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Mechanism of complement activation by radiographic contrast media*

C. M. ARROYAVE & E. M. TAN *Division of Allergy and Immunology, Scripps Clinic and Research Foundation, La Jolla, California, U.S.A.*

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SUMMARY

Activation of the complement system by radiographic contrast media (RCM) was demonstrated by *in vitro* haemolytic and immunological assays. Such activation was found to be a function of the RCM molar concentration. Iodipamide was the most active of five RCM tested. When RCM was incubated with normal human serum (NHS) in the presence of ethylene glycol–tetra-acetic acid and magnesium ions, conditions which block activation of the classical pathway but permit activation of the alternative pathway, haemolytically active C3, properdin and factor B were found to be decreased but haemolytically active C4 was normal. Using counterimmunoelectrophoresis, the activation of complement was further demonstrated by detection of C3 and factor B-split products. Finally, when radiolabelled complement proteins were reacted with RCM *in vitro* and studied by density-gradient ultracentrifugation, it was demonstrated that a large complex was formed with a sedimentation of 22S, similar in characteristics to the C5b–C9 complex. It was postulated that the mechanisms of *in vitro* consumption of complement by RCM was mainly through the alternative pathway.

INTRODUCTION

In a recent study in patients who were given radiographic contrast media (RCM) for diagnostic studies, it was demonstrated that a significant fall in total haemolytic complement (CH50) occurred during infusion of RCM (Arroyave, Bhat & Crown, 1976). Further analysis of the complement changes showed that falls in plasma CH50 were paralleled by falls in C3H50. There was reduction in properdin and factor B but no change in C1q and C4, suggesting that complement activation was by the alternative pathway. In this study we have investigated the *in vitro* effect of RCM on the complement system. It was found that different RCM are able to activate *in vitro* the complement system and this activation was found to be mainly through the alternative pathway. By sucrose density-gradient (SDG) ultracentrifugation experiments, it was found that during such activation a similar material to the C5b–C9 complex was formed.

MATERIALS AND METHODS

Buffers and solutions. In all complement determinations, gelatin–veronal buffer (GVB) was used and prepared as described previously (Mayer, 1961). Solutions without RCM, but identical in salt concentrations and pH, were made exactly according to the specifications of the commercial preparations in order to demonstrate that these solvents were not themselves activators of the complement system.

Reagents. Inulin was obtained from Pfanstiehl Laboratories (Waukeegan, Illinois). Five different RCMs were used during this study: (1) sodium and meglumine diatrizoate (Hypaque, Winthrop, New York); (2) sodium iothalamate (Conray 60, Mallinckrodt, St. Louis Missouri); (3) meglumine iodipamide (Cholografin, Squibb & Sons, Princeton, New Jersey); (4)

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Correspondence: Dr C. M. Arroyave, Division of Rheumatology, University of Colorado Medical Center, Denver, Colorado 80220, U.S.A.

sodium and meglumine diatrizoate (Renografin 60, Squibb & Sons, Princeton, New Jersey), and (5) meglumine diatrizoate (Reno-M-Dip, Squibb & Sons, Princeton, New Jersey).

Measurements of whole complement activity and individual complement components. We used a modification of the method of Mayer (1961) to determine whole haemolytic complement activity, and the results were expressed in terms of CH50 u/ml. Levels of haemolytically active C4 and C3 were quantitated by previously described techniques (Cooper & Müller-Eberhard, 1968; Müller-Eberhard *et al.*, 1966). Factor B and properdin haemolytic activities were assayed by modification of a previously described method (Martin *et al.*, 1976). The assay was made using 0·5% guinea-pig erythrocytes, properdin or factor B-depleted serum in the presence of 10 mM ethylene glycol–bis-amino-ethyl-tetra-acetic acid (EGTA) and 0·5 mM Mg^{2+}. The latter conditions permit activation of the alternative pathway but block that of the classical pathway (May, Rosse & Frank, 1973). The depleted serum was made by incubation of insolubilized anti-properdin or factor B (Avrameas & Ternyck, 1967) with fresh EDTA plasma. The absence of the specific protein was demonstrated both by double diffusion and haemolytic assays. All the haemolytic assays were performed in duplicate.

Purified proteins. Two different human C4 preparations were isolated by using a previously described method (Schreiber & Müller-Eberhard, 1974). Properdin, factor B and C6 were prepared as published (Götze & Müller-Eberhard, 1971, 1974; Arroyave & Müller-Eberhard, 1971). All the complement proteins were judged free of demonstrable contamination by chromatographic, electrophoretic and immunochemical criteria. Some of these proteins were radio-labelled with ^{125}I by the method of McConahey & Dixon (1966). Two ^{125}I-labelled C8 preparations were kindly supplied by Dr W. P. Kolb (Scripps Clinic and Research Foundation, La Jolla, California).

Immunochemical analyses. Immunoelectrophoresis (IEP) was performed according to Scheidegger (1955) in 1% agarose containing 10 mM ethylene dinitrilotetra-acetic acid (EDTA). IEP proceeded for 90–120 min at a potential gradient of 5 ma per slide and was developed with monospecific antisera to C3, C4 and factor B. Counterimmunoelectrophoresis (CIE) was performed as described previously (Arroyave & Tan, 1977). The test is based on the presence of breakdown products from C4, C3 and factor B after complement activation. The native molecules in plasma can be differentiated from its split products, since the latter's precipitin lines in CIE has more anodal positions. This test is rapidly performed and is more sensitive than the traditional haemolytic or immunoelectrophoretic assays. Monospecific antisera to C1q, C4, C3, C6, properdin and factor B were produced using highly purified proteins and by the method of lymph node immunization (Goudie, Home & Wilkinson, 1966). The antisera produced against C3, C4 and factor B were demonstrated to contain antibodies against the native proteins as well as against their respective split products. These are C3a, C3b, C3c and C3d for C3; a fragment with α mobility in C4 and α and γ mobility fragments for factor B. Anti-human C3a was kindly supplied by Dr Tony E. Hugli (Scripps Clinic and Research Foundation, La Jolla, California).

Activation of complement by RCM. In vitro activation of the complement system (classical or alternative) by RCM was studied by incubating different concentrations of RCM with fresh normal human serum (NHS), serum treated with 10 mM EGTA and 0·5 mM Mg^{2+} ions, or with genetically C2-deficient human serum (Wilson, Arroyave & Tan, 1976). 1 ml of serum was incubated for a period of 60 min at 37°C with a wide range of RCM concentrations in a final volume of 2 ml. Two tubes were included in this experiment. In the first tube after the incubation period, the reaction was stopped by the addition of 48 ml of cold GVB and immediately an aliquot was used for complement haemolytic determinations. The second tube was used for immunochemical analysis. All the haemolytic assays were made in duplicate. Solutions identical in salt concentration and pH but without RCM were prepared in order to demonstrate that these solvents were not themselves *in vitro* activators of the complement system.

Sucrose density-gradient ultracentrifugation (SDG). In order to determine if RCM would cause formation of C5b–C9 complex, 10–40% sucrose gradients in phosphate-buffered saline (PBS) pH 7·4, ionic strength 0·15 were formed in 5 ml cellulose tubes by using a Buchler automatic density-gradient maker (Buchler Instruments Division, Nuclear–Chicago Corporation, Fort Lee, New Jersey). The gradients were equilibrated to 4°C before the samples (100 or 200 μl) were applied. The samples consisted of a mixture of radiolabelled complement protein(s) and normal human serum. To activate the complement system, we incorporated in some of the mixtures either inulin or RCM and then incubated at 37°C for 180 min. Radiolabelled complement proteins incorporated in buffer or NHS were used as control. As markers, we used thyroglobulin 19S, human IgG, 7S and human haemoglobin 4·5S. Ultracentrifugation was performed in an SW50.1 rotor (Beckman Instruments, Incorporated, Spinco Division, Palo Alto, California) for 15 hr at 35,000 rev/min and 4°C. After ultracentrifugation, fractions were collected by puncturing the bottom of the tubes with a Buchler gradient-fractionation device. The ^{125}I-containing samples were analysed with a Nuclear–Chicago dual-channel, automatic, well-type gamma-counter (Nuclear–Chicago Corporation, Des Plaines, Illinois).

RESULTS

Haemolytic complement activity

Activation or consumption of the complement system by five different contrast media, as a function of molar concentrations, is shown in Fig. 1. A decrease in measurable CH50 u/ml was observed with all the RCM used. Iodipamide (Cholografin) was found to be the most potent activator of complement when compared with the other RCM. The other RCM required a higher molar concentration in the reaction with NHS to produce falls in complement haemolytic activity. Decrease in C3H50 u/ml was observed

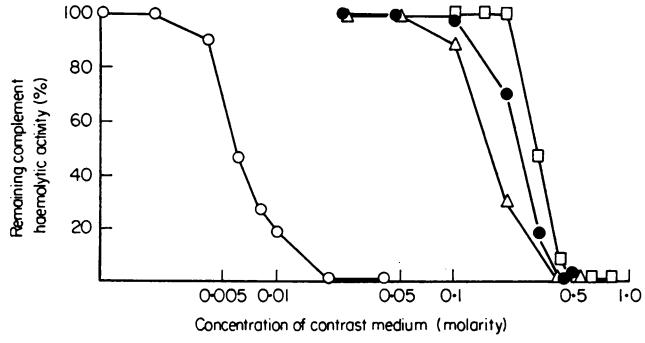

FIG. 1. Complement consumption by radiographic contrast media. NHS was incubated for 60 min at 37°C with different concentrations of RCM and the total haemolytic activity (CH50) was determined. The RCM used were: meglumine iodipamide (○) (Cholografin), sodium and meglumine diatrizoate (△) (Renografin 60), meglumine diatrizoate (●) (Reno-M-Dip) and sodium iothalamate (□) (Conray 60).

to be the same in NHS or NHS–EGTA–Mg^{2+}. It is important to mention that the buffer solutions in which the RCM-dyes are commercially available, were not able to produce any change in CH50 or C3H50 after they were incubated with NHS with or without EGTA–Mg^{2+} ions.

The effect of RCM on different serum haemolytic complement proteins are summarized in Table 1. In this particular experiment, the amount of RCM used in the reaction mixture was calculated to produce at least 50% inactivation of CH50 by using the data obtained in Fig. 1. Whole complement haemolytic activity (CH50) and C3H50 decreased considerably in all of the five RCM used; in contrast, the C4H50 values were within normal limits. However, haemolytically active properdin and factor B dropped around 30–70% in all the experiments in which a drop in CH50 was observed. A drop in C3H50, haemolytically active properdin and factor B was also observed when the five different RCM's were incubated in EGTA–Mg^{2+} serum or genetically C2-deficient serum.

Identification of split products from complement proteins

To determine if decrease in CH50 units was due to activation of the complement system with the formation of complement split products, mixtures of NHS–RCM were subjected to CIE and/or IEP after incubation at 37°C for 60 min. The antisera used were anti-C3, anti-C3a, anti-C4 and anti-factor B. As seen in Fig. 2, there are two different precipitin lines on the samples incubated with Cholografin and developed with antisera to C3 or factor B. The cathodal precipitin line corresponded to the mobility of native protein and the anodal precipitin line to its split products. No anodal split product precipitin line was detected when the antiserum used was anti-C4, suggesting that the C4 molecule was not cleaved.

TABLE 1. Effect of RCM on serum haemolytic complement activity*

RCM	Haemolytic activity (%)				
	CH50	C4H50	C3H50	Properdin	Factor B
Reno-M-Dip	43	95	50	45	36
Cholografin	35	92	42	50	45
Conray 60	40	94	60	46	60
Renografin	35	97	38	42	30
Hypaque	50	93	40	35	30
Control	100	100	100	92	95

* NHS was incubated at 37°C for 60 min with a concentration of RCM enough to produce approximately 50% inactivation of complement. After the incubation time the per cent consumption of the different complement components was determined.

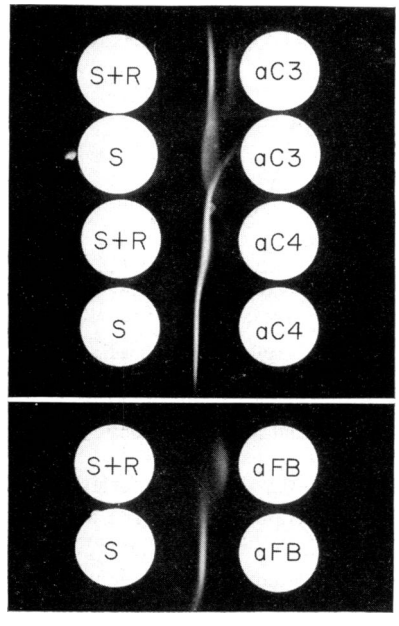

FIG. 2. Analysis by counterimmunoelectrophoresis of C3, C4 and factor B after incubation at 37°C for 60 min of NHS with Cholografin. In the cathodal well, NHS (S) or NHS incubated with Cholografin (S+R) were placed. Anti-human C3 (aC3), anti-human C4 (aC4) and anti-human factor B (aFB) were placed in the anodal well.

When the same reaction mixtures were developed with anti-C3a (IEP) (Fig. 3) a cathodal split product was observed that corresponded to C3a.

Formation of the stable C5b–C9 complex in free solution by activation of complement with inulin or RCM

A mixture of RCM and NHS containing ^{125}I-labelled C6 or ^{125}I-labelled C8 was incubated for 3 hr at 37°C and then subjected to SDG ultracentrifugation. As a positive control, we used serum incubated with inulin and as a negative control, serum incubated without RCM. Analysis of the distribution of ^{125}I-labelled C8 radioactivity in RCM and inulin-treated serum revealed the presence of a heavy component with an S-rate of approximately 22S corresponding to the mobility of the stable C5b–C9 complex (Fig. 4). No heavy ^{125}I-labelled C8 material was seen in the serum incubated without RCM or inulin. A similar pattern was observed when ^{125}I-labelled C6 was used.

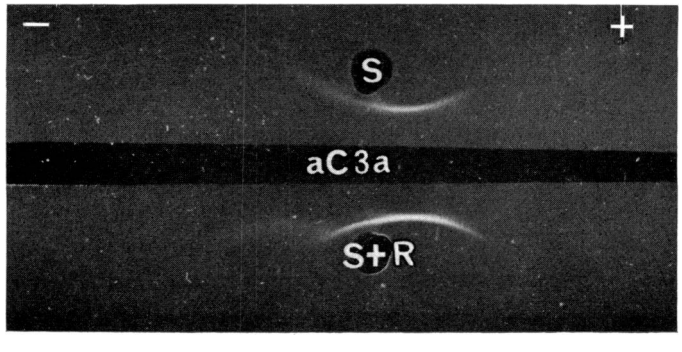

FIG. 3. Immunoelectrophoretic analysis of C3a in normal human serum (S) and meglumine iodipamide (Cholografin) treated serum (S & R) in the presence of EGTA-Mg^{2+} ions to block classical pathway activation (RCM). The antisera in the trough is monospecific against human C3a (aC3a).

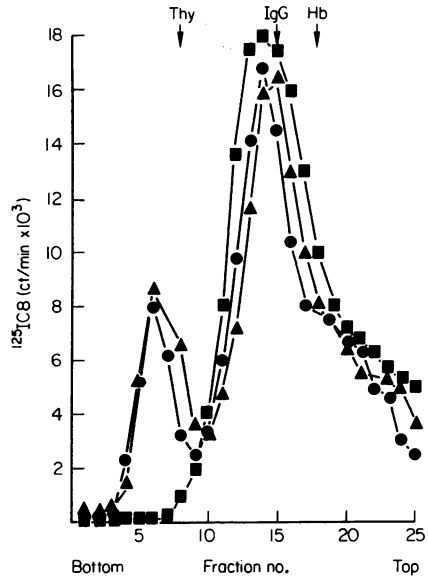

FIG. 4. Demonstration of the stable C5b–C9 complex in free solution. ^{125}I-labelled C8 was added to NHS before addition of inulin or RCM and sucrose density-gradient ultracentrifugation was performed on the mixture. A heavy complex is seen in fractions 5–10 in solutions containing inulin (▲) and RCM (●). The control experiment (■) contained serum and the radiolabelled protein without activator. The sedimentation markers were Thyroglobulin (19S), IgG (7S) and haemoglobin (4·5S).

DISCUSSION

We have shown that the complement system can be activated *in vitro* by different RCM. This activation was found to be dose-dependent. While this manuscript was in preparation, Lang, Lasser & Kolb (1976) also reported activation of complement by RCM in a similar fashion. In the present report, we present evidence that such activation was mainly through the alternative pathway, since haemolytically active C4 was normal in concentration but there was a significant decrease in haemolytically active C3, properdin and factor B. Activation of complement via the alternative pathway was also supported by the CIE and IEP experiments, in which precipitin lines corresponding to split products of C3 and factor B but not of C4 were detected. Activation of complement through the feedback mechanism (Lachmann & Nicol, 1973) is probably unlikely, if we consider that similar *in vitro* results (drop in C3H50, factor B and properdin levels but not C4H50) were obtained when RCM was incubated in genetically C2-deficient human serum or EGTA–NHS containing Mg^{2+} ions, conditions that prevent activation of the classical pathway, but permit activation of the alternative pathway.

The RCM activation of complement may have relevance to the production of clinical symptoms since C3a and C5a anaphylatoxins have been shown to be physiologically active peptides that produce contraction of smooth muscle, increase capillary permeability, release histamine from mast cells and many other cells (Osler & Sandberg, 1973; Bokisch, Müller-Eberhard & Cochrane, 1969). Preliminary *in vitro* experiments by Till *et al.* (personal communication) showed that incubation of NHS and RCM (meglumine diatrizoate or sodium meglumine ioglycamate) produce chemotactic locomotion of granulocytes and macrophages, which could be inhibited by addition of anti-C5. However, it is important to consider that the expression of various anaphylatoxin activities may be modulated by the action of the serum anaphylatoxin inactivator (Bokisch *et al.*, 1969).

Lang & Lasser (1962) reported complex formation between RCM and albumin. They noted that the binding of trypan blue to albumin was altered by the concomitant binding of Cholografin. It seems likely that the contrast media–protein interactions reflect the hydrophobic properties of the RCM. It is possible that in complement activation, a similar RCM–complement protein interaction may be the initiating event for triggering the alternative pathway reaction. This possibility is under investigation

in our laboratory. The presence of a 22S heavy material by sucrose density-gradient centrifugation in the reaction mixture containing NHS and RCM that is similar to the C5b–C9 stable complex described by Kolb & Müller-Eberhard (1973) is very suggestive that RCM is able to form such a complex.

Immunological events are not considered to be the major cause of RCM reactions (Lasser, 1968). Recent data showing direct histamine release and complement activation by contrast media also show the non-immunological nature of these reactions. During perfusion of canine liver with RCM, high levels of histamine release were shown in 3 min (Lasser *et al.*, 1971). Similar rises in plasma histamine were found during infusion of RCM in humans (Bhat, Arroyave & Tan, unpublished observations). Two different groups have demonstrated complement activation by RCM *in vivo*, Lasser and his co-workers in dogs, and in our laboratory in humans. With the demonstration of histamine release and complement activation by RCM, both *in vivo* and *in vitro*, the importance of these findings in untoward reactions to RCM would need to be investigated further.

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