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Glycosylation of polyclonal and paraprotein IgG in multiple myeloma

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It has previously been shown that in multiple myeloma (MM) each IgG paraprotein exhibits a unique oligosaccharide profile. It has been assumed that this results from a clone specific glycosylation machinery. However, the abnormal physiological environment of the bone marrow in this disease may also affect normal plasma cells producing polyclonal IgG. We present data to show that this is so and that, in two cases, the oligosaccharide profile of the polyclonal IgG reflected that of the paraprotein from the same patient rather than that of normal polyclonal IgG.

***Keywords*: Multiple myeloma, paraprotein IgG, polyclonal IgG, glycosylation, oligosaccaride**

Introduction

Glycosylation of protein molecules represents a major post-translational modification that may influence biological activity, stability, pharmokinetics, antigenicity etc. [1]. Oligosaccharides may be covalently bonded through the nitrogen atom (N-linked) of the amide group of asparagine or the oxygen atoms (O-linked) of the hydroxy amino acids serine and threonine. The structural motif that is permissive of N-glycosylation is Asn-X-Ser/Thr, where X may be any amino acid other than proline and N-linked oligosaccharides may be of the complex or high mannose type. The motif(s) for O-glycosylation is not defined. In naturally occurring molecules fidelity is generally observed for glycosylation at a given site, i.e. either complex or high mannose, however, heterogeneity results from differences in the outer arm or terminal sugars [2–5]. Changes in the sugar profiles of oligosaccharides are observed in numerous diseases and appear to be the primary defect in an increasing number.

It is evident that, in health, the oligosaccharide profile of a given glycoprotein is determined both by the tertiary structure of the molecule and the glycosylation machinery of the cells of the tissue in which it is biosynthesized. In some diseases it is evident that changes in oligosaccharide structures correlate with disease activity suggesting that the glycosylation machinery of tissues or individual cells is sensitive to their physiological environment. Similarly, the glycosylation of protein products produced by immortalized cell lines *in vitro* has been shown to be sensitive to culture conditions. A fuller understanding of parameters that influence glycosylation is required both to understand the natural relationship between structure and function and to enable the production of natural glycoforms of recombinant molecules *in vitro*. This is particularly so for human antibodies of the IgG class since the effector functions that they activate, but not antigen binding specificity, *in vivo*, are profoundly dependent on glycosylation [6–8]. Thus, control of N-glycosylation at a single site in the IgG molecule would allow antibodies of hundreds (thousands) of differing specificities to be applied to *in vivo* therapeutic modalities.

The human IgG molecule is composed of three compact protein moieties (two Fab and an Fc region) that each have rotational and translational mobility about a linear sequence referred to as the hinge region. The primary and quaternary structure of the Fab regions determines antibody specificity. The Fc region expresses numerous ligand interaction sites that determine essential biological properties e.g. activation of complement, Fc receptor mediated cellular activation etc. Glycosylation of the Fc is essential for the integrity of these effector sites. The N-linked oligosaccharide exhibits a considerable heterogeneity and results in the generation of multiple antibody glycoforms. Differences in biological activities of the various glycoforms have been reported; additionally, disease associated changes in glycoform profiles have been observed. It has been suggested, therefore, that overproduction of particular glycoforms of specific antibodies, particularly autoantibodies, may be related to the observed hypersensitivity.

Studies of monoclonal antibody production *in vitro* have shown that whilst the glycosylation profile is a unique property of the clone it can be modulated by the culture conditions [6] i.e. the nutritional environment and,

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presumably, specific activation signals received from that environment. Extrapolation to *in vivo* biosynthesis suggests that the physiological environment will influence IgG glycosylation and the possibility that in certain diseases changes in that environment will be reflected in the IgG glycosylation profile; essentially an acute phase response that reflects disease activity, i.e. is effect not cause. We have sought to investigate *in vivo* influences on IgG glycosylation in the disease multiple myeloma. In this disease plasma cells proliferate in the bone marrow in a grossly abnormal nutritional and cytokine environment. We have previously shown that the glycoform profile of the IgG paraprotein is unique, however, we now ask 'is the glycoform profile an independent characteristic of the neoplastic clone or does it also reflect the environment of the bone marrow in which plasma cells are proliferating and secreting IgG?'

We have selected myeloma sera containing an IgG3 paraprotein and purified both the paraprotein and the residual polyclonal IgG. Oligosaccharide analysis of the products from two sera show that the glycoform profiles of the paraprotein and polyclonal IgG closely mirror each other. Significantly, the glycoform profile of the polyclonal IgG differs substantially from that of normal polyclonal IgG.

Materials and Methods

Serum samples

Three samples were selected from patients with diagnosed multiple myeloma with an IgG3 paraprotein. The serum containing two paraproteins was selected from the patients entered in the Medical Research Council myeloma trials VI–VIII. One thousand records were scrutinized and nine accessed that had evidence of two paraproteins on routine zone electrophoresis. All samples had been preserved with azide and stored frozen.

IgG isolation

Sera were dialysed v 0.01 M phosphate buffer, pH 7.0 and passed over a DEAE(diethylaminoethyl)-cellulose column equilibrated with the same buffer. Further purification was effected using SpA(staphylococcal protein A)-affinity chromatography. Purity was assessed by SDS-PAGE.

Carbohydrate analyses

IgG from serum samples HA1025, HA1872, HA2858 were analysed as previously reported [7–9]. The glycosylation profile of IgG from serum 40274 was analysed, following release of the oligosaccharide with hydrazinolysis, by Matrix Assisted Laser Desorption Ionization – Time Of Flight (MALDI – TOF) mass spectrometry using gentisic acid as matrix.

Results

IgG isolated from sera HA1025, HA1872 and HA2858

Zone electrophoresis indicated that each serum contained polyclonal IgG in addition to the paraprotein. Passage of

Table 1. Oligosaccharide profiles of polyclonal (p) and monoclonal (m) IgG isolated from normal serum and sera of patients with multiple myeloma

	IgG preparation						
Saccharide	*poly IgG*	*pHA1025*	*mHA1025*	*pHA2858*	*mHA2858*	*pHA1872*	*mHA1872*
A	3.3	5.3	2.6	5.6	6.4	2.4	3.7
B	1.9	2.1	3.1	2.0	2.4	3.3	4.6
C	0.7	0.9	0.9	0.6	0.6	0.5	0.5
D	6.3	3.1	6.8	6.7	6.0	5.9	5.7
E	19.7	38.4	40.5	51.4	50.3	25.6	7.5
F	17.1	14.1	12.5	8.4	7.5	17.2	31.0
G	14.1	9.7	23.2	7.2	13.6	12.7	6.6
H	23.9	13.4	10.4	8.7	1.5	21.2	40.4
M	2.3	3.8	0.0	7.0	8.5	3.1	0.0
N	3.5	1.7	0.0	1.5	1.3	2.9	0.0
O	0.8	0.2	0.0	0.2	0.0	0.0	0.0
P	6.7	7.3	0.0	0.7	1.9	5.2	0.0

A.Protein-GlcNAc-GlcNAc-Man $\langle$ –Man-GlcNAc / –Man-GlcNAc.

B. as A with Gal on the Manα(1–6) arm; C. as A with Gal on the Manα(1–3) arm; D. as A with Gal on both arms; E. as A with fucose on the proximal GlcNAc; F. as E with Gal on the Manα(1–6) arm; G. as E with Gal on the Manα(1–3) arm; H. as E with Gal on both arms; M. as A with fucose on the proximal GlcNAc and bisecting GlcNAc; N. as M with Gal on the Manα(1–6) arm; O. as N with Gal on the Manα(1–3) arm; P. as N with Gal on both arms.

the dialysed sera over the DEAE column resulted in the isolation of both the polyclonal and monoclonal IgG. On passage of this material over the SpA-Sepharose column the polyclonal IgG was bound whilst the IgG3 paraprotein was recovered in the flow through fraction. The polyclonal IgG was eluted with citrate buffer, pH 3.0.

Haemagglutination analyses showed the IgG3 proteins to be free of polyclonal IgG but the polyclonal preparations did contain some IgG3 protein. This 'contaminating' protein was removed by immunoaffinity chromatography using a monoclonal anti-IgG3 antibody coupled to Sepharose.

IgG isolated from serum 40274

Zone electrophoretic analysis of nine sera suspected of having two paraproteins present were re-analysed by agarose and cellulose acetate electrophoresis. The presence of two paraproteins was most evident for serum 40274. Immunofixation tests revealed the presence of an IgG1 lambda paraprotein of gamma mobility and an IgG2 lambda paraprotein with beta mobility. Ion-exchange chromatography on DEAE-cellulose under conditions of 0.005 M phosphate, pH 7.0, allowed the isolation of the IgG1 paraprotein and elution with 0.05 M phosphate, pH 7.0, the IgG2 paraprotein. Zone electrophoresis and immunofixation revealed that the IgG1 protein preparation contained some polyclonal IgG and the IgG2 protein some other serum proteins. The IgG2 was further purified by passage over a SpA-Sepharose column; the bound IgG2 being released with citrate buffer, pH 3.0.

Oligosaccharide analyses of IgGs isolated from sera HA1025, HA1872 and HA2858

Following removal of terminal sialic acid residues the neutral sugar composition was determined for each of the paraproteins, residual polyclonal IgG and polyclonal IgG prepared from pooled normal serum (Table 1). It is evident that the polyclonal IgG isolated from sera HA1025 and HA2858 differ significantly from that obtained from pooled polyclonal IgG and are more similar to each other than to the pooled sample. Also, the profiles of the IgG3 paraproteins mirror those of the paired polyclonal IgG. The oligosaccharide profile of the polyclonal IgG isolated from serum HA1872 is more similar to that for pooled normal polyclonal IgG and differs markedly from that of the IgG3 paraprotein.

Oligosaccharide profiles of IgG1 and IgG2 paraproteins isolated from serum 40274

The MALDI-TOF profiles obtained for both the IgG1 and IgG2 paraproteins showed two peaks of mass 1481 and 1644. The proportions of the two peaks were similar for each sample. The 1481 peak corresponds to oligosaccharide structure E and the 1644 peak structures F and G (Table 1).

Discussion

Polyclonal IgG and IgG3 paraprotein were isolated from three myeloma sera. Importantly, following affinity chromatography purification, the polyclonal IgG was shown to contain $>0.1\%$ IgG3 protein; the presence of paraprotein might have unduly weighted the analysis of this polyclonal preparation. Oligosaccharide analysis, Table 1, showed that the polyclonal IgG and the IgG3 paraproteins isolated from serum HA1025 have similar profiles that are substantially different to that obtained for polyclonal IgG isolated from pooled sera. This is most evident for oligosaccharides E, F, G and H that account for $>75\%$ of total oligosaccharide. However, it is interesting to note that paraprotein HA1025 does not contain any oligosaccharides with bisecting GlcNAc whilst the polyclonal IgG isolated from the same serum does. Similarly, the oligosaccharide profiles of the polyclonal and monoclonal IgG proteins isolated from serum HA2858 mirror each other but differ from that of pooled polyclonal IgG; the matching levels of bisecting GlcNAc being particularly significant. These data suggest that in multiple myeloma glycosylation of the IgG is influenced by parameters of the disease, including the physiological environment of antibody secreting plasma cells, however, the glycosylation phenotype of the neoplastic clone is also evident. We have previously shown extreme divergence in IgG paraprotein glycosylation profiles and it will be of interest to re-investigate these sera and to compare glycosylation profiles for the polyclonal and monoclonal IgGs.

The oligosaccharide profile of the polyclonal IgG isolated from serum HA1872 is comparable to that of pooled polyclonal IgG whilst the profile for the paraprotein is very different. This suggests that whilst the paraprotein exhibits clone specificity in its glycosylation profile that of the polyclonal IgG is undisturbed. The state of clinical disease at the time of sample collection will be documented in future studies.

Further insight into factors determining IgG glycosylation was sought through analysis of two paraproteins isolated from a single serum. Serum 40274 contains an IgG1 and an IgG2 paraprotein. This may have arisen in one of several ways: i) isotype switching may have occurred within an originating neoplastic clone as an early event such that the two daughter clones expanded simultaneously. Whilst each secretes a different IgG isotype they will share a common clone specific glycosylation machinery; ii) two unrelated neoplastic clones may have arisen at a similar time point and expanded to result in the presence of two paraproteins at similar concentrations. The glycosylation profiles of proteins arising in this manner might allow determination of the influence of physiological environment on the glycosylation machinery of two different clones. Preliminary data indicate that the glycosylation profiles of the two paraproteins are similar whilst amino sequence analysis demonstrates that they are products of unrelated clones. Thus, the present data support the proposal that

physiological environments in multiple myeloma may profoundly influence IgG glycosylation for both the paraprotein and the normal polyclonal IgG being synthesized. This may be a somewhat extreme example that supports the view that disease associated changes in IgG glycosylation may reflect the disease process rather than be involved in its aetiology.

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