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THE IMMUNOENHANCING PROPERTY OF DIETARY WHEY PROTEIN CONCENTRATE

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Abstract—The plaque-forming cell response to sheep red blood cells was found to be enhanced in mice fed a formula diet containing 20 g lactalbumin / 100 g diet in comparison to mice fed equivalent formula diets of similar nutritional efficiency containing 20 g / 100 g diet of either casein, soy, wheat or corn protein, egg albumin, beef or fish protein, *Spirulina maxima*, or *Scenedesmus* protein, or Purina mouse chow. This effect was manifest after 2 weeks and persisted for at least 8 weeks of dietary treatment. Mixing lactalbumin with either casein or soy protein in a 20 g protein / 100 g diet formula significantly enhanced the immune response in comparison to that of mice fed diets containing 20% soy protein or casein.

Résumé—La formation de rosettes par des érytrocytes de moutons exposés à du sang de souris s'avère être stimulée si ses souris sont nourries avec une diète contenant 20 g de lactalbumine par 100 g de diète, par comparaison à des souris nourries avec des diètes équivalentes contenant 20 / 100 g de caséine, de soja, de protéine de blé ou de maïs, d'albumine, de protéine de porc, de poisson, de *Spirulina maxima* ou de *Scenedesmus* ou encore de moulées Purina pour souris. Cet effet se manifeste après 2 semaines et persiste 10 semaines après la modification diététique. L'effet de mélanger de la lactalbumine avec de la caséine ou du soja augmente également la réponse immune à comparaison à celle de souris qui sont nourries avec des diètes ne contenant pas de l'albumine.

Key words: dietary whey protein, humoral immune response.

INTRODUCTION

ALMOST THREE DECADES AGO, Schaedler and Dubos [1], reported that mice fed for 2–4 weeks formula diets containing either insufficient amounts of casein or plant protein, exhibited lesser growth or weight loss and increased susceptibility to *Staphylococcus aureus, Mycobacterium tuberculosis, Klebsiella pneumoniae*, in comparison to mice fed a 20% or 15% casein diet. Experimental [2–5] and clinical [6] data have since substantiated the concept that protein calorie malnutrition adversely affects various components of the immune system. A plausible explanation for these findings can be formulated on the light of current knowledge of amino acid metabolism. An adequate intake of essential amino acids is necessary because surplus amino acids are not stored and, for protein synthesis to proceed, all of the indispensable amino acids must be present simultaneously in the extracellular pool. With regard to the humoral immune response, clonal expansion and antibody production require rapid protein synthesis, so that amino acid restriction will inevitably interfere with these functions.

Our interest in the effect of amino acid intake upon the immune system was prompted by an observation made several years ago [7]. We fed mice a defined formula diet containing a free amino acid mixture duplicating casein. Another group of mice was fed a similar diet but with moderate restriction of phenylalanine and tyrosine compensated by a corresponding increment in the non-essential amino acid mixture. The second group of mice gained weight at the same rate as the mice fed the casein equivalent mixture or Purina mouse chow. However, when challenged with sheep red blood cells, these mice produced more antibodies and plaque forming cells against sheep red blood cells than Purina or casein equivalent-fed mice. A new concept thus emerged, namely, that changes in the amino acid profile of the diet can influence the immune response independently of any systemic effect on the nutritional status of the host. But, more importantly, changes in the amino acid profile, i.e. protein type, could conceivably enhance the humoral immune response beyond that which had traditionally been considered to represent a "normal" response. We subsequently assessed the effect on the immune response of different types of proteins in nutritionally adequate diets. Mice fed formula diets containing 20% or 28% lactalbumin were found to produce more plaque forming cells to sheep red blood cells than mice fed Purina mouse chow containing about 22% protein from various sources and of similar nutritional efficiency. The immune enhancing effect of lactalbumin was maximal at 20% concentration [8]. A 20 g net protein / 100 g diet provides a good method to assess the effect of protein

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type on the immune system. At this level most protein supplies the minimum daily requirement of all indispensable amino acids for the growing mouse [9] and this is important because the amino acid distribution, and not adequacy, is the variable under investigation. Moreover, it is preferable to avoid higher protein levels in order to prevent amino acid toxicity.

Lactalbumin is the term traditionally used to describe the group of milk proteins that remain soluble in "milk serum" or whey after precipitation of casein at pH 4.6 and 20°C, as in the manufacture of cheese. Beta-lactoglobulin, alpha-lactalbumin, serum albumin and immunoglobulin are considered to be the major components of the whey protein mixture [10]. Our studies have shown that mice fed diets containing 20 g/100 g of any one of the major components of lactalbumin developed immune responses to sheep red blood cells which were inferior to that of mice fed a diet containing 20 g lactalbumin/100 g of diet, leading to the assumption that the immunoenhancing effect of lactalbumin is dependent upon the overall amino acid pattern resulting from the contribution of all its protein components [11]. This hypothesis is substantiated by the observation that the divergent immune effect of lactalbumin and casein is essentially maintained when these proteins are replaced in the diet by an equal amount of a free amino acid mixture duplicating the amino acid profile of either lactalbumin or casein [11]. In the present study we have investigated the effect on the humoral immune response of practically all of the purified edible proteins commercially available at acceptable cost. In addition, we have assessed the kinetics of the observed effect of dietary protein type on the immune system.

MATERIALS AND METHODS

Animals

Male C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at 7 weeks of age.

Diets

The detailed composition of the common ingredients (vitamins and minerals) in all of the defined formula diets is given in Table 1. Diets are prepared in the following way: 20 g of selected pure protein, 56 g of product 80056 protein free diet powder (Mead-Johnson Co. Inc., U.S.A.), 18 g cornstarch, 2 g wheat bran; 0.5 g Nutramigen vit-iron premix (Bristol-Meyers, Ontario, Canada), 2.65 g KCl; 0.84 g NaCl. The only variable in the various purified diets was the type of protein. The formula diets contained 20 g/100 g diet of either lactalbumin, casein, soy protein, wheat protein (Bristol-Meyers of Canada), corn protein, egg albumin, beef protein, fish protein (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), *Spirulina maxima* or *Scenedesmus* protein (courtesy of Dr. G. Baudo, Milan, Italy). The lactalbumin (whey protein concentrate from cow's milk) used in our experiments is Lacprodan-80 (Danmark Protein, Worthington, Ohio, U.S.A.), containing 77%–80% net protein. The purified proteins were vitamin free. In other diets, the protein component (20 g/100 g diet) was a mixture of lactalbumin and casein or soy protein. The individual differences in pure protein content of the various protein powders were taken into consideration when preparing the various 20 g protein/100 g diets.

TABLE 1. VITAMIN AND MINERAL CONTENT OF TEST DIETS

The vitamin mixture plus the vitamins contained in the basal diet (Mead Johnson product 80056) provided *in milligrams per 100 g diet*: ascorbic acid, 65.0; niacin, 9.2; riboflavin, 0.69; thiamin, 0.63; folic acid, 0.12; vitamin B-6, 0.36; biotin, 0.058; pantothenic acid, 3.38; choline, 76 *and per 100 g diet*: retinyl palmitate, 1800 IU, ergocalciferol, 360 IU; vitamin E (*dl*-tocopheryl acetate), 18 IU; vitamin B-12, 0.054 mg; and vitamin K (phylloquinone), 90 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 ($K_2HPO_4 \cdot 2H_2$); 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).

Crude protein constitutes about 65% of dry weight of *Spirulina maxima* (Sosa Texcoco, Mexico). Hence *Spirulina*-based products have been utilized as a source of protein for feeding hungry people. The aminoacid pattern of *Spirulina* protein is similar to that of other micro-organisms and, in comparison to commonly used food proteins such as eggs or milk, it is relatively deficient in methionine, cysteine, and lysine [12]. The *Spirulina* powder product used in our experiments contains, in addition, 13% to 16% carbohydrates, 6% to 7% lipids and 6% ash. Unlike the other purified proteins used in this study, about 5% of the dry weight of *Spirulina* is constituted by cellular elements such as nucleic acids. This value is similar to that reported for unicellular algae such as *Scenedesmus*. In addition *Spirulina* contains a substantial amount of natural carotenes and other fat and water soluble vitamins [12]. The *Scenedesmus* powder used in our experiments (Sausal, Peru) contains approximately 60% crude protein. This level of protein in algae is exceeded only by *Spirulina*; hence *Scenedesmus* is also considered to be a good protein source [13].

Diets were continuously available in powder form from stainless-steel feeders 1.5 in high, designed to reduce spillage and spoilage. Other animals were fed a non-purified diet (Purina mouse chow, Ralston Purina Co., St. Louis, Missouri) containing an estimated 22 g protein from various sources/100 g diet. Seven week old mice were placed on the various diet regimens and immunologic studies commenced 1, 2 and 8 weeks later. Dietary treatment was continued throughout the experiments. Each dietary group comprised 10 mice or more.

Immunization for plaque assays

The diet-fed mice were immunized by an intravenous injection of 5×10^6 washed sheep red blood cells obtained weekly from Institut Armand-Frappier, Laval des Rapides, Quebec, Canada.

Plaque forming cell (PFC) assay

The method used for assaying IgM plaque forming cells was essentially the one described by Cunningham and Szenberg [14], with certain minor modifications. Spleen cell suspensions were prepared by gently tamping the spleen through a 50-mesh stainless steel screen, and collecting the cells in balanced salt solution (BSS) supplemented with 10% heat-inactivated calf serum (Grand Island Biological Company, Montreal, Quebec, Canada). The spleen cells were washed and made up to 15 ml with BSS. Sheep red blood cells were washed twice and made up to a 20% concentration. Guinea pig serum (Grand Island Biological Company, Montreal, Quebec, Canada) as a source of complement was diluted 1 / 15 with BSS. All stock solutions were kept on ice water until used. The test consisted of mixing 0.05 ml of spleen cells, 0.15 ml of sheep red blood cells and 0.75 ml of the complement solution in a test tube at 37°C. The whole mixture was immediately withdrawn and put into slide chambers, sealed with warm paraffin wax, and incubated at 37°C for 45–60 min. The number of plaque forming cells was counted and their total number per spleen estimated by multiplying the number of plaque forming cells in each sample (0.05 ml spleen cells) by 300. The values are expressed per total organ rather than per 10^6 spleen cells, since this appears to reflect more accurately the functional status of the spleen *per se*.

Mice were assayed for the plaque forming cell response to sheep red blood cells normally on the fifth day after immunization or, in the kinetic study, on days 3, 4, 5, and 6 post-immunization.

Statistics

The mean plaque forming cells values were compared among the dietary groups using either Student's t-test, when two groups were being compared, or the analysis of variance (ANOVA) for more than two groups. Because of the heterogeneity of variances among groups, the adjustment given by Brown and Forsythe [15] was used.

RESULTS

Nutritional data

In Table 2 data are reported on the nutritional efficiency of the different diets. Mice fed the defined formula diets and Purina chow increased in body weight by approximately the same amount with comparable caloric intake. Total serum proteins were also similar.

Humoral immune response

As indicated in Figure 1, mice fed the lactalbumin diet for 2 weeks exhibit a plaque forming cell response to sheep red blood cells which is higher than that of mice fed any other protein type or Purina mouse chow. The mean number of plaque forming cells per spleen 5 days after i.v. injection with 5×10^6 sheep red blood cells in the lactalbumin diet-fed mice was 487%, 494%, 736%, 927%, 309%, 284%, 230%, 214%, and 177% of that noted in casein, *Spirulina*, soy protein, wheat protein, *Scenedesmus*, corn protein, egg albumin, beef or fish protein diet-fed mice respectively, and 168% of that of Purina-fed mice. These differences are all statistically significant ($P = 0.0004$). The number of plaque forming cells per spleen in Purina-fed mice was 170% of that in corn protein diet-fed mice ($P = 0.0005$) and the value of the latter group was 171% of that noted in casein-fed mice ($P = 0.0005$). No significant difference was observed between fish protein diet-fed, beef protein diet-fed and Purina-fed groups.

The addition of lactalbumin to either soy protein or casein produced a significant increment in the humoral immune response of the host. In a 50:50 mixture with soy protein, lactalbumin induced a 4-fold increment in the immune response in comparison to a purely soy protein diet. In an 80:20 mixture with casein, lactalbumin induced a 3-fold increment and, in a 20:80 mixture with this protein, a 2-fold increase in the immune response was seen in comparison to a purely casein diet.

To establish the time course of the effect of dietary lactalbumin on the immune response, plaque forming cell assays were commenced, in selected experiments, after 1, 2, or 8 weeks of dietary treatment. The results of these experiments (Figure 2) show that the effect of the type of dietary protein on humoral immune response is evident after 2 weeks of treatment and persists for at least 8 weeks of dietary treatment (lactalbumin *vs.* casein fed after 2 and 8 weeks: $P < 0.0005$). The lactalbumin and casein diet-fed mice increased in body weight by approximately the same amount during the 2 month feeding period (73% gain for lactalbumin-fed and 76% gain for the casein-fed mice).

Figure 3 shows that the higher plaque forming cell response reported 5 days after injection of 5×10^6 sheep red blood cells in lactalbumin-fed mice, represents a real enhancement of the immune response produced by dietary protein type and not a shift in the timing of the peak response.

DISCUSSION

It is apparent from our data that the various 20 g protein / 100 g diets all sustain similar growth of mice with comparable food consumption and serum protein levels. These values were similar to those of Purina fed mice and could thus be construed as "normal". However, the humoral immune response to heterologous erythrocytes in the mice fed the lactalbumin diet was substantially greater than that of mice fed any other type of animal or plant protein, or Purina mouse chow. This marked enhancement of the immune response cannot be ascribed to presensitization of the lactalbumin diet-fed group with cross-reacting antigens present in lactalbumin because only very low numbers of plaque forming cells per spleen were found in non-immunized mice and, moreover, these did not differ between dietary groups [16]. In addition, we showed that the immunoenhancing property

TABLE 2. EFFECT OF 19 DAYS DIETARY REGIMEN ON FOOD CONSUMPTION, BODY GROWTH, TOTAL SERUM PROTEIN AND DEVELOPMENT OF SPLEEN*

Protein type	Avg. consumption (g / mouse / day ± SEM)[a]	Initial weight (g)[b]	Final weight (%.initial wt.)[c]	Serum protein (g / 100 ml)[d]	Average spleen	
					Wt(mg)[e]	# cells (10^6) ± SEM[f]
Lactalbumin	2.8 ± 0.1	22.6 ± 0.6	118.0 ± 3.2	5.8 ± 0.2	117 ± 2.1	194 ± 4.0
Casein	2.9 ± 0.2	23.0 ± 0.8	117.8 ± 4.6	6.1 ± 0.3	113 ± 3.6	150 ± 4.1
Spirulina maxima protein	2.9 ± 0.3	19.8 ± 0.9	121.0 ± 1.8	5.4 ± 0.5	104 ± 3.4	138 ± 6.0
Soy protein	3.1 ± 0.2	21.2 ± 0.3	114.1 ± 1.3	6.0 ± 0.4	107 ± 3.8	144 ± 4.3
Wheat protein	2.9 ± 0.2	20.0 ± 0.3	115.0 ± 2.2	5.9 ± 0.3	109 ± 2.6	139 ± 8.0
Scenedesmus protein	3.1 ± 0.4	23.0 ± 0.3	113.0 ± 3.0	6.1 ± 0.1	107 ± 4.0	152 ± 10.0
Corn protein	3.1 ± 0.2	22.8 ± 1.1	115.5 ± 5.4	5.6 ± 0.2	118 ± 3.2	162 ± 7.0
Egg albumin	3.0 ± 0.1	20.7 ± 0.6	116.0 ± 2.9	5.8 ± 0.3	114 ± 3.0	157 ± 6.0
Fish protein	2.8 ± 0.4	20.9 ± 0.3	117.1 ± 1.3	5.5 ± 0.1	105 ± 2.4	152 ± 6.0
Beef protein	2.9 ± 0.4	22.0 ± 0.3	113.0 ± 1.9	5.7 ± 0.3	109 ± 1.8	150 ± 5.0
Lactalbumin / Soy (50:50)	2.9 ± 0.3	20.7 ± 0.5	121.0 ± 4.7	5.8 ± 0.5	110 ± 8.0	180 ± 7.0
Lactalbumin / Casein (80:20)	2.7 ± 0.4	23.6 ± 0.4	121.0 ± 2.0	5.6 ± 0.4	112 ± 4.0	148 ± 4.9
Lactalbumin / Casein (20:80)	3.0 ± 0.2	23.4 ± 0.5	116.0 ± 2.0	6.0 ± 0.3	118 ± 4.0	145 ± 5.0
Purina mouse chow[g]	3.2 ± 0.3	21.1 ± 0.5	114.7 ± 1.8	5.8 ± 0.2	114 ± 1.9	189 ± 6.0

[a]The average food consumption over the 18 day feeding period was not considered different by ANOVA.

[b, c, d, e, f]The average initial body weight (b), increase in body weight (c), total serum protein (d), and spleen weight (e) were not considered different by ANOVA. The numbers of cells per spleen (f) in lactalbumin and Purina fed groups were higher by ANOVA ($p = 0.0001$) than the corresponding values in the casein, wheat, soy, and fish protein groups.

[g]Purina mouse chow, Ralston Purina Company, St. Louis, MO., (estimated 22 g protein from various sources per 100 g diet).

*Mice received 5×10^6 SRBC on day 14.

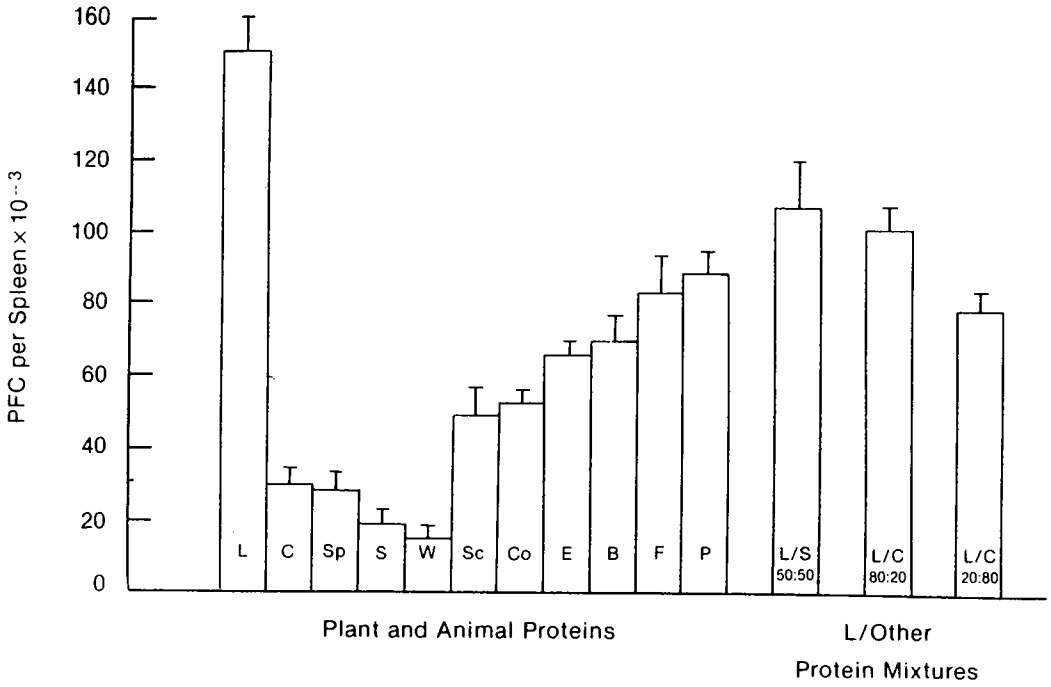

FIG. 1. Plaque forming cells/spleen (PFC) on the day showing peak production of PFC following immunization with 10^6 SRBC. Effect of 2 weeks of dietary treatment with 20 g/100 g diet of either lactalbumin (L), casein (C), *Spirulina maxima* protein (Sp), soy protein (S), wheat protein (W), *Scenedesmus* protein (Sc), corn (Co) protein, egg albumin (E), beef protein (B), fish protein (F), Purina mouse chow (P), or 20 g/100 g diet of a mixture containing 50% L and 50% S (L/S), or 80% L and 20% C, or 20% L and 80% C (L/C). Each value represents the mean ± SD. See text for statistical significance of differences.

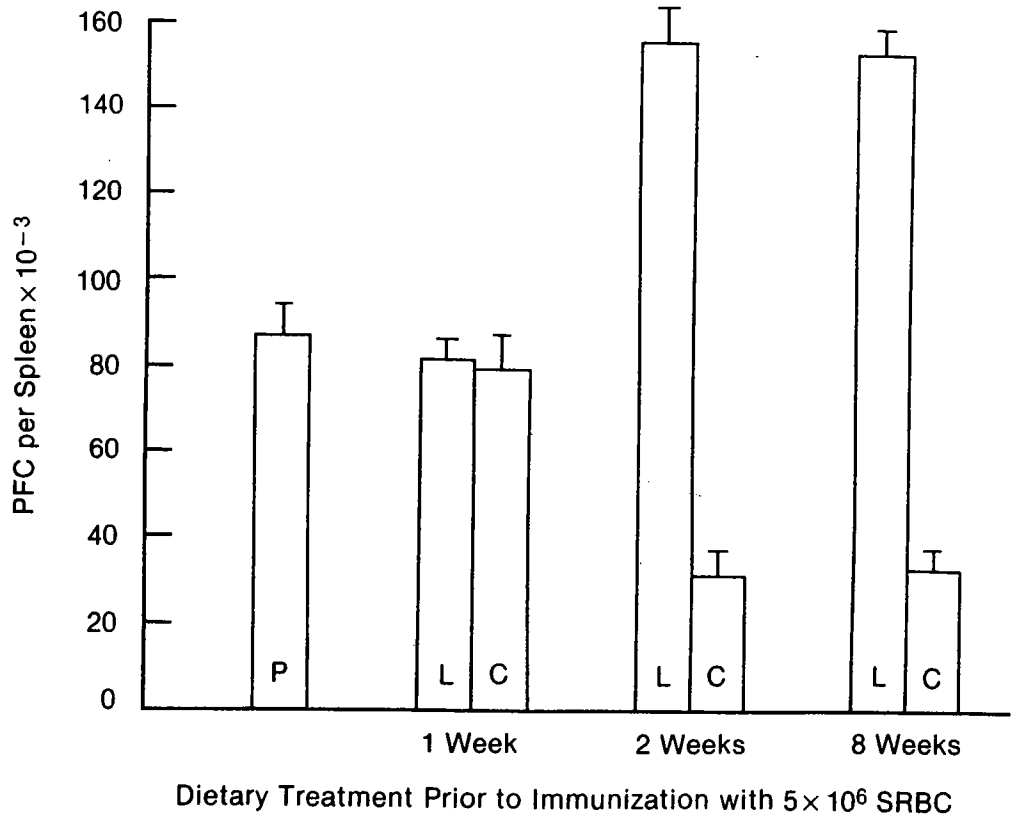

FIG. 2. Plaque forming cells/spleen (PFC) on the day showing peak production of PFC following immunization with 10^6 SRBC. Effect of 1, 2 or 8 weeks of dietary treatment with 20 g/100 g diet of either lactalbumin (L) or casein (C). Each value represents the mean ± SD. L *vs.* C after 2 weeks and 8 weeks: $P = 0.001$ or less.

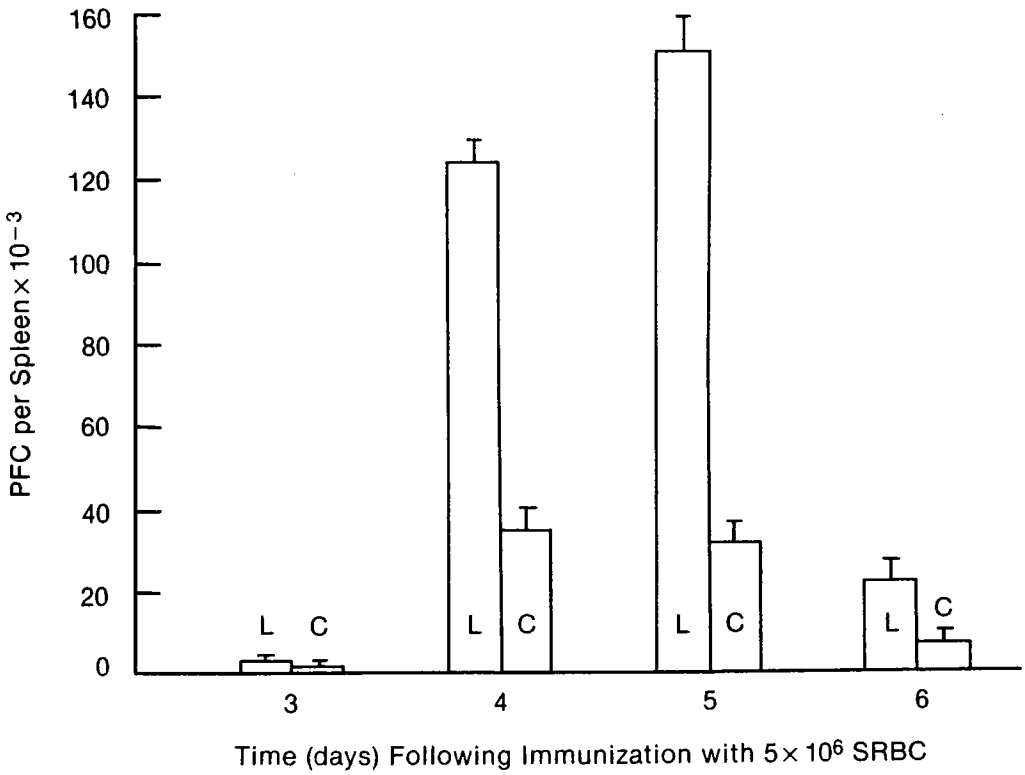

FIG. 3. Plaque forming cells / spleen (PFC) on days 3, 4, 5, and 6 following immunization with 10^6 SRBC. Effect of 2 weeks of dietary treatment with 20 g / 100 g diet of either lactalbumin (L) or casein (C). Each value represents the mean ± SD. L *vs* C on day 4 and 5 after immunization: $P = 0.001$ or less.

of lactalbumin in relation to casein was essentially maintained when these two proteins were replaced in diet by a free amino acid mixture duplicating the amino acid pattern of either lactalbumin or casein [11]. For these reasons we can assume that this phenomenon is not related to milk protein allergy or some other manifestation of oral immunization. The mean numbers of spleen cells in lactalbumin and Purina diet-fed groups were only 20% to 30% higher ($P < 0.0001$) than the corresponding values in casein, wheat, soy, fish and beef protein diet-fed groups. Thus the larger differences in the humoral immune response observed among different dietary groups could not be attributed to a non-specific effect of the diets on the total number of splenic lymphocytes. An incidental observation of note was that the immunoenhancing effect of lactalbumin was lost when we inadvertently added to the basal diet an expired vitamin supplement (Nutramigen vit-iron premix).

To our knowledge, only one other study has been reported on the influence of the type of protein in nutritionally similar and adequate diets on the immune response. Norton *et al.* [17] have recently shown that mice fed a corn protein diet produced, in response to sheep red blood cells, roughly twice the number of plaque forming cells as mice fed the corresponding casein diet, but the number was lower than that of mice fed Purina rodent chow. The same pattern of immune responses to sheep red blood cells in relation to corn protein, casein and Purina has been noted in our present experiments. Although the effect of lactalbumin was not investigated by these authors, the similarity between their results and ours for the other three dietary groups lends support to the validity of our current experiments. Studies performed at the Eppley Cancer Center in Nebraska [18, 19] are consistent with our findings on the immunoenhancing effect of dietary lactalbumin. Survival (resistance to spontaneous diseases) of hamsters of both sexes, measured over a 20 week period of feeding from four weeks of age, was best with 20 g lactalbumin / 100 g diet, in comparison with a 20 g / 100 g methionine and cysteine supplemented casein diet. Body weight gains were similar in both groups. In lifetime feeding studies, the mean and maximal longevity of female and male hamsters fed 10, 20, and 40 g lactalbumin / 100 g diet was increased in comparison with those fed commercial laboratory feed (estimated 24% protein from various sources). Survival was best with the 20% lactalbumin diet; in the males, longevity increased by 50%. No relationship was noted between food intake, maximal weight, and longevity. Here again it should be emphasized that the lactalbumin diet increased survival and longevity beyond that of "control" animals fed either of two nutritionally adequate reference diets, thus enhancing life expectancy beyond the limits traditionally assumed to be "normal".

Our current data on long term feeding (Fig. 2) are consistent with the concept that the immunoenhancing effect of dietary lactalbumin is not a short lived phenomenon and may indeed be felt as long as the protein is ingested.

As a practical consideration vis-à-vis using lactalbumin as a dietary supplement to enhance immunity in humans, we have evaluated the effect of mixing lactalbumin with other

proteins. Predictably, the addition of lactalbumin to either soy protein or casein significantly enhanced the immune response in comparison to that of mice fed 20 g of either soy protein or casein alone / 100 g diet. Our previous studies [11, 20] have indicated that the amino acid pattern of the ingested protein is an important factor in determining the effect of dietary protein type on the immune system. It is conceivable therefore, that the overall immunoenhancing effect of mixing different protein types in the diet would depend upon the ratio of each protein and the amino acid pattern of the individual protein. To the extent that the intensity of the immune response is influenced by dietary protein type, the presence of lactalbumin, beef and fish proteins in Purina chow could explain the relatively high immune response of Purina fed mice.

Our previous studies [20] have shown that bone marrow lymphocyte production is not stimulated by lactalbumin in comparison to casein feeding. The only significant effect that could be ascribed to the protein type was a change seen in the plasma amino acid profile, which essentially conformed to the amino acid composition of the ingested protein. This change was observed after 2 weeks of feeding, when the effect of lactalbumin on the immune response has become maximal.

Our previous studies have shown that the immunoenhancing effect of dietary lactalbumin is observed in at least 3 unrelated strains of mice [20, 21]. Moreover, a positive effect of dietary lactalbumin on resistance to spontaneous infection and longevity was reported in hamsters [18, 19]. These related observations seen in two different mammalian species suggest the possibility that the intake of lactalbumin might produce similar effects in man. No clinical trials have been reported so far on this subject although, since the time of Hippocrates and throughout the Middle Ages, whey has been prescribed in large doses (up to 2 litres / day, i.e. 12 g whey proteins) in the treatment of numerous ailments, especially acute septic conditions [22].

This concept appears to have been preserved in the following two proverbs* from the region of Florence: (1) if you want to live a healthy and active life, drink whey and dine early (chi vuol viver sano e lesto beve scotta e cena presto) and if everyone were raised on whey, doctors would be bankrupt (allevato con la scotta il dottore è in bancarotta).

In conclusion, mice fed a lactalbumin diet for at least 2 weeks exhibit a sustained enhancement of the humoral immune response to sheep red blood cells in comparison to mice fed most of the commercially available edible animal or plant proteins in formula diets of comparable nutritional efficiency. This effect persists as long as dietary treatment is continued (up to 2 months has been tested). Although the underlying mechanism remains unknown, the practical importance of these findings is self evident.

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Proverbi toscani, BELLONZI F, Giunti (Ed.), Florence, Italy, 1977.

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