Influence of Diet on the Induction of Experimental Autoimmune Thyroid Disease (44062)

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Abstract. **Immunization of CBA/J mice with thryoglobulin (Tg) emulsified in complete Freund's adjuvant induces experimental thyroiditis (EAT), a well-characterized model of Hashimoto's disease. Recent studies have suggested that dietary factors play a role in the modulation of the immune response and that diet can have a profound effect on the induction of autoimmune diseases. In this study, we examined the influence of diet on autoimmune thyroiditis in mice. EAT was induced in mice fed *ad libitum* one of the three diets, a standard maintenance chow (Agway H1000), Purina 5020 Breeding Chow, and Purina 5010 Autoclavable (unautoclaved) Diet.**

Tg-immunized mice fed the Agway 1000 diet were found to be resistant to the development of autoimmune thyroid disease, with only 4 out of 25 mice developing mild thyroiditis. In contrast, 16 out of 25 mice fed the Purina 5010 diet developed moderate to severe thyroiditis. Mice fed the 5020 diet were partly susceptible: 7 out of 25 developed a mild to moderate thyroiditis. Histologic examination of thyroid glands of diseased mice fed the 5010 and 5020 diets showed marked lymphocytic infiltration with destruction of follicles, compared with mice fed the Agway diet, the latter showing only mild infiltration with preservation of thyroid follicles. Titers of antibody to Tg did not differ among the groups, and there was no significant difference in the IgG isotype subclass usage.

The results demonstrate that diet can markedly affect the severity of autoimmune disease in the EAT model. In contrast, diet has little effect on the humoral autoimmune response in this system. These results implicate diet as a factor in the severity of cell-mediated autoimmune destruction and suggest that dietary modification could decrease pathology in some forms of autoimmune disease. [P.S.E.B.M. 1996, Vol 213]

Experimental autoimmune thyroiditis (EAT) is an animal model of Hashimoto's thyroiditis (HT). One form of EAT is induced by the injection of genetically susceptible strains of mice with mouse thyroglobulin (Tg) in complete Freund's adjuvant (1).

During the course of our studies on murine EAT, we noted a marked reduction in the incidence and severity of thyroiditis. Upon inquiring of any changes in animal care, we learned that there had been, during this time, a change in the rodent diet used in our animal facility. Therefore, to follow up on this interesting observation, we have directly compared the effects of three different rodent chows on the development of autoimmune thyroiditis and anti-Tg antibodies. The results of this study demonstrate that severity of thyroiditis is directly related to diet and suggest that diet may modulate cell-mediated autoimmune damage while having little influence on humoral immunity.

Materials and Methods

Mice. Six- to eight-week-old female CBA/J mice were purchased from the NCI Cancer Research Facility (Frederick, MD) and were housed, five per cage, in the Animal Care Facility of The Johns Hopkins University School of Hygiene and Public Health.

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Three different commercial diet preparations were prepared: Purina Autoclavable Rodent Chow (Diet 5010) (not autoclaved); Purina Breeding Mouse Chow (Diet 5020); and Agway Mouse Chow (Diet H1000). The last of these is the standard maintenance diet used at the JHU Animal Care Facility. Detailed composition of the diets is shown in Table I. For two of the diets, these values were independently validated by direct analysis at Hazleton Laboratories, Madison, WI (Table I). The animals were fed *ad libitum* one of the three diets for 3 weeks before the induction of thyroiditis.

Table I. Composition of the Three Diets Fed to Mice Subjected to EAT Induction

Ingredient	5010[a]	5020[b]	H1000[c]
Manufacturer determination			
Protein (%)	23.50	20.50	14.50
Fat (%)	6.00	9.00	6.50
Energy (kcal/g)	4.25	4.20	4.20
Calcium (%)	1.00	0.81	0.90
Phosphorus (%)	0.67	0.60	0.80
Potassium (%)	0.92	0.69	0.70
Magnesium (%)	0.22	0.17	0.23
Sodium (%)	0.28	0.26	00.40
Chlorine (%)	0.39	0.41	0.10
Fluorine (ppm)	35.00	N/A[d]	32.00
Iron (ppm)	184.00	164.20	280.00
Zinc (ppm)	124.30	114.40	50.00
Manganese (ppm)	115.00	136.20	55.10
Copper (ppm)	19.60	21.20	18.00
Cobalt (ppm)(	0.44	0.70	1.00
Iodine (ppm)	0.73	0.55	1.00
Chromium (ppm)	1.95	1.80	N/A
Selenium (ppm)	0.20	0.22	0.17
Carotene (ppm)	4.50	Trace	N/A
Menadione (ppm)	8.80	8.80	0.7
Thiamine (ppm)	86.50	8.50	7.7
Riboflavin (ppm)	8.00	8.00	7.6
Niacin (ppm)	100.00	55.00	56.32
Pantothenic acid (ppm)	25.40	21.00	17.18
Choline (ppm)	22.00	22.00	147.8
Folic acid (ppm)	6.00	2.10	0.89
Pyridoxine (ppm)	6.00	5.00	7.39
Biotin (ppm)	0.11	0.20	0.29
B12 (mcg/kg)	33.00	15.60	40.00
Vitamin A (IU/g)	46.00	30.00	15.70
Vitamin D (IU/g)	4.40	3.30	1.04
Vitamine E (IU/g)	66.10	66.00	0.05
Hazleton Laboratories Determination			
Calories (cal/100g)	374.00	—	369.00
Protein (%)	26.1	—	16.40
Moisture (100° vac. oven)	8.20	—	9.00
Total fat (%)	6.40	—	6.20
ASH (%)	6.40	—	6.40
Total carbohydrates (%)	52.90	—	62.00
Iodine (ppm)	0.915	—	1.23
Vitmin A (IU/g)	46.40	—	14.70

[a] Purina 5010 Autoclavable Rodent Chow.
[b] Purina 5020 Breeding Rodent Chow.
[c] Agway H1000 Standard Maintenance Rodent Chow.
[d] N/A, not applicable.

Food consumption was determined by weighing the unused food in the cage and subtracting it from the initial amount placed in each cage. Average daily food consumption per mouse was calculated by dividing the total consumption from the respective cage with the number of days and the number of mice in that cage. Furthermore, kilocalorie consumption was calculated from the information regarding kilocalories per gram of each diet provided in Table I. Mice were weighed at frequent intervals to determine the average weight gain.

Immunization. Mouse Tg was prepared from frozen mouse thyroid glands (Pelfreeze, Rogers, NJ), as described previously (2). Briefly, glands were thawed and separated from the trachea, homogenized in borate buffer (pH 8.3), and centrifuged at 100,000*g* for 1 hr. The clarified supernatant was separated in a Sephacryl-300 (Pharmacia, Piscataway, NJ) gel column. The first major peak, containing Tg was collected and the protein concentration calculated from the spectrophotometer reading at 280 nm. For immunization, Tg was emulsified with complete Freund's adjuvant (Difco, Detroit, MI) and injected subcutaneously at multiple sites on the back, at a dose of 100 μg/mouse in a 100-μl volume. Two immunizations were given 7 days apart. Animals were sacrificed 4 weeks after the first immunization. A control group of mice was immunized with mouse heart myosin emulsified with complete Freund's adjuvant.

Anti-Thyroglobulin Antibody Analysis. Blood was collected by cardiac puncture and sera analyzed by an enzyme-linked immunosorbent assay (ELISA). A modification of a previously described method was used (3). Briefly, Immulon 2 plates (Dynatech, Chantilly, VA) were coated with 2 μg/ml mouse Tg in carbonate-bicarbonate buffer (pH 7.2), overnight, at room temperature, then washed with phosphate buffered saline (PBS)-Tween and blocked with 1% bovine serum albumin (BSA) for 1 hr. Serum diluted in PBS-Tween was incubated in the plates for 2 hr at room temperature. After three washings with PBS-Tween and a rinse with PBS, alkaline phosphatase-conjugated goat anti-mouse IgG affinity-purified antibody (Cappell, Durham, NC) (1:500 dilution) was added for 1 hr. After repeated washings, plates were incubated with the substrate para-nitrophenol (Sigma Chemical Co., St. Louis, MO) 1 mg/ml in 10% diethanolamine buffer (pH 9.8). Absorbance was measured with a Dynatech ELISA reader at 405 nm.

IgG Subclass Analysis. IgG subclass isotypes IgG1, IgG2a, IgG2b, and IgG3 were analyzed by ELISA (4). Immulon 2 plates were coated with 2 μg/ml Tg in carbonate-bicarbonate buffer for 3 hr at room

temperature. Wells were washed with PBS-Tween and unoccupied regions of the plate were blocked with 1% BSA in PBS for 45 min. After the second wash, the mouse serum in different dilutions was incubated overnight at room temperature. Plates were washed again with PBS-Tween, rinsed with PBS, and incubated with alkaline phosphatase-conjugated secondary antibody (goat anti-mouse IgG1, 1:1500; IgG2a, 1:300; IgG2b, 1:1000; or IgG3, 1:300; Southern Biotechnology Associates; Birmingham, AL) for 6 hr. After the final wash, the substrate, para-nitrophenol 1 mg/ml in 10% diethanolamine buffer (pH 9.8), was added. Absorbance was measured with a Dynatech ELISA reader at 405 nm.

Histology. Thyroid glands were fixed in 10% buffered formalin and embedded in glycolmethacrylate. Sections were stained with toluidine blue and examined under the microscope for mononuclear cell infiltration and follicular destruction. Pathological index was determined by the following criteria: 0 = no infiltration in the thyroid gland; 1 = infiltration up to 25%; 2 = between 25% and 50% infiltration and follicle destruction; 3 = between 50% and 75% infiltration and follicle destruction; and 4 = between 75% and 100% infiltration and follicle destruction.

Immunohistochemistry. Thyroid glands from one series of experiments were immersed in OCT compound (Miles Inc., Naperville, IL) and snap frozen in dry ice cooled isopentane. Cryosections were used for immunohistochemical localization of lymphocytic phenotype. An indirect immunohistochemistry procedure (1) was used for identification of inflammatory cells in the thyroid. Tissue sections (5 μm) were placed on Histostik (Accurate Antibodies, Westbury, NY)-coated slides and acetone-fixed for 10 min. Endogenous peroxidase was inactivated by a 30-min incubation with 0.5% hydrogen peroxide in Tris-buffered saline (TBS)–milk (0.05 *M* Tris buffer in 0.85% saline, containing 0.5% skimmed dry milk [Carnation Company, Los Angeles, CA]) and 1% normal rabbit serum (NRS). This and all subsequent incubations were performed at room temperature with TBS-milk washes following each incubation period.

Inflammatory cells were identified by using tissue culture supernatants from the rat hybridoma clones M1/9.3.4.HL.2 (anti-common leukocyte antigen, CD45), GK1.5 (anti-CD4), and 53-6.72 (anti-CD8) from American Type Culture Collection (ATCC, Rockville, MD) followed by a 1-hr incubation with biotin-conjugated rabbit anti-rat IgG (Vector Laboratories, Burlingame, CA). Tissues were incubated for 1 hr with streptavidin horseradish peroxidase complex (Vectastain ABC; Vector Labs) and diaminobenzidine tetrahydrochloride substrate (Sigma) for 8 min. The slides were immersed in copper sulfate solution (0.5% copper sulfate in 0.15 *M* NaCl) for 2 min, followed by counterstaining with Mayer's modified hematoxylin (Polyscientific, Bay Shore, NY).

Results

Energy Intake and Weight Gain. Total caloric intake was correlated with the average weight gain in the three dietary groups of mice. Table II shows the average daily food intake, kilocalories consumed per day, and average weight gain in a week. The lowest average daily food consumption per mouse was recorded in the Purina 5010 diet–fed animals compared with the other diets. When calculated in terms of kilocalories per day per mouse, the 5010 diet resulted in a lower intake than the other two diets. Among the three diets, the average weight gain per week was the highest with the 5010 diet. Because the weight determinations were made before as well as after immunization, while the animals were being adapted to the respective diets, the weight differences were not a reflection of disease-associated hypothyroidism but of diet-associated metabolic changes. All mice appeared healthy during the course of the experiment, and there was no evidence of overt hypothyroidism or of infection.

Effect of Diet on the Induction of Thyroiditis. Table III presents a compilation of three separate experiments. A total of 25 animals in each group was immunized with Tg and the resulting number and percentage of animals that developed thyroiditis in each group are shown. Diagnosis was based on histological examination of the thyroid glands. Severity of the lesions was graded according to the nature of the cellular infiltrates and the amount of follicular destruction, following criteria described in Materials and Methods. Statistical analysis using the chi-square method shows that the difference in disease induction among the three dietary groups was significant at the level of $P < 0.002$ among groups. Table IV illustrates that induction of thyroiditis is antigen-specific as mice immunized with murine myosin or adjuvant alone do not

Table II. Weight Gain of Mice in Relation to Food Intake and Type of Diet

Diet[a]	Average food intake per mouse[b] (g)	Kcal per day[c]	Average weight gain per week (g)[d]
Purine 5010	2.53	10.75	1.8 ± 0.5
Purine 5020	2.71	11.38	1.1 ± 0.8
Agway H100	2.70	11.34	0.8 ± 0.3

[a] CBA/J mice fed on three different diets, described in Table I.
[b] Average food intake/mouse/day. See *Materials and Methods.*
[c] Calculated from the kcal/g information of each diet (Table I) and the average daily food intake per mouse ([b]).
[d] Numbers represent the mean ± SD of five mice per group.

Table III. Incidence of Thyroiditis Induction and Pathological Index in Mice Fed Three Different Diet Preparations

Diet	*n*	Animals with thyroiditis[a]	Path index[b]
Purina 5010	25	16 (64)	3.5
Purina 5020	25	7 (28)	1.5
Agway H1000	25	4 (16)	0.5

Note. Mice were preadapted to the diets indicated for 3 weeks prior to immunization with mTg in CFA on Day 0 and Day 7. Thyroids were harvested on Day 28.

[a] Chi-square analysis was performed and the differences in disease induction were found to be statistically significant ($P < 0.002$). Values in parentheses are percentages.

[b] Mean pathological index criteria: 0, no infiltration in the thyroid gland; 1, infiltration up to 25%; 2, infiltration and follicle destruction between 25% and 50%; 3, between 50% and 75% infiltration and follicle destruction; 4, between 75% and 100% infiltration and follicle destruction.

develop EAT. These controls further show that the diet alone does not induce thyroiditis. Figure 1 presents representative lesions found in the thyroid glands in mice fed on the different diets. Clearly, the 5010 diet was associated with massive cellular infiltrates, while the standard Agway diet was associated with few infiltrating cells, or only small foci. Interestingly, mice receiving the 5020 diet presented with an intermediate level of infiltrates and an intermediate frequency of animals with disease.

Immunostaining demonstrated that the phenotypic makeup of the mixed cellular infiltrate was mostly lymphocytes (i.e., $CD45^+$) with about 25% of the infiltrate comprised of $CD4^+$ cells and 10% of $CD8^+$ cells. In addition, plasma cells, macrophages, neutrophils, and eosinophils were seen.

Effect of Diet on Anti-Thyroglobulin antibody Titers. Figure 2 presents the ELISA results of an ELISA of pooled serum of mice taken 28 days after immunization. Anti-Tg antibody was present in all groups. Statistically, there was no significant difference among the groups in the overall antibody response to Tg ($P > 0.05$, by analysis of variance [ANOVA]).

Effect of Diet on Anti-Thryoglobulin Antibody IgG Isotype Subclass Analysis. Figure 3 presents the results of IgG1, IgG2a, IgG2b, and IgG3 subclass analysis of anti-Tg antibodies in the serum of the three different groups of mice. On Day 28, the subclass usage in the Tg antibodies of the three groups did not show any statistically significant correlation with the incidence on severity of thyroiditis in the three dietary groups ($P > 0.05$, by ANOVA), indicating that variation among the means of the IgG subclass titers was not significantly greater than expected by chance. However, the IgG2a subclass seemed somewhat higher in the mice fed Purina 5020 and much lower in the Agway-fed mice.

Discussion

There is growing evidence that diet has a profound effect on autoimmune disease (5). Investigation on the influence of caloric restriction on the lupus-prone NZB hybrid mouse has shown that in underfed mice the response of spleen cells to T-cell mitogens increased. The mice had better preserved cytotoxic and plaque-forming cell responses to allogeneic cells, diminished production of autoantibodies, and reduced deposition of gamma-globulins in capillaries of renal glomeruli (6). Qualitative differences in dietary fat intake have been shown to affect autoantibody isotypes. Saturated fat favors the IgM isotype, while unsaturated fats enhance titers of IgG autoantibodies (7). It has been proposed that fats affect autoimmunity by modulating the synthesis of prostaglandins and leukotrienes (8). Essential fatty acid deficiency has been shown to prevent multiple low-dose streptozotocin-induced diabetes in CD-1 mice (9). In addition, fish oil diet, rich in eicosapentaenoic acid—a minor precursor of arachidonic acid—prevented the induction of lupus-like autoimmune disease (10). In similar experiments, fish oil diet protected mice from collagen-induced arthritis (11). An understanding of the immunomodulatory role of various dietary factors can potentially lead to novel preventive and therapeutic approaches for the autoimmune diseases.

In the present study, we have demonstrated that differences in diet can modulate the *in vivo* induction of experimental autoimmune thyroiditis in susceptible mice. Among the three different diets used in this investigation, the incidence of thyroiditis is markedly reduced in animals fed the Agway H1000 diet. There was minimal focal infiltration in the thyroid glands of the mice fed Agway diet compared with the moderate and extensive cellular infiltration found with Purina diets 5020 and 5010, respectively. The humoral immune response was similar in all dietary groups; however, there was no significant difference due to diet in the total anti-Tg antibody titer or the IgG subclass-specific titers.

Our investigation demonstrates that the 5010 diet has factors that either directly contribute to target organ susceptibility to inflammatory cell infiltration, or potentiate the cellular immune response generated against the immunizing antigen. Potentially pertinent

Table IV. Thyroiditis Induction is Antigen-Specific

Treatment	*n*	Purina 5010	Purina 5020	Agway H1000
Adjuvant Only	10	0	0	0
mTG + Adjuvant	25	16	7	4
Myosin + Adjuvant	5	0	0	0

Note. The table shows the number of animals in each group and the resultant thyroiditis induction.

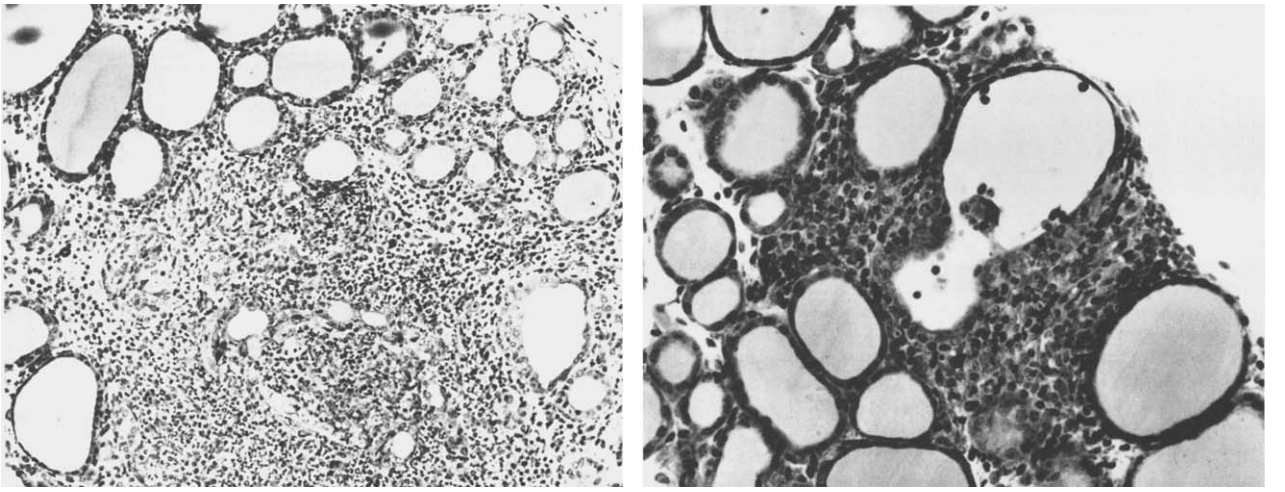

Figure 1. Histological section of thyroid gland from CBA/J mouse immunized with 100 μg/dose of mouse Tg and adjuvant. (A) The diffuse mononuclear cell infiltration of the thyroid gland from a mouse immunized with Tg and fed Purina diet 5010. (B) A focal area of mononuclear cell infiltrate in the thyroid gland of a similarly immunized mouse fed the Purina 5020 diet.

factors that may contribute to this effect are protein, zinc, manganese, carotene, menadione, thiamine, niacin, pantothenic acid, folic acid, vitamin A, vitamin D, and vitamin E, as these ingredients are shown to be quantitavely greater in the Purina 5010 diet than in the Agway H1000 diet (Table I). On the other hand, the components in the Agway diet may suppress the *in vivo* induction of a severe inflammatory response against the thyroid gland. Various factors that occur in higher amounts in the Agway diet are iron, cobalt, choline, iodine, biotin, and B12 (Table I). These ingredients may contribute to the protective effect of this diet on the induction of EAT. Mice fed Purina 5020 diet show thyroid lesions intermediate between those seen in mice fed the Purina 5010 and Agway H1000 diets. The dietary ingredients that are somewhat greater than the Agway H1000 diet are protein, zinc, cobalt, thiamine, pantothenic acid, folic acid, biotin, vitamin A, and vitamin D. Further studies are needed to characterize the factor or factors that modulate the immune response and contribute to the induction of autoimmune disease.

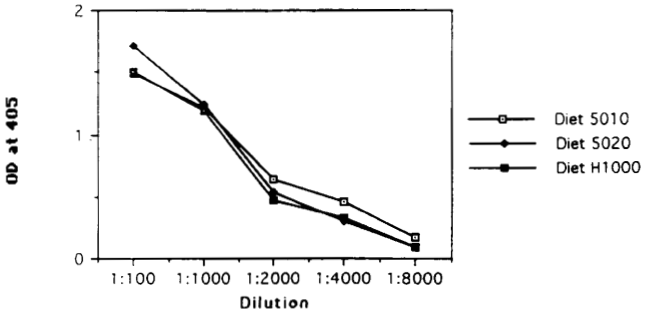

Figure 2. Anti-Tg antibody titer in the serum of CBA/J mice fed three different diets and immunized with 100 μg/dose mouse Tg and adjuvant. ELISA was performed on pooled serum from five mice in each dietary group. No significant difference was detected among the three groups ($P > 0.05$ at each dilution).

Previous studies have mainly concentrated on individual dietary factors. However, it is essential to understand the balancing effect resulting from the interplay of various components in the diet. A high-protein, low-fat diet has been shown to slow the process of autoimmune disease development in a variety of animal models (6). In our experiments, however, the results demonstrate an increased disease incidence in animals fed on diets containing comparatively higher amounts of protein within the three groups.

Among the minerals, iodine supplements have been shown to increase the immunogenicity of Tg and to produce a higher incidence of autoimmune thyroid

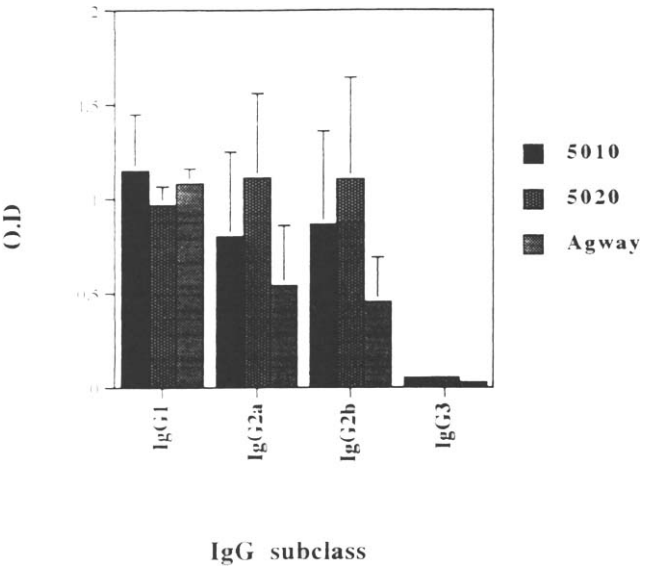

Figure 3. IgG isotype subclass analysis in the three different groups of mice was performed by ELISA on pooled serum from five mice in each dietary group. The results represent the mean of four sets of five different animals. No significant difference were observed ($P > 0.05$ for each subclass).

disease in animal models (12–14). Contrary to these reports, in our study higher iodine content, as seen in the Agway diet, was associated with a lower incidence of thyroiditis compared with the other two diets.

Zinc deficiency (>5 ppm) has been shown to protect from, or delay, autoimmune disease induction in mice (15). Our diets contained normal dietary level of zinc: 124.3, 114.4, and 50 ppm for 5010, 5020, and Agway diets, respectively (Table I). Although the three diets were not deficient in zinc, they differed considerably in their amounts of zinc. Moreover, these differences correlated with the incidence of disease outcome in our experiments. Therefore, zinc may be one of several dietary factors that are important in disease susceptibility. Reports suggest that a paucity of this mineral may impair the production of zinc-dependent thymic hormones, which are crucial for normal T-cell differentiation and may influence the development of autoimmune diseases (16, 17).

Vitamins A, C, D, E, and biotin have been independently shown to modulate the immune response (18, 19). In unpublished studies carried out with Dr. Alan Scott, we investigated the role of vitamin A in autoimmune thyroid disease induction, using two defined diets that contained either high or low levels of vitamin A. No difference was observed in the antibody levels and the thyroid pathology between the two groups. However, other components in the three diets could synergize with vitamin A to modulate the outcome of disease induction. One instance of the role of secondary vitamin A deficiency in mediating the effects of zinc deficiency on autoimmunity was investigated by Gershwin *et al.* (20) and by Suskind (21). Contrary to expectation, they found that vitamin A–deficient animals had more severe hypergammaglobinaemia than the control group. Other experiments demonstrated that mice chronically fed a diet very high in retinyl acetate had enlarged thymus and lymph nodes; a greater antibody response after sensitization with sheep red blood cells; and a more rapid rejection of skin grafts (21). This finding suggests that vitamin A deficiency itself could be another independent variable entering the complex process by which nutrition can influence the course of autoimmune disease. Vitamin A is known to potentiate the cell-mediated immune response (22), thereby indicating that an excess of vitamin A may also be involved as a co-factor in the enhancement of autoimmune disease in susceptible individuals.

Administration of vitamin D supplements have been shown to prevent experimental allergic encephalomyelitis in mice (23). However, in our experiments the vitamin D content was higher in the diet with the greatest disease incidence.

Since autoimmune diseases are multifactorial in origin, our results suggest that nutrition may play a vital role in modulating autoimmune or other cell-mediated inflammatory disease. Modification of diet has been shown to affect cell-mediated immunity, cytokine secretion, and responsiveness (24). It is possible that the differences in cellular infiltrates observed as a result of the three different diets in our investigation may be due to an impact of diet on various cytokines.

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