Alterations of serum protein N-glycosylation in two mouse models of chronic liver disease are hepatocyte and not B cell driven

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IN HUMANS, IMMUNOGLOBULIN G (IgG) is the most abundant liver disease are hepatocyte and not B cell driven. Alterations of serum protein glycosylation in two mouse models of chronic liver disease are hepatocyte and not B cell driven. N-glycosylation of immunoglobulin G (IgG) has an important impact on the modification of the total serum N-glycome in chronic liver patients. Our aim was to determine the role and magnitude of the alterations in which hepatocytes and B cells are involved in two mouse models of chronic liver disease. Common bile duct ligation (CBDL) and subcutaneous injections with CCl4 were induced in B cell-deficient and wild-type (WT) mice. IgG depletion was performed with beads covered with protein A/G and the depletions were evaluated by SDS-PAGE and Western blot analysis. N-glycan analysis was performed by improved DSA-FACE technology. Structural analysis of the mouse serum N-glycans was performed by exoglycosidase digests and MALDI-TOF mass spectrometry of permethylated glycans. The alterations seen in B cell-deficient mice closely resembled the alterations in WT mice, in both the CBDL and the CCl4 models. N-glycan analysis of the IgG fraction in both mouse models revealed different changes compared with humans. Overall, the impact of IgG glycosylation on total serum glycosylation was marginal. Interestingly, the amount of fibrosis present in CBDL B cell-deficient mice was significantly increased compared with CBDL WT mice, whereas the opposite was true for the CCl4 model as determined by Sirius red staining. However, this had no major effect on the alteration of N-glycosylation of serum proteins. Alterations of total serum N-glycome in mouse models of chronic liver disease are hepatocyte driven. Undergalactosylation of IgG is not present in mouse models of chronic liver disease.

B cells; glycomics; CCl4; CBDL; DSA-FACE

IN HUMANS, IMMUNOGLOBULIN G (IgG) is the most abundant glycoprotein in serum. IgG molecules consist of two Fab and one Fc fragment, which are linked through a flexible hinge region (9). N-glycosylation is present in the CH2 domain of the Fc fragment and ~30% of the circulating human IgG is also glycosylated in the Fab region. The majority of the Fab glycans are complex biantennary structures; multiantennary structures are very rare on IgG (13). This is in contrast to serum proteins synthesized by hepatocytes of which 5–15% of the glycans are multiantennary structures (unpublished data). The relative proportions of the other glycans on IgG are also completely different compared with glycans of liver-produced proteins. Especially agalactosylated glycans, that lack one or both galactoses in the glycan structure, are characteristic for IgG glycosylation and are rarely found on liver-produced proteins (29).

Alteration of N-glycosylation of serum proteins has been extensively studied by our group in various pathological states of the liver (GlycoFibroTest, GlycoCirrhoTest, and GlycoHCCTest; Table 1). In humans, these glycomic changes can be subdivided into alterations caused by B cells and alterations driven by the liver.

The GlycoFibroTest, that can be used as a follow-up tool for liver fibrosis patients, is mainly determined by IgG N-glycosylation. NGA2FB, an agalacto glycan, is present in the nominator. There is a linear increase of agalactosylated glycans (NGA2F and NGA2FB) on IgG in ascending Metavir stage (1), and we previously found that the correlation of NGA2FB with fibrosis was better than with NGA2F (29). This result indicates that there is also an increased N-acetylgalactosaminyltransferase III (GnT-III) activity in B cells during liver fibrosis cirrhosis and not just in regenerative liver nodules. This observation was confirmed by Klein et al. (11, 12).

NAG3 is present in the denominator of both the GlycoFibroTest and GlycoCirrhoTest. Biosynthesis of glycoconjugates necessitates the generation of activated monosaccharides. These activated monosaccharides take the form of nucleotide sugars (e.g., UDP-GlcNAc). The sugar nucleotides are generated starting from nonactivated monosaccharides that are either directly taken up from the extracellular environment or generated by the cell’s metabolism from other monosaccharides (6). In hepatocytes, the latter pathway is not present, and the N-acetylgalactosamine (GlcNAc) formed in these cells comes from lysosomal degradation of plasma glycoproteins that were taken up from the systemic circulation by surface receptors. This GlcNAc is then reused for the biosynthesis of the N-glycans of glycoproteins. As fibrosis progresses, collagen is deposited in the space of Disse and progressively less receptor-mediated uptake by the hepatocyte will be possible. Moreover, NA3 is formed by the GnT-V enzyme and it is known that the K_m values of GnT-V are much higher compared with those of other GlcNAc transferases (26). Therefore, if there is a limited supply of GlcNAc, other GlcNAc transferases will consume the introduced GlcNAc more readily and there will be less GlcNAc available for GnT-V to synthesize NA3. These two factors combined result in a lower NA3 production in ascending Metavir stage.

The GlycoCirrhoTest and GlycoHCCTest are diagnostic tests to respectively distinguish cirrhotic patients from noncirrhotic patients and patients with hepatocellular carcinoma.

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Table 1. N-glycome-derived biomarkers in various pathological states of the liver

<table>
<thead>
<tr>
<th>Marker Name</th>
<th>Marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>GlycoFibroTest*</td>
<td>[NGA2FB]log&lt;sup&gt;b&lt;/sup&gt; [NA3]&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GlycoCirrhoTest†</td>
<td>[NA2FB]log&lt;sup&gt;b&lt;/sup&gt; [NA3]&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>GlycoHCCTest‡</td>
<td>[NA3F]&lt;sup&gt;log&lt;/sup&gt; [NA2FB]&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The combination of these 3 markers is collectively called the GlycoHepatoTest. *Vanderschaeghe et al. 2009 (29); †Callewaert et al. 2004 (3); ‡Liu et al. 2007 (18).

(HCC) from cirrhotic patients (3, 18). These tests are determined by the competition of the GnT-III and the GnT-V enzyme for the same substrate (trimannose core). In cirrhosis, there is an upregulation of GnT-III in regenerative nodules and this enzyme is responsible for the formation of bisecting GlcNAc modified glycans. In this way, an increased abundance of these glycans on liver-produced proteins is observed in the serum of cirrhotic patients. However, it was recently shown that these bisecting glycans were particularly present on IgG and IgA in cirrhotic patients, indicating an also strong B cell involvement (11). In HCC, the opposite occurs. There is an upregulation of the GnT-V enzyme in tumor cells so that the balance is shifted toward more multiantennary glycans on liver-produced proteins. Next to GnT-V, α1–3 fucosyltransferase is also necessary to produce NA3FB, that is significantly increased in HCC patients. It has previously been shown that the branching fucose modification that is produced by this enzyme is strongly associated with HCC (27). As indicated earlier, B cell involvement in the GlycoHCCTest can also not be excluded because of the presence of NA2FB in the denominator. In summary, B cells are the dominant factor in the alteration of protein glycosylation in human liver patients.

N-glycosylation of serum proteins is somewhat species specific. On IgGs, the major difference with humans is that N-glycans on mouse IgGs terminate with N-glycolyneuraminic acid instead of N-acetyleneuraminic acid (24). On liver-produced proteins, the major difference with humans is the presence of α1–3 galactosylated glycoforms. The enzyme that produces these glycoforms has been lost in the evolution toward the human lineage (14). Furthermore, differences in expression of certain glycosyltransferases (for instance Mgat3) also exist between mice and humans. The glycosylation of mice IgG is barely described in literature, and the published papers do not indicate the presence of bisected N-acetylglucosamine at the difference of human IgG N-glycans (20, 21).

We previously did a glycomic research into the alterations that occurred in two mouse models of chronic liver disease, common bile duct ligation (CBDL) and chronic injections with CCl<sub>4</sub> (2). In summary, we saw an increased abundance of core-fucosylated glycans in CBLD mice and an increased abundance of glycans that are located at the end of the electropherogram in CCl<sub>4</sub> mice, most likely all multiantennary glycans. Our aim was to elucidate the role and magnitude that N-glycosylation of liver-produced proteins plays in these alterations by using B cell-deficient mice. In addition, IgGs were captured from the serum of wild-type (WT) CBLD and CCl<sub>4</sub> mice and the N-glycan fraction thereof was also determined.

**MATERIALS AND METHODS**

**Mouse Models of Chronic Liver Disease**

Male B10.129S2(B6)-Igh-6<sup>mu</sup>C<sup>gmu</sup>/1 mice (μMT-mice) were purchased from Jackson Laboratories (Bar Harbor, ME). B cell development is arrested in these mice at the stage of pre-B cell maturation by a deletion in the heavy chain region of IgM. As a consequence, these mice do not have mature B cells in their blood (10). This implies that isotype switching from IgM to IgG is not possible and no IgG is present in the serum of these mice. C57Bl/10 control mice were purchased from Harlan Laboratories (Horst, The Netherlands). B cell-deficient mice were housed in individually ventilated cages, whereas control mice were kept under normal housing conditions. Both mouse groups underwent a 12-h controlled light-dark cycle at a constant temperature and humidity. The Ethical Committee of experimental animals at the faculty of Medicine and Health Sciences, Ghent University, Belgium, approved the protocols.

CBDL, CCl<sub>4</sub>, and their respective controls were induced in B cell-deficient and control mice. The surgical procedure for CDBL was performed under sterile conditions in a laminar flow cabinet. Under isoflurane inhalation, a midline abdominal incision was made and the common bile duct was isolated. The common bile duct was clamped with a double ligature of a nonresorbable suture (silk cut 5-0). The first ligature was made below the junction of the hepatic ducts and the second was made above the entrance of the pancreatic duct. The common bile duct was sectioned between the two ligatures (15). Mice were euthanized 6 wk after CDBL (n = 8). In WT mice, this time point correlates with the cirrhotic stage. Sham-operated mice were used as control group and they were also euthanized after 6 wk (n = 10) (5).

The second mouse model was induced by chronic subcutaneous administration of CCl<sub>4</sub> (Merck, Darmstadt, Germany) twice weekly (1:1 dissolved in olive oil; 1 ml/kg) (7); 5% alcohol was added to drinking water. Mice were euthanized after 16 wk (n = 8). Again, this correlates with the cirrhotic stage in WT mice as validated in our laboratory (5). Control mice received a physiological saline solution (1 ml/kg) subcutaneously. They were euthanized after 16 wk (n = 10). No alcohol was added to the drinking water.

Body, liver, and spleen weight were determined in every mouse. Liver and spleen weight were expressed as percentage of body weight (relative liver and spleen weight). Blood samples were taken by placing a catheter in the arteria carotis. These samples were centrifuged at 10,000 rpm for 10 min, and 200 μl serum was taken off the clot for the determination of liver function tests [alanine aminotransferase (ALT), aspartate aminotransferase activity (AST), and total bilirubin (Tbiln)] in B cell-deficient and control mice. These were analyzed by routine photometric tests on a Hitachi 747 analyzer (Diagnostica, Boehringer Mannheim, Ingelheim, Germany). The remaining serum volume was used for N-glycan analysis in B cell-deficient and WT mice and for IgG depletion in WT mice. Finally, serum IgG concentration was determined in four samples of every B cell-deficient group and in five samples of every WT group (CBDL, CCl<sub>4</sub>, and their respective controls) by an ELISA (Immunology Consultants Laboratory, Newburg, OR). The ELISA was run according to manufacturer’s instructions and all analyses were done in duplo. A liver section of ~1 cm in diameter of the left, right, and middle lobes was taken for histological examination. Livers were fixed in 4% phosphate-buffered formaldehyde solution (Sigma, St. Louis, MO) and embedded in paraffin. From all tissue samples, 5-μm tissue sections were cut with a Leica RM 2145 sliding microtome (Leica Microsystems, Nussloch, Germany). The liver tissues sections were stained with 0.1% picrosirius red. The amount of fibrosis in the liver correlates with the amount of redness present on a liver tissue section. At an objective magnification of ×20, three regions were randomly
chosen per liver tissue section and the amount of redness was electronically quantified by using the imaging system Cell HD (Olympus, Hamburg, Germany). The mean value of these three regions was taken as a numeric description of the amount of fibrosis.

IgG Depletion

Serum samples of WT mice were depleted by using beads covered with protein A/G (Thermo Scientific, Waltham, MA): 100 μl serum was diluted with 100 μl binding buffer and subsequently incubated with 35 μl beads for 1 h. The mixture was transferred to a 96-well filter plate and centrifuged at 1,000 g for 15 s. The IgG depleted eluate was captured in a microtiter plate. Subsequently, after five wash steps with binding buffer, pure IgG was eluted from the beads with 0.1 M glycine pH 2 in the 96-well filter plate. After centrifugation at 1,000 g for 15 s, the eluate was neutralized with 1 M Tris pH 8.8. IgG-depleted serum and the IgG fraction were stored at −20°C for N-glycan analysis.

Proteomic Analysis: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Serum and IgG-depleted samples were diluted 1:20; the IgG fraction was processed undiluted. The samples were mixed with LDS sample buffer (NuPage; Invitrogen, Seattle, WA) and a sample reducing agent (dithiothreitol, Invitrogen). The proteins were denatured by heating at 70°C for 10 min, subsequently loaded into 10% Bis-Tris Gel (Invitrogen), and separated at a voltage of 170 V for 1 h. Finally, the gels were stained with Coomassie blue (Sigma-Aldrich, Bornem, Belgium) for 1 h and destained twice for 1 h and overnight.

For the Western blot analysis, the proteins were transferred to a nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) after separation in a 10% Bis-Tris Gel. Membranes were blocked in Tris-buffered saline containing 0.05% Tween and 5% nonfat milk. The membranes were incubated for 1 h with a secondary antibody (anti-IgG, dilution 1:10,000). Horseradish peroxidase detection was carried out with an enhanced chemiluminescence substrate (Roche Diagnostics, Indianapolis, IN).

Glycomic Analysis

The N-glycans present in 5 μl of serum were released from the proteins with peptide N-glycanase F (PNGase F). Subsequently, the glycans were fluorescently labeled and desialylated (Arthrobacter ureafaciens sialidase; Roche, Mannheim, Germany). DNA sequencer-assisted fluorophore-assisted capillary electrophoresis (DSA-FACE) technology was used to profile and analyze the labeled glycans. For an elaborate description of the protocol, we refer to Ref. 17. Eleven peaks were present in the electropherogram of every native and IgG-depleted mouse serum sample and nine peaks were present in the electropherogram of every mouse IgG sample. The peak height of every peak was quantified to obtain a numerical description of the profiles. These data were analyzed with SPSS 16.0 software (SPSS, Chicago, IL). The peak heights were normalized to the total intensity of the measured peaks (represented as a percentage of the total peak height).

Structural analysis of aminopyrene trisulfonic acid-labeled serum N-glycans was performed by digesting appropriate amounts with exoglycosidases: Streptococcus pneumoniae β-1,4-galactosidase, jack bean β-N-acetylgalactosaminidase, bovine kidney α-fucosidase (all from Prozyme, San Leandro, CA). DSA-FACE was used to analyze the digestion products.

Matrix-Assisted Laser Desorption Ionization Tandem Time-of-Flight Mass Spectrometry

Derivatization of samples. The released N-glycans were purified by chromatography on a Sep-Pak C18 cartridge (Waters, Milford, MA) to separate the released, hydrophobic N-glycans from the remaining hydrophobic compounds. The Sep-Pak columns were conditioned by eluting sequentially with 5 ml methanol, 5 ml dilute acetic acid, 5 ml propan-1-ol, and 15 ml dilute acetic acid. Dried samples were resuspended in a small volume of dilute acetic acid, loaded onto the column, and eluted stepwise with 5 ml of dilute acetic acid as previously described (8).

The purified N-glycans were subsequently methylated by using the sodium hydroxide permethylation procedure as described previously (8). Briefly, five to seven NaOH pellets were ground to fine powder and mixed with 2–3 ml anhydrous dimethylsulfoxide (Romil, Cambridge, UK) before being added to each dried sample. This was followed by the addition of 0.6 ml of methyl iodide and vigorous shaking at room temperature for 15 min. Permethylated glycans were extracted with chloroform and then purified by using Sep-Pak C18 cartridges. The cartridges were successively conditioned with methanol (5 ml), water (5 ml), acetonitrile (5 ml), and water (15 ml). Each sample was dissolved in 200 μl of methanol-water (1:1) solution before being loaded onto the cartridges. The cartridges were washed with 5 ml of water and then eluted sequentially with 3 ml of each 15, 35, 50, and 75% acetonitrile solution in water. The 35, 50, and 75% acetonitrile-water fractions were collected and then concentrated with a Savant SpeedVac and subsequently lyophilized.

MS and MS/MS analyses of permethylated glycans. Matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF) mass spectrometry (MS) data were acquired on a Voyager-DE STR mass spectrometer (Applied Biosystems, Foster City, CA) in the reflectron mode with delayed extraction. Permethylated samples were dissolved in 10 μl of matrix [20 mg/ml 2,5-dihydroxybenzoic acid in 70% (vol/vol) aqueous methanol], spotted onto a target plate, and dried under vacuum.

Further MS/MS analyses of peaks observed in the MS spectra were carried out by using a 4800 MALDI-TOF/TOF (Applied Biosystems) mass spectrometer in positive ion mode (M + Na)+.

The collision energy was set to 1 kV, and argon was used as collision gas. Samples were dissolved in 10 μl of methanol, and 1 μl was mixed at a 1:1 ratio (vol/vol) with 2,5-dihydroxybenzoic acid (20 mg/ml in 70% methanol in water) as matrix.

Analysis of MALDI data. The MS and MS/MS data were processed using Data Explorer 4.9 Software (Applied Biosystems). The mass spectra were baseline corrected (default settings) and noise filtered (with correction factor of 0.7) and then converted to ASCII format. The processed spectra were then subjected to manual assignment and annotation with the aid of a glycobioinformatics tool known as GlycoWorkBench (4).

Peak picking was done manually, and proposed assignments for the selected peaks were based on molecular mass composition of the 12C isotope together with knowledge of the biosynthetic pathways.

Statistical Analysis

Data analysis was performed with SPSS version 16.0 (SPPS). The data were statistically processed with the Mann-Whitney U-test unless stated otherwise. P values less than 0.05 (2-tailed probability) were considered as significant.

RESULTS

Clinical Data

Clinical data of the WT and B cell-deficient mice in both models of chronic liver diseases are summarized in Table 2.

Histological Analysis

CBDL B cell-deficient mice showed significantly more fibrosis compared with CBDL WT mice. The mean surface of fibrosis was quantified in CBDL B cell-deficient mice as 41,955.6 vs. 32,687.4 μm² in CBDL WT mice (P = 0.04, two-tailed t-test). Interestingly, the CCl₄ model showed the opposite result: there was significantly more fibrosis present in WT mice chronically injected with CCl₄ compared with B
Table 2. Clinical data and liver function tests of B cell-deficient and wild-type mice

<table>
<thead>
<tr>
<th></th>
<th>CBDL 6w μMT</th>
<th>Sham 6w μMT</th>
<th>CBDL 6w WT</th>
<th>Sham 6w WT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>19.6 ± 2.0c</td>
<td>28.9 ± 2.1</td>
<td>19.9 ± 2.3c</td>
<td>26.0 ± 1.1</td>
</tr>
<tr>
<td>Rel liver weight, %</td>
<td>10.3 ± 2.2c</td>
<td>4.6 ± 0.4</td>
<td>9.5 ± 2.8c</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Rel spleen weight, %</td>
<td>0.45 ± 0.4c</td>
<td>0.12 ± 0.02</td>
<td>0.46 ± 0.1</td>
<td>0.4 ± 0.08</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>500 ± 447c</td>
<td>43 ± 60</td>
<td>422 ± 201c</td>
<td>136 ± 52</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>264 ± 122c</td>
<td>38.7 ± 11.2</td>
<td>284.3 ± 141.3c</td>
<td>68 ± 44</td>
</tr>
<tr>
<td>TBiln, mg/dl</td>
<td>17.0 ± 6.6c</td>
<td>0.06 ± 0.02</td>
<td>22.9 ± 4.6c</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>CCl4 16w μMT</td>
<td>Saline 16w μMT</td>
<td>CCl4 16w WT</td>
<td>Saline 16w WT</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>28.9 ± 2.8</td>
<td>32.0 ± 4.2</td>
<td>30.3 ± 1.8a</td>
<td>28.0 ± 3.2</td>
</tr>
<tr>
<td>Rel liver weight, %</td>
<td>5.9 ± 0.7b</td>
<td>4.9 ± 0.4</td>
<td>5.6 ± 0.4b</td>
<td>5.0 ± 0.4</td>
</tr>
<tr>
<td>Rel spleen weight, %</td>
<td>0.12 ± 0.02</td>
<td>0.11 ± 0.09</td>
<td>0.37 ± 0.11</td>
<td>0.31 ± 0.07</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>73 ± 37b</td>
<td>70 ± 81</td>
<td>98 ± 26b</td>
<td>67 ± 29</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>61 ± 28b</td>
<td>34 ± 20</td>
<td>88 ± 34b</td>
<td>28 ± 10</td>
</tr>
<tr>
<td>TBiln, mg/dl</td>
<td>0.08 ± 0.02c</td>
<td>0.09 ± 0.03</td>
<td>0.19 ± 0.02</td>
<td>0.21 ± 0.05</td>
</tr>
</tbody>
</table>

Values are means ± SD; n = 8 subjects per group. CBDL, common bile duct ligation; CCl4, carbon tetrachloride; μMT, B cell deficient; WT, wild type; 6w, euthanized at 6 wk; 16w, euthanized at 16 wk; Rel, relative; AST, aspartate aminotransferase; ALT, alanine aminotransferase; TBiln, total bilirubin. *P < 0.05; **P < 0.01; ***P < 0.001 compared with control mice in the B-cell-deficient and WT models. dP < 0.001 compared with WT mice.

The optical density measured in all B cell-deficient mice, irrespective from induced liver damage, rarely exceeded background value. As expected, this indicates that no IgG was present in the serum of B cell-deficient mice.

In agreement with our previous study, IgG concentration in WT mice was low compared with human serum samples and ranged between 0.5 and 1.5 mg/ml (2). The IgG concentration was comparable in CBDL, CCl4, and Sham-operated WT mice [mean value of 0.75 ± 0.1 mg/ml]. The relative high IgG concentration in Sham-operated mice can be explained by the invasive character of this procedure and the fact that cholestatic liver damage in mice does not seem to result in further IgG elevation.

Proteomic Evaluation of the IgG Depletion and the IgG-Fraction

Each IgG molecule is a dimer and consists of four polypeptides, two identical heavy chains (molecular mass 53 kDa) and two identical light chains (molecular mass 22.5 kDa). The pure IgG fraction was clearly shown on one-dimensional PAGE, and comparison with native samples revealed that not all IgG was removed from the sample in the first depletion (Fig. 2). The definitive answer was given by Western blot
analysis, after three consecutive depletions, all samples were IgG free and very little pure IgG (a fraction of the light chain) could be recovered in the third depletion (Fig. 2).

Glycomic Analysis

Structural analysis of the mouse serum N-glycans. Exoglycosidase digests provided structural information on most of the peaks, but it could not unambiguously determine the precise glycan structure (see supplemental figures online; the online version of this article contains supplemental data). In contrast, MALDI-TOF analysis revealed the precise glycan structures of the most abundant peaks in the IgG and total serum electropherogram (Fig. 3), but since it is a less sensitive technique compared with capillary electrophoresis, not all peaks present in the electropherogram were detected in the MS spectra. Moreover, structural analysis with MALDI-TOF MS was performed without sialidase digest; therefore, multiple peaks in the total serum MS spectrum correspond to one peak in the total serum electropherogram.

By combining the exoglycosidase digests and MS data, the following peaks could be deduced in the IgG electropherogram: peak 1 is an agalacto, core-α-1,6-fucosylated biantennary (NGA2F), peaks 3 and 4 are single agalacto, core-α-1,6-fucosylated biantennaries (NG1A2F), and peak 7 is a bialacto, core-α-1,6-fucosylated biantennary glycan (NA2F). Similarly, the following peaks could be deduced in the total serum electropherogram: peak 1 is an agalacto, core-α-1,6-fucosylated biantennary (NGA2F), peak 5 is a bialacto, biantennary glycan (NA2), peak 7 is a bialacto, core-α-1,6-fucosylated biantennary glycan (NA2F), and peak 9 is triantennary glycan (NA3). From the latter structure (NA3), it could be deduced that peaks 10 and 11 are also two multiantennary glycans. The remaining peaks in the IgG and total serum electropherogram could not be definitely determined, particularly because the precise glycan structure was not assessed by MS analysis. This was mostly the case for the low abundant peaks in the electropherograms. Importantly, the fucosidase digest confirmed that peaks 1, 6, and 7 are core fucosylated glycans, and the MS
Fig. 3. Matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF) mass spectrometry (MS) of \( N \)-glycans released from mouse IgG and total serum by PNGase F digestion. Glycans were permethylated prior to MALDI-TOF analysis. Structures were assigned taking into account the molecular weight value and the fragment ions obtained upon MS/MS analysis. A: profile of total permethylated \( N \)-glycans released from a pool of 72 mouse IgG samples. Annotated structures correspond to core fucosylated complex-type biantennary glycans (m/z 1893, 2098, 2301), and core-fucosylated complex-type mono- and biantennary glycans with \( N \)-glycolylneuraminic acid (m/z 2185, 2489, 2693, 2839, 3026) and \( \alpha \)-linked galactose (m/z 2839) as capping sugar. B: profile of total permethylated \( N \)-glycans released from a pool of 60 mouse serum samples. Annotated structures correspond to high-mannose-type \( N \)-glycans (m/z 1636 and 1841), core fucosylated complex-type biantennary glycans (m/z 1893, 2098, and 2301), complex-type mono- and biantennary glycans with \( N \)-glycolylneuraminic acid as capping sugar (m/z 2315, 2519, 2910), and core fucosylated complex-type mono- and biantennary glycans with \( N \)-glycolylneuraminic acid as capping sugar (m/z 2489, 2693 and 3084). One triantennary \( N \)-glycan was also detected with 2 \( N \)-glycolylneuraminic acid residues at m/z value 3359.
analysis also confirmed the absence of bisecting GlcNAc-modified glycans in both IgG and total serum electropherogram.

**General aspects.** Peak 1 (NGA2F) is an IgG-specific but not exclusive glycan. In B cell-deficient mice, peak 1 is still present, but in very small quantities, whereas it is one of the most abundant glycans on IgG. In WT mice, peak 1 is one of the minor peaks in the electropherogram, reflecting the low IgG concentration in serum of mice. In contrast, peaks 8 and 11 were never present on IgG, indicating that these glycans only appear on liver-produced protein (Figs. 4–6). Glycomic analysis was done on the IgG fraction that was captured in the first depletion.

**WT and B cell-deficient mice.** CBDL 6 wk vs. Sham 6 wk. First, we looked at significant N-glycan alterations in total serum by comparing WT CBDL mice with WT sham-operated mice. A significant increase in peak height of peaks 1 (NGA2F), 6, 7 (NA2F), and 11 and a significant decrease in peak height of peaks 2, 5 (NA2), and 9 (NA3) were observed.

Subsequently, we compared the peak heights of CBDL B cell-deficient mice with the peak heights of Sham-operated B cell-deficient mice. In this analysis, specifically the N-glycan alterations on liver-produced protein are observed and the pattern of alterations closely reflected those of total serum. A significant increase in peak height of peaks 1 (NGA2F), 6, 7 (NA2F), and 11 and a significant decrease in peak height of peaks 2, 3, 4, 5 (NA2), and 9 (NA3) were observed (Fig. 4).

Despite the close resemblance of the glycomic profile between WT and B cell-deficient mice, two peaks (peaks 2 and 3) were supplementarily decreased in the B cell-deficient CBDL model. We related the relative peak height of these peaks with the Sirius stain values to determine whether the difference in fibrotic development between the WT and B cell-deficient mouse is responsible for the additional alterations. However, no correlation could be found between the Sirius red stain values and the relative peak heights (Spearman’s rank correlation test, \( P = 0.76 \) and 0.42, respectively).

The same analysis was performed for all other peaks in the electropherogram, in both the WT and B cell-deficient mice. Only in the CCl4 WT model we found significant correlations between Sirius red stain values and the relative peak heights (see following section).

**CCl4 16 wk vs. Saline 16 wk.** In total serum, we observed a significant increase in peak height of peaks 9 (NA3) and 11 and a significant decrease in peak height of peaks 2, 3, 4, 6, and 10. In analogy of CBDL, the pattern of alterations on liver-produced protein closely resembled those of total serum. When comparing CCl4 injected B cell-deficient mice with saline injected B cell-deficient mice, we observed a significant increased peak height of peaks 8, 9, and 11 and a significant decreased peak height of peaks 1 (NGA2F), 2, 3, 4, 6, and 10 (Fig. 5).

Similar to the CBDL model, two peaks were supplementarily significantly altered in the B cell CCl4 model. Peak 1 (NGA2F) was additionally significantly decreased and peak 8 was additionally significantly increased. We also examined whether these extra alterations were due to the significant less fibrotic development in the B cell-deficient CCl4 model. Again, no correlation could be found between the Sirius red stain values and peak 1 (NGA2F) and peak 8 (Spearman’s rank correlation, \( P = 0.73 \) and \( P = 0.57 \), respectively).

Surprisingly, many relative peak heights in the CCl4 WT model correlated very well with the Sirius red stain values. There were significant correlations with peak 3 (\( P = 0.007 \)), peak 4 (\( P = 0.021 \)), peak 5 (\( P = 0.007 \)), peak 7 (\( P = 0.021 \)), peak 8 (\( P = 0.004 \)), peak 9 (\( P = 0.028 \)), and peak 11 (\( P = 0.028 \)) (Spearman’s rank correlation). For the most part, these are also the peaks that were significantly altered in peak height in the CCl4 model.

**Immunoglobulin G.** CBDL 6 wk vs. Sham 6 wk. We compared the height of the nine peaks in the IgG electropherogram of CBDL mice (\( n = 6 \)) with those of Sham mice (\( n = 8 \)). Only one peak was significantly altered. Peak 2 was significantly increased in the IgG electropherogram of CBDL mice (\( P = 0.029 \)) (Fig. 6).

CCl4 16 wk vs. Saline 16 wk. The same analysis was performed on the IgG electropherograms of CCl4 (\( n = 8 \) and
There were more significant alterations on IgGs of mice chronically injected with CCl4. Peaks 1 and 2 are significantly decreased in peak height and peak 3 is significantly increased in peak height. The decrease of peak 1 and increase of peak 3 are not found in the total serum electropherogram, reflecting the low IgG concentration in mice (0.5–1.5 mg/dl) compared with humans (8–20 mg/dl) (2).

In general, IgG N-glycosylation has a more significant impact in humans than in mice. Not only is there a higher IgG concentration, but the number and magnitude of alterations on saline (n = 8) injected mice. Three peaks were significantly altered in height. Peak 1 (NGA2F) and peak 2 were significantly decreased on the IgGs of CCl4 mice ($P = 0.036$ and $P = 0.027$). In contrast, peak 3 (NG1A2F) was significantly increased on the IgGs of CCl4 mice ($P = 0.036$) (Fig. 6).

**DISCUSSION**

N-glycan alterations observed in total serum can reflect a changed B cell and/or a changed hepatocyte physiology. In mouse models of chronic liver disease, the dominant factor is clearly the change in hepatocyte N-glycan homeostasis. The pattern of N-glycan alterations in total serum closely resemble the alterations seen on liver-produced protein, in both the CCl4 and the CBDL model.

Significant N-glycan alterations on IgG were scarce. On IgGs of CBDL mice, only peak 2 was significantly increased. In total serum, peak 2 is not significantly altered; there could be a balanced effect between a decrease on liver-produced protein and an increase on IgGs.
IgG during liver fibrosis is more elaborate and this clearly has an important influence on the total serum N-glycome of cirrhotic patients (11). Interestingly, undergalactosylation of IgG, an important feature on the IgGs of human liver patients, was not observed in these mouse models of chronic liver disease. This does not necessarily mean that IgG undergalactosylation does not occur in mice. It is well known that undergalactosylation of IgG is also present in rheumatoid arthritis patients (16) and, in contrast to our study, this undergalactosylation could be extrapolated to a mouse model of rheumatoid arthritis (28).

It must be noted that there are important variations in terminal galactosylation of IgGs between mice and humans: 70% of human IgG oligosaccharides are galactosylated, whereas only ~45% of mouse IgG oligosaccharides contain galactose residues. This was confirmed by the MS analysis of the pooled IgG fraction (24). Moreover, the change in agalacto glycans on IgG is functionally important. In the context of undergalactosylation, complement-dependent cytotoxicity (CDC) is especially affected: less terminal galactose on IgG results in reduced CDC activity. However, undergalactosylation also results in the exposure of terminal GlcNAc residues that have been shown to bind to the serum protein mannose binding protein and activate the alternative complement cascade (25). To what extent this has an impact on the IgG functionality of CCl4 and CBDL mice remains to be elucidated.

An interesting observation was the degree of fibrosis present in the mouse models of chronic liver disease induced in B cell-deficient mice. In the CCl4 model, there was significantly less fibrotic development in B cell-deficient mice, whereas the opposite situation was present in the CBDL model. In the CCl4 model, B cells seem to have an impact on fibrosis in an antibody- and T cell-independent manner (23). The main hypothesis is that macrophages that contribute to recovery from inflammatory scarring are preferentially activated in the absence of B cells. Moreover, soluble factors produced by stimulated B cells can induce collagen synthesis by hepatic stellate cells.

In contrast, in a model of primary biliary cirrhosis, B cell-deficient mice developed a more severe form of cholangitis than controls and had a significant greater frequency of activated CD4+ and CD8+ T cells in the liver (22). They also had reduced frequency of Foxp3+ regulatory T cells in the hepatic CD4+ T cell population and natural killer T cells in hepatic inflammatory cell infiltrates. In this model, B cells have a suppressive effect on the inflammatory response.

These results indicate that the discrepancy in fibrotic development between the two models can mainly be explained by the interaction between B and T cells. In the CCl4 model, B cells have an impact on fibrosis independently from T cells, whereas in the CBDL model the T cell population is significantly altered in the absence of B cells. A detailed clarification of this difference in fibrotic development between the two models falls beyond the scope of this study.

In the B cell-deficient CBDL model, peak 2 and 3 are supplementarily significantly decreased compared with WT mice, and in the CCl4 model peak 1 and peak 8 were also additionally significantly altered in B cell-deficient mice. However, no correlation between the Sirius red stain values and the peak heights of these peaks could be observed, indicating that the difference in fibrotic development between WT and immunocompromised mice is not responsible for the extra glycemic alterations. In a previous study (2), we established that the majority of N-glycan alterations in the CCl4 and CBDL model were present at an early stage in the fibrotic development. Since we looked at the end stage of fibrotic development in both models, the impact of these histological differences on N-glycan alterations are minimal.

The serum N-glycosylation pattern of a WT serum sample that was depleted three times was identical to that of a B cell-deficient serum sample. This was an extra confirmation that the B cell-deficient mice did not contain IgGs. By combining the data from the B cell-deficient mice with the fucosidase digest, it was confirmed that the increase of α1–6 fucosylated glycans (peaks 1, 6, and 7) in CBDL mice is hepatocyte and not B cell dependent. This is important because this feature could be extrapolated to human liver patients with hyperbilirubinemia (2). The most likely hypothesis is that fucosylation of N-linked glycans within polarized hepatocytes directs glycoproteins to the apical surface and into bile (19). In cholestatic conditions, the hepatocyte becomes depolarized and the fucosylated glycoforms end up in the systemic circulation instead of bile. Moreover, the increase of multiantennary glycans (peaks 9 and 11) in CCl4 was also confirmed as determined in our previous study (2). Although another multiantennary glycan (peak 10) was significantly decreased in CCl4 mice, this glycan is fucosylated (most likely branch fucosylated), as indicated by the fucosidase digest, and this will influence its behavior in chronic liver disease. Finally, in agreement with previous reports (30), we could not identify any bisecting GlcNAc-modified glycans in total serum indicating no expression of the Mga13 gene in normal liver and B cell. This supports the evidence of a nonhepatic action of NgnT-III in promoting hepatocyte proliferation after partial hepatectomy as well as the progression of diethylnitrosoamine-induced tumors (30).

In conclusion, alterations of serum protein N-glycosylation in mice are mainly hepatocyte driven. Alterations of N-glycans on IgG are less abundant and do not reflect an undergalactosylation status. This in combination with the low IgG concentration in mice minimizes the impact of IgG N-glycosylation in mouse models of chronic liver disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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