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Prevention of immune precipitation by purified components of the alternative pathway

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SUMMARY

The role of the alternative pathway in complement-mediated prevention of immune precipitation has been investigated by the use of BSA–anti-BSA immune complex (IC) purified components. For immune precipitation to be prevented all six alternative pathway components (C3, factors B D̄, P, H and I) were required. In the absence of one or both of the control proteins H and I, excessive fluid phase turnover of C3 occurred with precipitation of IC. Kinetic studies showed that in the presence of the control proteins, an initial phase of precipitation occurred, and was followed by a phase of resolubilization of IC. When the efficiency of classical and alternative pathways in the prevention of immune precipitation was compared it was found that the classical pathway proteins were more effective than the alternative pathway components. A reaction mixture containing the components of both pathways was no better than the classical pathway protein alone. ^{125}I-C3 was bound to IC which had been rendered soluble in the presence of classical or alternative pathway components. A molar ratio of two molecules C3b: five molecules IgG was calculated. Other complement components which were bound to IC which had been formed in the presence of serum were C1q, C4, C2, C3, C5, P and H. Factors B and I were not detected. Our findings suggest that the alternative pathway is of secondary importance to the classical pathway in the prevention of immune precipitation.

Keywords immune precipitation complement alternative pathway

INTRODUCTION

Antigen–antibody complexes (IC) formed at equivalence or in antibody excess in the presence of serum, remain soluble. This phenomenon, the prevention of immune precipitation, is dependent upon complement activation (Schifferli, Bartolotti & Peters, 1980). Unlike the solubilization of insoluble IC which is largely dependent upon the alternative pathway (Takahashi, Tack & Nussenzweig, 1977), prevention of immune precipitation relies upon an intact classical pathway (Schifferli, Woo & Peters, 1982). Although the use of purified classical pathway components has shown that C1. C4, C2 and C3 are sufficient to prevent immune precipitation (Naama *et al.*, 1984), earlier studies using serum reagents depleted of factors B or D̄ suggested that the alternative pathway also played a role in this process (Schifferli *et al.*, 1980; Naama *et al.*, 1983). In order to resolve this inconsistency we have investigated the effects of the purified alternative pathway components C3, factors B and D̄, P (properdin), I (C3b inactivator) and H (β1H), on immune precipitation.

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MATERIALS AND METHODS

Complement components. C1 (Gigli, Porter & Sim, 1976), C4 (Bolotin *et al.*, 1977), C2 (Kerr & Porter, 1978), C3 (Tack & Prahl, 1976), B (Hunsicker, Ruddy & Austen, 1973), D̄ (Fearon, Austen & Ruddy, 1974), P (Fearon & Austen, 1975), H (Whaley & Ruddy, 1976) and I (Fearon, 1977) were purified by published techniques. C3 was immunoadsorbed with Sepharose 4B (Pharmacia) to which anti-H had been conjugated by the cyanogen bromide method (Goetzl & Metzger, 1970). This immunadsorption step was necessary as C3 preparations are frequently contaminated with traces of H.

For some experiments C3 was radiolabelled with ^{125}I using Enzymobeads (Biorad). The specific activity of ^{125}I-C3 was 0·14 μCi/μg. ^{125}I-C3 had the same haemolytic activity as unlabelled C3. Antisera to C1q, C4, C3, C5, B, P, H and I were produced by the immunization of rabbits. Rabbit antiserum to C2 was a gift from Dr Michael Kerr.

IC. These were prepared at equivalence using ^{125}I-labelled bovine serum albumin (BSA, Sigma) and immunadsorbent purified rabbit anti-BSA (McPhaden & Whaley, 1982).

Prevention of immune precipitation. The assay is similar to that described by Naama *et al.* (1983). The reaction volume (115 μl) consisted of isotonic veronal-buffered saline (containing $MgCl_2$, 1 mmol/l; $CaCl_2$ 150 μmol/l and gelatin 0·1% wt/vol.), serum or a mixture of purified complement components, ^{125}I-BSA (5 μl containing 0·5 μg) and anti-BSA (10 μl containing 1·5 μg). The reaction mixture, with the exception of the anti-BSA, was prepared at 0°C and then warmed to 37°C. Anti-BSA was then added to the warmed mixture at time 0. For dose–response studies the proportion of complexes remaining soluble after 60 min was determined by removing 50 μl of the reaction mixture and adding it to 1 ml of ice cold phosphate-buffered saline (PBS), in an Eppendorff tube. Soluble complexes were separated from insoluble complexes by centrifugation (Beckman Microfuge) and the precentage of complexes which remained in solution was calculated (Naama *et al.*, 1983).

For kinetic assays 15 μl samples were removed from the reaction mixture at 3, 5, 10, 15, 30 and 60 min. The samples were processed as described above.

In all studies the effect of purified components on the rate and extent of immune precipitation was compared with that of normal serum. The serum was obtained from one of us (JKN), and stored in aliquots at −70°C. The concentrations of C1q, C4, C3, B, P, H and I were measured by radial immunodiffusion (Mancini, Carbonara & Heremans, 1965), and C1 and C2 by haemolytic assay (Rapp & Borsos, 1970). For dose–response curves the serum was diluted 1:5 initially, and then titrated by doubling dilution. The concentrations of purified components were adjusted to be the same as those present in each serum dilution. For kinetic assays serum was diluted 1:5.

C3 consumption by IC. IC were formed in the presence of (a) NHS (1:5 dilution). (b) C1q (C1 40 μg/ml), C4 (100 μg/ml), C2 (2,000 u/ml) and C3 (300 μg/ml, (c) B (50 μg/ml), D (100 u/ml), P (6 μg/ml) and C3 (300 μg/ml) or (d) B, D, P, C3, H (50 μg/ml) and I (10 μg/ml) under the conditions described for measuring prevention of immune precipitation.

Residual C3 activity was then measured by haemolytic assay using EAC 14 ^{oxy}2 cells and potassium thiocyanate treated guinea pig serum to which C5 (100 u/ml) had been added (Cooper & Müller Eberhard, 1970).

Binding of ^{125}I-C3 to IC. Complexes were formed in the presence of serum or in reaction mixtures containing purified components, which contained ^{125}I-C3. The proportion of ^{125}I-C3 to total C3 was the same in both situations.

After incubation at 37°C for 1 h, the insoluble complexes were precipitated by centrifugation. After the addition of protein A-Sepharose (10 μl packed beads, Pharmacia) to the supernatant and a further incubation step at 37°C for 30 min, the protein A-Sepharose was pelleted by centrifugation. Following three washes in PBS containing Nonidet P40 (0·1% wt/vol.), sodium deoxycholate (0·5% wt/vol.) and EDTA (10 μmol/l), the radioactivity bound to the beads was counted. The control for this experiment was ^{125}I-C3 in buffer alone. In two parallel experiments IC were formed in the presence of identical concentrations of individual components, but the C3 was not radiolabelled. In one set of experiments ^{125}I-labelled BSA was used, and in the other ^{125}I-labelled anti-BSA was

employed. These experiments were performed in the same way as the experiments using ^{125}I-C3, and were designed to show how much of the antigen and antibody in the supernatants could be precipitated by protein A-Sepharose.

Determination of complement components which are bound to complexes. ^{125}I-BSA–anti-BSA complexes were formed in the presence of serum in 10 separate tubes. Following the removal of insoluble complexes by centrifugation, antisera (heat inactivated at 56°C for 30 min) to C1q, C4, C2, C3, C5, B, P, H and I were added so that optimal precipitation occurred. After washing the insoluble pellets three times the amount of radioactivity was measured. Heat-inactivated normal rabbit serum was added to the tenth tube as a negative control.

RESULTS

Alternative pathway components

Individual components. B (50 μg/ml), D (100 u/ml), P (6 μg/ml), C3 (300 μg/ml), H (50 μg/ml) or I (10 μg/ml) alone did not prevent immune precipitation.

Combinations of components. Combinations containing any two, three, four or five of the six components in the concentrations shown above did not prevent immune precipitation. Only when all six components were present together was any inhibition of immune precipitation observed (Fig. 1). When the six components were present at the same concentrations as were present in serum immune precipitation was reduced (Fig. 1). However the combination of purified components was not as effective as serum in preventing immune precipitation.

Kinetics of immune precipitation

Although there was little difference in the proportion of IC which had precipitated after 1 h in buffer, or in reaction mixtures containing C3, B, D̄ and P, C3, B, D̄, P and H, and C3, B, D̄, P and I,

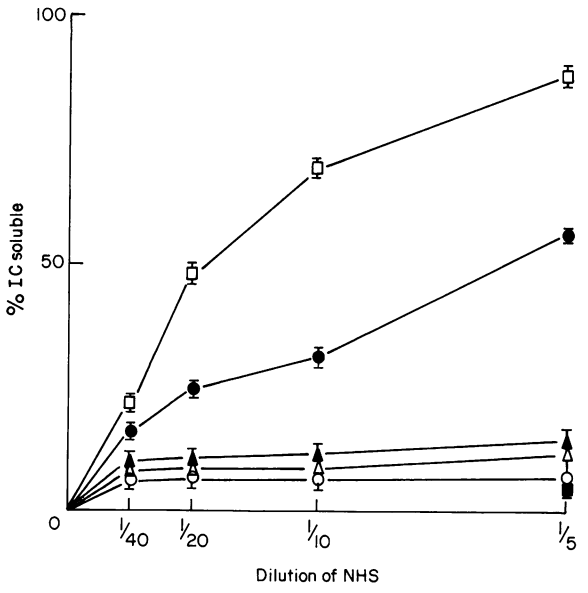

Fig. 1. Dose–response curves for the inhibition of immune precipitation by normal serum (□——□) and reaction mixtures containing C3, B, P and D̄ (○——○), C3, B, P, D̄ and H (△——△), C3, B, P, D̄ and I (▲——▲) and C3, B, P, D̄, H and I (●——●). The concentration of the alternative pathway components were adjusted so that they were the same as those which were present in the serum dilutions. A 1:5 dilution of serum contained 300 μg C3, 50 μg B, 6 μg P, 50 μg H and 10 μg I. D̄ was added to the highest concentration at 100 u/ml. Each point represents the mean (±s.e.) of three determinations.

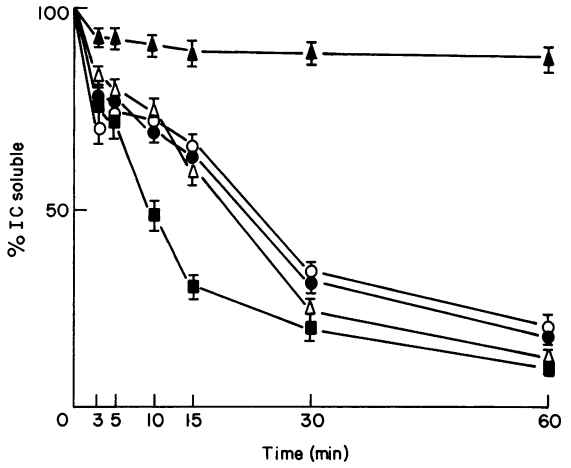

Fig. 2. Kinetics of immune precipitation in the presence of serum diluted 1:5 (▲——▲); C3 (300 μg/ml), B (50 μg/ml), P (6μg/ml) D̄ (100 u/ml) (△——△); C3, B, P, D̄ H (50 μg/ml) (●——●); C3, B, P, D̄, I (10 μg/ml) (□——□) and buffer (■——■). The concentration was adjusted to give the same concentrations as those present in diluted serum. Each point represents the mean (±s.e.) of three determinations.

all of these combinations appeared to slow the rate of immune precipitation to a minor extent (Fig. 2). However only when C3, B, D̄, P, H and I were present together was immune precipitation reduced at the end of the incubation period, when this combination of components was present the pattern of precipitation was different to that observed with the incomplete combinations described above. Instead of early slowing of the rate of precipitation, during the first 10 min the rate of precipitation was the same as that observed in the buffer control. However, after this initial phase of precipitation, a phase of resolubilization occurred (Fig. 3).

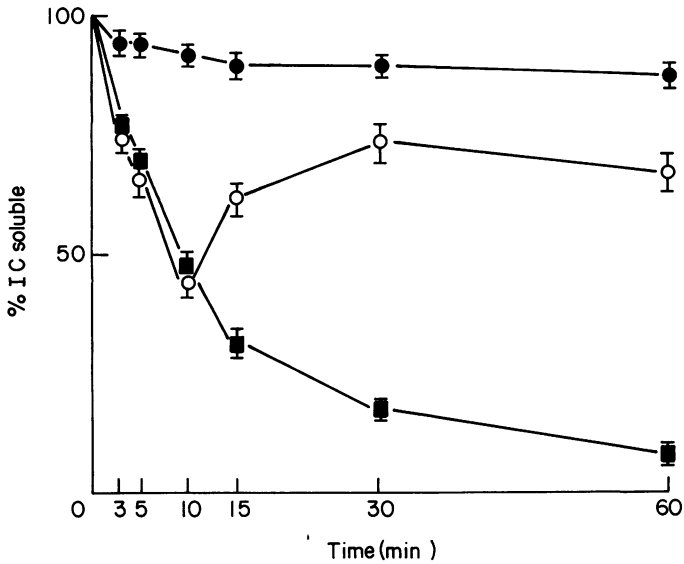

Fig. 3. Kinetics of immune precipitation in the presence of serum diluted 1:5 (●——●) and purified alternative pathway complement components, C3 (300 μg/ml), B (50 μg/ml), P (6 μg/ml), D̄ (100 u/ml), H (50 μg/ml), I (10 μg/ml) (○——○) and buffer (■——■). The concentration was adjusted to give the same concentration as those present in diluted serum. Each point represents the mean (±s.e.) of three determinations.

Comparison of the efficiency of C3 in the classical and alternative pathways

Reaction mixtures containing mixtures containing varying amounts of C3 and (a) the classical pathway components, C1 (C1q 40 μg/ml), C4 (100 μg/ml) and C2 (2,000 u/ml), (b) the alternative pathway components B (50 μg/ml), D̄ (100 u/ml). P (6 μg/ml). H (50 μg/ml) and I(10 μg/ml) or (c) the classical and alternative pathway components together, were assessed for their ability to prevent immune precipitation. C3 was more effective in the presence of the classical pathway than the alternative pathway components (Fig. 4). The reaction mixture containing both classical and alternative pathway components was no more effective than that containing classical pathway components alone.

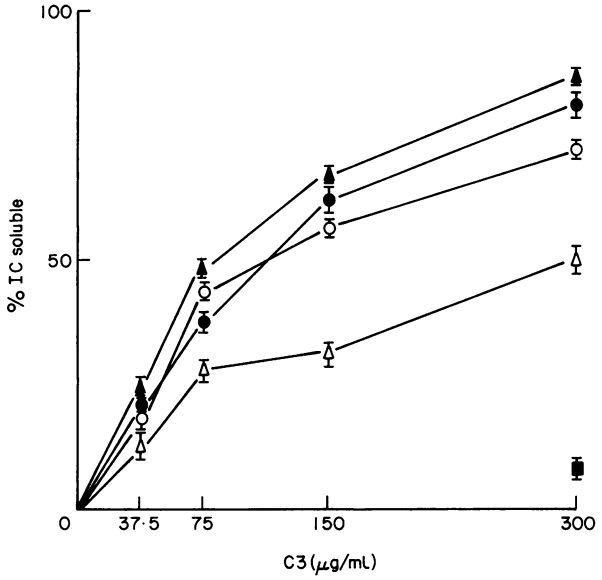

Fig. 4. Dose–response curves of the inhibition of immune precipitation by serum diluted 1:5 (▲——▲) and by using various amounts of C3 with classical and alternative pathway components; C1 (C1q 40 μg/ml), C4 (100 μg/ml). C2 (2,000 u/ml) B (50 μg/ml), P (6 μg/ml), H (50 μg/ml), I (10 μg/ml) (●——●); classical pathway alone (C1 C1q 40 μg/ml), C4 (100 μg/ml), C2 (2,000 u/ml) (○——○); alternative pathway alone B (50 μg/ml), D̄ (100 u/ml), P (6 μg/ml); H (50 μg/ml), I (10 μg/ml) (△——△); buffer control (■). The concentration of complement proteins was adjusted to give the same concentration as those in diluted serum. C3 concentrations are as shown on the abscissa. Each point represents the mean (±s.e.) of three determinations.

C3 consumption by IC

When IC were formed in serum 57·3% of C3 activity was consumed (Table 1). IC formed in the isolated classical pathway proteins were associated with a loss of 52·2% of C3 activity. C3 consumption was only 34·3% when IC were formed in the presence of C3, B, D̄, P, H and I. In the absence of the control proteins H and I (i.e. C3, B, D̄, P), C3 turnover was 76·3% in the absence and 74·9% in the presence of complexes. When the six alternative pathway proteins (C3, B, D̄, P, H and I) were incubated in the absence of complexes there was no loss of C3 haemolytic activity.

Binding of complement components to IC

When IC were formed in reaction mixtures consisting of the classical pathway components (C1 [C1q 40 μg/ml], C4 [100 μg/ml], C2 [2,000 u/ml]) and ^{125}I-C3 (300 μg/ml), the alternative pathway components (B [40 μg/ml], D̄ [100 u/ml], P [6 μg/ml], H [50 μg/ml], I [10 μg/ml] and ^{125}I-C3 [300 μg/ml]) or both classical and alternative pathway components, small but significant quantities of radioactivity were precipitated by protein A-Sepharose (Table 2).

Table 1. C3 consumption by IC

Reaction mixture	C3 u/ml	%C3 consumed
NHS (1:5 dilution)+IC	2,466	57·3
NHS (1:5 dilution)	6,250	Nil
C1, C4, C2, C3+IC	2,823	52·2
C3, B, D̄, P	1,400	76·3
C3, B, D̄, P+IC	1,483	74·9
C3, B, D̄, P, H, I	5,916	Nil
C3, B, D̄, P, H, I+IC	3,881	34·3
C3+buffer	5,907	Nil

Table 2. Binding of ^{125}I-C3 to IC

Reaction mixture	C3 bound		IgG soluble		Ratio C3:IgG
	%	μg	%	μg	
C1, C4, C2	1·8	0·62	85	1·28	1:2·4
B, D̄, P, H, I	1·3	0·45	64	0·96	1:2·5
C1, C4, C2, B, D̄, P, H, I	1·8	0·62	85	1.28	1:2·4

Molar ratios of C3b and IgG are based on mol. wts of 178,500 for C3b (Hugli & Müller-Eberhard, 1978) and 1,500,000 for IgG (Stanworth & Turner, 1978)

Table 3. Precipitation of soluble IC antisera to complement components

Antiserum	% precipitation*
C1q	55·6±1·4†
C4	88·0±0·6
C2	41·0±2·1
C3	90·7±0·3
C5	29·0±0·5
B	0
P	34·1±0·1
H	31·7±2·3
I	0

* Precipitation achieved with the opitmal volume of antiserum. No precipitation of B or I was produced even when different volumes of antisera were added.

† Mean±s.e. of three experiments.

The C3 input in these experiments was 300 μg/ml (34·5 μg/tube) and the input of anti-BSA was 150 μg/ml (1·5 μg/tube). In the mixture containing classical pathway components alone, or classical pathway plus alternative pathway components, 1·8% (0·62 μg) of the C3 was bound specifically to the soluble complexes.

In a parallel series of experiments in which unlabelled C3 and ^{125}I-anti-BSA were used, 85% of the complexes remained in solution, and were precipitated entirely by protein A-Sepharose. Thus 0·62 μg of C3b were bound to 1·28 μg of antibody in the form of soluble complexes. Assuming mol. wts of 178,500 for C3b (Hugli & Müller-Eberhard, 1978) and 150,000 for IgG (Stanworth & Turner, 1978) these data indicate that 1 molecule of C3 is bound for every 2·4 molecules of IgG in the soluble complex. When the alternative pathway components were studied on their own only 1·3% (0·45 μg) of C3b was bound specifically to the complexes. In a parallel experiment only 64% (0·96 μg) ^{125}I-anti-BSA was in solution, and this was precipitated entirely by protein A-Sepharose. Thus 1 molecule of C3b was bound for every 2·4 IgG molecules in the soluble complexes. The number of C3b molecules bound to IgG in the mixture of classical and alternative pathway components was the same as that observed when the classical pathway components alone were present.

In a second series of experiments in which IC consisting of ^{125}I-BSA and anti-BSA were formed in serum, almost all of the soluble complexes present after 1 h incubation at 37°C were precipitable with anti-C3 (90·7%) and anti-C4 (88·0%). Antisera to C1q (55·6%), C2 (41·0%), C5 (29·0%), P (34·1%) and H (31·7%) precipitated smaller quantities of IC, whereas normal rabbit serum, and antisera to B and I did not precipitate any complexes (Table 3).

DISCUSSION

In a previous paper we showed that purified classical pathway components were as effective as whole serum in the prevention of immune precipitation (Naama *et al.*, 1984). This finding suggests that the alternative pathway does not play a significant role in this complement-mediated activity. This conclusion is contrary to the findings reported earlier by Schifferli *et al.* (1980) and ourselves (Naama *et al.*, 1983), who noted that immune precipitation occurred in sera which had been depleted of B or D̄. The results of the experiments reported here show that the six purified alternative pathway components (C3, B, D̄, P, H and I) were able to reduce immune precipitation. The presence of both of the regulatory proteins H and I was essential for this reduction in immune precipitation to be observed (Figs 1 and 2). This observation is compatible with the findings of Fujita, Takata & Tamura (1981) who found that both H and I were essential for the solubilization of immune precipitates by the alternative pathway components. They also are in agreement with the observations of Fearon & Austen (1977) who showed that binding of C3b to particulate activators of the alternative pathway occurred only when H and I were present in mixtures of alternative pathway components. When H and/or I were omitted from the reaction mixtures, excessive fluid phase turnover of C3 occurred which was accompanied by immune precipitation (Table 1 & Fig. 2). The presence of H and I prevented fluid phase turnover in the absence of IC and limited C3 turnover in the presence of IC. This limited C3 turnover was associated with reduced immune precipitation.

The kinetic studies showed that in the presence of H and I immune precipitation occurred at the same rate as in the buffer control, however, the alternative pathway proteins resolubilized the newly formed precipitates (Fig. 3). In contrast, in the absence of one or both of the alternative pathway regulatory proteins, situations in which excessive fluid phase C3 turnover occurred, the initial rate of immune precipitation was slowed. We have not explained this finding but it suggests that perhaps some C3b became bound to IC. If so, then the quantity of bound C3b must have been insufficient, or the sites of binding were not those which were essential for the prevention of immune precipitation.

An antisera to C3 and C4 were able to precipitate the vast majority of the soluble IC, it is probable that these two components are present in greater quantities that the other components. These studies also showed that significant amounts of C1q, C2, P, H and C5 were bound to soluble IC. P, H and C5 were presumably bound to C3b. Failure to precipitate soluble complexes with antiserum to I probably occurs because this enzyme does not have a high affinity for C3b (Pangburn, Schreiber & Müller-Eberhard, 1977). It has been shown previously that I is not

consumed during classical or alternative pathway activation (Whaley, Ward & Ruddy, 1978). The failure to precipitate soluble complexes with anti-B suggests that B is not bound to C3b when complexes are formed in serum.

Previous studies Naama *et al.*, 1983, 1984) have shown that the critical step in the prevention of immune precipitation is the activation of C3. The binding of C3b to IC presumably prevents the formation of large complexes (Takahashi, Takahashi & Hirose, 1980) as well as dissociating antigen–antibody bonds (Miller, 1977). Our data show that an average of two C3b molecules are incorporated for every five IgG molecules in the form of soluble complexes (Table 2). This ratio is the same whether the complex is formed in the presence of the classical pathway components, the alternative pathway components or a mixture of both. It is lower than the estimate (1 molecule C3b: 1 molecule IgG) of Fujita *et al.* (1981) who were studying the solubilization of immune precipitates. We are unable to explain this discrepancy.

Although the alternative pathway proteins were able to maintain complexes in solution, this occurred only after an initial phase of precipitation. This pattern of precipitation followed by resolublization is very similar to that observed in C1q deficient serum in which only the alternative pathway is functioning (Schifferli & Peters, 1983).

The following facts indicate that in whole serum the classical pathway is of greater importance than the alternative pathway in the prevention of immune precipitation: (1) the purified classical pathway components are as effective as whole serum (Naama *et al.*, 1984); (2) purified components of the alternative pathway are (a) less effective than the classical pathway proteins and (b) only resolubilize following an initial phase of immune precipitation; (3) the mixture of classical and alternative pathway proteins is no more effective than the classical pathway components alone and (4) Factor B, an essential component of the alternative pathway C3 convertase does not appear to be present on complexes rendered soluble by serum whereas C2, The equivalent protein of the classical pathway, can be detected.

Thus, our evidence suggests that the alternative pathway is of secondary importance to the classical pathway in the prevention of immune precipitation. It is probable that the alternative pathway provides a backup system to which limits immune precipitation in patients who have inherited deficiencies of the classical pathway proteins. In addition, patients with diseases such as systemic lupus erythermatosus or rheumatoid arthritis in which complement activation occurs with selective depletion of classical pathway components, the alternative pathway may prevent excessive immune precipitation.

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