ± 18.3[b]
IgG3 (µg/mL)	<0.0001	49.8 ± 12.4[a]	14.6 ± 2.5[b]	14.1 ± 0.7[b]	9.8 ± 1.0[b]
IgE (ng/mL)	<0.0001	3.9 ± 1.7[a]	6.7 ± 1.0[a]	28.8 ± 2.5[b]	29.1 ± 3.6[b]

Numbers in a row not sharing a common superscript are significantly different.

to those previously obtained in relation to this experimental system in which weight loss on the part of the LP group was attributable to decrements in both lean and fat tissue.[10,14] The wasting disease produced in the present study was similar to the condition shown in previous studies of the same experimental system[10] to be associated with profound depression in cell-mediated and humoral acquired immunity.

The cumulative food intake of the malnourished group over the 14-day experimental period was 43% of that exhibited by the CG group (Table 1). On a daily basis, however, this represented an intake of dietary energy and micronutrients comparable, on a body weight basis, to that of the CG group (data not shown). The wasting disease of the LP group, on the other hand, resulted from dietary imbalance rather than from an insufficient intake of energy or of nutrients other than protein.

Quantification of serum immunoglobulin levels

Though serum levels of total IgG were higher in two known metabolically-depressed animal models compared to the control group, only the difference between LP group and control group was significant (Table 2). This finding is in contrast with some reports of diminished serum immunoglobulin levels in protein-energy restriction, notably kwashiorkor,[15,16] which might be corrected by appropriate nutritional care.[17] In one animal study, energy restriction caused a significant decrease in the serum concentration of total IgG as well as of IgA, C3, and the number of circulating natural killer (NK) cells.[18] On the other hand, there are some reports of the normal and even increased serum levels of IgG in energy-protein restriction.[19]

DISCUSSION

It has been shown that calorie restriction might suppress Th1 and Th2 cytokines- induced immunoglobulin production in young and old C57BL/6 cultured submandibular glands.[20] Generally speaking, reductions in energy availability can impair humoral immunity.[21] However, the humoral immunity may respond to malnutrition in various ways depending on the type of malnutrition induced (such as acute vs. chronic, protein malnutrition vs. energy restriction). As table 2 and figure 1 show, serum levels of Th2-type immunoglobulins (IgG1, IgG2b and IgE)[22] are significantly increased in severely malnourished animals compared to the control group and this increment for IgE is in the order of magnitude. As for IgG2b, though the LP and RG mice showed higher serum levels than CG, the difference was just for LP significant. In this study, we used the ratio of the sum of the Th2- to Th1-type immunoglobulins (IgG1+IgG2b

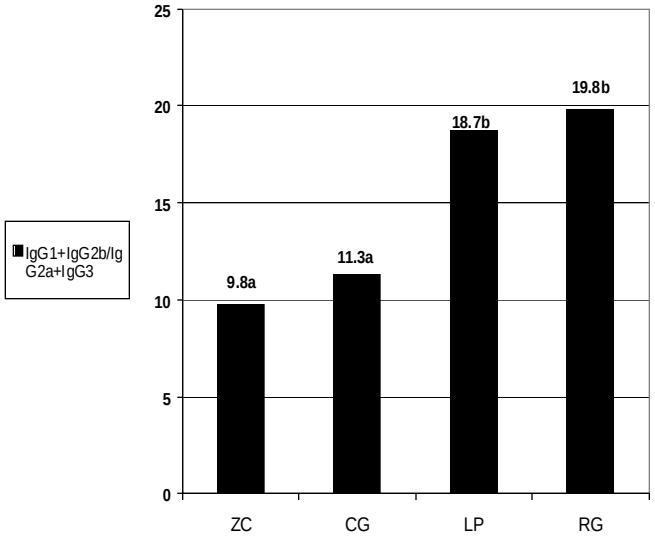

Figure 1. The ratio of Th1-/Th2-type IgG subclasses in two animal models of acute malnutrition. Numbers not sharing a common letter are significantly different.
ZC: zero-time control group; CG: age-matched control group; LP: low-protein group; RG: energy-restricted group

/ Ig2a+IgG3) as an indicator of Th2 arm activation versus Th1. As it is seen in table 2 and figure 1. This ratio was significantly higher in both severely malnourished groups compared to the CG and ZC. The systemic shift towards Th2 arm during protein malnutrition, however, may be impaired if parasitic infections are superimposed.[23] On the other hand, it is not just protein deficiency that may lead to Th2 arm activation as it has been shown that protein hypernutrition may also cause Th2 immune functions become predominant.[24] Protein nutrition has therefore a very critical role in the immune function, not all aspect of which fully understood.

In ZC mice serum levels of total IgG, as well as IgG1, IgG2b and IgG3 were significantly higher than those in the other three groups. The increased serum levels of immunoglobulins in ZC mice, was probably due to the intestinal uptake of those proteins from maternal milk.

Though increased serum levels of IgE in protein-energy deficiency has previously been reported in a human study,[16] in a recent study conducted in Brazil children with moderate PEM and without associated parasitic infections were found to have significantly lower serum IgE levels than healthy children (69.3 ng/ml vs 95.97 ng/ml, respectively).[25] On the other hand, in one study it was shown that the production of IgE on challenge with allergen was lower in protein-calorie deficient mice than in normal mice and the levels of antigen-specific IgG1, IgG2a and IgE were all significantly depressed by malnutrition.[26] The production of specific antibodies may therefore be affected in the context of malnutrition. Some evidence shows that though serum levels of some Th2 cytokines such as interleukin (IL)-4 and total IgE may be increased in malnutrition, the serum levels of specific anti-Ascaris lumbricoides IgE may be decreased in infected malnourished children.[27]

The increased ratio of Th2- / Th1-type immunoglobulins, if it reflects an overall shift of Th1/Th2 balance towards Th2, may have some important implications. In animal studies on immunobiology, response to vaccination, immunity to antigens and so on, the weight gain of animals must be highly taken into consideration as some treatments may affect energy intake and hence the immune response in the experimental group(s). On the other hand, in some auto-immune disorders, such as systemic lupus erythematosous (SLE), there is some evidence of imbalance towards Th1 predominance (28-30) and there are some reports of disease alleviation through energy restriction in such autoimmune disorders as rheumatoid arthritis.[8,31] It is therefore probable that in some autoimmune disorders with Th1 predominance, dietary manipulation may help in treatment.

To the best of our knowledge, this is the first report of the effects of acute protein-energy restriction on the serum concentrations of total IgG subclasses. We concluded that during acute uncomplicated malnutrition, Th1/Th2 balance is apparently shifted towards Th2 arm. This deviation seems to be more prominent during acute protein deficiency. This finding may have some important experimental as well as clinical implications.

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