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Complement-Fixing Properties of Human IgA Antibodies

Alternative Pathway Complement Activation by Plastic-Bound, But Not Specific Antigen-Bound, IgA

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The complement-fixing properties of human IgA antibodies bound to specific antigen, or coated directly on plastic surfaces, were examined in comparison with those of IgG antibodies. Use was made of antigen-binding (anti-staphylococcal α-toxin) IgA and IgG monoclonal antibodies and normal polyclonal IgA and IgG, purified >99.9% by avoidance of denaturing processes. Complement-fixation ELISA was used, with a high density of biotin-conjugated staphylococcal α-toxin bound to avidin-coated plates for the efficient capture of antibodies, and conditions were adjusted for the assessment of classical and alternative pathways of complement activation. Although IgA coated directly on plastic surfaces activated the alternative complement pathway in a dose-dependent manner, IgA antibodies bound to antigen failed to fix complement by either classical or alternative pathways. In contrast, IgG antibodies, either bound to antigen or coated directly on plastic, activated complement mainly by the classical pathway. It was concluded that the complexation of IgA antibodies with antigen is insufficient to elicit complement activation: rather a degree of denaturation seems to play a part in the expression of alternative complement pathway-activating properties by IgA.

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The anti-infectious properties of systemic antibodies depend largely upon their ability to engage the phagocytic and complement systems. Whereas IgG and IgM are well known for their ability to activate these systems and hence fulfil a major anti-microbial defence function, the biological properties of serum IgA are less well understood. Despite its abundance, serum IgA does not appear to play a prominent role in defence against infection, and its ability to activate complement under physiological conditions is controversial (for review see Ref. 19). While it is known that IgA does not bind C1q [1, 12, 16] and hence does not activate the classical complement pathway (CCP), activation of the alternative complement pathway (ACP) by artificially aggregated, denatured, or chemically modified IgA has been reported by several workers [5, 11–13]. Most of these investigations have used human IgA myeloma proteins of unknown antigen-binding specificity, as it has been difficult to obtain large quantities of human IgA of known specificity, free of other isotypes. Although human secretory IgA antibodies [8], and naturally occurring human IgA immune complexes [15] have been shown not to activate the ACP, studies using mouse or rat IgA monoclonal antibodies (MoAb) have demonstrated ACP activation by antigen-complexed IgA [24, 26, 28, 29].

The availability of human myeloma sera containing MoAb of IgA and IgG isotype that recognize defined bacterial antigens [21] enabled us to examine the complement-activating properties of human IgA complexed with corresponding antigen. The complement-fixation enzyme-linked immunosorbent assay (ELISA) of Baatrup *et al.*

Petitioner Merck, Ex. 1203, p. 175
Merck Sharp & Dohme LLC v. Halozyme Inc.
PGR2025-00046

[3] was used, and conditions were adjusted for assessing both CCP and ACP.

MATERIALS AND METHODS

Immunoglobulins. Myeloma sera containing monoclonal Ig that neutralize staphylococcal α-toxin (SAT) were previously described [21]. Serum Tin contained an IgA1λ protein (31.5 mg/ml anti-SAT IgA), Fug contained both IgG1κ and IgA1κ components (8.75 and 3.14 mg/ml, anti-SAT IgG and IgA, respectively), and Kal and JJF contained IgG1κ proteins (3.70 and 7.10 mg/ml anti-SAT IgG, respectively). The sera were fractionated on DEAE–Sepharose CL6B (Pharmacia Biotechnology, Uppsala, Sweden) equilibrated in 0.01 M Tris HCl, pH 8.2. Stepwise elution with 0.1 M Tris HCl, pH 7.65, and 0.1 M Tris HCl, 0.1 M NaCl, pH 7.65, yielded the IgG- and IgA-rich fractions respectively. IgG-Fug was further purified on protein A–Sepharose CL4B (Pharmacia) in 0.01 M phosphate-buffered saline (PBS), pH 7.4, and eluted with a gradient of 0.15 M citrate-phosphate buffer, pH 5.0, to 0.1 M citric acid, pH 2.2 [14]. After neutralization, it was passed sequentially over immunosorbent columns containing anti-human IgM and anti-human IgA (both Dakopatts, Glostrup, Denmark) linked to CNBr-activated Sepharose 4B (Pharmacia), and finally concentrated by re-chromatography on DEAE–Sepharose. IgA-Tin and IgA-Fug were further purified on jacalin–agarose (Vector Laboratories, Burlingame, Calif., USA) and eluted with 0.8 M galactose in PBS [20], passed through columns of anti-IgM and anti-IgG (Dakopatts) coupled to CNBr-Sepharose, and also concentrated by re-chromatography on DEAE–Sepharose. Normal human serum (Nor, source unknown) was fractionated in the same manner to obtain pure normal IgG and IgA, and IgG was purified from another serum (Hi) having a high level of anti-SAT IgG antibodies, estimated at 300 μg/ml.

The concentrations of total and anti-SAT IgA and IgG in these preparations were determined by ELISA (see below) and are given in Table I, which indicates minimal contamination of each preparation with unwanted Ig isotypes. On sodium dodecyl sulphate–polyacrylamide gel electrophoresis after reduction, the preparations revealed two polypeptide bands corresponding to the expected H and L chains. When analysed by high-performance liquid chromatography (HPLC) on a 60×0.75 cm column of TSK-G3000SW (LKB Products, Bromma, Sweden) in 0.1 M phosphate buffer, pH 6.8, IgG-Fug and IgG-Nor eluted with single peaks at the elution volume expected of IgG, IgA-Fug and IgA-Nor both eluted at the elution volume expected of monomeric IgA with only minor peaks indicative of polymeric forms, while IgA-Tin revealed peaks corresponding to polymeric IgA and little monomeric IgA.

ELISA. Immunoglobulins were estimated by ELISA on polystyrene microtitre plates (Nunc, Roskilde, Denmark) coated with anti-human IgG, IgA, or IgM (Dakopatts) diluted 1:1000 in borate-buffered saline, pH 8.4. The plates were blocked in 0.5 M NaCl, 0.01 M phosphate, pH 7.4, 0.15% Tween 20, 0.02% NaN_3, which also served as wash buffer and diluent. Duplicate samples serially diluted twofold were incubated in the wells overnight, and bound Ig were revealed by development with peroxidase-conjugated anti-IgG, anti-IgA, or anti-IgM (Dakopatts; 1:1000 in azide-free diluent). The substrate was 0.5 mg/ml *o*-phenylenediamine, 1 mM H_2O_2 in 0.1 M citrate–phosphate, pH 5.0; colour development was stopped after 5–15 min with 1 M H_2SO_4, and absorbance at 492 nm was measured in a Titertek Multiskan photometer. Calibrations were performed with appropriate dilution series of Serum Protein Calibrator X908 (Dakopatts), and standard curves for interpolation of unknowns were constructed by linear regression of log mean absorbance (less background given by wells lacking sample) on log concentrations of IgG, IgA, or IgM.

In the assay of anti-SAT antibodies, plates coated with SAT in the ordinary way were found incapable of binding the antibodies, possibly because of conformational changes induced in SAT by attachment to the hydrophobic plastic, or because the relevant epitopes were otherwise obscured. This difficulty was satisfactorily overcome by assaying anti-SAT antibodies on plates coated first with avidin (2 μg/ml; Sigma Chemical Co., St Louis, Mo., USA) in borate-buffered saline, pH 8.4,

TABLE I. Assay of IgA and IgG preparations

Ig preparation	IgA (μg/ml)		IgG (μg/ml)		IgM (μg/ml)		Contamination (%)*	
	Total	Anti-SAT	Total	Anti-SAT	Total	Anti-SAT	As Ig	As anti-SAT
IgA-Tin	50	79	<0.02	<0.002	0.042	<0.012	~0.1	<0.02
IgA-Fug	47	43	<0.004	<0.004	0.051	<0.01	~0.1	<0.03
IgG-Fug	0†	0	57	53	0	0	0	0
IgA-Nor	15.8	ND	<0.01	ND	0.013	ND	~0.1	–
IgG-Nor	0	ND	82	ND	0	ND	0	–
IgG-JJF	ND	ND	1340	693	ND	ND	–	–
IgG-Kal	ND	ND	1060	121	ND	ND	–	–
IgG-Hi	<8	0	8420	190	1.6	0	~0.1	0

* Percentage of undesired Ig or antibody isotype present.
† 0 = complete absence of detectable reaction.
ND = not determined.

and then with biotinylated SAT (5 μg/ml) in ELISA diluent. Biotinylated SAT was prepared by treating 1 mg of SAT (Calbiochem, San Diego, Calif., USA) dissolved in 0.25 ml of 0.1 M $NaHCO_3$ with 50 μg of biotinyl-ε-aminocaproic acid *N*-hydroxysuccinimide ester (Sigma) in 50 μl of dimethylsulphoxide, for 4 h at room temperature, and then dialysing against PBS. Wells coated with avidin alone served as controls. The remainder of the ELISA procedure was as described above. The slopes of the dilution curves were similar to those of the corresponding standards in the total Ig assays, and interpolation on calibration curves in most cases gave concentrations of antibodies similar to concentrations of Ig in the purified preparations (see Table I). IgA-Tin consistently gave a result in the antibody assay approximately 1.6 times that in the IgA assay, for reasons that are not clear, but that may be related to its polymeric molecular form.

Complement-fixation ELISA. This was a minor modification of the procedure described by Baatrup *et al.* [3]. In this assay, plates coated with a high density of antigen are treated with antibody and then exposed to fresh serum as a source of complement. C3b bound to the solid phase during activation is detected by means of an enzyme-conjugated anti-C3c or anti-C3d reagent. Microtitre plates were coated with avidin (5–10 μg/ml) followed by biotinylated SAT (10–20 μg/ml), and treated overnight with Ig preparations serially diluted twofold. Fresh-frozen (−70°C) human serum having a low level (~10 μg/ml) of IgG anti-SAT was used as the complement source. For assaying CCP activation, this was diluted 1:25 in 0.01 M PBS, pH 7.4, containing 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 15 mM NaN_3 (PBS^{2+}) and incubated in the antibody-treated wells for 20 min at 37°C. For assaying ACP activation, the complement source was diluted 1:5 in PBS containing 10 mM $MgCl_2$ and 10 mM ethylene glycol-bis(β-aminoethyl ether) tetraacetic acid (EGTA) and incubated in the wells for 1 h at 37°C. Bound C3b was revealed by development with peroxidase-conjugated anti-C3c (Dakopatts) 1:500 in azide-free diluent, followed by peroxidase substrate as described above. In some experiments, peroxidase-conjugated anti-C3d, prepared by coupling anti-C3d (Dakopatts) to horseradish peroxidase (Sigma; type VI) by the two-step glutaraldehyde procedure [2], was used.

In preliminary trials, CCP activity was inhibited by the addition of 10 mM Mg EGTA, and ACP activity was lost on dilution of the complement beyond 1:10. Complement fixation due to anti-SAT antibody was abrogated if the avidin-coated plates were not treated with biotinylated SAT.

To examine complement fixation by Ig directly bound to plastic, the plates were coated with purified IgA or IgG serially diluted in borate-buffered saline by incubation overnight. Complement-fixation ELISA was then continued as described above, for either CCP or ACP activation.

All experiments were performed in duplicate or triplicate, and the results are given as the mean of the values so obtained, which were in very close agreement, less the mean of the appropriate background values. Standard errors were therefore calculated as the standard error of the difference of these means (SED), and were typically in the range 0.005–0.02, which is too small to register on the graphs presented. Each experiment was repeated on at least two occasions, and the results were highly reproducible.

RESULTS

Complement activation by immunoglobulins bound to antigen-coated surfaces

When serially diluted monoclonal IgA-Tin or IgA-Fug, or normal polyclonal IgA, were incubated on plates coated with avidin and biotinylated SAT at the level used for antibody assays, dose-dependent binding of IgA was observed, but there was no fixation of C3 by the ACP associated with bound IgA antibody (Fig. 1). To enhance the binding of the IgA antibodies further, the assay was repeated using plates coated with a higher density of antigen (5 μg/ml avidin and 10 μg/ml of biotinylated SAT). Under these conditions, there was also no activation of the ACP due to increasing doses of bound IgA, but high background colour development in the absence of IgA was observed (Fig. 2). Since this colour development was dependent upon the addition of biotinylated SAT to avidin-coated wells, and upon treatment with the complement source, it appeared that SAT itself induced ACP activation. This could be observed by coating wells directly with SAT alone. Attempts were made to separate the ACP-activating property from biotinylated SAT by HPLC on a TSK-G3000SW column, but the complement activating component co-chromatographed with antigenically detectable SAT. In spite of this problem, IgA antibodies bound to SAT had no discernible effect on ACP activation (Figs 1 and 2). These findings were in marked contrast to those obtained with IgG antibodies, or with the same IgA antibodies coated directly on plastic (see below).

No activation of the CCP was observed by any of the IgA preparations, even when applied to plates coated with antigen at high density (Fig. 2). Individual IgG MoAb often fail to activate the CCP, but show a synergistic effect when mixed together (Ref. 4; and see below). However, if IgA-Tin and IgA-Fug were mixed together and applied to avidin–SAT-coated wells, no enhancement of ACP or CCP activation was observed (Fig. 2).

Results were similar when complement fixation was assayed by means of peroxidase-conjugated anti-C3d or anti-C3c (Fig. 2). This indicated that failure to observe bound C3b was not due to

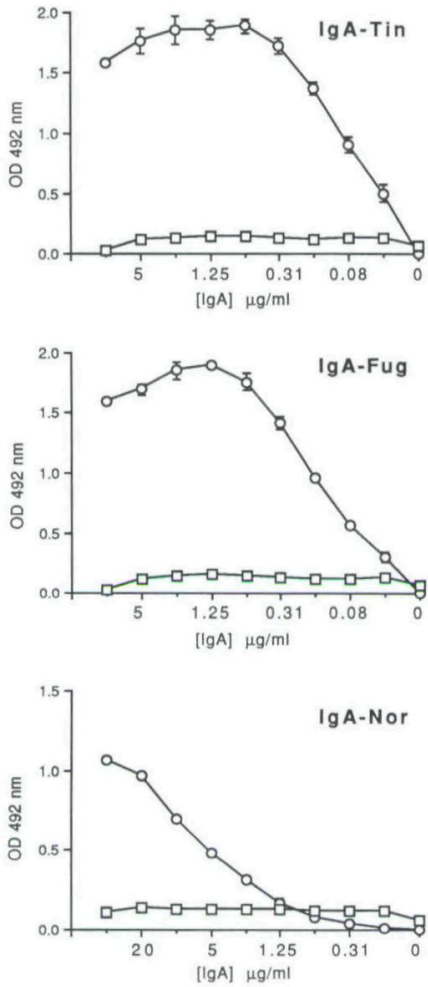

FIG. 1. ACP activation by IgA antibodies bound to plates coated with avidin (2 µg/ml) and biotinylated SAT (5 µg/ml). (□) ACP (revealed with anti-C3c); (○) bound IgA. Each point shows the mean of duplicate experiments (less the mean obtained on control wells coated with avidin only) ± standard error of the difference of the means (SED), which in most cases is too small to show.

cleavage and removal of the C3c fragment by factor I.

In marked contrast to IgA, when polyclonal IgG with high antibody activity was applied to avidin–SAT-coated plates, CCP activation could be demonstrated in relation to the dose of IgG (Fig. 3). Individual IgG anti-SAT MoAb failed to demonstrate much complement-fixing activity, but mixtures of any two were able to activate the CCP, and all three together had an effect approaching that shown by polyclonal IgG (Fig. 3). Polyclonal IgG antibodies bound to avidin–SAT-coated plates activated the ACP in a dose-dependent manner (Fig. 4), but less actively than the CCP. Thus, the assay was capable of revealing ACP activation by antibodies bound to avidin–SAT-coated plates, despite the high background due to ACP activation by the antigen itself.

Since the complement source contained a low level of IgG anti-SAT antibodies, it is possible that these contributed to the activation of complement. Absorption of the complement serum on a column of CNBr–Sepharose-linked SAT in the presence of 10 mM ethylenediamine tetraacetate at 4°C, followed by restoration of Ca^{2+}, eliminated the anti-SAT antibody and preserved 50% of the complement activity, but this only slightly diminished the background complement activation.

Complement activation by immunoglobulins directly coated on plastic

When purified IgA preparations were coated directly on plastic plates, ACP activation dependent on the dose of IgA was observed by means of anti-C3c (Fig. 5). The effect was greatest with polymeric IgA-Tin, lower with monomeric IgA-Fug, and least with normal IgA which was predominantly monomeric. Similar results were obtained with anti-C3d reagent (not shown). These results are in marked contrast to those obtained when the same IgA antibodies were bound by specific antigen to a comparable extent (compare Figs 1 and 2 with Fig. 5). No CCP activation was demonstrated by any of the IgA preparations under these conditions (Fig. 5). For comparison, single monoclonal IgG-Fug or normal IgG directly bound to plastic induced C3 fixation by the CCP, and to a lesser extent by the ACP (Fig. 6). These results are essentially similar to those obtained when polyclonal or mixed monoclonal IgG antibodies were bound specifically to antigen, except that CCP activation by plastic-bound monoclonal IgG did not depend on synergism between different antibodies.

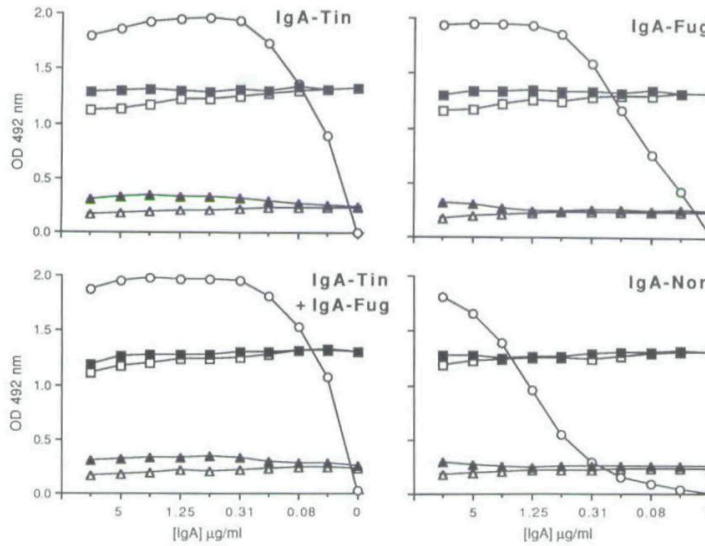

FIG. 2. C3 fixation by IgA antibodies bound to plates coated with avidin (5 µg/ml) and biotinylated SAT (10 µg/ml). (□) ACP revealed with anti-C3c; (△) CCP revealed with anti-C3c; (■) ACP revealed with anti-C3d; (▲), CCP revealed with anti-C3d; (○) bound IgA. Each point shows the mean of triplicate experiments less background (without IgA); SED values were too small to show.

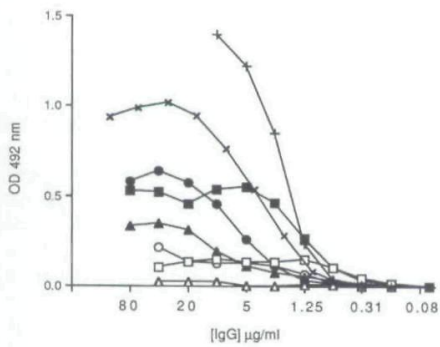

FIG. 3. CCP activation by IgG antibodies bound to avidin–SAT-coated plates. (○) IgG-Fug; (□) IgG-JJF; (△) IgG-Kal; (■) IgG-Fug plus IgG-JJF; (●) IgG-Fug plus IgG-Kal; (▲) IgG-JJF plus IgG-Kal; (×) IgG-Fug plus IgG-JJF plus IgG-Kal; (+) IgG-Hi (polyclonal). Each point shows the mean of duplicate experiments less background (without IgG), which was 0.103 ± 0.003 (SD, $n=6$) for IgG-JJF and IgG-Kal, and 0.135 ± 0.003 (SD, $n=6$) for the others. SED values are omitted for clarity, but typical values were 0.005–0.020, and in all cases <0.035.

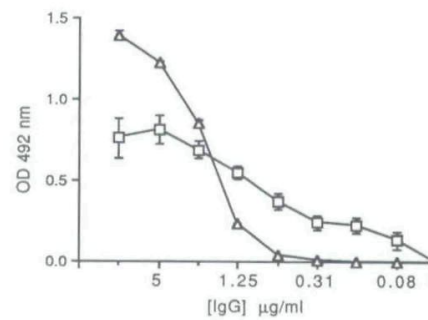

FIG. 4. ACP activation, revealed with anti-C3c, by polyclonal IgG antibodies bound to avidin–SAT-coated plates, compared with CCP activation (data replotted from Fig. 3). (□) ACP; (△) CCP. Each point shows the mean ± SED of triplicate experiments. Note that background ACP activity in the absence of added IgG (0.472 ± 0.020, SD) has been subtracted to facilitate comparison between curves.

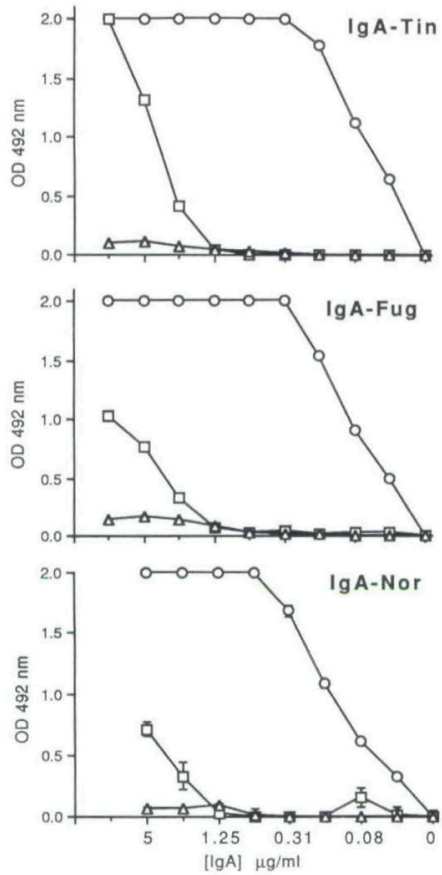

FIG. 5. C3 fixation by purified IgA bound directly to plastic plates. (□) ACP; (△) CCP; (○) bound IgA. Each point shows the mean of duplicate experiments less background (without IgA) ± SED. ACP activation by IgA-Nor was done with complement serum diluted 1:4.

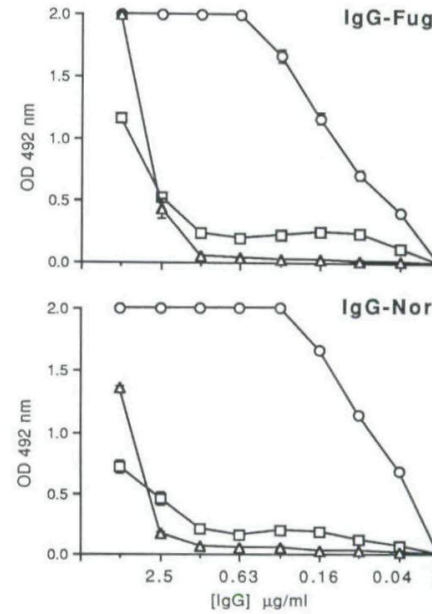

FIG. 6. C3 fixation by purified IgG bound directly to plastic plates. (□) ACP; (△) CCP; (○) bound IgG. Each point shows the mean of duplicate experiments less background (without IgG) ± SED.

DISCUSSION

The present results show that highly purified human serum IgA coated on plastic surfaces could induce complement fixation by the ACP, but that the same IgA antibodies bound to antigen failed to do so. Since adsorption to hydrophobic surfaces frequently induces conformational changes in protein structure, it seems likely that similar denaturation of IgA might be responsible for its ACP-activating property, although it is not known what these changes are. Great care was taken in the purification of IgA to avoid any procedure such as precipitation, ultrafiltration, or exposure to denaturing solvents that might induce conformational change. Failure of plastic-bound (or antigen-bound) IgA to fix complement by the CCP in the present assay agrees with previous findings that IgA does not activate the CCP nor bind C1q [1, 12, 16]. IgA does not possess the amino-acid residues that are highly conserved at three critical positions in the CH2 sequence of IgG for C1q binding [9, 18].

The behaviour of IgA is in marked contrast to that of IgG, which was able to activate the CCP, and to a lesser extent the ACP, upon binding to antigen. Antigen-bound IgG MoAb showed synergism in CCP activation [4], in accordance with the known need for a critical proximity of Fc regions for efficient C1q binding [6]. The need for an analogous juxtaposition of Fc regions for ACP activation seems unlikely, as IgG-mediated ACP activation appears to involve structures in the $F(ab')_2$ fragments [10, 27]. Nevertheless, indi-

vidual monoclonal IgA proteins, when adsorbed to plastic, could activate the ACP, and individual monoclonal IgG proteins could activate both pathways under these conditions. Even the binding to SAT of both monoclonal IgA proteins together, one of which (Tin) was predominantly polymeric, did not result in IgA-dependent complement fixation. In this context, it cannot be argued that IgA-Tin and IgA-Fug compete for binding to SAT for the following reason. Whereas IgG-Fug and IgA-Fug are mutually competitive in binding to SAT, as might be expected because they are probably derived from heavy chain-switch variants of the same neoplastic clone, IgA-Tin and IgG-Fug do not inhibit each other's binding (M.W. Russell *et al.*, unpublished observations). Thus, IgA-Tin and IgA-Fug can bind independently to different determinants on SAT, as also revealed by the additive nature of their binding to SAT seen at low doses (Fig. 2).

Failure to observe C fixation with an anti-C3c reagent in these experiments could not be due to accelerated breakdown of fixed C3b–C3bi by factor I [25], because an anti-C3d reagent gave the same results. Although fluid-phase release of C3b would not be measured, it must be noted that bound C3b is a component part of the C3b.Bb complex that constitutes the C3 convertase of the ACP [25]. This means that there could be no fluid-phase release of C3b due to solid-phase IgA in the complement-fixation ELISA without the binding of at least some C3b. Johnson *et al.* [17] found that complement did not prevent or reverse precipitation of antigen by mouse IgA antibody, implying a lack of complement activation and C3b fixation. On the other hand, Rits *et al.* [28, 29], using rat IgA antibody–antigen complexes and homologous complement, found evidence of C3b fixation as revealed by solubilization of precipitates and by haemolysis. Furthermore, Hiemstra *et al.* [13] observed ACP-mediated haemolysis by human IgA that was treated with the heterobifunctional reagent succinimidyl 3-(2-pyridyldithio)propionate, and then bound to erythrocytes coated with monoclonal mouse IgG1 antibody to IgA. In this case, it might be argued that nascent C3b was able to bind to the IgG or modified red cells, or even to functional groups introduced by the chemical modification of IgA, as treatment of IgA with other cross-linking agents or with heat failed to achieve the same effect [13]. As our results show that only plastic-bound IgA could activate the ACP and bind C3b, it seems likely that ACP activation by human IgA depends on conformational changes induced by aggregation or denaturation on hydrophobic surfaces, or on chemical modification.

In these studies, purified normal IgA having antibody activity against SAT also activated the ACP when bound to plastic, but not when bound to antigen. ACP activation by plastic-bound polymeric IgA-Tin was greater than with monomeric IgA-Fug or IgA-Nor, as noted by others [11, 13, 26, 29]. However, only IgA proteins of subclass 1, which predominates in serum and is selectively bound by jacalin [20], were studied. The primary structure of IgA2, apart from the hinge region, differs from IgA1 by only 20 amino-acid residue substitutions in the constant domains [22]. Whether these could result in different properties with regard to complement activation is not known.

The present results support the concept that serum IgA is a non-inflammatory isotype of antibody whose biological functions, though unclear, are other than defence against infectious agents [19]. A physiological role for IgA can be envisaged in the protection of the internal milieu from the aggressive effects of other immune effector mechanisms [23]. Non-inflammatory IgA antibodies could be involved in restoring homeostasis after an infectious episode, and in alleviating autoimmune conditions. IgA antibodies of appropriate specificity could also be important in controlling inflammatory diseases, including periodontal disease, which develops at the gingival margin. This is a complex interface between the systemic and mucosal compartments of the immune system, where secretory IgA derived from the saliva and serum IgA confront other Ig and cellular elements derived from the circulation. On the other hand, in IgA nephropathy, Henoch-Schönlein purpura, and dermatitis herpetiformis, renal or cutaneous deposits of IgA, probably in the form of immune complexes, are partially associated with the deposition of C3 [7], and have been implicated in the disease process through activation of the ACP. However, it is difficult to discern the role of IgA in complement activation within these lesions, because IgA and C3 deposits do not necessarily coincide, and IgM or IgG may also be present [30]. Our findings and those of Imai *et al.* [15] suggest that the immunopathology of these conditions is not due to complement fixation by IgA immune complexes.

However, since altered or denatured human IgA can activate the ACP, it must be considered whether there are physiological or pathological circumstances in vivo that can cause IgA to develop this property, and it becomes important to determine the nature of the structural changes involved.

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