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Influence of severe protein malnutrition on rat lacrimal, salivary and gastrointestinal immune expression during development, adulthood and ageing

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SUMMARY

The objective of the present study was to examine and to compare the impact of severe protein malnutrition during development, adulthood and ageing on secretory immune expression in the eye, mouth and small intestine. In addition, we sought to determine whether potential abrogation of mucosal immunity by protein deprivation might be reversed by the administration of a balanced diet. Weanling, adult and aged rats were provided isocaloric diets containing 24% (control), 19%, 14%, 10%, 6% and/or 3·2% protein levels for defined periods and various immunological parameters were evaluated before, during and after the dietary regimen. Our results demonstrated the following. (1) Severe protein malnutrition (3·2%) dramatically suppressed the secretory immune system in eyes of weanling rats. After 8 weeks of protein insufficiency, tear IgA concentrations in young rats had undergone a precipitous decrease, such that IgA could not be detected in tears. This response was paralleled by a significant decline in the tear volume, tear secretory component (SC), IgG and total protein content, number of IgA-containing cells in lacrimal tissue, as well as the amounts of SC and/or IgA in saliva, intestinal secretions and serum. In contrast, the immunological effects of protein malnutrition in adult or aged animals varied considerably depending upon the specific mucosal site. (2) The influence of protein deprivation was dose dependent and reversible: maintenance of weanling rats on 10%, 6% or 3·2% protein diets interfered with the establishment of ocular and intestinal mucosal immunity, but later administration of optimal diets to these malnourished animals permitted a rapid immune recovery. (3) The impact of protein malnutrition on tear IgA levels in weanling animals, as shown by pair-feeding experiments, appeared to reflect primarily protein deficiency and not caloric restriction. Overall, these findings show that dietary protein plays a significant, site-specific role in the developmental expression of the secretory immune system.

INTRODUCTION

Over the past 4 decades, considerable research effort has been focused upon the inter-relationship between nutrition and systemic immunity. These studies have demonstrated that the functional integrity of the immune system is extremely dependent upon optimal nutrition.[1,2] Thus, severe malnutrition (e.g. protein–calorie) may significantly diminish both cell-mediated and humoral immunity and dramatically decrease resistance of the host to bacterial, viral, fungal and parasitic infections.[1,2] In turn, infectious disease may exacerbate the impact of concurrent malnutrition.[1] Consequently, given estimates that over 500 million people suffer from diverse nutritional deficiencies,[3] it is not surprising that protein–calorie malnutrition is the most common form of acquired immunodeficiency world-wide.[2]

The influence of malnutrition on systemic immunity appears to be substantially increased in immunologically compromised individuals, such as very young children and the elderly.[2,4] Ageing, for example, is accompanied by a degeneration of lymphoid tissue, a marked decline in systemic immunity and a progressive increase in the incidence of infectious and autoimmune disease.[5] In fact, a striking parallel has been observed between immune dysfunction secondary to ageing and that resulting from nutritional deficiencies.[5] This synergism, when expressed in immunologically impaired elderly populations, in whom various forms of malnutrition are increasingly prevalent, may enhance vulnerability to infection and predispose to a fatal outcome.[1]

However, despite the importance of nutrition in systemic immune function, relatively few reports have addressed the

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impact of dietary disorders on secretory immunity, which protects mucosal surfaces against invasive and toxic organisms.[6] This latter nutritional research has concentrated almost entirely on protein deprivation in young children or animals: these populations may have incomplete development of their secretory immune system[7] and are highly susceptible to infectious disease.[8,9] In contrast, the question of whether malnutrition and ageing synergize to erode defensive barriers of the secretory immune system remains to be clarified. Moreover, whether nutritional disorders uniformly, or selectively, affect immunity in different mucosal sites has yet to be fully explored.

Therefore, the objective of the current study was to examine and to compare the influence of severe protein malnutrition during development, adulthood and ageing on secretory immune expression in the eye, mouth and small intestine. In addition, we sought to determine whether potential suppression of mucosal immunity by malnutrition might be reversed by the administration of a balanced diet.

MATERIALS AND METHODS

Animals and dietary provisions

Male Sprague-Dawley rats were obtained from Zivic-Miller Laboratories, Inc. (Allison Park, PA) and housed in constant temperature rooms with light/dark intervals of 12 hr duration. For experimentation, three age groups were utilized: weanling (21 days old), adult (3·5 months old) and aged (16·5 months old). Weanling animals were removed from foster mothers at 21 days of age, immediately before the provision of designated diets. Purified test diets were obtained from Purina Mills, Inc. (Richmond, IN) and contained vitamin-free casein, sucrose, solka floc, vitamin and mineral mixtures, DL-methionine, choline chloride, lard, corn oil and dextrin. All diets were isocaloric (4·16 kcal/g digestible energy), consisted of 3% fibre, and were specially prepared to provide varying amounts of protein, including 24% (control), 19%, 14%, 10%, 6% and 3·2% (contained no DL-methionine) protein. To maintain identical caloric levels in these different diets, the contents of fat and carbohydrate were appropriately adjusted. In addition, to permit analysis of dietary intake, the weights of ingested food by the various groups were recorded throughout the experiments. For storage purposes, test diets were kept in closed receptacles at 4°.

In most experiments, animals' food consumption was unrestricted. However, to conduct pair-feeding studies, weanling rats (21 days old) were isolated from foster mothers, placed in separate cages, given group designations (i.e. '3·2%', '24% pair-fed', '24% *ad lib*') and all administered 50 g of 3·2% protein-containing diets (day 0). Thereafter, the amount of food/day ingested by a given '3·2%' rat determined the weight of '24%' diet provided to a specific pair-fed rat on the next day. For comparative purposes, additional animals were included in these 'paired' experiments and exposed to 24% protein diets *ad libitum*.

General procedures

Tears were obtained from the eyes of etherized rats, then measured and processed according to a reported protocol.[10] Anaesthetized rats were then injected subcutaneously with pilocarpine nitrate [0·5 mg/100 g body weight (BW)] to enhance the flow, and facilitate the collection, of saliva.[11] Blood was collected by cardiac aspiration and allowed to clot at room temperature. After rat death, small intestinal secretions were obtained by surgically removing and then flushing the intestinal (proximal 254 mm) lumen with 3 ml of 0·15 M saline.[11] Tears, saliva, intestinal fluids and blood were centrifuged at 10,000 *g* for 4 min and supernatants were stored at −20°.

To process lacrimal glands for the immunofluorescent analysis of IgA plasma cells, tissues were cleared of adherent debris, weighed and placed in glass vials containing St Marie's fixative (19 parts 100% ethanol: 1 part glacial acetic acid) at 4°. After overnight fixation, tissues were dehydrated in ethanol and xylene, embedded in paraffin and cut into 5 μm sections. Sections were transferred to gelatin-coated slides, then deparaffinized prior to staining.

Protein concentrations in mucosal secretions and serum were measured by the Hartree method,[12] using bovine serum albumin (BSA) (Calbiochem-Behring Corp., La Jolla, CA) as the standard. Unless otherwise indicated, statistical analysis of the data were performed with the two-tailed, Student's *t*-test.

Immunofluorescence techniques

To determine the number of IgA-containing cells in lacrimal gland sections, tissues were processed for indirect immunofluorescence microscopy, stained with appropriate immunological reagents and overlaid with glycerol-paraphenylenediamine mounting media as previously reported in detail.[13] For quantitative measurement of IgA-containing cell density, two to 10 sections/tissue and approximately 15 microscopic fields (312·5× magnification)/section were examined with a Zeiss Photoscope II fluorescence microscope, equipped with epilluminination and fitted with a xenon lamp, a 460–490 nm excitation filter and a 528 nm barrier filter. To calculate the total number of IgA-containing cells in lacrimal glands, the mean cell density/microscopic field was multiplied by gland weight (mg) and by a correction factor (326·7), which compensated for such variables as cell size, microscopic field volume and tissue density.[14] To confirm immunofluorescence findings, selected tissues were reanalysed and yielded essentially identical results.

Immunoassays for IgA, free secretory component (SC) and IgG

The IgA and free SC[15] levels in tear, salivary, intestinal and/or serum samples were measured with specific, double antibody radioimmunoassays (RIA), according to detailed procedures described previously.[10,11] The assay for SC detected primarily free SC;[11] consequently, SC findings are reported in terms of free SC content. It should be noted that all SC assays of intestinal secretions were performed within 2–6 weeks of fluid collection, in order to minimize any potential effect of proteolysis. To quantitate the amount of IgG in experimental samples, a previously described ELISA was utilized.[16] Standard curves were included with each assay and evaluated by log logit transformation. Assay sensitivities, defined as the lowest amount of antigen measurable and equivalent to the quantity two standard deviations above the zero dose response, were 3·3 ng IgA,[10] 0·44 ng free SC[11] and less than 0·1 ng IgG.[16]

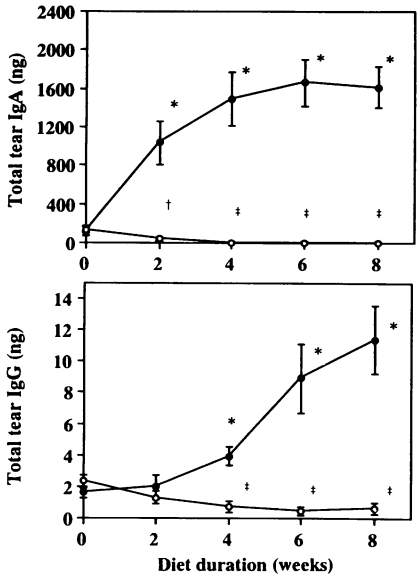

Figure 1. Effect of severe protein deprivation on tear IgA and IgG levels in weanling (21 day) rats. Animals ($n = 10$/treatment group) were given isocaloric diets containing control [24% (●)] or low [3·2% (○)] protein contents for 8 weeks. Tears were collected at 2-week intervals and processed for the measurement of IgA and IgG. Numbers represent the mean ± SE. * Significantly greater than week 0 ($P < 0.005$) and age-matched '3·2%' group ($P < 0.001$) values; † significantly ($P < 0.05$) less than week 0 amount; ‡ significantly ($P < 0.005$) lower than week 0 value. For comparison, a similar 8-week period of protein deficiency (3·2%) in adult (3 months) and aged (16·5 months) rats ($n = 9$–11/treatment group) had no effect on the tear IgA or IgG content, relative to that in '24%' protein diet controls (data not shown).

RESULTS

Influence of severe protein malnutrition on immune expression in the eye, mouth, intestine and serum of weanling, adult and aged rats

To determine the effect of severe protein malnutrition during development and ageing on the ocular, salivary and intestinal secretory immune systems, as well as serum immunoglobulin concentrations, weanling (21 days old), adult (3·5 months old) and aged (16·5 months old) male rats ($n = 9$–11/treatment group) were fed isocaloric diets containing either low (3·2%) or control (24%) protein levels. This nutritional schedule was continued for 8 weeks and tears were collected prior to initiation of the diet (day 0) and at successive 2-week intervals, whereas saliva, intestinal secretions and lacrimal glands were obtained at experimental termination.

Ocular immune system

Provision of a control protein diet to weanling rats resulted in a pronounced, 13-fold increase in the tear levels of IgA during the experimental time-course (Fig. 1). This significant ($P < 0.0001$) rise occurred irrespective of whether IgA amounts were expressed in terms of concentration or total protein. In contrast, exposure of weanling animals to a protein-deficient diet led to a progressive decrease in tear IgA content, such that by 4 or 6 weeks, only 10% of rats had measurable tear IgA concentrations. In fact, following 8 weeks of protein starvation, no IgA could be detected in tears (Fig. 1). This suppressive influence of protein malnutrition on tear IgA was associated with a parallel, but less extensive, decline in the volume of tears (Table 1), and protein level in tears (Table 2).

The impact of protein deprivation on tear IgA in weanling rats coincided with a precipitous drop in the tear free SC concentration (Table 2), as well as the total number of IgA-containing cells in lacrimal tissue (Table 1). After 8 weeks of protein insufficiency, the total free SC amounts, free SC concentrations and free SC/protein ratios in tears were 94-fold ($P < 0.0005$), 35-fold ($P < 0.0001$) and sevenfold ($P < 0.005$) less than those in tears of control animals. Moreover, by the end of this experimental period, 75% of protein-malnourished rats had undetectable tear SC levels. With regard to lacrimal IgA-containing cells, the number of these cells in protein-deprived animals was 33-fold lower than enumerated in control lacrimal glands and appeared to be due to significant ($P < 0.001$) reductions in IgA plasma cell density and lacrimal tissue weight (Table 1). Of interest, the size of lacrimal glands in protein-malnourished rats, as a function of body weight, was slightly, but significantly ($P < 0.01$), less than that of controls (Table 1).

Maintenance of weanling rats on low dietary protein also significantly ($P < 0.005$) attenuated the total amount of tear IgG, relative to pretreatment or control levels (Fig. 1). However, this response seemed to reflect alterations in tear volume and protein content, because no consistently significant differences were evident in the IgG concentration or IgG/protein ratio of tears during the malnutrition interval.

Although an 8-week administration of the 3·2% protein diet dramatically curtailed the development of mucosal immune expression in eyes of weanling rats, a similar, low-protein exposure exerted minimal impact on ocular immune parameters in adult and aged rats. Thus, following 8 weeks of protein malnutrition, no significant changes occurred in the volume of, or total IgA, free SC, IgG or protein in, tears of these older animals (Fig. 1, Tables 1, 2, additional data not shown). The tear IgA concentration did decrease in protein-starved adult rats (Table 2), but this effect appeared related to tear volume fluctuations and no variation was noted in the tear IgA/protein ratio, compared to control. Protein insufficiency was also associated with a significant ($P < 0.01$) reduction in the total number, but not the density (i.e. cells/microscopic field), of IgA-containing cells in the adult lacrimal gland; this difference may be attributed to the diminished weight of lacrimal tissue in protein-deprived, adult rats (Table 1). A striking finding in these studies, which has previously been observed,[17,18] was the relative level of IgA, IgG and total protein in tears of aged rats. Tear protein concentrations in these elderly animals were considerably reduced, compared to those of adult rats, and consequently resulted in the highest IgA/protein (e.g. up to 8%) and IgG/protein ratios recorded in all age groups.

Salivary, intestinal and serum immune expression

Protein malnutrition in weanling rats led to a profound decrease in the IgA, free SC and total protein concentrations in small intestinal fluids, a twofold decline in salivary IgA and free SC levels and a 57% drop in serum IgA and protein amounts (Table 2). In contrast, protein starvation in weanling animals had no significant effect on salivary or serum IgG concentrations. Of particular interest, the magnitude of the malnutrition-induced suppression of mucosal immune parameters was greatest in the eye and lowest in the mouth (Fig. 1, Table 2).

Table 1. Influence of severe protein malnutrition on the body weight, tear volume, lacrimal gland weight and total number of IgA-containing cells in lacrimal tissues of weanling, adult and aged rats

	Body weight (g)		Tear volume (μl)		Lacrimal gland weight (mg)		Total IgA-containing cells ($\times 10^5$)/lacrimal gland	
Age group	24%	3·2%	24%	3·2%	24%	3·2%	24%	3·2%
Weanling	540±25	63±2[a]	5·0±0·8	0·8±0·2[a]	142±2·7	15±0·5[a]	1·67±0·23	0·05±0·01[a]
Adult	794±35	565±15[a]	5·1±0·9	7·3±0·9	132±2·3	95±2·4[a]	3·10±0·41	1·71±0·27[b]
Aged	911±33	718±33[b]	5·1±1·5	5·3±0·7	118±5·5	87±3·0[a]	2·59±0·51	1·83±0·36

Weanling (W; 21 days), adult (A; 3 months) and aged (Ag; 16·5 months) rats (n=9–11/treatment group) were administered isocaloric control (C; 24%) or low (M; 3·2%) protein diets for 8 weeks. At experimental termination, lacrimal tissues (1 gland/rat) were processed for the immunofluorescent analysis of IgA-containing cells. Approximately 15 microscopic fields/tissue section and between 259 and 540 microscopic fields/treatment group were evaluated. Values represent the mean±SE of measured parameters after the 8-week interval. For comparison, at the start of the experiment, body weights (g) equalled: WC=60±2; WM=58±1; AC=618±27; AM=609±17; AgC=849±34; AgM=866±25, and tear volumes (μl) equalled: WC=2·1±0·2; WM=1·9±0·3; AC=5·9±1·0; AM=5·0±0·9; AgC=5·7±1·3; AgM=4·5±1·3. [a] Significantly (P<0·0001) less than age-matched control value; [b] significantly (P<0·01) less than age-matched control value.

Table 2. Effect of protein deprivation on IgA, IgG, free SC and protein concentrations in tears, saliva, intestinal secretions and serum of weanling, adult and aged rats

	Protein (mg/ml)		IgA (μg/ml)		IgG (μg/ml)[a]		SC (μg/ml)	
Group	24%	3·2%	24%	3·2%	24%	3·2%	24%	3·2%
Tears								
Weanling	34·9±3·0	6·8±2·3[b]	403±69	0±0[b]	2·52±0·35	1·06±0·62	86·8±16·7	2·5±1·7[b]
Adult	23·1±1·9	21·1±1·9	299±74	139±21[c]	3·81±0·64	2·61±0·30	35·9±2·7	32·8±4·3
Aged	11·8±3·2	9·2±3·1	375±25	395±59	5·76±1·75	4·72±1·80	22·8±4·2	23·2±2·9
Saliva								
Weanling	7·36±0·28	6·47±0·40	36·5±5·5	14·6±3·4[f]	0·29±0·08	0·51±0·15	4·69±0·89	2·38±0·23[e]
Adult	7·90±0·80	6·67±0·74	50·2±16·9	33·1±3·3	1·21±0·23	2·10±0·52	5·10±1·09	6·13±0·77
Aged	7·65±0·71	5·19±0·41[c]	54·5±5·0	43·2±4·9	1·56±0·69	2·46±0·43	6·84±0·96	3·94±0·86[e]
Intestine								
Weanling	1·57±0·25	0·31±0·04[b]	354±57	43±5[b]			1·81±0·31	0·29±0·07[b]
Adult	2·42±0·31	0·94±0·05[b]	523±60	360±44[e]			2·71±0·19	2·35±0·42
Aged	2·05±0·21	0·85±0·07[d]	694±93	393±97[e]			3·06±0·35	1·06±0·27[c]
Serum								
Weanling	80·6±1·4	51·2±2·4[b]	148±13	94±10[f]	7·02±1·66	4·42±0·62		
Adult	80·2±1·3	69·0±1·0[b]	325±61	230±30[e]	8·94±0·66	11·19±0·78[g]		
Aged	70·7±0·7	68·0±1·6	376±69	1022±246[g]	8·44±0·77	10·38±1·02		

Tears, saliva, intestinal secretions and serum were collected after weanling (21 days), adult (3 months) and aged (16·5 months) rats (n=9–11/treatment group) were exposed to control (24%) or low (3·2%) protein diets for 8 weeks. Numbers equal the mean±SE. [a] Serum IgG levels are mg/ml; [b–f] significantly (P<0·0001[b]; <0·01[c]; <0·0005[d]; <0·05[e]; <0·005[f]) less than value of age-matched control; [g] significantly (P<0·05) greater than value of age-matched control.

Maintenance of adult or aged rats on low-protein diets resulted in a continued deficit in intestinal protein, IgA and free SC (in aged) concentrations (Table 2). However, with the exception of diminished protein and free SC levels in aged rat saliva, salivary concentrations of IgA, IgG, free SC and protein in older animals appeared to be refractory to the influence of chronic protein malnutrition (Table 2). In serum from adult rats fed minimal protein, the concentrations of protein and IgA were lessened, whereas the levels of IgG were increased, relative to controls. In aged animals, though, restricted protein intake augmented IgA concentrations, but did not alter serum protein or IgG amounts (Table 2).

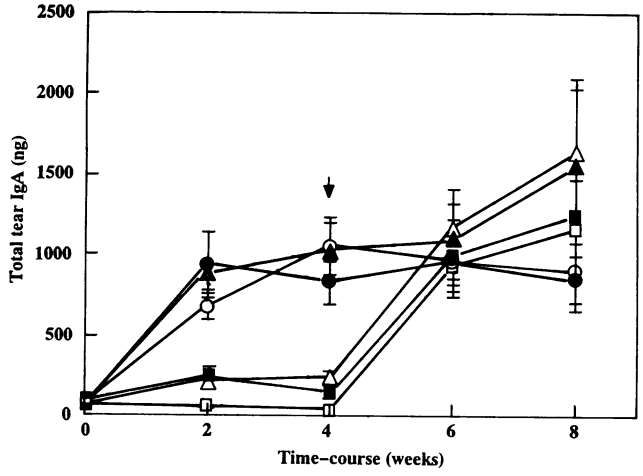

Figure 2. Influence of varying degrees of protein malnutrition, followed by the administration of a control protein diet, on tear IgA content in weanling rats. Young animals (21 days old; $n=6$–14/treatment group) were given isocaloric diets containing 24% [control (●)], 19% (○), 14% (▲), 10% (△), 6% (■) or 3·2% (□) protein composition for 4 weeks, then groups were administered (time designated by arrow in graph) 24% protein diets for an additional 4 weeks. Numbers equal the mean ± SE. Tear IgA levels increased significantly ($P<0·005$) during the first 4 weeks in rats on 24%, 19%, 14% and 10% protein diets, and during the second 4-week period ($P<0·001$) in animals initially exposed to 6% and 3·2% protein diets.

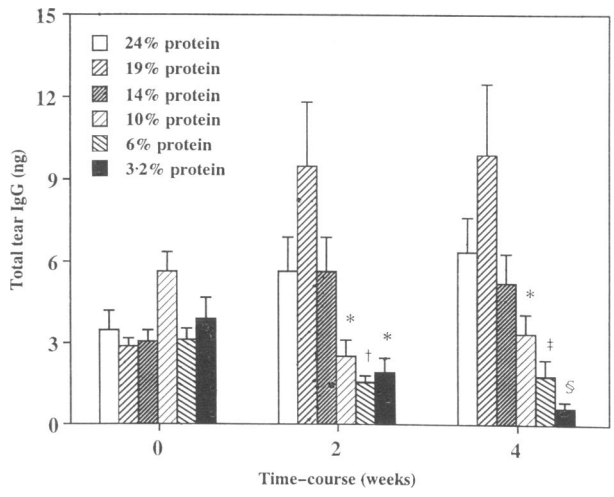

Figure 3. Impact of dietary protein variations on tear IgG levels in weanling rats. Young animals (21 days old; $n=13$–14/treatment group) were provided isocaloric diets containing 24%, 19%, 14%, 10%, 6% or 3·2% protein content for 4 weeks. Tears were obtained at 2-week intervals. Numbers represent the mean ± SE. Significantly (*$P<0·05$; †$P<0·01$; ‡$P<0·005$; §$P<0·001$) less than age-matched '24%' group value.

These findings demonstrate that the immunological impact of protein malnutrition may vary significantly depending upon the specific mucosal site and chronological age of the animal.

Impact of varying degrees of protein malnutrition, followed by the provision of a balanced diet, on immune parameters in the eye, intestine and serum

To assess whether the effects of protein malnutrition on immunity are dose dependent, and possibly reversible by dietary improvement, the following studies were performed. Weanling (21-day-old) rats ($n=6$–14/treatment group) were administered isocaloric diets containing 24%, 19%, 14%, 10%, 6% or 3·2% protein for 4 weeks, and then provided with control (24%) protein diets for an additional 4 weeks. Tears were collected immediately before the initiation of various diets and thereafter at biweekly intervals. Small intestinal fluids, serum and lacrimal glands were obtained at either 4 or 8 weeks.

Our results demonstrated that the magnitude of immunological suppression during protein malnutrition clearly depended upon the extent of protein deprivation, as well as the mucosal location. Within 2 weeks of dietary implementation, the total levels (Fig. 2) and concentrations of tear IgA were significantly reduced in groups exposed to 10%, 6% and 3·2% protein diets, as compared to control. By 4 weeks, these same groups had markedly diminished levels of tear IgA (Fig. 2), IgG (Fig. 3), free SC and total protein (Fig. 4). The malnutrition effect on tear IgG appeared to be due to tear volume fluctuations, because consistently significant reductions in tear IgG concentration were not found in different experiments. In lacrimal tissue, administration of 6% and 3·2% protein diets to rats caused a significant ($P<0·05$) decline in the total number (Table 3), but not the density, of IgA-containing cells. This lymphocytic

Table 3. Impact of initial protein deficiency (4 weeks), followed by the secondary administration of a control protein diet (4 weeks), on the number of IgA-containing cells in, and the weight of, lacrimal glands of weanling rats

Dietary protein (%)		Lacrimal gland weight (mg)		Total IgA-containing cells ($\times 10^5$)/lacrimal gland	
1° diet	2° diet	Post-1° diet	Post-2° diet	Post-1° diet	Post-2° diet
24	24	105 ± 5·4	156 ± 6·0[a]	1·12 ± 0·25	1·86 ± 0·17
19	24	112 ± 2·9	159 ± 5·3[a]	2·02 ± 0·29	2·11 ± 0·18
14	24	111 ± 2·2	148 ± 6·4[a]	1·55 ± 0·13	2·31 ± 0·33
10	24	63 ± 3·0[b]	124 ± 4·6[a,c]	1·12 ± 0·10	1·33 ± 0·17[d]
6	24	40 ± 1·9[b]	131 ± 2·8[a,c]	0·46 ± 0·05[d]	1·09 ± 0·15[e]
3·2	24	15 ± 1·0[b]	125 ± 4·4[a,c]	0·28 ± 0·06[e]	1·07 ± 0·31[d]

Weanling rats (21 days old; $n=6$–8/treatment group) were administered varied protein diets (1° diet) for 4 weeks, then exposed to a control protein diet (2° diet) for an additional 4 weeks. Lacrimal glands were collected after the first or second dietary regimen. To determine the number of IgA-containing cells, 49 ± 2 (1° diet) or 42·7 ± 1·7 (2° diet) microscopic fields/tissue (2–10 sections/tissue) were examined by immunofluorescence. Measurements equal the mean ± SE. [a] Significantly ($P<0·0001$) greater than weight after 1° diet; [b] significantly ($P<0·0001$) less than 24% 1° diet value; [c] significantly ($P<0·001$) less than 24% 2° diet value; [d] significantly ($P<0·05$) less than 24% diet value; [e] significantly ($P<0·01$) less than 24% diet value.

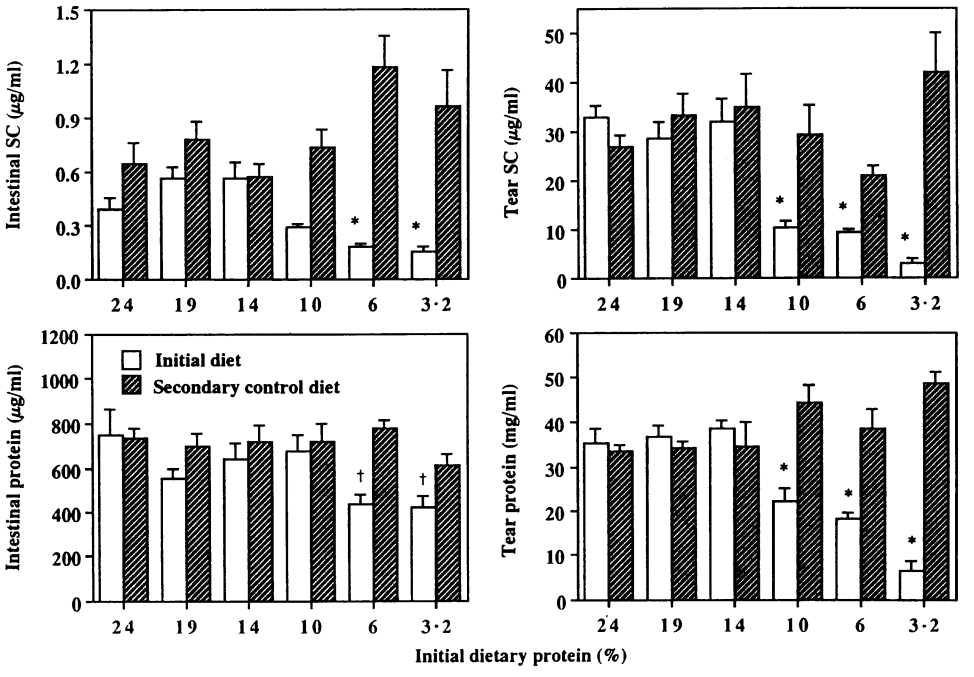

Figure 4. Influence of different dietary protein regimens on the free SC and total protein concentrations in tears and intestinal secretions of weanling rats. Twenty-one-day-old animals ($n=6$–14/treatment group) received isocaloric diets containing 24%, 19%, 14%, 10%, 6% or 3·2% protein 'Initial diet' for 4 weeks, and then were administered control (24%) protein diets 'Secondary diet' for the following 4 weeks. Values equal the mean ± SE. Significantly (*$P<0·001$; †$P<0·05$) less than age-matched '24%' group concentration.

alteration was superimposed upon a protein dose-related decrement in lacrimal gland weight (Table 3).

As concerns intestinal secretions and serum, the 4-week exposure to varying protein diets did not curtail IgA concentrations. In contrast, serum protein levels were significantly ($P<0·005$) lowered in the 10%, 6% and 3·2% groups, and intestinal free SC and protein concentrations (Fig. 4) were significantly decreased in rats receiving the 6% and 3·2% protein diets.

The impact of protein insufficiency on mucosal immune parameters was almost completely reversed by dietary improvement. Thus, provision of 24% protein diets to malnourished rats for 2 or 4 weeks resulted in the total recovery of tear IgA (Fig. 2), free SC and protein levels (Fig. 4), as well as intestinal free SC and protein concentrations (Fig. 4), and serum protein content (except in the original 3·2% group), when compared to control amounts. In addition, the total number of IgA-containing cells in lacrimal glands of rats initially exposed to 6% and 3·2% protein diets underwent a significant ($P<0·05$) increase after the administration of control diets (Table 3). The extent of this lymphocytic rise, though, did not achieve the IgA-containing cell levels expressed in control glands (Table 3).

Kinetics of the tear IgA response in protein-malnourished rats following dietary correction

Our previous results demonstrated that the malnutrition-induced suppression of tear IgA levels may be reversed by a 2-week dietary improvement. To determine the kinetics of this response, weanling rats ($n=8$–10/treatment group) were maintained on either 24% (C) or 3·2% (M) protein diets for 8 weeks, then provided (day 0) 24% dietary protein for an additional 15 days. Tears were collected prior to the dietary alteration on day 0, as well as on days 1, 4, 7, 10 and 15, and then processed for IgA and total protein measurements.

As illustrated in Fig. 5, administration of enriched-protein diets to protein-malnourished rats stimulated a rapid and progressive rise in tear IgA content. Within 4 days of dietary correction, tear IgA levels had increased significantly ($P<0·05$) in protein-deprived rats (day $0=0\pm0$ μg IgA/ml; day $4=115\pm34·1$ μg IgA/ml). During the ensuing 3 days, tear IgA amounts doubled and by day 10 tear IgA content equalled control. This IgA response was paralleled by a dramatic rise in both the volume and protein levels of tears (Fig. 5). Of interest, despite the recovery in various tear parameters, the body ($C=531\pm13·8$; $M=203\pm5·7$; $P<0·0001$) and lacrimal gland ($C=151\pm3·1$; $M=86·5\pm2·1$; $P<0·0001$) weights of 'protein-deficient' rats had not reached control weights after 15 days of the 24% protein dietary regimen. In contrast, by day 15, the lacrimal gland/body weight ratio of originally malnourished rats significantly ($P<0·0001$) exceeded that of controls ($C=0·028\pm0·001$; $M=0·043\pm0·001$).

The rapidity of the tear IgA recovery in protein-malnourished rats was also observed in another study, wherein weanling rats ($n=8$–10/treatment group) were placed on 24% or 3·2% protein diets for 4 weeks, then uniformly given 24% protein. Tear IgA levels, which were undetectable after 4 weeks of protein deprivation, increased significantly after 3 ($68·6\pm24·9$ ng tear IgA; $P<0·05$) and 6 ($400\pm78·9$ ng tear IgA; $P<0·001$) days of optimal nutrition. The tear IgA response at 3 days coincided with a rise in total tear protein, but preceded a later increase in tear volume.

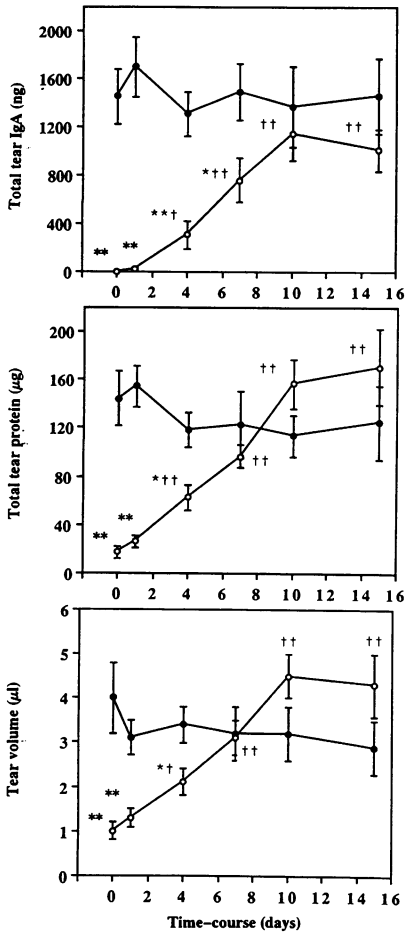

Figure 5. Effect of the administration of a control (24%) protein diet to protein-malnourished rats on the volume of, and total protein and IgA content in, tears. Weanling rats (21 days old; $n = 8$–10/treatment group) were given isocaloric low [3·2% (○)] or control [24% (●)] protein diets for 8 weeks, then provided (day 0) 24% protein diets for 15 days. Numbers represent the mean ± SE. Significantly (*$P < 0.05$; **$P < 0.005$) less than age-matched control value; significantly (†$P < 0.05$; ††$P < 0.005$) greater than 'protein-deficient' day 0 value.

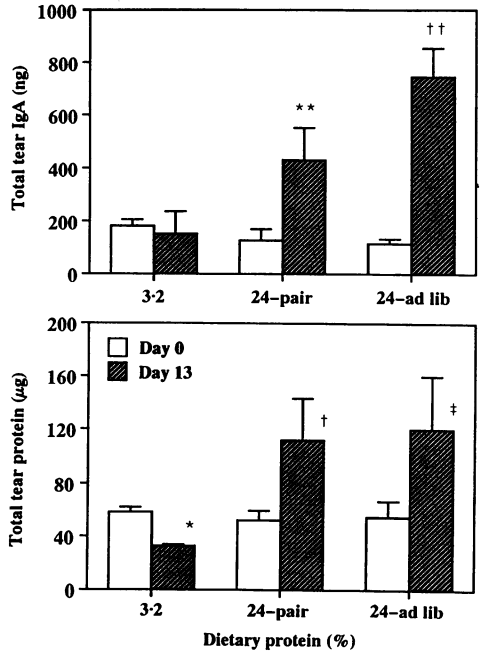

Figure 6. Impact of protein-deficient or pair-fed control diets on the IgA and total protein levels in weanling rats. Twenty-one-day-old animals ($n = 5$–6/group) were maintained in separate cages and given isocaloric: (a) low protein (3·2%) diets *ad libitum*; (b) control (24%) protein diets under pair-fed conditions (24-pair), i.e. the weight of provided food to each animal was identical to that ingested by a paired 'low-protein' rat; and (c) control protein diets *ad libitum* (24-ad lib). Tears were collected prior to the initiation (day 0), and at the end (day 13), of the dietary interval. Numbers equal the mean ± SE. * Significantly ($P < 0.005$) less than day 0 amount; significantly (†$P < 0.05$; ‡$P < 0.05$, one-tail) greater than '3·2' day 13 level; ** significantly ($P < 0.05$) higher than day 0 IgA content; †† significantly ($P < 0.005$) greater than day 0, and day 13 '3·2' values.

Effect of pair-feeding low and control protein diets on tear IgA levels in weanling rats

During the course of these nutrition studies, it was recognized that weanling animals placed on a 3·2% protein diet ate significantly less food than those provided a 24% protein diet. For example, during the first 2 weeks of dietary exposure, protein-deprived rats ingested approximately 50% of the food intake (by weight) of control rats. Consequently, although all diets were isocaloric, the malnourished animals received both decreased protein and diminished calories. Therefore, to assess whether the malnutrition-related decline in tear IgA levels was due to protein restriction, or a combined protein–calorie deficiency, a pair-feeding experiment was conducted. As outlined in the Materials and Methods, weanling rats were pair-fed diets containing either 3·2% ($n = 5$) or 24% ($n = 5$) protein and, for comparison, another group of animals ($n = 6$) was allotted a 24% protein diet *ad libitum*. Tears were obtained immediately before the initiation of the study (day 0) and after 13 days, then assayed for IgA and total protein.

These results showed that tear IgA levels increased significantly in rats administered either 24% pair-fed or 24% *ad libitum* protein diets (Fig. 6). In contrast, this developmental rise in tear IgA did not occur in animals placed on a 3·2% protein diet. Moreover, the total amount and concentration of tear protein significantly decreased in '3·2%' animals during the time–course of the experiment, relative to levels expressed on day 0 or in age-matched rats fed 24% protein (Fig. 6). Given these findings, as well as the absence of significant changes in tear volume during this study, it would appear that the early tear IgA deficit following malnutrition is due primarily to dietary protein deficiency and not to caloric restriction.

DISCUSSION

The present study demonstrates that severe protein malnutrition during development, but not necessarily adulthood or senescence, exerts a tremendous impact on the expression of the

secretory immune system. Protein insufficiency appeared to suppress completely the maturation of ocular mucosal immunity in weanling rats. Similarly, protein deprivation dramatically interfered with the establishment of intestinal immunity and significantly diminished salivary immune expression. These findings indicate that dietary nutrients play an essential and determinant role in the ontogeny of the secretory immune system.

In support of this hypothesis, previous research has shown that the structure and function of the secretory immune system in very young children or animals may be significantly impaired by prolonged intervals of malnutrition. In general, nutritive deficiency in several species may lead to a selective and significant decrease in total IgA and SC concentrations in external secretions,[2,19,20] a diminished number of IgA-bearing cells, IgA plasma cells, specific IgA antibody-containing cells, intraepithelial lymphocytes and helper, suppressor and total T cells in mucosal tissues,[21-24] a reduction in IgA^+ cell migration to secretory sites,[25] a blunted sIgA antibody response to viral and bacterial antigens,[2,22,23,25] atrophy of mucosal-associated lymphoid tissue[25] and alterations in epithelial surface morphology and permeability.[1,24] The extent of these malnutrition-associated effects may vary significantly among different mucosal locations (this study).

Several explanations may account for the profound immune susceptibility to malnutrition during development. *First*, marked protein deprivation during the post-weaning period is well known to retard growth severely. Consequently, given that the activation of rat mucosal immunity begins at approximately 21 days of age,[7,26-28] the concurrent provision of a protein-deficient diet might be anticipated to inhibit this maturational process. *Second*, reduced protein intake diminishes antigenic exposure, which is of critical importance in the recruitment, proliferation and function of B- and T-lymphocyte populations in mucosal tissues of young animals.[7,29] *Third*, malnutrition delays puberty,[30] curtails hypothalamic and pituitary activity[31] and decreases circulating levels of androgens and thyroid hormones.[30-32] These malnutrition-induced disruptions in the endocrine environment might well account for the pronounced suppression of secretory immunity in the eyes of weanling rats, because lacrimal gland development[33] and ocular secretory immune expression[16,34] are extremely dependent upon hormones from the thyroid gland and/or the hypothalamic–pituitary–gonadal axis. As an additional consideration, given the nature of endocrine–immune interactions in the salivary gland,[35] intestinal tract and liver,[28,36,37] it is quite possible that malnutrition-related changes in glucocorticoid, insulin or thyroid hormone levels could influence the establishment of salivary or intestinal immunity, or the considerable biliary flow of IgA and SC into upper intestinal secretions,[38] in weanling animals. *Fourth*, chronic malnutrition may depress sympathetic nervous activity[32] and alter neuropeptide gene expression.[39] Such responses could theoretically affect mucosal immune development, given the significant, site-specific and bidirectional inter-relationship between the nervous and secretory immune systems.[40]

In contrast to the impact on weanling animals, protein malnutrition had minimal or no effect on various mucosal immune indices in adult or aged rats. This relative absence of nutritional influence was unexpected, particularly since ageing itself is associated with an attenuation in nasal IgA concentrations, hepatic IgA clearance and SC expression, salivary IgA responses to antigenic challenge, lymphocyte interactions in mucosal tissues and IgA-containing cell density in mesenteric lymph nodes (literature in refs 17,41). However, it is also true that the influence of ageing on mucosal immunity is site dependent[41] and may not compromise total or polymeric IgA concentrations in tears, saliva or intestinal secretions (refs 17,42–45; this study). Thus, it appears that mature immune systems may be relatively resistant to the stress of nutritional deprivation.[2] Consistent with this observation, researchers have reported a lack of effect of prolonged malnutrition on biliary IgA levels in adult female rats,[46] milk sIgA concentrations in nursing mothers[47,48] and salivary IgA content in variably aged groups of children.[49] Our studies did demonstrate that severe protein insufficiency reduces intestinal SC and/or IgA amounts in the small intestine of adult and aged rats. This finding, which may be species related,[50] correlates with the negative effect of decreased antigenic exposure on IgA plasma cell numbers in the adult intestine.[51] Yet, secretory immune expression in the eye and mouth of older animals appeared somewhat refractory to nutritional deficiency. Whether malnutrition during adulthood or senescence, as during childhood,[2,25] might significantly ameliorate mucosal immune responsiveness to toxic agents or microbial invasion remains to be determined.

During the course of our experiments, we found that extreme protein malnutrition causes fluctuations in the serum concentrations of IgA and IgG. The explanation for these changes, which varied according to age, are unclear. In fact, malnutrition does not seem to predictably influence circulating IgA or IgG levels, given that previous studies have noted increases, decreases or no change in these immunoglobulin concentrations.[2,52,53] It may be that the nature of the serum IgA or IgG responses may depend upon the length, type and severity of the nutritional imbalance, as well as the chronological age, existing immune status and species.

The immunosuppression induced by protein malnutrition in weanling rats was almost completely reversed by the administration of a balanced diet. The kinetics of this immune restoration, the capacity for which has been observed in various mucosal sites,[2,19-22] was exceedingly rapid in the eye. Within 3–4 days after nutritional correction, tear IgA levels had risen tremendously in previously malnourished animals. The speed of this immune recovery most likely reflects a dietary influence on the lacrimal gland microenvironment and IgA transport capability, given that IgA-containing cell populations were only partially restored following 1 month of renutrition.

In summary, protein deprivation significantly impairs the maturation of the secretory immune system during development, especially in the eye. It is possible that this later mucosal immune susceptibility to malnutrition may play a role in the high incidence of ocular infectious disease (e.g. *Chlamydia trachomatis*);[54] in undernourished children.

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