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Characterization and functional analysis of cellular immunity in mice with biotinidase deficiency

Kirit Pindolia [a,b], Hong Li [c,d], Cisley Cardwell [a], Barry Wolf [a,b,*]

[a] Department of Medical Genetics, Henry Ford Hospital, Detroit, MI 48202, USA
[b] Center for Molecular Medicine and Genetics, Wayne State University, Detroit, MI 48201, USA
[c] Division of Genetic and Metabolic Disorders, Children's Hospital of Michigan, Detroit, MI 48201, USA
[d] Department of Human Genetics, Emory University School of Medicine, Decatur, GA 30033, USA

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ABSTRACT

Biotinidase deficiency is an autosomal recessively inherited metabolic disorder that can be easily and effectively treated with pharmacological doses of the vitamin, biotin. Untreated children with profound biotinidase deficiency may exhibit neurological, cutaneous and cellular immunological abnormalities, specifically candida infections. To better understand the immunological dysfunction in some symptomatic individuals with biotinidase deficiency, we studied various aspects of immunological function in a genetically engineered knock-out mouse with biotinidase deficiency. The mouse has no detectable biotinidase activity and develops neurological and cutaneous symptoms similar to those seen in symptomatic children with the disorder. Mice with profound biotinidase deficiency on a biotin-restricted diet had smaller thymuses and spleens than identical mice fed a biotin-replete diet or wildtype mice on either diet; however, the organ to body weight ratios were not significantly different. Thymus histology was normal. Splenocyte subpopulation study showed a significant increase in CD4 positive cells. In addition, *in vitro* lymphocyte proliferation assays consistently showed diminished proliferation in response to various immunological stimuli. Not all symptomatic individuals with profound biotinidase deficiency develop immunological dysfunction; however, our results do show significant alterations in cellular immunological function that may contribute and/or provide a mechanism(s) for the cellular immunity abnormalities in individuals with biotinidase deficiency.

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1. Introduction

Biotinidase (EC 3.5.1.12) is the enzyme responsible for recycling the vitamin, biotin, by cleaving and releasing the biotin from various biotin-dependent carboxylases [1]. Biotinidase deficiency (OMIM 253260) is an autosomal recessively inherited metabolic disorder [1,2]. Individuals with profound biotinidase deficiency (less than 10% of mean normal serum activity) cannot recycle biotin, and without exogenous biotin will become biotin deficient. Supplementation with biotin can prevent and reverse most of the clinical symptoms, if initiated early.

The clinical manifestations of biotinidase deficiency typically do not appear until several months of age and in some cases may not occur until adolescence [3]. Untreated children usually exhibit breathing abnormalities such as hyperventilation and stridor. Some children also develop neurological abnormalities such as seizures in addition to hypotonia, lethargy, stridor, apnea and ataxia. In severe cases optic atrophy and high frequency sensory neuronal hearing loss are also manifested [3,4]. Skin rash and alopecia are also common in these children. Developmental delay is a concern in those children who were not diagnosed and treated early. Most untreated children will ultimately exhibit metabolic ketoacidosis, organic acidemia/uria and mild hyperammonemia. Although these children do not generally acquire bacterial infections, some develop opportunistic fungal infections. Mucosal and cutaneous candidiasis are the most common fungal infections reported in children with biotinidase deficiency [1,5]. Cellular immunological dysfunction has been implicated for the susceptibility to candida infection in symptomatic children with biotinidase deficiency [6–9]. The immunological alterations have been variable and contradictory; nevertheless, it is evident that some children with biotinidase deficiency exhibited cellular immunological abnormalities which resolved with biotin therapy.

With the advent of newborn screening for biotinidase deficiency children with the disorder are being identified and treated with biotin prior to the development of symptoms [2]. Therefore, newborn screening for the disorder has essentially eliminated our opportunity to study symptomatic children. In order to understand better the immunological dysfunction in biotinidase deficiency, we are characterizing various cellular immunity parameters in our recently developed knock-out mouse with profound biotinidase deficiency.

* Corresponding author at: Department of Medical Genetics, Henry Ford Hospital, 3031 West Grand Blvd., Suite 700, Detroit, MI 48202, USA. Fax: +1 313 916 1730.
E-mail address: bwolf1@hfhs.org (B. Wolf).

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2. Material and methods

2.1. Mouse with profound biotinidase deficiency

The development and characterization of the biotinidase-deficient (BD) mouse used in this study have been described previously [10]. Briefly, the chimera with the gene knock-out for biotinidase was generated followed by initial backcross with C57BL/6 background mouse for ten generations to obtain the biotinidase gene knock-out mouse with C57BL/6 genetic background. Approval was obtained from the Institutional Animal Care and Use Committee (IACUC) for maintaining the breeding colonies. Standard breeder diet (Harlan Teklad, Madison, WI) was fed to the mice used for breeding until the litters were weaned and used for specific experiments described below. Both the BD and wildtype (WT) mice were obtained from the breeding colonies.

2.2. Animal care, animal housing and study design

IACUC approval was obtained for all procedures performed in this project. All mice were maintained under standard housing conditions in a 12-hour light–dark cycle with free access to food and water. All mice, regardless of their biotinidase activity status, remain asymptomatic when fed a standard rodent diet that contains the daily requirement of 0.35 mg/kg of biotin. This diet is designated as a biotin-supplemented "+Btn diet". A custom-formulated, biotin-deficient diet used in these experiments contains essentially no biotin (less than the detection limit of 0.007 mg/kg) which is prepared with alcohol-extracted casein as the sole source of protein. This diet is designated as a biotin-deficient "−Btn diet".

Three-week old mice were placed on either "+Btn diet" or "−Btn diet" for a total of two weeks. A two-week period for feeding the animals a biotin-deficient diet was chosen because at longer times the animals exhibited severe neurological and metabolic compromise. After two weeks on this diet the animals were just beginning to exhibit mild symptoms, which simulate the conditions observed in symptomatic children with biotinidase deficiency. The experiments were performed in four groups of mice: first, WT mice on +Btn diet (WT + Btn); second, WT mice on −Btn diet (WT−Btn); third, BD mice on biotin supplemented diet (BD + Btn); and fourth, BD mice on biotin-deficient diet (BD−Btn). We had placed five mice in each group. This study was designed to investigate the effects of biotinidase and/or biotin deficiency on immunological function in these mice.

2.3. Weight measurements

Three-week-old WT and BD mice were placed on the normal biotin-supplemented (+Btn) or biotin-deficient (−Btn) diet immediately after being weaned. All mice were weighed on regular intervals and monitored for their overall health and growth.

2.4. Organ to body ratio measurement

Immune organs, thymus and spleen, were harvested from anesthetized mice at the end of the experiments. These organs were weighed to the nearest tenth of a milligram. The organ weights were normalized to the body weights of mice to account for the body weight variations. The ratios of organ weights/100 g body weights were calculated and compared among the four groups of mice.

2.5. Cryo-sectioning and histopathologic evaluation of thymus

For histologic study of thymus, mice were anesthetized after two weeks on specific diets. Intact thymuses from anesthetize mice were removed and snap frozen in liquid nitrogen. The snap frozen thymuses were embedded in OCT compound (Sakura Finetek USA Inc., Torrance, CA) in cryo-molds. Frozen blocks were stored at −80 °C until used for sectioning. Six micrometer thick serial sections were cut using cryostat set at −25 °C followed by mounting them on superfrost plus slides. The slides were stored at −20 °C until used for staining. The slides were stained with hematoxylin and eosin (H & E) for pathological evaluation of overall thymus architecture and structural abnormalities.

2.6. Characterization splenic lymphocyte subpopulations

A single cell splenocyte suspension was prepared by teasing the spleen with sterile needles on a Petri dish. Red cells were lysed and removed using ACK lysing buffer kit (from Life Technologies, Carlsberg, CA) following guidelines provided with the kit. In brief, one volume of cell suspension was mixed with ten volumes of ACK lysing buffer followed by incubation at room temperature for 3 to 5 min. Splenocytes were then collected using centrifugation at 300 ×*g* for 5 min at room temperature followed by supernatant aspiration while making sure that the pellet remains undisturbed. Splenocyte samples were washed with cold 1× PBS (phosphate buffered saline). Splenocyte numbers were determined using a hemocytometer.

Single-cell suspensions of splenocytes were then labeled with a mouse T and B lymphocyte antibody cocktail (BD Biosciences; San Diego, CA) using multiplex labeling procedure in a single reaction tube for three different cluster determinants (CDs). Both T- and B-lymphocyte cocktail solutions were incubated with splenocytes at 4 °C for 50 min, after which the cells were washed with PBS solution, fixed with 4% paraformaldehyde, and analyzed using the Accuri C6 Multi Color Flow Cytometer (Accuri, Ann Arbor, MI).

For T-lymphocyte analysis, antibody mixer used contained anti-mouse CD8a, anti-mouse CD3e, and anti-mouse CD4. Antibody against CD8 was used for the detection of cytotoxic T-lymphocytes [11,12], antibody against CD4 was used to detect T-helper cells [13,14], and antibody against CD3 was used for the detection of all mature T-cells [15,16].

For B-lymphocyte analysis, antibody cocktail contained anti-mouse surface IgM (sIgM), anti-mouse CD45R/B220, and anti-mouse CD23. Antibody against sIgM (μ chain) was used to detect activated B-cells, including plasma cells [17], antibody against CD23 was used for identifying mature resting B-cells [18], and antibody against CD45 was used for the detection of B-lymphocytes of all stages, from pro-B through mature antibody secreting B-lymphocytes and memory B-cells [19].

2.7. Functional characterization of splenocytes

To characterize the proliferation response of splenocytes, ^{3}H thymidine incorporation and the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) cleavage assay were performed. ^{3}H thymidine incorporation assay is used to determine the extent of DNA synthesis and cell division in response to a stimulatory signal [20]. MTT assay on the other hand is an indirect measure of cell growth and proliferation; it is a complementary assay to the ^{3}H thymidine incorporation assay for cell proliferation. During MTT assay, a yellow tetrazole is reduced to purple formazan by the mitochondrial enzyme in living cells. The amount of solubilized purple formazan generated is then quantified by measuring absorbance at 550 nm by spectrophotometry. The absorbance represents the degree of cell growth and proliferation [21].

The culture media used contained DMEM with 10% dialyzed Fetal Bovine Serum (free of unconjugated biotin), 1% non-essential amino acids, 0.2% 0.5 M sodium pyruvate, 1% antibiotic/antimycotic, and 0.07% 14 M beta-mercaptoethanol.

2.7.1. ^{3}H thymidine incorporation

2×10^{5} cells were cultured in 0.2 ml of the media in a 96-well microtiter tissue culture plate. Each sample was setup in triplicates and the average of three results was used as a representative result for the particular sample. The culture was stimulated by phytohemaglutination antigen (PHA) (20 μg/ml), or Concanavalin A (ConA) (1.5 μg/ml), or

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