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All Forms of Human IgA Antibodies Bound to Antigen Interfere with Complement (C3) Fixation Induced by IgG or by Antigen Alone

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Polyclonal human secretory IgA1 and IgA2 antibodies to a bacterial protein antigen *Streptococcus mutans* AgI/II, and polyclonal human serum IgA1 and IgA2 antibodies to staphylococcal α-toxin, were found to interfere with antigen-mediated C3b fixation. In fluid phase, immune complexes of antigen and IgA failed to fix C3b, whereas antigen-IgG complexes did fix C3b. Partial removal of glycan chains with *Streptococcus mitis* SK96 glycosidases diminished the capacity of IgA antibodies to interfere with antigen-mediated C3b fixation by the alternative complement pathway. The authors conclude that native serum or secretory IgA antibodies suppress C3b fixation, and that the glycan chains play a significant role in maintaining this property.

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INTRODUCTION

It has been shown previously that human monoclonal IgA1 (obtained from the sera of myeloma patients) having antibody activity against staphylococcal α-toxin (SAT) and fails to fix C3 by either the classical or alternative pathway (CCP and ACP, respectively), when bound to solid-phase antigen [1, 2]. Furthermore, these human IgA1 antibodies interfere strongly with C3 fixation by IgG antibodies to the same antigen, regardless of whether or not IgA1 and IgG antibodies are mutually competitive in binding to the antigen, and independent of the polymeric (p) or monomeric (m) molecular form of IgA1 [2]. Yet the same IgA1 preparations bound directly to plastic fix C3 under conditions appropriate for ACP activation, in accordance with numerous other reports involving interfacially denatured, heat-aggregated, or chemically modified human IgA of various sources [3–6]. Most of the literature reports similarly show a lack of complement activation by human IgA antibodies bound to antigen [7, 8]. However, several studies, especially those using experimental animal IgA monoclonal antibodies (MoAbs), show complement activation by the ACP [9–11]. This has been reported also for a chimeric human-rat IgA2 antibody [12]. One problem with MoAbs and myeloma proteins is that they may be physiologically abnormal, and unrepresentative of normal polyclonal antibodies. Furthermore, human IgA is heterogeneous in molecular form (mIgA, pIgA, and secretory IgA [SIgA]) and occurs in two subclasses (IgA1 and IgA2), the latter of which has two allotypes. The physiological properties of these different forms have not been fully elucidated, but they are known to differ in certain respects [13].

Therefore, the objective of the studies reported in this paper was to determine whether all forms of normal polyclonal human IgA antibodies, bound to antigen, behave in the same way as monoclonal IgA1 myeloma-derived antibodies with respect to fixation of C3. The authors have determined also the effect of carbohydrate depletion on the ability of human IgA antibodies to interfere with C3b fixation.

MATERIALS AND METHODS

Immunoglobulins. Monomeric and polymeric IgA of both subclasses were separated from myeloma plasma (provided by Dr J. Mestecky). IgA also was obtained from normal human serum and secretory IgA of both subclasses from normal human colostrum. Serum from a patient with subacute bacterial endocarditis due to *Streptococcus mutans* [14] was used as a source for isolation of IgG and IgA with antibody activity to *S. mutans* protein AgI/II [15].

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Serum IgG and IgA were separated by chromatography on a Mono Q column (Pharmacia LKB Biotechnology, Piscataway, NJ, USA) with a gradient of 0–0.5 M NaCl. The IgA-rich fractions were freed of IgM and IgG impurities on anti-IgM and protein G HiTrap affinity columns (Pharmacia). The anti-IgM column was prepared by coupling rabbit anti-human IgM antibody (Dako Corp., Carpinteria, CA, USA) to a HiTrap NHS-activated Sepharose column (Pharmacia). To separate the IgA1 and IgA2 subclasses, a jacalin affinity column was prepared by coupling jacalin (Vector Laboratories Inc., Burlingame, CA, USA) to a HiTrap NHS-activated Sepharose column, and equilibrated with 0.175 M Tris.HCl pH 7.5 as running buffer. IgA2 was collected from the effluent, and IgA1 was eluted with 0.1 M melibiose in Tris buffer pH 7.5 [16]. The IgG fractions were chromatographed on a protein G HiTrap column in 0.02 M phosphate buffer, pH 7.4, and eluted with 0.2 M glycine HCl, pH 2.2, into tubes containing 0.1 volume of 1 M Tris.HCl pH 9.5.

SIgA was isolated from normal human colostrum. Cells and lipids were removed by centrifugation and the samples were depleted of casein by acidification to pH 4.2 with 2% acetic acid, followed by centrifugation and neutralization. Then the samples were precipitated with 50% saturated ammonium sulfate, centrifuged and dialysed. Lactoferrin was removed by affinity chromatography on a Heparin-Sepharose HiTrap column (Pharmacia) [17]. The IgA-rich fractions obtained by chromatography on a Mono Q column as described above were subjected to jacalin-affinity chromatography to separate SIgA1 and SIgA2, as described above. Traces of IgM and IgG were removed on anti-IgM and protein G columns as described above.

Ig and antibody concentrations were determined by ELISA. Anti-IgA, anti-IgG and anti-IgM antisera (Dako) (dilution 1:1000) or *S. mutans* Ag I/II (5 μg/ml) were used to coat plates in borate-buffered saline pH 8.2 (BBS). Appropriate dilutions of the samples were applied to the plates and incubated overnight at room temperature. After several washings with 0.01 M phosphate-buffered saline pH 7.3 (PBS) containing 0.05% Tween 20, anti-IgA, anti-IgG or anti-IgM antibodies conjugated to peroxidase (Dako) were used as developing reagents. The colour developed with *o*-phenylenediamine/H_2O_2 substrate was read at 490 nm. As a standard, serum with defined Ig isotype concentrations was used (The Binding Site, Birmingham, UK).

Fluid-phase immune complex formation. Equimolar quantities of AgI/II and IgG or IgA antibodies respectively, were incubated in PBS for 1 h at 37°C, then applied to a Superose 6 FPLC gel filtration column (Pharmacia). The collected fractions were tested for the presence of antigen-antibody complexes by ELISA. The peak fractions showing the presence of both were treated for 1 h at 37°C with normal human serum at 1/5 dilution, as a source of complement. After chromatography on the same column the fractions were tested for the presence of AgI/II, IgG or IgA respectively, and C3b by ELISA.

ELISA for immune complex detection. Plates were coated overnight with anti-IgA or anti-IgG (Dako) at 1:1000 dilution, washed in PBS-Tween and the fractions applied to them. After overnight incubation at room temperature, bound immune complexes on replicate plates were developed with anti-AgI/II conjugated to peroxidase or anti-C3b (Dako) conjugated to peroxidase. The colour formed with OPD/H_2O_2 as substrate was read at 490 nm.

Enzyme treatment. Streptococcus mitis SK96 (donated by Dr M. Kilian, University of Aarhus, Denmark) was grown in semi-defined culture medium to stationary phase [15]. The 2-l culture supernatant was precipitated with 50% saturated ammonium sulfate, centrifuged and dialysed against PBS to a final volume of 6 ml. Purified samples of 0.5 mg of SIgA1 and SIgA2 were treated overnight at 37°C with 0.2 volume of *S. mitis* enzyme preparation, known to contain neuraminidase and other glycosidase activities, but no protease activity [18]. After this treatment the samples were subjected to gel filtration on a TSK-GEL G-3000SW column (30 × 0.75 cm; TosoHaas, Montgomeryville, PA, USA), and eluted with 0.02 M phosphate, 0.5 M Na_2SO_4, pH 6.7, to separate the SIgA from enzyme and cleavage fragments. In parallel SIgA was incubated overnight in PBS at 37°C, subjected to the same chromatography procedure, and used as a control in ELISA and carbohydrate analysis. The antibody activity of enzyme-treated and control samples was measured by ELISA as described above, and both preparations were adjusted to the same antigen-binding activity.

Analysis of carbohydrates. The monosaccharides were determined as trifluoroacetates of methyl glycosides by gas chromatography as described [19], with a few modifications. Aliquots of IgA and IgG preparations (20 and 50 μg, respectively) were methanolyzed in 0.5 M HCl in methanol and methyl glycosides were derivatized with a mixture of ethyl acetate, and trifluoroacetic anhydride (85:15) in 0.5 ml teflon-capped reagent vials. The analyses were performed with a Hewlett-Packard model 5890 gas chromatograph equipped with a 25 μm fused silica (0.22 mm inner diameter) OV-1701 WCOT column, electron capture detector, and model 3396 integrator.

Complement fixation assays. ELISA plates were coated overnight with 10 μg/ml of *S. mutans* AgI/II, or for 4 h with 5 μg/ml avidin followed by washing and coating with 10 μg/ml biotinylated SAT (Calbiochem, San Diego, CA, USA). After several washings with PBS-Tween, serial two-fold dilutions of IgG, IgA1, or IgA2, starting at specific antibody concentrations of 5 μg/ml for IgG or 2 μg/ml for IgA1 or IgA2, were added to the plates. When inhibition of C3b fixation was tested, IgA1 or IgA2 was added before, together with, or after the IgG samples. To assess C3b fixation under conditions appropriate for CCP activation, the samples were treated with normal human serum as a source of complement at a final dilution of 1:25 in 0.01 M PBS pH 7.4 containing 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 15 mM NaN_3 and incubated at 37°C for 20 min. For assaying C3b fixation under conditions appropriate for ACP activation, the complement source was diluted at 1:5 in PBS containing 10 mM EGTA (ethylene glycol-bis-(β-aminoethyl ether) tetra-acetic acid) and 10 mM $MgCl_2$ and incubated for 1 h at 37°C [5]. The bound C3b was revealed by development with peroxidase conjugated anti-C3c (Dako) or anti-C3b (Janssen Biochimica, Accurate Chemical and Scientific Corp., Westbury, NY, USA).

To examine the effect of IgA on complement activation by the antigen itself, the plates were coated with 5 μg/ml of AgI/II in BBS overnight at room temperature. After washing in PBS-Tween, IgG, SIgA1 or SIgA2 antibodies to AgI/II were added in serial two-fold dilutions starting at 2 μg/ml concentration. The Ig preparations were incubated overnight, and after washing in PBS-Tween the plates were processed for ACP activation as described above.

All assays were performed in duplicate and the results are given as the mean of the values obtained (OD 490 nm). Each experiment was repeated at least four times with two different donors of serum as sources of complement and two different polyclonal anti-C3b reagents. In preliminary trials of the method, essentially similar results were obtained with monoclonal anti-C3c and anti-C3d reagents (Quidel, San Diego, USA).

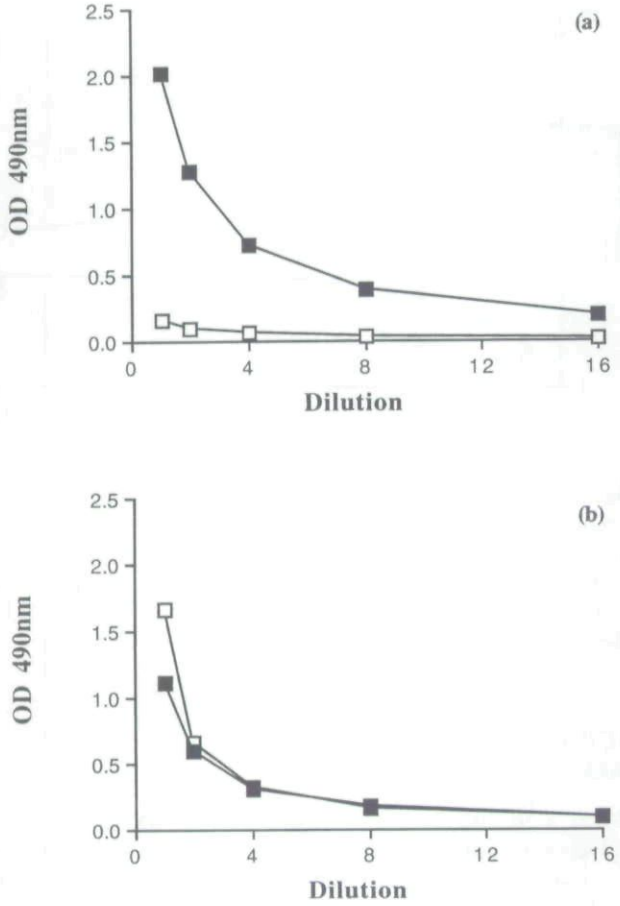

Fig. 1. Solution-phase fixation of C3b by immune complexes consisting of (a) IgA antibody and AgI/II or (b) IgG antibody and AgI/II. Purified complexes were exposed to fresh human serum and serial dilutions were applied to ELISA plates coated with anti-IgA (a) or anti-IgG (b) and developed with peroxidase-conjugated anti-C3b or anti-AgI/II. Each point represents mean OD 490 nm ±SD, which are too small to show. —□—, a-C3b; —■—, a-AgI/II.

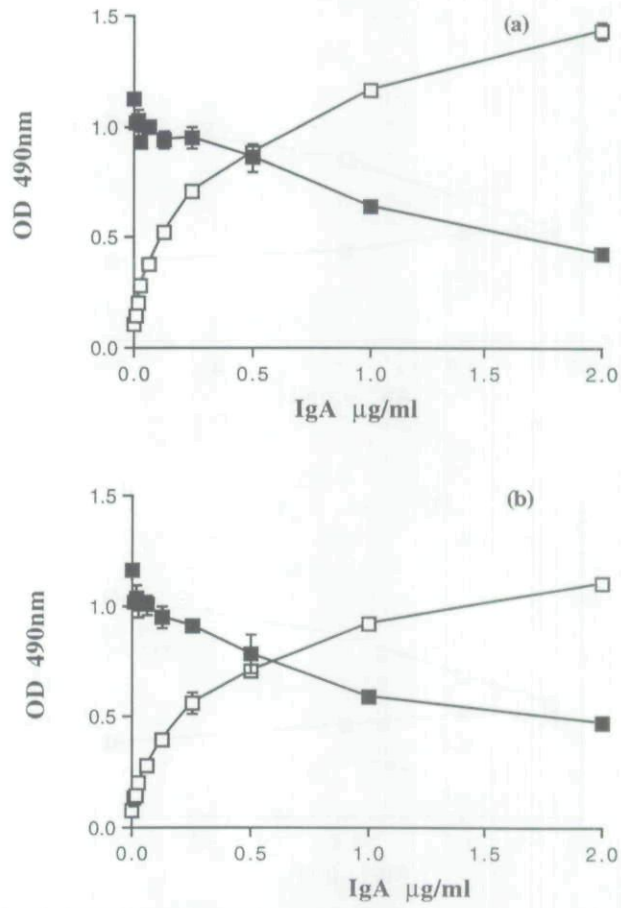

Fig. 2. Inhibition of IgG-initiated CCP activation by (a) SIgA1 or (b) SIgA2 antibodies bound to solid-phase AgI/II. Bound SIgA or fixed C3b were detected with peroxidase-conjugated anti-IgA or anti-C3b respectively. Each point represents mean OD 490 nm ±SD, which may be too small to show. —□—, Bound IgA1; —■—, Fixed C3b.

RESULTS

Interaction of fluid-phase immune complexes with the complement system

When separated soluble immune complexes of IgA1 antibody and AgI/II were exposed to complement, fixation of C3b to the immune complexes was minimal (Fig. 1a). In contrast, similar immune complexes of IgG antibody and AgI/II fixed C3b strongly in the fluid phase (Fig. 1b). The conditions of complement activation for this experiment (serum diluted 1:5 in PBS) would have allowed both CCP and ACP activation. The authors have shown previously that human monoclonal IgA1 antibodies bound to antigen-coated surface failed to fix C3b under conditions favouring either CCP or ACP activation [2]. When serially diluted SIgA1 and SIgA2 antibodies to AgI/II were incubated on plates coated with AgI/II, dose-dependent binding of IgA1 and IgA2 was observed, but there was no fixation of C3b in experiments designed to assess CCP activation. The assay was repeated using higher densities of antigen for coating up to 20 μg/ml but no CCP activation by SIgA1 and SIgA2 was observed under these conditions. These findings were in marked contrast to those obtained with IgG antibodies to the same antigen, isolated from the serum of a patient with endocarditis and used in the same concentrations as these of IgA isotype (data not shown). The authors have never observed C3b fixation by intact human IgA antibodies bound to antigen, regardless of the subclass or molecular form of IgA, whether the antigen is soluble or solid-phase bound, or whether conditions favour CCP or ACP activation.

When either SIgA1 or SIgA2 anti-AgI/II antibodies isolated from human colostrum were titrated into 5 μg/ml IgG antibodies and applied to plates coated with AgI/II, C3b fixation by the CCP was inhibited in relation to the dose of IgA1 or IgA2 antibody applied and the amount of IgA1 or IgA2 bound (Fig. 2a, b). Apparently, binding of IgG antibodies, as measured by ELISA, was not affected. Both SIgA1

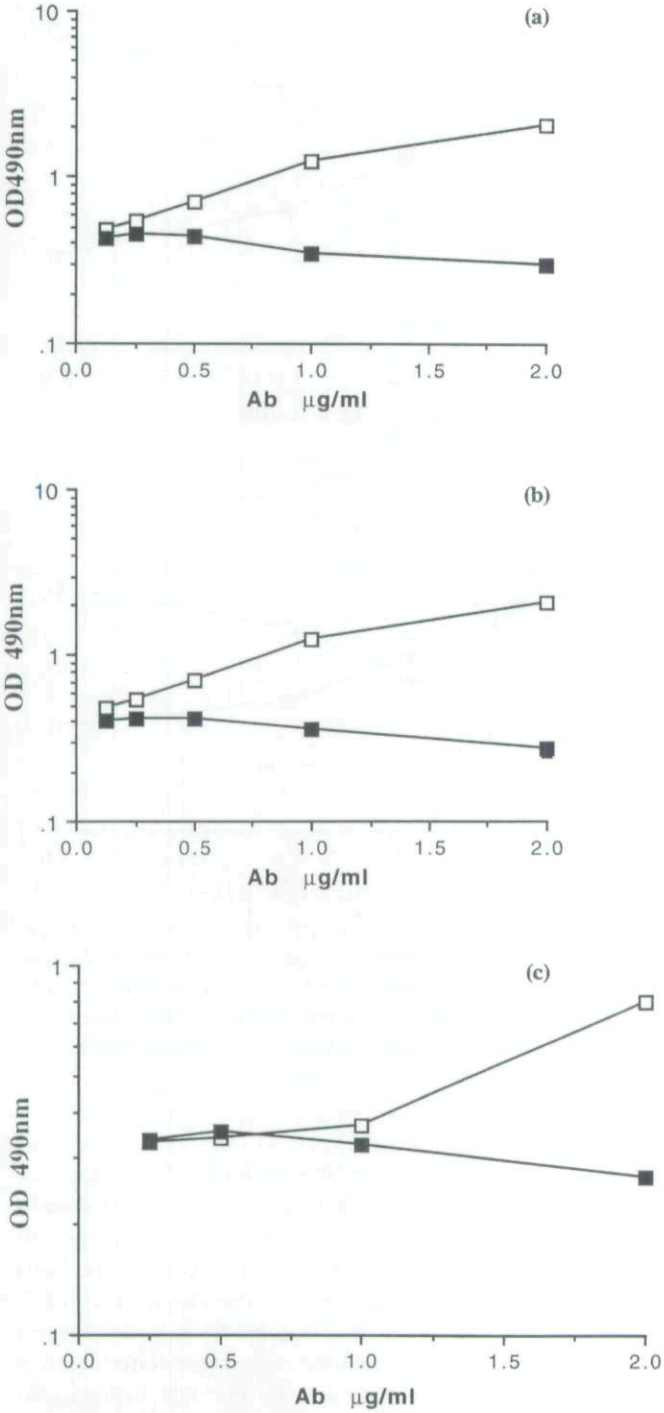

Fig. 3. Inhibition of antigen-mediated C3b fixation under conditions favouring ACP activation by (a) SIgA1, (b) SIgA2, and (c) serum IgA1 antibodies, compared to IgG antibodies. Each point represents mean OD 490 nm ± SD (which are too small to show), obtained with peroxidase-conjugated anti-C3b. (a) —□—, IgG, —■—, SIgA1. (b) —□—, IgG; —■, SIgA2. (c) —□—, IgG, —■—, IgA1.

and SIgA2 revealed similar inhibitory effects, and the time of their application, before, together with, or after the IgG preparation made no difference to the inhibition.

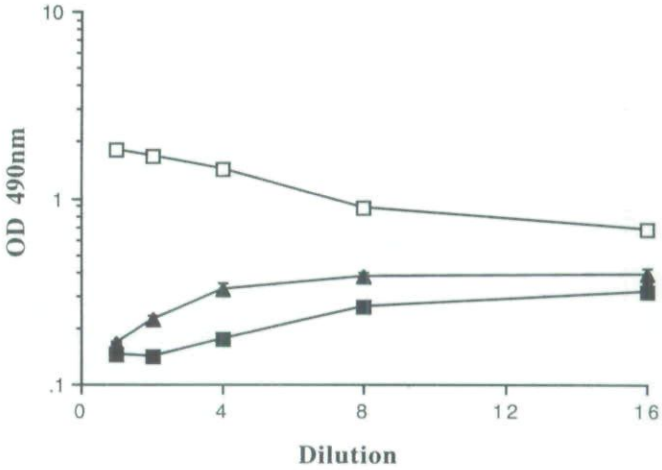

Fig. 4. Inhibition of antigen-mediated C3b fixation under conditions favoring ACP activation by SIgA antibodies treated or untreated with *S. mitis* SK96 enzymes, compared to IgG antibodies. Each point represents mean OD 490 nm ± SD, obtained with anti-C3b conjugate. —■—, S-IgA1/2 control; —▲—, S-IgA1/2 SK96; —□—, IgG.

ACP inhibition by secretory IgA1 and IgA2 polyclonal antibodies

When C3b fixation by the ACP by SIgA1 and SIgA2 polyclonal antibodies bound to AgI/II-coated surfaces was assayed, both SIgA1 and SIgA2 antibodies inhibited antigen-mediated C3b fixation (Fig. 3a, b). The effect was concentration-dependent, and no difference between the two IgA subclasses was noted. A similar result was obtained with polyclonal serum IgA1 with antibodies to SAT (Fig. 3c). In contrast, IgG antibodies enhanced C3b fixation (Fig. 3).

Influence of the oligosaccharides on SIgA antibody-dependent ACP inhibition

Certain oral streptococci have been shown to secrete glycosidases (which include neuraminidase and other glycosidase activities) capable of removing oligosaccharides from human IgA [18]. The authors found that IgA treated with glycosidases from *S. mitis* SK96 (which does not produce IgA1 protease [18]) and coated directly on plastic, display enhanced C3b fixation by the ACP (EB Niklova *et al.*, unpublished observation). To determine whether similar treatment of SIgA antibodies to AgI/II would reverse inhibition of antigen-mediated C3b fixation, the authors treated SIgA antibodies with *S. mitis* SK96 enzymes overnight, chromatographed them to remove the enzymes and performed the same ACP assay for antigen-bound SIgA antibodies. Enzyme-treated SIgA had diminished capacity to interfere with C3b fixation (Fig. 4). Carbohydrate analysis of SIgA after enzyme treatment and the control samples is shown in Table 1. The treatment with *S. mitis* SK96 enzymes resulted in partial removal of N-linked sugar residues and depletion of more

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Table 1. Depletion of carbohydrates from SIgA treated with *S. mitis* SK96 glycosidases

Removal of sugar residues (%)					
Fuc	Man	Gal	GlcNAc	GalNAc	NANA
32	20	24	24	17	56

than half of the sialic acid. Despite incomplete removal of the glycan chains, the ability of the treated SIgA antibodies to interfere with C3b fixation was diminished.

DISCUSSION

These results with polyclonal SIgA1 and SIgA2 antibodies to *S. mutans* AgI/II extend previous findings with monoclonal myeloma IgA1 and IgG antibodies to SAT [2], thereby showing that the capacity of human IgA antibodies to interfere with CCP activation does not depend on molecular form, and is not an abnormal property of pathological myeloma proteins. As C1q binding by IgG has been located in three highly conserved amino-acid residues in the CH_2 domain [20] that are not present in IgA1 or IgA2, the substitution of IgA for IgG molecules in an immune complex would be expected to diminish CCP activation, and the authors showed previously that C1q binding to IgG antibodies was inhibited when IgA antibody competitively displaced IgG from binding to antigen [2]. Furthermore, inhibition was independent of the order in which IgG and IgA were applied, as reported also by Jarvis *et al.* [21]. However, as IgA-mediated inhibition of C3b fixation was not necessarily dependent upon displacement of IgG and inhibition of C1q binding, there must be additional mechanisms involved. Such mechanisms may include binding of C1q without activation of C1, as shown by Hiemstra *et al.* [22], or possibly interference with subsequent steps leading to C3 convertase formation [2, 23]. IgA1 antibodies were reported to be more effective than IgA2 in the inhibition of IgG-initiated CCP-mediated bacteriolysis of *Neisseria meningitidis* [21], but IgA2 antibodies are more likely to be directed at the capsular polysaccharide of this organism [13] rather than at components of the outer membrane where the membrane attack complex is assembled. In the authors' system, by measuring C3b deposition, the authors observed essentially no difference in interference by equivalent amounts of SIgA1 and SIgA2 antibodies on the same antigenic surface.

The authors' results differ also from those obtained by Valim & Lachmann [12], using a chimeric monoclonal human-rat IgA2 anti-hapten antibody which activated the ACP when bound to haptenated protein. Several factors may explain these different findings. The authors used deposition of C3b on the solid phase as the measure of activation, and it is possible that further C3 conversion occurred in the fluid phase. However, C3b deposition on the surface is important for the major anti- microbial effector activities of complement activation, i.e. opsonization or the assembly of the membrane attack complex to cause cytolysis, and stabilization of C3 convertase would be necessary for IgA to permit fluid-phase activation of C3. Several reports [9–11] have shown that various species of animal monoclonal anti-hapten IgA antibodies can activate the ACP. It is possible that there are significant differences between human and animal IgA in this regard. In addition, the ability of heavily haptenated protein antigens to activate the ACP [24] also must be considered, as the binding of an antibody to a haptenated protein may result in conformational changes that would enhance the ACP-activating property of the antigen. However, other reports have shown that animal IgA antibodies either fail to activate or inhibit activation of the ACP [25–29], and most reports concerning intact human IgA antibodies physiologically bound to conventional antigens fail to show complement activation [1, 7, 8, 30]. In the purification of IgA antibodies, the authors were careful to avoid procedures that might result in aggregation or denaturation, such as the use of lyophilization, or desorption from immunoaffinity columns by means of denaturing solvents. The authors consider this important, because, purified IgA antibodies that fail to activate the ACP when bound to antigen do activate it when deposited on a solid plastic surface [1], or aggregated, denatured, or chemically modified IgA [3, 4, 31]. Although it is not known currently what alterations in the structure or conformation of the IgA molecule are responsible for these effects, partial deglycosylation by enzymes derived from *S. mitis* [20] suggests a role for oligosaccharide chains which constitute approximately 12% of the serum IgA molecule. Comparison between normal serum IgA1 and IgA1 myeloma proteins reveal different carbohydrate contents of these molecules of normal and pathological origin [32], and a recent report shows that glycosylation of chimeric human-rat antibodies produced in transfectoma cell lines may be abnormal [33]. Studies of many glycoproteins have illustrated the frequent importance of carbohydrate groups for proper folding [34]. Mutant α heavy chains are folded differently from the wild-type counterpart, and the absence of carbohydrate from CH1 or the CH3 domains alters the assembly of the IgA molecule [35].

However, the results presented here and in other published reports support the authors' contention that native human serum and secretory IgA antibodies have anti-inflammatory properties by suppressing C3b fixation.

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