# Biotin deficiency induces changes in subpopulations of spleen lymphocytes in mice[1–3]

*Armida Báez-Saldaña, Georgina Díaz, Bertha Espinoza, and Enrique Ortega*

**ABSTRACT** Biotin deficiency is known to affect immune function in both humans and experimental animals. In this study, we determined the effect of biotin deficiency on 4-wk-old Balb/cAnN mice during 20 wk of experimentation. The growth rate of mice slowed significantly during the first 6 wk of consumption of a diet designed to induce biotin deficiency; thereafter, from weeks 7 to 20 there was progressive weight loss in the mice receiving the biotin-deficient diet. In the livers of biotin-deficient mice, the specific activities of two biotin-dependent enzymes—pyruvate carboxylase and propionyl-CoA carboxylase—decreased by as much as 75% and 80%, respectively, and in spleen lymphocytes the specific activities of these two enzymes decreased by 63% and 75%, respectively. With respect to the effects of biotin deficiency on the immune system, we observed statistically significant changes in both the absolute number of spleen cells and in the proportions of spleen cells carrying different phenotypic markers: after 16 wk the percentage of cells expressing surface immunoglobulin (sIg) decreased from 47% (control and supplemented) to 27% (deficient) and $CD3^+$ cells increased from 42% (control and supplemented) to 54% (deficient). The mitogen-induced proliferation of spleen cells from deficient mice was lower than that of spleen cells from the control mice. These findings suggest that biotin could have an important role in lymphocyte maturation and responsiveness to stimulation, and consequently in the capacity of the immune system to respond to an antigenic challenge. *Am J Clin Nutr* 1998;67:431–7.

**KEY WORDS** Biotin deficiency, pyruvate carboxylase, propionyl-CoA carboxylase, immune system, mice, spleen, lymphocyte subsets

## INTRODUCTION

Biotin is a water-soluble vitamin of the B complex that serves as a cofactor of four enzymes: propionyl-CoA carboxylase (PCC), pyruvate carboxylase (PC), methylcrotonoyl-CoA carboxylase, and acetyl-CoA carboxylase. The first three of these enzymes are found in mitochondria and the fourth in the cytoplasm. These four enzymes participate in various pathways in the metabolism of proteins, lipids, and carbohydrates (1).

Studies of patients with impaired biotin metabolism and of biotin-deficient animals have established that normal biotin intake and metabolism are important for the defense mechanisms of the organism. Two defects in the biotin utilization cycle are known in humans; abnormalities in the activity of either holocarboxylase synthetase (the enzyme responsible for covalently attaching biotin to specific lysine residues on the carboxylases) or in biotinidase (the enzyme responsible for detaching biotin from biocytin) result in multiple carboxylase deficiency, which has been reported to be associated with clinical manifestations of immune dysfunction, including mucocutaneous candidiasis, hypergammaglobulinemia with immunoglobulin A (IgA) deficiency, overwhelming infections, and a diminished antibody response to *Candida* or pneumococcal antigens (2, 3). Some clinical symptoms of multiple carboxylase deficiency have been reported to disappear after the oral administration of biotin (4).

Likewise, biotin-deficient rats showed a weak antibody response against diphtheria toxoid (5) as well as a lower number of antibody-forming cells in the spleens of the rats immunized previously with sheep erythrocytes (6). In another study, an important reduction in the size and cellularity of the thymus was found in biotin-deficient rats, which also had a depressed immune response against sheep erythrocytes and did not develop experimental allergic encephalomyelitis after immunization with myelin basic protein (7). Biotin has also been shown to be essential for the in vitro generation of cytotoxic T lymphocytes from mouse spleen (8). In guinea pigs, biotin deficiency induced an increase in the number of circulating neutrophils and a decrease in lymphocytes carrying B and T cell markers (9).

The clinical and experimental evidence summarized above indicates that biotin deficiency has significant effects on the ability of the organism to mount an adequate immune response. However, the precise functions that are affected, the possible differences in biotin metabolism among species, as well as the pathogenesis of the immune deficiency remain to be established.

We studied the effect of biotin deficiency on indicators of the immune function in mice. We assessed the functional state of biotin in the same experimental animals, as reflected in the serum concentration of free biotin and in the specific activity in liver and spleen of two biotin-dependent enzymes: PC and PCC.

[1] From the Department of Immunology, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de Mexico, Mexico.

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[3] Address reprint requests to E Ortega, Department of Immunology, Instituto de Investigaciones Biomédicas, UNAM, Ap Postal 70228, CP 04510, México DF.

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## MATERIALS AND METHODS

### Mice

Male Balb/cAnN mice were obtained from the breeding colony of the Instituto de Investigaciones Biomédicas, UNAM. Two different lots of animals were used for this study, one consisting of 54 animals and another of 24 animals. Each lot of animals was divided into three experimental groups: control, biotin supplemented, and biotin deficient. After weaning (at 3–4 wk of age) and for up to 20 wk the control group was fed a commercial, nonpurified diet (8626 Teklad Mouse Breeder Diet; Harlan Teklad, Madison, WI); animals in the deficient group received a biotin-depleting diet lacking biotin and with 30% dried egg white as a protein source (catalog no. TD-81079; Harlan Teklad), whereas animals in the supplemented group received a modified biotin-depleting diet, which contains 0.004 g biotin/kg diet (catalog no. TD-96075; Harlan Teklad). Egg white contains avidin, a glycoprotein that binds biotin, forming a noncovalent complex that is not absorbed into the blood (1). Animals were maintained on 12-h light and dark cycles and allowed water and the respective food ad libitum.

At the indicated times after beginning the respective diet, mice from the three experimental groups were bled from the axillar plexus under ethilic ether anesthesia. After bleeding, the animals were killed by cervical dislocation and the spleen and liver of each animal was removed for subsequent use.

The guidelines followed were those of an institutional committee for animal care and use, composed of scientists and the veterinarian in charge of the animals, in accordance with international standards of experimentation in animals.

### Determination of free biotin in serum

Sera were separated by centrifugation at 1200 × *g* at room temperature for 10 min and were kept frozen at –20 °C until used. For determination of free biotin, the vitamin was extracted with four volumes of absolute ethanol, and the ethanolic extract was dried in a Speed-Vac concentrator-evaporator (Savant Instruments, Farmingdale, NY). Dried extracts were kept at 4 °C until used. The concentration of biotin was determined by a competitive radioassay as follows: immediately before measurement the dried extracts were resuspended in a volume of a solution of 44.5 mmol tris-HCl/L, 44.5 mmol sodium borate/L, 1 mmol EDTA/L, and 200 mg sodium azide/L in water, pH 7.4 (TBE), equal to the original volume of serum. From this, an appropriate aliquot was taken and diluted with TBE to obtain a final volume of 200 μL, to which 0.22 kBq *d*-[8,9](-$^3$H(*N*)-biotin (specific activity: 1665 GBq/mmol; DuPont NEN Research Products, Boston) and 0.16 pmol avidin (Sigma Chemical Co, St Louis) were added. The reaction mixture was then incubated for 24 ± 2 h at 4 °C in an orbital shaker at 200 rpm. Anti-avidin polyclonal antibodies coupled to Sepharose (Pharmacia, Uppsala, Sweden) were added and incubation continued for another 24 ± 2 h. After centrifugation at 130 × *g* for 3 min at 4 °C, half of the supernate was taken to determine the concentration of unbound [$^3$H]biotin by scintillation counting in an LS6500 liquid scintillation counter (Beckman, Fullerton, CA). The percentage of [$^3$H]biotin bound was calculated by the difference between total and unbound [$^3$H]biotin. The biotin concentration in each sample was assessed by interpolation in a logit-log standard curve drawn by using the percentage of maximal [$^3$H]biotin bound observed for solutions containing from 0 to 320 pg biotin in 200 μL TBE. Nonspecific binding (in the absence of avidin) was in the range of from 0% to 6% ($n$ = 25) and the maximum binding was adjusted to between 70% and 85 %. Under these conditions the assay can properly determine from 20 to 300 pg biotin per reaction tube. The interassay CV of a pooled sample assayed in parallel was 15%. The recovery efficiency in sera to which known amounts of biotin were added was 96.5 ± 10% ($n$ = 20).

### Determination of enzymatic activity of PCC and PC

After 4, 8, 12, 16, and 20 wk of experimentation, the liver and spleen of three animals from each experimental group were removed as described above. The livers were frozen immediately and kept at –70 °C until used. The spleens were placed in ice-cold phosphate-buffered saline (PBS) and a cell suspension was prepared. After the cells were washed with PBS, erythrocytes were hemolyzed by hypotonic shock as described elsewhere (10). The cell suspension was centrifuged at 400 × *g* for 5 min at 4 °C and the pellet was kept frozen at –70 °C until used.

To determine the enzymatic activities of PCC and PC, the livers were thawed and homogenized with a polytetrafluoroethylene piston in seven volumes of ice-cooled PBS. The homogenate was centrifuged at 1200 × *g* for 10 min at 4 °C and the supernate was discarded. The erythrocytes were destroyed by pouring 1 mL ammonium chloride lysing buffer onto the cell pellet for 2 min (10), followed by the addition of 10 volumes of cold PBS. After centrifugation, the supernate was discarded and eight volumes of cold PBS were added. This cell suspension was diluted 1:4 with tris lysis buffer (50 mmol tris/L, 0.025 mmol EDTA/L; pH 8.0) and cells were disrupted with an Ultrasonic Homogenizer (4710 Series; Cole Parmer Instrument Co, Chicago) at 24 W (two cycles of 20 × 1 s). Enzymatic activity was determined in 10 μL of the sonicated samples by a radioenzymatic method (11). The assay was linear up to 4 μg protein for 120 min. Aliquots of the homogenates containing 2 μg protein in 10 μL were used and the reactions were incubated for 60 min. Protein concentrations in the sonicates were determined by the Bradford method (12).

To determine enzymatic activities of PCC and PC in spleen cells, the frozen pellets were thawed, resuspended in 1 mL lysis buffer/20×10$^6$ cells, and sonicated as described above for the liver. PC and PCC activities were determined by the same radioenzymatic method (11).

### Concanavalin A–induced proliferation of spleen cells in vitro

Proliferative assays of spleen cells stimulated with the T cell mitogen concanavalin A (ConA) was performed essentially as described (10). The tissue culture medium was Dulbecco's modified Eagle's medium supplemented with 10% biotin-free fetal calf serum (FCS), 2 mmol L-glutamine/L, 100 mg streptomycin/L, and 100 × 10$^3$ U penicillin/L. Biotin-free FCS was prepared by incubating FCS (GIBCO BRL, Gaithersburg, MD) with avidin-Sepharose (Sigma Chemical Co) for 16 h at 4 °C in an orbital shaker at 200 rpm. Before being added to the medium, the serum was separated by centrifugation at 150 × *g* for 5 min at 4 °C and sterilized by filtration through a 0.22-μm filter membrane (Millipore Corp, Bedford, MA). Biotin concentrations in the FCS before and after incubation with avidin-Sepharose were 106 and < 0.4 nmol/L, respectively. Because avidin-Sepharose chromatography has been shown to retain quantitatively carboxylases with covalently bound biotin from various sources (13, 14), it is assumed that the procedure removes all biotin (free,

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reversibly bound, and covalently bound) present in the FCS.

Three mice per group were killed and their spleens were removed. Spleen cell suspensions were prepared in Dulbecco's modified Eagle's medium supplemented with 10% biotin-free FCS and seeded in microwells ($2.5 \times 10^5$ cells/well). The cells were incubated with 0, 10, and 800 nmol biotin/L and 0, 1.0, and 5.0 mg ConA/L (Sigma Chemical Co) in a total volume of 200 μL. Each experimental condition was assayed in triplicate wells. At the end of a 48-h period, [$^3$H]thymidine (18.5 kBq/well, catalog no. NET-027, specific activity: 247.9 GBq/mmol; DuPont NEN Research Products) was added and incubation continued for 24 h. [$^3$H]thymidine incorporation was determined after the cells were harvested automatically onto filter papers with a cell harvester (Skatron Instruments, Sterling, VA). Radioactivity in the filters (Printer Filter-mat A; Pharmacia) was quantitated by scintillation counting in a 1205 Betaplate (Wallac Oy, Turku, Finland).

### Determination of lymphocyte subpopulations

At the indicated times, three mice from each group were killed and their spleens removed as described above. Single-cell suspensions of spleen cells from each mouse were stained (15) with fluorescein anti-mouse Ig (Zymed Laboratories, South San Francisco), fluorescein anti-mouse CD3-ϵ (Boehringer Mannheim, Mannheim, Germany), fluorescein anti-mouse CD4, or phycoerythrin anti-mouse CD8 (GIBCO BRL) for 60 min on ice. The cells were washed, fixed with 1% paraformaldehyde, and analyzed in a FACScan cytofluorometer (Becton Dickinson, San Jose, CA). The results are expressed as the percentage of positive cells for each marker (eg, sIg$^+$).

### Statistical analysis

Results are reported as means ± SDs. The statistical analysis of the differences among the three experimental groups was performed by two-way analysis of variance (ANOVA) followed by an all pairwise multiple comparison by the Student-Neuman-Keuls method. SIGMASTAT software (Jandel Scientific Software, Rafael, CA) was used for this analysis.

## RESULTS

The data presented in this section are the combined data obtained from two different lots of male Balb/cAnN mice studied.

### Effect of biotin deficiency on body weight

Mice in the control, supplemented, and deficient groups were weighed individually every week; the results are shown in **Figure 1**. Mice of the control group showed a steady weight gain during the 20 wk of the study, from 17.3 ± 2.36 g at week 0 ($n$ = 26) to 31.4 ± 1.4 g at week 20 ($n$ = 3). The growth curve of the supplemented mice was similar to that of the control group, increasing from 17.2 ± 2.0 g at week 0 ($n$ = 26) to 30.1 ± 0.7 g at week 20 ($n$ = 3). The growth curve of mice that received the biotin-depleting diet showed a biphasic pattern: from 17.1 ± 2.3 g at week 0 ($n$ = 26) it reached a maximum of 23.6 ± 1.4 g at week 6 ($n$ = 20) and decreased thereafter to 17.0 ± 2.3 g at week 20 ($n$ = 3). A two-way ANOVA was performed with weight data at 0, 2, 4, 8, 12, 16, and 20 wk for the three mice of each group that were killed at week 20 (the rest of the mice in each group were killed at different times). Significant differences were found between the deficient and the control groups and between the deficient and the supplemented groups ($P < 0.05$), whereas no significant difference was found between the control and supplemented groups. Also, there was a significant interaction between diet and time ($P < 0.001$).

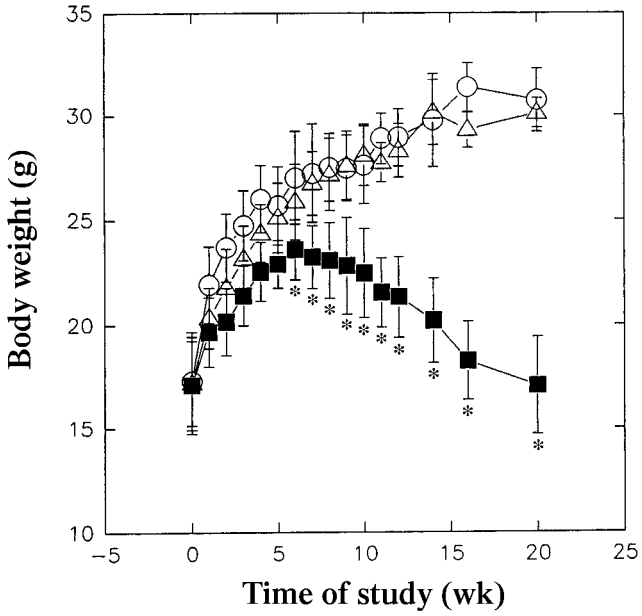


**FIGURE 1.** Mean (± SD) corporal weight of male Balb/cAnN mice fed a commercial nonpurified diet (○, control group), a biotin-depleting diet supplemented with biotin (△, supplemented group), or a biotin-depleting diet (■, deficient group). For the three groups, $n$ = 26 (weeks 0–4), $n$ = 20 (weeks 5–8), $n$ = 14 (weeks 9–12), $n$ = 9 (weeks 13–16), $n$ = 6 (weeks 17–18), and $n$ = 3 (weeks 19–20). *Significantly different from the control and supplemented groups, $P < 0.05$.

### Clinical symptoms associated with biotin deficiency

Mice were observed every week by the same veterinarian to determine any pathologic signs. In addition to the smaller size of mice in the deficient group, some of these mice showed alopecia, which was more severe in the areas surrounding the mouth and anus. The biotin-deficient group also adopted a squatted position. No other pathologic signs were detected by the veterinarian.

### Concentration of free biotin in sera

Concentrations of free biotin in sera of the experimental animals were measured 4, 8, and 16 wk after the study started. The concentration of free biotin in the sera of control mice was 40.5 ± 11.8 nmol/L ($n$ = 12). The concentration of free biotin in the sera of mice from the supplemented group was 54.5 ± 17.0 nmol/L ($n$ = 12). The concentration of free biotin in sera of mice in the deficient group was 3.66, 3.87, and 2.97 nmol/L at 4, 8, and 16 wk, respectively, yielding a global average of 3.46 ± 0.77 nmol/L ($n$ = 12). Thus, the concentration of free biotin in the sera of mice from each experimental group remained fairly constant during the study.

### Effect of biotin deficiency on enzymatic activities of carboxylases

The enzymatic activities of PCC and PC in liver homogenates and in spleen cells of mice from the three experimental groups were determined at the beginning of the study and at 4, 8, 12, 16,

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and 20 wk of experimentation (**Figure 2**). On the whole, specific activities of both enzymes were higher in the liver than in the spleen. In the liver of the control mice, the specific activity of PC was twofold higher than that of PCC; in contrast, in spleen cells the specific activity of PCC was 5–8-fold higher than that of PC. In general, the specific activities of both enzymes in the organs of mice in the supplemented group were similar to the corresponding values of the control group whereas the activities of both enzymes in mice from the deficient group were smaller.

A two-way ANOVA was performed to analyze differences in the specific activity of each carboxylase in both liver and spleen. No significant differences were found between the activities of both enzymes in mice from the control and supplemented groups. In contrast, there were significant differences in the specific activities of each enzyme in both liver and spleen between the deficient and control groups and between the deficient and supplemented groups ($P < 0.05$). A significant interaction between time and diet was observed for PC in the liver and for PCC and PC in the spleen ($P = 0.018$, $P = 0.006$, and $P = 0.014$, respectively). No significant interaction between time and diet was found for PCC in the liver ($P = 0.061$)

### Concanavalin A–induced proliferation of spleen lymphocytes

The spleens of mice in the deficient group were consistently smaller than the spleens of the supplemented and control mice, which was reflected in the lower number of cells obtained from them. The average number of cells obtained from the spleen of control and supplemented mice was $120 \times 10^6$ cells; from the spleen of deficient mice the average number was $54 \times 10^6$ cells (**Table 1**).

At different times after the specific diets began, the spleen cells from three mice from each group were used to measure proliferation induced by the T cell mitogen ConA. To assess the possible effect of biotin during the proliferative assay, the cells from each mouse were simultaneously assayed in culture media containing exogenously added biotin at concentrations of 0, 10, or 800 nmol/L. In all experiments conducted, maximum [$^3$H]thymidine incorporation was observed at 5 mg ConA/L. The results from a representative proliferative assay performed 18 wk after the biotin-depleting diet started are summarized in **Table 2**. Despite variations in the net mean becquerels incorporated after ConA stimulation in different experiments (ranging from 64 to 327 Bq), the results from experiments with spleen cells obtained at 4, 8, 12, or 18 wk after the specific diets began showed essentially the same pattern: the spleen cells from the supplemented and control groups showed a similar [$^3$H]thymidine uptake whereas the net uptake by cells from deficient mice was smaller by 20–25%. However, two-way ANOVA revealed no significant differences among the three groups. Note that in all experiments the presence or absence of biotin in the culture medium had no effect on the proliferation of spleen cells from animals belonging to any of the experimental groups (Table 2).

### Effect of biotin deficiency on subpopulations of lymphocytes

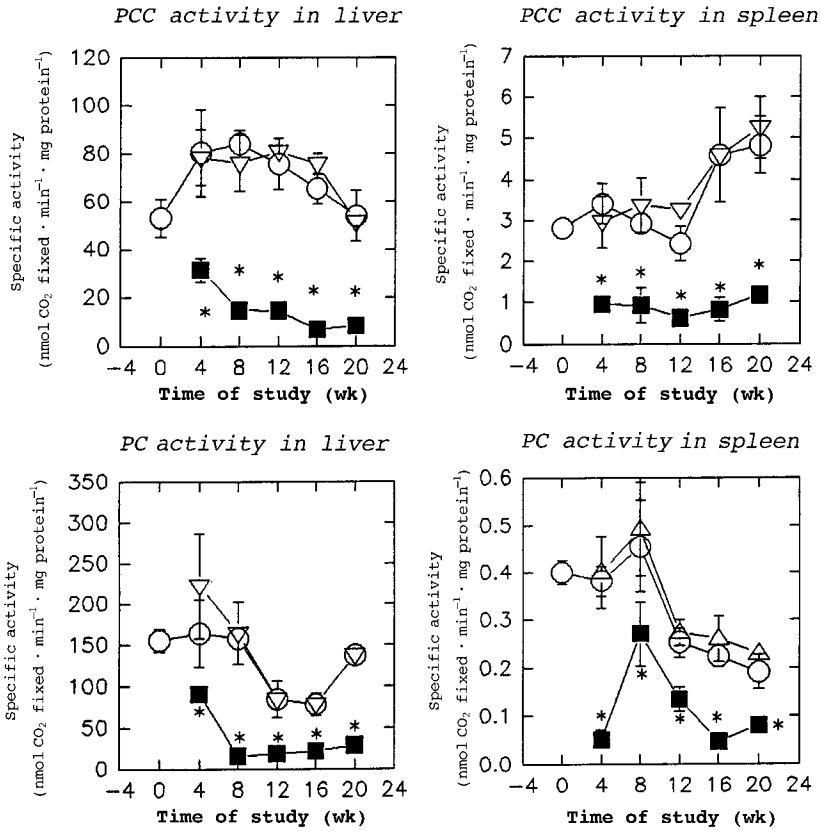


**FIGURE 2.** Specific activity of propionyl-CoA carboxylase (PCC) and pyruvate carboxylase (PC) in liver and spleen. Mice aged 3–4 wk were fed a commercial nonpurified diet (○, control group), a biotin-depleting diet supplemented with biotin (△, supplemented group), or a biotin-depleting diet (■, deficient group). Results are reported as the mean ± SD of three animals per group for each time point. *Significantly different from the control and supplemented groups, $P < 0.05$.

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**TABLE 1**
Number of cells in the spleen of mice in the three groups[1]

| Time of study | Control | Supplemented | Deficient |
|---|---|---|---|
| 4 wk | 120 ± 19 [3] | 117 ± 31 [3] | 59 ± 18 [3] |
| 8 wk | 126.5 ± 16 [3] | 121.6 ± 7 [3] | 61.5 ± 12 [3] |
| 12 wk | 108.9 ± 17 [3] | 98.8 ± 20 [3] | 48 ± 9 [3] |
| 16 wk | 114 ± 6 [6] | 137.6 ± 25 [6] | 55.6 ± 11 [6] |
| 20 wk | 137 ± 17 [3] | 128 ± 13 [3] | 45.6 ± 9 [3] |

[1] $\bar{x}$ ± SD (millions of cells); *n* in brackets. Mice started receiving the corresponding diet after weaning. At the indicated times, the spleens were removed and disgregated in Dulbecco's modified Eagle's medium. The number of cells obtained were counted in a hemocytometer.

### in spleen

Percentages of cells expressing CD3-ϵ, CD4, CD8, and surface immunoglobulin (sIg) in the spleens of mice from the three experimental groups are shown in **Figure 3**. The percentage of CD3 , CD4, and CD8 cells was higher and the percentage of $sIg^+$ cells lower in the deficient mice than in the control and supplemented mice. Two-way ANOVA showed that for each of the four markers there were significant differences between the deficient and control groups as well as between the deficient and supplemented groups ($P < 0.05$). There were no significant differences in any of the four markers between the supplemented and control groups. There was a significant interaction between time and diet for $sIg^+$, $CD4^+$, and $CD8^+$ markers ($P < 0.001$).

## DISCUSSION

Fragmented pieces of evidence have suggested that biotin deficiency has a deleterious effect on several immune phenomena, including a higher susceptibility to infections (particularly fungal infections), a diminished antibody response against various antigens, and a decrease in circulating lymphocytes (2, 16). However, the mechanisms underlying these observations are unknown. This study was undertaken to gain insight into the mechanism by which biotin deficiency affects the immune status of mice. This species was chosen because mice are, after humans, the mammals whose immune system has been most studied. In contrast, the metabolic alterations induced by biotin deficiency have been studied mainly in rats. Thus, we started by determining some indicators of the functional state of biotin in control, supplemented, and biotin-deficient BALB/cAnN mice. These indicators were the concentration of free biotin in serum and the specific activity of PC and PCC in liver and spleen.

Biotin deficiency was induced by giving mice (deficient group) a commercial diet with dried egg white as the protein source and without biotin. An equivalent group of mice was fed a similar diet but supplemented with 0.004 g biotin/kg diet (supplemented group). This amount of biotin was in excess of the biotin that the avidin in the diet could potentially bind, to make sure that the biotin intake was in excess of the biotin requirements recommended by a Subcommittee on Laboratory Animal Nutrition of the National Research Council (17). An equivalent group of mice that received a commercial nonpurified diet served as the control. No significant differences were found between the control and supplemented mice for any of the indicators studied, including growth, free biotin in serum, specific activity of PC and PCC in liver and spleen, ConA-induced [$^3$H]thymidine uptake by spleen lymphocytes, and the relative proportions of $sIg^+$ (B cells) and CD3-$\epsilon^+$ (T cells) spleen cells. This indicates that when supplemented with biotin, the commercial diet given to these mice contained all the necessary nutrients for proper growth and development. Because the same diet (but without biotin supplementation) was given to the mice in the deficient group, it can be suggested that the differences observed between these mice and those in the control and supplemented groups were caused by the absence of biotin in the diet.

So significantly did biotin deficiency affect the growth of the mice that whereas the mice in the control and supplemented groups almost doubled their weight during the 20 wk study, those in the deficient group had a weight increase of 36.4% during the first 6 wk but lost weight subsequently, returning to their original weight by the end of the study. Biotin is an essential growth factor and thus the effect of biotin deficiency on growth was expected. The initial period of weight gain (although smaller than that of control mice) could have reflected the time it takes to deplete the biotin stored in tissues.

The average concentrations of free biotin in sera of control and supplemented mice were 40 and 54 nmol/L, respectively, which were not significantly different. We do not know of any study in which free biotin was determined in mice. This concentration is similar to that found by Fields et al (18) in the sera of normal Sprague-Dawley rats. In contrast, the average concentration of free biotin in sera of deficient mice was only 6% of that in sera of control and supplemented mice. As mentioned above, the method used to determine free biotin in this study is based on avidin binding. It was reported recently that only about half of the total avidin-binding substances in human sera correspond to biotin, the rest being biotin sulfoxide and bisnorbiotin (19). We do not think that this could invalidate our conclusion that mice in the control and supplemented groups had similar concentrations

**TABLE 2**
[$^3$H]Thymidine incorporation by spleen cells[1]

| Biotin | Control | | Supplemented | | Deficient | |
|---|---|---|---|---|---|---|
| | Unstimulated | Stimulated | Unstimulated | Stimulated | Unstimulated | Stimulated |
| | *Bq* | | | | | |
| 0 nmol/L | 0.50 ± 0.14 | 183 ± 36 | 0.73 ± 0.28 | 163 ± 44 | 0.54 ± 0.13 | 132 ± 29 |
| 10 nmol/L | 0.62 ± 0.29 | 195 ± 42 | 0.72 ± 0.31 | 233 ± 60 | 0.54 ± 0.22 | 123 ± 31 |
| 800 nmol/L | 0.68 ± 0.37 | 166 ± 39 | 0.66 ± 0.23 | 172 ± 45 | 0.45 ± 0.19 | 119 ± 20 |

[1] $\bar{x}$ ± SD; $n = 3$ per group. Cells from each animal were assayed in triplicate. Mice were sacrificed after 18 wk of receiving the specific diet for each group. Spleen cells ($2.5 \times 10^5$/well) were incubated without (unstimulated) or with (stimulated) 5 mg concanavalin A/L, in Dulbecco's modified Eagle's medium containing the indicated biotin concentration. After 48 h, [$^3$H]thymidine was added and after 24 h the radioactivity incorporated per well was measured.

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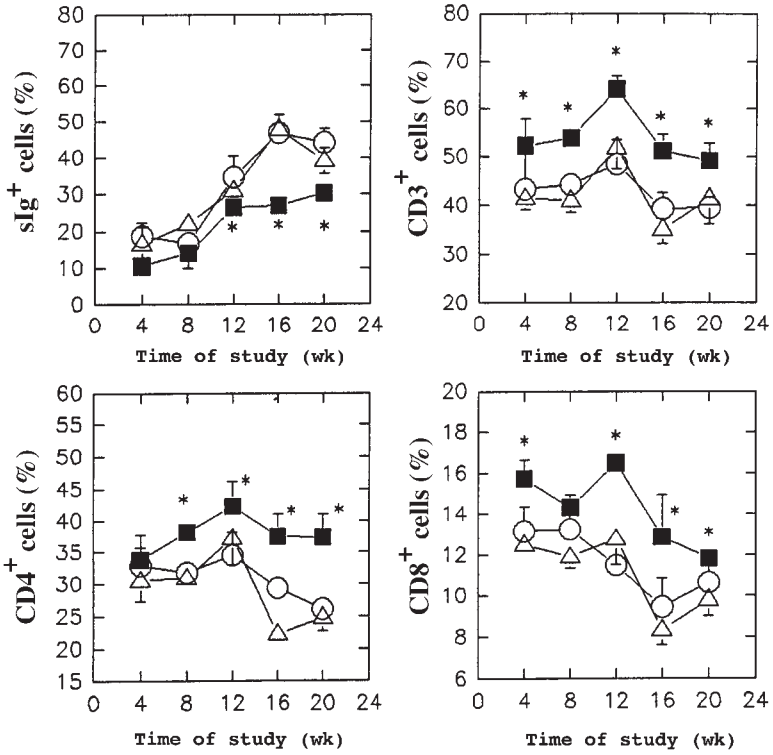


**FIGURE 3.** Percentage of cells expressing surface immunoglobulin (sIg$^+$), CD3-ϵ, CD4, and CD8 markers in the spleens of mice. Mice aged 3–4 wk were fed a commercial nonpurified diet ( ○, control group), a biotin-depleting diet supplemented with biotin (△, supplemented group), or a biotin-depleting diet (■, deficient group). Mice from each group were killed at the indicated times and the percentage of cells expressing each marker was determined by flow cytometry. Results are reported as the mean ± SD of three to six animals per group for each time point. *Significantly different from the control and supplemented groups, $P < 0.05$.

of free biotin in serum and that this concentration was significantly higher than that found in sera of mice in the deficient group. The lower concentrations of free biotin in sera of mice in the deficient group could suggest that, in general, the cells of these mice had a limited supply of biotin for the synthesis of biotin-dependent enzymes. However, it was pointed out by Bonjour (20) that the proper interpretation of serum concentrations of biotin remains to be determined.

The mean specific activities of PC and PCC in liver of control and supplemented mice were 130 and 72 nmol $CO_2$ fixed·min$^{-1}$·mg protein$^{-1}$, respectively. These specific activities of PC and PCC found in the livers of mice in the control group were 25 and 8 times higher, respectively, than those found in the livers of rats (21). This could reflect differences in enzyme content among different species or among different strains within the same species. Another factor that may have contributed to the higher specific activities found in this study was the method used to eliminate erythrocytes (hypotonic shock).

As reflected by both the concentration of free biotin in sera and the specific activities in liver and spleen of two of the enzymes that utilize biotin as a cofactor, PC and PCC, the biotin-depleting diet caused a significant change in the functional state of biotin in mice. Overall, the specific activity of PCC in the liver dropped by 80% and by 75% in the spleen whereas the specific activity of PC in the liver dropped by 73% and by 63% in the spleen. In the liver, although at 4 wk there was already a significant decrease in the activities of both enzymes, the maximum decrease was observed after 8 wk and remained at about the same level for the rest of the study. In contrast, the specific activity of both PCC and PC in the spleen was already diminished after 4 wk of experimentation.

After 4 wk and for up to 16 wk of experimentation, the spleens of mice in the deficient group yielded only 50% of the number of cells obtained from spleens of the control mice; at 20 wk the percentage dropped to only 34%. The weight of the spleens showed the same relative differences (data not shown). This effect of biotin deficiency on spleen size was not proportional to the effect on corporal weight, which at 4, 8, 12, and 16 wk was 92%, 82%, 60%, and 55% of the respective weight of mice in the control and supplemented groups.

Despite the pronounced differences in the specific activity of PCC and PC in spleen lymphocytes, ConA-stimulated [$^3$H]thymidine incorporation by splenocytes from the mice of the deficient group was only 25–30% lower than that of control mice. However, biotin deficiency was shown to induce a relative enrichment of the T cell subpopulation: in the spleens of control animals, an average of 42% of the cells were T cells whereas in the spleens of deficient mice T cells accounted for ≈54% of the cells (Figure 3). Because ConA is a T cell–specific mitogen, one can calculate that for control mice, 42% of $2.5 \times 10^5$ cells incorporated an average of 181 Bq (1.73 mBq/cell) whereas for deficient mice, 54% of $2.5 \times 10^5$ spleen cells incorporated 124.5 Bq (0.92 mBq/cell). Thus, on a cell per cell basis, [$^3$H]thymidine incorporation by T cells from biotin-deficient mice was diminished by ≈46% compared with that in control animals. We believe that this diminished response was not due to the low spe-

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cific activity of the biotin-dependent carboxylases during the proliferative assay because the addition of 800 nmol biotin/L to the incubation medium increased the activity of both enzymes (data not shown) yet had no effect on the incorporation of [$^3$H]thymidine. Rather, this may have resulted from an improper development of lymphoid cells in the animals subjected to the biotin-depleting diet, which cannot be reversed by incubation in a medium that contains biotin.

The percentage of sIg$^+$ cells (B cells) in the spleen was significantly lower in the deficient group (27%) than in the control and supplemented mice (average for both groups: 47%) after 16 (30%) and 20 (43%) wk of the study, with a concomitant increase in the percentage of T cells (54% compared with 42%), mainly of the helper (CD4$^+$) phenotype (22). These results suggest that biotin depletion affects the production, maturation, or both of B and T cells differently. A relatively lower number of B cells could correlate with the diminished antibody response and low number of plaque-forming cells found in biotin-deficient rats (5–7).

In summary, we characterized the effects of a biotin-depleting diet in mice. Growth was severely affected and the activities of PC and PCC in the spleen and liver diminished significantly. In addition, we showed that biotin deficiency had important effects on the immune system of mice. Although a detailed investigation of other possible indicators of immune dysfunction is needed, the data presented in this study have already identified two effects that are thought to affect the ability of the immune system to mount an adequate immune response against an antigenic challenge. These are the depressed T cell proliferative response to a mitogenic stimulus and the low number of B cells in the spleen. This animal model should be useful for exploring the mechanisms underlying these phenomena in particular and the effects of biotin deficiency on the immune system in general.

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