INDEPENDENCE OF THE BINDING OF DOMAIN-SPECIFIC LIGANDS TO FAB AND FC SUGGESTS THAT ANTIGEN-INDUCED EFFECTS IN IGG ANTIBODIES ARE NOT ALLOSTERIC

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1. Introduction

Alternate mechanisms for the appearance of effector functions in the Fc part of antibodies (e.g., triggering of complement fixation, histamine release, binding to cell receptors) upon antigen binding to the Fab moiety have been proposed [1,2]. One explanation suggested that aggregation of antibody by antigen simply produced large complexes which by virtue of multiple binding interactions exhibited enhanced binding to other multivalent partners such as membrane receptors or the first complement component. A contrasting hypothesis posited that hapten and antigen induced an allosteric change in the conformation of the Fc region of the immunoglobulin.

An allosteric role for the antigen has been recently favored by certain spectroscopic studies [3–5], by complement fixation data [6] and the reactivity of the Fc-bound J-chain in IgM as an index of Fc activation [7]. A recently proposed allosteric model of antigen action extrapolated from a composite X-ray structure [8], involving the formation of new contacts between the CH_1 and CH_2 domains of IgG upon antigen binding is incompatible with recent experimental findings: we have demonstrated that the binding of hapten or antigen to rabbit anti-pneumococcal polysaccharide antibodies did not alter the conformation of the hinge peptides, as evidenced by the unchanged susceptibility of the hinge region to attack by thiols and proteolytic enzymes [9].

Spectroscopic studies have indicated significant conformational changes upon hapten or protein A binding [10,11]. Are the effects of such domain-specific ligands localized within the domain itself, or are they propagated throughout other domains of the IgG antibody? If the allosteric mode of antigen binding to antibody IgG were correct, we would expect the association constant of hapten bound to be altered in the presence of bound protein A to the Fc domain; reciprocally, the dissociation constant of protein A from Fc should vary in the presence of monovalent or polyvalent antigen binders in the Fab domain.

Using a spectroscopic approach, we have attempted to detect the possible existence of allosteric interactions between the Fab and Fc portions of the immunoglobulin molecule by observing the mutual effects of hapten or antigen binding in the Fab region on one hand and the binding of *Staphylococcus aureus* protein A in the Fc domain on the other.

2. Materials and methods

The antibodies used were homogeneous and electrophoretically restricted proteins specific for

type II and III pneumococcal polysaccharide antigen as evidenced by analytical 1EF technique. Whole polysaccharide antigens and smaller oligosaccharide haptens obtained by partial acid hydrolysis were prepared as in [4,12]. Active monovalent protein A fragments were isolated from a partial tryptic digest by affinity chromatography on human IgG–Sepharose 6 B column. The acid-eluted material was further purified by gel-filtration in G-75 Sephadex. An av. mol. wt 6500 was computed from sedimentation equilibrium data. Fragments were monovalent in the Fc reaction as evidenced by inhibition of hemagglutination and by their inability to produce IgG–protein A fragment complexes larger than 6.6–7 S species as determined in the analytical ultracentrifuge. Antibody–protein A fragment dissociation constants were determined by fluorescence titration at 25°C in 0.2 M sodium phosphate buffer, pH 7.4. Fluorescence of antibody solutions (concentrations of free or antigen-liganded antibody, approx. 1–2 μM) was excited by irradiation at 295 nm. Small corrections for the inner-filter effect were applied. On hapten titrations, haptens were added to a final conc. 14–25-fold molar excess over antibody combining site concentration. The highest final concentration of protein A fragments in the titration samples was 8–10 μM. Fluorescence measurements were performed on a Schoeffel fluorimeter equipped with dual monochromators in the excitation light path and a 1000 W Xenon lamp. The fluorescence at the antibody emission maximum, 330–333 nm, was digitalized and averaged in quadruplicate.

3. Results

Because of the absence of tryptophanyl residues in protein A [13], the binding of protein A to rabbit antibodies could be followed by the quenching of antibody tryptophan fluorescence. Preliminary experiments showed that the addition of protein A to large soluble preformed antigen–antibody complexes rendered these aggregates insoluble. To circumvent this problem, active, monovalent tryptic fragments of protein A were prepared [14,15] and used in titration experiments of soluble antigen–antibody complexes. Three different monoclonal antibodies (45–394, GE-10(A) and GE-10(C)) and one antibody of restricted heterogeneity (30–267) to two non-crossreactive pneumococcal polysaccharide antigens (SII and SIII) were used and their reaction with oligosaccharide haptens could be also followed by quenching or enhancement of antibody fluorescence.

The binding of monovalent protein A fragment to large soluble antigen–antibody aggregates was compared to protein A fragment binding to antibody alone (table 1). Antibodies, which exhibited different spectroscopic responses to hapten binding, i.e., quenched (ab 45–394), unaltered (ab 30–267), and enhanced (ab GE 10 A and C) fluorescence (see table 2), apparently underwent similar fluorescence changes upon protein A fragment binding not affecting tryptophanyl residues near the hapten binding sites. The removal of Fab fragment from IgG by papain digestion did not result in a decrease of protein A-induced fluorescence quenching. On the contrary, the greater extent of quenching in the isolated Fc fragment upon protein A binding (20–22%) suggests that the tryptophanyl residues whose fluorescence is quenched are restricted to the Fc region of the IgG molecule. The invariance of dissociation constants of protein A fragments from the Fc moiety of various antibodies in the presence and absence of haptens or antigens strongly suggest a lack of mutual interactions between Fab and Fc regions.

The reciprocal experiment shown in table 2 was to study spectroscopically the binding of a nonasaccharide hapten to two electrophoretic components of the anti-SII pneumococcal polysaccharide antibody GE 10 and the binding of an octasaccharide hapten to anti-SIII pneumococcal polysaccharide antibody 45–394, in the presence and absence of protein A. Table 2 indicates that hapten binding was unaffected with respect to association constants and fluorescence changes upon binding of protein A to any of the 3 antibodies studied.

4. Discussion

The most striking observation which emerges from the set of experiments summarized here is that the binding of hapten or antigen to the Fab region of rabbit antibody and the binding of protein A to the Fc region seem to occur independently (i.e., no mutual Fc–Fab interactions), as judged from fluores-

Table 1
Binding of monovalent protein A fragments to rabbit antibodies and to their hapten antigen complexes[b]

Antibody	Specificity	Hapten or antigen[a] present ($S_{20,w}$)	K_d[b] (μM)	Fluorescence[c] quenching (%)
30–267	SIII	–	0.89 ± 0.09	10
		27 μM octasaccharide	0.98 ± 0.07	9
		Ag–Ab complexes (11–15 S)	0.81 ± 0.10	11
		Ag–Ab complexes (22–44 S)	0.91 ± 0.08	10
45–394	SIII	–	0.99 ± 0.10	9
		25 μM octasaccharide	0.89 ± 0.08	11
		Ag–Ab complexes (6, 13–24 S; 1:4)	0.94 ± 0.11	10
		Ag–Ab complexes (16–24 S)	1.07 ± 0.09	9
		Ag–Ab complexes (6, 28–45 S; 1:4)	0.87 ± 0.12	10
GE 10 (A)	SII	–	0.82 ± 0.07	11
		44 μM octasaccharide	0.93 ± 0.09	9
		Ag–Ab complexes (19–26 S)	0.86 ± 0.08	9
		Ag–Ab complexes (6, 21–36 S; 1:5.5)	0.82 ± 0.10	10
GE 10 (C)	SII	–	0.95 ± 0.11	11
		44 μM octasaccharide	0.88 ± 0.10	9
		Ag–Ab complexes (6, 15–27 S; 1:6.5)	0.97 ± 0.07	9
		Ag–Ab complexes (18–29 S)	0.99 ± 0.10	9
Fragment nonimmune Fc	IgG	–	0.83 ± 0.08	20–22

[a] Complexes of antibody and hapten were formed by the addition of hapten to solutions of antibody to yield the final concentration of hapten indicated. In some cases, monomeric antibody was removed from large antigen–antibody complexes by passage over a Sepharose 6B-CL column. The parenthetical figures are $S_{20,w}$ values obtained from ultracentrifugal analysis in 0.2 M phosphate buffer. Where present, an accompanying ratio indicates relative amounts of monomeric to antigen-aggregated antibody

[b] Protein A fragment dissociation constants were obtained from evaluation of the quenching data in Scatchard plots assuming a valence of 1 for protein A fragments and 2 for antibody and Fc fragments. Error limits represent the standard deviations

[c] Where necessary, a correction compensating for antigen- or hapten-induced fluorescence changes was applied. No correction for ligand binding to antibody 30–267 was necessary, since ligand binding was not accompanied by a fluorescence change. In this case, hapten binding had been confirmed by equilibrium dialysis. The fluorescence change due to multivalent antigen binding was assumed to be the same as that for haptens at saturation

cence titration data. Even in large antigen–antibody complexes, no discernable alteration in the dissociation constant of protein A fragments could be detected in 4 different antibodies. A subtle allosteric change whose alteration of the binding parameters was on the order of the cited experimental precision, would escape detection. The present data are consistent with [9] in which the conformation of the hinge peptides which form the covalent link between Fab and Fc regions was shown to be unchanged upon binding of monovalent and polyvalent antigens. One of the antibodies examined here (45–394) has already been extensively studied by a variety of independent techniques. Circular polarization of fluorescence changes, assigned to a putative conformational change in the Fc region, appeared significantly upon binding of a polyvalent 16-sugar hapten or of the whole SIII polysaccharide antigen [4]. However, these induced

Table 2
Binding of haptens to rabbit antibodies and to their antibody–protein A complexes

Antibody	Specificity	Hapten[b]	Protein A[a] (μM)	Size of free[b] antibody–protein A complexes	Fluorescence change (%)[c]	K_a (× 10^5)[d]
45–394	SIII	octasaccharide	0	–	– 9	3.7 ± 0.3
			9.6	9–25 S	– 9	3.3 ± 0.2
GE 10 (A)	SII	nonasaccharide	0	–	+ 14	2.2 ± 0.2
			8.7	8–29 S	+ 14	2.0 ± 0.1
GE 10 (C)	SII	nonasaccharide	0	–	+ 13	1.6 ± 0.1
			8.7	9–31 S	+ 14	1.8 ± 0.1

[a] At the indicated concentrations of protein A and antibodies, soluble complexes were formed
[b] Sedimentation coefficients of antibody–protein A complexes in 0.2 M phosphate buffer, pH 7.4 corrected to $S_{20,w}$ values
[c] A quenching of 10 ± 1% was observed for protein A–antibody interaction. This was corrected for in the evaluation of the hapten-induced fluorescence change
[d] Hapten association constants obtained from evaluation of fluorescence titration data in Scatchard plots (± standard deviation)

structural changes putatively attributed to the Fc fragment did not correlate with the complement activation of the same immune complexes. Significant complement binding was observed only with aggregates formed with a 21-sugar unit [16], while complexes with the 16-sugar unit marginally bound complement. Using the sensitive Fc-bound gadolinium (III) probe, only a change in the enhancement of the water proton relaxation rate in polymeric immune complexes formed with a 28-sugar oligosaccharide was demonstrated [17]. Moreover, on binding dinitrophenyl antigen to specific antibody no large conformational change in the CH_2 domain was detected as employing a spin label attached to the terminal portion of the oligosaccharide in the CH_2 domain [18]. A recently obtained low-resolution X-ray structure of the Dob immunoglobulin is also incompatible with the essential aspects of a 2-state allosteric antibody model [19]. The appearance of an alleged conformational change in the Fc region as judged by spectroscopic and magnetic resonance techniques cannot be unambiguously separated from the effects of aggregation.

The results presented here and numerous recent studies in the field (reviewed [2]), are not consistent with any model employing an antigen-induced allosteric effect transmitted to the Fc domain. Rather the appearance of an alledged conformational change in Fc posited to be necessary to trigger effector functions, cannot be unambiguously separated from the effect of simple crosslinking of Fc domains by multivalent antigenic determinants.

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