CHAPTER 6

LONG CHAIN INULIN-TYPE FRUCTANS BUT NOT SHORT CHAIN INULIN-TYPE FRUCTANS ENHANCE HEPATITIS B VACCINATION RESPONSE IN YOUNG ADULTS

Leonie M. Vogt\textsuperscript{1}, Marijke M. Faas\textsuperscript{1,2}, Paul de Vos\textsuperscript{1}

\textsuperscript{1}Immuoendocrinology, Division of Medical Biology, Department of Pathology and Medical Biology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, the Netherlands
\textsuperscript{2}Department of Obstetrics and Gynaecology, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9700 RB Groningen, the Netherlands
ABSTRACT

Objective. Inulin-type fructans have shown immune modulation in human cells, cell lines, and in supplemented experimental animals, but this has not been confirmed in healthy, immunocompetent human populations so far. Enhancing vaccination efficacy by supplementing with nutritional compounds is an accepted method for studying nutritional immune effects. This study was aimed to analyze the vaccine potentiating effect of inulin-type fructans, and to study whether their degree of polymerization is a determinant in the strength of the immune response. By analyzing peripheral blood lymphocyte subsets, concomitant changes in these populations may give insight in underlying mechanisms of a stimulated immune response.

Design. In this randomized double-blind placebo-controlled supplementation study, 40 healthy volunteers aged 18-29 (17 males, 23 females), were supplemented for 14 days with inulin-type fructans of DP10-60 (long chain), or inulin-type fructans of DP2-25 (short chain), or fructose as placebo (n=13, 13, 14 per group, 8 g in single dose per day). On day 7, all volunteers were vaccinated against hepatitis B (Engerix-B). Blood samples were collected at day 0 (basal samples), 7, 14, 21, and 35. Anti-HBsAg titer at day 0, 14, 21, and 35 was analyzed with an Abbot Architect immunoanalyzer. At all five time points, the percentages of different B cell, T cell, NK cell, and NKT cell populations within the total lymphocyte population and subsets within populations were analyzed using multi-parameter flow cytometry.

Results. DP10-60 fructans stimulated the vaccine-specific antibody response at day 35. This was not observed with DP2-25 fructan. In addition, two responders were identified in the DP10-60 fructan group vs. no responders in either the DP2-25 fructan group or the placebo group. In the placebo group, but not in the fructan supplemented groups, at day 21, the percentage of IgM⁺ non-class switched memory B cells was increased as compared to basal samples. In the DP10-60 group and the placebo group at day 14 and 21, the percentage of transitional B cells was increased compared to basal samples, but not in the DP2-25 group. The percentage of CD161⁺ NK cells was increased at day 21 and day 35 for each supplement. In the DP10-60 group, the percentage of TBET⁺ Th1 cells was increased at day 35 compared to basal samples. The increase in CD45RO⁺ cells as percentage of CTLs was not significant in the placebo group, and occurred earlier in time in the DP10-60 group (day 14)
compared to the DP2-25 group (day 35). Finally, the percentage of CD45RO$^{\text{hi}}$ cells in the Th population was increased at day 14 and 35 compared to basal samples in the placebo group but was not significantly increased in the fructan supplemented groups.

**Conclusion.** This study demonstrates clear structure-effector relationships for dietary fibers in immune responses in humans. In addition, it shows that using less efficacious vaccination protocols to prove efficacy of food components on immunity may be a more feasible approach than using efficacious vaccination protocols. Finally, it is demonstrates that orally taken bioactive ingredients can influence systemic responses against pathogens during vaccination.
INTRODUCTION

6.1 Background
Dietary fibers are considered an essential part of healthy nutrition. The health benefits of sufficient fiber intake comprise prevention of colorectal cancer \(^1\), type 2 diabetes \(^2\), cardiovascular disease \(^3\), reduced risk of hyperlipidemia, hypercholesterolemia and hyperglycemia \(^4\) \(^5\), and regulation of bowel habit \(^6\)\(^-\)\(^10\). Because the role of the immune system in these protective effects is not completely clear, there is a call for evidence of immune modulation by ingestion of dietary fibers in human studies, and for structure–function studies \(^11\) \(^12\).

Inulin-type fructans are prebiotic dietary fibers with many health benefits. They are oligomers and polymers of fructose subunits, and often terminate in a glucose molecule \(^13\). Besides the established effects on gut health and metabolism \(^11\) \(^12\) \(^14\), evidence for immunostimulatory effects of inulin-type fructan consumption is accumulating. Results from ex vivo and in vitro experiments in human cells, cell lines, and from animal studies, support the notion that inulin-type fructans exert immune modulating effects. For an extensive overview of these features the reader is referred to a collection of literature reviews \(^15\)-\(^19\). The underlying mechanisms for immune modulation by inulin-type fructans have been attributed to the selective stimulation of beneficial bacteria in the intestine, and their SCFA fermentation products \(^17\). In addition, previous results from our group support the notion that these fibers can also exert direct effects on immune cells, by activating Toll-like receptors (TLRs) on monocyctic cells, and inducing cytokine production in human peripheral blood mononuclear cells (PBMCs) upon in vitro stimulation \(^20\). The degree of polymerization (DP, or chain length) of inulin-type fructans ranges between 2 and 60, and commercially available inulin-type fructan powders often contain mixtures of different DP fructans. Interestingly, in our studies the DP of inulin-type fructans proved to be an important determinant in skewing PBMC cytokine profiles; short chain enriched inulin-type fructans (DP2-25) induced an anti-inflammatory IL-10/IL-12 ratio, whereas the long chain enriched inulin-type fructans (DP10-60) produced a more proinflammatory, or immunostimulatory IL-10/IL-12 ratio. In addition, TLR2, which was dose dependently activated by the fructans, demonstrated increased activation with increasing average fructan DP. Based on these differences in in vitro direct effects on immune cells, we selected the DP2-25 fructans and DP10-60 fructans as supplements to
study in vivo effects in a human trial. So far, inulin-type fructan supplementation trials which were aimed at boosting the human immune system, commonly target immunocompromised populations such as infants and elderly. We hypothesize that inulin-type fructan supplementation can also be beneficial for healthy immunocompetent populations. A means to study immune modulation is by studying the effects of inulin-type fructan supplementation on building immunity against pathogens such as during an infection or during vaccination, where an immune reaction is evoked without inducing disease. These types of studies are also recommended by regulatory agencies such as the European Food Safety Authority (EFSA) to prove immune efficacy of food components.21

Up to now, human vaccine efficacy studies with oligo-, or polysaccharide food supplements have mainly been done with efficacious vaccination protocols such as measles22, tetanus23, influenza2324, or pneumococcal24 vaccination. Demonstrating efficacy of a food component in these protocols requires high numbers of volunteers, and effects will always be modest. Vaccines which by themselves do not induce an immediate strong antibody response are to our opinion a more attractive option for application in nutritional immunity studies. Hepatitis B vaccination belongs to this category. Common vaccination programs use hepatitis B surface antigen (HBsAg) to immunize subjects, and require booster injections to reach protective antibody titers (i.e. above 100 IU/ml)25. We hypothesize that by supplementing young adults with inulin-type fructans from 7 days before, until 7 days after the first injection of an HBsAg vaccination program, the antibody response against the vaccine will be improved compared to placebo supplemented participants. This could be represented by an earlier onset of titer development, an increased titer response on the final measuring time point, and/or the presence and number of ‘responders’, characterized as subjects with titers equal to or above 10 IU/mL. Due to the previous results in our group mentioned above, we expect that DP10-60 fructans will stimulate the immune system and enhance the vaccine-induced response as compared to DP2-25 fructans and placebo. Besides the vaccination efficacy, peripheral blood lymphocyte subsets of the supplemented subjects were studied using multi-parameter flow cytometry. Concomitant changes in these populations may give an indication of underlying mechanisms of a stimulated immune response and highlight possible differences induced in lymphocyte populations, related to difference in DP of fructan supplements.
CHAPTER 6. LONG CHAIN INULIN-TYPE FRUCTANS BUT NOT SHORT CHAIN INULIN-TYPE FRUCTANS ENHANCE HEPATITIS B VACCINATION RESPONSE IN YOUNG ADULTS.

METHODS

6.2 Investigational compounds
Inulin from chicory is a polydisperse mixture of linear fructan oligomers and polymers coupled by means of β(2-1) bonds, and mostly with a terminal glucose unit. The number of fructose units in the chain (degree of polymerization, or DP) can vary naturally between 2 and 60. Supplement A (Frutafit®TEX! Sensus, Roosendaal, the Netherlands), is a natural powdered food ingredient based on chicory inulin, and known for its texturizing properties. The DP ranges between 10 and 60. Supplement B (Frutafit®CLR Sensus), is a powdered fructo-oligosaccharide (FOS) produced by partial hydrolysis of chicory inulin. Frutafit®CLR is a highly soluble food ingredient with a DP ranging mainly between 2 and 25. Supplement C consists of fructose, which is a powdered carbohydrate, and serves as placebo because it consists of the monomer building blocks of fructans and does not have β(2-1) bonds. The DP profiles of supplement A and B are depicted in Figure 1.

![Figure 1. DP profiles of supplement A and supplement B.](image)

Figure 2. Schematic overview of experimental procedures in time.

![Figure 2. Schematic overview of experimental procedures in time.](image)
6.3 Volunteers and interventions

This study was approved by the ethical board of the University Medical Center Groningen, Medisch Ethische Toetsingscommissie University Medical Center Groningen, and documented in the approved application METC_097. It has been registered in the national Dutch trial register, Nederlandse Trial Register (NTR41644). Written informed consent was obtained from all participants, and data was analyzed and presented anonymously. Hepatitis B vaccination and blood sampling was conducted within the University Medical Center Groningen, in the Netherlands. All clinical investigation was conducted according to the principles expressed in the Declaration of Helsinki.

A randomized double-blind placebo controlled human dietary intervention trial was designed to study the effect of inulin-type fructans on vaccination efficacy and peripheral blood lymphocyte populations. To study whether the degree of polymerization (DP) of inulin-type fructans can influence whether different types of immune reactions are stimulated, three groups were included in the study. Healthy volunteers without a history of gastrointestinal symptoms and free of medication, aged 18-29 (17 males, 23 females), were supplemented for 14 days with inulin-type fructans of DP10-60, or with inulin-type fructans of DP2-25, or fructose (Sigma-Aldrich, the Netherlands) as placebo (n=13, 13, and 14 per group respectively, 8g/d in one dose per day), and vaccinated against hepatitis B (Engerix-B, GlaxoSmithKline Biologicals s.a, Belgium) on day 7. The volunteers consumed their habitual diet and filled out a nutrition diary for the 35 days of the study. Blood samples were collected at day 0, 7, 14, 21, and 35, see Figure 2 for a schematic overview of experimental interventions.

6.4 Peripheral blood lymphocyte isolation and multi-parameter flow cytometry

Multi-parameter flow cytometry was performed to measure percentages of different B cell, T cell, NK cell, and NKT cell populations within the total lymphocyte population and subsets within populations. Blood was drawn from the inner cubital vein and collected in 10 mL lithium heparin Vacutainer tubes (BD, Plymouth, UK). All subsequent steps were performed at 4°C. Whole blood (1.5 mL) was centrifuged at 2000 g for 15 min, and plasma supernatant was aliquotted and stored at -20°C for anti-hepatitis B antibody titer analysis. The remaining whole blood was separated into two 50 mL tubes and erythrocytes were lyzed by incubating twice with 40 mL of ammonium chloride per tube for 10 min. Cell pellet
was collected by centrifuging for 5 min at 1800 g. After washing the cell pellet twice with 15 mL FACS buffer (2% fetal bovine serum in phosphate buffered saline, FBS in PBS), cells were counted on a coulter counter (Beckton Dickinson, the Netherlands) and 1 x 10⁶ cells per well were transferred to a round bottom 96 wells plate. After pelleting the cells for 5 min at 1800 g and discarding the supernatant, cell pellets were resuspended in 50 µl of blocking buffer (20% normal rat serum, Jackson laboratories, in FACS buffer) and incubated for 20 min. Cells were pelleted as described above, resuspended in 50 µl of extracellular antibody mix consisting of extracellular antibodies, 5% normal rat serum, and FACS buffer (antibodies are listed in Table 1-3), and were incubated in the dark for 30 min. After washing the cells which were stained for B cell and NK cell markers, twice with 200 µl FACS buffer per well, cells were incubated with 200 µL of FACS-lysing buffer (Beckton Dickinson BV, Breda, the Netherlands) for 30 min. Cells were then washed three times with FACS buffer, resuspended in 200 µl FACS buffer per well, and stored at 4°C in the dark until analysis. After incubation with the extracellular antibody mix, the cells stained for T cell markers were washed three times with 200 µl of permeabilization buffer (eBioscience, Vienna, Austria) per well. Cells were resuspended in 50 µl of intracellular blocking buffer (20% normal rat serum in permeabilization buffer) and incubated for 20 min. After pelleting the cells as described above and discarding the supernatant, cells were incubated with 50 µL of intracellular antibody mix, consisting of intracellular antibodies, 5% normal rat serum, and permeabilization buffer (antibodies are listed in Table 2), and incubated for 30 min. Cells were washed three times with permeabilization buffer, resuspended in 200 µl of FACS buffer, and stored at 4°C in the dark until analysis on an LSR II flow cytometer (Beckton Dickinson BV). The corresponding isotype control antibodies were purchased from the same company as the target antibodies, and isotype stainings were used to set positive gates, using 1% margins. UltraComp eBeads (eBioscience, Vienna, Austria) were applied to set the appropriate compensation values for each antibody panel. FlowJo VX software (FlowJo, Oregon, USA) was used to analyze lymphocyte subsets. Basal sampling at day 0 for every individual allowed repeated measures analysis on the flow cytometry data.

Flow cytometry gating strategies are described in Figure 3.1, 3.2 and 3.3. Within the B cell population we used the markers CD19, CD21, CD27, CD38, IgA, IgD, IgG, and IgM to identify naïve non-class switched B cells, class switched memory B cells (IgG⁺ and IgA⁺), non-class switched memory B cells, transitional B cells, and plasmablasts/plasma cells. Using
CD3, CD56, and CD16 as markers we identified NK-\(^\text{-}\) and NKT cells, and within the NK cell population we identified cytotoxic NK cells (CD56\(^+\),CD16\(^{\text{hi}}\))\(^{26}\) and cytokine producing NK cells (CD56\(^{\text{hi}},\)CD16\(^{\text{dim}}\) or CD56\(^{\text{hi}},\)CD16\(^{-}\))\(^{26}\). We determined the percentage of CD161\(^+\) and CD335\(^+\) cells within the cytotoxic-, and cytokine producing NK cells. CD161 is a cell surface marker which can be present on different subsets of NK cells\(^{27}\). Ligation of CD161 is known to cause activation of Pi3K, PkB, Akt and ERK pathways and is important in the regulation of NK and NKT cell function\(^{27}\). CD335 is a natural cytotoxicity-triggering receptor also known as PCR1 or NKp46\(^ {28}\). We used CD3 and CD8 as markers to distinguish Th cells and CTLs, and within the Th cell population we identified memory cells (CD45RO\(^+\)), Th1 cells (TBET\(^+\)), Th2 cells (CD294\(^+\)), Th17 cells (RoRγT\(^+\)), and FoxP3\(^+\) Th cells which may represent regulatory cells. It is generally believed that for the time frame following challenge with a virus or a viral vaccine, human CD8\(^+\) memory cells are principally found within the CD45RO\(^{\text{hi}}\) population \(^{29}\), therefore we also gated and analyzed this population.

**6.5 Anti-HBsAg titer analysis**

Anti-HBsAg titers were analyzed at day 0, 14, 21, and 35. Per sample, 500 \(\mu\)l of plasma was aliquotted in Architect tubes (Abbott, Illinois, U.S.A.), and analyzed for Anti-HBsAg titers using an Architect Immunoassay Analyzer (Abbott Diagnostics) following the manufacturer’s instructions.

<table>
<thead>
<tr>
<th>Antibody and label</th>
<th>company</th>
<th>cat#</th>
<th>dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19-PE</td>
<td>ITK, Biolegend</td>
<td>302208</td>
<td>10x</td>
</tr>
<tr>
<td>CD21-PE-Cy7</td>
<td>ITK, Biolegend</td>
<td>354912</td>
<td>100x</td>
</tr>
<tr>
<td>CD27-BV421</td>
<td>ITK, Biolegend</td>
<td>302824</td>
<td>50x</td>
</tr>
<tr>
<td>CD38-A700</td>
<td>ITK, Biolegend</td>
<td>303524</td>
<td>100x</td>
</tr>
<tr>
<td>IgA-FITC</td>
<td>DAKO</td>
<td>F0188</td>
<td>20x</td>
</tr>
<tr>
<td>IgD-APC</td>
<td>ITK, Biolegend</td>
<td>348222</td>
<td>20x</td>
</tr>
<tr>
<td>IgG- PerCP-Cy5.5</td>
<td>ITK, Biolegend</td>
<td>409312</td>
<td>7.5x</td>
</tr>
<tr>
<td>IgM-BV605</td>
<td>ITK, Biolegend</td>
<td>314524</td>
<td>10x</td>
</tr>
</tbody>
</table>
Figure 3.1. Gating strategies for peripheral blood B lymphocyte populations. Single cells are gated using FSC-H vs. FSC-A scatter plot (A). Single cells are gated to a FSC vs. SSC plot, and lymphocyte gate is set (B). Within the lymphocyte population, B cells (CD19+ ) are identified as a separate population (C). The CD21 isotype is used to set the gate margin on 1% positive cells (D) and this gate is copied to the CD21 stained sample (E, upper plus lower gate), identifying CD21+ cells. Within this population, the CD27 isotype is used to set the gate margin for 1% positive cells (F, upper gate) and the CD27 population (F, lower gate) and this gate is copied to the CD27 stained sample (G). Within the CD27 population (G), IgD isotype is used to set the gate margin on 1% positive cells (H) and this gate is copied to the IgD stained sample (I), identifying the naïve non-class switched B cells. Within the CD19+ population, CD27 isotype is used to set the gate margin on 1% positive cells (J) and this gate is copied to the CD27 stained sample (K), identifying CD27+ cells. Using the IgD isotype, the 1% positive gate and negative gate are set (L), and the negative gate is copied to the IgD stained sample (M) to identify the IgD- class switched memory population. Within this
population, isotypes are used to set 1% positive gates for IgG (N) and IgA (Q), which are copied to IgG and IgA stained samples to identify IgG⁺ and IgA⁺ class switched memory cells (panel O and R). The isotype gate set in panel L is copied to the IgD stained sample to identify CD19⁺ CD27⁻ IgD⁺ cells (P). Within this population, the IgM isotype is used to set a 1% positive gate (S), which is copied to the IgM stained sample to identify the IgM⁺ non-class switched memory B cells (T). Within the CD19⁺ population, the CD38 isotype is used to set a 1% positive gate (U), which is copied to the CD38 stained sample to identify CD38⁺ and CD38hi cells (V). Within the CD38hi population the IgM isotype is used to set a 1% positive gate (W), which is copied to the IgM stained sample to identify IgMint cells (X*) and the IgMhi transitional B cell population (X**). The CD21lo population as set in panel E is copied to this IgMint cell population in panel X, identifying the plasmablasts and plasma cells (Y).

Figure 3.2. Gating strategies for peripheral blood NK and NKT lymphocyte populations. Single cells and lymphocytes are gated as described for B lymphocytes (A,B). Within the lymphocyte population, CD56⁺ CD3⁻ cells are gated (C, **), indicating the NKT cells. Within the lymphocyte population, the CD3⁺ population is gated (C,*), and plotted in a panel with CD56 on the x-axis and CD16 on the y-axis (D). In panel D, left, the cytokine producing cells are gated [CD56hi CD16⁺], and the cytotoxic cells are also gated, panel D, right [CD56int CD16lo]. Within these populations, isotype for CD161 (E and I), and isotype for CD335 (G and K) were used to set 1% positive gates, which were copied to the CD161 stained samples (F and J) and to the CD335 stained samples (H and L) respectively.
CHAPTER 6. LONG CHAIN INULIN-TYPE FRUCTANS BUT NOT SHORT CHAIN INULIN-TYPE FRUCTANS ENHANCE HEPATITIS B VACCINATION RESPONSE IN YOUNG ADULTS.

Figure 3.3. Gating strategies for peripheral blood T lymphocyte populations. Single cells and lymphocytes are gated as described for B lymphocytes (A, B). The T cell population within lymphocytes are gated in a CD3 vs. CD8 plot (C). CTLs are identified and gated based on expression of CD3 and CD8 (D, upper population). The CD45RO isotype is used to set the gate margin on 1% positive CTLs (E). This gate is copied to the CD45RO stained sample, now demonstrating the CD45RO+ population, and the upper cell cluster now indicates CD45ROhi cells (F). Th cells are identified based on expression of CD3 and no expression of CD8 (D, lower population). Isotype controls are used to set the gate margins on 1% positive cells for CD45RO, TBET, and RoRγT (G, I, L). These gates are copied to the CD45RO-, TBET-, and RoRγT stained samples, now demonstrating the positive populations (H, J, M). A cross-gate is applied to identify FoxP3+ Th cells (*) and CD294+ Th2 cells (**) in panel K.
Table 2. Antibodies applied for flow cytometry of NK/NKT lymphocytes.

<table>
<thead>
<tr>
<th>Antibody and label</th>
<th>company</th>
<th>cat#</th>
<th>dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3-PerCP</td>
<td>ITK, Biolegend</td>
<td>300428</td>
<td>30x</td>
</tr>
<tr>
<td>CD16-E450</td>
<td>eBioscience</td>
<td>48-0168-42</td>
<td>10x</td>
</tr>
<tr>
<td>CD56-APC</td>
<td>eBioscience</td>
<td>17-0569-42</td>
<td>25x</td>
</tr>
<tr>
<td>CD335-PE</td>
<td>ITK, Biolegend</td>
<td>331908</td>
<td>30x</td>
</tr>
<tr>
<td>CD161-PE-Cy7</td>
<td>eBioscience</td>
<td>25-1619-42</td>
<td>20x</td>
</tr>
</tbody>
</table>

Table 3. Antibodies applied for flow cytometry of T lymphocytes.

<table>
<thead>
<tr>
<th>Antibody and label</th>
<th>company</th>
<th>cat#</th>
<th>dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3-Pacific Blue</td>
<td>BD</td>
<td>558117</td>
<td>25x</td>
</tr>
<tr>
<td>CD8-PerCP</td>
<td>BD</td>
<td>345774</td>
<td>25x</td>
</tr>
<tr>
<td>CD45RO-biotin</td>
<td>Biolegend</td>
<td>304220</td>
<td>25x</td>
</tr>
<tr>
<td>Streptavidin-PAC</td>
<td>LifeTechnologies</td>
<td>S32365</td>
<td>100x</td>
</tr>
<tr>
<td>FoxP3-APC</td>
<td>eBioscience</td>
<td>17-4776-42</td>
<td>25x</td>
</tr>
<tr>
<td>TBET-PE-Cy7</td>
<td>eBioscience</td>
<td>25-5825-80</td>
<td>150x</td>
</tr>
<tr>
<td>CD294-PE</td>
<td>Miltenyi</td>
<td>130-098-879</td>
<td>40x</td>
</tr>
<tr>
<td>RoRγT-PE</td>
<td>eBioscience</td>
<td>12-6981-80</td>
<td>50x</td>
</tr>
</tbody>
</table>

**Statistical analysis**

GraphPad Prism 5.0 was used for statistical analysis of all data. Flow cytometry data sets were analyzed for normal distribution using a d’Agostino Pearson test. To test for anti-HBsAg antibody titer development per supplement, statistical significance levels were determined with a Friedman test and Dunn’s multiple comparison test, comparing T14, T21, and T35 to T0 (basal samples), and Mann Whitney test was used for differences between supplements. Repeated measures ANOVA and Tukey’s multiple comparison test or a Friedman test and Dunn’s multiple comparison test were used to analyse the flow cytometry data, comparing T14, T21, and T35 to T0 (basal samples). P-values < 0.05 were considered statistically significant, and p = 0.1 was considered representative for a statistical trend.
RESULTS

6.6 Supplementation with DP10-60 fructans increased anti-HBsAg antibody production compared to supplementation with DP2-25 fructans and placebo

Effects of nutritional supplementation with DP10-60 fructans vs. DP2-25 fructans on vaccination efficacy were studied by analyzing vaccine-induced anti-HBsAg antibodies in peripheral blood plasma samples of supplemented subjects using Architect Immunoassay analysis (Figure 4). Subjects supplemented with DP10-60 fructans developed antibody titers from time point T21 (0.6 ± 0.34 IQR) and reached significantly elevated levels at time point T35 compared to basal samples at T0 (2.93 ± 0.75 IQR, p < 0.05). In contrast, in plasma of subjects supplemented with DP2-25 fructans or placebo, no increases in antibody titers were observed at T35 as compared to basal samples at T0.

Individuals developing an antibody titer against any vaccination, which is above a predetermined threshold value are indicated as so-called responders, vs. individuals who do not reach titers above this value, which are indicated as non-responders. This threshold value for the anti-HBsAg applied in the present study is generally set at 10 IU/mL. There were no responders in the placebo groups and the DP2-25 fructan group at T35. This was different in the DP10-60 fructan group in which two responders were present at time point T35.

These results combined, demonstrate that supplementing young adults with a daily single bolus of 8 g DP10-60 fructans in the 14-day period around the first injection enhanced the efficacy of the anti-hepatitis B vaccination.

6.7 Flow cytometry analysis of peripheral blood B cell subsets

Peripheral blood lymphocyte subsets of the supplemented individuals were analyzed by multi-parameter flow cytometry to study the effects of different DP fructans. Specifically, we were interested whether changes in T cell, B cell, NK cell, and NKT cell subsets were induced. As B cells are essential in mounting antibody responses against vaccines, we will first describe the results of B cell subsets in peripheral blood of subjects in the experimental groups. The subsets and their corresponding markers of identification are summarized in Table 1, gating strategies are shown in Figure 3, and results of B lymphocyte flow cytometry are shown in Figure 5. The percentage of B cells within the lymphocyte population was
analysed, and subsequently, the percentage of the following populations within the lymphocyte population or within the B cell population were analysed and compared to basal samples at T0; 1) Naïve non-class switched B cells [CD19+ CD21+, CD27- IgD+], 2) Class-switched memory B cells [CD19+ CD27+ IgD-; within this population the percentages of IgA+...
and IgG² cells were analysed], 3) Non-class switched memory B cells [CD19⁺ CD27⁺ IgD⁻ IgM⁺], 4) transitional B cells [CD19⁺ CD38hi IgMhi], and 5) plasmablasts or plasma cells [CD19⁺ CD38hi IgMint CD21lo].
The percentages of B cells in the total lymphocyte population did not change as compared to basal samples (Figure 5.I). Naïve non-class switched B cells (Figure 5.II), class switched memory B cell populations (Figure 5.III), and non-class switched memory B cells (data not shown) did not differ in time compared to basal samples as percentage of lymphocytes or B cells. The percentage of IgM$^+$ cells in the non-class switched memory B cell population (Figure 5.IV) was increased for the placebo group at T21 compared to the basal samples (p < 0.05), but in the DP10-60 group and DP2-25 group no changes were observed as compared to basal samples. The percentage of transitional B cells and the percentages of plasmablasts or plasma cells did not differ in time as percentage of lymphocytes or B cells (Figure 5.V and 5.VII). However, in the DP10-60 fructan group and the placebo group, the percentages of IgM$^{hi}$ cells within the CD38$^{hi}$ transitional B cells were significantly increased at T14 and T21 as compared to basal samples (p < 0.05). This effect was absent in the DP2-25 fructan group (Figure 5.VI).

6.8 Flow cytometry analysis of peripheral blood NK-, and NKT cell subsets

Human NK cells (CD56$^+$ CD3$^-$) are part of the first line of defense against viral pathogens and their activation can modulate the outcome of the adaptive immune response.$^{30}$ Similar to NK cells, NKT cells (CD56$^+$ CD3$^+$) are also implicated in the response against hepatitis B vaccine antigens.$^{30}$ The subsets and their corresponding markers of identification are summarized in Table 2, gating strategies are depicted in Figure 3, and results of NK lymphocyte flow cytometry are depicted in Figure 6. Within the lymphocyte population and within the NK cell population, the percentages of cytokine producing (CD56$^{hi}$ CD16$^{int}$) or cytotoxic (CD56$^{int}$ CD16$^{hi}$) cells were determined (Figure 6.I and 6.IV), and within the cytotoxic and cytokine producing cells, the percentages of CD161$^+$ and CD335$^+$ cells were analysed. No differences were observed for either cytotoxic or cytokine producing NK cell populations as percentage of the lymphocyte population. In the DP2-25 group and placebo group (Figure 6.II), an increased effect was observed for CD161$^+$ cytokine producing NK cells at all time points compared to basal samples (T0) but in the DP10-60 group this effect was only observed on time points T21 and T35 compared to basal samples (p < 0.05). No changes were observed for CD161$^+$ cytotoxic NK cells as compared to basal samples irrespective of the treatment. The percentage of CD335$^+$ cells did not differ in time within
either NK cell population. In the DP10-60 group (Figure 6.VII), NKT cells tended to show a decrease on time point T14 compared to basal samples, expressed as percentage of the lymphocyte population (p = 0.1), but no changes were observed in the DP2-25 and placebo group.

Figure 6. Percentages of cytokine producing NK cells and cytotoxic NK cells, and percentages of CD161+ and CD335+ cells within these populations, and NKT cells within the lymphocyte population in time, plotted per supplement. A) DP10-60 fructans, B) DP2-25 fructans C) placebo. Repeated measures ANOVA and Tukey’s post test or a Friedman test and a Dunn’s post test were used to analyze time effect per supplement. Median and IQR are plotted as percentage of the indicated cell populations, n=13, 13, and 14 for supplement DP10-60, DP2-25, and placebo respectively. P = 0.1 indicates a statistical trend, * represents statistical difference with p < 0.05, ** represents statistical difference with p < 0.01, and *** represents statistical difference with p < 0.001.
6.9 Flow cytometry analysis of peripheral blood T cell subsets

Gating strategies are depicted in Figure 3, and T cell antibodies and labels are listed in Table 4. Results of T lymphocyte flow cytometry are shown in Figure 7. Within the T cell population, we analyzed the percentages of Th1 cells, Th2 cells, FoxP3+ Th cells, Th17 cells, Th memory cells, and CTL memory cells. Percentages of Th2 cells, FoxP3+ Th cells, and Th17 cells at T35 did not differ from basal samples at T0 (Figures 5.II, 5.III, and 5.IV). At time point T35, the percentage of TBET+ (Th1) cells in the DP10-60 fructan group was significantly increased as compared to basal levels (p < 0.05), but this effect was not observed in the DP2-25 group or placebo group (Figure 5.I). Compared to basal samples at T0, the percentage of CD45ROhi CTLs expressed as percentage of CTL memory cells was increased at time point T14 and T35 in the DP10-60 group (p < 0.05), and at time point T35 in the DP2-25 group (p < 0.05), but no significant increases were observed in the placebo group (Figure 5.V). In addition to the CTLs, Th cells also typically demonstrated changes in the CD45ROhi population. However, only the placebo group demonstrated significant increases in CD45ROhi Th cells as percentages of the Th memory population (Figure 5.VI), at time point T14 and T35 as compared to basal samples at T0 (p < 0.05).

DISCUSSION

Previous studies from others and us have shown that inulin-type fructans can impact immunity by either serving as microbiota accessible fiber 17 or by directly binding to immune cells 20, but scientific evidence in humans of related immunological benefits were largely lacking. Building on in vitro results in our studies with inulin-type fructans, we hypothesized that DP10-60 fructans would stimulate vaccine responses in vivo due to their predominant induction of proinflammatory cytokines in PBMCs, including a relatively low IL-10/IL-12 ratio 20, in combination with their strong ability to activate TLR2 20 31. We observed that supplementation with DP10-60 fructans significantly enhanced the titer response (T35 of the study) as compared to the DP2-25 fructan supplemented group, and that a strong increased trend was present as compared to the titer development in the placebo group. Another observation that supports the immune stimulating effects was the identification of two responders in the DP10-60 group vs. no responders in either the DP2-25 group or the placebo group. Inulin-type fructans have been studied in several infant vaccination trials, but in
the majority of the fructans were only studied in combination with GOS and/or pectic oligosaccharides. In one study though, long term supplementation with a mixture of oligofructose/inulin, i.e. short chain inulin-type fructans combined with long chain inulin-type fructans,

Figure 7. T cell subsets expressed as percentages of Th cells or T memory cells in time, plotted per supplement. A) DP10-60 fructans, B) DP2-25 fructans C) placebo. A Friedman test and a Dunn’s post test were used to analyze time effect per supplement. Median and IQR are plotted as percentage of the indicated cell populations, n=13, 13, and 14 for supplement DP10-60, DP2-25, and placebo respectively. * represents statistical difference with p < 0.05.
enhanced the vaccination responses. In this study Saavedra et al. observed an increase in blood IgG levels after measles vaccination in a 10 week supplementation study with oligofructose (OF)/inulin (7/3, 0.2 g/kg BW/d) in 7-9 months old infants. In a study by Duggan et al. in which 6-12 month old infants were supplemented with OF, i.e. only short chain inulin-type fructans (0.7 g/d), no effect was observed on antibody response after vaccination with H. influenza type B vaccine. Strikingly, other studies in infants with prebiotic mixtures did not induce vaccine potentiating effects. It should be noted that the applied fructans in these mixtures are often - if not always - of a short chain nature. Although this body of evidence is still relatively small, it is tempting to speculate that the DP10-60 fructans are indeed more suitable to apply for the purpose of potentiating vaccination programs.

Dietary fibers can differ substantially in their molecular composition, and even within categories such as inulin-type fructans, different effects on the body and microbiota can be elicited. To study the effect of different DP of fructans on changes in peripheral lymphocytes, we analyzed B cell-, T cell-, NK cell, and NKT cell subsets of supplemented individuals for their percentage of the total lymphocyte population, percentage of relevant subpopulations, and for differences in activation marker expression. Class-switching is one of the hallmarks of activation and maturation of B cells. If the peripheral blood is representative for the immune responses occurring after the vaccination, the increased titer response at T35 would be expected to coincide with a decrease in naive B cells, and increases in transitional B cells or even plasma cells. The percentage of transitional B cells which are in the process of maturation (CD38hi IgMhi) was significantly increased in the DP10-60 group and the placebo group for time points T14 and T21 compared to the basal samples but not in the DP2-25 group. Increased percentages of this population indicate that B cells are activated and stimulated to differentiate into antibody producing plasma cells, which is a functional objective of vaccination. The fact that this population was stimulated by DP10-60 fructan supplementation and not by DP2-25 fructan supplementation underscores the effective differences of these two supplements. The induction of memory B cells is important for the ability to mount an efficient secondary immune response and protection against infection upon encountering the relevant antigen. In the non-class switched memory cells, the percentage of IgM+ cells was slightly increased for the placebo group at T21 compared to basal samples while the fructan groups did not. It is possible that the chosen time points for
sampling are too early after vaccination to observe the induction of B memory cells\textsuperscript{36} and that these cells arise after the 35 day period.

NK and NKT cells did not demonstrate clear supplement dependent effects, suggesting that B cells and especially T cells may be more involved in the boosting of the vaccination response via dietary inulin supplementation, or that B cell and T cell effects may be better detectable in peripheral blood. Supplementation with DP10-60 fructans induced striking differences in T cell populations in time, the increased titer response for the long chain group on T35 was associated with an increased percentage of (TBET\textsuperscript{+}) Th1 cells compared to basal samples. Contrary to Th1 cells, (CD294\textsuperscript{+}) Th2 cells did not change in time for either treatment. These results suggest that in the DP10-60 group, a shifted Th1/Th2 balance may have been induced which was skewed towards Th1 cell responses. These results are corroborated by literature reports mentioned in several reviews \textsuperscript{15-19}, which subscribe that inulin-type fructans can shift the balance towards Th1 reactions instead of Th2 mediated responses. Although the in vitro results from our group \textsuperscript{20}, and others \textsuperscript{34} led to expect increased Treg percentages at T35 in the DP2-25 group as compared to the DP10-60 group and the placebo group, no changes in regulatory T-cell populations were observed. Future vaccination studies may also include regulatory B cells in flow cytometry analysis, as these cells may also play a role in the success or absence of anti-HBsAg vaccination responses \textsuperscript{35}. It would be interesting to study whether these Bregs are stimulated by short chain inulin-type fructans as opposed to the Tregs which did not show increases in this study. Th17 populations also showed no changes in time or differences due to treatment. The induction of memory cells is important in the efficacy of vaccinations, as these cells are responsible for fast recognition of a second encounter with the relevant antigen and mounting an adequate immune response \textsuperscript{36}. The results indicate that both fructan treatments stimulate the induction of memory CD45RO\textsuperscript{hi} CTLs but the placebo did not. On the other hand, the CD45RO\textsuperscript{hi} Th cells were only enhanced in the placebo group at T35. It would be interesting in follow up studies to also include CD45RA and CCR7, to study the transitions into T cell memory or effector cells in more depth \textsuperscript{37}.

The fact that we observed clear differences for the different DP compounds suggests that DP is an important factor which determines the reaction of immune cells in the body. This could be explained by several mechanisms. Upon consumption, inulin-type fructans of different DP may selectively stimulate different populations of the microbiota \textsuperscript{38-40}, and
these different populations could influence the immune system either in a stimulating or an attenuating manner. In addition, short chain fibers could be fermented into different products than long chain fibers, thus inducing different SCFA profiles in the intestine, qualitatively and quantitatively. However, most effects of SCFA on the immune system are attenuating, contrary to the immune stimulation results observed in the current study. It is more likely that the effects are the result of the sum of indirect and direct effects on immune cells in the intestine. It is striking that an oral supplement can stimulate a systemic response to an intramuscular vaccination, and this warrants further studies into the way this process of antigen uptake and presentation can be impacted by orally taken supplements. Generally, the antigens of an intramuscular vaccination such as the applied hepatitis B vaccination, are thought to be detected by circulating dendritic cells, which then recruit other immune cells and migrate towards a draining lymph node, where antigen is presented to B- and T cells followed by a primary immune response. Because of the natural surveillance function exerted by DCs, they circulate through the body and mount immune responses against antigens which are encountered. Ligation of innate immune receptors on DCs followed by antigen presentation toward effector cells, such as B cells, T cells, and NK cells locally, or in specialized lymphoid structures could be a mechanism which explains the induction of pro- and anti-inflammatory cytokines as observed in many prebiotic studies. Due to the fact that DCs can ‘sample’ the gut lumen and they are migratory cells, they are one of the candidate cell types to mediate dietary fiber-induced immune effects occurring in the periphery.

In conclusion, this is the first in vivo study which demonstrates a structure-function relation of a dietary fiber on a human immune response. We demonstrate that events in the gut by supplementing with a dietary fiber, have consequences for systemically induced changes in the immune system. The in vivo immunostimulatory potential of long-chain enriched inulin-type fructans subscribes an important nutritional health claim that these fibers can be beneficial for the immune system. Finally we feel that the use of a low efficacy vaccination model such as Hepatitis B may be more instrumental to demonstrate immunological effects of a nutritional supplement than the very efficient vaccination models. These human studies can be done with relatively low numbers of subjects and still with a high statistical power.
ACKNOWLEDGEMENTS

The research was jointly financed by the European Union, European Regional Development Fund and the Ministry of Economic Affairs, Peaks in the Delta, the Municipality of Groningen, the Provinces of Groningen, Fryslan and Drente, as well as the Dutch Carbohydrate Competence Center (CCC WP2a), and by Royal Cosun.

Within the framework of the Carbohydrate Competence Center (CCC WP2a) to which Royal Cosun contributed both financially and intellectually, the data obtained in the course of this study and reported in this chapter/article are considered proprietary data of the parties to CCC WP2a, patent pending.
REFERENCES


CHAPTER 6. LONG CHAIN INULIN-TYPE FRUCTANS BUT NOT SHORT CHAIN INULIN-TYPE FRUCTANS ENHANCE HEPATITIS B VACCINATION RESPONSE IN YOUNG ADULTS.


(25) Hoebe CJ, Vermeiren AP, Dukers-Muijirers NH. Revaccination with Fendrix(R) or HBVaxPro(R) results in better response rates than does revaccination with three doses of Engerix-B(R) in previous non-responders. *Vaccine* 2012 Nov 6;30(48):6734-6737.


