The commonplace observation that inadequate diets either predispose to, or exacerbate infectious disease has been verified in many experimental model systems (1–5). Several host defensive mechanisms are affected. In particular inflammatory responses (6), phagocytic capacity (7, 8), and antibody production (9–11) have all been found to be either reduced or absent in protein deficient hosts. Conversely, dietary protein deficiency has been found to reduce the incidence of spontaneous tumors (12, 13) and increase resistance to transplantable tumors (14). Recent work has suggested that this phenomenon may be due to a preferential suppression of antibody production in animals fed diets containing moderate amounts of protein (15). This failure to produce tumor-specific “blocking” antibody is reflected in what appear to be heightened cell-mediated immune responses against tumor antigens (16). The evidence of these studies suggests that a primary deficit in the production of antibody may provide a common mechanism for the apparently opposite in vivo effects by which protein energy malnutrition produces both a lack of resistance to bacterial infections and increased resistance to tumors.

Despite the many studies which have been carried out on antibody production and hemolytic plaque-forming cells little is known of the effects of protein malnutrition on the various lymphoid cell populations. Although B cells are acknowledged to be antibody-producing cells (17, 18) and T cells are known to be essential for the normal development and manifestation of cell-mediated immunity (18, 19), both populations must normally be present for an adequate development of full immune capacity (17, 19). However, in vivo tests for their presence or function in malnourished animals may prove negative if either antigen-processing or inflammatory responsiveness (6) are inadequate. Ideally, quantitative tests which can be performed either in vitro or in normal “second-party” animals should be used as these are not subject to the above limitations.

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The graft-vs.-host (GVH) reaction offers a test which can be used to quantitate the presence of T cells and which simultaneously tests their functional capacity. The Simonsen spleen weight assay for GVH reactions (20) has been used in this study to examine the pattern of change in T-cell numbers induced by a diet which contained 4% protein by weight.

Materials and Methods

Animals. Inbred male and female BALB/c mice were obtained from the preclinical Animal House, University of Western Australia on the day of weaning (17 or 18 days of age). The only strain used in GVH assays was the F1 progeny resulting from a C57BL male × BALB/c female cross.

Diets. The normal diet contained approximately 20% protein (Wesfarmers W.A.), deficient diets comprised 56% wheat starch, 20% sucrose, 4% salt mixture, 10% peanut oil, 0.5% wheat germ oil, 1.5% cod liver oil, 4% agar, 4% egg albumin as protein, and a complete vitamin supplement. On the day of weaning mice were randomly assigned to two groups, one of which was fed the protein-deficient diet ad libitum and one which was fed the commercially produced mouse cube diet ad libitum. The intake of the deficient diet was low immediately after weaning but increased with time and the animals grew slowly, although hypotrophy of lymphoid organs was observed1 during this time period. The possible effects of dietary intake restriction were analyzed by means of pair-feeding experiments in which weaning mice were restricted to the same daily intake of normal diet as that consumed by age-matched mice fed the protein-deficient diet.

Preparation of Cell Suspensions. The spleen, thymus, and mesenteric lymph node were removed from 3–10 normal or deprived mice using aseptic procedures. Tissues were gently rubbed through a 50 gauge nickel mesh in Hanks' balanced salt solution (HBSS) and washed twice in HBSS before being made up to the desired concentration. Peyer's patches were removed by dissection and the tissue was then rinsed with HBSS containing streptomycin, penicillin, and neomycin at 100 μg/ml before a single cell suspension was prepared as described above. All suspensions were injected in 0.1 ml of HBSS and control littersmates received 0.1 ml of HBSS alone.

GVH Reactions (GVHR). The assay used was based on that of Simonsen (20). Litters of 3– to 5-day old F1 mice were used as recipients of donor BALB/c cell suspensions. All recipients were weighed 8 days after parental cell injection and their spleens removed, trimmed of fat and connective tissue, and weighed. A ratio of spleen weight to body weight was computed for HBSS-injected controls and compared with the same ratio in test mice. This formed the basis for the splenomegaly assay; in practice, only when the ratio cell-injected:HBSS-injected exceeded 1.3 were the results taken as being positive. All results represent the mean index of not less than four separate recipient litters.

Adrenalectomy. Mice were adrenalectomized on the day of weaning. A dorsal midline incision was made through the skin then two lateral incisions through the peritoneal musculature were made anterior to the kidneys. The exposed adrenal glands were grasped with forceps, then excised, and the skin incision was then closed with silk sutures. Sham adrenalectomy was performed by exteriorizing the adrenal gland but excision was not completed. Adrenalectomized animals were given 0.9% NaCl as drinking water and placed on normal or deprived diets as appropriate.

Antithymocyte Serum. This was prepared according to the method of Levey and Medawar (21). A pool of serum collected from three rabbits and absorbed with mouse red blood cells was used to inactivate mouse spleen cell suspensions. Using an in vitro cytotoxic assay the pooled absorbed antiserum at a dilution of 1:20 was found to inactivate 95% of thymus cells, 80% of spleen cells, and 45% of bone marrow cells.

Results

Effect of Malnutrition on GVH Reactivity in Different Lymphoid Organs. The pattern of change in cellular reactivity for spleen, mesenteric lymph node,

1 Bell, R., and Lee A. Hazell. Influence of dietary protein restriction on immune competence. II. Effect on lymphoid tissue. Manuscript in preparation.
Peyer's patch, and thymus cells is shown in Figs. 1 and 2. In these experiments, cells were harvested from mice which had been fed their respective diets for 14–28 days after weaning. Significantly increased reactivity was shown by cells from each of the different lymphoid organs of deprived mice, however, the degree of increase varied in each organ and it was only with spleen and mesenteric lymph node cells that a parallel line comparison was valid. In these groups deprived mice showed a fourfold increase in spleen and a 50% increase in mesenteric lymph node GVHR activity. Cells from the thymus and Peyer's patches showed approximately two- and tenfold increases respectively.

Neither deprived or normal bone marrow cells showed GVH reactivity at any age. The GVH capacity of cells from lymph nodes other than the mesenteric could not be tested due to the general hypotrophy of these organs in deprived mice.

**Ontogeny of GVH Reactivity.** Recent experiments have shown that in mice full capacity for various immune reactions may not be reached until a
considerable time after birth (22). It seemed possible that ontogenetic effects could influence the experiments described above and to test this the development of GVH capacity in the spleen cell population was examined in normal and deprived mice of varying ages. The results (Fig. 3) show that the capacity to mount a positive GVH reaction appeared in the spleen at or just before weaning. Full adult reactivity was attained by 10 days after weaning and, in all, complete maturation of GVH-reactive potential took approximately 4 wk from birth in this strain of mouse. Within 7 days of being placed on a deficient diet (i.e. 7 days after weaning) deprived mice exceeded the capacity of both their weaning controls and of normal adults to elicit GVH reactions. The evidence also suggested that there may have been a slight rise in GVH reactivity with increasing time on the deprived diet.

Influence of Adrenalectomy. The splenic lymphocytes responsible for the initiation of GVH reactions have been shown to be resistant to the lympholytic and immunosuppressive effects of corticosteroids (23). A stress-induced corticosteroid hypersecretion could, therefore, by selective lympholysis of the susceptible population, produce artificially high GVH indices in the lymphoid organs of deprived mice. To determine whether any such effect had contributed to the observed increase in GVH reactions the effect of adrenalectomy on the GVH
capacity of cells from the spleens of deprived mice was investigated. The results are shown in Table I and adrenalectomized animals both 8 and 15 days later can be seen to have increased GVH reactions. From this data it appears clear that the increase in GVH reactivity found in deprived mice cannot be mediated solely by the selectively lympholytic effects of increased corticosteroid production.

**Paired-Feeding Trials.** Food intake in mice fed low protein diets ad libitum was lower than that consumed by mice fed normal high protein diets ad lib. A part of the increased GVH activity noted with deprived mice could have been due to a restricted food intake which would, in turn, lead to a reduction in overall caloric intake in addition to the existing protein deficiency. Pair-feeding trials were therefore instituted to examine the possible effect of intake restriction on mice fed a normal diet. The results of GVH assays using spleen cells from mice pair-fed the normal high protein diet are shown in Table II. A significant increase in GVH-reactive potential above normally fed controls was evident in the spleen but this increase was, nevertheless, significantly lower than the reactivity of mice fed a deprived diet. The reactivity of thymocytes was not increased above the normal level nor was the extreme hypotrophy of lymphoid organs as evident in this experimental group.

**The Effect of Nutritional Repletion.** GVH-reactive capacity of spleen cells from deprived mice which had been returned to a normal (20% protein) diet is shown in Fig. 4. Within 5 days of the return to a normal diet a severe loss of GVH-inducing capacity appeared. This dramatic loss persisted until 16 days

![Fig. 3. The development of GVH reactivity in spleen cells in normal and deprived mice.](image)

(○—○), Spleen cells from normal mice; (◇—◇), spleen cells from deprived mice. The arrow at 17 days represents the day of weaning and the first day of feeding normal and deprived diets. Positive responses (spleen index greater than 1.3) are not detected until the day of weaning but deprived mice rapidly exceed the capacity of normal controls to initiate GVH reactions.
**Table I**

**Influence of Adrenalectomy on GVH Reactivity of Spleen Cells from Deprived Mice**

| Experimental procedure | Splenomegaly index |
|------------------------|--------------------|
|                        | 8 Days after       |
|                        | 15 Days after      |
| Adrenalectomized-deprived $5 \times 10^8$ | $1.97 \pm 0.10 (P < 0.05*)$ | $2.36 \pm 0.08 (P < 0.001)$ |
| Sham deprived $5 \times 10^8$ | $2.06 \pm 0.11 (P < 0.05)$ | — |
| Normal $5 \times 10^8$ | $1.68 \pm 0.13$ | — |
| Deprived $5 \times 10^8$ | $2.36 \pm 0.06$ | — |

* Significance assessed by Student's $t$ test and vs. normal in each case.

**Table II**

**Effect of Quantity Restriction (Pair-Feeding) of a Normal Diet on Capacity to Initiate GVH Reactions**

| Treatment                  | Spleen index | Significance* |
|----------------------------|--------------|---------------|
| Pair-fed spleen $5 \times 10^8$ | $2.05 \pm 0.09$ | — |
| Normal spleen $5 \times 10^8$ | $1.68 \pm 0.13$ | Pair-fed vs. normal ($P < 0.05$) |
| Deprived spleen $5 \times 10^8$ | $2.36 \pm 0.06$ | Pair-fed vs. deprived ($P < 0.005$) |
| Pair-fed thymus $2 \times 10^7$ | $1.39 \pm 0.09$ | — |
| Normal thymus $2 \times 10^7$ | $1.37 \pm 0.09$ | Pair-fed vs. normal (NS) |
| Deprived thymus $2 \times 10^7$ | $2.06 \pm 0.14$ | Pair-fed vs. deprived ($P < 0.01$) |

* Significance assessed by Student's $t$ test.

After refeeding commenced and then returned to approximately normal levels by about day 26. This loss of activity was not shared by other organs, for example, cells from the mesenteric lymph node showed a gradual decline in GVH reactivity to normal adult values and never entirely lost this basic capacity.

**The Effect of Antithymocyte Serum.** Spleen cells from normal and deprived mice were incubated with rabbit antimouse thymocyte serum and guinea pig complement (absorbed with mouse thymocytes) immediately before their inoculation into 3- to 5-day old F, recipients. GVH-reactive potential was completely abrogated in cells from both donors using this antiserum. Cells treated with normal rabbit serum and complement retained entirely normal levels of activity.

**Discussion**

The effect of malnutrition on immune responses has generally been measured either in the malnourished host or in vitro on cell populations which have undergone differentiation to a mature effector cell in the same environment. While such assays may clearly show the total defensive capacity of any organism in a particular nutritional state they do not locate the site or sites of breakdown.
or failure in complex immune mechanisms. This is particularly so where the interaction of several cell types may be a prerequisite for normal responses. Such experiments also leave unanswered the question of whether any specific dietary deficiency (such as protein) may act to preferentially limit precursor cells, cell division after successful antigenic stimulation, or the synthesis of antibody, all of which events require protein synthesis.

The experiments outlined here have shown a rather unexpected increase in the capacity of cells from the lymphoid organs of deprived mice to initiate GVHR. The origin of GVH-reactive cells in the thymus has previously been shown by several authors (24, 25) and in this study it was shown that both normal and deprived lymphoid cell populations have their GVH-reactive component destroyed by rabbit antimouse thymocyte serum. The reactivity of cell populations from deprived mice cannot therefore be ascribed to some novel effect of protein deprivation on antibody production or to the sudden acquisition by some non-T-cellular component of GVHR capacity. The conclusion that the increase in reactivity of lymphoid cells from deprived mice is due to a relative increase in the numbers of T cells present in these organs seems clear. McFarlane and Hamid (11) and Aschkenasy (10) both concluded from their observations that dietary protein deficiency produced a greater impairment of T cells than of B cells. Neither sets of results are directly comparable with those reported here due to the very severe protein restriction imposed in their studies and to the use of monkeys and rats, whose dietary requirements may differ considerably from those of mice, as experimental animals.

The experiments of Cooper, Mariani, and Good (26) utilized both mice and dietary protein intakes very similar to those used in this study. They found that skin grafts were rejected more rapidly in deprived mice than in normal controls, thus suggesting intact or even increased T-cell function. Jose and Good (15)
attributed reduced tumor growth in protein-deprived hosts to a clearly defined reduction in blocking antibody production, however, cell-mediated immunity as assayed by an in vitro test was also found to be increased, thus implying that this arm of the immune response was relatively normal. Similarly, in vitro responses to PHA and to various antigens have been found to be normal or even elevated in marasmic children (27). These results have also been confirmed in this laboratory (R. G. Bell, unpublished observations). This evidence thus suggests that the phenomenon of normal or elevated T-cell numbers may be a common finding in the moderate to severe protein-malnutrition syndrome which is typified by the clinical pattern of marasmus.

The GVH reactivity of the normal cell population from each of the organs studied fell into two different categories. The secondary lymphoid organs, mesenteric lymph node and spleen, had high intrinsic GVH reactivity which was increased relatively little, (50% and fourfold respectively) in malnourished mice. Conversely, the cells from normal mouse thymus and Peyer's patches possessed only marginal reactivity even in very high concentration. The effect of malnutrition was to change the slope of their dose-response curves (Figs. 1 and 2) so that they more closely resembled secondary lymphoid organs in their reactivity patterns. Because these dose-response curves are no longer parallel it is impossible to compute precisely the increase in reactivity but this was quite large in both cases. The simplest explanation of the change in the pattern of responses in all organs was the loss of a population or populations of cells which were nonreactive in GVH assays. Histologically there was a very great but not complete loss of the thymic cortex and total cell yields from the thymus often fell to 10% or less of the numbers found in normally nourished controls; a similar proportionate loss of cell numbers was also found in the Peyer's patches. Since the major cellular population in both organs is lymphoid, it is likely that the cells lost correspond to either B lymphocytes or immature cells in the case of Peyer's patches and to immature T cells in the thymus. The relatively small change in reactivity of the mesenteric lymph node cell population reflected a maintenance of this organ's weight (both organ weight and cell yield only fell to 40% of that of control mice). The conservation of mesenteric lymph node mass and cellularity appeared to be mainly due to the antigenic challenge delivered to the mesenteric lymph node from the gut by draining lymphatics.

The very great inhibition of cellular proliferation, evident in the reduction of body growth caused by dietary protein restriction provides the most likely explanation for these results. Since there's considerable evidence that some lymphoid cell populations, particularly B cells, have a short life span (28), it is reasonable to assume that recruitment into this population would be more rapidly diminished than into the long-lived T-cell population. The capacity to induce GVH reactions is a function of long-lived recirculating T cells (24) and the persistence of this function demonstrates that attrition of this cell type was slow under moderate nutritional stress. The studies of Spear and Edelman (22, 29) have shown that both T and B cells may be present in the spleen of neonatal mice but that they were incapable of mounting immune responses in mice of that age. Some further maturation steps appeared necessary before this population gradually acquired full adult immune reactivity. These authors have also shown
that at birth the percentage of T cells in the neonatal spleen is approximately 73% of adult values and this finding has been confirmed for other lymphoid organs of BALB/c mice (30) and is also probably true for the spleen in this strain of mouse. In deprived mice the contribution of newly divided thymocytes to the peripheral population must fall dramatically immediately after weaning as the thymus displays a rapid loss of weight which is quite disproportionate to changes in body weight. Little de novo contribution to the peripheral T-lymphocyte pool could therefore be expected and the rapid increase in activity of spleen cells from deprived mice shown in Fig. 3 probably contains a large maturation component in addition to a loss of non-T cells. The very rapid dilution of GVH reactivity in the spleen cell population of nutritionally repleted mice (Fig. 4) also suggested either a very rapid division rate within the spleen or an influx into the spleen of cells lacking GVH reactivity in these mice. This phenomenon was organ-specific and similar changes in reactivity were not observed in mesenteric lymph node or thymus cells of nutritionally repleted animals.

The persistence of long-lived T cells and the possibility that these may continue to be produced (albeit at a lower rate) in nutritionally deprived hosts offers another possible explanation for the observed decrease in the incidence of tumors in malnutrition (12-14). Cells derived from the thymus are believed to be important in tumor surveillance (31) and the results cited here offer a mechanism which could bring about more efficient tumor surveillance in malnutrition.

Summary

The effect of dietary protein restriction in mice on the capacity of their lymphoid cells to induce graft-vs.-host responses (GVHR) was studied. Mice were fed diets containing 4% or 20% protein ad libitum. The GVHR capacity of cells from all lymphoid organs of deprived mice was increased on a cell-for-cell basis over that of their normally fed counterparts. The slope of the dose-response curves did not change for spleen and mesenteric lymph node cells although their reactivity was increased by fourfold, and 50% respectively. The slope of the curves for thymus and Peyer's patches was changed indicating fundamental changes in the reactive cellular populations of these organs. Changes in GVH reactivity of cell populations from deprived mice were not mediated by increased corticosteroid production as adrenalectomy did not reduce their GVH responses.

An explanation for the results was sought in a general decrease in production of short-lived cells with a rapid turnover such as most B cells. Long-lived T cells appear to persist and retain their reactivity for quite long periods in the face of nutritional insults.

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References

1. Schaedler, R. W., and R. J. Dubos. 1956. Reversible changes in the susceptibility of mice to bacterial infections. II. Changes brought about by nutritional disturbances. J. Exp. Med. 104:67.
2. Schaedler, R. W., and R. J. Dubos. 1959. Effect of dietary proteins and amino acids on the susceptibility of mice to bacterial infections. J. Exp. Med. 110:921.
3. Boyd, F. M., and H. M. Edwards, Jr. 1963. The effect of dietary protein on the course of various infections in the chick. J. Infect. Dis. 112:53.
4. Watson, M. 1937. Studies on the influence of diet on resistance to infection. II. The effect of various diets on the resistance of mice to bacterial infection. J. Hyg. 37:420.
5. Scrimshaw, N. S., C. E. Taylor, and J. E. Gordon. 1968. Interactions of nutrition and infection. World Health Organization, Monograph Series. 57.
6. Taylor, P. E., C. Tejada, and M. Sanchez. 1967. The effect of malnutrition on the inflammatory response. As exhibited by the granuloma pouch of the rat. J. Exp. Med. 126:539.
7. Guggenheim, K., and E. Buechler. 1948. Nutrition and resistance to infection. The effect of quantitative and qualitative protein deficiency on the bactericidal properties and phagocytic activity of peritoneal fluid of rats. J. Immunol. 58:133.
8. La Via, M. F., P. A. Barker, and R. W. Wissler. 1956. A study of the correlation of antigen phagocytosis and the splenic histologic reaction with antibody formation in protein-depleted rats. J. Lab. Clin. Med. 48:237.
9. Kenney, M. A., C. E. Roderuck, L. Arnrich, and F. Piedad. 1968. Effect of protein deficiency on the spleen and antibody formation in rats. J. Nutr. 95:173.
10. Aschkenasy, A. 1973. Differing effects of dietary protein deprivation on the production of rosette-forming cells in the lymph nodes and the spleen and on the levels of serum haemagglutinins in rats immunized to sheep red cells. Immunology. 24:617.
11. McFarlane, H., and J. Hamid. 1973. Cell mediated immune response in malnutrition. Clin. Exp. Immunol. 13:153.
12. Rous, P. 1914. The influence of diet on transplanted and spontaneous mouse tumors. J. Exp. Med. 20:433.
13. White, J., and H. B. Andervont. 1943. Effect of a diet relatively low in cystine on the production of spontaneous mammary-gland tumors in strain C3H female mice. J. Natl. Cancer Inst. 3:449.
14. Theuer, R. C. 1971. Effect of essential amino acid restriction on the growth of female C57BL mice and their implanted BW 10232 adenocarcinomas. J. Nutr. 101:223.
15. Jose, D. G., and R. A. Good. 1971. Absence of enhancing antibody in cell mediated immunity to tumor heterografts in protein deficient rats. Nature (Lond.). 231:323.
16. Jose D. G., and R. A. Good. 1973. Quantitative effects of nutritional essential amino acid deficiency upon immune responses to tumors in mice. J. Exp. Med. 137:1.
17. Miller, J. F. A. P., and G. F. Mitchell. 1969. Thymus and antigen reactive cells. Transplant. Rev. 1:3.
18. Johnston, J. M., and D. B. Wilson. 1970. Origin of immunoreactive lymphocytes in rats. Cell. Immunol. 1:430.
19. Katz, D. H., and B. Benacerraf. 1972. The regulatory influence of activated T cells on B cell responses to antigen. Adv. Immunol. 15:1.
20. Simonsen, M. 1962. Graft versus host reactions. Their natural history, and applicability as tools of research. Prog. Allergy. 6:349.
21. Levey, R. H., and P. B. Medawar. 1966. Nature and mode of action of anti lymphocyte serum. Proc. Natl. Acad. Sci. U.S.A. 56:1130.
22. Spear, P. G., and G. M. Edelman. 1974. Maturation of the humoral immune response in mice. J. Exp. Med. 139:249.
23. Cohen, J. J., and H. N. Claman. 1971. Hydrocortisone resistance of activated initiator cells in graft versus host reactions. Nature (Lond.). 229:274.
24. Cantor, H., and R. Asofsky. 1972. Synergy among lymphoid cells mediating the
graft-vs.-host response. III. Evidence for interaction between two types of thymus-derived cells. *J. Exp. Med.* 135:764.

25. Stutman, O., E. J. Yunis, and R. A. Good. 1970. Cooperative effect of thymic function and lymphohemopoietic cells in restoration of neonatally thymectomized mice. *J. Exp. Med.* 132:583.

26. Cooper, W. C., T. Mariani, and R. A. Good. 1970. Effects of chronic protein depletion on immune response. *Fed. Proc.* 29:364.

27. Jose, D. G., J. S. Welch, and R. L. Doherty. 1970. Humoral and cellular immune responses to streptococci and other antigens in Australian Aboriginal school children. *Aust. Paed. J.* 6:192.

28. Sprent, J., and A. Basten. 1973. Circulating T and B lymphocytes of the mouse. II. Life span. *Cell. Immunol.* 7:40.

29. Spear, P. G., A. L. Wang, V. Rutishauser, and G. M. Edelman. 1973. Characterization of splenic lymphoid cells in fetal and newborn mice. *J. Exp. Med.* 138:557.

30. Raff, M. C., and J. J. T. Owen. 1971. Thymus-derived lymphocytes: their distribution and role in the development of peripheral lymphoid tissues of the mouse. *Eur. J. Immunol.* 1:27.

31. Burstein, N. A., and A. C. Allison. 1970. Effect of anti lymphocytic serum on the appearance of reticular neoplasms in SJ1-J mice. *Nature (Lond.)*. 225:1139.