

ACTIVATION OF THE GUINEA PIG ALTERNATIVE  
COMPLEMENT PATHWAY BY MOUSE IgA  
IMMUNE COMPLEXES\*

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Activation of the complement system by IgA has been a controversial subject since Ishizaka et al. (1) and South et al. (2) observed that C1 was not fixed by IgA. Lack of complement activation has also been inferred from reports that secretory IgA antibodies were unable to opsonize or promote bacterial killing in the presence of complement (3–6). On the other hand, there is evidence that some complement-dependent functions occur in the presence of IgA antibodies. Adinolfi et al. (7) showed that *Escherichia coli* could be lysed by complement in the presence of secretory IgA and lysozyme. Knop et al. (8) and Henson et al. (9) demonstrated stimulation of phagocytosis by neutrophils in the presence of IgA and complement. After “interfacial” aggregation, secretory IgA was able to consume C3-9 without fixing C1; removal of the secretory component together with the Fc portion of the molecule did not alter the mode or extent of complement consumption (10). Also, chemically aggregated IgA myeloma proteins and their fragments have been reported to activate complement by both the classical and alternative pathways (11–15). However, only one previous study has demonstrated activation of complement by IgA myeloma proteins acting as antibodies (16). In this study solubilization of immune complexes prepared with unfractionated mouse ascites fluid induced by antigen-binding myelomas was used as an indirect measure of activation of the alternative pathway in mouse and guinea pig serum.

Activation of the alternative complement pathway by IgA has also been suggested in some clinical situations. Soter et al. (17) reported a patient with IgA cryoglobulinemia who showed low levels of C3 and factor B (also known as C3 activator) concurrent with their increased catabolism. By immunofluorescence, IgA, C3, and factor B have been detected at the dermal-epidermal junction in patients with dermatitis herpetiformis (18) and in the glomeruli in Berger’s disease (19). Early complement components were not observed in either case.

Although some of the results in the above experimental studies could have been due to contamination of IgA with other classes of immunoglobulins, the presence of complement components in secretions (20–23) where IgA is the primary immunoglobulin (24) could be taken to suggest the possibility of interaction. The present work

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deals with the interaction in vitro of serum complement and a purified mouse myeloma IgA protein with specific antigen-binding capacity. The use of such a protein as antibody both minimizes contamination with other classes of immunoglobulins and allows formation of antigen-antibody complexes, which are likely to be more relevant to natural immune phenomena than artificially aggregated myeloma proteins.

### Materials and Methods

**Buffers.** Borate-buffered saline, pH 8.0 (BBS),<sup>1</sup> and phosphate-buffered saline, pH 7.4 (PBS), were used for chromatography and gel filtration, and barbital buffer, pH 8.6, was used for Pevikon block electrophoresis for the purification of the IgA. Chromatography and gel filtration buffers contained 0.02% NaN<sub>3</sub>. Gelatin-veronal-buffered saline, pH 7.5, 0.145 M NaCl containing 0.015 M CaCl<sub>2</sub> and 0.005 M MgCl<sub>2</sub> (GVB<sup>++</sup>); dextrose-gelatin-veronal-buffered saline, pH 7.5, 0.075 M NaCl containing 0.015 M CaCl<sub>2</sub> and 0.005 M MgCl<sub>2</sub> (DGVB<sup>++</sup>); and 0.01 M or 0.04 M EDTA-GVB, pH 7.5, 0.145 M NaCl were prepared according to established methods (25, 26) and used for complement titrations.

**Materials.** Sepharose 6B and 4B and protein A-Sepharose were obtained from Pharmacia Fine Chemicals (Div. of Pharmacia Inc., Piscataway, N. J.). Pevikon was purchased from Accurate Chemical & Scientific Corp. (Westbury, N. Y.). *p*-Nitrophenylphosphorylcholine (Biosearch, San Rafael, Calif.) and palladium-on-charcoal catalyst (Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y.) were used for the synthesis of reactive phosphorylcholine (PC) hapten. PC-Cl, used for specific elution of the myeloma protein, was purchased from Calbiochem-Behring Corp. (American Hoechst Corp., San Diego, Calif.). Agarose used in immunoelectrophoresis and crossed immunoelectrophoresis (CIEP) was from Behring Diagnostics (Somerville, N. J.). Dithiothreitol (DTT) and iodoacetamide (IAA) (both from Sigma Chemical Co., St. Louis, Mo.) were used for reduction and alkylation for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Electrophoresis grade SDS and acrylamide were from Bio-Rad Laboratories (Richmond, Calif.). Bovine serum albumin (BSA) was purchased from Miles Laboratories, Inc. (Elkhart, Ind.) and pooled normal guinea pig serum for Pel-Freez Biologicals (Rogers, Ariz.). Reagent grade KSCN and NH<sub>4</sub>OH were from Fisher Scientific Co. (Pittsburgh, Pa.). BALB/c mice (6–8 wk old) were from Charles River Laboratories, Inc. (Wilmington, Mass.) and rabbits from Camm Research Lab Animals (Wayne, N. J.). Pristane, used for priming the mice before intraperitoneal injection of tumor cells to produce ascites, was from Aldrich Chemical Co. (Milwaukee, Wis.). Complete Freund's adjuvant for immunization of mice and rabbits was from Difco Laboratories (Detroit, Mich.). Anti-guinea pig C3 was purchased from N. L. Cappel Laboratories, Inc. (Cochranville, Pa.), and anti-guinea pig factor B was a gift from Dr. V. Nussenzweig, New York University Medical Center. Na<sup>125</sup>I was from Amersham Corp. (Arlington Heights, Ill.). Lactoperoxidase was purchased from Worthington Biochemical Corp. (Freehold, N. J.) and hydrogen peroxide from Fisher Scientific Co.

**Preparation of PC-BSA and Sepharose Conjugates.** *p*-Diazoniumphenylphosphorylcholine was synthesized from *p*-nitrophenylphosphorylcholine (27) and used to prepare PC-Sepharose immunoadsorbent (27) and PC-BSA (28) with three to five PC groups per molecule BSA, calculated by the method of Gearhart et al. (29).

**Antisera.** Antisera to mouse  $\alpha$ ,  $\mu$ , and  $\gamma$  chains were from McWilliams et al. (30). Rabbit antiserum to BSA was prepared by immunizing rabbits with BSA in complete Freund's adjuvant in the footpads and giving regular boosters intradermally with BSA in saline. BALB/c mouse anti-BSA was prepared by injecting female mice (6–8 wk old) with 500  $\mu$ g BSA in

<sup>1</sup> *Abbreviations used in this paper:* BSA, bovine serum albumin; BBS, borate-buffered saline; CIEP, crossed immunoelectrophoresis; C4DGPS, C4-deficient guinea pig serum; DTT, dithiothreitol; EA, sheep erythrocytes coated with antibodies to sheep erythrocytes; factor H,  $\gamma$ 1H globulin; factor I, formerly C3b inactivator; IAA, iodoacetamide; NGPS, normal guinea pig serum; <sup>125</sup>I-C3<sup>sp</sup>-NGPS, NGPS trace-labeled with <sup>125</sup>I-C3<sup>sp</sup>; NHS, normal human serum; oxy C2, oxidized human C2; PBS, phosphate-buffered saline; PC, phosphorylcholine; SDS-PAGE, sodium dodecyl sulfate-gel electrophoresis. (Complement nomenclature conforms to WHO Bulletin. 1968. 39:395.)

complete Freund's adjuvant in the footpads and bleeding 4 wk later (31). Rabbit anti-sheep and anti-bovine erythrocytes were prepared according to Campbell et al. (32). The 50% saturated  $(\text{NH}_4)_2\text{SO}_4$  precipitation fraction of anti-sheep erythrocyte antiserum was used to prepare sheep erythrocytes coated with antibodies to sheep erythrocytes (EA) for complement titrations (see below).

*Complement Reagents and Complement Titrations.* C4-deficient guinea pig serum (C4DGPS) obtained from our own colony was pooled after C4 activity was checked in the individual sera. Human serum was obtained from normal donors and pooled. Blood from male BALB/c mice was drawn into 5 mM  $\epsilon$ -aminocaproic acid, centrifuged, and the pooled serum used as the source of mouse complement.

Human and guinea pig C1, C2, and C4, and oxidized human C2 (oxy C2) were prepared from human and guinea pig sera (33–35). Guinea pig C3 was purified from pooled, freshly drawn guinea pig serum to which diisopropylfluorophosphate (Calbiochem), phenylmethylsulfonylfluoride, and benzamidine (Sigma Chemical Co.) were added to inhibit proteolysis. The serum was passed through a lysine-Sepharose column (Pharmacia Fine Chemicals) (36) and the C3 was purified by the method of Nelson et al. (33), except that at each step inhibitors of proteolysis were added (DFP, PMSF, benzamidine). C3<sup>gp</sup> purified by this method was hemolytically active and gave in SDS-PAGE a single band at 180,000 mol wt that yielded two bands upon reduction, 115,000 mol wt (C3 $\alpha$ ) and 64,000 mol wt (C3 $\beta$ ). The C3 was labeled with  $^{125}\text{I}$  by lactoperoxidase (37). There was minimal loss of hemolytic activity and the specific activity was  $6.25 \times 10^8$  cpm/mg. When labeled C3<sup>gp</sup> was reduced, electrophoresed in 5–15% gradient acrylamide SDS slab gels, and autoradiographed on Kodak XR-5 film (Eastman Kodak Co., Rochester, N. Y.), the  $\alpha$  chain was labeled more strongly than the  $\beta$  chain; trace-labeled contaminants that did not stain with Coomassie Blue appeared at 72,000 and 50,000 mol wt. Guinea pig C5 was purchased from Cordis Laboratories, Inc. (Miami, Fla.). Reagents deficient in C5 (R5) and C3 (R3) were prepared by treatment of NGPS with KSCN or  $\text{NH}_3$  (38–40).

Titration of whole complement (CH50) and C3-9, both human and guinea pig, were done according to Kabat and Mayer (25) and Rapp and Borsos (26). Mouse C3-9 were titrated with EAC1<sup>hu</sup>4<sup>hu2oxy</sup> prepared with purified components. Human and guinea pig C4 were titrated with EA and C4DGPS (41). Titration of mouse C4 was carried out with EA prepared with bovine erythrocytes and antibody, C4DGPS, and oxy C2 (42). Human C3 and guinea pig C3 and C5 were measured with EAC1<sup>hu</sup>4<sup>hu</sup> and EAC1<sup>gp</sup>4<sup>gp</sup> (43) with oxy C2<sup>hu</sup> or C2<sup>gp</sup>, respectively, and reagents deficient in the corresponding component. Factor B as antigen was measured by CIEP on 50  $\times$  50-mm glass plates (44).

*SDS-PAGE.* Electrophoresis for assessing the purity of the mouse IgA was done in cylindrical 5.0% gels in 0.1 M Tris acetate, pH 7.4, with 0.02 M EDTA and 1% SDS. Slab gel electrophoresis was performed according to Laemmli (45), and two-dimensional slab gel electrophoresis according to Law and Levine (46). Hydroxylamine treatment of gel strips was done according to Law et al. (47).

*Preparation of Immune Complexes.* IgA and IgG immune complexes were prepared near equivalence as determined by precipitin curve analysis (48). Because the IgA myeloma protein is a low affinity antibody, the equivalence zone was broad. The midpoint was taken as equivalence.

*Purification of TEPC-15 IgG Myeloma Protein.* 10<sup>6</sup> TEPC-15 tumor cells were injected intraperitoneally into 6–8-wk-old BALB/c mice. After 14–18 d ascites fluid and serum were collected, pooled, and stored at  $-20^\circ\text{C}$ .

The IgA myeloma protein was purified by a modification of the method of Chesebro and Metzger (27). Serum or ascites fluid was dialyzed against BBS, pH 8.0, diluted 1:3 in BBS, and passed over a PC-Sepharose immunoabsorbent column. After washing, PC-specific protein was eluted with 0.001 M PC-Cl in BBS, concentrated by ultrafiltration, dialyzed against PBS, pH 7.0, and then passed over a calibrated Sepharose 6B column to separate the IgA into monomeric, dimeric, and polymeric fractions. The dimeric fraction was passed through a protein A-Sepharose column to remove any contaminating IgG (49) and through an anti- $\mu$  Sepharose column to remove any contaminating IgM. The resultant dimeric IgA was free of contaminating proteins as assessed by immunoelectrophoresis and SDS-PAGE under reduced and nonreduced conditions. J chain was demonstrated in the dimeric IgA by alkyl urea-PAGE (50). Because

dimeric IgA was the most abundant form and is the one most closely related to secretory IgA, the primary antibody of secretions, it was used in the experiments to be presented. A limited number of experiments were also performed with polymeric and monomeric IgA, and the results were similar to those with dimeric IgA. However, monomeric IgA activated complement less efficiently.

### Results

*Activation of the Complement System by Dimeric IgA Anti-PC-BSA Immune Complexes.* Initial analysis of complexes prepared with the IgA myeloma and PC-BSA showed that maximum consumption of C3 in NGPS occurred near equivalence. To determine whether IgA complexes at equivalence consumed other complement components, suspensions of IgA immune complexes were mixed with equal volumes of guinea pig, mouse, or human serum and incubated at 37°C. The complexes were then removed, and the residual activities of the supernatants titrated. IgA immune complexes consumed 40% of the hemolytic activity of C3-9 in guinea pig serum and 30% in mouse serum (Table I). The controls showed no complement consumption. The loss of hemolytic activity in guinea pig serum was associated with a 47% reduction in C3 activity without loss of C4 or C2 activities. That the C3 consumption in NGPS was indeed due to activation by IgA immune complexes was demonstrated by CIEP with an anti-C3<sup>9P</sup> antiserum, which showed the characteristic shift in mobility of C3 to a more anodal position (data not shown). When human serum was used as the source of complement, none was consumed. This findings could not be explained by a general incompatibility between mouse immunoglobulins and normal human serum (NHS) as evidenced by the capacity of mouse IgG anti-BSA immune complexes to activate human complement (Table I). Because of limitations in techniques for measuring individual components of complement in mouse serum and the observation that immune complexes prepared with mouse immunoglobulins activated guinea pig complement as well as mouse complement, guinea pig complement was used for further experiments.

TABLE I  
*Complement Consumption by IgA and IgG Immune Complexes  
in Various Sera\**

Ig class	Source of serum	Percent complement consumption			
		C3-9	C4	C2	C3
IgA	Guinea pig	40	0	0	47
	Mouse	30	0	ND‡	ND
	Human	0	0	0	0
IgG	Guinea pig	100	59	45	94
	Mouse	16	100	ND	ND
	Human	36	35	74	ND

\* 0.2-ml samples each of a suspension of IgA-anti-PC-BSA or IgG-anti-BSA immune complexes at a concentration of 2 mg antibody/ml (plus controls of dimeric IgA alone [2 mg/ml], PC-BSA [0.1 mg/ml], or saline) were mixed with an equal volume of undiluted NGPS, mouse serum, or NHS and incubated at 37°C, with shaking, for 1 h. The samples were then chilled in ice, the complexes removed by centrifugation, and the residual complement activities of the supernatant sera measured.

‡ Not done.

The lack of C4 and C2 utilization shown in Table I suggested that consumption of hemolytic C3 by IgA immune complexes takes place via the alternative pathway of complement activation. Further evidence was obtained in experiments that demonstrated depletion of factor B in both NGPS and C4DGPS, a source of complement in which only the alternative pathway is available. The IgA complexes consumed ~50% of the factor B and C3, and essentially no C5 (Table II and Fig. 1). A sample incubated with zymosan showed 53% depletion of C3 hemolytic activity and 70% depletion of factor B antigen. In contrast to the lack of C5 consumption by IgA complexes, zymosan activated 29% of the C5. Similar results were obtained in NGPS, except that the availability of the classical pathway allowed more efficient activation by zymosan (90% of the factor B and 100% of the hemolytic C3) whereas the IgA activation remained the same as in C4DGPS (not shown). These results demonstrate differences in alternative pathway activation between IgA immune complexes and zymosan. Furthermore, they illustrate the contribution of the classical pathway in complement activation by zymosan but not by IgA.

*Time, Temperature, and Ion Requirements for Activation of Guinea Pig Complement by IgA Immune Complexes.* To determine the time required to achieve maximum C3 activation, IgA immune complexes and NGPS were incubated at 37°C. Samples were taken

TABLE II  
Activation of the Alternative Pathway by IgA Immune Complexes  
and Zymosan in C4DGPS\*

	Percent consumption		
	B	C3	C5
IgA complexes	50	48	9
Zymosan	70	53	29
Control	0	0	0

\* 0.2 ml of C4DGPS was incubated at 37°C for 1 h with equal volumes of either a suspension of IgA immune complexes at 0.5 mg antibody/ml, a suspension of 0.5 mg/ml of zymosan, or saline. Factor B antigen was measured by CIEP; C3 and C5 activities were measured hemolytically.

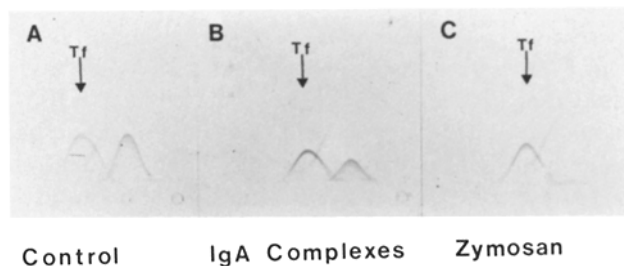


FIG. 1. Depletion of factor B in NGPS by mouse IgA immune complexes and zymosan measured by CIEP. 0.2 ml of NGPS was incubated with 0.2 ml of IgA complexes (0.5 mg/ml), 0.2 ml of zymosan (0.5 mg/ml), or 0.2 ml of saline for 1 h at 37°C. After centrifugation, 5  $\mu$ l of each supernatant was loaded into a well in 1.2% agarose gel for 2 h of electrophoresis at 200 V in EDTA-barbital buffer, pH 8.6. At the end of the first dimension, the excess agarose was cut off and 2.6 ml of agarose containing 10  $\mu$ l of anti-GP factor B antiserum was poured onto the plate. A second electrophoresis was run overnight (50 V) perpendicular to the first. The plates were then washed, dried, and stained with Coomassie Blue to reveal the precipitation peaks. Transferrin-anti-transferrin (TF) was included as a mobility marker in each slide.

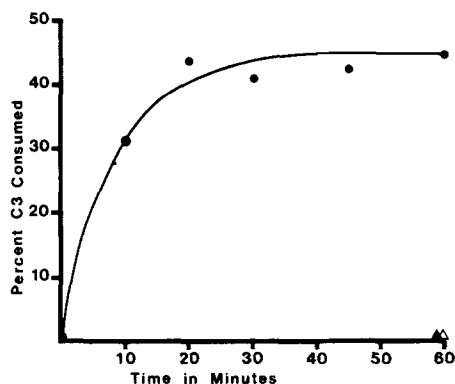


FIG. 2. Time-course of C3 activation in NGPS by IgA immune complexes. 0.6 ml of IgA anti-PC-BSA immune complexes at 0.5 mg antibody/ml was mixed with 0.6 ml of NGPS. Equal volumes of saline and NGPS were also mixed and incubated at 37°C as a control. Samples were incubated at three different temperatures. At the given times, 0.2-ml samples were removed, diluted with 0.8 ml ice cold GVB<sup>++</sup>, and centrifuged. The residual C3 hemolytic activities of the supernatants were titrated. ●, C3 consumed at 37°C; ▲, at 0°C, and Δ, at 17°C.

TABLE III  
Cation Requirements for C3-9 Consumption by IgA Immune Complexes in Guinea Pig Serum\*

Serum treatment	Percent C3-9 consumption
Saline	30
EGTA + Mg <sup>++</sup>	36
EDTA	0

\* To 0.2 ml of NGPS, made 0.02 M in EGTA and 0.002 M in MgCl<sub>2</sub>, or 0.01 M in EDTA, was added 0.2 ml of a suspension of IgA immune complexes at 0.5 mg antibody/ml. After 1 h at 37°C the C3-9 hemolytic activities of the supernatant sera were measured.

at intervals and the residual C3 hemolytic activities of the supernatant sera were compared with the saline control. After 20 min of incubation, ~45% of the C3 hemolytic activity had been depleted and no further consumption was observed (Fig. 2). When the reaction was performed at 17°C or 0°C (even up to 16 h), no C3 was utilized. Guinea pig C3-9 was consumed in the presence of EGTA and Mg<sup>++</sup>, but not in EDTA, indicating that Mg<sup>++</sup> but not Ca<sup>++</sup> is required (Table III).

*Complement Activation as a Function of Immune Complex Dose.* Experiments were next performed to examine in more detail the effect of different doses of IgA and IgG immune complexes on C3 and C5 in both NGS and C4DGPS. Suspensions of varying concentrations of immune complexes were incubated with equal volumes of undiluted NGPS or C4DGPS. The residual C3 and C5 hemolytic activities of the supernatant sera were measured. Increasing C3 consumption by IgA immune complexes occurred in both NGPS and C4DGPS, reaching a maximum of 40-50% (Fig. 3). In contrast, C3 consumption approaching 100% was observed with the IgG immune complexes in NGPS (Fig. 3A). However, when C4GDPS was used, the pattern of utilization was the same as that obtained with IgA immune complexes (Fig. 3B). Complexes prepared with IgA were unable to consume substantial C5 activity in either NGPS or C4DGPS (Fig. 4). However, activation of C5 by IgG complexes in NGPS displayed the expected

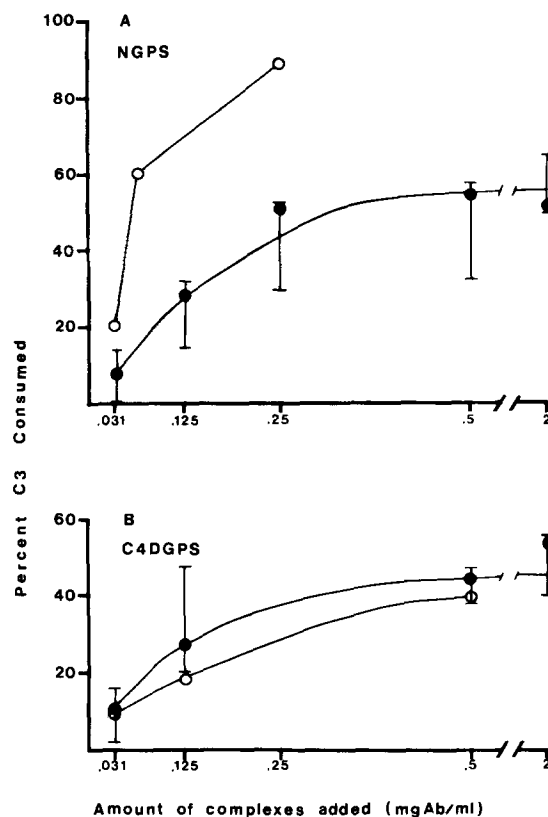


FIG. 3. Activation of C3 by immune complexes. 0.2 ml of IgA anti-PC-BSA immune complexes or IgG anti-BSA immune complexes prepared at equivalence at the given concentrations were mixed with an equal volume of either NGPS or C4DGPS and incubated at 37°C for 1 h. 0.2 ml of saline was incubated with an equal volume of either serum as controls. The residual hemolytic activities of the supernatant sera were measured after centrifugation. Each curve indicates a representative experiment: ○, IgG complexes; ●, IgA complexes. Brackets depict the range of C3 activation in different experiments with the IgA immune complexes.

dose-dependent consumption (Fig. 4 A). Similar to the alternative pathway activation by IgA immune complexes, IgG immune complexes were unable to diminish C5 activity when only the alternative pathway was available (Fig. 4 B). In this case, alternative pathway activation by IgA and IgG immune complexes was limited to C3 and resulted in a maximum of 50% C3 consumption, even with 2.0 mg/ml of immune complexes.

To investigate whether the limited C3 consumption by IgA complexes was due to insufficient functional convertase-binding sites on the complexes, the following experiment was performed. A suspension of IgA immune complexes was incubated with NGPS. After incubation, the supernatant fluid was separated and the hemolytic C3 activity found to be 53% consumed (Table IV). When the complexes removed from the original reaction mixture were suspended in fresh NGPS, C3 was activated to the same extent as in the initial serum (47% C3 consumed). Since the IgA immune complexes retained their capacity to activate C3, it was considered that activation of the residual C3 in the supernatant serum was impaired. Therefore, the supernatant

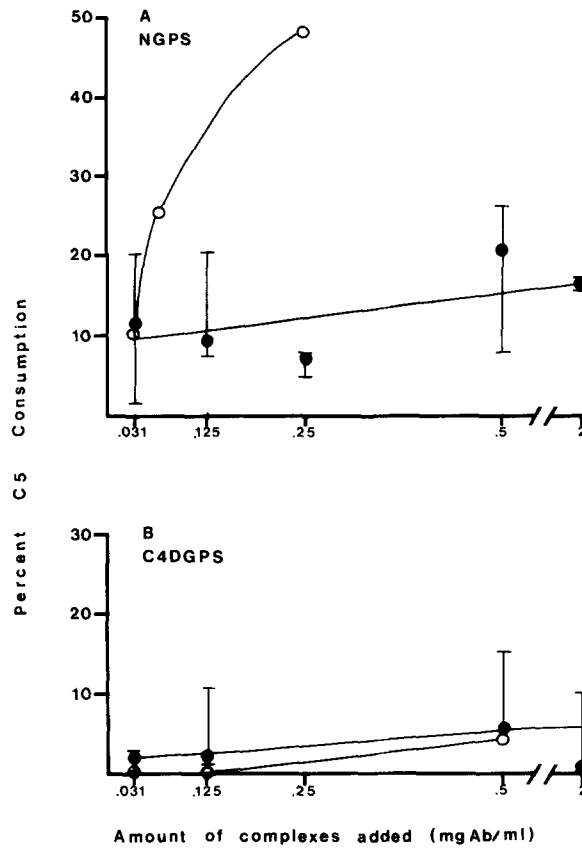


FIG. 4. Activation of C5 by immune complexes. 0.2 ml of IgA anti-PC-BSA immune complexes or IgG anti-BSA immune complexes prepared at equivalence at the given concentrations were mixed with an equal volume of either NGPS or C4DGPS and incubated at 37°C for 1 h. 0.2 ml of saline was incubated with an equal volume of either serum as controls. The residual hemolytic activities of the supernatant sera were measured after centrifugation. Each curve indicates a representative experiment: ○, IgG complexes; ●, IgA complexes. Brackets depict the range of C5 activation in different experiments with the IgA immune complexes.

of the original reaction mixture was divided into three equal samples that were added to fresh IgA immune complexes, fresh IgG immune complexes, or saline. These samples were incubated, the complexes removed, and the residual hemolytic C3 activity measured. Fresh IgA immune complexes failed to activate C3 further, whereas fresh IgG complexes completely consumed the remaining C3 activity (Table IV).

This further consumption of C3 by IgG could be explained by activation of either the classical or alternative pathways. This question was investigated in the next experiment. IgA immune complexes were incubated with C4DGPS and the supernatant divided into equal samples that were reincubated with suspended pellets of fresh IgA or IgG immune complexes or zymosan. In the C4DGPS supernatant, neither fresh IgA nor IgG complexes could further activate factor B, C3, or C5 (Table V). Only zymosan utilized these components in C4DGPS supernatant. The complexes from the original mixture ("activated" IgA complexes) were treated as in the previous experiment, and the results were similar (Table V).



TABLE IV  
*Ability of IgA Complex-Activated NGPS to be Further Activated  
 by Subsequent Treatment\**

First stage		Second stage	
Treatment of fresh NGPS	Percent C3 consumption	Treatment of activated NGPS	Percent‡ C3 consumption
Fresh IgG complexes	53	Saline	0
Activated IgA complexes	47	Fresh IgA complexes	0
		Fresh IgG complexes	100

\* 0.4 ml of fresh NGPS was incubated with 0.4 ml of 0.5 mg/ml IgA immune complexes for 1 h at 37°C. After centrifugation, 0.3-ml aliquots of the supernatant were incubated with fresh pellets of the indicated immune complexes equivalent to 0.15 ml of 0.5 mg/ml complexes for another hour at 37°C. The remaining supernatant was carried as the saline control. The equivalent of 0.2 ml of 0.5 mg/ml of the activated IgA immune complexes was incubated at the same time with fresh NGPS. Residual C3 activities of the resulting supernatant sera were measured hemolytically.

‡ Percent of the 47% residual C3 activity of the first supernatant.

TABLE V  
*Ability of IgA Complex Activated C4DGPS to be Further Activated  
 by Subsequent Treatment\**

First stage				Second stage			
Treatment of fresh C4DGPS	Percent consumption			Treatment of activated C4DGPS	Percent consumption‡		
	B	C3	C5		B	C3	C5
Fresh IgA complexes	50	48	9	Saline	0	0	0
Activated IgA complexes	41	50	3	Fresh IgA complexes	0	0	7
				Fresh IgG complexes	0	2	9
				Zymosan	100	52	30

\* 0.5 ml of C4DGPS were incubated for 1 h at 37°C with 0.5 ml of IgA immune complexes at 0.5 mg/ml. After centrifugation, 0.2 ml aliquots of the supernatant were mixed and incubated with saline or pellets of the equivalents of 0.1 ml of either IgA immune complexes, IgG immune complexes, or zymosan, all at a concentration of 0.5 mg/ml, for 1 h at 37°C. 0.2 ml of the resuspended activated IgA complex pellet was mixed and incubated at the same time with fresh C4DGPS. Residual C3 and C5 activities of the resulting supernatant sera were measured hemolytically; factor B antigen was measured by CIEP.

‡ Of the residual activities of the first supernatant.

*Binding of  $^{125}\text{I-C3}^{\text{gp}}$  to Immune Complexes.* Because binding of C3b to a suitable surface may be required for efficient C3/C5 alternative pathway convertase activity, it was considered that the IgA immune complexes might not supply such a surface and that therefore alternative pathway activation was limited. To determine whether C3 binds to the IgA complexes during complement activation,  $^{125}\text{I-C3}^{\text{gp}}$  was added to NGPS to trace label the C3. IgA or IgG immune complexes were mixed with an equal volume of  $^{125}\text{I-C3}^{\text{gp}}$ -NGPS and the reactions carried out as described previously. The precipitates were washed until the wash buffer counts reached background. To

TABLE VI  
Binding of  $^{125}\text{I-C3}^{\text{SP}}$  to Immune Complexes\*

	IgA complexes	IgG complexes
Percent counts per minute bound	0.5	5.9
Percent C3 activated	40.6	80.8

\* To 0.2-ml samples of  $^{125}\text{I-C3}^{\text{SP}}$ -NGPS, 0.08 ml of 0.2 M EDTA or 0.08 ml of GVB<sup>++</sup> was added in the cold. Then 0.2 ml of immune complexes at 0.5 mg antibody/ml was added and the mixtures incubated at 37°C for 1 h with shaking. Each tube was counted to determine the total counts per minute (cpm) added. The percent cpm bound to the washed precipitates in EDTA was subtracted from the percent cpm bound to the precipitates in the absence of EDTA to yield the percent cpm bound to the precipitates due to activation. C3 activation was measured by hemolytic titration.

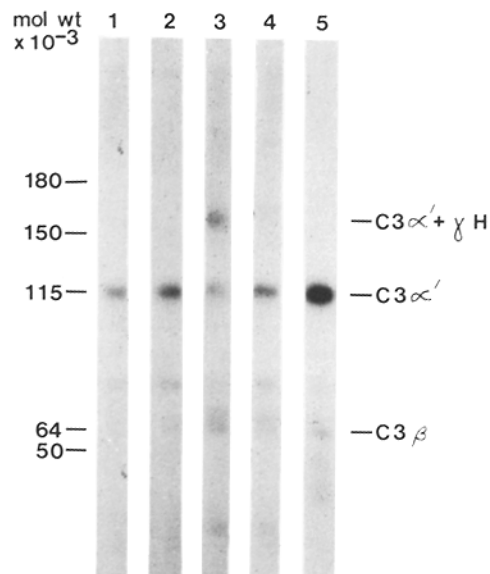


FIG. 5. Binding of  $^{125}\text{I-C3}^{\text{SP}}$  to immune complexes. After incubation in NGPS to which  $^{125}\text{I-C3}^{\text{SP}}$  had been added, immune precipitates were solubilized in SDS, reduced with DTT, and run on an SDS 5-15% polyacrylamide slab gel. An equal amount of radioactivity was loaded in each track. Autoradiography of the dried gels was then performed. Track 1, IgA complexes; track 2, IgA complexes in the presence of EDTA (no complement activation); track 3, IgG complexes; track 4, IgG complexes in the presence of EDTA; track 5,  $^{125}\text{I-C3}^{\text{SP}}$  in NGPS and EDTA showing differential labeling of  $\alpha$  and  $\beta$  chains. Molecular weights are indicated on the left. The slowest band (mol wt  $\pm 165,000$ ) in track 3 is interpreted as a covalent combination of the  $\gamma$  chain of IgG and the  $\alpha$  chain of C3.

measure nonspecific binding, a control sample of  $^{125}\text{I-C3}^{\text{SP}}$ -NGPS was incubated with 0.04 M EDTA before addition of the immune complexes to prevent complement activation. The IgG immune complexes added to the  $^{125}\text{I-C3}^{\text{SP}}$ -NGPS activated twice the amount of C3 but bound 12 times more C3 than the IgA immune complexes (Table VI).

The nature of the binding of  $^{125}\text{I-C3}^{\text{SP}}$  to the immune precipitates was studied by autoradiography of polyacrylamide slab gels in which samples of the washed, SDS-solubilized, reduced precipitates had been electrophoresed (Fig. 5). There is a band of

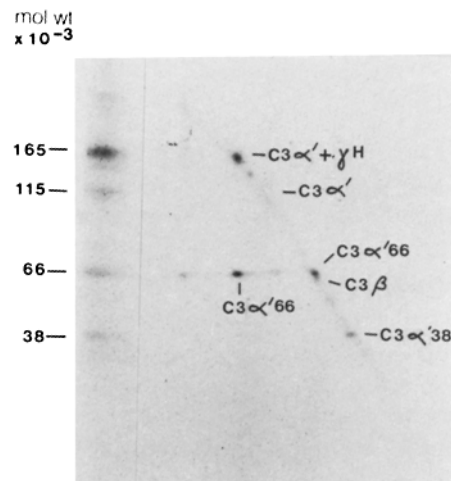


FIG. 6. Two-dimensional electrophoresis of solubilized IgG complexes with bound  $^{125}\text{I-C3}^{\text{BP}}$ . After electrophoresis of the solubilized, reduced IgG complexes with bound  $^{125}\text{I-C3}^{\text{BP}}$  in an SDS 5–10% polyacrylamide gel, a strip was cut out, treated with 1 M hydroxylamine, and reduced with DTT a second time. This strip was then placed on top of an SDS 5–15% gradient polyacrylamide gel and electrophoresed along with a sample of the original material not exposed to hydroxylamine and containing the same number of counts (shown vertically on the left). Locations of some C3 products are indicated; others cannot be precisely identified.

165,000 mol wt in the case of IgG complexes (track 3). No comparable high molecular weight band is seen with the IgA complexes (track 1). The 165,000 mol wt band could correspond to the  $\gamma$  chain of IgG (55,000 mol wt) covalently bound to the labeled  $\alpha'$  chain of C3 (115,000 mol wt). Thus, it appears that classical pathway activation results in binding of  $\text{C3}^{\text{BP}}$  to the IgG immune complexes, whereas IgA immune complex activation of the alternative complement pathway does not lead to stable binding of C3.

Since the putative bond between the  $\alpha$  chain of C3 and the  $\gamma$  chain of IgG was not sensitive to DTT, the possibility of an ester linkage was considered. After electrophoresis, the gel was treated with hydroxylamine and then with reducing sample buffer (47) to attempt to cleave the proposed link. A second electrophoresis perpendicular to the first dimension was then performed. The 165,000-mol wt spot, after hydroxylamine treatment and reduction, upon electrophoresis in the second dimension gave rise to some radioactive material of 66,000 mol wt (Fig. 6). Because this is slightly larger than the  $\text{C3}^{\text{BP}}$   $\beta$  chain and is strongly labeled, whereas the  $\text{C3}^{\text{BP}}$   $\beta$  chain does not label efficiently (cf. Fig. 5, track 5), the 66,000 mol wt band is likely to be derived from the  $\text{C3}\alpha'$  chain. The remaining 165,000 mol wt material could result either from incomplete cleavage by hydroxylamine or intact  $\text{C3}\alpha'$  chain bound to the  $\gamma$  chain of IgG by a nonester bond. As a control for the hydroxylamine treatment,  $\text{EAC1}^{125}\text{I-C4}^{\text{hu}}$  ghosts were solubilized, electrophoresed, and treated in the same manner as were the IgG immune complexes (51). After electrophoresis in the second dimension, a radioactive high molecular weight spot ( $\text{C4}\alpha'$  that had been bound to a cell surface protein) was completely displaced to the location of  $\text{C4}\alpha'$  chain, 90,000 mol wt (not shown). These experiments with radioactive C3 suggest that efficient convertase formation requires covalent binding of C3 to a surface. This binding can be supplied by the classical

pathway but neither IgA nor IgG appears to support an appropriate binding site for the alternative pathway.

### Discussion

A model system using a mouse myeloma protein as antibody, complexed with the antigen PC-BSA, was used to investigate activation of the complement system by IgA. IgA immune complexes were able to activate C3-9 in mouse and guinea pig but not human serum. Activation occurred via the alternative pathway as evidenced by lack of C4 and C2 activation in both NGPS and C4DGPS. Moreover, consumption of C3 by IgA immune complexes required the presence of  $Mg^{++}$  but not  $Ca^{++}$ , and factor B, required for the formation of the alternative pathway C3 convertase (52), was depleted.

Because of the limited C3-9 consumption in spite of substantial C3 activation (50%) by the IgA immune complexes in guinea pig serum, it was considered that the late acting components C5-9 were not used. Although increasing C3 activation occurred to a maximum of 50% with greater doses of IgA immune complexes, C5 consumption remained low. Without activation and cleavage of C5, the assembly of the terminal components (C5b-C9) cannot take place (53), and total complement consumption remains limited.

Because there was always  $\pm 50\%$  of the C3 activity left in the supernatant serum even when very large amounts of IgA complexes were used, it was of interest to know whether this C3 was susceptible to further activation. The results in Tables IV and V demonstrate that this C3 could be consumed under certain conditions. The supernatant from NGPS previously incubated with IgA complexes could be further activated by fresh IgG complexes (Table IV), which activate the classical pathway resulting in consumption of the residual C3. Whether there could be additional alternative pathway activation in the supernatant serum was examined with C4DGPS. The results of these experiments demonstrate that in the absence of a classical pathway neither the residual C3 activity nor factor B could be further depleted by IgG complexes, and, as previously shown, IgA complexes were without effect. However, zymosan, an alternative pathway activator with a "protected" surface (54, 55) was capable of consuming the remaining factor B, and substantial amounts of C3 and C5.

The action of the control proteins (factor I [formerly C3B inactivator] and factor H [B1H globulin]) during alternative pathway activation by IgA immune complexes may be an important factor limiting C3 consumption. The C3 activated could be primarily in the fluid phase; it is quickly inactivated and therefore cannot participate in assembly of the C3bBb convertase on the IgA immune complexes. Whereas the classical pathway has a molecule, C1, which is specifically bound to and activated by IgG or IgM antibody-antigen complexes or aggregates, and leads to consistent, relatively stable C3/C5 convertase formation, the alternative pathway convertase seems to be less specific in its binding, and deposition of C3 is left much more to chance. For C5 cleavage to take place it has been suggested that the C3b generated by either the classical or alternative pathway convertase must bind to a suitable surface and in turn bind C5 to enable the C3 convertase to acquire C5 cleaving function (56). Zymosan provides a suitable surface for efficient alternative pathway activation (54, 55) and C5 consumption whereas the IgA immune complexes do not

(Table II), nor do IgG complexes in C4DGPS (Fig. 4). Without a protected surface, the alternative pathway is more susceptible to the actions of factors I and H.

The complete lack of activation of complement by mouse IgA complexes in NHS was not a result of total species incompatibility because mouse IgG immune complexes were able to activate the classical pathway in NHS. Whether the lack of activation could be due to rigorous control remains to be investigated.

The data presented here on the binding of radioactive C3<sup>6P</sup> to mouse IgG complexes during classical pathway activation further support the requirement for binding of activated C3 to an appropriate surface to establish efficient C3/C5 convertase function (Table VI). The relative lack of binding of C3<sup>6P</sup> to the IgA complexes implies that this type of alternative pathway activation is inefficient and subject to rigorous control.

In the case of the classical pathway activation of C3 by IgG complexes, the bond between the C3 $\alpha'$  chain and the  $\gamma$  chain of IgG appears to be covalent, not disulfide but at least partly ester in nature because some can be dissociated by hydroxylamine and resolved into a C3 $\alpha'$  fragment of 66,000 mol wt (Fig. 6). The 165,000-mol wt spot that remains on the diagonal should contain intact C3 $\alpha'$  chain. It is possible that the part of the C3 responsible for the convertase activity remains intact and covalently bound to the immune complexes while the inactivated portion can be removed. Since IgA complexes do not bind an appreciable amount of C3, all of the C3b generated into the fluid phase is rapidly inactivated.

In view of recent reports of synergism by different antibody classes in alternative pathway activation (57, 58), it could be considered that IgA requires the participation of another class of antibody to create more effective alternative pathway activation. However, these studies used zymosan or erythrocytes as the surface on which C3b is deposited. Analogy with immune complexes is difficult because the structures available for C3b binding are different. In the case of immune complexes, a second class of antibody could enhance the amplification of the alternative pathway in a manner similar to that proposed for solubilization (59) rather than increase the initial rate of C3b deposition as has been reported for IgG antibody enhancement of alternative pathway activation by zymosan (57). Experiments involving cooperation by antibody classes in complement activation as applied to protein antigens in the form of immune complexes have yet to be done.

In conclusion, activation of the alternative or classical pathways by immunoglobulins may depend on their abilities to bind C1 and subsequently C3 in appropriate conformation for efficient convertase formation and function. The binding of C1 to IgG or IgM may cause a change in the antibody molecule, allowing C3b to bind. If the antibody does not bind C1, as is the case for IgA, the alternative pathway may be activated, but without a C3b binding site the C3b in the fluid phase would be quickly inactivated by factors I and H, thereby limiting the activation. The control of C3 activation in these cases might not be overcome until and unless either an appropriate surface is provided to stabilize the alternative pathway convertase, or the classical pathway can be activated. It is possible that cooperation between immunoglobulin classes could fulfill either or both of these requirements.

### Summary

Activation of the complement system by IgA was investigated with immune complexes containing a mouse IgA myeloma protein with specificity for phosphoryl-

choline linked to bovine serum albumin (PC-BSA). These IgA anti-PC-BSA immune complexes activated the alternative complement pathway in mouse and guinea pig serum, while human complement was not affected. The activation proceeded with consumption of C3 but little or no consumption of C5. C3 did not bind to the IgA immune complexes during complement activation although it did bind covalently to IgG immune complexes. It is suggested that IgA immune complexes do not supply a suitable surface for C3 binding and effective alternative pathway convertase assembly; therefore, cleavage is limited and occurs primarily in the fluid phase. Without C3 binding, C5 cleavage does not occur nor can the alternative pathway activation proceed to the amplification step.

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