

## Increased sialylation of oligosaccharides on IgG paraproteins—a potential new tumour marker in multiple myeloma

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### Abstract

**Aims**—To investigate whether changes in carbohydrate structure of IgG are related to malignancy and stage of disease in myeloma and monoclonal gammopathy of uncertain significance (MGUS).

**Methods**—61 patients were studied at diagnosis: 14 with MGUS, nine with stage I multiple myeloma, 11 with stage II, 21 with stage III, and five with solitary plasmacytoma. IgG was extracted from serum by protein G affinity chromatography. Oligosaccharides were cleaved from the protein backbone enzymatically by N-glycosidase F. Oligosaccharide analysis was performed by high pressure anion exchange chromatography with pulsed electrochemical detection (HPAE-PED).

**Results**—Up to 15 oligosaccharide peaks were identified in three major fractions: neutral, monosialylated, and disialylated. Patients with myeloma showed an increase in the proportion of sialylated oligosaccharides in comparison with patients with MGUS. The ratio of neutral to sialylated oligosaccharides (N:S) was reduced at all stages of myeloma compared with MGUS: MGUS, 11.35; myeloma stage I, 7.6 ( $p = 0.047$ ); stage II, 5.20 ( $p = 0.035$ ); stage III, 3.60 ( $p = 0.0002$ ); plasmacytoma, 7.5 ( $p = 0.046$ ). The N:S ratio was independent of paraprotein concentration ( $r = 0.05$ ).

**Conclusions**—The ratio of neutral to sialylated oligosaccharides may act as a new marker of malignancy in IgG paraproteinaemia and warrants further investigation.

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Immunoglobulin G is enzymatically N-glycosylated at Asn-297 in the CH2 domain of each heavy chain.<sup>1-3</sup> Variation in the terminal residues of each oligosaccharide leads to much heterogeneity, with up to 30 separate oligosaccharides being identified.<sup>4</sup> However, the oligosaccharide pattern within healthy individuals remains relatively constant.

Human IgG contains 2.8 N linked oligosaccharides on average, of which two are located in the Fc region. Additional oligosaccharides can be located within the variable region (Fab) dependent on the presence of an N-glycosylation site.<sup>5</sup> In general Fc linked oligosaccharides are not disialylated, have a low incidence of monosialylation (10%), and have no galactose

residue on the  $\alpha$  1-3 arm of at least one oligosaccharide.<sup>5</sup>

Alterations in glycation of IgG have been reported in a variety of disease states including rheumatoid arthritis,<sup>6</sup> Crohn's disease, tuberculosis,<sup>7</sup> and Castleman's disease.<sup>8</sup> The most common abnormality described is that of hypogalactosylation, in which the proportion of oligosaccharide structures bearing an outer arm galactose is reduced, with a corresponding increase in those terminating in N-acetylglucosamine (IgG G0).

Paraproteinaemia is a common finding in the population, especially with increasing age. Previous studies have indicated that the incidence of paraproteinaemia is 3% at 60 years, rising to 11.7% between the age of 80 and 90 years.<sup>9</sup> Monoclonal paraproteins are the hallmark of malignant plasma cell disorders, but are also associated with a variety of non-myelomatous conditions. Plasma cell disorders form a spectrum of disease states ranging from the clinically benign monoclonal gammopathy of unknown significance (MGUS) to the highly malignant plasma cell leukaemias.<sup>10</sup>

The current criteria used to differentiate MGUS from myeloma reflect the lack of a single diagnostic test to make a reliable distinction between these entities. The plasma cell labelling index (PCLI) measures the proliferation rate of plasma cells and is regarded as the best indicator of active disease, differentiating between MGUS, smouldering myeloma, and active myeloma.<sup>11</sup> Serum  $\beta$ -2 microglobulin, interleukin-6 (IL-6), soluble IL-6 receptor, C reactive protein, and plasma cell surface markers have all been advocated as prognostic markers in plasma cell malignancies.<sup>11-14</sup> However, in clinical practice prolonged follow up of patients over many years may be the only certain way of identifying those with progressive disease.

There has been little work to date on the analysis of the carbohydrate moieties of IgG at different stages of myeloma. In one study of 29 subjects with MGUS and myeloma, 11 of 17 subjects with stage II and III myeloma showed reduced binding of IgG to the lectin from *Ricinus communis* with increased binding to concanavalin A. High pressure liquid chromatographic analysis of released oligosaccharides revealed that the change in lectin binding affinity was associated with a decrease in galactosylation of IgG in these subjects. Plasma cells from these patients revealed a low galactosyl-

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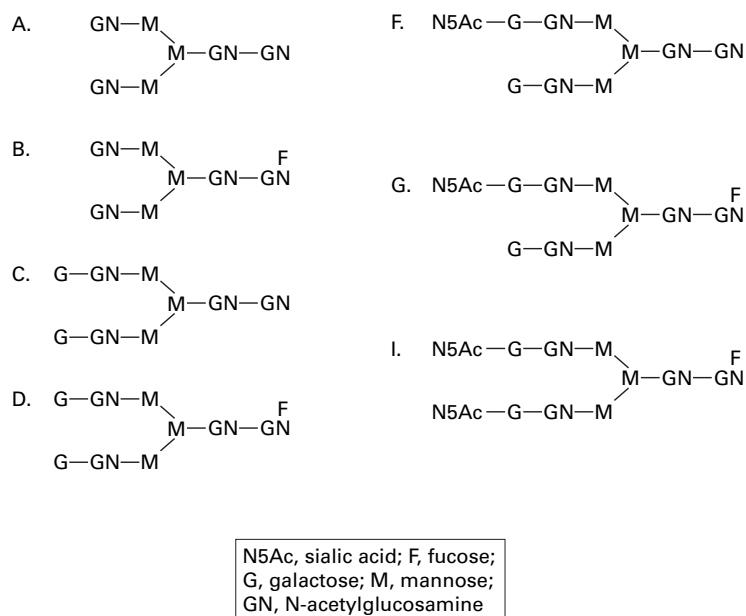


Figure 1 Diagrammatic representation of some of the major oligosaccharides identified from IgG using high pressure anion exchange chromatography. A to E represent neutral oligosaccharides; F and G represent monosialylated forms; I represents the disialylated form.

transferase to N-acetylglucosaminyl transferase III ratio, which reflected hypogalactosylation of IgG.<sup>15</sup>

A further study by the same group identified four myeloma subjects with highly sialylated, fucosylated, and bisected biantennary sugar chains within the F(ab)<sub>2</sub> region of their IgG paraproteins.<sup>16</sup>

We undertook this study to determine whether change in oligosaccharide structure of IgG in multiple myeloma and MGUS is a marker malignancy and whether this can differentiate between MGUS and multiple myeloma.

## Methods

### SUBJECTS

Sixty one subjects with IgG paraproteins (30 male and 31 female, median age 70 years

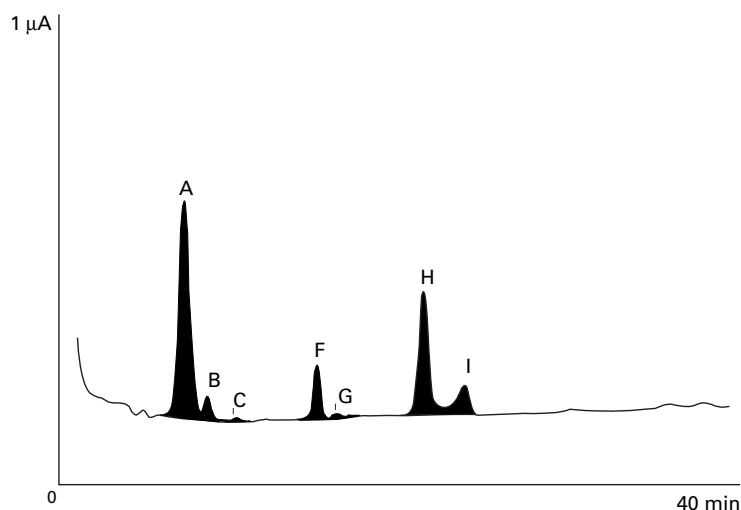


Figure 2 Chromatogram of oligosaccharides of IgG from a patient with stage III myeloma. Neutral oligosaccharides are represented by peaks A, B, and C; monosialylated forms are represented by peaks F and G; disialylated form are represented by peaks H and I. There is a large increase in the proportion of disialylated oligosaccharides in this sample.

(range 50 to 88)) were studied at the time of diagnosis. Subjects were classified according to the Salmon and Durie criteria.<sup>17</sup> Fourteen patients had MGUS, nine had stage I multiple myeloma, 11 had stage II, 21 had stage III, and five had a solitary plasmacytoma. All the secreted paraproteins were of an IgG isotype that was confirmed by routine immunofixation. Twenty age matched control subjects were selected from patients attending the Royal Victoria Infirmary. None of these patients had paraproteinaemia and all had normal immunoglobulin concentrations as confirmed by routine electrophoresis and specific assays for immunoglobulins. The study was approved by the joint ethics committees of Newcastle University, Newcastle Health Authority, and South West Durham Health Authority.

Patients with Salmon and Durie stage II and II myeloma were treated with various chemotherapy regimens that involved oral melphalan and prednisolone, VAD, and ABCM. Patients were followed for between two and 37 months from diagnosis.

Venous blood was taken at the time of diagnosis and serum separated by centrifugation. Serum was stored at  $-70^{\circ}\text{C}$  until analysis. Immunoglobulin G was extracted from serum using protein G affinity chromatography (Pharmacia) and dialysed against deionised water. Purity of the preparations was checked using SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

Two hundred micrograms of IgG were freeze dried overnight and redissolved in 50  $\mu\text{l}$  of ammonium formate buffer (50 mM, pH 8.8.) N linked oligosaccharides were cleaved from IgG using N-glycosidase F (Boehringer Mannheim) by the method of Mann *et al.*<sup>18</sup> Following overnight digestion (16 hours), oligosaccharides were separated by micro-ultrafiltration (Whatman) and freeze dried overnight. Bovine fetuin and human serum albumin underwent the same procedure and acted as positive and negative control glycoproteins for the procedure.

### CHROMATOGRAPHIC ANALYSIS

Analysis of oligosaccharide profiles was undertaken using high pressure anion exchange chromatography with pulsed electrochemical detection (Dionex UK). Elution of oligosaccharides was based on the method of Townsend *et al.*<sup>19</sup>

Separation of oligosaccharides was achieved using a Carbobac PA-100 (40  $\times$  250 mm) anion exchange column (Dionex UK), using 100 mM sodium hydroxide base eluant and a sodium acetate gradient. Eluant conditions were: 0–5 minutes, 100 mM NaOH, 20 mM sodium acetate; 5–15 minutes, 100 mM NaOH, 20–30 mM sodium acetate; and 15–40 minutes, 100 mM NaOH, 30–100 mM sodium acetate. The method was standardised on a series of neutral, monosialylated, and disialylated biantennary N linked oligosaccharides available from a commercial source (Oxford Glycosystems). Twenty five microlitres of reconstituted oligosaccharides were injected onto the column and run at a full scale

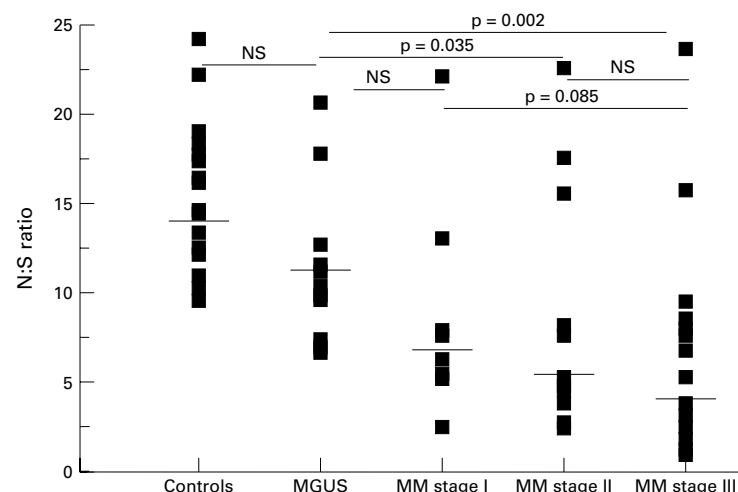
**Table 1** The ratio of neutral to sialylated oligosaccharides (N:S ratio),  $\beta$ -2 microglobulin ( $\beta$ -2M), C reactive protein (CRP), and interleukin-6 (IL-6) concentrations by each subject group

Category	N:S ratio	$\beta$ -2M (mg/l)	CRP (mg/l)	IL-6 (ng/ml)
Controls (n=20)	14.50 (9.6–24.2)	1.80 (0.4–3.8)	3.30 (0.7–8.3)	Not measured
MGUS (n=14)	11.35 (9.06–18.5)	3.05 (2.5–4.33)	2.40 (0–12)	9.25 (7.95–12.75)
Stage I (n=9)	7.60 (5.35–10.45) p=0.047	4.40 (3.2–5.9) p=0.156	8.0 (3.9–30.5) p=0.084	24.5 (12.5–77.3) p=0.031
Stage II (n=11)	5.20 (3.80–15.50) p=0.035	4.20 (3.8–6.3) p=0.051	15.0 (4.8–50.0) p=0.035	11.0 (8.95–18.0) p=0.278
Stage III (n=21)	3.60 (1.81–7.90) p=0.0002	7.85 (5.55–10.0) p=0.0001	18.0 (4.2–45.3) p=0.025	14.0 (9.3–31.5) p=0.036
Plasmacytoma (n=5)	7.50 (3.05–9.68) p=0.047	3.70 (3.10–6.60) p=0.314	50.0 (0–63.0) p=0.183	13.5 (13.0–14.0) p=0.232

Values are expressed as medians with the 95% confidence intervals. Significance values are calculated between MGUS and the other stages using one way analysis of variance. MGUS, monoclonal gammopathy of uncertain significance; stage I to III refers to multiple myeloma staging.

sensitivity of 300 nC. Data were collected on a Dionex 4400 integrator.

Neutral, monosialylated, and disialylated oligosaccharides were identified by retention times against a calibrated series of oligosaccharides from a commercial source (Oxford Glycosystems). All the oligosaccharides shown in fig 1 were prepared by hydrazinolysis<sup>20</sup> from a variety of human proteins. The purity and structural integrity of each oligosaccharide was assessed by a combination of 500 MHz one dimensional <sup>1</sup>H nuclear magnetic resonance,<sup>21</sup> gel filtration chromatography,<sup>22</sup> and high performance anion exchange chromatography with pulsed amperometric detection.<sup>19</sup> Each oligosaccharide was run under identical eluant conditions; a single peak was identified and its retention time recorded. Elution profiles could therefore be identified for neutral, monosialylated, and disialylated oligosaccharides. In addition a library of oligosaccharides prepared from murine IgG (Oxford Glycosystems) was also run to confirm the elution profiles



**Figure 3** Neutral to sialylated oligosaccharide (N:S) ratio represented graphically in each subject group. Horizontal bars represent median in each group.

obtained. The oligosaccharides prepared from patients' IgG were then eluted under identical conditions, and the relative structures identified by their elution profile. Each identified peak had its area measured by triangulation and was expressed as a percentage of the total area of oligosaccharides eluted. Total percentage areas for neutral, monosialylated, and disialylated oligosaccharides were calculated and expressed as a neutral to sialylated oligosaccharide ratio (N:S) for each sample.

Serum  $\beta$ -2 microglobulin and C reactive protein were measured by radial immunodiffusion (Binding Site) and immunoreactive IL-6 was measured by a sandwich enzyme linked immunosorbent assay technique (Quantikine R+D systems).

#### STATISTICAL ANALYSIS

Non-parametric statistical methods were used in the data analysis. One way analysis of variance (ANOVA) and the Mann-Whitney U test were employed to compare subject groups. Results are expressed as medians, with semi-interquartile range. Correlations were performed using Pearson product moment correlation (Minitab Inc).

#### Results

Chromatographic analysis of all samples revealed up to 15 major peaks eluting in three distinct groups—neutral, monosialylated, and disialylated. The major oligosaccharides identified are shown in fig 1. A chromatogram of a subject with myeloma stage III is shown in fig 2. There is a large increase in the late eluting disialylated oligosaccharides at the end of the chromatogram.

Subjects with myeloma (all stages) had a significantly lower N:S oligosaccharide ratio than subjects with MGUS and controls (table 1; fig 3). The median N:S ratio showed a progressive decline with disease stage, indicating an increase in the proportion of sialylated oligosaccharides. However, there were no significant differences between stages I, II, and III (fig 3). Eighteen of 21 subjects (85%) with stage III multiple myeloma, eight of 11 (73%) with stage II, seven of nine (78%) with stage I, and four of five (80%) with a solitary plasmacytoma had an N:S ratio below the 95% confidence limit (9.06) of the subjects with MGUS. Two of 14 subjects (14.3%) with MGUS had an N:S ratio less than 9.0.

The N:S ratio showed no correlation with paraprotein concentration ( $r = 0.05$ ) at any stage of disease.

There was a progressive increase in monosialylated and disialylated oligosaccharides at each disease stage (fig 4) This only reached statistical significance at stage III ( $p = 0.02$ ) for disialylated glycans and at stage II and III for monosialylated oligosaccharides (stage II,  $p = 0.043$ ; stage III,  $p = 0.002$ ). The percentage increase in all sialylated oligosaccharides achieved statistical significance only in stage III disease ( $p = 0.0015$ ).

$\beta$ -2 Microglobulin was increased at all stages of myeloma as compared with MGUS (table 1), although the differences between MGUS

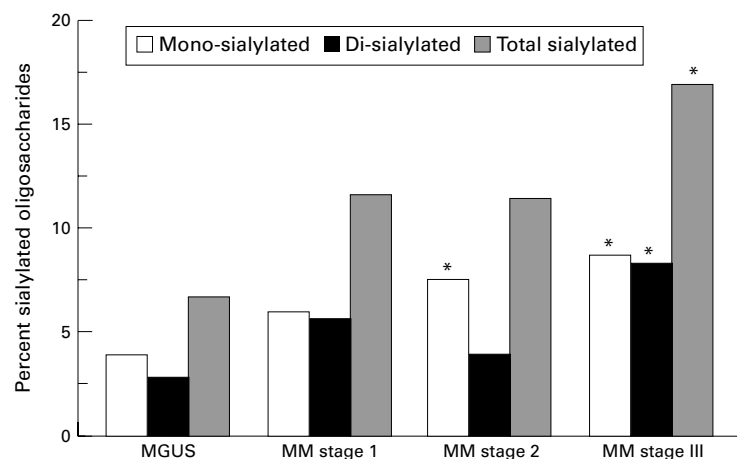


Figure 4 Graphical representation of the percentage of mono and disialylated oligosaccharides of IgG at each disease stage. \*Stages at which there was a significant increase ( $p < 0.05$ ) over the values in monoclonal gammopathy of uncertain significance (MGUS).

and stage I multiple myeloma and solitary plasmacytoma were not significant. Using a cut off value of 3.0 mg/litre, 20 of 21 stage III multiple myeloma subjects, 10 of 11 stage II subjects, seven of nine stage I subjects, and all three plasmacytoma subjects had a  $\beta$ -2 microglobulin value of more than 3 mg/litre. However seven of 14 subjects with MGUS also had a value of more than 3 mg/litre.

C reactive protein concentrations were increased at all stages of malignant disease, although the increase was not significant in stage I multiple myeloma. Using a cut off value of 6 mg/litre, C reactive protein was increased in 14 of 21 subjects with stage III multiple myeloma, in eight of 11 with stage II, in five of nine with stage I, and in two of the three plasmacytoma subjects. A C reactive protein concentration greater than 6 mg/litre was recorded in three of the 14 MGUS subjects.

IL-6 was raised in patients with myeloma but the variation tended to be wide. Values were significantly raised in stage I and stage III disease. Using a cut off value of 12.8 ng/l (upper 95% confidence limit), three of 14 of the subjects with MGUS, eight of nine of those with stage I multiple myeloma, four of 11 with stage II, and eight of 21 with stage III had a raised serum IL-6 concentration. There was a reasonable correlation between CRP and IL-6 ( $r = 0.229$ ) but there was no correlation between IL-6 and N:S ratio in serum.

### Discussion

Paraproteins are a common finding in the population, varying in prevalence between 3% at 60 years to almost 12% at 80 to 90 years,<sup>9</sup> and are the hallmark of clonal plasma cell diseases. It is important to be able to distinguish between MGUS and multiple myeloma and to have robust prognostic indicators in myeloma. Several clinical staging systems have been developed, the most common in use being the Salmon and Durie system.<sup>17</sup> Staging systems use multiple criteria, which include paraprotein concentration, percentage of plasma cells in the bone marrow, Bence Jones protein excretion, the presence of lytic lesions on skel-

etal survey, and haemoglobin concentration. More recently  $\beta$ -2 microglobulin, interleukin-6, and C reactive protein have been advocated as prognostic indicators.<sup>11-13</sup> The most promising in clinical use have been the plasma cell labelling index and measurement of serum  $\beta$ -2 microglobulin.<sup>14</sup>

Immunoglobulin G paraproteins are the most common paraprotein isotype in myeloma, accounting for over 50% of patients. IgG is a glycoprotein containing two N linked oligosaccharide chains at ASN-297 in the Fc region. Previous studies have indicated that there are at least 30 possible variants, leading to much microheterogeneity.<sup>1-5</sup> Many of the early studies on the structure of IgG were performed on IgG extracted from myeloma patients. However, in most analytical procedures IgG was pretreated with sialidase to remove terminal N-acetylneuraminic acid (sialic acid) residues in order to simplify the analytical procedure and subsequent interpretation.

Nishiura *et al* investigated glycoprotein changes in myeloma and reported differential binding of IgG paraproteins to the lectins concanavalin A and RCA 120,<sup>15</sup> indicating hypogalactosylation of IgG, particularly at the late stages (II and III) of myeloma, but did not, however, analyse the contribution of sialylation. There was reduced N-galactosyltransferase activity in bone marrow cells from five patients with multiple myeloma, which supported their findings. They also undertook high performance liquid chromatography (HPLC) analysis of the carbohydrate chains which were removed from the parent IgG by hydrazinolysis. However, the derivatised sugar chains were pretreated with neuraminidase to remove sialic acid before HPLC separation and so no information on sialylation was available. A further report by this group<sup>16</sup> indicated that in one or two patients with myeloma, highly sialylated forms of oligosaccharide could be identified.

In the present study we only studied IgG paraproteins. IgM and IgA paraproteins are described in myeloma and other lymphoproliferative disorders,<sup>23</sup> but are less common than IgG. The glycation of IgM and IgA is far more complex than that of IgG. Immunoglobulin M exists as a pentamer with an associated J chain and has at least five N-glycation sites per heavy chain.<sup>24</sup> Immunoglobulin A contains both N and O linked oligosaccharides which can be particularly numerous when it is in its dimeric form.<sup>25</sup> Thus it was beyond the analytical capability of the present study to analyse IgM and IgA paraproteins.

The role of sialic acid and cell surface glycoproteins in malignancy has been long debated<sup>26-28</sup> but there have been few studies in myeloma. Frithz *et al* reported an increase in sialyltransferase and fucosyltransferase activities in serum in 19 untreated patients with myeloma.<sup>29</sup> Patients at the later stages of disease showed higher activity than those at earlier stages. Six subjects followed for 20 months on treatment had a fall in sialyltransferase activity that correlated with the fall in paraprotein concentration. Patients being

treated with alkylating agents and prednisolone in general had lower sialyltransferase activities. Sialyltransferase activity did not correlate with serum creatinine concentration.

Further work by Cohen *et al* showed an increase in sialyltransferase activity in peripheral blood mononuclear cells in 14 patients with myeloma, compared with healthy subjects and those with MGUS.<sup>30</sup> Sialyltransferase activity was assayed in bone marrow cells (50–100% plasma cells) of five patients with myeloma and was found to be up to 19 times higher in those subjects with non-malignant disorders (plasma cells < 1.0%). Total sialic acid in mononuclear cells was measured by thiobarbituric acid (TBA) method<sup>31</sup> and was found to be similar in all groups studied.

In a follow up study by the same group,<sup>32</sup> sialyltransferase activity was determined in mononuclear bone marrow cells of 10 patients with myeloma and in one patient with a solitary plasmacytoma. Enzyme activity was found to be up to 12 times higher in the patients with myeloma. Activity was also increased in the plasma cells from the patient with the plasmacytoma compared with normal spleen and tonsillar tissue from the same patient.

The results of our studies support the hypothesis that increased sialylation of glycoproteins occurs in plasma cells from patients with multiple myeloma. We found that the IgG paraprotein secreted from the malignant plasma cell is more heavily sialylated in subjects with myeloma than in subjects with MGUS and in normal controls. This finding is consistent with previous reports of increased sialyltransferase activity in the mononuclear cells from such patients.<sup>29 30 32</sup>

Previous workers have not been able to detect significant increases in either total serum sialic acid or mononuclear cell sialic acid concentrations. This could be explained as follows. Total serum sialic acid will measure contributions from all the serum glycoproteins, including the acute phase proteins, and this is likely to diminish any contribution from an IgG paraprotein. Secondly, previous assays used for sialic acid measurement have been relatively non-specific and may suffer from interference.<sup>31</sup> In this study we only measured sialylated oligosaccharides associated with IgG and by the highly sensitive and specific method of high pressure liquid chromatography with pulsed electrochemical detection.<sup>19</sup>

The data presented here showed a significant decrease in the ratio of neutral to sialylated oligosaccharides (N:S) in myeloma. This ratio showed a progressive decrease with disease stage. All samples were analysed at the time of diagnosis and so were not affected by subsequent treatment. The sensitivity of the N:S ratio in differentiating between MGUS and myeloma was 80.4%, with a corresponding specificity of 85.7%. In contrast  $\beta$ -2 microglobulin had a higher sensitivity (90–100%, depending on stage) but a lower specificity (50%). The N:S ratio showed no correlation with paraprotein concentration suggesting that the extent of sialylation of IgG oligosaccharides is independent of paraprotein concentration.

In this study, C reactive protein and IL-6 performed less well than  $\beta$ -2 microglobulin and the N:S ratio in terms of sensitivity and specificity in differentiating between MGUS and myeloma. The IL-6 was particularly high in stage I disease. This may have been influenced by an individual patient who had a very high IL-6 concentration of 180 ng/litre associated with a high C reactive protein of 83 mg/litre.  $\beta$ -2 Microglobulin was normal (0.5 mg/litre) and the patient had a survival of over 30 months on completion of the study. The N:S ratio in this individual was 5.5. It is possible that the high values for IL-6 and C reactive protein seen in this patient could be attributed to secondary infection rather than to active myeloma.

Interleukin-6 is a major plasma cell growth factor in myeloma,<sup>13</sup> but it also appears to have a role in the modulation of glycation of acute phase proteins. Under cell culture conditions, IL-6 stimulation of Hep 3B cells has been shown to alter the glycation patterns of the secreted proteins  $\alpha$ -1 protease inhibitor, caeruloplasmin, and  $\alpha$ -fetoprotein.<sup>33</sup> Nakao *et al* studied the potential of IL-6 to modulate glycation changes in a non-secreting human myeloma cell line.<sup>34</sup> This group stimulated the OAM-1 cell line with IL-6 and reported a decrease in N-acetylglucosaminyl transferase III activity from these cells. They also studied cell surface oligosaccharides using FACS analysis with fluorescein isothiocyanate (FITC) labelled datura-stramonium lectin (DSL), which preferentially recognises asialo-tetra-antennary N linked oligosaccharides. There appeared to be increased binding of DSL to the cell surface in IL-6 stimulated cultures, indicating increased surface glycoprotein. Cells in these experiments were pretreated with sialidase to simplify analysis, and so data on sialylation are not available.

Further work by Cohen *et al* showed that sialyltransferase activity could be altered by both dexamethasone and recombinant interferon- $\alpha$  in a human myeloma cell line.<sup>35</sup> It is apparent that cytokines such as IL-6 may modulate glycation of secreted glycoproteins in vitro; one could postulate that such modulation may occur in vivo and may reflect the changes in sialylation of IgG paraproteins that we have observed. We found no correlation between IL-6 concentration in serum and the N:S ratio on IgG.

In conclusion an increase in sialylation of IgG oligosaccharides in subjects with myeloma compared with subjects with MGUS is reported in this cross sectional study. The N:S ratio may act as an additional marker of malignancy in MM and warrants further investigation.

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