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Treatment of Guillain-Barré syndrome with *Bifidobacterium infantis* through regulation of T helper cells subsets

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ABSTRACT

Background: Guillain-Barré syndrome (GBS) is a rare, autoimmune-mediated disease. The use of *Bifidobacterium* is reportedly effective in alleviating GBS since they act by regulating T helper (Th) cells.
Objectives: In this study, we explored the differentiation of T helper cell subsets in patients with GBS. We also evaluated the effect of GBS on *Bifidobacterium* levels in patients and the likely protective influence of this bacterium in alleviating the disease in an animal model.
Materials and methods: We used flow cytometry, and real-time polymerase chain reaction (PCR) to determine the T cell subsets differentiation among 30 GBS patients and 20 healthy controls (HC). The concentration of *Bifidobacterium* was assayed by real-time PCR. Experimental autoimmune neuritis (EAN) animal model was established to support the protective role of *Bifidobacterium* in GBS.
Results: The expression of Th cells, Th2 and Th17 in the patients was significantly higher than that in the HC, while Treg cells decreased substantially. Moreover, the levels of *Bifidobacterium* in the GBS patients were considerably lower than those in the HC, the concentration of *Bifidobacterium* correlating with Th2 and Th17 subsets negatively. Treatment with *Bifidobacterium* significantly reduced the levels of Th2 and Th17 and promoted the levels of Treg cells.
Conclusions: We concluded from this study that *Bifidobacterium* alleviated GBS by regulating Th cells, although in-depth studies might be required to fully understand the mechanism of action.

1. Introduction

Guillain-Barré syndrome (GBS) is an acute and inflammatory autoimmune polyneuropathy that affects the nerve roots and at times in conjunction with peripheral nerves. The disease is one of the most severe instigators of acute symmetric flaccid paralysis worldwide, which is the leading cause of sudden neurological injury [1], with high disability and mortality incidences, mostly in young adults. 10% of GBS patients have acute sequelae, and about 5% of them die of respiratory failure, lung infections, and other complications. Plasmapheresis, intravenous human immunoglobulin, and other immunotherapies do not provide satisfactory treatments, and there have been no adequate measures to block the development of the disease till now. There is an approximate increase of 0.1 million GBS cases in China every year, bringing enormous financial and psychological burdens to the families and the society. So, it is of immense importance to investigate its pathogenesis and to develop more effective treatment approaches for improving the patients' quality of life and for reducing social burden.

Studies on immunological mechanisms of GBS have shown that $CD4^+$ T cell subsets (also known as Th cells) including Th1, Th2, Th17, and $CD4^+CD25^+$ T cells (Treg) and other immune effector cells and cytokines, such as interleukins (IL), interferons (IFN), and tumor necrosis factors (TNF) form complicated linkages involved in the immunoregulation of GBS. These linkages purportedly influence the occurrence, development, and prognosis of the disease [2, 3]. Meanwhile, reports suggest that the expression of Th17 cell-related cytokines in the peripheral blood and cerebrospinal fluid of patients with GBS increases and that of the Treg cells decreases. With different post-immunotherapy results, the mechanism of action of Th cell subsets on autoimmunity and peripheral myelin insulation damage remains unclear. Further studies are, therefore, needed.

Recent molecular biology and immunology evaluations have found that probiotics have significant effects on human health [4]. There is growing evidence to support the claim that probiotics activate and

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modulate host immune systems through the intestinal immune system, and inhibit abnormal autoimmune responses, playing an essential role in the treatment of abdominal and other autoimmune diseases characterized by inflammation, such as Crohn's disease, etc. [5]. Also, in vitro, and in vivo studies revealed that *Bifidobacterium infantis* (*B. infantis*), one of the critical probiotics in the intestinal tract, has an undeniable influence on the differentiation of Th cell subsets [6]. Gastrointestinal perfusion with *B. infantis* could improve the imbalance of increased expression of Th17 cell-related factors and decreased expression in the level of Treg cells in the peripheral blood in rat models with inflammatory bowel diseases [7]. We showed, in our previous work, that the unbalanced expression and the rebalancing of Th cell subsets (Th1, Th2, Th17) and Treg cells play a crucial role in the damage and repair of GBS. Subsequently gastrointestinal perfusion with a quantitative *B. infantis* was proven to change the disproportion of Th cell subsets in peripheral blood lymphocytes and increase their autoimmune inflammatory responses. It has been proposed that there are many different Th cell subsets which may contain different functional subsets (for neuroprotection and nerve impairment) in the local or systemic microenvironment before and after the onset of GBS, but the mechanism is still unknown.

In the present study, we explored the correlation between the differentiation, imbalanced expression of Th cells, and the pathogenesis and clinical symptoms of GBS and observed the effect of *B. infantis* in improving the imbalance of Th subsets, thereby ameliorating GBS symptoms. We studied the role of Th subset differentiation in the occurrence and development of GBS in humans and investigated the efficacy of gastrointestinal-infused *B. infantis* on GBS in a rat model.

2. Materials and methods

2.1. Subjects

All patients with GBS who accepted medical services in the First Affiliated Hospital of Bengbu Medical College from January 2017 to December 2018, 30 patients in all, were enrolled in this study. The patients were diagnosed according to the National Institute of Neurological Disorders and Stroke (NINDS) diagnostic criteria [8]. All cases fell into two categories: acute inflammatory demyelinating polyneuropathy (AIDP) and acute motor axonal neuropathy (AMAN). Acute motor-sensory axonal neuropathy (AMSAN), Miller-Fisher syndrome, and chronic inflammatory demyelinating peripheral neuropathy (CIDP) were excluded. Experienced neurologists evaluated the severity of individual patients with GBS using the GBS disability scale scores (GDSS). Scores were graded 0 - regular neurological status; grade 1 - minor symptoms, able to run; grade 2 - limb weakness, able to walk 5 m unaided; grade 3 - able to walk 5 m only with aid; grade 4 - chair- or bed-bound; grade 5 - requiring assisted ventilation; and grade 6 - death. Written informed consent was obtained from individual participants, and the experimental protocol was approved by the Ethical Committee of Bengbu Medical College. Their demographic and clinical characteristics are summarized in Table 1. Individual patients were treated daily with the intravenous administration of 0.4 g/kg/d of immunoglobulin for 7 consecutive days.

Table 1
the demographic and clinical characteristics of participants.

	Healthy controls	GBS patients
Number	20	30
Age	37.5 ± 8.8	39.1 ± 6.7
Gender(M/F)	10/10	19/21
WBC in CSF (10^6/L)	0.63 ± 0.33	0.67 ± 0.41
WBC in plasma (10^9/L)	5.02 ± 0.98	6.33 ± 1.56
Albumin in CSF(g/L)	0.22 ± 0.07	0.714 ± 0.16**
Albumin in plasma (g/L)	39.65 ± 5.37	40.66 ± 5.13

** $p < 0.01$ vs. HC.

2.2. Detection of Th1, Th2, Th17 and Treg cells by flow cytometry

We obtained informed consent from all subjects before blood collection. Peripheral blood samples were collected from all the patients directly into containers, as were control samples. The blood collected was diluted at a ratio of 1:1 with an equivalent volume of PBS and a 1:1 ratio of an appropriate amount of human lymphocyte separation medium (Haoyang, Tianjing, China) was taken according to the amount of the blood sample with anticoagulants and added to a 15 mL centrifuge tube. After a 20 min centrifugation at 1500 rpm, the white blood cells, which formed a white film-like layer under the supernatant, were collected and pippeted into a sterile centrifuge tube and centrifuged at 10000 rpm for 5 min. Following a 3-time wash, the total cell number and the percentage of living cells were counted using the trypan blue staining.

The cells (10^6/tube) were washed with the FACS Buffer. Surface marker antibodies CD4 and CD25 (eBioscience, CA, USA) were then added and left for incubation for 20 min at room temperature, followed by FACS Buffer washing. After incubating with Fix/Perm Buffer for 30 min at 4 degrees, either of IL-4, IL-17 or FOXP3 (eBioscience, CA, USA) antibodies was added and the cells incubated for 60 min at room temperature. 1 × Perm Wash Buffer and an appropriate FACS Buffer were used for the next washing. Following the wash, the fluorescence positive cells of $CD4^+IFN\text{-}\gamma^+$, $CD4^+$ $IL\text{-}4^+$, $CD4^+$ $IL\text{-}17^+$ or $CD25^+$ $Foxp3^+$, representing the Th1, Th2, Th17 and Treg cells, respectively, were determined by flow cytometry. Data analysis was conducted using CELLQUEST or Win MDI software. A minimum of 30,000 cells was counted for each sample, and specific homologous control antibodies were used for eliminating the nonspecific staining.

2.3. The detection of B. infantis by real-time polymerase chain reaction (PCR)

Two gram of fresh stool were thoroughly mixed with 18 mL PBS and centrifuged at 400 ×*g* for 5 min. The top content of the centrifuged mixture was collected and thrice centrifuged at 400 *g* for 5 min. 1 mL of the upper portion of the second centrifuged mixture was collected and centrifuged at 9000 *g* for 3 min, washed 4 times with PBS, and redissolved in 0.1 mL distilled water. After a 5 min heating at 100 °C, the cells were immediately placed in ice water.

A pure culture of *B. infantis* (10^2–10^8 CFU/mL) was used as a positive template, and its fluorescence curve was used as a standard curve. The primers used for *B. infantis* were 5′-CTCCTGGAAACGGGTGG-3′ and 5′-GGTGTTCTTCCCGATATCTACA-3′. Real-time PCR was carried out at 95 °C for 15 s, followed by 40 cycles each of 94 °C for 10 s, 55 °C for 10 s, 74 °C for 35 s, and then 74 °C for 2 min.

2.4. Detection of transcription factors by real-time PCR

Total RNA from lymphocytes (10^6 cells) was extracted using Trizol (Invitrogen, CA, USA) and reverse transcription was carried out with a Reverse Transcriptase kit (Takara, Dalian, China). Real-time PCR was performed with the following primers: T-bet (sense 5′-GGACCCAACT GTCAACTGC-3′, anti-sense 5′-TGTCGCCACTGGAAGGA-3′); GATA-3 (sense 5′-GCCATTCGTACATGGAAGC-3′, anti-sense 5′-CGGAGGGTAA ACGGACAG AG-3′); RORγτ (sense 5′-GCAGCAACAGGAACAAGTGG-3′, anti-sense 5′-GCTTTGCCTCGTTCTGGACT-3′), Foxp3 (sense 5′-GCAGC AACAGGAACAAGTGG-3′, anti-sense 5′-GCTTTGCCTCGTTCTGG ACT-3′), and β-actin (sense 5′-GTGGACATCCGCAAAGAC-3′, anti-sense 5′-AAAGGGTGTAACGCAACTAA-3′). PCR was run in a 7300 real-time PCR System (Applied Biosystems, CA, USA) using a general SYBR green fluorescence detection for 10 min at 94 °C, followed by 45 cycles each of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The calculation of relative quantitative expression was done using $2^{-\Delta\Delta CT}$ method [9].