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Activation of mouse complement by different classes of mouse antibody

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Summary. The capacity of mouse IgM, IgG1, IgG2 and IgA anti-dinitrophenyl (DNP) antibodies to activate mouse or guinea-pig complement was studied, using a sensitive haemolytic assay and two-dimensional immunoelectrophoresis to detect cleavage of mouse C3. Three monoclonal IgM antibodies, and a heterogeneous IgM fraction, lysed trinitrophenylated erythrocytes in the presence of guinea-pig C, but failed to produce lysis in the presence of mouse C, and only activated mouse C3 very inefficiently. A monoclonal IgG1 antibody did not produce haemolysis in the presence of guinea-pig or mouse C, but cleaved mouse C3 via the alternative pathway. Two IgA myeloma proteins (M315 and M460) had similar properties. A heterogeneous IgG2 antibody fraction produced haemolysis in the presence of both mouse and guinea-pig C, and was shown to activate both the classical and alternative pathways of mouse C.

INTRODUCTION

The capacity of different classes of antibody to activate complement has been widely studied. It is generally assumed that rabbit and human IgM antibodies against cell surface antigens activate C more efficiently than IgG, although the reverse may be true of antibodies to soluble antigens (Borsos & Rapp, 1965; Ishizaka, Tada & Ishizaka, 1968). Many studies addressed to this question have used C from heterologous species (especially guinea-pig), while less has been done with homologous C, except in the human. In particular, little is known about the capacity of mouse antibodies to activate mouse C (C^{Mo}), presumably because of the poor haemolytic activity of C^{Mo} in comparison with guinea-pig C (C^{GP}). Winn (1965) reported that mouse IgM antibodies fixed C^{Mo} very poorly, if at all, and emphasized that the C-fixing properties of different antibody classes could depend on the species source of C used. As part of our studies on the role of C in generation of immunological memory (Klaus & Humphrey, 1977; Klaus, 1978), we have re-investigated the activation of C^{Mo} and C^{GP} by different classes of mouse antibody.

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MATERIALS AND METHODS

Monoclonal antibodies

E4, H1 and H3 are three monoclonal IgM anti-DNP antibodies which were generated by fusing anti-DNP plaque-forming cells from BALB/c mice immunized with dinitrophenylated keyhole limpet haemocyanin (DNP–KLH), with the drug-resistant myeloma cell line P3-NSI-1-Ag-4-1 (Köhler & Milstein, 1976) using

polyethylene glycol. A monoclonal IgG1 antibody (A3) was produced by fusing anti-DNP PFC from CBA/Ca mice primed and boosted with DNP–KLH with the same myeloma cell line. The hybrid cell lines were cloned in agar once or twice, and were then maintained as subcutaneous tumours in BALB/c mice (for E4, H1 and H3) or in (BALB/c × CBA/Ca)F_1 mice (for A3). The sera from these mice were used as sources of antibody, after inactivation (56°/30 min) and absorption with sheep erythrocytes.

The two IgA anti-DNP myeloma proteins used (M460 and M315) were maintained as ascitic tumours in BALB/c mice. The proteins were isolated (without reduction and alkylation) by binding them to a column of DNP_{17}-bovine serum albumin linked to Sepharose 4B (Pharmacia, Uppsala, Sweden), eluting with 0·2 M glycine-HCl pH 2·6, and immediately neutralizing the eluate with 1 M Tris–HCl, pH 8·6. Both proteins gave strong precipitin reactions with various DNP-substituted proteins.

Heterogeneous antibodies

Mouse anti-DNP antibodies were prepared as described previously (Klaus, 1978). The serum was further separated into IgG1 and IgG2 fractions using Staphylococcal protein A (SpA) coupled Sepharose 4B by a modification of the method of Ey, Prowse & Jenkin (1978). In brief, the serum was dialysed against 0·1 M Tris–HCl, pH 8·6, and the IgG was adsorbed to the column in this buffer. When the E_{280} of the effluent had fallen to $<0·1$, IgG1 was eluted with 0·1 M citrate buffer, pH 6·0. Finally, IgG2 was eluted with 0·2 M glycine–HCl, pH 2·6, into 1 M Tris–HCl, pH 8·6. By immunodiffusion analysis (at 3 mg/ml) using specific rabbit anti-γ_1 and anti-γ_2 Fc antisera, the IgG1 fraction contained no detectable IgG2 while the IgG2 contained trace amounts of IgG1. We found it advisable to re-run the 'dropthrough' peak to maximize recovery of IgG1 which, in our hands, bound rather weakly to SpA, even at pH 8·6.

A heterogeneous IgM anti-DNP fraction was prepared from 20 C3H/He mice given 50 μg DNP-Ficoll i.v., and bled 7, 14 and 21 days later. The serum pool was run through a Sephadex G200 column and the 19S peak subsequently run through the SpA column at pH 8·6. The dropthrough peak from this column contained no IgG by immunoelectrophoretic analysis. Rabbit anti-DNP antibody (A^R) was obtained by hyperimmunization with DNP–bovine serum albumin.

Complement

Guinea-pig C (C^{GP}) was from random-bred Hartley guinea-pigs. It was absorbed with SRBC, and in some experiments trinitrophenylated (TNP) SRBC, and stored at −70°. Mouse C was obtained from 4 to 6 month old male CBA/Ca mice, which were bled from the axilla. The blood was kept on ice for 20–30 min prior to removal of serum, which was stored in small aliquots under liquid nitrogen, and was used without absorption.

This method was adopted after initial experiments indicated that allowing pooled mouse blood to clot at room temperature could result in significant cleavage of C3. Other groups have also found that clotting on ice is the preferred method for preparing C^{Mo}, since clotting at room temperature can lead to substantial losses in haemolytic C activity (Borsos & Cooper, 1961; Andrews & Theofilopoulos, 1978). If blood is not pooled before clotting, however, the two methods yield sera with similar titres of haemolytic C activity (unpublished data).

Precipitin and haemagglutinin assays

Dinitrophenylated keyhole limpet haemocyanin (DNP–KLH, containing 60 DNP groups/10^6 mol. wt protein) was radiolabelled with ^{125}I (specific activity 0·5 μCi/μg). Increasing amounts of antigen were added to a constant amount of antibody, and the maximal amount of antigen precipitated (equivalence point) determined in a standard precipitin assay.

Haemagglutinin titres were determined using TNP–SRBC (Rittenberg & Pratt, 1969) coupled with 10 mg trinitrobenzene sulphonic acid/ml of packed SRBC. Assays were done using 200 μl aliquots of doubling dilutions of antibody in veronal-buffered saline pH 7·3, with 0·1% gelatine, together with 100 μl aliqots of 1% TNP–SRBC. Agglutination titres are given as + + + (complete agglutination) to a + (partial agglutination).

Haemolytic C assay

TNP–SRBC were washed in gelatin-veronal buffer (GVB: see below) and 100 μl packed cells were labelled with 150 μCi [^{51}Cr]-Na_2CrO_4 (Radiochemical Centre, Amersham) for 60 min at 37°, and 4° overnight. Finally, the cells were washed three times and were made up to 0·2% in GVB.

The haemolytic test was done essentially as described by Berden, Hagemann & Koene (1978) in capped, disposable plastic tubes. In brief, 100 μl 0·2% TNP–SRBC-^{51}Cr (E) in GVB (0·02 M veronal buffer,

pH 7·3, containing 0·13 M NaCl, 0·1% gelatin, 1·0 mM $MgCl_2$ and 0·15 mM $CaCl_2$) were incubated for 10 min at 37° with 100 μl of doubling dilutions of antibody (A). Then 300 μl of appropriately diluted C^{Mo} or C^{GP} were added and the tubes were incubated for 90 min at 30°. After addition of 1 ml of ice-cold GVB the tubes were centrifuged, and 1 ml supernatant was removed for ^{51}Cr counting in a gamma spectrometer. Each point was assayed in triplicate.

Each experiment included the following controls. (i) Cells incubated with 1:100 rabbit anti-DNP (haemagglutinin titre 1:5120) antibody and C^{GP} or C^{Mo} (EA^RC^{GP} or EA^RC^{Mo}, respectively). Both lots of C were previously titrated to give *ca* 60% lysis of EA^R. Thus C^{GP} was used at dilutions of 1:1000–1:2500, and C^{Mo} at 1:10–1:25 (details given with individual experiments). (ii) Cells lysed with water (total ^{51}Cr release). (iii) Cells incubated in GVB alone (spontaneous ^{51}Cr release), and (iv) cells incubated in C alone ($E+C^{Mo}$ or $E+C^{GP}$).

Percentage lysis was calculated by:

$$\frac{\text{c.p.m. test serum} - \text{spontaneous release}}{\text{c.p.m. total lysis} - \text{spontaneous release.}} \times 100$$

C3 cleavage

Cleavage of mouse C3 was assayed by two-dimensional immunoelectrophoresis of C^{Mo} after treatment with antigen–antibody precipitates formed at equivalence. Appropriate amounts of DNP–KLH and antibody were incubated at 37° for 30 min and at 4° overnight. The precipitates were washed, resuspended in 50 μl veronal-buffered saline containing either 1·0 mM $MgCl_2$ plus 0·15 mM $CaCl_2$, or 25 mM $MgCl_2$ plus 25 mM ethylene-glycol tris-(aminoethyl)-tetraacetic acid (EGTA). The latter buffer provides conditions for activation of the alternative C pathway only (Sandberg & Osler, 1971). Fifty microlitres of C^{Mo} were added and tubes were incubated at 37° for 45 min. Each experiment included a buffer only control, and a control containing DNP–KLH alone. Antigen–antibody crossed electrophoresis (Laurell, 1965) was performed in agarose gels containing 0·01 M EDTA, the second dimension being run in the presence of a specific sheep anti-mouse C3 serum (Pryjma, Humphrey & Klaus, 1974).

Conglutination

Samples of TNP_{10}–SRBC were incubated with subagglutinating doses of various anti-DNP antibodies, washed thrice and made up to 1% (v/v) in veronal-buffered saline containing Ca^{2+} and Mg^{2+}. Two hundred and fifty microlitre aliquots of EA were incubated for 15 min at 37° with either 100 μl C5-deficient C^{Mo} (from AKR mice) or 30 μl C6-deficient rabbit C. Then 100 μl aliquots of EAC were added to 200 μl bovine conglutinin diluted 1:10 or 1:100, or buffer alone. The mixtures were incubated for 30 min at 37° and 4° overnight, and then read for agglutination. The conglutinin (kindly provided by Dr Dianne Scott) was prepared from bovine serum by the method of Lachmann & Hobart (1978).

RESULTS

Haemolysis by different antibody classes and C^{Mo} or C^{GP}

IgM antibodies. Figure 1 shows that the three monoclonal IgM antibodies H1, H3 and E4 all lysed TNP–SRBC in the presence of C^{GP} but not in the presence of C^{Mo}. H1 and E4 gave substantial haemagglutinin titres. H3 agglutinated TNP–SRBC very weakly, and also failed to precipitate DNP–KLH for unknown reasons. There were substantial differences in the amount of C^{GP} required for comparable degrees of lysis using these three antibodies. Thus, E4 required 0·12 μl C^{GP}/tube, H3 0·3 μl, and H1 > 1·2 μl for 60% lysis. The reasons for this are unknown, but may relate to differences in anti-complementary activity in the three sera. Increasing the amount of C^{Mo} to 100 μl/tube did not lead to significant lysis with any of these antibodies (not shown). Varying the amount of trinitrobenzene sulphonic acid from 2 to 50 mg/ml packed SRBC did not produce significant lysis with C^{Mo}.

To examine the possibility that these monoclonal antibodies might for some reason by atypical IgM molecules, we repeated this experiment with a heterogeneous IgM antibody fraction. The results in Fig. 2 show that this also produced haemolysis in the presence of C^{GP} but not in the presence of C^{Mo}.

IgG antibodies. The monoclonal IgG1 antibody A3 did not lyse TNP–SRBC with either C^{Mo} or C^{GP} (Fig. 3). The results with the heterogeneous IgG1 fraction were somewhat different. Although this also failed to give haemolysis with C^{GP}, it activated C^{Mo} weakly, but significantly. In a subsequent experiment (not shown)

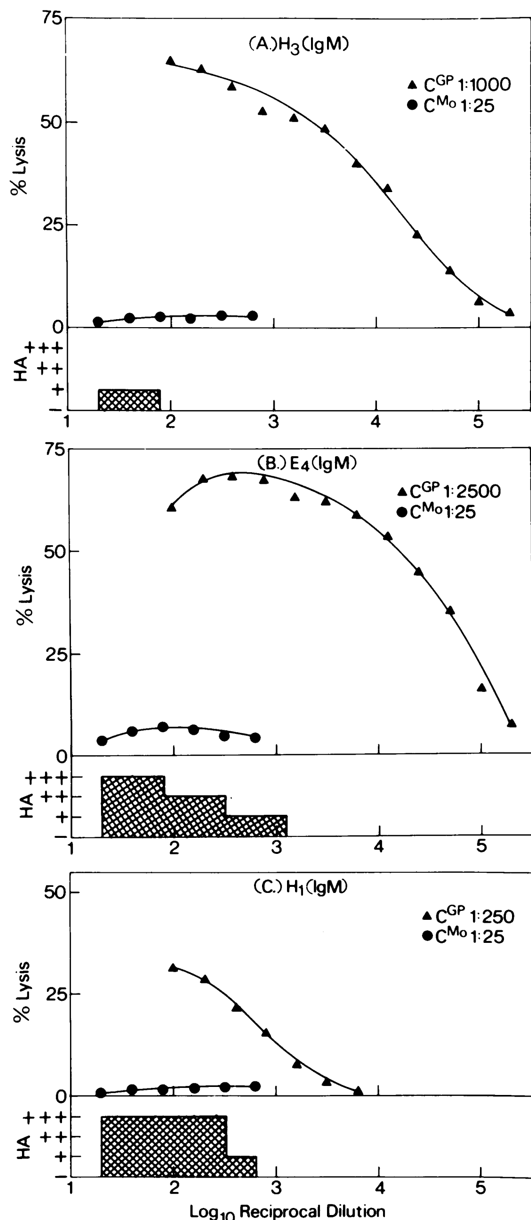

Figure 1. Haemagglutinin (HA) and haemolysis titrations of three monoclonal IgM antibodies H3, E4 and H1. Controls: H3: $EA^{R}C^{Mo}$, 59·2%; $EA^{R}C^{GP}$, 84·1%; $E+C^{Mo}$, 1·8%; $E+C^{GP}$, 0·4%; E4: $EA^{R}C^{Mo}$, 59·2%; $EA^{R}C^{GP}$: 59·6%, $E+C^{Mo}$: 2·2%, $E+C^{GP}$, 1·7%. H1: $EA^{R}C^{Mo}$, 59·2%; $EA^{R}C^{GP}$, 95·9%, $E+C^{Mo}$, 1·8%; $E+C^{GP}$, 2·8%. The dilution of C added to each tube is given on each panel.

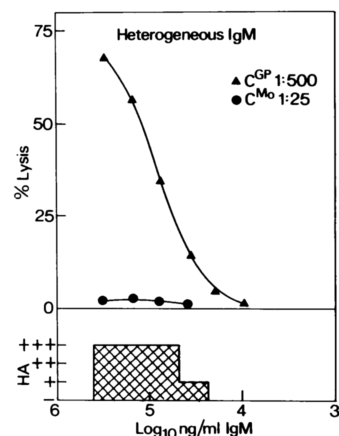

Figure 2. Haemagglutinin (HA) and haemolysis titrations of a heterogeneous IgM anti-DNP antibody fraction. Controls: $EA^{R}C^{GP}$, 95·6%; $EA^{R}C^{Mo}$, 20·3%; $E+C^{GP}$, 0%; $E+C^{Mo}$, 0·27%. The dilution of C added to each tube is shown.

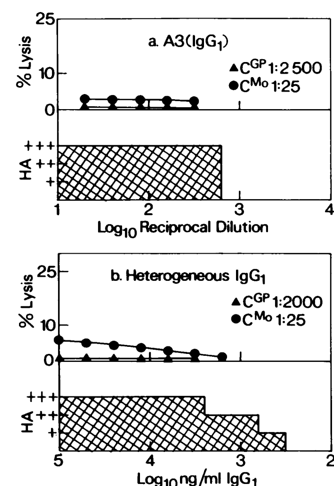

Figure 3. Haemagglutinin (HA) and haemolysis titrations of (a) A3 monoclonal IgG1 antibody and (b) heterogeneous IgG1 antibody. Controls: A3: $EA^{R}C^{GP}$, 59·6%; $EA^{R}C^{Mo}$, 59·2%; $E+C^{GP}$, 1·7%; $E+C^{Mo}$, 2·2%. Heterogeneous IgG1: $EA^{R}C^{GP}$, 31·5%; $EA^{R}C^{Mo}$, 25·7%; $E+C^{Mo}$, 1·0%; $E+C^{GP}$, 0%. The dilution of C added to each tube is given in each panel.

this antibody produced 30% lysis of TNP–SRBC in the presence of 30 μl C^{Mo}.

Figure 4 illustrates that the heterogeneous IgG2 antibody fraction lysed TNP–SRBC in the presence of both C^{Mo} and C^{GP}. The rather low percentage of lysis

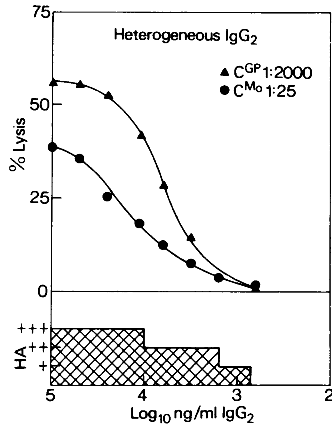

Figure 4. Haemagglutinin and haemolysis titration of a heterogeneous IgG2 antibody fraction. Controls: EA^RC^{Mo}, 25·7%, EA^RC^{GP}, 31·5%, $E+C^{Mo}$, 1·5%; $E+C^{GP}$ 1·0%.

with C^{Mo} (also reflected in the results with EA^RC^{Mo}) in this experiment is unexplained. Subsequently, we retitrated this antibody in the presence of C^{Mo} and in the presence or absence of Ca^{2+} (Fig. 5). The results show that lysis of TNP–SRBC by IgG2 and C^{Mo} requires Ca^{2+} and therefore presumably occurs via the classical C pathway. The weak activation of C^{Mo} by the heterogeneous IgG1 fraction was also Ca^{2+} dependent (not shown).

IgA antibodies. Neither M315 nor M460 at concentrations from 1·5 to 100 μg/ml caused lysis of TNP–SRBC in the presence of either C^{Mo} or C^{GP} (data

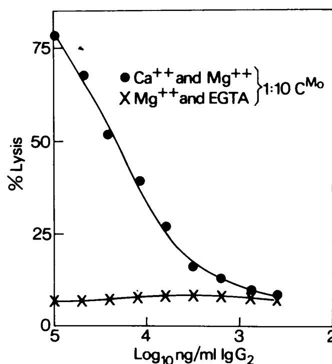

Figure 5. Calcium dependence of haemolysis by IgG2 and C^{Mo}. The antibody fraction used in Fig. 4 was retitrated either in GVB (containing Ca^{2+} and Mg^{2+}) or in GVB containing 25 mM $MgCl_2$ and 25 mM EGTA. Controls: EA^RC^{Mo} (in GVB) 67·2%; EA^RC^{Mo} (in GVB, Mg^{2+}, EGTA). 2·0; $E+C^{Mo}$, 6·3%.

not shown). M315 agglutinated TNP–SRBC down to concentrations of 2·0 ng/ml, while M460 agglutinated down to 75 ng/ml.

Cleavage of mouse C3 by different antibody classes

C3 cleavage was detected by antigen–antibody crossed electrophoresis of C^{Mo} incubated with immune precipitates formed at equivalence. Figure 6 shows that 12 μg (wt of antigen) DNP–KLH–A3 (IgG1) complexes produced substantial C3 cleavage. Since the degree of cleavage was independent of the presence of Ca^{2+}, this reflects activation of the alternative C pathway. In contrast, 50 μg of DNP–KLH–E4 (IgM) or DNP–KLH–H1 (IgM) complexes did not cause significant C3 cleavage. H3 IgM could not be tested since it is a non-precipitating antibody.

Figure 7 shows that the heterogeneous IgG1 and IgG2 fractions caused substantial cleavage of C3 in the presence of Mg^{2+} and EGTA. Hence both fractions presumably activate the alternative C pathway. Similarly, complexes containing M315 (IgA) or M460 (IgA) also cleaved C3 via the alternative pathway. It is possible that the two IgA proteins were less active than IgG1, although it should be stressed that the present technique does not lend itself to accurate quantification of the degree of C3 cleavage.

C3-fixation by IgM antibodies

As an additional test of the capacity of IgM anti-DNP antibodies to activate murine C3, TNP–SRBC sensitized with H1, H3, E4 or heterogeneous IgM antibodies were incubated with mouse or rabbit C and then with bovine conglutinin.

As shown in Table 1 these antibodies only gave weak conglutination in the presence of C^{Mo}, while the rabbit anti-DNP antibody was more strongly positive. In marked contrast, all the antibodies produced strong conglutination in the presence of rabbit C, even using conglutinin diluted to 1:100.

DISCUSSION

The present study shows that mouse IgG2 anti-DNP antibodies activate both the classical and alternative pathway of C^{Mo}, while IgG1 probably only activates the alternative pathway, as does IgA. The most striking finding is that IgM antibodies do not produce haemolysis in the presence of C^{Mo}, although it is

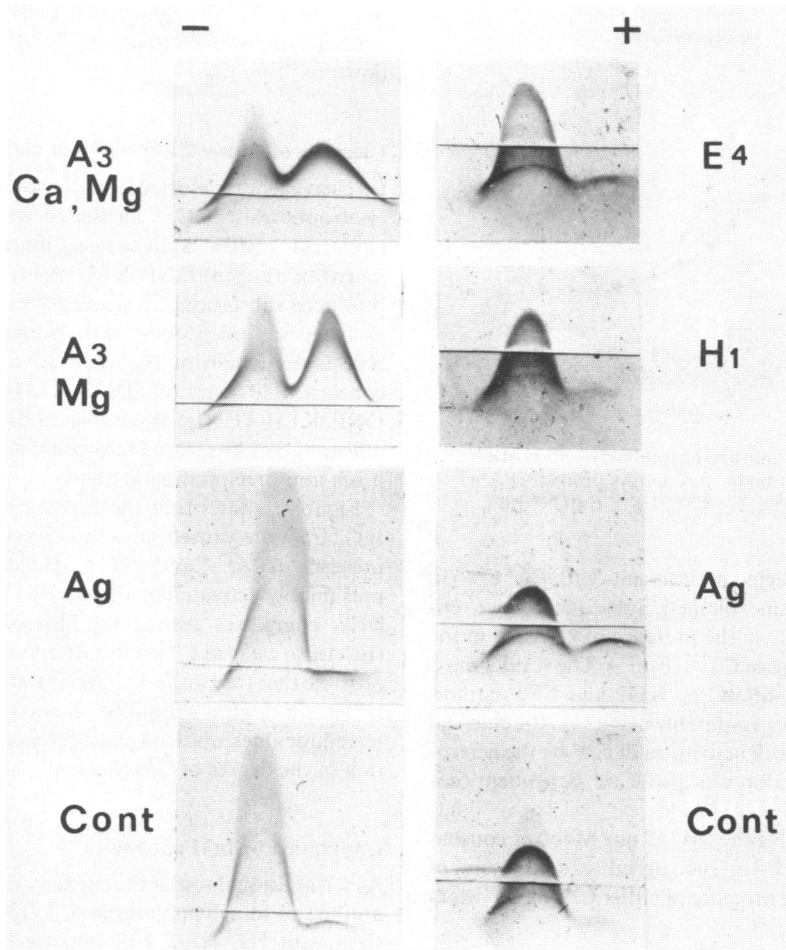

Figure 6. Assay for C3 cleavage by IgM and IgG1. In the left-hand panel aliquots of mouse C were incubated with 12 μg DNP–KLH–A3 IgG1 complexes in the presence of Ca^{2+} and Mg^{2+} or in the presence of Mg^{2+} and EGTA. Controls received 12 μg DNP–KLH (Ag) or buffer alone (cont.). In the right-hand panel mouse C was incubated with 50 μg DNP–KLH–E4 (IgM), DNP–KLH–H1 (IgM) complexes or 50 μg DNP–KLH (Ag) alone, all in the presence of Ca^{2+} and Mg^{2+}. The origin is to the left, and uncleaved C3 is the slowest moving peak.

commonly accepted such antibodies activate C^{GP} very well. This corroborates the findings of Winn (1965) and of Berden *et al.* (1978), who came to similar conclusions concerning the haemolytic properties of mouse IgM. It also agrees with data from Ruszkiewicz (1975) who found that 19S rat alloantibodies to histocompatibility antigens only lysed lymphocytes in the presence of heterologous, but not in the presence of rat C, while 7S antibodies were lytic regardless of the source of C.

Bovine conglutinin binds to fixed C3 (which has reacted with KAF (C3b inactivator)) of several species, including that of the mouse (Linscott, Ranken & Triglia, 1978). The IgM anti-DNP antibodies gave only weak conglutination with C^{Mo}, and were strongly positive with rabbit C, while the rabbit (IgG) anti-DNP antibody gave strong conglutination with both rabbit and mouse C (Table 1). The two monoclonal IgM antibodies failed to produce detectable cleavage of murine C3, as assayed by crossed electrophoresis (Fig. 6). In addition, a 19S antibody fraction from mice given SRBC failed to lyse SRBC in the presence

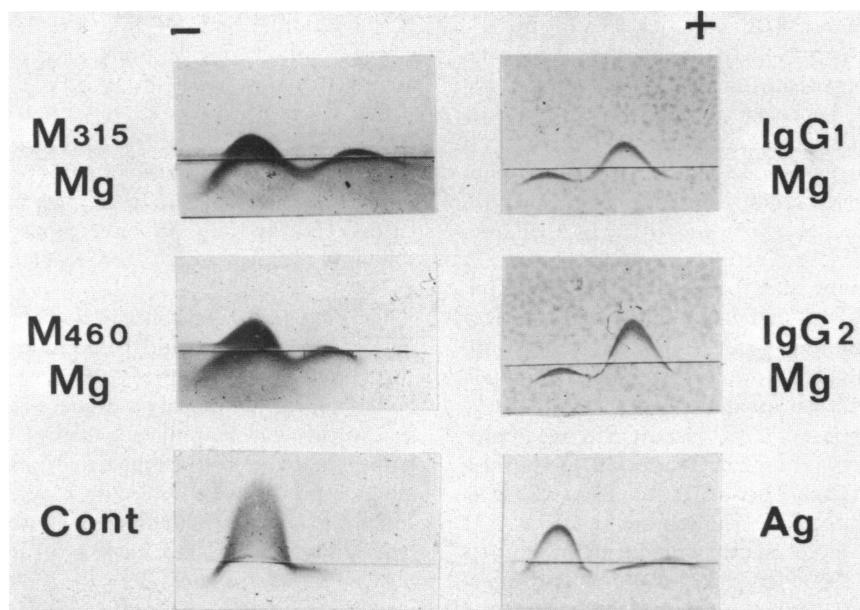

Figure 7. C3 cleavage by IgG1, IgG2 and IgA. In the left-hand panel, aliquots of mouse C were incubated with 20 μg DNP–KLH–M315 or 20 μg DNP–KLH–M460 complexes, in the presence of Mg^{2+} and EGTA. In the right-hand panel, mouse C was incubated with 20 μg DNP–KLH–IgG1 (heterogeneous) or 20 μg DNP–KLH–IgG2 (heterogeneous) complexes in the presence of Mg^{2+} and EGTA. Controls (conts) are C^{Mo} incubated with buffer alone. Origin to the left: the slowest moving peak is native C3.

Table 1. C3 fixation by mouse IgM anti-DNP antibodies assayed by conglutination

		Antibody				
C source:*	Conglutinin	E4	H1	H3	IgM†	A^{Rb}‡
Mouse	1:10	±	±	±	+	++
	1:100	−	−	−	−	−
	None	−	−	−	−	−
Rabbit	1:10	+++	+++	+++	+++	+++
	1:100	+++	+++	+++	+++	+++
	None	−	−	−	−	−

* C5-deficient (AKR) C^{Mo} or C6-deficient rabbit C.
† Heterogeneous IgM anti-DNP.
‡ Rabbit (IgG) anti-DNP.

TNP-SRBC were sensitized with subagglutinating concentrations of various antibodies, washed, incubated with mouse or rabbit C, and then with bovine conglutinin diluted 1:10 or 1:100 (−) to (+++) denotes the degree of agglutination.

of seven-fold more C^{Mo} than was required to give 60% lysis with IgG anti-SRBC antibodies (data not shown). We conclude that mouse IgM antibodies activate murine C3 inefficiently, and are essentially inactive in producing haemolysis in the presence of C^{Mo}. It is unlikely that the monoclonal anti-DNP IgM antibodies represent a non-haemolytic sublcass of IgM, since a conventional IgM anti-DNP fraction had similar properties (Fig. 2, Table 1).

Borsos & Rapp (1965) demonstrated that rabbit IgM anti-SRBC antibodies are more efficient at activating C^{GP} than IgG. Later studies with rabbit anti-Forssman antibodies, however, showed that although IgM is more efficient in *initiating* the fixation of C^{GP} (Borsos & Rapp, 1965), IgG is more effective in producing haemolysis (Frank & Gaither, 1976). Furthermore, while IgG anti-Forssman antibodies are equally effective in producing haemolysis and cytolysis, IgM antibodies are more efficient in inducing haemolysis than cytolysis (Spooner & Sell, 1966). Non-complement-fixing IgM antibodies with diverse specificities have been described in various species (Cowan, 1973; Rosse, 1968). In the mouse, Plotz, Colten & Talal (1968) described IgM antibodies that failed to lyse SRBC in the presence of C^{GP}. In man, aggregated IgM myeloma proteins fix human C less efficiently than IgG1,2 and 3 (Spiegelberg, 1974), and human IgM has been reported not to activate C^{Mo} (Van Snick & Masson, 1978). Human IgM myeloma proteins also do not activate the alternative pathway of human C (Spiegelberg & Götze, 1972).

The structure of the antigen may influence the capacity of IgM antibodies to fix C (Ishizaka *et al.*, 1968). Cunniff & Stollar (1968) concluded that C fixation by IgM may require an antigen of relatively large molecular volume, and with critically spaced antigenic determinants. We examined possible epitope density effects on the haemolytic capacities of the IgM antibodies used in the present study: increasing the amount of trinitrobenzene sulphonic acid from 2 to 50 mg/ml packed SRBC had no significant effect on the lysis of IgM-coated cells by C^{Mo}, but did increase the lysis of cells by C^{GP} (data not shown).

Although A3 monoclonal IgG1 antibody failed to activate the classical pathway in C^{Mo}, a heterogeneous IgG1 antibody fraction gave some haemolysis with C^{Mo} (Fig. 3). This may have been due to contamination of the IgG1 with IgG2, since two additional monoclonal IgG1 antibodies (provided by Drs M. Shulman and G. Köhler) also failed to lyse TNP–SRBC in the presence of C^{Mo} (data not shown). It is uncertain if both IgG2a and IgG2b activate the classical pathway of C^{Mo}, although IgG2a is known to activate the classical pathway of C^{GP} (Spiegelberg, 1974).

The finding that mouse IgA antibodies initiate cleavage of C3 via the alternative pathway, agrees with similar results with human IgA (Götze & Müller-Eberhard, 1971), and also with the capacity of C^{Mo} to solubilize antigen-mouse IgA complexes (Miller, 1976).

The biological significance of non-complement-fixing IgM antibodies could be considerable. As discussed by Ruzkiewicz (1975), the fact that IgM antibodies appear first during a primary response could lead to their blocking the activities of C-fixing antibodies, or indeed of cell-mediated immune effector mechanisms. We have studied the capacity of immune complexes prepared with different classes of antibody to localize in splenic lymphoid follicles and to generate B memory cells in mice. Both these phenomena are strictly dependent on C activation (Klaus & Humphrey, 1977; Papamichail, Gutierrez, Embling, Johnson, Holborow & Pepys, 1975). Complexes of DNP–KLH–E4 or H1 IgM antibody neither localize in lymphoid follicles, nor do they elicit memory, while complexes prepared with IgA, IgG1 and IgG2 antibodies are effective (Klaus, 1979). These results strongly suggest that the inefficiency of C activation by mouse IgM is not an *in vitro* artefact.

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