Oral vitamin B\textsubscript{12} supplement is delivered to the distal gut, altering the corrinoid profile and selectively depleting \textit{Bacteroides} in C57BL/6 mice

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ABSTRACT

Vitamin B\textsubscript{12} is a critical nutrient for humans as well as microbes. Due to saturable uptake, high dose oral B\textsubscript{12} supplements are largely unabsorbed and reach the distal gut where they are available to interact with the microbiota. The aim of this study was to determine if oral B\textsubscript{12} supplementation in mice alters 1) the concentration of B\textsubscript{12} and related corrinoids in the distal gut, 2) the fecal microbiome, 3) short chain fatty acids (SCFA), and 4) susceptibility to experimental colitis. C57BL/6 mice (up to 24 animals/group) were supplemented with oral 3.94 µg/ml cyanocobalamin (B\textsubscript{12}), a dose selected to approximate a single 5 mg supplement for a human. Active vitamin B\textsubscript{12} (cobalamin), and four B\textsubscript{12}-analogue (ADE-CN-Cba, 2Me-ADE-CN-Cba, 2MeS-ADE-CN-Cba, CN-Cbi) were analyzed in cecal and fecal contents using liquid chromatography/mass spectrometry (LC/MS), in parallel with evaluation of fecal microbiota, cecal SCFA, and susceptibility to dextran sodium sulfate (DSS) colitis. At baseline, active B\textsubscript{12} was a minor constituent of overall cecal (0.86%) and fecal (0.44%) corrinoid. Oral B\textsubscript{12} supplementation increased active B\textsubscript{12} at distal sites by >130-fold (cecal B\textsubscript{12} increased from 0.08 to 10.60 ng/mg, fecal B\textsubscript{12} increased from 0.06 to 7.81 ng/mg) and reduced microbe-derived fecal corrinoid analogues ([ADE]CN-Cba, [2Me-ADE]CN-Cba, [2MeS-ADE]CN-Cba, CN-Cbi). Oral B\textsubscript{12} had no effect on cecal SCFA. Microbial diversity was unaffected by this intervention, however a selective decrease in \textit{Bacteroides} was observed with B\textsubscript{12} treatment. Lastly, no difference in markers of DSS-induced colitis were detected with B\textsubscript{12} treatment.

Introduction

Vitamin B\textsubscript{12} is synthesized exclusively by microbes.\textsuperscript{1} In the human gut, such synthesis occurs distal to the site of absorption, therefore this nutrient must be obtained from exogenous sources. The quantity of vitamin B\textsubscript{12} required by adults, 2 µg/day, is lower than any other micronutrient.\textsuperscript{2} Commercially available supplements contain up to 5 mg/tablet, greatly exceeding the daily requirement. Unlike other water-soluble vitamins, which are largely absorbed and enter the circulation, receptor-mediated B\textsubscript{12} absorption in the ileum becomes saturated around 2 µg/meal\textsuperscript{3} and only a small fraction of high dose oral supplements are absorbed by non-specific pathways. Following supplement ingestion, non-absorbed vitamin B\textsubscript{12} reaches the large intestine where it is available to interact with the colonic microbiota. Microbes require vitamin B\textsubscript{12} or related corrinoids for their own functions. It is estimated that 20\% of gut bacteria are able to synthesize vitamin B12, yet >80\% of gut bacteria require this nutrient for their own metabolic purposes.\textsuperscript{3,4} Further, microbes produce various B\textsubscript{12} analogues by altering the lower ligand base (Figure 1 (a)), and only a small fraction of the corrinoid in human feces is active B\textsubscript{12}.\textsuperscript{5} Auxotrophic bacteria rely on B\textsubscript{12} transporters for acquisition, even encoding multiple transporters with varied preference for different B\textsubscript{12} analogues to gain competitive advantage.\textsuperscript{3} B\textsubscript{12} destined for utilization by the host is chaperoned throughout the digestive tract and then during circulation by glycoproteins (haptocorrin, intrinsic factor, transcobalamin II), possibly as a means of preserving
the importance of \( B_{12} \) for microbial physiology is illustrated by early \( B_{12} \) assays which derive \( B_{12} \) concentration from the rate of bacterial growth. Collectively, these observations led us to hypothesize that high dose oral \( B_{12} \) could fundamentally alter the corrinoid economy and microbial composition in the distal gut.

Two known \( B_{12} \)-dependent enzymes have been described in humans, but at least 15 \( B_{12} \)-dependent enzymes exist among gut microbes. \( B_{12} \) has additional roles in microbial gene regulation, functioning in \( B_{12} \)-dependent riboswitches (regulatory RNA elements), and as a cofactor for gene regulatory proteins. L-Methylmalonyl-CoA mutase is a \( B_{12} \)-dependent enzyme shared by humans and microbes (though the direction of product-substrate is reversed). Anaerobic microbes use this enzyme to generate CO\(_2\), a critical electron acceptor in the anaerobic lumen. Propionate, a short-chain fatty acid (SCFA) generated as a byproduct of this reaction, is important in host physiology and immunity. In one study, the addition of \( B_{12} \) to

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**Figure 1.** \( B_{12} \) supplement alters the corrinoid profile in the cecum and feces. (a) Base moieties of the most abundant corrinoids. Cobinamide lacks the base as well as ribose and phosphate moieties present in other corrinoids. (b) Cecal corrinoid profile overall and (c) by individual corrinoid in control (n = 5) and \( B_{12} \) treated (n = 4) mice by unpaired two-tailed Student’s t-test. (d) Fecal corrinoid profile overall and (e) by individual corrinoid at baseline and after 16-day \( B_{12} \) supplementation (ADE n = 16/group, others n = 22/group, paired two-tailed t-test).
Results

As a starting point to profile B₁₂ metabolites in mice, we measured the influence of oral B₁₂ supplementation (3.94 µg/ml) on corrinoids in the distal gut. Cobalamin, and four other corrinoid analogues were selected for analysis based on their abundance in human feces and the availability of standards for LC/MS. These included cobalamin, [ADE]CN-Cba, [2Me-ADE]CN-Cba, [2MeS-ADE]CN-Cba, and CN-Cbi, which differ from cobalamin in the lower ligand base moiety (Figure 1(a)). Cobalamin was found to be a minor constituent representing just 0.86% of cecal and 0.44% of fecal corrinoid at baseline (Figure 1(b–d)). Supplemented animals had 132-fold higher cobalamin concentration in cecal contents (Figure 1(c). 0.08 vs 10.60 ng/mg, p = 0.0005) and 136-fold higher concentration in fecal contents (Figure 1(e). 0.06 vs 7.81 ng/mg, p = 0.0001) with Bonferroni-corrected α of 0.0125 and 0.01 respectively. Likewise, MeS-Ade was 5.6-fold higher in cecal contents of supplemented animals (Figure 1(b,c). 0.26 vs 1.45 ng/mg) but this was non-significant after Bonferroni correction. In contrast, there was a robust decrease in cobamidine analogues (Figure 1(d,e)) in feces of supplemented mice except for cobinamide which was increased. To control for change over time we compared corrinoids in non-supplemented control animals at days 1 vs 16, and found a marginal decrease in fecal cobalamin, which was non-significant after Bonferroni correction (α = 0.01; Fig S1).

Given the widespread microbial requirement for exogenous corrinoid, we hypothesized that B₁₂ supplementation would select for microbes whose growth was otherwise limited by this nutrient. Stool samples were collected for 16S rRNA gene sequencing at baseline (T1) and after 16 days (T2) of treatment. Including samples from DSS colitis experiments, described below, 54 stool and cecal samples were collected for microbiome analysis, of which 52 were successfully profiled (two animals given DSS had inadequate cecal contents to generate adequate 16S rRNA gene amplicons). In total 3,242,801 sequences were obtained (average 60,051 per sample). Goods coverage exceeded 99.8% for all samples analyzed demonstrating adequate sequencing. Beta diversity analysis at phylum level (Figure 2(a)) showed similar overall composition before and after B₁₂ supplementation, while a significant difference was seen at the genus level (Figure 2(b), p = 0.048). Additionally, this analysis yielded one conspicuous finding: the closely related genera Bacteroides (2.50% OTU baseline vs 0.54% OTU post, p = 0.027) was significantly reduced after B₁₂ supplementation (Figure 2(c)). Furthermore, while alpha diversity in these samples demonstrated no difference by treatment, principal coordinates analysis (PCoA) revealed that the control vs. B₁₂ groups post-supplementation differed significantly (Fig S2).

Culture experiments have shown increased propionate synthesis by some bacteria with the addition of B₁₂ to culture media. Therefore, we tested the hypothesis that B₁₂ would alter cecal SCFA concentrations. Cecal contents rather than stool was used for SCFA analysis because a large portion of SCFA produced in the gut is absorbed in the colon before luminal contents are expelled as fecal pellets. This analysis did not identify any effect of B₁₂ on cecal acetate, propionate, or butyrate levels (Figure 2(d)).

Published work implicates commensal Bacteroides species in the pathogenesis of murine colitis, in contrast, lower levels of Bacteroides are associated with human inflammatory bowel disease (IBD). Given our finding that oral B₁₂ supplementation decreased the proportion of Bacteroides in feces, we sought to determine the effect of oral B₁₂ in murine colitis. The DSS model of experimental colitis was chosen because prior studies using this model demonstrated a role for SCFA-mediated signaling. As expected, induction of experimental colitis with the addition of 2.25% DSS in drinking water resulted in lower weight, shorter colon length, and increased gut permeability as reflected by the appearance of serum...
fluorescence following gavage of FITC-dextran (Figure 3(a–c)). However, B₁₂ supplementation, which was sustained during DSS administration, did not significantly influence these endpoints (Figure 3(a–c)). Similarly, there was no significant difference in IL-1β, TNF-α, IL-6, IL-10, IL-12p70, IFN-γ, or murine KC in colonic mucosal scrapings with oral B₁₂ supplementation in DSS colitis (Figure 3(d)). Given the potential for host B₁₂ status to influence response to colitis,²⁰ we included a control group that received intraperitoneal B₁₂ injection (parenteral B₁₂) – an intervention that did not alter fecal corrinoids (Fig S3).

**Discussion**

It is predicted that 80% of gut microbes require B₁₂ or similar corrinoids, though a minority harbor the B₁₂ biosynthetic pathway.⁸ Therefore, most gut microbes rely on acquisition of exogenous corrinoid or precursor molecules from their environment and successful competition confers a survival advantage.³ Therefore, we hypothesized that oral supplementation would deliver excess B₁₂ to the distal gut, disrupting the corrinoid economy and thereby alter the microbiome, SCFA, and resistance to colitis.

To our knowledge, this is the first study to profile corrinoids in the distal gut of mice since the LC/MS method was originally described by Allan and Stabler.⁵ Importantly, it demonstrates that active B₁₂ is a minor constituent of the fecal corrinoid pool in mice (0.44%, 59 ng/g stool) which is similar to published results from human feces (1.4%, 19 ng/g stool).⁵ We show that oral B₁₂ supplementation in mice, at a level corresponding to human consumption of a 5 mg daily supplement, increased B₁₂ in the distal gut >130 fold. B₁₂ supplementation decreased the absolute concentration of several non-B₁₂ corrinoids. Cobinamide was increased in fecal contents which indicates microbial modification of cobalamin salvaging the corrin ring. Given the ability of microbes to sense and acquire B₁₂³,⁹ this likely
reflects negative regulation of microbial corrinoid synthesis in the setting of environmental excess.

Our analysis did not reveal major alterations of the fecal microbiome with B$_{12}$ supplementation. Use of samples from replicate experiments reduced the technical error in this analysis, the study likely was limited in its power to detect changes in the microbiome. The primary finding was the selective decrease in *Bacteroides*, a highly prevalent constituent of the mammalian gut microbiome. *Bacteroides* require exogenous B$_{12}$, so the depletion of this genus with supplementation is paradoxical. *Bacteroides* derive competitive advantage from redundant B$_{12}$ transporters that enable them to acquire environmental corrinoid. This leads us to speculate that *Bacteroides* may lose this advantage in an environment replete with B$_{12}$. Conflicting data exist regarding the role of *Bacteroides* in colitis. *Bacteroides* in general and enterotoxigenic *B. fragilis* specifically are associated with IBD in humans and contribute to pathogenesis of colitis in animal models. To the contrary, monocolonization of germ free mice with *B. fragilis* is protective in DSS colitis, likely through immunomodulatory influences of its capsular polysaccharide-A and modulating secreted outer membrane vesicles. Given this selective decrease of *Bacteroides*, we subjected B$_{12}$ supplemented mice to DSS colitis to evaluate their susceptibility to disease. Despite some trends toward B$_{12}$-mediated protection of barrier function, no significant influence of B$_{12}$ supplementation was discernable during DSS administration (Figure 3 (a–d)-D). These findings agree with recent data regarding the role of cyanocobalamin in murine colitis. Zhu et al. show that cyanocobalamin supplementation during DSS administration had a negligible effect on colitis outcomes, including weight loss and colon length reduction. Furthermore, cyanocobalamin supplementation during DSS did not have a significant effect on microbial alpha diversity, similar to our findings.

![Figure 3. B$_{12}$ supplement in DSS colitis. (a) Vitamin B$_{12}$ had no effect on weight loss in DSS colitis (sum of three replicate experiments, Control (n = 15), B$_{12}$ (n = 15), DSS (n = 23), DSS/B$_{12}$ (n = 24) by ANOVA and Dunnett’s multiple comparisons (Control vs DSS or DSS/B$_{12}$: p < 0.0001, but DSS vs DSS/B$_{12}$: p = ns) or measures of disease including (b) colon length, Control (n = 15), B$_{12}$ (n = 15), DSS (n = 20), DSS/B$_{12}$ (n = 19) by ANOVA and Tukey’s multiple comparisons, or (c) enteral administered FITC-dextran detected in circulation of Control (n = 14), B$_{12}$ (n = 15), DSS (n = 20), DSS/B$_{12}$ (n = 19) by ANOVA and Tukey’s multiple comparisons. (d) Colon tissue cytokines were not significantly different comparing DSS vs DSS/B$_{12}$ by ANOVA and Tukey’s multiple comparisons. Unique letter represents p < 0.05.](image)
This is in contrast with work in other organ systems, which has shown that cyanocobalamin exhibits anti-inflammatory effects against acute and chronic inflammation in mice. \(^{31}\)

The finding that cecal SCFA concentrations are unaffected by B\(_12\) supplementation (Figure 2(d)) contrasts with results of bacterial culture experiments in which addition of B\(_12\) increased propionate production. \(^{13}\) This may reflect resilience of the metabolic network in the distal gut lumen. \(^{11}\) Alternately, an influence of B\(_12\) on cecal SCFA may have been obfuscated in the background of B\(_12\) provided by the standard diet that all animals received. The decision to use standard B\(_12\)-containing chow and the selection of the B\(_12\) dose for these studies was intended to reflect typical human exposures. One strength of this work is the number of mice included and replication of experiments. For example, we report that DSS reduces butyrate in cecal contents (Figure 2(d), \(n = 20\)/group). This finding corroborates results from other studies that employed just five animals/group. \(^{32,33}\)

In summary, we report that oral B\(_12\) supplement, as cyanocobalamin, was effective in delivering B\(_12\) to the distal gut of C57BL/6 mice as well as modulating B\(_12\) analogues and selectively depleting Bacteroides, but did not appreciably influence cecal SCFA levels or protection in DSS colitis.

**Methods**

**Mice and B\(_12\) treatment**

All experimental protocols were approved by the University of Colorado Institutional Animal Care and Use Committee. Male and female C57BL/6 mice were bred and maintained in a specific-pathogen-free facility and used at age 8–16 weeks. Animals were evenly distributed amongst treatment groups by age and sex. Mice were fed a standard diet (Teklad Global Soy Protein-Free Extruded Rodent Diet) containing .08 mg/kg vitamin B\(_12\) as cyanocobalamin. Mice were supplemented with B\(_12\) in the form of cyanocobalamin (Sigma-Aldrich, St. Louis, MO, USA) in drinking water at a concentration of 3.94 µg/mL, which provided 1.025 µg B\(_12\)/g, or on average, 30.46 µg B\(_12\) per mouse, based on water disappearance. This is the human equivalent of a single 5,000 µg oral supplement based on published data on C57BL/6 water consumption using standard body surface area based conversion between human and mouse. \(^{34,35}\)

Mice were supplemented with B\(_12\) throughout the duration of the experiment with baseline and post supplement samples collected at day 1 and day 16. Water bottles containing B\(_12\) were replaced every other day. As a control in colitis experiments, parenteral B\(_12\) (via intraperitoneal injection) at a dose of 2.65 µg/mouse, every 3 days, was provided to the control group to account for potential systemic influences of B\(_12\) on colitis. \(^{20}\) This dose was selected to provide an optimistic 2.9% absorption/day. \(^{36}\)

**Corrinoid analysis**

Cobalamin and B\(_12\) analogues were measured in cecal contents and stool by stable isotope dilution liquid chromatography/mass spectrometry (LC/MS) as previously described by Allen and Stabler. \(^{5}\) Briefly, frozen samples were thawed and 1 mL 0.1M KPO\(_4\) buffer at pH 8.0 was added with stable isotope labeled internal standards. Samples were heated to 90°C for 45 min, then cooled on ice and centrifuged and eluted fraction added to prepared C18 reverse phase resin columns. The eluant was dried overnight before rehydration in 1 mL H\(_2\)O and then passed through R-protein resin which was prewashed with 1 mL phenol, 3 mL H\(_2\)O and 1 mL 0.5M glycine. Sample was eluted with 100 µl phenol, and the aqueous layer was extracted and analyzed by LC/MS as detailed previously. \(^{5}\)

**Microbiome and SCFA analysis**

Bacterial profiles were determined by broad-range amplification and sequence analysis of the bacterial 16S rRNA gene V3V4 variable region following our previously described methods. Fecal pellets and cecal contents were collected directly into sterile Eppendorf tubes and flash frozen at −80°C until samples were processed for DNA isolation and sequencing. Bacterial DNA from cecal and fecal samples were isolated using the Power Fecal DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA). Paired-end sequencing was performed on the Illumina MiSeq platform using the 600-cycle v3 kit. Sequences were merged and quality filtered as previously described. \(^{37,38}\) Merged sequences were
aligned and classified with SINA (1.3.0-r23838).\textsuperscript{39,40} Operational taxonomic units (OTUs) were produced by clustering sequences with identical taxonomic assignments. Explicit software (v2.10.5) was used for microbiome analyses, including calculation of alpha and beta diversity measures such as Goods Coverage index.\textsuperscript{41} Short chain fatty acids were analyzed by stable isotope GC/MS using an adaption of a published method.\textsuperscript{42} Briefly, cecal samples were collected directly into pre-weighed, sterile Eppendorf tubes and flash frozen at $-80^\circ C$ until processing. Samples were then subject to an alkylation procedure in which sample and alkylating reagent were added, vortexed for 1 min, and incubated at 60$^\circ C$ for 15 min. Following cooling and addition of \textit{n}-hexane to allow for separation, 200 $\mu L$ of the organic phase was transferred to glass inserted and analyzed by GC/MS. Results were quantified by reference to a standard curve and normalized to sample weight.

**Experimental colitis**

Colitis was induced using 2.25% dextran sodium sulfate (DSS), (MW 36,000–50,000, MP Biochemicals, Santa Ana, CA, USA) added to drinking water for 9 days, as previously reported.\textsuperscript{43} DSS was made fresh daily. Animals were weighed daily and on the day of euthanasia were gavaged with 3-5KD FITC dextran (Sigma Aldrich, St. Louis, MO, USA). Serum was collected at euthanasia, four hours after gavage, and fluorescence quantified. Mucosal scrapings from the colon were placed in phosphate buffered solution containing Halt Protease Inhibitor Cocktail (Fisher Scientific) and stored at $-80^\circ C$. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Tissue cytokines were quantified as previously reported\textsuperscript{44} using the Mouse Proinflammatory 7-Plex Tissue Culture Kit and according to manufacturer instructions (Meso Scale Discovery, Rockville, MD, USA).

Statistical Analyses were performed using GraphPad Prism 7 (GraphPad Software, La Jolla, CA, USA). Tests include paired and unpaired two-tailed Student’s \textit{t}-test and ANOVA with Tukey’s and Dunnett’s multiple comparisons as indicated in figure legends. Statistical differences reported as significant when $p < 0.05$ with Bonferroni correction applied when multiple comparisons were made.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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