Evaluation of a novel probiotic Bacillus coagulans (Ganeden BC30™) cell wall and metabolites: Anti-inflammatory and immune modulating effects in vitro.

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Purpose of this study

This study was undertaken to evaluate whether the biological effects caused by the novel probiotic, spore-forming strain of Bacillus coagulans GBI-30 (PTA-6086, Ganeden BC30™) [1] were due to cell wall components alone, or whether secreted substances from the live bacteria had additional biological properties. This investigation was conducted to address specific aspects of the ongoing discussion as to whether probiotics only act via the cell walls, or whether they may provide different bioactive compounds when alive and metabolically active.

Cell wall fraction versus metabolites produced by actively growing bacteria

Bacillus coagulans spores were heat-activated, and bacterial cultures were established. Subsequently, the culture medium in which the active bacteria had grown was harvested as a source of metabolites (MET), and the bacteria were used to isolate cell wall fragments (CW). Both of these fractions were compared in a series of in vitro bioassays using human peripheral blood mononuclear cells (PBMC) and polymorphonuclear (PMN) cells.

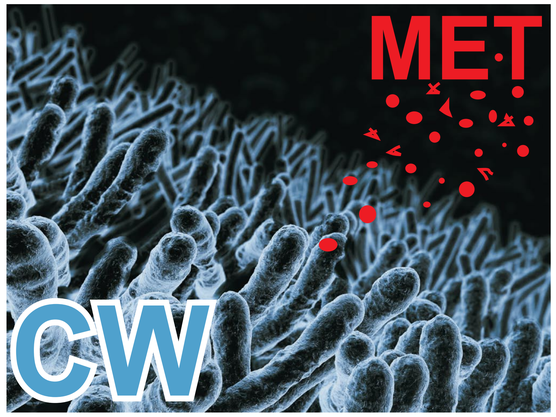

Figure 1. Scanning electron micrograph of the gram-positive bacteria *Bacillus coagulans*. Bacteria cell walls (CW) are highlighted in blue. Red dots simulate secreted metabolites (MET) in the culture medium.

Anti-inflammatory effects

Reduction of ROS formation: PMN cells can produce reactive oxygen species (ROS) as an anti-bacterial defense mechanism. However, under conditions of oxidative stress, their formation of ROS is a strongly contributing factor in the acceleration of inflammatory conditions. Both MET and CW inhibited spontaneous and oxidative stress-induced ROS formation in human PMN cells. The reduction in spontaneous ROS formation was greatest with CW treatment.

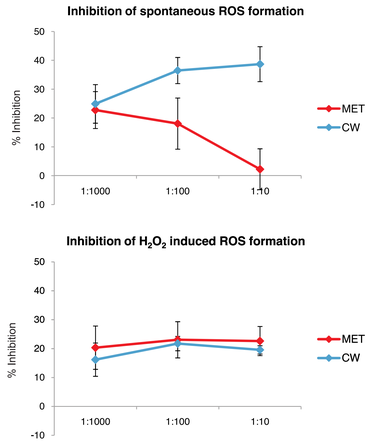

Figure 5. *PMN cell ROS formation:* Human PMN cells were exposed to either the metabolite fraction (MET) or the cell wall fraction (CW) and reactive oxygen generation measured in either the absence (spontaneous ROS formation) or presence of hydrogen peroxide (induced ROS formation) using an indicator dye that becomes fluorescent when oxidized. The data is presented as % inhibition of ROS formation and reflects a comparison between ROS generation in treated versus untreated PMN cells. A nearly 40% decrease in spontaneous ROS formation was seen following treatment with CW ($p<0.02$). Data reflect averages of cultures performed in quadruplicate for each test condition.

Reduced migration towards inflammatory chemoattractants
The migratory behavior of PMN cells towards IL-8 and LTB4 was strongly inhibited by treatment of cells with either MET or CW. Anti-inflammatory effects were greatest with CW treatment and this response extended to very low concentrations of CW.

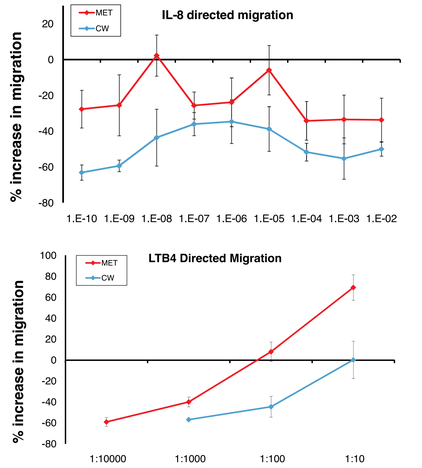

Figure 6. *PMN cell migration, IL-8 and LTB4 directed:* Effects of BC fractions on human PMN cell migration towards 2 different pro-inflammatory chemical signals were evaluated. PMN cell chemotactic migration towards the cytokine IL-8 was decreased in the presence of MET and CW. The cell wall fraction (CW) triggered inhibition of PMN cell migration towards IL-8 down to dilutions of 10^{-10} ($p<0.02$). The metabolite fraction (MET) showed similar but weaker inhibition across the same dose range. PMN cell chemotactic migration towards the inflammatory mediator LTB4 was decreased by both the metabolite fraction and the cell wall fraction, indicating an anti-inflammatory effect. PMN cell migratory responses were assayed in quadruplicate for each test condition and compared to the average migration of cells that were not treated with MET or CW.

Immune modulation and shift towards Th2 cytokine profile

Direct effects on cytokine production: Both fractions directly modulated cytokine production in 5 day PBMC cultures, inducing expression of the Th2 cytokines IL-4, IL-6, and IL-10, and inhibiting expression of IL-2.

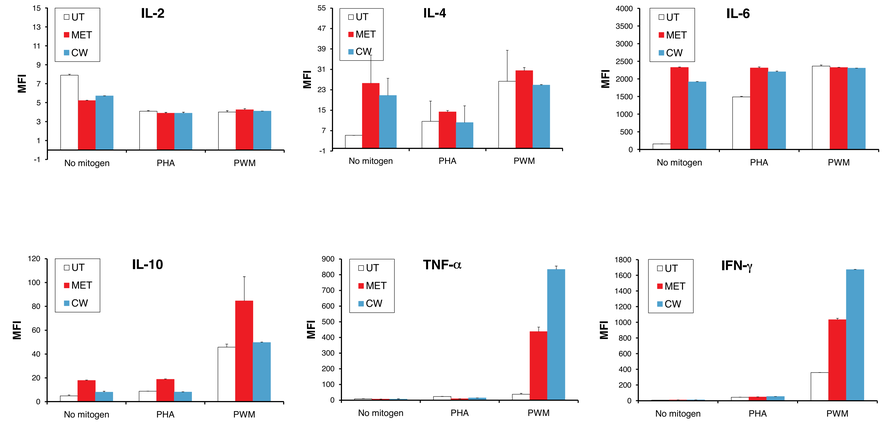

Figure 7. *PBMC culture cytokine production:* The effects of the metabolite fraction (MET) and cell wall fraction (CW) on cytokine production were evaluated in human PBMC cultures. Treatment of PBMC with MET and CW resulted in reduced IL-2 production. There was no effect on mitogen-induced IL-2 production. Treatment of PBMC with MET and CW resulted in increased IL-4 production. There was no effect on mitogen-induced IL-4 production. Treatment of PBMC with MET and CW resulted in a dramatic increase in IL-6 production. Both MET and CW further enhanced the PHA-induced IL-6 production. In contrast, neither MET nor CW modulated the PWM-induced IL-6 production. Treatment of PBMC with MET, and to a lesser extent CW, resulted in an increase in IL-10 production. MET but not CW further enhanced the PHA-induced as well as the PWM-induced IL-10 production. Both MET and CW treatment of PBMC resulted in a mild reduction in TNF-α production, both in the absence of mitogens, and in the presence of PHA. In contrast, co-stimulation of PBMC with PWM and either MET or CW resulted in a strong increase in TNF-α production. No changes in IFN-γ production were seen following treatment of PBMC with MET or CW in the absence of mitogen or presence of PHA. However, very large increases were seen in the presence of PWM, which mimics the adaptive immune responses dependent on complex cellular interactions between macrophages and T/B lymphocyte subsets.

Support of anti-bacterial and anti-viral aspects of the immune system

Increased phagocytosis of bacteria-like fluorescent beads: Treatment of PMN cells with either MET or CW resulted in increased phagocytosis of fluorescent beads mimicking bacteria. This enhancement of the innate immune response was greatest following treatment with MET.

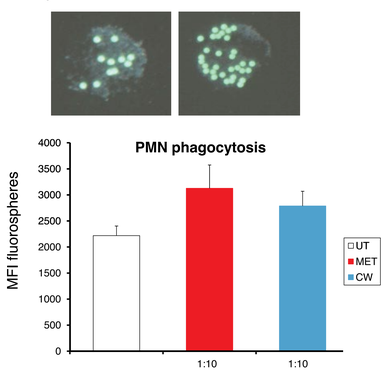

Figure 2. Human polymorphonuclear (PMN) cells were evaluated for phagocytic activity, measured by the uptake of green-fluorescent beads. Flow cytometry analysis showed an increase in mean fluorescence intensity (MFI) of PMN cells treated with either MET or CW. The increase was statistically significant. Microscopy showed that the level of bead uptake typical of an untreated PMN cell (left photo) was increased in PMN cells treated with CW (right photo).

Increased immune surveillance and anti-bacterial migratory behavior: Both BC fractions exhibited dose-dependent effects on PMN cell migration. The support of PMN cell immune surveillance (random migration) and anti-bacterial defense mechanisms (f-MLP-directed migration) was greatest for MET.

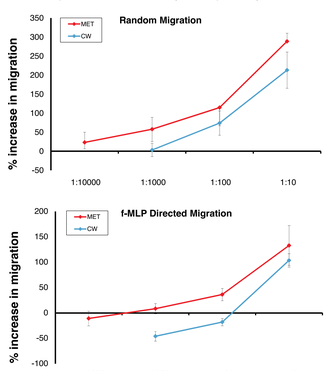

Figure 3. *PMN cell migration, random and f-MLP directed:* Effects of BC fractions on human PMN cell migration were evaluated using Transwell migration plates. Both MET and CW treated PMN cells showed greatly increased random migration behavior. The increase was highly statistically significant for both MET ($p<0.001$), and CW ($p<0.005$). PMN cells treated with MET or CW also showed increased migratory behavior in response to the bacterial peptide f-MLP.

NK cell activation and increased cytotoxic activity: Both MET and CW induced the expression of the CD69 activation marker on human natural killer (NK) cells, and enhanced the cell surface expression of CD107a when NK cells were exposed to K562 tumor cells in vitro. The effect of MET and CW on this aspect of innate immunity suggests an increase in NK cell cytotoxicity, i.e. increased scavenging and killing transformed (virus-infected and malignant) cells.

NK cells can kill tumor cells and virus-infected cells either via cell-cell contact or by secretion of substances such as Perforin, which perforates the cell membrane on the target cell. During this process, the CD107a receptor expressed on the interior of vacuoles in the cytoplasm of NK cells is transiently brought to the cell surface. Thus, CD107a expression on NK cells is a measure of their cytotoxic activity.

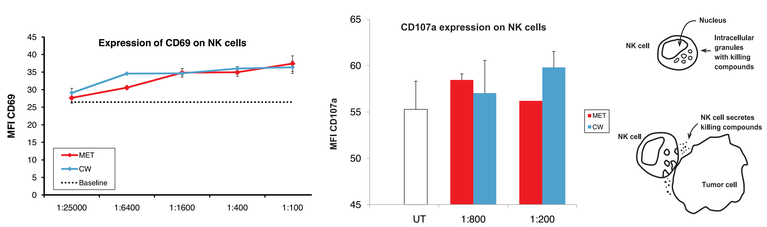

Figure 4. *Natural killer cell activation:* Effects of the metabolite fraction (MET) and cell wall fraction (CW) on NK cell activation status (LEFT) and cytotoxic activity (RIGHT) was evaluated using human PBMC. Both MET and CW fractions induced the expression of the activation marker CD69 on CD3 negative, CD56 positive NK cells in a dose-dependent manner. The effect was statistically significant for the 1:400 dilution of both MET and CW. *Natural killer cell cytotoxicity:* NK cells can kill tumor cells and virus-infected cells either via cell-cell contact or by secretion of substances such as Perforin, which perforates the cell membrane on the target cell. During this process, the CD107a receptor expressed on the interior of vacuoles in the cytoplasm of NK cells is transiently brought to the cell surface. Thus, CD107a expression on NK cells is a measure of their cytotoxic activity. Expression of CD107a on NK cells pretreated with MET or CW showed a mild increase over baseline. The graphs show the averages and standard deviations of each culture condition performed in triplicate.

Conclusions

- A broad assessment of immune function was examined in vitro using a panel of bioassays. It was found that MET and CW had overlapping effects but did not perform identically.
- CW showed the greatest anti-inflammatory effects: very low doses suppressed migration of PMN cells towards the inflammatory mediators IL-8 and LTB4. This is surprising given that the cell wall of bacteria usually elicits a pro-inflammatory response through the engagement of Toll-like receptors on immune cells [2].
- MET showed the greatest effect on enhancing anti-microbial defense mechanisms: increasing phagocytosis and random and f-MLP-directed migration of PMN cells.
- Both fractions caused an increase in several markers for NK cell activity. This was seen both as an increase in the CD69 marker and the CD107a marker. The effect of MET and CW on this aspect of innate immunity would support increased NK cell activity in terms of scavenging and killing transformed cells. This suggests a beneficial role for BC30™ in supporting the immune responses to virus-infected and malignant cells.
- Both fractions had direct effects on cytokine production in PBMC cultures: the decrease in IL-2 and increase in IL-4, IL-6 and IL-10 suggest immune modulating properties that direct cells towards a Th2 anti-inflammatory response.
- The 4-fold increase in IL-10 production in the presence of MET is particularly noteworthy given the important role this anti-inflammatory cytokine plays in the maintenance of mucosal immunity [3]. This result also suggests a potential benefit arising from intestinal colonization of BC30™ and the release of metabolites. Other probiotic strains have been developed by genetic modification to secrete IL-10 to the gut mucosal immune system, specifically in clinical conditions where IL-10 may help relieve inflammatory conditions such as Crohn's disease [4]. Due to the increased IL-10 production by BC30™ components, it is likely that BC30™ would have beneficial effects on inflammatory bowel disease.
- In contrast to the direct anti-inflammatory effect on cytokine profile, BC30™ strongly enhanced responses to the known stimulus Pokeweed mitogen (PWM), which requires the collaboration of macrophages and different lymphocyte subsets. This suggests that BC30™ metabolites and cell wall may enhance the response to invading pathogens, for example, in Peyer's Patches, where different cell types work in concert to produce innate and adaptive immune defense reactions.

References

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Acknowledgements

This study was conducted at NIS Labs, an independent contract research laboratory specializing in natural products testing. The work was sponsored in part by NIS Labs, in part by AIBMR Life Sciences, and in part by Ganeden Biotech Inc.

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