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THE INFLUENCE OF FEEDING FREQUENCY AND PROTEIN SOURCE ON PROTEIN STATUS AND IMMUNE RESPONSE

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The aim of the present study was to examine if different feeding frequencies have an influence on protein status and immune response in rats. Protein status of animals was characterised by [1-^{14}C] leucine oxidation in postabsorptive phase and reflected by body growth rate and amount of titers in the serum after immunisation with thyreoglobuline. The feeding frequency tends to influence weight gain in groups fed with casein based diet: meals provided twice a day were more efficient. Breath test measurements showed no significant differences in $^{14}CO_2$ recovery for different meal frequencies but the tendency for higher values in groups fed with 6 meals was observed. The rodents fed with casein based diet showed larger amount of antibodies than soya based diet groups but it was significant only in the case of 2 meals. Increasing the feeding frequency from 2 to 6 meals significantly improved the immune response in soya protein fed groups. However there was no difference in body mass gain between these two groups. It could suggest that improvement of protein status resulted in better availability of amino acids for metabolism but the composition of these amino acids allowed only the increase of antibody production and not for productive purposes (growth). It might also suggest that at the marginal level of protein intake, the immune response (antibody production) was favourable for growth development when the meal frequency was increased.

INTRODUCTION

It is well known that nutrition status has an influence on the immune system. Thus, poor nutrition deprives immune functions, not only in individuals with general protein-energy malnutrition in developing countries, but also in undernourished people in developed nations – hospitalised patients, alcoholics, the elderly and food faddist [Chandra, 1989, 1990]. In fact, tests of immunocompetence are included in protocols for the assessment of nutritional status [Garrow & James, 1993].

The majority of studies investigating the relationship between nutrition and immune factors are focused on protein-energy malnutrition (PEM) in children in developing countries, and on debilitated hospitalised patients in industrial nations. Investigations of the effect of deficiency of single nutrient on immunity have generally been performed on experimental animals, as single-nutrient imbalances are relatively rare in humans. Moreover, human malnutrition is characterised by multiple nutrient deficiencies and frequently complicated by the presence of infections, with its own significant impact on immune function [Keusch, 1991].

Studies on laboratory animals showed that early malnutrition, when induced in utero or within the first few weeks of life, may result in prolonged or permanent impairment of immune response [Jose *et al.*, 1973]. The offsprings of protein- or energy-deprived mice also manifest reduced immune responses, even when offsprings themselves are given adequate nutrition after birth [Chandra, 1975; Beach *et al.*, 1983]. When the diets were restricted to 60% of normal intake, the weaning mice demonstrated a reduction in the number of antibody-forming cells in mitogenic (proliferative) responses, and in a delayed type of hypersensitivity skin responses, for as long as two years. Thus, early malnutrition may affect diverse maturational events, including the development or selection of T- and B-cell repertoires, which comprise the pre-programmed immune responsivity of the host. Moderate restricted protein diets also lead to enhanced cell-mediated immune responses, even though serum immunoglobulin levels and specific antibody production may be diminished [Keusch, 1991]. Another study performed by Barett [1988] suggests that protein-calorie malnutrition seems to stress the T-cell more than the B-cell system but low-protein diets alone are not invariably detrimental. However, when animals are fed energy-restricted diets they experience a decreased incidence of autoimmune disease compared to controls fed *ad libitum*, and the 'deprived' animals apparently live longer [Weindruch *et al*., 1982, 1983].

Considering the type of protein in the diet Bounous and Kongshavn [1985] observed that it has a significant effect on development of humoral immunity to T cell-dependent antigens. Thus, the humoral immune response of mice fed a lactalbumin diet was found to be nearly five times greater

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than that of mice fed corresponding casein, soya or wheat protein diets. The humoral immune response of mice fed casein, soya and wheat diets was substantially lower than that of mice fed nonpurified diets, whereas that of mice fed lactalbumin diet was higher. Bounous *et al.* [1985] postulated that the type of protein in the diet directly influences the intrinsic capacity of the B-lymphocytes to respond to an immunogenic stimulus. It was suggested that the principal factor responsible for the observed different effect of dietary lactalbumin and casein on humoral immunity was not the availability or concentration of single essential amino acids but rather the composite effect of the specific amino acids distribution in the protein. The amino acid composition of the ingested protein, indicate that the diet--dependent changes in plasma amino acid profile might represent the crucial factor responsible for the observed effect of protein type on the B cell response.

Feeding frequency and meal size are important nutritional factors that can influence protein metabolism of the body. Studies on amino acid utilization in rats [Schiffelers *et al.*, 1996; Bujko *et al.*, 1997] and in pigs [Batterham & Bayley, 1989] suggest that the same marginal daily amount of food served as small and more frequent meals might improve protein utilization compared to less frequent larger meals. In the case of lower postprandial oxidation more amino acids remain available in the body till postabsorptive phase to support maintenance processes, productive processes (*e.g.* growth, lactation) or other functions according to the actual physiological demands of the body. All these functions can be vital, but in the case of amino acid deficiency and depending on the physiological situation of the animal (*e.g.* stress, immunisation), some functions may have a higher priority at the expense of others.

The main aim of the present study was to examine the effect of feeding frequency on protein status of animals as characterised by excretion of metabolic end products from [^{14}C] labelled leucine. In the case of fixed marginal level of daily protein intake large *vs.* small meals due to higher postprandial oxidative losses were expected to affect body weight as well as other physiological functions (like immune response). The rats fed with the same, marginal amount of protein divided into different number of meals had to 'choose' the way of using proteins (*e.g.* to maintain body weight or other functions). Therefore we hypothesized that the immune response of an individual animal might be influenced by protein status as the immune response requires additional amino acids for net synthesis of immune proteins and cells. In consequence more proteins might be used for immune response and less would be available for growth.

MATERIALS AND METHODS

Male WU-Wistar rats (n=44, Center for Small Laboratory Animals, Wageningen University, Wageningen, The Netherlands) were randomly divided into 4 experimental groups (n=11; casein 2, casein 6, soya 2, soya 6) and caged individually at 22–23°C and 70% humidity with 16 h of artificial light and 8 h of red light (9.00–17.00). Tap water was available *ad libitum* during the experiment. After weaning period the animals had *ad libitum* access to commercial diet (RHM-B Hope Farms, Woerden, The Netherlands). Starting from 8 weeks of age all rats were fed with a protein restricted diet (75 g/kg) containing 1 gram of protein (casein or soya isolate) and 219 kJ ME per day. Total daily amount of feed during the experiment was set at 13.3 g and divided into 2 *vs.* 6 meals introduced during the red light period. Two large meals were given for 1 h each at 9.00 a.m. and 4 p.m.. Six small meals were served at 9.00 a.m., 10.30 a.m., 12.00 a.m., 1.30 p.m., 3.00 p.m. and 4.30 p.m. for 30 min each. Body weight of individual animals was determined 3 times a week prior to the first feeding in the morning (8.30–9.00 a.m.) and statistical differences on day 60 were determined with ANOVA (Statistica). After 6 weeks of experimental feeding schedule, protein status of animals was determined by postabsorptive $^{14}CO_2$ breath test technique. At 8 p.m. rats received an intraperitoneal injection of 1 μCi [1-^{14}C] leucine dissolved in 200 μL of water. Immediately after injection the rats were placed into individual macrolon cages equipped for collecting CO_2. The radioactivity recovered as $^{14}CO_2$ in the breath during 4 h with sampling intervals of 30 min was used as a parameter for protein status estimation [Schreurs *et al.*, 1992]. The values for recovery of metabolic end products in breath from labelled amino acid were expressed as the percentage of injected doses and tested for differences with Student t-test.

Thereafter all rodents were subject to an immunisation procedure with thyreoglobuline (Thyreoglobuline Bovine, Sigma, Chemical Company, USA) injected subcutaneously. Thyreoglobuline is a large protein recognised by the immune system of an animal as non-self and elicited to the release of antibodies. The primer consisted of a thyreoglobuline (250 μg) diluted with 250 μL Freund complete oil. One week later all animals were injected with the booster (the same amount but diluted with incomplete oil), which elicited secondary immune response. Ten days after the booster injection blood samples from all rats were taken to estimate relative values of antibody titers (IgG) by an indirect antigen coated plate (IACP) enzyme linked immuno sorbent assay (ELISA) according to the method LMA [1993] with some modifications. Conjugate specific for the rat IgG (Anti-Rat IgG, Sigma, chemical company, St. Louis, USA) and alkaline phosphatase (4-nitro-phenylphosphate in substrate buffer) were used. The extinction was measured after 1-h incubation with a spectrophotometer (405 nm). The titer was determined as the dilution factor closest to the 50% extinction value related to the plateau value (100%). To determine distribution differences, a Kolmogorov-Smirnov two-sample test was done for pairs of groups. Larger dilution factor meant larger amount of antibodies and comparisons of data gave information about the relative amount of titers in the blood serum of animals.

RESULTS

Results of the immunisation experiment are presented in Table 1. The highest response measured as titers number in blood sample was observed in groups fed with casein--based diet with frequency of 2 meals, the lowest values were found in group fed soya-based diet twice a day.

The body weight development expressed as the per cent of initial (day 0 of feed restriction) weight is presented in Figure 1. The average weight of all animals at the beginning of the experiment was 300 g (SD 25 g). During the first 7–10 days of protein restriction a substantial fall in body mass was

TABLE 1. Number of rats with titers on different dilution factors per experimental group.

Dilution factors	Groups:			
	Casein 2^{A}	Casein 6^{AB}	Soya 2^{B}	Soya 6^{AC}
1 : 1280	-	2	2	-
1 : 2560	2	4	6	3
1 : 5120	7	3	1	4
1 : 10240	1	2	-	4

Groups with different capital letters are significantly different ($p<0.05$).

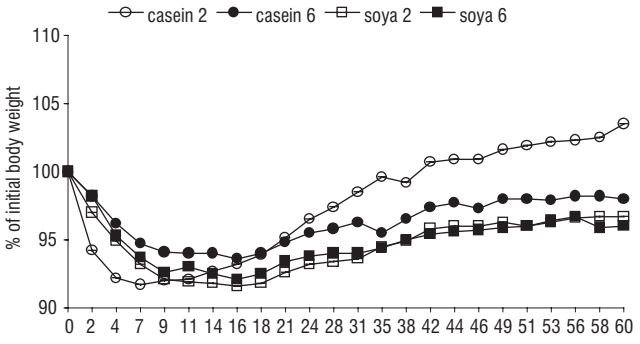

FIGURE 1. Average body weight expressed as percentage of initial weight of rats fed with frequency of 2 *vs.* 6 meals on casein or soya protein based diets during the experiment.

observed in all experimental groups. Thereafter, the rodents started to regain their body mass. There were no differences in body weight development in groups fed with soya-based diet with frequency of 2 *vs.* 6 meals (97% and 96% of initial weight, respectively). Rats fed with casein--based diet with frequency of 2 meals per day reached higher body mass (103% of initial weight) compared to the group fed the same diet with frequency of 6 meals (98%), however, the differences were not significant.

Results of [1-^{14}C] leucine breath test measurements are presented in Table 2. Results indicate that considering the same frequencies of feeding (2 and 6 meals) animals reached a significantly better protein status on casein-based diets compared to soya-based diets. Within the same protein-based diets (casein and soya) values for 6 meals feeding frequency were higher, however not significantly.

TABLE 2. Cumulative recovery of $^{14}CO_2$ in the rats' breath 4 hours after intraperitoneal injection of [1-^{14}C] leucine (% of dose).

Cumulative recovery of [1-^{14}C] leucine	Groups:			
	Casein 2	Casein 6	Soya 2	Soya 6
% of dose, mean±SD	19.2 ± 2.5^{A}	20.9 ± 1.7^{A}	15.1 ± 1.5^{B}	16.4 ± 1.9^{B}

Groups with different capital letters are significantly different ($p<0.05$).

DISCUSSION

The relationship between protein metabolism and immunology is important considering that malnutrition or even marginal feeding in animal and human nutrition can have adverse effect on mechanism used for host defence [Keusch, 1992]. Protein-energy malnutrition causes widespread atrophy of lymphoid tissues and dysfunction of cell--mediated immunity [Heatley, 1995]. As a consequence it could be suggested that better protein status might be reflected by better immune response. In this case more amino acids are available and can be used for immune response (antibodies and cells production). Inversely, infection might results in reduced growth in young animals or a loss of weight in mature animals. In many instances this repression in body weight is not only due to a reduction in feed intake, but may also represent a decrease in efficiency of feed utilisation or net degradation of tissue proteins. Especially, when the protein intake is marginal, an animal has to make a selection how to use its dietary protein: for maintenance, growth or for functions such as an immune response. As a consequence a better immune response was expected in animals with better protein status reflected in the present study by a higher level of leucine oxidation in postabsorptive phase. Higher oxidation in post absorptive phase together with body weight growth or maintaining body mass (without catabolism) is supposed to reflect higher body protein degradation which is replenish by higher postprandial synthesis. As a consequence protein turnover is higher and protein status better [Bujko *et al.*, 1997]. Chandra [1977] noticed that children with higher protein intake had higher antibody response. Studies on animals [Bounous & Kongshavn, 1985] showed that the type of protein in the diet directly influenced the intrinsic capacity of the B-lymphocytes to respond to immunogenic stimuli. Bounous *et al.* [1985] found that immune response of mice fed a lactoalbumine diet was nearly five times greater than that of mice fed corresponding diets based on casein, soya or wheat protein. The present study clearly showed the influence of protein status on immune response. It is evident that both, feeding frequency and protein source influenced the level of antibody production.

Generally, animals conditioned on casein diets responded better than these on soya protein but only in the case of two-meal frequency was the response significant. It could be suggested that casein has more adequate amino acid concentrations to fulfil the body requirements for the antibody synthesis. The worst protein status created by feeding soya--based diet twice a day led to the lowest titers amount in the serum. An increase in the meal frequency on soya diet to 6 meals improved the immune response to the level obtained on both casein diets. Increasing the meal frequency on better protein quality (casein) diets had no significant influence on antibody production. This indicates that casein protein, even fed in 2 meals, was adequate for the immune response in experimental conditions. This could also explain that statistically significant difference in titers in serum between casein and soya diets was observed only in two--meal frequency.

Our results indicate the influence of meal frequency and protein source on physiological strategy of animals. The worst protein status (rats fed on soya pellets twice a day) limited the antibody production but simultaneously had no effect on the body weight. After increasing the meal frequency to six meals animals improved their protein status and responded better to immunisation but failed to regain the body weight. The better protein status (rats fed on casein pellets) resulted in higher titers production independently of meal frequency. It seems that different amino acids had limited the immune response and the body weight development. The worse amino acid availability had

a greater impact on antibody production than on the body weight gain. It could be pointed out that at the marginal level of protein intake, the change in protein source or feeding frequency had a greater influence on the immune response than on the body mass gain.

CONCLUSIONS

1. At the fixed, marginal level of protein intake the shift in feeding frequency from 2 to 6 meals in the case of soya protein improved the antibody production in rats.

2. An increase in the feeding frequency from 2 to 6 meals at the marginal level of dietary protein intake caused a tendency to improve protein status of animals measured by postabsorptive leucine oxidation.

3. At the marginal level of protein intake the immune response functions were in favour of body mass gain when meal frequency or protein source was changed.

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