

Glycosylation of IgA is required for optimal activation of the alternative complement pathway by immune complexes

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SUMMARY

To investigate the effect of carbohydrate on activation of the alternative pathway of complement by IgA immune complexes, aglycosylated monoclonal IgA was made biosynthetically in the presence of tunicamycin. When immune complexes were incubated with normal human serum (NHS), the aglycosylated IgA immune complexes caused less depletion of the alternative pathway activity of the serum. They also bound less C3 and produced less terminal complement complexes. The binding of C3 to both immune complexes was mainly through hydroxylamine sensitive ester bonds. C3 did not bind to free IgA.

INTRODUCTION

The various classes and subclasses of immunoglobulins differ in their ability to activate complement. For classical pathway activation, initiated by C1q binding to the Fc region of immunoglobulins, IgG1 and IgG3 are good activators, IgG2 and IgM are weaker and IgG4 and IgA (which lacks a C1q binding site) are not active. In contrast, IgG1, IgG3 and IgM do not significantly activate the alternative pathway, whereas IgA is a much better activator of the alternative pathway.^{1,2}

An important step in alternative pathway activation is the deposition of C3b, produced by fluid phase C3 convertase, on to a receptive surface. When C3b deposits on a non-activator surface, it is quickly inactivated by factor I in collaboration with factor H. When C3b deposits on an activator surface, it becomes more resistant to cleavage by factor I due to a lower affinity of factor H for the activator surface. Then, an amplification C3 convertase, C3bBb, forms and more C3b molecules are produced and deposit on to the activator surface. There are a variety of activators of the alternative pathway, such as zymosan, agarose, viruses, bacteria, parasites, and some tumour cells.³ A common feature of complement activators in the alternative pathway is the presence of carbohydrate.⁴

IgA is heavily glycosylated. IgA1 has two potential *N*-linked glycosylation sites and five *O*-linked sites on its heavy chain. IgA2 does not have *O*-linked sites but has more *N*-linked sites.

In addition, IgA J chain contains 8% carbohydrate and the secretory component contains 22% carbohydrate.⁵ This carbohydrate could be important in complement activation.

To understand the role of carbohydrate in complement activation by IgA immune complexes (IC), an aglycosylated IgA was made biosynthetically using tunicamycin, an antibiotic which inhibits *N*-glycosylation by blocking the dolichol-dependent, asparagine-linked glycosylation pathway.^{6,7} Then, the properties of glycosylated and aglycosylated IgA IC in complement activation were compared.

MATERIALS AND METHODS

Preparation of IgA2 and aglycosylated IgA2 (IgA2cf)

IgA2 and IgA2cf were produced by a monoclonal cell line, JW393/A (European Collection of Animal Cell Cultures, Salisbury, U.K.). These cells produce IgA2 chimeric anti-5-iodo-4-hydroxy-3-nitrophenacetyl (NIP) antibody which has mouse variable regions and human constant regions.⁸ To produce IgA2, the cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Sigma, Poole, U.K.) containing 10% heat-inactivated fetal calf serum (FCS; Globepharm Limited, Surrey, U.K.) until saturated. To produce IgA2cf, the cells were grown to 2 litres and harvested by centrifugation. Then the cells were continually grown in 2 litres of DMEM containing 2.5 µg/ml tunicamycin (Boehringer Mannheim GmbH, Mannheim, Germany) for another 48 hr. Supernatants were collected and concentrated by ultrafiltration. IgA2 and IgA2cf were affinity purified through a NIP-caproate-*o*-succinimide (Cambridge Research Biochemicals, Cambridge, U.K.) cross-linked AH-Sepharose (Pharmacia, Middlesex, U.K.) column and eluted by 3 M potassium thiocyanate (KSCN). Further separation of IgA2cf from IgA2 was achieved through a concanavalin A (Con A)-agarose column (Sigma) in 5 mM sodium acetate buffer containing 0.1 M NaCl, 1 mM CaCl₂ and 1 mM MnCl₂, pH 5.2. IgA2 bound but IgA2cf did not bind to the column.

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Abbreviations: APB, alternative pathway buffer; BSA, bovine serum albumin; IC, immune complexes; IgA2cf, aglycosylated IgA2; NHS, normal human serum; NIP, 5-iodo-4-hydroxy-3-nitrophenacetyl; OD, optical density; PEG, polyethylene glycol 6000; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCC, terminal complement complex.

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NIP-bovine serum albumin (NIP-BSA)

NIP-caproate-*o*-succinimide (10 mg/ml) was mixed with an equal volume of 5 mg/ml BSA in 50 mM carbonate-bicarbonate buffer containing 0.5 M NaCl, pH 9.6 at 4° overnight. NIP-substituted BSA was separated from free NIP by a Sephadex G-25 (Pharmacia) gel filtration column. The molar ratio of NIP to BSA was 15.6 to 1 by measurement of optical density (OD) at 430 nm for NIP with a molar extinction coefficient of 4900. BSA concentration was measured by the Folin method.

Western blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli.⁹ Proteins were transferred on to nitrocellulose paper (Schleicher & Schuell, Dassel, Germany) by MilliBlot™-SDE Electroblots Apparatus (Millipore, Watford, U.K.). To visualize carbohydrate, the nitrocellulose paper was stained by peroxidase-conjugated Con A using 4-chloro-1-naphthol (Sigma) as substrate. To detect IgA and its Fab fragment, ¹²⁵I-labelled goat anti-human IgA (Sigma) or goat anti-mouse IgG (Fab-specific) antibody was used. To visualize all proteins, the nitrocellulose paper was stained using amido black.

Affinity of IgA2 and IgA2cf

A modified Farr test¹⁰ was used for measurement of the affinity of IgA2 and IgA2cf. One hundred and fifty microlitres IgA2 or IgA2cf was mixed with an equal volume of NIP-CAP-OH containing trace amounts of N[¹²⁵I]P-CAP-OH. Fifteen microlitres normal human serum (NHS) was included as carrier protein for the following precipitation. The final antibody concentration was 50 nM and the final hapten concentration ranged from 10 nM to 2500 nM. After 1 hr incubation at room temperature, bound hapten was precipitated by 50% saturated ammonium sulphate. The supernatant and the precipitate were counted and the affinity constant of IgA2 and IgA2cf was calculated from the Scatchard plot.

IC

The antigen and antibody ratio at equivalence was determined using an Ouchterlony assay. All IC were formed at equivalence ratio in the following experiments. IgA IC and IgAcf IC are barely soluble even in five times antigen excess. To make preformed IC, NIP-BSA and antibody were mixed at 4° overnight. The precipitate was obtained by centrifugation.

Haemolytic assay

Alternative pathway activity of NHS was determined using a haemolytic assay.¹¹ Eighty micrograms antibody was mixed with NIP-BSA at equivalence ratio and serially double-diluted in 200 μ l APB (veronal-buffered saline with 1 mM MgCl₂ and 10 mM EGTA, pH 7.4) containing 10% NHS. The mixture was incubated at 37° for 1 hr and 40 μ l 1% suspension of rabbit erythrocytes was added. The mixture was then incubated at 37° for another 30 min. The reaction was stopped by adding 0.5 ml cold veronal-buffered saline containing 10 mM EDTA. After centrifugation, the OD of the supernatant was read at 412 nm. Haemolytic activity of NHS incubated with IC was compared with that of NHS in the absence of IC. Samples were tested in duplicate.

Detection of C3 binding and terminal complement complex (TCC) production by ELISA

Forty micrograms IgA2 and preformed IC (40 μ g IgA2 or IgA2cf with an equivalent amount of NIP-BSA) in 200 μ l of a 1 in 6 dilution of NHS in APB were incubated at 37° for 1 hr. To measure TCC, a 96-well plate was coated with 1.5 μ g/well monoclonal anti-neo-C9 antibody (a kind gift from Dr R. Würzner, Innsbruck, Austria).¹² The serum mixture was serially double-diluted in 2% BSA/PBS/Tween 20. TCC was detected by biotin-labelled anti-C6 antibody (a kind gift from Dr R. Würzner)¹² followed by peroxidase-conjugated streptavidin (Sigma). To measure C3 deposition, the serum mixture was precipitated with 6% polyethylene glycol 6000 (PEG) (Merck Ltd, Lutterworth, U.K.)/10 mM EDTA at 4° for 1 hr in order to reduce background.² After washing with 6% PEG/10 mM EDTA, the precipitate was redissolved in 100 μ l PBS. A 96-well plate was coated with 2 μ g/well anti-mouse IgG (Fab specific) antibody. C3 bound to IC was detected by sheep anti-human C3 antibody (produced in our own laboratory) conjugated with alkaline phosphatase. To measure IgA2 and IgA2cf in the precipitate, a 96-well plate was coated with anti-mouse IgG (Fab-specific) antibody and bound antibody was detected by alkaline phosphatase-labelled anti-mouse IgG (Fab-specific) antibody.

Detection of C3 binding on SDS-PAGE

Twenty micrograms IgA2 and preformed IC (20 μ g IgA2 or IgA2cf with an equivalent amount of NIP-BSA) in 100 μ l of a 1 in 4 dilution of NHS in APB were incubated at 37° for 1.5 hr. [¹²⁵I]IgA2, [¹²⁵I]IgA2cf and [¹²⁵I]human C3 were used as tracers. Then 10 mM EDTA and 5 μ l Sepharose CL-4B beads coupled with anti-mouse (Fab-specific) antibody or monoclonal anti-C3c antibody (clone 4) (produced in our own laboratory)^{13,14} were added and left at 4° overnight. After thoroughly washing with PBS/NP-40/10 mM EDTA, the beads were boiled for 3 min in 0.12 M Tris/HCl, pH 6.5 containing 1% SDS and 2% 2-mercaptoethanol (Merck Ltd) and the supernatant was directly loaded on to 5–15% SDS-PAGE. The gel was autoradiographed at –70°.

Hydroxylamine treatment

Two-dimensional SDS-PAGE was used to check the bond between C3 and IC. The first-dimensional gel was the same as above except that two tracers ([¹²⁵I]IgA2 and [¹²⁵I]C3 or [¹²⁵I]IgA2cf and [¹²⁵I]C3) were used together. The gel with the band of C3-IgA2 or C3-IgA2cf was cut and soaked in 1 M hydroxylamine-0.1 M NaHCO₃-0.1% SDS, pH 10, for 2 hr at 37°. Then the gel pieces were equilibrated with stacking gel buffer before being loaded on to the second-dimensional gel.

RESULTS**Inhibition of carbohydrate synthesis by tunicamycin**

Tunicamycin, an *N*-glycosylation inhibitor, can be used to make aglycosylated IgA2 through biosynthesis as IgA2 only has *N*-linked oligosaccharide side chains.^{5,15} After purification, IgA2cf showed no detectable carbohydrate by Con A on Western blots. The heavy chain molecular weight decreased to 53,000, about 7,000 less than glycosylated IgA2 heavy chain (Fig. 1). The affinity constants of IgA2 and IgA2cf were $2.51 \times 10^7/M$ and

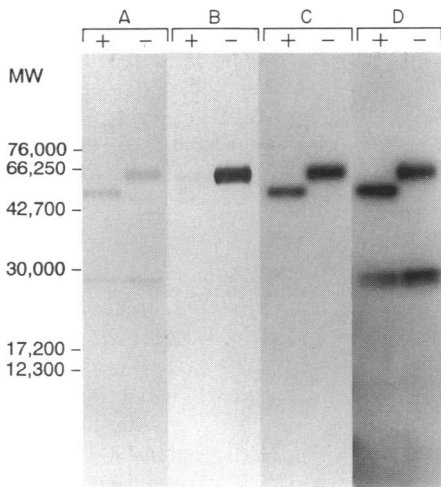


Figure 1. SDS-PAGE (10–20%) under reducing conditions. Western blots of IgA2 produced in cells grown with (+) or without tunicamycin (-). (A) stained by amido black; (B) detected by Con A-peroxidase conjugate; (C) detected by [¹²⁵I]anti-human IgA antibody; (D) detected by [¹²⁵I]anti-mouse IgG (Fab-specific) antibody.

2.79 × 10⁷/M respectively. The affinity retained after deglycosylation suggested that the protein folding was probably correct.

Consumption of complement by IC

The level of alternative complement pathway activity which remained in NHS after incubation with IC was measured using a haemolytic assay. Rabbit erythrocytes incubated with a 1 in 10 dilution of NHS in the absence of IC were used as control, which gave 65% lysis of total cells. NHS incubated with IgA2 IC retained less complement activity than when incubated with IgA2cf IC (Fig. 2). IgA2 IC appear to be at least five times as potent as IgA2cf IC in depleting alternative pathway activity in NHS.

TCC production

TCC production was quantified by ELISA as an indicator of complement activation. When incubated in NHS, IgA2 IC

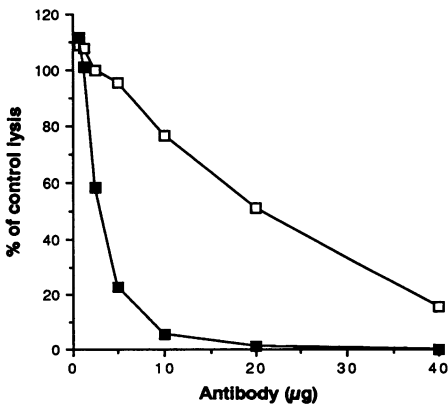


Figure 2. The effect of IgA2 IC and IgA2cf IC in alternative pathway activation. Remaining haemolytic activity in NHS incubated with IC is compared with that of NHS in the absence of IC and expressed as the percentage OD of lysed rabbit erythrocyte. (—■—) IgA2-NIP IC; (—□—) IgA2cf-NIP IC.

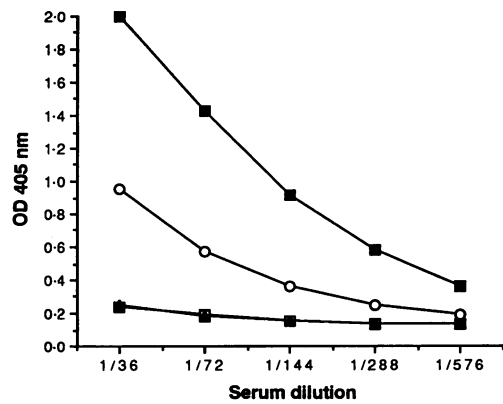


Figure 3. Detection of TCC formation by ELISA. IgA2 and preformed IC in NHS were incubated at 37° for 1 hr. The serum mixture was serially double diluted and then assayed for TCC formation as described in Materials and Methods. (—■—) IgA2 IC + NHS; (—○—) IgA2cf IC + NHS; (—▲—) IgA2 + NHS; (—□—) NHS.

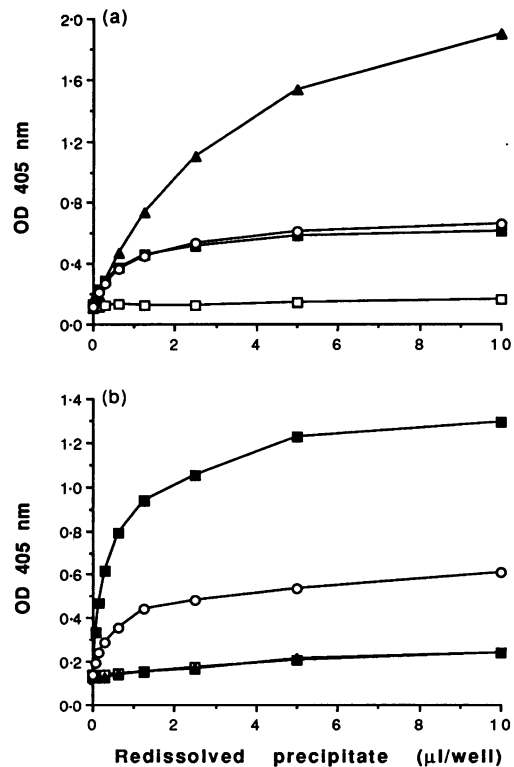


Figure 4. Detection of C3 binding by ELISA. Each well received redissolved precipitate (see Materials and Methods) as indicated in a volume of 100 µl in 2% BSA-PBS-Tween 20. (a) Detected by alkaline phosphatase-conjugated anti-mouse IgG (Fab-specific) antibody; (b) detected by alkaline phosphatase-conjugated sheep anti-human C3 antibody. (—■—) IgA2 IC + NHS; (—○—) IgA2cf IC + NHS; (—▲—) IgA2 + NHS; (—□—) NHS.

generated approximately four times as many TCC as did the same amount of IgAcf IC. IgA2 alone did not promote TCC production (Fig. 3).

C3 deposition on IC

C3 deposition on IgA2 IC and IgA2cf IC was quantified by ELISA. Equal amounts of IgA2 IC and IgA2cf IC were obtained by precipitation with 6% PEG (Fig. 4a). Free IgA could also be precipitated in 6% PEG and gave stronger signals than IgA IC when detected by anti-mouse antibody. The lower signal given by IC IgA may be caused by steric interference with the accessibility of the anti-mouse IgG antibody. However, there was no detectable C3 deposition on free IgA2 and at least twice as much C3 bound to IgA2 IC as to IgA2cf IC (Fig. 4b).

Direct evidence for C3 binding to IC was shown by SDS-PAGE. After IgA2 IC were incubated with NHS, a common 175,000 MW band appeared on SDS-PAGE under reducing conditions when traced by [¹²⁵I]C3 and [¹²⁵I]IgA2 (Fig. 5). This indicated the covalent binding of IgA2 heavy chain (60,000 MW) to either α' chain (110,000 MW) of C3b or to two 68,000 MW chains of iC3b. Similarly, after IgA2cf IC were incubated with NHS, a common band also appeared when traced by [¹²⁵I]C3 and [¹²⁵I]IgA2cf. The band, however, had a lower molecular weight (167,000 MW). Free IgA2 did not show any covalent binding of C3 after incubation with NHS. Some C3 might non-covalently associate to the beads and did not wash off in the experimental conditions (lane 5). Very strong heavy- and light-chain bands of IgA2 IC and IgA2cf IC still remained after affinity purification by anti-C3 antibody. These IgA2 and IgA2cf were non-covalently associated with C3 through IC.

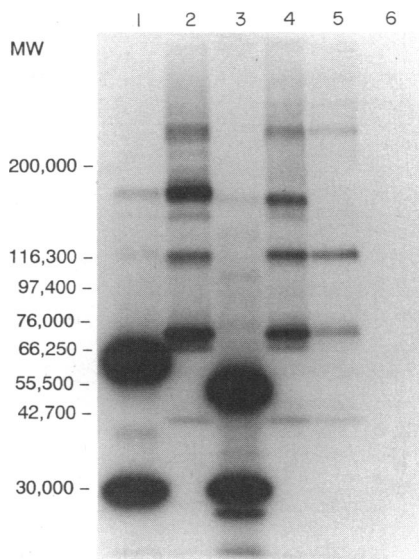


Figure 5. Detection of C3 binding on SDS-PAGE (5–15%) under reducing conditions. Lanes 1 and 2, IgA2 IC; lanes 3 and 4, IgA2cf IC; lanes 5 and 6, IgA. Samples were incubated with NHS and affinity purified by monoclonal anti-C3c antibody beads (lanes 1, 3, 6) or anti-mouse IgG (Fab-specific) antibody beads (lanes 2, 4, 5). [¹²⁵I]IgA2 (lanes 1 and 6), [¹²⁵I]IgA2cf (lane 3) and [¹²⁵I]human C3 (lanes 2, 4 and 5) were used as tracers.

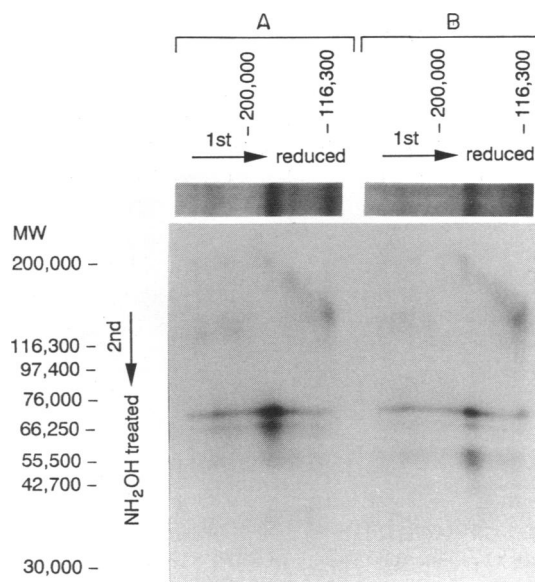


Figure 6. Two-dimensional SDS-PAGE of C3-IgA2 and C3-IgA2cf after treatment with hydroxylamine (see Materials and Methods). (A) C3-IgA2 complex containing strip; (B) C3-IgA2cf complex containing strip. First-dimensional gel was 5–15% and second-dimensional gel was 6–16% under reducing conditions.

The bond between C3 and IC

Hydroxylamine treatment was used for analysing the bond between C3 and IC. On a two dimensional gel, both C3-IgA and C3-IgAcf complexes were cleaved into two main bands, i.e. a 68,000 MW fragment band of α -chain of iC3b (the product of factor I-mediated cleavage of C3b, also observed by others^{16,17}) and a heavy-chain band from IgA2 or IgA2cf (Fig. 6). Therefore during the 1.5hr incubation, the initially bound C3b has been subsequently inactivated to iC3b by the combined action of factors I and H. The sensitivity of C3-IC to hydroxylamine cleavage indicated that the bond between C3 and IC was an ester.

DISCUSSION

Carbohydrates play a very important role in the biological functions of immunoglobulin including solubility, diversity, conformation, blood clearance, complement fixation and Fc receptor binding.^{18–23} In complement activation by the classical pathway, removal of carbohydrate from mouse IgG2a caused a threefold reduction in affinity for C1q binding.²⁴ Similarly, a mutant of anti-NIP antibody (IgG2b) in which the normally glycosylated asparagine at position 297 in the CH₂ domain was substituted by alanine became non-lytic to sheep red blood cells coated with NIP.²⁵ In this paper, we demonstrate that alternative pathway activation by IgA2 IC greatly depends on its carbohydrate component. Without carbohydrate, IgA2 IC are much weaker activators of complement. C3 binding and TCC production decreased dramatically.

Both the classical and alternative pathways of complement activation need C3 activation. Although both nascent C3b and C4b use their opened thiolester bond to bind suitable groups on receptive surfaces, they differ in their preference for groups. One isotype of C4b, C4A, forms an amide bond with an NH₂ group

while the other isotype, C4B, forms an ester bond with an OH group.^{26,27} C3b mainly forms the ester bond.^{16,28} In this paper, we show that C3 binding to carbohydrate-free IgA immune complexes is also mainly through an ester bond. So it is not surprising that carbohydrate, as it can offer abundant OH groups, is important for complement activation, especially for the alternative pathway.

Although carbohydrate is very important in complement activation, it is not the only substance which can activate alternative complement pathway. In this paper, we show that removal of carbohydrate from IgA does not totally abolish its ability to activate complement, even though the activation becomes very weak. In addition, IgG2, an immunoglobulin which is not heavily glycosylated, is also a good activator of the alternative pathway.¹

It has been shown by other authors that IgM immune complexes do not activate the alternative pathway although IgM is heavily glycosylated.^{1,8} Compared to other myeloma immunoglobulins, IgM differs in oligosaccharide structure. Its peripheral *N*-acetylglucosamine residue is substituted at position 6 rather than at position 4.²⁹ The significance of this difference in complement activation is not known.

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