The effect of aglycosylation on the binding of mouse IgG to staphylococcal protein A

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Aglycosylated IgG produced by hybridoma cells cultured in the presence of tunicamycin was compared with normal IgG for its ability to bind to staphylococcal protein A. No differences were found in binding or elution profiles. It is concluded that aglycosylation does not produce major structural alterations at the C_H2–C_H3 interface of the Fc region of IgG.

Mouse IgG2a *Staphylococcal protein A* *Tunicamycin* *Role of carbohydrate* *Aglycosylation* *IgG secondary function*

1. INTRODUCTION

Staphylococcal protein A is a cell wall component which occurs in many strains of *Staphylococcus aureus* and is found to interact with most mammalian IgGs [1,2] and also IgA and IgM of certain species and subclasses ([3] and references therein). The interaction is of high affinity [4,5] and is specific for the Fc region of the immunolobulin [6]. The protein A molecule comprises 5 domains; 4 are highly homologous and are Fc binding whereas the fifth, C-terminal domain, acts to anchor the protein to the cell wall and does not interact with Fc [7,8]. Partial tryptic digestion produces univalent low-M_r fragments which retain the binding ability of the parent molecule [9]. One of the fragments (fragment B) has been co-crystallized with human $Fc\gamma_1$ and shown to bind at the C_H2–C_H3 domain interface [10,11], a position also implicated from work on binding of protein A to IgG fragments [4]. The binding of protein A to IgG is therefore the most structurally well characterized of all the IgG secondary interactions.

The role of the conserved C_H2 domain oligosaccharide of IgG is not clear, although glycosidase treatment has been reported to interfere with IgG secondary functions [12,13]. The location of the carbohydrate between the two C_H2 domains has led to proposals that it acts to separate these two domains [14] and/or to stabilize the native Fc structure [15,16]. Due to its unique specificity protein A has been widely used as an immunological tool (review [1,2]). Its defined binding site on IgG also allows it to be used as a structural probe for the integrity of this site. This paper compares the interaction with protein A of normal IgG and aglycosylated IgG (produced by hybridoma cells cultured in the presence of tunicamycin – an antibiotic which specifically inhibits *N*-glycosylation [17,18]) and therefore allows an assessment of whether any gross structural changes occur in the Fc region in the absence of oligosaccharide attachment.

Abbreviations: DNP, 2,4-dinitrophenyl; BSA, bovine serum albumin; FCS, foetal calf serum; PBS, phosphate buffered saline; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; CPB, citrate-phosphate buffer containing BSA

2. MATERIALS AND METHODS

2.1. *Materials*

Protein A–Sepharose CL-4B was obtained from Pharmacia. Tissue culture media were obtained

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from Gibco, tissue culture vessels from Falcon. L-[4,5-^{3}H]Leucine (197 Ci/mmol) and D-[1-^{14}C]-glucosamine hydrochloride (57.9 mCi/mmol) were purchased from Amersham International. BSA and Dowex 1X8-400 were from Sigma. Protosol was from New England Nuclear. Tunicamycin was generously provided by Dr M. Potter, NIH, Bethesda.

2.2. *Cell culture*

Radio-labelled IgG was produced by culturing the hybridoma cell line K3 (a DNP-binding mouse IgG2a hybridoma, generously made available by Dr B.A. Askonas, Mill Hill) in RPMI 1640/10% FCS/2 mM glutamine/5% CO_2 ± tunicamycin in the presence of 2 μCi/ml [^{3}H]leucine (to label protein) and 1 μCi/ml [^{14}C]glucosamine (to label amino sugars) essentially as in [18]. Cells (2×10^6/ml) were pre-incubated for 4 h ± tunicamycin before the addition of radiolabel, and the supernatant harvested 36 h later. Preliminary experiments had indicated that under these conditions a tunicamycin concentration of 2 μg/ml (for the particular batch of tunicamycin used) produced total inhibition of glycosylation and so this was the concentration used.

2.3. *Isolation of radiolabelled IgG*

Radiolabelled IgG was isolated on columns of DNP–lysine–Sepharose. After washing with PBS the IgG was eluted with 50 mM DNP–glycine pH 7.2, which was subsequently removed on a Dowex 1X8-400 column equilibrated in PBS.

2.4. *SDS–PAGE*

Radiolabelled IgG samples were run on 9% SDS–PAGE [19]. Reduction of samples was with 2-mercaptoethanol. Gels were sliced into 2-mm sections and the radioactivity solubilized into 0.3 ml of Protosol:water (9:1) overnight before counting in tolulene-based scintillant.

2.5. *Protein A–Sepharose chromatography*

For the protein A–Sepharose chromatography the buffer system used was 0.05 M citric acid/0.1 M Na_2HPO_4/BSA (1 mg/ml) (CPB). The BSA (which does not interact with protein A) was found to be necessary to prevent non-specific adsorption of the IgG at such low concentrations to the glassware. The column of protein A–Sepharose (16 mm × 4.4 mm) was equilibrated with CPB (pH 8) and the labelled IgG loaded in the same buffer. After washing with this buffer, the column was eluted with a linear gradient of CPB (pH 8) to CPB (pH 3) (25 ml + 25 ml) at a flow rate of 30 ml/h. The fractions (0.5 ml) were counted in a tolulene-based scintillant.

3. RESULTS

The SDS–PAGE profiles of K3 IgG radiolabelled in culture in the presence and absence of 2 μg/ml tunicamycin are shown in fig.1. As has been demonstrated [18,20,21], the IgG produced is still secreted from the cells and from fig.1 it is seen that under the conditions used it is totally aglyco-

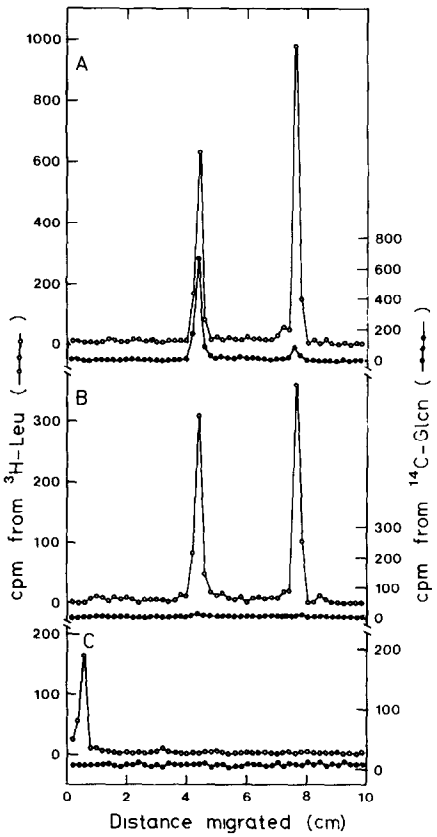

Fig.1. SDS–PAGE (9% acrylamide) of IgG synthesized by the K3 cell line cultured in medium containing [^{3}H]leucine and [^{14}C]glucosamine. (A) in the absence of tunicamycin, (B,C) in the presence of 2 μg/ml tunicamycin. In (A) and (B) the samples were reduced with 2-mercaptoethanol; (C) is unreduced.

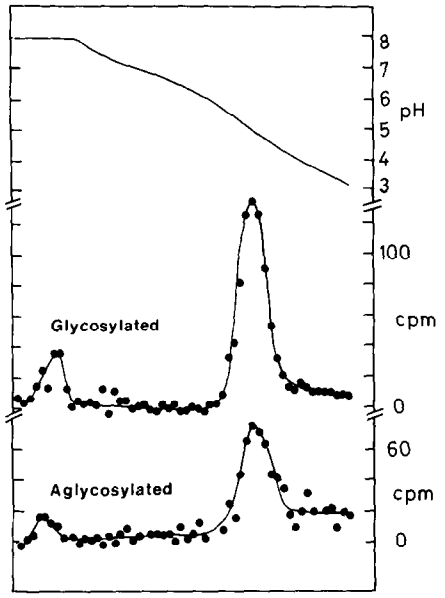

Fig.2. The binding and pH elution profiles of radiolabelled glycosyl and aglycosyl IgG to protein A–Sepharose.

sylated. In the absence of reducing agents the aglycosylated IgG migrates as a single 150 000 M_r band indicating that assembly to a disulphide-linked H_2L_2 unit occurs normally in the absence of carbohydrate. Since the aglycosylated IgG still binds to the DNP–Sepharose affinity column hapten binding is also not affected. The binding/elution profiles of these biosynthetically radiolabelled glycosylated and aglycosylated forms of K3 IgG to protein A–Sepharose are shown in fig.2. Both IgGs are found to bind equally well to the column. Moreover, both glycosylated and aglycosylated proteins elute with the gradient in an identical manner, at the expected position for a mouse IgG2a [22].

4. DISCUSSION

Immobilized staphylococcal protein A has been widely used in the affinity purification of IgG. More recently it has been found that the conditions for elution of IgG from protein A–Sepharose differ for different IgG subclasses. This has been used in the purification of IgG subclasses of many species [22–27]. Perhaps the best example is with mouse IgG [22], where each of the main subclasses (1, 2a and 2b) may be separated from each other by their position of elution from protein A–Sepharose using a series of buffers of descending pH. Since no other mouse Ig class binds, this affords a rapid and effective purification protocol which would be extremely difficult by more conventional techniques. By implication therefore, the position of elution from a protein A–Sepharose column by a descending pH gradient reflects the differing structure/sequence around the protein A binding site. This is well illustrated in the case of human IgG3 which is not usually considered to bind protein A. The lack of binding is explained by the substitution of His-435 in the protein A binding site by an arginine residue; restoring the histidine as is found in a γ3 allotypic variant, restores binding [28]. The binding to, and conditions of elution from protein A–Sepharose therefore offer an indication of any structural changes around the protein A binding site. Such an approach has been used to show that the hinge-deleted human IgG1(Dob) binds protein A normally [29], reflecting the normal Fc structure found in X-ray crystallographic studies on this protein [30].

The results presented here show that aglycosylated IgG also binds to protein A in a manner which is indistinguishable from normal IgG. The implications of this are 2-fold. Firstly, protein A–Sepharose may be used for purification of aglycosylated IgG in the same manner as for the parent IgG, if required. More importantly however, these results indicate that the conformation of the C_H2–C_H3 domain interface as sensed by protein A remains the same upon aglycosylation.

The protein A binding site is formed by the unique longtitudinal interaction of the C_H2 and C_H3 domains of IgG [10,11]. Any disruption of this quaternary interaction would be expected to result in a loss of binding (individual C_H2 and C_H3 domains do not retain any affinity for protein A [4]). Therefore it is implied that the characteristic (i.e., 'split' C_H2) orientation of the C_H2 and C_H3 domains found in the glycosylated Fc is retained in aglycosylated IgG, since any perturbation to their quaternary interaction would be expected to affect the protein A binding region. It should be noted however, that although gross structural effects upon aglycosylation seem likely in the light of these results, minor structural alterations or destabilization [15,16] localized to the immediate area of the carbohydrate may not be reflected in altered protein A binding.

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