Intravenous immunoglobulin G (IVIg): Properties of dimeric and sialylated IgG
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Chapter 5

Development of a fluid phase assay to measure sialylated IgG in intravenous immunoglobulin G (IVIg) products

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Abstract

Immune modulating therapy of acute and chronic autoimmune diseases has become a second major clinical indication for IVIg therapy in the past three decades. For this purpose, high doses (1.0 - 2.0 g/kg) of IVIg are required. One possible explanation for the dose requirement is that only a fraction of IVIg is causing the immune modulating effect. A recent study reported that the sialylated IgG fraction might be this immune modulating fraction in IVIg. To measure the degree of IgG sialylation in IVIg under native conditions we developed a fluid phase assay. Using this lectin-based inhibition ELISA mainly Fab sialylation was measured since the sialic acid residues attached to Fc were only accessible to the lectin under non-native conditions, i.e. protK digestion. We found a similar degree of total IgG sialylation in the four commercially available IVIg products. The ELISA results show that the IVIg products are comparable with respect to the degree of total IgG sialylation, so there is no enrichment or depletion of sialylated IgG in the IVIg products due to the IVIg manufacturing process. In addition, using mass spectrometric analysis we demonstrated that the total degree of Fc sialylation in the studied IVIg products was similar.
Introduction

Intravenous immunoglobulin (IVIg) is widely used for treatment of autoimmune and inflammatory diseases. Over the past years an increase in the off label use of IVIg is observed. For this purpose, often a high IVIg dosage, i.e. 1.0 - 2.0 g/kg, is administered. An increased usage of high dose IVIg might cause IVIg shortage in the future. If only a fraction of IVIg is causing the desired effects for a given indication, and the remaining material would be more suited for different indications, identification of this fraction of IVIg would possibly allow the development of a more efficient use of IVIg.

The sialylated IgG fraction of IVIg is a possible candidate. IgG molecules are large glycoproteins containing N-linked glycosylation sites in the Fab and the Fc region. From total amount of attached IgG glycans respectively 16 and 3% are monosialylated or disialylated. The degree of Fab and Fc sialylation is not equal. The Fab glycosylation sites are not conserved and depend on the presence of glycosylation motifs in the hypervariable region amino acid sequence. Most of the Fab glycosylation sites are located on the exposed loop, meaning that they contain fully processed glycan moieties with a high degree of sialylation, i.e. monosialylation (32%) as well as disialylation (14%) 7,9,10. Besides Fab glycosylation, each IgG contains two conserved N-linked glycosylation sites in the Fc region (Asn297). Each of the two N-linked glycans may differ in the degree of glycosylation resulting in a wide diversity of Fc glycosylation 7,10. From the Fc glycans approximately 15% contain sialic acid residues, either in a monosialylated form (~ 14%) or disialylated form (~ 1%) 7.

It is known that the degree of Fab and Fc sialylation influences the biological function of IgG. Fab glycans can significantly change the IgG antigen binding. Attached glycans can either increase the affinity for the antigen or can completely abrogate the antigen binding due to steric blocking of the interaction 7. In contrast, Fc glycans are required to maintain the quaternary structure and thermodynamic stability of the Fc fragment 11. Besides that recent studies have shown that the sialylated IgG fraction, i.e. in particular the Fc sialylated IgG fraction, in IVIg is responsible for the biological effects of IVIg 12-15. Kaneko et al. and Schwab et al. showed that IVIg enriched for sialylated IgG was more protective compared to IVIg in a murine model of arthritis and thrombocytopenia 12,15. In contrast, Leontyev et al. demonstrated that IVIg enriched for sialylated IgG had no enhanced efficacy using a comparable murine model of thrombocytopenia 14. In their model the protective effect of IVIg was independent from either Fc- or Fab-sialylation. In line with the results of Leontyev et al. we found a loss of a protective effect of a bolus injection of IVIg enriched for sialylated IgG in a murine thrombocytopenia model 13.

The found differences in biological activity of the IVIg enriched for sialylated IgG might be caused by the use of different IVIg products as starting material to enrich IVIg
for sialylated IgG. Therefore, the aim of this study was to determine the degree of IgG sialylation in IVIg under native conditions. For this purpose, we developed a lectin-based ELISA to compare the degree of IgG sialylation in four commercially available IVIg products of that three were used in aforementioned in vivo studies.

Materials and methods

Immunoglobulin products

Four IVIg products were studied: Nanogam® (Product A), a 5% IgG solution (Sanquin, Amsterdam, The Netherlands), Multigam® (Product B), a 5% IgG solution (CAF-DCF, Brussels, Belgium), Gamunex® (Product C), a 10% IgG solution (Talecris, Frankfurt am Main, Germany) and Octagam® (Product D), a 5% IgG solution (Octapharma AG, Lachen, Switzerland). Three of the four IVIg products (Product A, C and D) were used in vivo studies as described earlier. An overview of the biological effects of the IVIg products after enrichment for sialylated IgG (IVIg-SA (+)) is summarized in Table 1.

Table 1: Overview of the IVIg products used in earlier described in vivo studies to study the effect sialylated IgG in IVIg.

<table>
<thead>
<tr>
<th>Product</th>
<th>Study</th>
<th>Murine model</th>
<th>Biological effects of IVIg-SA (+) compared to IVIg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Guhr et al. 13</td>
<td>ITP</td>
<td>Loss of protective effect</td>
</tr>
<tr>
<td>C</td>
<td>Leontyev et al. 14</td>
<td>ITP</td>
<td>Similar protective effect</td>
</tr>
<tr>
<td>D</td>
<td>Kaneko et al. 12</td>
<td>Arthritis</td>
<td>Enhanced protective effect</td>
</tr>
</tbody>
</table>

Reagents

Biotinylated Sambucus nigra agglutinin (SNA) lectin which recognizes α-2,6 linked sialic acid residues with a binding capacity of 1.5 mg fetuin/ml gel and biotinylated Ricinus communis agglutinin (RCA) lectin which recognize galactose residues were obtained from Vector Laboratories (Burlingame, CA, USA).

Papain digestion of IVIg

To determine the site of sialylation, Fab and Fc fragments were prepared by treating IVIg with DTT pre-activated papain (Sigma-Aldrich, St. Louis, MO, USA) at an enzyme : antibody ratio of 1:400 in phosphate buffered saline (PBS) pH 7.4 with 3 mM EDTA at 37 °C for 3 hours. The reaction was stopped by adding fresh iodoacetamide (Sigma-Aldrich, St. Louis, MO, USA) to a final concentration of 5 mM. The digested material was separated from the undigested material by size exclusion chromatography using a HiLoad 16/600 Superdex200 column (60 cm, 124 ml, GE Healthcare, Uppsala, Sweden) connected to a HPLC system (GE Healthcare, Uppsala, Sweden). The Fab fragments
were separated from the Fc fragments using a MonoQ 5/50 GL column (GE Healthcare, Uppsala, Sweden). After the addition of sodium azide (NaN<sub>3</sub>) to a final concentration of 0.05% the fragments were stored at 4 °C.

**Proteinase K digestion of IVIg**

To determine the degree of total sialylation (Fab- and Fc-linked glycans containing sialic acid residues as well as accessible and hidden sialic acid residues), IVIg (Product A), Fab and Fc fragments derived from Product A were digested with protK (proteinase K, Sigma-Aldrich, St. Louis, MO, USA). The IgG solutions were dialyzed for 16 - 20 hours against PBS at 4 °C, followed by incubation with protK (ratio 0.6 U/mg protein, overnight, at 50 °C). The protK was inactivated by incubating the digest at 80 °C for 3 minutes according to the manufacturer’s protocol. After the addition of sodium azide (NaN<sub>3</sub>) to a final concentration of 0.05% the digested IVIg product was stored at 4 °C.

**Determination of IgG sialylation**

**Western blotting**

To visualize the presence of lectin-binding glycans on IgG fragments, 2.5 µg of the non reduced IVIg products, Fab and Fc fragments were applied to a Bis-Tris SDS-PAGE 4 - 12% gradient gel. After running the gel, proteins were transferred to a nitrocellulose membrane according to the manufacturer’s instructions (NuPAGE system, Invitrogen, Carlsbad, CA, USA). Non-specific sites were blocked by incubating the membrane for one hour at room temperature (RT) in Western Blocking Reagent (Roche, Basel, Switzerland). The blot was probed with biotinylated SNA or RCA (2.5 µg/ml) for one hour at RT. After subsequent washing streptavidine-horseradish peroxidase conjugate was added (strep-HRP, Amersham, Uppsala, Sweden) and incubated the blot for one hour at RT. The bound peroxidase was detected by using the Supersignal Substrate system (Pierce, Rockford, IL, USA) as recommended by the manufacturer.

**Lectin ELISA**

To measure the degree of IgG α-2,6-sialylation in IVIg we used an enzyme-linked immunosorbet assay (ELISA). In an initial experiment Maackia Amurensis (MAA) binding (specific for α-2,3-sialylation) was only detectable under non – native conditions (data not shown). So, based on this result and the literature findings describing that IVIg contains mainly α-2,6 linked sialic acid residues no further experiments with MAA were performed in this study.

First, the optimal concentration of the lectin was established by testing a serial dilution of biotinylated SNA in phosphate buffered saline (PBS) containing 0.1% Tween20 was added to a microtiter plate (Maxisorp; Nunc, Roskilde, Denmark) coated with 5 µg/ml
IVIg. After one hour incubation at RT, the plates were washed and streptavidin-HRP (Amersham, Uppsal, Sweden) diluted 1:4000 in PBS/0.1% Tween20 was added. After one hour incubation at RT the plates were washed and 100 µl/well TMB substrate buffer (Interchim, Montluçon Cedex, France), diluted with an equal volume of distilled water was added. The reaction was stopped with 2 M H₂SO₄ after 5 minutes and the absorbance was measured at 450/540 nm with a Titertek multiscan. Next, the optimal concentration of biotinylated SNA (0.5 µg/ml) was added to a microtiter plate (Maxisorp; Nunc, Roskilde, Denmark) coated with a serial dilution of IVIg and processed as described.

To determine the specificity of the SNA binding the IVIg coat was oxidized by adding 10 mM sodium periodate in 50 mM citrate buffer pH 4 for 3 minutes prior to the incubation with biotinylated SNA. Furthermore, we treated IVIg with neuraminidase (New England Biolabs, Ipswich, MA, USA). After dialysis for 16 - 20 hours at 4 °C against 50 mM sodium citrate buffer pH 6.0, 700 Units neuraminidase were added to 100 mg IVIg and the mixture was incubated for 6 hours at 37 °C. The neuraminidase reaction was stopped by dialyzing the mixture for 16 - 20 hours against PBS.

**Lectin inhibition ELISA**

To measure the sialylated IgG content in IVIg, an enzyme-linked immunosorbent inhibition assay (ELISA) was used. Biotinylated SNA diluted to 0.5 µg/ml in phosphate buffered saline (PBS) containing 0.1% Tween20 was pre-incubated with the inhibitors (IVIg products or IVIg-derived fragments) for one hour at RT. After adding the mix to an IVIg coated microtiter plate (5 µg/ml) (Maxisorp; Nunc, Roskilde, Denmark), the plate was incubated for one hour at RT, which was processed as described above.

Relative potencies in the SNA inhibition ELISA were calculated by parallel line analysis 17. A value above 1 for a fraction indicates that this fraction is more potent, thus contain more (or more accessible) sialylated glycans per IgG molecule.

**Mass spectrometric analysis of IgG Fc glycosylation**

By mass spectrometry, we specifically measured Fc sialylation without any interference from Fab glycosylation 18,19. IVIg samples were subjected to trypsin treatment, and resulting glycopeptides were analyzed by nano-LC-quadrupole-time-of-flight-ion trap-MS as described elsewhere 19. Glycopeptides were assigned in terms of peptide and oligosaccharide composition as described before 20. IgG2 and 3 Fc glycoforms were not distinguished as they have identical tryptic N-glycosylated peptide moieties 19. Relative intensities of the glycopeptide species (Table 1) derived from IgG1 (20 glycoforms), IgG4 (10 glycoforms), and IgG2/3 (20 glycoforms) were obtained by integrating and summing three isotopic peaks of the triple protonated as well as the double protonated species followed by normalization to the total IgG subclass specific glycopeptide intensities. On
the basis of the normalized intensities the levels of galactosylation, sialylation, bisecting N-acetylglucosamine, and core fucosylation were calculated as described previously. The non-fucosylated species of IgG4 remained below the limit of detection and were, therefore, not included in the IgG4 calculations.

### Results

**Western blotting**

We determined the presence of sialylated IgG in four commercially available IVIg products by Western blotting using biotinylated SNA and RCA (Figure 1). A similar binding of the biotinylated SNA to IgG was found. In addition, RCA binding was visible indicating a similar degree of IgG sialylation since RCA only binds to galactose residues in the absence of sialic acid residues. No differences between the IVIg products were detectable.

![Figure 1: Binding of biotinylated SNA and RCA to monomeric IgG (detection of sialylated and galactosylated IgG in the studied IVIg products (Product A-D)).](image)

To determine the binding site of the lectins, we measured the SNA and RCA binding to Fab and Fc fragments (Figure 2). Both biotinylated SNA and RCA bound to Fab as well as to Fc fragments derived from Product A and B. There was no difference in SNA and RCA binding between the two IVIg products. However, there was one difference: an additional 75 kD protein band was visible in Product B. In Product B 4% IgG4 is present whereas Product A contains almost no IgG4 (< 1%). From the literature it is known that IgG4 molecules can be present as non-covalent dimers of half-molecules. These half-molecules are visible on SDS as a 75 kD protein band, so most likely sialylated IgG4 half-molecules were detected using this assay. In addition to the aforementioned experiments performed under non-reducing SDS-PAGE, we also analyzed blots obtained by SDS-
PAGE under reducing conditions. The SNA binding was comparable to the non-reduced conditions and no differences were found between the two IVIg products or their Fab/Fc fragments (data not shown).

<table>
<thead>
<tr>
<th>SNA</th>
<th>RCA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Product A</strong></td>
<td><strong>Product B</strong></td>
</tr>
<tr>
<td>IVIg Fab Fe IVIg Fab Fe</td>
<td>IVIg Fab Fe IVIg Fab Fe</td>
</tr>
</tbody>
</table>

![Figure 2: Determination of Fab- and Fc-sialylation by using SNA and RCA.](image)

SNA and RCA binding to Product A, B and their corresponding Fab and Fc fragments.

**Lectin ELISA**

To determine the degree of IgG sialylation in IVIg, we developed a direct lectin ELISA using Product A and biotinylated SNA (Figure 3). We found SNA binding to the IVIg coat. Since not all sialic residues might be available for lectin binding, we pre-treated IVIg with proteinase K (protK). The SNA binding to IVIg/protK was somewhat lower compared to IVIg that is in contrast to our earlier experiments using an inhibition ELISA (data not shown). This suggests that the glycopeptides resulting from the protK digestion does not bind to the ELISA plate as well as intact IgG.
Next, we measured the SNA binding using serial dilutions of two IVIg products (Product A and B) as coat. We observed no difference in SNA binding to the IVIg coats (Figure 4A). To show the specificity of SNA for the IgG coat the IVIg coat was oxidized before SNA incubation. This oxidation resulted in a complete abrogation of the SNA binding (Figure 4B) demonstrating the glycan-specificity of the SNA binding. The specificity of the SNA binding to sialic acid residues was confirmed by an abrogation of the SNA binding to IVIg after neuraminidase treatment of IVIg that removes all the sialic acid residues (Figure 4C). In addition, IVIg buffer had no influence on the SNA binding, i.e. similar SNA binding patterns were observed after buffer exchange to PBS or 50 mM sodium citrate buffer pH 6.0 (data not shown).
Figure 4: SNA lectin binding to IVIg under various conditions (IVIg coat concentration 5 µg/ml). A) Comparison of SNA binding to a serial dilution of Product A (◊) and Product B (♦); B) Influence of oxidation: SNA binding to Product A (♦), Product B (■), oxidized Product A (◊) and oxidized product B (○) and C) treatment of neuraminidase: SNA lectin binding to Product A (♦) and Product A treated with neuraminidase (◊).
Lectin inhibition ELISA
To compare the degree of IgG sialylation in four IVIg products, we measured the SNA binding to IgG after pre-incubation with the IVIg. All IVIg products inhibited the binding of biotinylated SNA to the IgG coat in a comparable concentration dependent manner (Figure 5A). Product C was approximately 1.6 fold more potent to inhibit SNA binding to IgG than Product A, whereas the inhibition by Product B and D was similar to Product A (Table 2).

Table 2: Potencies (depicted as mean with corresponding 95% confidence intervals) of the four IVIg products (A – D) to inhibit SNA binding to IgG.

<table>
<thead>
<tr>
<th>Product</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1.3 (1.0 - 1.6)</td>
<td>1.6 (1.4 - 1.8)</td>
<td>1.0 (0.9 - 1.2)</td>
</tr>
<tr>
<td>B</td>
<td>1.2 (0.9 - 1.6)</td>
<td>1.1 (0.6 - 2.1)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>1.4 (1.1 - 1.8)</td>
<td></td>
</tr>
</tbody>
</table>

To measure Fab and Fc sialylation under native conditions, we measured the SNA binding to IgG after pre-incubation with Fc and Fab fragments and their protK digests derived from Product A. No inhibition of the SNA binding to IgG was found using Fc fragments whereas a concentration dependent inhibition was found for Fab fragments. After protK digestion, Fc fragments were able to inhibit the SNA binding to IgG (Figure 5B). Unexpectedly, the reactivity of the Fab fragments decreased upon proteolytic digestion.
Figure 5: Measurement of the degree of IgG sialylation using SNA inhibition ELISA.
A) SNA binding to IgG after pre-incubation with a serial dilution of the four IVIg products: Product D (♦), Product A (Δ), Product B (□) and Product C (●); B) SNA binding to IgG after pre-incubation with a serial dilution of Fc (Δ), Fab (◊), Fc/protK (▲) and Fab/protK (●) derived from Product A. Data represents mean ± SEM, n = 2.
Mass spectrometric (MS) analysis
To specifically measure Fc glycosylation without any interference from Fab glycosylation we used MS analysis. Fc sialylation, galactosylation, fucosylation and bisecting GlcNAc levels of all IgG subclasses, i.e., IgG1, IgG2/3 and IgG4, were similar in the IVIg products (A, C and D) and IgG subclasses, though some minor differences were observed (Table 3). For Product B this analysis was not performed.

Table 3: Comparison of the Fc glycosylation features of three IVIg products.
Each IgG Fc glycopeptides sample was analyzed five times by mass spectrometry, and mean values ± 95% confidence intervals are given after normalization to the sum of total glycoforms per subclass. Percentage (%) values are given throughout. n.d. not determined

<table>
<thead>
<tr>
<th>Product</th>
<th>Galactosylation</th>
<th>Sialylation</th>
<th>Bisecting GlcNAc</th>
<th>Fucosylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>55</td>
<td>16</td>
<td>21</td>
<td>91</td>
</tr>
<tr>
<td>C</td>
<td>55</td>
<td>18</td>
<td>18</td>
<td>91</td>
</tr>
<tr>
<td>D</td>
<td>58</td>
<td>19</td>
<td>19</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>IgG2/3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>46</td>
<td>16</td>
<td>16</td>
<td>95</td>
</tr>
<tr>
<td>C</td>
<td>47</td>
<td>18</td>
<td>14</td>
<td>94</td>
</tr>
<tr>
<td>D</td>
<td>49</td>
<td>18</td>
<td>16</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>IgG4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>48</td>
<td>18</td>
<td>24</td>
<td>n.d.</td>
</tr>
<tr>
<td>C</td>
<td>52</td>
<td>22</td>
<td>14</td>
<td>n.d.</td>
</tr>
<tr>
<td>D</td>
<td>52</td>
<td>21</td>
<td>18</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Discussion
The aim of this study was to develop a fluid phase assay to measure the degree of IgG sialylation under native conditions. In initial experiments using Western blotting we found SNA binding to IgG, Fab and Fc fragments showing the presence of α-2,6 sialylated IgG molecules in IVIg. Further analysis using a direct lectin ELISA assay confirmed the presence of α-2,6 sialylated IgG molecules in IVIg. In addition, using this ELISA assay no MAA binding (specific for α-2,3 sialylated IgG) was detectable confirming literature findings describing that IVIg contains mainly α-2,6 linked sialic acid residues 16. Therefore, no further experiments with MAA were performed in this study.

To avoid artifacts induced by blotting or by solid phase coupling in general, we developed an inhibition ELISA assay to measure the binding of the SNA to sialic acid residues in fluid phase (native conditions). Using this assay, we observed a concentration dependent inhibition pattern of SNA binding to IgG. This inhibition pattern was comparable for the 3/4 IVIg products. The amount of sialylated IgG was similar in Product...
A, B and D whereas Product C was slightly more potent to inhibit the SNA binding to IgG suggesting a higher degree of total IgG sialylation compared to the other IVIg products (A, B and D). To determine the binding site of SNA to IgG, we used Product A derived Fab- and Fc fragments as well as their protK digests to inhibit the SNA binding to IgG. We found an inhibition of the SNA binding by Fab fragments whereas Fc fragments inhibited SNA binding only after protK digestion suggesting that Fc linked sialic acid residues are not available for SNA binding under native conditions. In contrast protK digested Fab fragments showed an unexpected lower capacity to inhibit SNA binding. A decreased binding affinity of the SNA to the release sialic acid residues might be the reason. Another possible explanation is a loss of protein due to precipitation caused by the chosen protK inactivation protocol (protK inactivation at – 80 °C). However, further research is needed to explain these results. Using MS analysis that measures the total amount of Fc sialylation, i.e. accessible and hidden sialic acid residues attached to Fc, we found a similar degree of Fc sialylation in the IVIg products (A, C and D) with only slightly lower Fc sialylation levels for Product A. Since the degree of Fc sialylation in the IVIg products is similar it is unlikely that the contradictory results obtained with IVIg-SA (+) in the different model systems is caused by differences in Fc sialylation in the starting material of the SNA affinity chromatography, i.e. IVIg 12-14. However, further research is needed to determine the degree Fc sialylation in the different IVIg products that is accessible for SNA binding under native conditions using the lectin-based inhibition ELISA assay.

It is known that α-2,6 linked sialic acid residues on N-linked glycans can bind to Siglec-2 (CD22) which is an inhibitory receptor exclusively expressed on B cells 23-25. Recent studies demonstrated that the binding of sialylated IgG to Siglec-2 caused a change of the B cell activation threshold and thereby in the regulation of antibody production 24,26. In autoantibody mediated immune diseases this might be the desirable effect of IVIg enriched for sialylated IgG. Based on the lectin-based inhibition ELISA results the studied IVIg products would expected to behave similarly in vivo after administration, i.e. show a similar binding pattern to receptors such as sialic-acid-binding immunoglobulin like lectins (Siglecs). The binding would presumably be Fab mediated since the sialic acid residues attached to the Fc are not accessible assuming a similar steric constrains as found with SNA. However, further research is required to measure the binding capacity of sialylated IgG to Siglecs to confirm this hypothesis.

In summary, we have developed a fluid phase assay that measures the degree of IgG sialylation in native therapeutic antibody products such as IVIg. Our results show that the commercially available IVIg products tested in this study have a similar degree of total IgG sialylation. Fc sialylation was only measurable under non-native conditions, i.e. protK digestion, indicating that the sialic acid residues are not accessible for SNA
binding. Hence, the MS data show that degree of total Fc sialylation in IVIg is similar. In addition, as the IVIg products are comparable with respect to the degree of IgG sialylation it appears that there is no enrichment or depletion of sialylated IgG in the IVIg products due to the IVIg manufacturing process.

Acknowledgements
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References


