The effects of oxygen free radicals on the carbohydrate moiety of IgG

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The CH2-linked glycoform of rheumatoid IgG is abnormal in having a reduced galactose content. This has been postulated to be a synthetic defect due to a decrease in the level of rheumatoid B cell galactosyltransferase. However, more recent work has indicated that agalactosylation may be common to chronic inflammatory diseases. In this work we have investigated the effect of oxygen free radicals (OFRs), which are generated by activated phagocytic cells at inflammatory sites, on the carbohydrate moiety of IgG. Radiolytically generated peroxy (ROO') and hydroxyl radicals (OH') but not superoxide anion radicals (O2-) were found to destroy galactose on IgG. After OH' attack, this was associated with an increase in the availability of N-acetylgalcosamine, possibly due to its presence as a terminal residue. These results suggest that the agalactosylation associated with chronic inflammation may not only be synthetic in nature, but may also be a consequence of post-synthetic degradation by OFRs.

Oxygen free radical; Galactosylation; Immunoglobulin G; Inflammation

1. INTRODUCTION

Immunoglobulins are a major class of serum glycoproteins whose function lies in the specific interaction with and clearance of antigens. The structure of the immunoglobulin G molecule (IgG) is illustrated in fig.1. Antigenic specificity is achieved via amino acid variability in VH and VL, whilst antigenic clearance relies on the binding of complexed immunoglobulin to Fc receptors. The latter requires the interaction of specific amino acid residues in the CH2 region [1], and also the presence of an intact carbohydrate moiety [2] which maintains the structural integrity of the CH2 domain.

In rheumatoid arthritis (RA) IgG dysfunction has been reported; it behaves not only as an antibody, but also as a putative antigen for rheumatoid factor [3]. This may arise as a consequence of molecular modification(s) and studies aimed at elucidating differences between rheumatoid IgG and normal IgG have described an altered conformation in the hinge region [4], the appearance of a new visible fluorescence in the molecule [5] and alterations to the carbohydrate moiety [6].

Early studies on the glycoform of IgG in RA demonstrated a decrease in galactose content [6], and further sequence analysis has revealed that galactose loss occurs on terminal residues in the CH2 region, revealing N-acetylgalacosamine as the terminal sugar [7]. This has been postulated to be a synthetic defect due to decreased levels of the enzyme, B cell galactosyltransferase, which catalyses the post-synthetic addition of galactose onto terminal N-acetylgalcosamine [8]. An independent study, however, has demonstrated that decreased galactosylation of IgG is associated with other chronic inflammatory diseases such as Crohns and systemic lupus erythematosus [9]. This evidence suggests that the decrease in galactosylation of IgG occurring in RA is a phenomenon associated with inflammation and could be the consequence of a

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non-specific degradative effect of an inflammatory mediator.

Inflammatory sites are characterised by the presence of large numbers of phagocytes, which release a variety of glycolytic enzymes [10] and oxygen free radicals (OFRs) [11]. Previous studies from this group have shown that OFRs can induce a new visible fluorescence in IgG indistinguishable from that found in rheumatoid IgG [4]. Here, we have investigated the effect of such OFRs on the carbohydrate moiety of IgG to ascertain whether they could mediate the post-synthetic degradation of terminal galactose on IgG.

2. MATERIALS AND METHODS

2.1. Materials

Lyophilised polyclonal human IgG, peroxidase-conjugated lectins (Frythina crysostagalli and Triticum vulgaris), endoglycosidase H from Streptomyces griseus, streptomycin, hydrogen peroxide, o-phenylenediamine (OPD) tablets and oxidised glutathione were obtained from Sigma (Poole, England). 96-well Nunc immuno plates were used for the modified ELISA procedure and were from Gibco (Denmark). All other general laboratory reagents were of Analar grade and were obtained from Sigma or BDH (Poole).

2.2. Irradiation procedures

OFRs were generated in free solution using a 60Co γ source [12]. Polyclonal IgG was irradiated at 1 mg/ml in 40 mM potassium phosphate buffer (pH 7.2) in the presence or absence of either 100 mM formate or 10 mM phenylalanine. In the absence of any solute OH− is produced in solution, whereas in the presence of formate, pure O2− is generated in solution and in the presence of phenylalanine a peroxy radical (ROO′) is formed [13]. Doses of up to 2000 Gy were used at a dose rate of −15 Gy per min. Tubes were agitated every 20 min to re-oxygenate the solutions, and the samples were immediately frozen prior to analysis.

2.3. Neutral sugar analysis by HPLC

To release the oligosaccharide moiety, the pH was reduced to 5 and irradiated IgG was incubated with 10 μM/ml endoglycosidase at 37°C in the presence of streptomycin (10 μg/ml) as a bacteriostat for 72 h. All protein was then precipitated using 5% trichloroacetic acid, and the carbohydrate-containing supernatant analysed by ion-exchange chromatography of their borate complexes on a strong ion-exchange resin (Aminex A-28): the mixture of sugars to be analysed was injected onto a resin bed in a solution of 0.13 M boric acid. A series of borate buffers of increasing pH and ionic strength was then used to elute the sugar-borate complexes. The sugars were then visualized by mixing the eluent with a stream of orcinol/conc. H2SO4 reagent, heated at 100°C for 15 min followed by detection in a flow cell at 425 nm.

2.4. Modified ELISA to detect terminal carbohydrate residues

This procedure is based on the ELISA technique described in [14]. Briefly, 50 μl IgG (50 μg/ml in carbonate buffer at pH 9.6) was coated onto the wells of a 96-well immuno plate in triplicate and allowed to bind at 37°C for 1 h. Following 3 washes in PBS containing 0.05% Tween 20, 0.5% SDS in PBS was added to each well to destroy any secondary and tertiary conformation (omission of this step, reduced lectin binding to N-acetylgalactosamine by 75%, and to galactose by 25%). After 5 min, this was washed as before, and any exposed sites remaining on the plate were blocked with oxidised glutathione (2 μg/ml in PBS). Peroxidase-conjugated lectins [T. vulgaris (TV) at 0.1 μg/ml and E. crysostagalli (EC) at 2 μg/ml] were allowed to react with bound IgG for 1 h at room temperature.
Their specificities were confirmed as being N-acetylglucosamine for TV and galactose for EC by inhibition studies using free sugars. Following 3 washes, IgG-bound lectin was incubated with H$_2$O$_2$ and OPD at pH 5, for 30 min. The reaction stopped by the addition of 2 M H$_2$SO$_4$ and was visualized at 492 nm.

Results were expressed as the ratio N-acetylglucosamine/galactose in terms of TV binding/EC binding. Polyclonal IgG was included on each plate as an internal standard and given the arbitrary value of 1 for TV/EC. The coefficient of variation within batches was calculated as 4%.

3. RESULTS

Following analysis of neutral sugars by HPLC, galactose, mannose and also glucose were detected in the carbohydrate moiety of IgG; glucose arising from the boric acid catalysed release of the amino group from N-acetylglucosamine.

Activated phagocytes release a variety of OFRs (reviews [11,15]) and therefore, in order to assess the effects of each of these species on the glycoform of IgG, steady-state $\gamma$ radiolysis was employed to generate specific OFRs in solution.

Following exposure to radiolytically generated OFRs, the mannose content of IgG was not significantly affected (see fig.2) irrespective of the radical species generated. Whilst O$_2^-$ did not cause a significant loss of either galactose or glucose, 2000 Gy ROO$^-$ destroyed 40% of galactose without any effect on glucose. OH$^-$, on the other hand, after a dose of 2000 Gy, destroyed 38% of galactose on IgG, but in addition there was a highly significant increase in the glucose peak. OH$^-$ had no detectable effect on the carbohydrate at doses equal to or below 500 Gy.

**Fig.2.** The effects of specific OFRs on the neutral sugars of IgG, as measured by HPLC. Each line represents the mean of three experiments.

**Fig.3.** The effects of OFRs on the ratio of N-acetylglucosamine (Glc-NAc) to galactose (Gal) as measured by the binding of the lectins Triticum vulgaris and Erythina cristagalli, respectively, in a modified ELISA. Results are expressed as means ± SD of five separate experiments; *p < 0.005.
The use of lectins of defined specificity enabled the effects of OFRs on both galactose and N-acetylglucosamine to be studied. Fig. 3 illustrates a significant dose-dependent increase in available N-acetylglucosamine following exposure to OH\(^-\) associated with the loss of galactose, but this was not true for ROO\(^-\) attack. Again O\(_3^-\) had not significant effect.

4. DISCUSSION

One of the few studies addressing the effects of OFRs on hexose sugars has demonstrated that the treatment of free sugars with Fe(II) in the presence of oxygen liberates malondialdehyde [16]. Glucuronic acid was found to be the most susceptible hexose sugar, but at high Fe(II) concentrations, both galactose and sucrose also reacted with thiobarbituric acid to produce a detectable chromophore (indicative of malondialdehyde formation) [16].

The results described herein have clearly demonstrated that hexose sugars within the overall structure of a glycoprotein can also be degraded by OFRs and be released from the core structure. The direct measurement of neutral sugars has shown that OH\(^-\) and ROO\(^-\) can be seen to cause a significant loss of galactose but not mannose. Lectin analysis revealed that in addition to the loss of galactose OH\(^-\) also induced a corresponding increase in the availability of N-acetylglucosamine for TV. When this is interpreted in the context of the glycoform (see fig.1), carbohydrate loss appears to occur at the terminal residue. In rheumatoid IgG there is an absence of disialylated structures and a low incidence of monosialylated structures (10%) in the CH2 N-linked oligosaccharides of IgG [7], thus exposing a reducing end of galactose for oxidative modification.

Although little work has been done on galactose, its C4 epimer, glucose, has been shown to undergo carbon-bound proton abstraction after OH\(^-\) attack [17]. This yields primary carbohydrate radicals which can undergo a number of elimination and rearrangement reactions. The elimination of CO can occur, giving rise to carbohydrates of shorter chain length or rearrangement of the C4 radical on galactose could yield glucose, and therefore explain the observed increase in yield of glucose following OH\(^-\) attack. Removal of galactose from the glycoform core could also potentially occur via glycosidic bond cleavage [17], however, if such a reaction mechanism was occurring, then no loss of galactose would be detected by neutral carbohydrate analysis.

These results indicate that, not only may a decrease in the levels of B cell galactosyltransferase be a cause of the reduced galactosylation of IgG in RA, but also that an oxidative, OH\(^-\)-mediated, process may play an important role. Further evidence for the inflammatory degradation of IgG has been provided by the observation that agalactosylation is elevated in synovial fluid IgG, the primary site of inflammation and phagocyte sequestration [18].

The carbohydrate moiety present on glycoproteins appears to be essential for their function and interaction with other biomolecules [18] and therefore these findings have important implications for all glycoproteins at inflammatory sites. By the nature of OH\(^-\) as highly reactive radical species, they cannot be specific and thus the higher the concentration of a particular biomolecule, the greater its chance of OH\(^-\)-induced damage. Within the rheumatoid joint, immunoglobulin synthesis is markedly elevated [19], and would therefore be a predicted target for OH\(^-\). Since galactose recognition by hepatic parenchymal cells is an important mechanism in IgG clearance [20], its absence on rheumatoid IgG would be expected to prolong its half-life and further increase the probability of OH\(^-\) attack.

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