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IMMUNOENHANCING PROPERTY OF DIETARY WHEY PROTEIN IN MICE: ROLE OF GLUTATHIONE†

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Abstract—The spleen cells immune response to sheep red blood cells of C3H/HeJ mice fed a 20 g whey protein/100 g diet is substantially higher than that of mice fed an equivalent casein diet of similar nutritional efficiency. The present study indicates that the observed immunoenhancing effect of the whey protein mixture is dependent on the overall amino acid pattern resulting from the contribution of all its protein components. Whey protein contains substantially more cysteine than casein. Dietary cysteine is considered to be a rate limiting substrate for the synthesis of glutathione which is necessary for lymphocyte proliferation. Our studies show that enhancement of host humoral immune response is associated with greater and more sustained production of splenic glutathione during the antigen driven clonal expansion of the lymphocyte in whey protein fed mice in comparison to mice fed the equivalent casein or the cysteine-enriched casein diet. Hence the efficiency of dietary cysteine in inducing supernormal glutathione levels is greater when it is delivered in the whey protein than as free cysteine. Administration of S-(n-butyl) homocysteine sulfoximine, which reduces splenic glutathione level by half, produces a 4–5 fold drop in the humoral immune response of whey protein diet-fed mice. This is further evidence of the important role of glutathione in the immunoenhancing effect of dietary whey protein.

Résumé—La réponse immunitaire de cellules spléniques de souris C3H/H_eJ à l'exposition à des érythrocytes de mouton est significativement plus prononcée lorsque les animaux reçoivent une diète contenant 20 g de protéines de petit lait que lorsque les animaux reçoivent une diète équivalente contenant de la caséine. Notre étude démontre que l'effet immunostimulant est relié au profil d'acides aminés du petit lait, c'est-à-dire de sa composition protéique. Celle-ci contient plus de cystéine que la caséine. L'apport diététique de cystéine représente une étape limitant la synthèse de glutathion, un facteur nécessaire à la prolifération lymphocytaire. Notre étude démontre que la stimulation de la réponse humorale est associée à une production plus marquée et plus soutenue de glutathion par les cellules spléniques au cours de l'expansion clonale déterminée par l'exposition antigénique, lorsque celle-ci est étudiée chez des animaux nourris de petit lait *vs* des animaux nourris de caséine enrichi de cystéine. La stimulation due à la cystéine est donc plus marquée lorsque celle-ci est administrée avec les protéines du petit lait. L'administration de S (n-butyl)-homocystéine sulfoximine reduit le niveau splénique de glutathion de moitié et réduit la réponse humorale de 4 à 5 fois chez les animaux nourris au petit lait. Ces résultats confirment le rôle central du glutathion dans l'effet immunostimulant du petit lait.

Key words : immunoenhancement, dietary whey protein, splenic glutathione

INTRODUCTION

OUR INTEREST in the effect of dietary amino acids on the immune system was prompted by the observation that minor changes in the amino acid profile of a defined formula diet can influence the immune response without having any significant effect on the nutritional status of the host [1]. It was subsequently discovered that indeed the type of protein (i.e. amino acid profile) in nutritionally adequate and similar diets can influence the intensity of the immune response. The humoral immune response (number of plaque forming cells to sheep red blood cells) is significantly higher in mice fed a 20 g whey protein concentrate/100 g diet than in mice fed formula diets containing 20 g/100 g diet of any other type of commercially available semipurified food protein such as casein, soy, wheat, corn, egg white, fish, beef protein, *Spirulina maxima, Scenedesmus* algae protein or Purina mouse chow [2–7]. This immunoenhancing effect of whey protein was observed in at least five unrelated strains of mice [3–8]. In mice not challenged with an immunogenic stimulus, the type of protein was found to have little or no effect on body growth, food consumption, serum levels of protein, minerals and trace metals, circulating leukocytes [3–6] and,

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TABLE 1. AMINO ACID COMPOSITION OF TEST PROTEINS* (G / 100 G PROTEIN)

Amino acid	Casein¶	Whey protein concentrate**
Phenylalanine	5.3 ± 0.2	3.4 ± 0.3
Tryptophan	1.4 ± 0.2	2.1 ± 0.0
Glycine	2.0 ± 0.1	2.0 ± 0.2
Serine	6.2 ± 0.5	5.2 ± 0.4
Leucine	10.0 ± 0.4	10.4 ± 0.7
Isoleucine	6.0 ± 0.6	6.1 ± 0.8
Valine	7.1 ± 0.3	5.8 ± 0.8
Methionine	2.9 ± 0.2	2.1 ± 0.3
Cysteine	0.3 ± 0.1	2.3 ± 0.3
Aspartic acid	7.3 ± 0.1	10.7 ± 0.7
Glutamic acid	22.9 ± 0.3	18.8 ± 0.7
Histidine	3.0 ± 0.1	2.0 ± 0.2
Tyrosine	6.0 ± 0.1	3.0 ± 0.4
Proline	11.6 ± 0.4	6.1 ± 0.7
Arginine	4.0 ± 0.1	2.8 ± 0.3
Alanine	3.1 ± 0.3	4.9 ± 0.4
Lysine	8.2 ± 0.1	9.2 ± 0.5
Threonine	4.6 ± 0.3	6.8 ± 1.3

*Value expressed as Mean ±S.D. of data from reliable sources¶,**.

¶Reference 14, 15, and 16.

**Reference 14, 17, 18, and 19.

more specifically, the genesis of bone marrow B lymphocytes [6]. Recently we have demonstrated inhibition of the carcinogenic effect of dimethylhydrazine in mice on whey protein [8]. The exact mechanism responsible for the observed effects of dietary whey protein on the immune system is not known, but to date has been detected most clearly during the antigen-driven clonal expansion of the B-lymphocytes, in the development of humoral immunity. The mode of anticarcinogenic action of whey protein has not been investigated.

The present study was designed to investigate the mechanism and the factors responsible for the observed immunoenhancing effect of dietary whey protein. Despite the fact that whey protein contains about eight times more cysteine than does casein (Table 1), there is no elevation in plasma cysteine in whey protein-fed mice in comparison to the casein-fed counterpart, even though the plasma profile of most other amino acids was found to essentially conform to that of the ingested protein [6–7]. Glutathione is a tripeptide that is central in cellular protection against oxygen radicals and a wide spectrum of xenobiotics. It also maintains cellular proteins in a functional state. Glutathione is synthesized in a series of enzymic steps. The rate limiting enzyme is γ-glutamyl cysteine synthetase and intracellular cysteine is a rate limiting substrate [9]. It is thus conceivable that cysteine in the whey protein diet affects glutathione synthesis and storage. It has been shown that adequate intracellular levels of glutathione are necessary for lymphocyte proliferation [10] and lymphocytes exposed to sulfhydryl oxidizing agents have a decreased proliferative response to mitogen [10, 11]. More recently, dietary glutathione was found to reverse age-associated decline in the immune-responsiveness of mice [12].

We have assessed the effect of whey protein diet compared to casein diet in mice on plaque forming cells response and splenic glutathione. A third diet studied consisted of casein with added free L-cysteine adequate to mimic the cysteine content of the whey protein diet. To further confirm the impact of whey protein on splenic glutathione and its relation to plaque forming cells response, we have observed the effect of glutathione depletion by buthionine sulfoximide (BSO) on the plaque forming cells response in whey protein fed mice. BSO inhibits γ-glutamyl cysteine synthetase specifically.

Whey proteins represent the group of milk proteins that remain soluble in "milk serum" or whey after precipitation of casein at pH 4.6 and 20°C, in the manufacture of cheese. Beta-lactoglobulin, alpha-lactalbumin, serum albumin, and immunoglobulin are considered to be the major components of the bovine whey protein mixture [13]. We have thus investigated the effect of each major component of whey protein on the plaque forming cells response as well as the effect on the immune response of whey protein when fed as a pancreatic hydrolysate or in the form of free amino acid mixture.

MATERIALS AND METHODS

Animals

Male C3H/HeJ mice were obtained from Jackson Laboratories (Bar Harbor, Maine) at seven weeks of age and were maintained five per cage in a temperature controlled 12 hour light-dark cycle room.

Diets

The amino acid composition of bovine whey protein concentrate and casein is given in Table 1, which shows the grand mean of all data from reliable sources including the samples used in our study [14–19]. The detailed composition of the common ingredients (vitamins and minerals) in all of the defined formula diets is given in Table 2. Diets were

TABLE 2. VITAMIN AND MINERAL CONTENT OF FORMULA 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, 53.3; niacin, 5.1; riboflavin, 0.38; thiamin, 0.34; folic acid, 0.063; vitamin B-6, 0.26; biotin, 0.031; pantothenic acid, 1.93; choline, 44; *and per 100 g diet*: retinyl palmitate, 1295 IU; ergocalciferol, 260 IU; vitamin E (*dl*-tocopheryl acetate), 11.6 IU; vitamin B-12, 0.001 mg; and vitamin K (phylloquinone), 0.06 mg. The mineral content of ions or cations (expressed in milligrams per 100 g diet) and the actual chemical compounds fed were:

Ca, 350 ($CaHPO_4 \cdot 2H_2O$ and $Ca_3(C_6H_5O_7)_2 \cdot 4H_2O$); P, 260 ($K_2HPO_2 \cdot 2H_2O$); Fe, 7.9 ($FeSO_4 \cdot 2H_2O$); Mg, 63.2 (MgO); Cu, 0.31 ($CuSO_4$ $5H_2O$); Zn, 3.5 ($ZnSO_4 \cdot 7H_2O$); Mn, 0.48 ($MnSO_4$); Cl, 1108 ($C_5H_{14}CINO$); K, 997 ($K_2HPO_4 \cdot 2H_2O$); Na, 232 (NaCl).

prepared in the following way: 20 g of selected pure protein, 56 g of product 80056 protein free diet powder containing corn syrup, corn oil, tapioca starch, vitamins and minerals (Mead-Johnson Co. Inc., Indiana, USA), 18 g cornstarch, 2 g wheat bran, 0.05 g Nutramigen vit-iron premix (Bristol-Meyers, Ontario), 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: bovine whey protein concentrate, Lacprodan-80 (Danmark Protein, Worthington, Ohio); casein (Bristol-Meyers of Canada, Ontario); casein with the addition of 2.6 g of L-cysteine HCl H_2O/100 g casein (Sigma Chemical Co. Ltd., MO) in order to raise the cysteine level of casein to that found in whey protein (for this, the relative weight of the HCl and H_2O moities was taken into account); beta-lactoglobulin (βL) (Sigma Chemical Co. Ltd., MO); alpha-lactalbumin (αL) (courtesy of the Dairy Bureau of Canada); gamma-globulin (γG) (Sigma Chemical Co. Ltd., MO); or bovine serum albumin (SA) (Sigma Chemical Co. Ltd., MO). All purified proteins were vitamin free. The net protein content of the different protein powders was taken into consideration when preparing the various 20 g protein/100 g diets. In other experiments whey protein and casein in diet were replaced by an equal amount of the corresponding enzymatic hydrolysate (40% free amino acid and 60% oligopeptides [450–1000 mwt]) or by a free amino acid mixture duplicating the composition of either whey protein or casein. Diets were continuously available in powder form from stainless steel feeders, 1.5 in high and especially designed to reduce spillage and spoilage. Mice were placed on the various diets and immunologic studies or spleen glutathione assays commenced 3 weeks later.

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.

Plaque forming cell (PFC) assay

The method used for assaying IgM plaque forming cells was as described by Cunningham and Szenberg [20] with 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). 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) 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 the total number of plaque forming cells per spleen estimated by multiplying the number of plaque forming cells in each sample (0.05 ml spleen cells) by 300. Plaque forming cells have been 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 normally assayed for the plaque forming cells response to sheep red blood cells on the fifth day after immunization, when the response was shown to peak, or in the kinetic study, on days 3, 4, 5, and 6 post-immunization.

Spleen glutathione content

Ninety milligrams of mouse spleen were weighed using a Mettler PM-300 balance and samples varied from 90 mg by less than 5 mg (<5%). The samples were then homogenized in 5-sulfosalicylic acid (5% w/v). Homogenates were centrifuged for 5 min in a microfuge at 10,000 × g. The assay was carried out using the supernatants on the same day according to the methods of Anderson [21]. Values are expressed as μmol per g/wet tissue.

Buthionine sulfoximine experiments

In some experiments, following three weeks of whey protein feeding and one day prior to immunization with sheep red blood cells, mice were injected i.p. with 450 mg/kg of buthionine sulfoximine (BSO) (S-[n-butyl] homocysteine sulfoximine), a specific inhibitor of γ-glutamylcysteine synthetase. At the same time 20 mM of BSO was added to the drinking water.

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 [22]. Each dietary group comprised at least ten mice.

RESULTS

In Table 3 data are reported on the nutritional efficiency of the various diets. Mice fed these diets increased in body weight by approximately the same amount. Total serum proteins were also similar.

As indicated in Figure 1, mice fed the whey protein diet for three weeks exhibit an immune response significantly higher than that of mice fed other protein types. The mean number of plaque forming cells per spleen at 5 days (peak response) after i.v. injection with 5×10^6 sheep red blood cells in the whey protein diet-fed mice was 499% and 403% of that noted in casein and casein + cysteine diet-fed mice respectively. These differences are all statistically significant ($p = 0.0004$). No significant difference was noted between casein diet-fed and casein + cysteine diet-fed mice. When protein

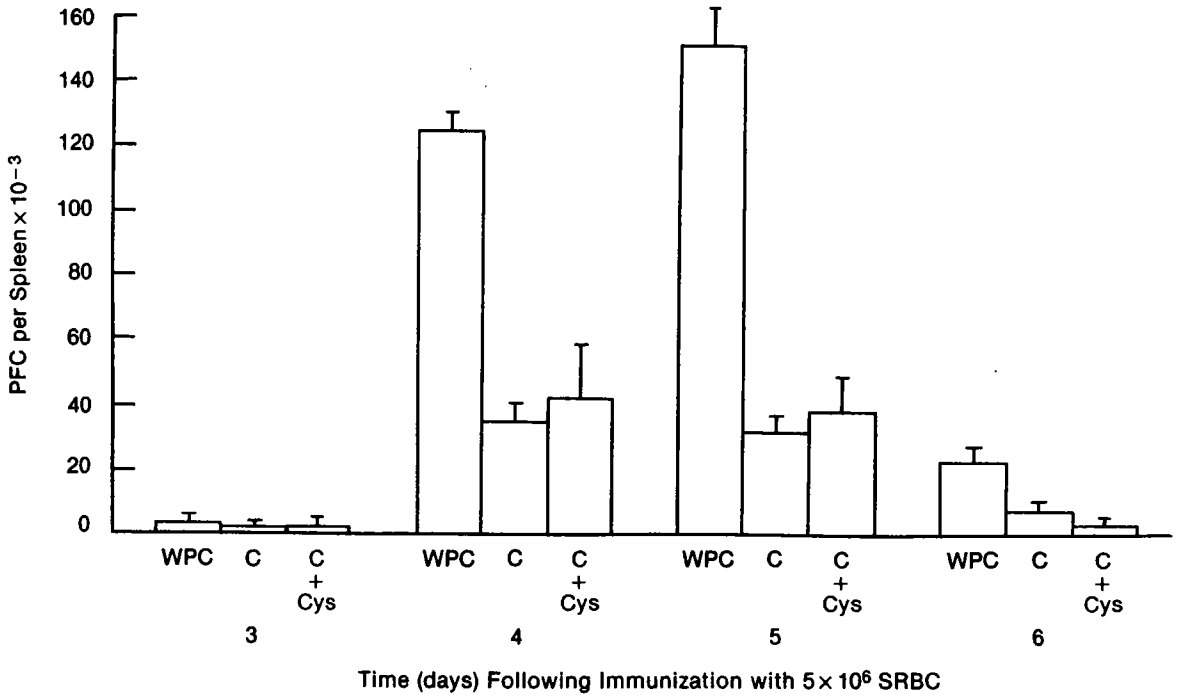

FIG. 1. Plaque forming cells / spleen (PFC) on day 3, 4, 5, and 6 following immunization with 10^6 sheep red blood cells (SRBC). Effect of 3 weeks of dietary treatment with 20 g / 100 g diet of either whey protein concentrate (WPC), casein (C), or casein and cysteine (C + Cys). Each value represents the Mean ± Standard Deviation. Whey protein *vs* casein or casein + cysteine on days 4 and 5 after immunization: $P < 0.001$.

hydrolysate was given, the plaque forming cells response in mice fed the whey protein diet was found to be 504% of that noted in the casein diet-fed mice ($p = 0.0004$) (Fig. 2). When free amino acid mixture was given, the plaque forming cells response in mice fed the whey protein amino acid diet was found to be 332% of that of the casein amino acid diet-fed counterpart ($p = 0.0001$) (Fig. 2). Because milk immunoglobulin is not commercially available we have instead used bovine gamma globulin (γG), since all immunoglobulins in milk are normally in serum, although their relative concentration may vary [13]. Our results (Fig. 2) indicate that animals fed diets containing 20 g/100 g diet of any one of the four major components of whey protein (βL, αL, γG, SA) developed a plaque forming cells response to sheep red blood cells inferior to ($p = 0.0002$) that of mice fed a diet containing 20 g whey protein/100 g diet.

No significant differences were seen among dietary groups in splenic glutathione level of unimmunized mice (3.12 ± 0.32 [Mean ± Standard Deviation], 3.07 ± 0.33, 3.11 ± 0.29, μmol/g in whey protein diet-fed, casein diet-fed or casein + cysteine diet-fed mice respectively). However on days 2, 3, 4, and 6 after immunization, the glutathione levels in whey protein diet-fed mice were 13% ($p < 0.005$), 7% ($p < 0.025$), 21% ($p < 0.0005$), and 21% ($p < 0.0005$) higher than the corresponding values in casein diet-fed mice. On days 4 and 6 after immunization glutathione levels in whey protein diet-fed mice were actually 12% ($p < 0.005$) and 17% ($p < 0.0005$) above levels in whey protein fed unimmunized mice, and at no time were they found to be below levels of unimmunized mice fed the whey protein diet. In the casein + cysteine diet-fed mice glutathione values were above the corresponding casein diet-fed mice values only on day 4 (23%, $p < 0.0005$) post-immunization. The addition of cysteine to the casein diet increased the glutathione level only

TABLE 3. EFFECT OF THREE WEEKS DIETARY REGIMEN ON FOOD CONSUMPTION, BODY GROWTH AND TOTAL SERUM PROTEIN*

Protein type	Average food consumption	Average body weights		Average serum protein
(20 g / 100 g diet)	(g / mouse / 24 hr)	Original weight (g)	Final weight¶%	(g / dl)
Whey protein concentrate	3.1 ± 0.1	20.3 ± 1.6	134.2 ± 12.5	5.8 ± 0.2
Casein	2.9 ± 0.3	20.5 ± 1.1	130.6 ± 9.1	6.1 ± 0.4
Casein and cysteine	3.2 ± 0.5	21.5 ± 1.2	126.2 ± 9.8	6.0 ± 0.3
Whey protein concentrate + buthionine sulfoximine	2.8 ± 0.2	19.7 ± 1.0	125.0 ± 8.1	5.9 ± 0.4

*Values expressed as Mean ± Standard Deviation.
¶As percentage of original weight (grand Mean of all experiments).

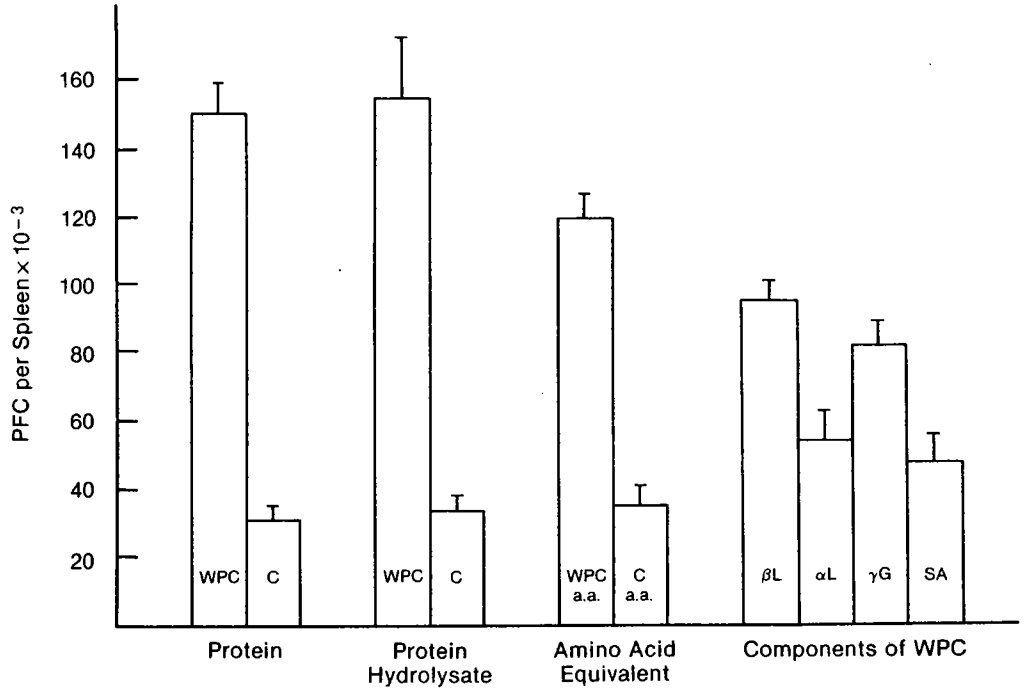

FIG. 2. Plaque forming cells / spleen (PFC) on day showing peak production of plaque forming cells following immunization with 10^6 sheep red blood cells (SRBC). Effect of 3 weeks of dietary treatment with 20 g / 100 g diet of either whey protein concentrate (WPC), casein (C), whey protein concentrate hydrolysate, casein hydrolysate, whey protein concentrate amino acid equivalent (WPC.a.a.), casein amino acid equivalent (C.a.a.), beta-lactoglobulin (βL), alpha-lactalbumin (αL), gamma-globulin (γG), or bovine serum albumin (SA). Each value represents the Mean ± Standard Deviation. See text for statistical significance.

on day 4 post-immunization, compared to the value of corresponding unimmunized mice (14%, $p < 0.005$), and on day 3 it was actually 7% below preimmunization level. In the casein diet-fed mice, glutathione values declined, on day 2 following immunization, to 93% ($p < 0.05$) of preimmunization level and never arose above values in unimmunized mice. It is noteworthy that at the time of peak plaque forming cells response (day 5 after immunization), no difference was seen among dietary groups in splenic glutathione values (Fig. 3). We have no explanation for this phenomenon at present. Statistical analysis was done on absolute glutathione concentration.

The administration of BSO to whey protein diet-fed mice resulted in a 50% ($p < 0.0005$) drop in splenic glutathione on the day showing highest glutathione values after immunization and a 4–5 fold ($p < 0.0005$) decrease in plaque forming cells response on days showing highest values after immunization (Fig. 4). There was a marginal effect on body growth (7% drop, $p < 0.005$) but no effect on spleen weight. The plaque forming cells response in mice fed whey protein diet + BSO is similar to that of casein diet-fed mice (Fig. 4). No significant difference was seen amongst dietary groups in average spleen weight (data not shown).

DISCUSSION

Our previous studies have shown that mice fed the above described 20 g protein/100 g diets or Purina mouse chow [4–7], or 20 g protein hydrolysate [3] or amino acid equivalent [7]/100 g diet, increased in body weight by approximately those same amounts, with similar food consumption and serum protein values. The data presented in Table 3 are consistent with the concept that the test diets are nutritionally similar and are adequate in terms of body weight growth and serum protein maintenance. However, after challenging mice with an immune stimulus and measuring the specific humoral immune response to sheep red blood cells (Fig. 1), it was observed that the humoral immune response of mice fed the whey protein diet was almost five times greater than the corresponding values for mice fed the casein diet and the cysteine enriched casein diet. This impressive enhancement of the plaque forming cells response cannot be ascribed to presensitization of the whey protein diet-fed group with cross-reacting antigens present in whey protein, because our previous studies [4] showed only very low numbers of plaque forming cells per spleen in non-immunized mice and, moreover, these did not differ between dietary groups. In addition, our current studies show that the immunoenhancing effect of whey protein in comparison to casein is maintained when these two proteins are replaced in formula diets by either a hydrolysate (free amino acid and oligopeptides with mwt < 1000) or, to a lesser degree, a free amino acid mixture duplicating the amino acid pattern of either whey protein or casein (Fig. 2). These related observations appear to obviate the likelihood that any protein component or fragment other than the actual concentration and type

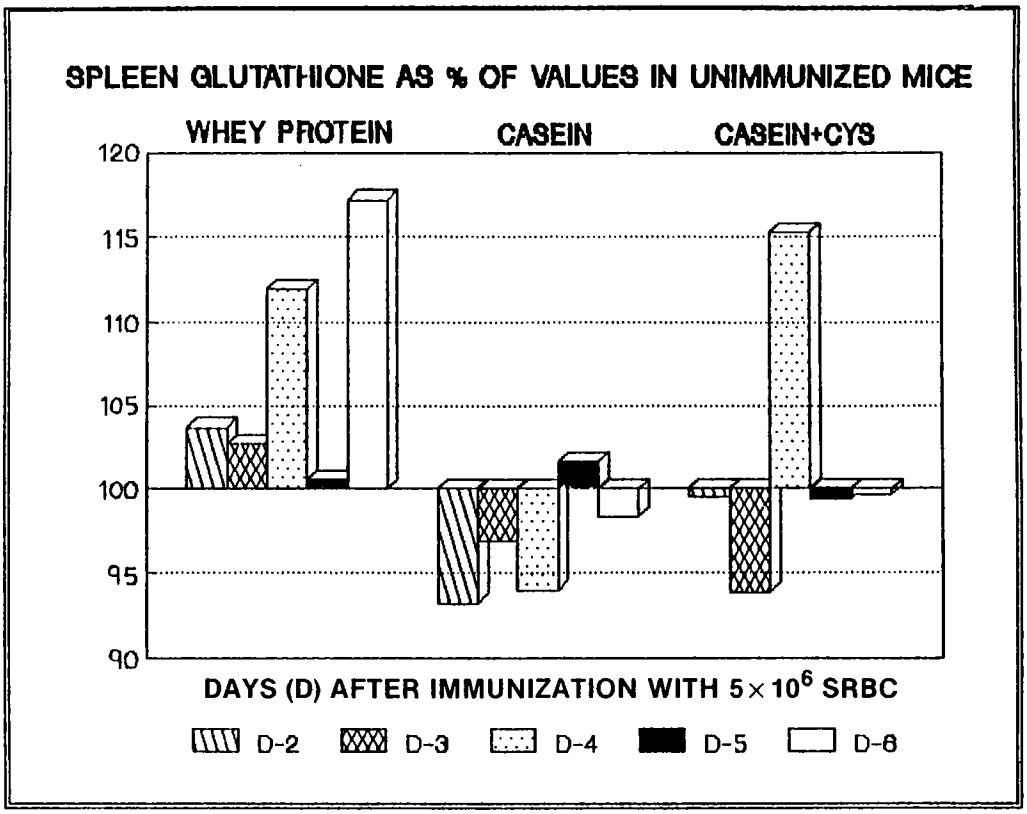

FIG. 3. Spleen glutathione levels expressed as % of values in unimmunized mice fed the corresponding diet for 3 weeks. Effect of 3 weeks dietary treatment with 20 g / 100 g diet of either whey protein concentrate (WPC), or casein (C), or casein + cysteine (C + cysteine). D: day following immunization with 5×10^6 sheep red blood cells (SRBC). Each value represents the Mean of 10 mice. See text for statistical analysis.

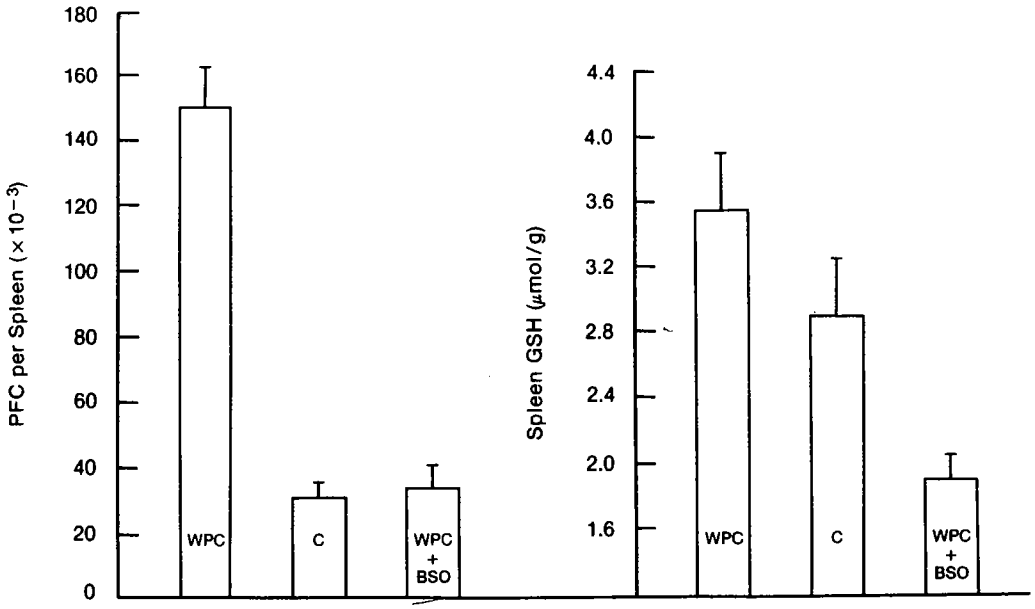

FIG. 4. Left: Plaque forming cells / spleen (PFC) on day 5 showing peak production of plaque forming cells following immunization with 10^6 sheep red blood cells (SRBC). Effect of 3 weeks of dietary treatment with 20 g / 100 g diet of either whey protein concentrate (WPC), whey protein concentrate + buthionine sulfoximine (BSO) (WPC + BSO) or casein (C). Each value represents the Mean ± Standard Deviation: whey protein *vs* whey protein + BSO or casein: $P < 0.0005$. Right: Spleen glutathione (GSH) on day 4 showing peak levels of glutathione following immunization with 5×10^6 sheep red blood cells (SRBC). Effect of 3 weeks of dietary treatment with 20 g / 100 g diet of either whey protein concentrate (WPC), whey protein concentrate + BSO (WPC + BSO) or casein (C). Each value represents the Mean ± Standard Deviation. Whey protein *vs* whey protein + BSO or casein: $P < 0.0005$.

of amino acid or some very small peptides acts as a crucial factor in inducing the enhanced immune responsiveness associated with the whey protein diet. Our results also indicate that mice fed diets containing any one of the four major protein components of the whey protein mixture developed a plaque forming cells response to sheep red blood cells significantly less than that of mice fed the corresponding whey protein mixture. We can thus conclude that the observed immunoenhancing effect of whey protein is dependent upon the overall amino acid pattern resulting from the contribution of all its protein components, and is not related to milk protein allergy or some other manifestation of oral immunization.

We have attempted to identify the changes induced by dietary protein type which might directly or indirectly affect the humoral immune responsiveness. In mice not challenged with an immunogenic stimulus, the type of protein in the diet was found to have little or no effect on a variety of parameters examined, including body growth, food consumption, serum protein, minerals and trace metals, and circulating leukocytes; more specifically, the genesis of bone marrow B lymphocytes were all within normal limits [3–7]. This confirms that at 20 g/100 g diet concentration, the proteins provide an adequate daily supply of essential amino acid for the growing mice [23]. As previously reported [6, 7], the only significant effect of protein type is a change in plasma amino acid profile which conforms to the amino acid composition of the ingested protein, except for cysteine. In spite of an 8-fold higher cysteine content in the whey protein mixture, the plasma level of cysteine in whey protein diet-fed mice was not different from that in their casein diet-fed counterparts. This observation is consistent with clinical data showing that in infants fed either a whey predominant formula or a casein predominant formula, no significant differences in plasma cysteine concentration, unlike that of other amino acids, were observed among groups over any time period [24]. Dietary cysteine is a rate limiting substrate for the synthesis of glutathione [9], which is necessary for lymphocyte proliferation. The redox state of the lymphocyte can modulate the intracellular concentration of cyclic GMP [25], which is known to be intimately involved in lymphocyte proliferation [26]. Recently, Fidelus [27], with the use of cysteine delivery agents like 2-oxothiazolidine-4-carboxylate (OTZ) and 2-mercapto-ethanol, was able to enhance *in vitro* both intracellular glutathione concentration and mouse splenic lymphocyte Con-A induced proliferation. These data suggest that modulation of intracellular glutathione may indeed affect immune responsiveness. With increasing age, there is a corresponding decrease in both intracellular glutathione concentrations and response to mitogenic stimulation of lymphocytes. Enhancement of intracellular glutathione by glutathione feeding appears to reverse the age associated decline in immune responsiveness in mice [12].

In our studies we demonstrate a significant difference between casein and whey protein diets' effects on splenic glutathione concentration during the oxygen requiring antigen driven clonal expansion of the lymphocyte, and following that expansion, in the development of humoral immunity. This might reflect the ability of the lymphocytes of whey protein diet-fed mice to offset potential oxidative damage, thus responding more fully to the antigenic stimulus. When free cysteine is added to the casein diet to mimic the whey protein cysteine level, glutathione elevation in the spleen occurs only on day 4 following immunization. The observed enhancement of plaque forming cells response is associated with greater and/or more consistent production of splenic glutathione in immunized mice fed whey protein diet in comparison to mice fed the casein or casein + cysteine diet. The efficiency of dietary cysteine in inducing supernormal and immune effective glutathione levels appears, then, to be superior when delivered in the whey protein rather than as free cysteine. Administration of BSO specifically inhibits γ-glutamylcysteine synthetase [28], thus lowering cellular glutathione content. Our data clearly indicate that BSO treatment, while reducing in half spleen glutathione level, produces a 4–5 fold drop in the immune response of whey protein diet-fed mice, thereby abolishing the difference in immune response between whey protein diet and casein diet-fed mice. The effect of BSO is further evidence of the predominant role of glutathione in the immunoenhancing effect of whey protein diet. Our results are consistent with recent data by Furukawa *et al.* [12], who studied the effect of glutathione feeding in aging mice. These authors showed that dietary glutathione supplementation failed to significantly alter the glutathione content of unchallenged spleen cells. However, 68 hours after incubation with Con A, splenocytes from glutathione-fed mice had about 40% more glutathione than spleen cells from control mice that exhibited, instead, a drop in glutathione content after mitogen challenge. Moreover, dietary glutathione supplementation partially reversed the age-associated decline in immune responsiveness. Hence, it appears that lymphocyte glutathione levels and immune responsiveness can be influenced by feeding either the rate limiting precursor of glutathione in whey protein or the actual tripeptide. To the extent that cysteine can be considered a factor in promoting higher immune response and glutathione tissue levels, our studies show that the administration of cysteine in the whey protein mixture is far more effective than when administered as free cysteine. It is conceivable that the specific amino acid profile of whey protein or a cysteine containing peptide is an important factor in determining the fate of the ingested cysteine. This assumption is supported by the observation that the immunoenhancing effect of whey protein is essentially maintained when administered as hydrolysate, whereas the addition of free cysteine to a casein diet fails to produce any significant effect on immune responsiveness.

This newly discovered immunoenhancing mechanism of dietary whey protein does not appear to be a short-lived phenomenon of little consequence. Indeed, it was found to reach its peak after two weeks and to persist as long as dietary treatment is continued (tested up to two months) [29]. Since glutathione is central in a variety of reactions detoxifying potentially toxic and/or carcinogenic xenobiotics, the impact of whey protein on this system could have potential implications beyond the immune system alone.

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REFERENCES

1. BOUNOUS G, KONGSHAVN PAL: The effect of dietary amino acid on immune reactivity. *Immunology* **35**: 257–66, 1978.
2. BOUNOUS G, STEVENSON MM, KONGSHAVN PAL: Influence of dietary lactalbumin hydrolysate on the immune system of mice and resistance to *Salmonellosis*. *J Infect Dis* **144**: 281, 1981.
3. BOUNOUS G, KONGSHAVN PAL: Influence of dietary proteins on the immune system of mice. *J Nutr* **112**: 1747–55, 1982.
4. BOUNOUS G, LETOURNEAU L, KONGSHAVN PAL: Influence of dietary protein type on the immune system of mice. *J Nutr* **113**: 1415–21, 1983.
5. BOUNOUS G, KONGSHAVN PAL: Differential effect of dietary protein type on the B-cell and T-cell immune response in mice. *J Nutr* **115**: 1403–8, 1985.
6. BOUNOUS G, SHENOUDA N, KONGSHAVN, PAL, OSMOND DG: Mechanism of altered B-cell response induced by changes in dietary protein type in mice. *J Nutr* **115**: 1409–17, 1985.
7. BOUNOUS G, KONGSHAVN PAL: Influence of protein type in nutritionally adequate diets on the development of immunity. In: FRIEDMAN M, ed. Absorption and utilization of amino acids. Boca Raton, Florida: CRC Press. 1989: in press.
8. BOUNOUS G, PAPENBURG R, KONGSHAVN PAL, GOLD P, FLEISZER D: Dietary whey protein inhibits the development of dimethylhydrazine induced malignancy. *Clin Invest Med* **11**: 213–7, 1988.
9. TATEISHI N, HIGASHI T, SHINYA S, NARUSE A, SAKAMOTO Y: Studies on the regulation of glutathione level in rat liver. *J Biochem* **75**: 93–103, 1974.
10. NOELLE RJ, LAWRENCE DA: Determination of glutathione in lymphocyte and possible association of redox state and proliferative capacity of lymphocytes. *Biochem J* **198**: 571–9, 1981.
11. CHAPLIN DD, WEDNER HJ: Inhibition of lectin-induced lymphocyte activation by diamide and other sulfhydryl reagents. *Cell Immunol* **36**: 303–11, 1978.
12. FURUKAWA T, MEYDANI SN, BLUMBERG JB: Reversal of age-associated decline in immune responsiveness by dietary glutathione supplementation in mice. *Mechanisms of Aging and Development* **38**: 107–17, 1987.
13. EIGEL WN, BUTLER, JE, ERNSTROM CA, FARRELL, HM, HARWALKAR VR, JENNES R, WHITNEY R: Nomenclature of protein of cow's milk. Fifth rev. *J Dairy Sci* **67**: 1598–631, 1984.
14. Dairy Bureau of Canada, 1987.
15. US Department of Agriculture: Values calculated from amino acid content of foods. 1957.
16. FAO: Amino acid content of foods. FAO Nutritional Studies **24**. Rome: FAO, 1970.
17. Agricultural Research Service. Composition of foods: dairy and egg products – raw, processed, prepared. Agricultural Handbook **1**. Washington, D.C.: US Department of Agriculture, 1976.
18. Ross Laboratories: Meeting of the nutritional needs of full-term infants. Columbus, Ohio: Ross Laboratories, 1985.
19. Danmark protein. Dairy Specialty. Worthington, Ohio, 1981.
20. CUNNINGHAM AJ, SZENBERG A: Further improvements in the plaque technique for detecting single antibody-forming cells. *Immunology* **14**: 599–600, 1968.
21. ANDERSON ME: Tissue glutathione. In: CRC Handbook of Methods for Oxygen Radical Research. Boca Raton, Critical Review: 1985: 317–29.
22. BROWN MB, FORSYTHE AB: The small sample behaviour of some statistics which test the equality of several means. *Technometrics* **16**: 129, 1974.
23. JOHN AM, BELL JM: Amino acid requirements of the growing mouse. *J Nutr* **106**: 1361–7, 1976.
24. JANAS LM, PICCIANO MF, HATCH TF: Indices of protein metabolism in term infants fed human milk, whey-predominant formula, or cow milk formula. *Pediatrics* **75**: 775–84, 1985.
25. GOLDBERG ND, GRAFF G, HADDOX MK, STEPHENSON JH, GLASS DG, MOSER ME: *Adv. Cyclic Nucleotide Res* **9**: 101–3, 1978.
26. STROM TB, LUNDIN AP, CARPENTER GB: The role of cyclic nucleotides in lymphocyte activation and function. *Prog Clin Immunol* **3**: 115–53, 1977.
27. FIDELUS RK, TSAN MF: Glutathione and lymphocyte activation: A function of aging and auto-immune disease. *Immunology* **61**: 503–8, 1987.
28. GRIFFITH OW, ANDERSON ME, MEISTER A: Inhibition of glutathione biosynthesis by prothionine sulfoximine (S-n-propylhomocysteine sulfoximine), a selective inhibitor of γ-glutamylcysteine synthetase. *J Biol Chem* **254**: 1205–10, 1979.
29. BOUNOUS G, KONGSHAVN PAL, GOLD P: The immunoenhancing property of dietary whey protein concentrate. *Clin Invest Med* **11**: 271–8, 1988.