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CHANGES IN THE DEGREE OF GALACTOSYLATION OF RABBIT IgG DURING LONG-TERM IMMUNIZATION WITH BOVINE SERUM ALBUMIN

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The galactose content of isolated IgG was analyzed before immunization and at intervals during prolonged immunization over 16 months. It was found that IgGs from non-immunized and immunized animals differed in galactose content and that the galactose content of serum IgG varied during the course of the immune response. These results suggest possible intraclonal variations or the emergence of subclones of cells secreting IgG that is differently galactosylated relative to the IgG of non-immunized animals. Since isotypic polymorphism of IgG (subclasses), which determines the effector potential(s) of IgG antibodies, does not exist in rabbits, glycosylation may be what defines functional subsets of isotypically uniform antibodies produced during an immune response in these animals.

Key words: IgG, galactose content, immune response

INTRODUCTION

Immunoglobulin G (IgG) is a multifunctional glycoprotein with, on average, 2.8 mol of oligosaccharides (glycans) per mol of IgG (Rademacher *et al.*, 1996). Most of the carbohydrate moiety is present on the Fc region where complex biantennary oligosaccharides are covalently linked to the conserved Asn-297 in both CH2 domains. Glycosylation of the Fc region appears to be essential for the conformation of the IgG molecule (Masuda *et al.*, 2000; Krapp *et al.*, 2003) and, consequently, for full expression of the effector functions, such as activation of the classical complement pathway, binding to the Fc receptors on the cell surface, induction of antibody-dependent cellular cytotoxicity, rapid elimination of antigen-antibody complexes from the circulation and feedback immunosuppression (Jefferis *et al.*, 1998). Each oligosaccharide chain, attached to the Fc region is composed of a core heptasaccharide structure with variable additions of fucose (Fuc) and outer arm sugars, such as galactose (Gal), bisecting N-acetylglucosamine (GlcNAc) and sialic acid (NeuAc) (Fig 1).

The oligosaccharides attached to the Fc region are mostly neutral (without sialic acid) and are classified into three types, G0, G1 and G2, according to the number of terminal Gal residues. Additional heterogeneity results from the presence or absence of Fuc and/or bisecting GlcNAc, such that one of 16 different neutral, asialoglycans may be attached at the Asn-297 residue (Rademacher *et*

al., 1996). Therefore, IgG can be regarded as a population of glycosylated variants or glycoforms, each conveying different physical and biochemical properties (Wormald *et al.*, 1997, Yamaguchi *et al.*, 1998) which may result in functional diversity (Jefferis and Lund, 1997). The relative proportions of IgG glycoforms have been shown to alter, both in relation to and as a result of physiological changes. A shift towards a particular IgG glycoform(s) has been observed to occur in some human chronic inflammatory diseases (Youings *et al.*, 1996; Watson *et al.*, 1999), as well as in autoimmune prone mice (Bodman *et al.*, 1994; Thompson *et al.*, 1996), suggesting that the carbohydrate moiety might be involved in disease pathogenesis. Changes in the proportion of IgG glycoforms under different physiological or pathological conditions most probably reflect a shift in the distribution of specific antibodies of the IgG class during the ongoing, often chronic, humoral immune response to a given antigen. However, little is known about the dynamics of changes in the glycosylation of IgG during such a response, and particularly about the appearance of glycoforms that may be of pathogenetic relevance. So far, galactosylation changes at different time points during a normal immune response has been studied only in mice (Lastra *et al.*, 1998).

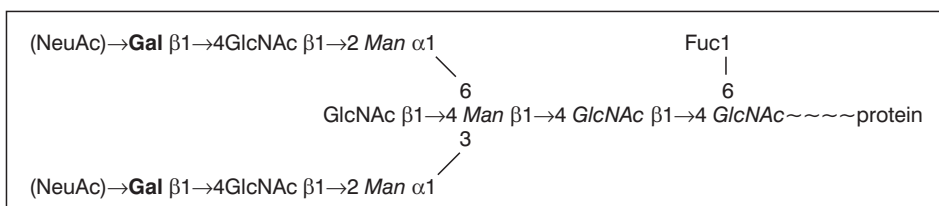


Figure 1. Structure of the N-linked oligosaccharide chain, attached to Asn-297 in the CH2 domain of the Fc region in the IgG molecule

This paper describes an experiment in which chronological variations in the IgG glycosylation status were analysed during long term immunization of rabbits with bovine serum albumin (BSA). The results showed that a long lasting humoral anti-BSA response is followed by permanent changes in the galactose content of serum IgG.

MATERIAL AND METHODS

Animals. The experimental model has been previously described in detail (Milošević-Jovčić *et al.*, 1995). Briefly, ten male Chinchilla rabbits weighing 2,5-3 kg each were immunized with BSA (twice crystallized, Miles Laboratorie) in two phases lasting three months each. After the first phase animals had a rest period of seven months. For the first inoculation in each phase 20 mg of albumin-complete Freund's adjuvant (FCA) mixture was administered intradermally into each animal. Further injections of 10 mg of antigen in saline were given

subcutaneously at various time intervals. Non-immunized rabbits served as controls. Six control animals were kept during the whole period of immunization under the same conditions as the immunized rabbits.

Serum samples used for analysis originated from blood taken by ear vein puncture on day 0 (before immunization) and at monthly intervals during the course of immunization. Serum samples were stored frozen at -80°C . After thawing the samples were centrifuged at 10000 rpm before being used for IgG isolation.

IgG was isolated from pooled rabbit sera by protein G chromatography. Eleven IgG samples representing the eleven different time points of prolonged immunization were prepared.

The *galactosylation profile* of isolated rabbit IgGs was determined by using RCA-I and GS-II lectins in a competitive lectin-binding ELISA (ELBA). They bind to galactose (Gal) and N-acetylglucosamine (GlcNAc) residues respectively when sialic acid is absent. In this assay system normal rabbit IgG (nIgG) from preimmune sera, bound to a solid phase, served as a receptor for sugar specific lectins, while solute IgG from different phases of immunization (ilgG) served as inhibiting ligands of the lectin-receptor interactions. The concentration of ilgG necessary to achieve 50% inhibition of lectin binding to immobilized nIgG was taken as the measure (KI50) of the relative abundance (expression) of accessible sugars.

RESULTS

Galactose content of isolated IgG was analysed before immunization and at intervals during prolonged immunization for 16 months. It was found that IgGs from nonimmunized and immunized animals differed in galactose content and that the galactose content of serum IgG varied during the course of the immune response (Fig 2).

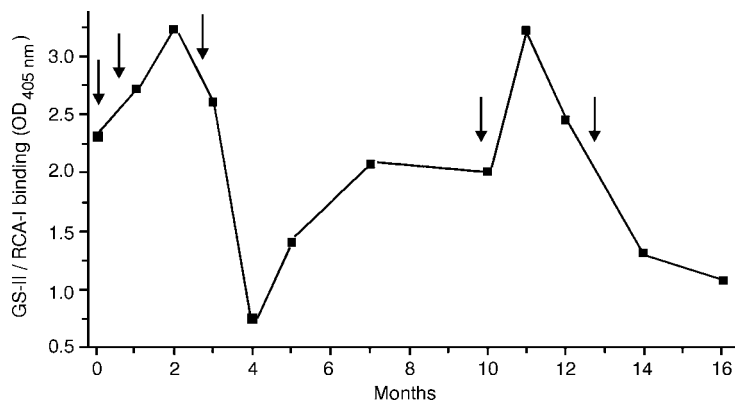


Figure 2. Change with time in the degree of galactosylation of IgG in hyperimmunized rabbits. The data are the means of all immunized animals at each time point. Arrows indicate BSA injections

Within the first month of immunization, an increase in galactose content of serum IgG was registered. Then, between days 30 and 120, the galactose content of serum IgG declined gradually, a 3,7-fold lower value relative to that observed for IgG before immunization. The galactose content of IgG climbed back slowly during the rest period of seven months and at 10 months approached the value registered prior to immunization. Repetitive immunization led to fluctuations in IgG galactosylation similar to those observed in the first phase. After an initial rise within the first month of the second immunization phase, the degree of IgG galactosylation fell. Somewhere about this time, up to 18 months when they were sacrificed, all rabbits showed clear clinical signs of arthritis, such as swelling and redness in all joints, and became almost completely immobilized. Histological evidence of arthritis is however, not available, since histopathology of the joints was not examined.

DISCUSSION

Glycosylation of the Fc region of IgG is of vital importance for the full expression of the proper Fc effector functions. There are many different glycoforms of IgG, each probably with a unique profile of effector functions. Antibodies of the IgG class are produced during the secondary immune response and the proportions of each IgG glycoform within the specific response to a given antigen could differ widely. Although differences in the functional activity of differently glycosylated IgG has been observed and pathogenicity of glycoforms devoid of galactose (G0) has been demonstrated, the dynamics of changes in carbohydrate content of IgG during the course of humoral immune responses remains largely unsolved. At present little is understood about the mechanisms that regulate changes in the glycosylation profile of IgG during immune responses.

The results of our work showed that variations in galactose content of serum IgG occurred during the humoral anti-BSA response in rabbits. A change in IgG galactose content was seen soon after the initial injection, as well as after the later boosts. In relation to IgG obtained before immunization, both increased and decreased galactose content was registered in IgG over the entire immunization period studied. However, a certain tendency of galactosylation to fluctuate in a special way was manifested. Namely, after an initial, rather rapid rise within the first month, galactose content declined gradually within the next two months, a trend shown to be similar in both phases of prolonged immunization, which lasted three months each and which were separated with a rest period of seven months. These findings are very similar to those obtained by Lastra *et al.* (1998) in mice immunized with BSA. The humoral anti-BSA response in their experimental model was shown to be accompanied by an initial rise and later fall in IgG galactose content. IgG antibodies became less galactosylated as anti-BSA titers increased and more galactosylated as titers fell. The authors interpreted such findings as to suggest that "at some time points and in some circumstances a decrease in IgG galactosylation can occur at the time IgG synthesis is greatest, and conversely as the rate of IgG synthesis decreases IgG galactosylation increases". The same

could be seen in our model; the galactose content of IgG climbed slowly during the rest period of seven months and returned to the values registered prior to immunization. However, the rapid transient rise in galactose content (decrease in G0 level) at the beginning of immunization, seen in both models, remains unclear. It may be interesting to consider an old experiment of De Vos-Cloetens *et al.* (1971), who showed that immunization of rabbits with BSA or TMV stimulates concomitant synthesis of specific antibodies and immunoglobulins which are unable to react with the injected antigen but are in some way specifically related to it. The stimulation of these non-specific immunoglobulins was most marked in the early phase of either the primary or anamnestic response. The fact that serum anti-BSA antibodies in our experiment were detected in the majority of animals 15 to 18 days after antigen injection, and 5 to 10 days after antigen injection IgG had a galactose content higher than before immunization, may suggest the possibility that the population of IgG with increasing galactose content belonged to these non-specific, antigen related, immunoglobulins. In our experiment this "early" IgG with increased galactose content was detected only after the first dose of antigen which, in both immunization phases, was emulsified in FCA. Further inoculations were without FCA, and were followed by IgG with decreased galactose content (increase in G0 level) regardless, however, of the intervals of antigen injection. Although exposure to mycobacteria has been observed to result in increased serum G0 in arthritis-prone mice (Rook *et al.*, 1991; Bodman *et al.*, 1994), other data showed an initial fall in %G0 in mice immunized with antigen in FCA (Pilkington *et al.*, 1996). Rapid transient lowering of %G0, detectable in the ELISA, in the serum of mice 3-4 hours after a single injection of collagen dissolved in acetic acid and emulsified in FCA has been ascribed to the bacterial-mimetic characteristic of G0 IgG and the possible ability of some of the ingredients of the adjuvant to induce the mechanism (other than FcR binding) by which IgG terminating with GLcNAc on the Fc oligosaccharides may be removed from the circulation (Pilkington *et al.*, 1996).

It is not clear why changes in the relative extent of galactosylation occur during the immune response. IgG galactosylation is known to be controlled by a galactosyl transferase that mediates the transfer of galactose to an acceptor, so agalactosyl IgG production has generally been ascribed to a decrease in galactosyl transferase activity of B lymphocytes (Alavi and Axford, 1996). However, our findings confirm that during the immune response the glycoform profile fluctuates, and particularly that galactosylation of specific IgG antibodies can vary independently of the total IgG galactosylation, which remains relatively constant (Lustra *et al.*, 1998). This is suggestive of heterogeneity within the proliferative antibody-producing clone. The mechanism governing this heterogeneity is, however, not known. The fluctuating glycoform profile may reflect clonal fluctuation in relative levels of galactosyltransferase (Nishiura *et al.*, 1990), or postsecretory degradation of N-linked oligosaccharides (Parekh *et al.*, 1989), selection by different turnover rates among IgG molecules with different degrees of galactosylation (Newkirk *et al.*, 1996), or altered microenvironments, particularly concerning cytokines released in inflammatory reactions (Hitsumoto *et al.*, 1992) or in "immunization stress" (Bodman *et al.*, 1994). These may be the

underlying cause of the dominance of a particular glycoform at some time points during the response. On the other hand, there is evidence that the structure of the IgG molecule itself is an important parameter which affects the type of oligosaccharide attached, and the arm preference for galactosylation was seen to be related to the IgG subclass and, even, heavy chain allotypes in man (Jefferis *et al.*, 1998). It is, therefore, possible that the fluctuations in IgG galactosylation during the immune response reflect the temporal distribution of isotypic or allotypic variants of anti-BSA antibodies galactosylated in a subclass/allotype-specific manner. Rabbits, however, unlike other mammalian species, which usually have several IgG subclasses, possess only one IgG isotypic variant (Nezlin, 1998). IgG-Fc carries two group e allotypes and the glycans are characterized by having a relatively high level of hypogalactosylated structures, approximately 35% G0 in the Fc region (Rademacher *et al.*, 1996). In rabbit Fc, the opposing oligosaccharides in the CH2 domains possess different sequences and conformations (one of the $\alpha 1 \rightarrow 3$ arms is devoid of galactose, and the other can either be devoid of galactose or terminate in a single galactose residue), but only certain oligosaccharides "fit together following the steric requirements of the protein-carbohydrate and carbohydrate-carbohydrate interactions within the CH2 domains" (Taniguchi *et al.*, 1985). Whether or not e allotypes, which correlate with a Thr (e14) to Ala (e15) interchange at position 309 (Kehoe and Seide-Kehoe, 1979), which is near the attachment point for oligosaccharide, influence the local conformational changes and, thus, the G0-type structures is not clear. Murray and Brown (1988) found that conformational changes in the Fc region of autologous IgG occur during hyperimmunization of rabbits, which they suggested could be due to glycosylation pattern alterations, but serologic analyses performed to detect e and other allotypic markers during hyperimmunization, failed to reveal any alteration in preexisting allotypic expression. It may be, therefore, that the fluctuations in the degree of galactosylation that we observed in IgG of hyperimmune rabbits at different time points actually reflect intracloonal variations or the emergence of subclones of cells secreting IgG that is differently galactosylated from the IgG of nonimmunized animals. Variations were observed in the neutral oligosaccharide profile of IgG secreted by murine hybridomas (Rothman *et al.* 1989), as well as of individual IgG paraproteins at different stages of the disease in multiple myeloma in man, whilst that of the polyclonal IgG component remained stable (Farooq *et al.*, 1997). This suggested that intracloonal changes of glycan composition may occur within the proliferative clone. However, in contrast to these non-physiological situations where the culture medium nutrients and pH, proliferative state of the cells (malignancy) or other extreme conditions were identified as the sources of variability in glycosylation phenotype, galactose variations in IgG during the humoral response in immunized rabbits might be a part of the subtle, naturally occurring, transient alterations in carbohydrate structure, by which the glycans contribute to the different IgG-Fc effector functions within the single IgG isotype. It has been established that glycosylation of IgG-Fc is essential for recognition and activation of IgG-Fc receptors differently expressed on leucocytes (Jefferis *et al.* 1998), and that conformation and effector activities of antibodies may be modulated by removal

of particular sugar residues (Krapp *et al.*, 2003). Since isotypic polymorphism of IgG (subclasses), which determines the effector potential(s) of IgG antibodies (Jefferis *et al.*, 1998), does not exist in rabbits, glycosylation may be what defines functional subsets of isotypically uniform antibodies produced during an immune response in these animals.

The obtained results may be of particular interest since hyperimmune rabbits in our experiment have been shown to produce autoanti-IgG antibodies of the rheumatoid factor type reactive with anti-BSA antibodies (Milošević-Jovčić *et al.*, 1995), and to develop arthritis. It has been shown that IgG with a decreased content of terminal galactose (G0IgG) is a target for rheumatoid factor (Newkirk, 1996), which is the characteristic autoantibody associated with rheumatoid arthritis in man.

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**PROMENE U STEPENU GALAKTOZILACIJE IgG KUNIĆA TOKOM DUGOTRAJNE
IMUNIZACIJE GOVEĐIM SERUMSKIM ALBUMINIMA**

ĆIRIĆ DRAGANA I MILOŠEVIĆ-JOVČIĆ NADEŽDA

SADRŽAJ

Ugljeni hidrati su strukturni element imunoglobulina G (IgG), bitan za konformaciju i efektorne funkcije molekula. Ugljenohidratni sadržaj čine oligosaharidni lanci (glikani) vezani kovalentno za konzervirani Asn 297 u svakom CH2 domenu Fc regiona IgG molekula. Sadržaj pojedinih šećera koji ulaze u sastav glikana nije isti u svim molekulima IgG. Imunoglobulini G su populacija različitih glikoformi čiji sadržaj varira pod fiziološkim, a naročito pod raznim patološkim uslovima. Predpostavlja se da su te varijacije odraz promena u distribuciji specifičnih antitela IgG klase tokom tekućeg, često hroničnog, imunog odgovora na neki antigen. O dinamici tih promena se, međutim, malo zna, a posebno o pojavi glikoformi tokom imunog odgovora koje bi mogle imati patogenetski značaj. U ovom radu su analizirane promene u glikozilaciji serumskih IgG tokom imunog odgovora kunića dugotrajno imunizovanih goveđim serumskim albuminima. Rezultati su ukazali da je taj odgovor praćen permanentnim promenama u sadržaju terminalne galaktoze. S obzirom da su molekuli IgG kunića, za razliku od drugih sisara, izotipski uniformni, tj. nemaju podklase (kojima je determinisana brojnost efektorne funkcije IgG), razlike u galaktozilaciji bi mogle determinisati pojavu funkcionalnih subsetova IgG antitela stvorenih tokom imunog odgovora kod ove vrste.