The Effect of Dietary Protein Depletion on Immunocompetence:

The Importance of Nutritional Repletion Prior to Immunologic Induction

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MANY SURGICAL PATIENTS are found preoperatively to be malnourished or protein-depleted as a result of sudden starvation secondary to acute conditions of the gastrointestinal tract precluding oral feedings or as a consequence of chronic illnesses such as malignancy, cirrhosis, and inflammatory bowel disease. Studley[18] found that in patients undergoing surgery for peptic ulcers, there was a 33% mortality in those with a preoperative weight loss of over 20% body weight as compared to the 3.5% mortality in those with better nutritional status. Many cases ending in fatalities in this series were attributable to an infectious etiology. Rhoads and Alexander[15] had reported on the increased incidence of post-operative infections in surgical patients with hypoproteinemia.

Although malnutrition seems to increase the susceptibility of the host to infection, the exact relationship between protein depletion and host defense mechanisms is unclear. Previous data in the literature have yielded conflicting results but recent studies in children suggested the presence of depressed humoral and cell-mediated immunity in severe protein-calorie malnutrition.[12,19] Investigations designed to study the association between nutrition and immuno-competence in patients have always been handicapped by the heterogeneity of the patient population, the presence of coexisting infections, the simultaneous presence of vitamin or mineral deficiencies, and the difficulty of precisely quantitating protein-calorie malnutrition.

The advantages of using an animal model to study the problems of malnutrition and immunity lie in the ease of establishing controlled protein-depletion as well as in the absence of coexisting illnesses which may interfere with immunologic assessment. Many past studies in protein-depleted animals have demonstrated impaired antibody production.[4,5,10,13,20] There have also been reports of increased susceptibility of these animals to viral and bacterial infections.[21,22] Some recent work has been done on cellular immune mechanisms but results have been conflicting. The demonstration of impaired thymus-dependent immunity in protein-deficiency states by some investigators[21,23] have been challenged by reports demonstrating enhanced cell-mediated immunity.[11]

Recently Dudrick *et al.* were capable of inducing true weight gain by means of intravenous hyperalimentation in patients with conditions usually associated with significant catabolism.[8,9] The possible advantages of nutritional repletion of the protein depleted surgical patient for purposes of improving immune competence are the ultimate goal of this study. The object of the present study is to use the rat model to evaluate the various immune parameters in chronic protein depletion. A comparison will be made between starting protein

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repletion 48 hrs. before antigenic challenge and starting repletion 48 hrs. after immunization. This will provide valuable information concerning the importance of nutritional status during the inductive early recognition phases of the immune response.

Materials and Methods

A. *Methods of Immunologic Assessment*

1. Serum Anti-KLH Titration. KLH (Pacific Biomarine Co., Venice, California) was purified according to the method described by others.[7] Two hundred μg were given to all rats intramuscularly on day zero. Sera were collected at day of sacrifice, 10 days after immunization, for the determination of anti-KLH antibodies using the tanned sheep cell hemagglutination technique.[7]

2. Spleen Cell Hemolytic Plaque Assay. Rats were given sheep erythrocytes according to the protocol described below. Spleens were collected from rats at time of sacrifice, in chilled medium-199 (Microbiological Assoc., Bethesda, Md.). Spleens were cut into small fragments and cell suspensions were prepared by passage of fragments through size 50 wire mesh. Lymphoid cells were counted. The direct plaque assay for IgM producing cells was then performed exactly as described earlier by us.[2] For the development of the indirect IgG hemolytic plaques, 1 ml of 1:100 dilution of goat anti-rat globulin (Hyland Laboratories, California) was added to the plates at time of plating the cells and then allowed to incubate for one hour prior to addition of the guinea pig complement.

3. In Vitro Lymphocyte Response to Mitogens. At the time of sacrifice, cervical lymph nodes were collected in medium 199, using a sterile technique. Cell suspensions were prepared by passage of nodes through sterile No. 50 wire mesh. Cell counts were then done and cell suspensions were adjusted to contain 2×10^6 lymphocytes/ml. The technique of *in vitro* cell culture was as described earlier by us.[1] In all cases 10% fetal serum was used.

B. *Animal Groups*

7–15 week-old female Sprague-Dawley rats (Charles River, Wilmington, Mass.) weighing 150–350 grams were used for this study. The animals were housed in group cages, fed water and either normal rat chow or protein-free diet ad libitum. Rats were divided into 4 groups in such a way that the mean weights would be approximately equal after potein depletion. Protein-depleted rats were those that were maintained on a protein-free diet for six weeks before initiating the studies.

All animals followed the same protocol for immunologic evaluation. At day 0, each rat was given 0.2 or 2.0 mg KLH intramuscularly, 0.1 ml complete Freund's adjuvant intradermally in the foot pad, and 1×10^8 sheep erythrocytes were injected intraperitonially. On day 2, 0.5×10^8 sheep erythrocytes were re-injected. On day 9, animals were skin tested with 50 μg PPD intradermally. Skin tests were read 24 hours later and animals were then sacrificed. Sera, spleens and lymph nodes were obtained as described.

A total of 127 rats were studied and these were divided into the following four groups according to diet schedule:

I. Normal: maintained on regular rat chow (Wayne-Lab Blox, General Biochemicals, Chagrin Falls, Ohio).

II. Depleted: this group was protein-depleted for 6 weeks before immunizations and was continued on the protein-free diet (Biological Research Products Mod. Cat. #170590, Chagrin Falls, Ohio) until sacrificed. In this diet, protein has been replaced isocalorically with corn starch.

III. Repleted A: this group was protein-depleted for 6 weeks but was repleted using the regular rat chow 48 hrs. before immunizations. They were maintained on the regular diet until sacrificed.

IV. Repleted B: this group was protein depleted for 6 weeks but was refed a regular diet 48 hrs. after immunizations until sacrificed.

To quantitate the degree of protein depletion and repletion, weights at various times were recorded. Re-

TABLE 1. *Weights at Various Times of Study*

Group	No. Rats Studied	Mean Initial Wt. (gm) (Mean & Range)	Mean Wt. (gm) after Protein Depletion (Mean & Range)	% Wt. Loss	Mean Wt. (gm) Gain During Repletion	Mean Wt. (gm) on Day of Sacrifice (Mean & Range)
Normal	40	191 (160–333)	—	—	—	219 (175–331)
Depleted	30	253 (178–370)	174 (111–277)	31%	—	163 (104–253)
Repleted A*	30	232 (187–286)	160 (132–194)	32%	84	244 (204–286)
Repleted B†	27	248 (201–336)	173 (142–258)	31%	69	242 (211–325)

* Protein depleted rats that were started back on regular rat chow 48 hrs *prior* to sensitization
† Protein depleted rats that were started back on regular rat chow 48 hrs *after* sensitization

sults are found in Table 1. The normal group of rats had a mean weight gain of 28 gms during the 10 day period of experimentation. The depleted group had a mean initial weight of 253 gms and had a mean weight of 163 gms at the time of sacrifice. This represents an average weight loss of 31%. The group designated repleted-A had an initial weight of 232 gms, and experienced a 32% weight loss during the 6-week period of protein restriction. Their mean weight gain during the 12 day period of protein repletion before their sacrifice was 84 gms. Repleted group B had an initial weight of 248 gms, and lost 31% of their initial weight. Their mean weight gain during the 8 days of protein repletion was 69 gms. (Table 1).

Results

Anti-KLH Titers (Table 2)

Anti-KLH titers in depleted animals failed to show a significantly depressed response, less than 2 tubes dilution difference, compared to normals. A subnormal response is suggested, however, with both antigenic doses. The highest titers were recorded in the group repleted 48 hrs. prior to immunization and antibody titers at both sensitizing doses were significantly different from titers of the depleted group ($p < 0.02$). The anti-KLH response of repleted group B is comparable to that of normals. Titers of the depleted group or the repleted group B were not significantly different from the normal group ($p > 0.05$).

Hemolytic Spleen Plaque Assay (Table 3)

Protein-depleted rats were shown to have mean direct and indirect plaque counts per spleen only approximately half that of normals. The animals which were protein repleted 48 hrs. prior to antigenic stimulation, however, demonstrated twice normal values of IgM producing cells and almost 3 times normal IgG producing cells. Repleted group B showed a near normal response. When plaque counts were calculated on the basis of 10^6 lymphoid cells plated instead of per spleen, depression was not demonstrated in the depleted group although twice normal response was still evident in repleted group A.

TABLE 2. *Primary Sensitization with Keyhole Limpet Hemocyanin (KLH)*

Group Tested	Anti-KLH Titres† Following Sensitization with KLH‡ 2.0 mg Mean§ & Range	No. of Rats Tested	0.2 mg Mean§ & Range	No. of Rats Tested
Normal	64(64–128)	11	8(1–20)	10
Depleted	32(4–128)	8	4(1–10)	8
Repleted A*	128(32–256)	16	16(10–40)	9
Repleted B*	64(16–256)	13	8(1–10)	8

* Depleted rats that were repleted 2 days before (Group A) or 2 days after (Group B) primary sensitization.
† Reciprocal of hemagglutination titers × 10^3.
‡ Rats were injected with either 0.2 mg or 2.0 mg KLH i.m. 10 days before determination of serum anti-KLH titers.
§ Geometric mean titers.

PPD Skin Tests (Table 4)

Skin test results demonstrated that the highest percentage of positive skin tests was in the group repleted 48 hrs. prior to injection of complete Freunds' adjuvant and the lowest percentage was in the depleted group. Only 5% of depleted animals showed a skin induration > 10 mm compared to 23% of the normal controls and 48% of repleted group A. If the criteria for positive skin test were changed to > 5 mm, the relative percentages in the 4 groups become 67, 5, 95 and 47 as shown in Table 4.

In Vitro Lymphocyte Proliferation (Table 4)

The mean specific incorporation (S. I.) of tritiated thymidine by lymph node lymphocytes from normal rats in response to 0.1 ml of 1:10 dilution of phytohemagglutinin-M (PHA) was 5.9 compared to 2.2 of the depleted group. Repleted group A and repleted group B showed S.I. of 8.3, and 4.7 respectively. With a larger amount of PHA (0.3 ml) added to the cell culture, the highest mean S.I. of 18.4 was again recorded in repleted group A which had received 12 days of nutritional repletion. Normal and depleted groups responded with specific incorporations of 9.2 and 3.9 respectively. Repleted group B which had received 8 days of protein repletion before the lymphocytes were cultured yielded an intermediate response with a S.I. of 7.4. Lymphocyte stimulation with pokeweed mitogen demonstrated a mean S.I. of 5.6 for the normal group as compared with 1.2 of the depleted group. Repleted group A and B had mean S.I. of 5.8 and 3.8 respectively.

Discussion

Evaluation of the bone marrow dependent system in this study was accomplished by hemolytic splenic plaque assays, measurements of anti-KLH antibodies, and assessment of *in vitro* lymphocyte proliferation in response to pokeweed mitogen. Thymus-dependent immune response was evaluated by the use of PPD skin tests and *in vitro* lymphocyte proliferation in response to phytohemagglutinin. Results indicate impairment of both B and T cell immunity in rats which have lost approximately 30% of their body weight over a 6-week period via dietary protein restriction. More importantly, this study demonstrates that adequate nutritional status is important for the successful induction of the immune response. Animals which were started on protein repletion 48 hrs. before immunologic challenge showed supernormal immune responses. However, animals which were sensitized at a depleted state and repleted 48 hrs.

TABLE 3. *Hemolytic Splenic Plaque Assay Following Immunization with Sheep Erythrocytes*

Group	Number of Plaques (Mean & Range)† Per Spleen Direct (× 10^3)‡	Per Spleen Indirect (× 10^3)‡	Per 10^6 Lymphoid Cells Plated Direct‡	Per 10^6 Lymphoid Cells Plated Indirect‡	Mean # of Lymphoid Cells ×10^6 Per Spleen (Mean & Range)
Normal	6.6(1.7–13.5) [7]	5.0(3.5–7.8) [4]	12.7(7–25) [7]	11.7(6–20) [4]	531(330–750)
Depleted	2.5(1.0–4.4) [9]	2.6(1.0–4.2) [4]	16.6(6–33) [9]	14.0(11–19) [4]	166(90–300)
Repleted (A)*	12.5(4.5–20.4) [5]	14.5(10.3–22.3) [3]	24.4(15–40) [5]	25.0(20–31) [3]	498(300–720)
Repleted (B)*	5.7(1.7–12.9) [6]	8.3(0–13.0) [4]	11.3(1–25) [6]	13.7(0–23) [4]	525(270–900)

* Depleted rats that were repleted two days before (Group A) or two days after (Group B) the first intraperitoneal administration of sheep erythrocytes (day 0).

† Rats were given 1 × 10^8 & 0.5 × 10^8 erythrocytes on days 0 & 2 respectively. All rats were sacrificed for the plaque assay on day 10 Numbers between [ ] represent numbers of rats tested.

‡ Direct and indirect represent number of plaques in absence of or presence of goat anti-rat immunoglobulin antiserum respectively .

later demonstrated a partial improvement in their immune responses.

Many studies in the past have demonstrated impaired antibody production in protein-depleted rats, mice, and rabbits.[4,5,10,13,20] The repletion of malnourished animals with optimal diets have been shown to reverse the increased susceptibility to viral or bacterial infection observed during malnutrition[21,22] while improvement of antibody production has also been seen after successful protein repletion of previously depleted animals.[10,20] Kenney *et al.* have recently demonstrated that protein malnutrition in adult Wistar rats is associated with smaller spleens with less nitrogen and RNA.[13] Using the method of hemolytic spleen, plaque assay, we have demonstrated in this study that the total number of antibody-forming cells in depleted animals was only one-third of normal, but antibody production per a fixed number of lymphoid spleen cells was not decreased. This would indicate that the defect is due to a decreased number of lymphoid cell and not due to failure of the antibody forming cell to secrete antibodies. It seems that malnutrition depleted both the IgM and IgG producing cells.

In this study, B cell mediated immune function as demonstrated by serum anti-KLH titer following sensitization was also evaluated. We were able to demonstrate a depression of the anti-KLH titers in the depleted group, though the difference in the titer was not significantly different from that of the control normal group. We considered 2 dilutions difference in the geometric mean titer to be significant.

The recent studies of thymus dependent function (cell-mediated immunity) in malnourished animals have yielded interesting and conflicting results. Atrophy of lymphoid tissue and thymus gland in marasmic mice was evident in studies of Woodruff,[21,22] and this was shown to be reversible by conversion to an optimum diet for one month. In addition, the increased susceptibility of marasmic mice to Coxsackie virus B_3 infection can be reduced by transfer of lymphoid cells from healthy mice

TABLE 4. *PPD Skin Tests and In Vitro Proliferative Response of Lymph Node Lymphocytes to Phytomitogens*

Group	% of Rats with Positive Skin Test† [No. Tested] 10 mm	5 mm	Specific Incorporation‡ in Presence of PHA 1:10 (0.1 ml) (Mean and Ranges) & [No. Tested]	PHA 1:10 (0.3 ml)	PWM 1:10 (0.1 ml)
Normal	23 [27]	67 [27]	5.9 (0.3–21.7) [17]	9.2 (4.4–12.3) [7]	5.6 (0.7–13.2) [12]
Depleted	5 [20]	5 [20]	2.2 (0.5–6.4) [11]	3.9 (0.6–9.9) [9]	1.2 (0.6–2.9) [5]
Repleted A*	48 [21]	95 [21]	8.3 (0.7–28.6) [6]	18.4 (0.9–43.9) [6]	5.8 (0.6–13.9) [6]
Repleted B*	32 [19]	47 [19]	4.7 (1.2–11.9) [6]	7.4 (1.7–20.3) [6]	3.8 (0.8–8.8) [6]

* Depleted rats that were repleted two days before (Group A) or two days after (Group B) primary sensitization.

† Rats were injected with 0.1 ml complete Freunds' adjuvant and tested with 50 μg PPD ID 9 days later.

‡ Specific incorporation is the ratio of counts/min (cpm) of three days cell cultures to which the phytomitogen was added to cpm of cultures to which no mitogen was added (control cutlures). The latter cultures gave 169 (92–312) cpm. Means, ranges, and no. of experiments from different rats are shown.

recovering from infection.[23] Other authors,[11] however, have claimed that protein deficiency may enhance cell-mediated immunity as demonstrated by enhanced graft rejection. In this study thymus dependent function was assessed by delayed skin reactivity to tuberculoprotein and by the *in vitro* lymphocyte responses to PHA and was found to be suppressed in protein malnutrition. The enhanced T-cell function demonstrated by others[11] in malnutrition was thought to be due to a depressed production of a blocking antibody.

A significant finding in this study is the supranormal response recorded in every immune parameter studied in the group of animals (Group A) protein-repleted 48 hrs. prior to sensitization (Tables 2–4). Rats (Group B) which received protein repletion 48 hrs. after sensitization demonstrated only near-normal immunocompetence. This significant difference in the magnitude of the response between repleted Group A and B indicates that nutritional status during the induction and/or proliferative phase of the immune response is crucial, and points to the immunologic advantage of early nutritional repletion. The rebound type of supranormal response recorded in repleted Group A is apparently not unique to our study model. Supranormal rates of hepatocyte synthesis of albumin have been observed during early protein repletion of previously depleted animals.[14,17]

Despite the wide use of modern antibiotics in recent years, it is evident that satisfactory prevention and treatment of infections in surgical patients have not been attained.[3] Better understanding of host defense mechanisms and factors which alter them can provide new insights to the problems of infections in the surgical patient. Data from this study and those of others[4,5,10,12,13,19,20] demonstrate that nutritional status is one of the determinants of host immuno-competence and the recognition that malnourished patients have poor host defenses is important in surgical practice.

Malnutrition is commonly observed in many patients on a surgical service. Oral feedings are denied to patients with bowel fistulae and those with prolonged intestinal obstruction or ileus. Patients with severe trauma or burns and those on respirators or with depressed central nervous system function are also unable to feed by mouth for long periods. In addition, necessary surgical intervention in some of these already malnourished patients can represent a further metabolic insult. The recognition that protein malnutrition is associated with impaired host defense mechanisms therefore urges adequate nutritional therapy in surgical patients both pre-and post-operatively. Improvement of patients' immunocompetence may yield improved surgical morbidity and mortality. In patients whose conditions preclude the effective use of the gastrointestinal tract for feeding, the use of total parenteral hyperalimentation should be considered.[8,9] Data from this study would also suggest that early or prophylactic nutritional repletion may be more beneficial as it would permit successful immunologic induction upon antigenic challenge.

There have been many reports of infectious complications, especially fungal, in patients receiving parenteral nutrition.[6] This could be one of the manifestations of depressed cellular immunity in patients with poor nutritional status but does not really constitute a contraindication to intravenous hyperalimentation. Meticulous care of the subclavian catheter, the catheter site, and nutritional solutions will ensure that severe infectious complications are rare even in depressed hosts. The proper use of parenteral hyperalimentation or other means of nutritional therapy can theoretically break the vicious cycle of malnutrition and infection and equip the patient with optimal host defense mechanisms.

Summary

The use of serum anti-KLH antibody assay, the hemolytic spleen plaque assays, delayed skin tests to tuberculin, and in vitro lymphocyte transformation in response to mitogens, have demonstrated the depression of humoral and cell-mediated immunity in young adult rats who were protein-depleted by being fed protein-free diet for 6 weeks. Protein repletion of the protein depleted rats 48 hrs. before antigen administration resulted in significant supranormal responses in all immune parameters tested. Those animals whose repletion started 48 hrs. after sensitization still demonstrated subnormal responses. It is concluded that nutritional status during the induction and/or proliferative phase of the immune response is important for the full expression of the immune response.

Malnutrition, a frequent finding among surgical patients, may be responsible for increased susceptibility to infection and early prophylactic nutritional repletion may have a vital role in restoring immune competence. In many surgical patients whose conditions preclude the use of the gastrointestinal tract for feeding, the use of intravenous hyperalimentation deserves special consideration.

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