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Activation of human complement by mouse and mouse/human chimeric monoclonal antibodies

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SUMMARY

The complement (C)-activating capabilities in human serum of 32 mouse and 10 mouse/human chimeric MoAbs of different isotypes, and their fragments, were tested *in vitro*. Activation of C via the classical pathway (CP) was performed in 1% factor D-deficient serum in gelatin containing Veronal buffer in the presence of calcium and magnesium (GVB^{++}), while activation of the alternative pathway of C (AP) was assessed in 10% C1q-depleted serum in the presence of 5 mM $MgCl_2$ in GVB^{++}. The C-activating ability of MoAbs was expressed relative to the degree of activation of complement by aggregated IgG for the CP and relative to mouse IgG1 for the AP. All of seven mouse IgG2a MoAbs were potent activators of the CP. The results of CP activation by IgG1, IgG2b and IgG3 isotypes were different for individual MoAbs. Only three (two IgG1 and one IgG3) of 32 mouse MoAbs were potent activators of the AP. IgG2a and IgG2b were relatively poor AP activators. There were a few MoAbs which activated both the AP and CP. Of 10 chimeric MoAbs, two IgG1, one IgG2 and one IgG4 were poor or non-activators of the CP. On the other hand, IgG2 and IgG4 were good AP activators. IgG3 was the most potent AP activator. Most of the $F(ab')_2$ fragments were activators of the AP and displayed no activation of the CP. Fc fragments only activated the CP, whereas Fab' did not activate the CP or the AP. These studies suggest that the route of complement activation by class and subclass MoAbs can not always be predicted in advance and based only on their subclass identity.

Keywords monoclonal antibodies complement activation

INTRODUCTION

MoAbs are used as reagents for the analysis of immune mechanisms [1–3], the diagnosis of some diseases [4,5], or as drugs for the therapy of patients with malignant tumours [6–8].

In addition to the antigen specificities of MoAbs, it is important to know whether they can activate complement (C), because the capability to activate C may increase the usefulness of these MoAbs, especially in enhancing interactions with complement receptors on specific cell types. It now appears to be established that C activation via the classical pathway (CP) is induced by IgG1, IgG3 and IgM, but poorly by IgG4 or IgA [9–11]. There are also studies suggesting that IgM, IgG1 and IgG3 do not significantly activate the alternative pathway (AP), but that IgG2 and IgA are able to activate the AP efficiently [11,12]. It has been reported that mouse MoAbs of the IgG2a subclass and IgM are potent activators of the CP, while IgG1 is less efficient. In addition, all IgG subclasses and IgM were reported to be poor activators of the AP [13]. Since we had preliminary evidence that not all mouse MoAbs activate C in human serum to the same extent, we systematically investigated the complement-activating abilities of 32 mouse and 10 human/mouse chimeric MoAbs, and their fragments.

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MATERIALS AND METHODS

Buffers

Isotonic Veronal-buffered saline containing 0·1% gelatin (GVB), half-isotonic GVB supplemented with 3% dextrose, 0·5 mM $MgCl_2$ and 0·15 mM $CaCl_2$ ($DGVB^{++}$), GVB containing 0·5 mM $MgCl_2$ and 0·15 mM $CaCl_2$ (GVB^{++}), 5 mM $MgCl_2$ and 10 mM EGTA (MgEGTA-GVB) were used as diluents in the haemolytic assays or in the complement activation assays by ELISA. PBS, PBS containing 0·5% bovine serum albumin (BSA), 0·01 M EDTA and Tween 20 and 0·1 M phosphate buffer, pH 5·0 were used as diluents in the ELISA assays.

Sera and plasma

Normal human sera (NHS) were obtained from normal volunteers, pooled, aliquotted in portions of 1 ml and frozen at −80°C until use. Factor D-deficient serum (D def) with an

intact classical complement pathway was obtained from a patient with hereditary D deficiency [12]. C1q-deficient plasma (C1q def), which was used as a complement source for the measurement of AP activation, was prepared by immunoadsorption of freshly drawn normal human plasma using MoAb anti-C1q bound to Biogel A5. EDTA plasma with an inhibitor cocktail containing 20 mM benzamidine, 50 mM EDTA, 500 mM ε aminocapuroic acid, 2·5 mM phenylmethylsulphonyl flouride (PMSF) and 5 U/ml trasylol, was applied to the immunoadsorbent column. Fractions were collected at a rate of 1 ml/h at 4°C, and the fall-through was pooled, concentrated to the original volume, dialysed against isotonic Veronal-buffered saline, aliquotted in portions of 0·5 ml and frozen at −80°C until use. The C1q-deficient serum had no CH_{50} activity at a dilution of 1:10, but exhibited full AP_{50} activity. Levels of B, D̄, P and C3 were 88%, 79%, 82% and 81% relative to the original untreated serum, respectively. CH_{50} activity of the C1q-deficient serum could be restored fully with 5 μg of purified C1q/ml of serum.

Monoclonal antibodies

Thirty-two mouse MoAbs with specificity for tumour or bacterial antigens (17 IgG1, seven IgG2a, five IgG2b and three IgG3) were used [14–19]; five of them were modified to 10 chimeric antibodies (five IgG1, one IgG2, one IgG3, one IgG4 and two IgM) as described [17], yielding a total of 42 MoAbs. These 42 MoAbs were numbered from 1 to 42 and were tested for their capacity to activate the complement system in human serum. In addition, fragments of four other mouse MoAbs were used. These were numbered from 43 to 46.

Purification of MoAbs

The IgG MoAbs were purified by protein A-Sepharose chromatography. After loading, the column was rinsed with 1 M glycine, pH 8·9, and bound IgG was eluted with 0·1 M citrate buffer, pH ranging from 3·5 (IgG2a and IgG2b) to 5·5 (IgG1). The eluted IgG were loaded on a Q-Sepharose FF column in 0·05 M Tris pH 8·0, containing 0·15 M NaCl to remove the contaminating DNA and endotoxin. IgG was obtained from fall-through fractions, and kept at 4°C.

Digestion of MoAbs

The purified preparations of IgG were digested at pH 4·0 with 1% pepsin to yield $F(ab')_2$. Digestion was monitored by high performance liquid chromatography (HPLC) gelfiltration on TSK-3000 and the digestion was allowed to proceed until residual IgG was less than 5% of the starting material. The digest was neutralized with 1 M Tris and applied on Protein A-Sepharose to remove residual IgG and Fc fragments. Final purification was achieved by gelfiltration on Superdex S200 Prepgrade columns.

Fab′ fragments were prepared by digestion of the IgG preparations with papain. The digestion was monitored by HPLC gel filtration and was stopped by adding Iodoacetamide to the reaction mixture. The Fab′ and Fc fragments were isolated by Protein A chromatography in 1·5 M glycine containing 3 M NaCl, followed by elution of the proteins with 0·1 M citrate buffer using a continuous pH gradient from 5·5 to 3·5. Final purification was accomplished by gel filtration on Superdex S200 Prepgrade.

Complement haemolytic assays

CH_{50} and AP_{50} were measured by previously described methods, respectively [20,21].

Binding of MoAbs to ELISA plates

Per cent binding of MoAbs and their fragments to ELISA wells was calculated as follows. A standard curve was prepared using dilution series of mouse IgG2a. The bound IgG was detected in ELISA using horseradish peroxidase (HRP) coupled to monoclonal rat anti-mouse κ chain.

The amount of binding of other MoAbs and fragments was obtained by comparing OD_{492} relative to the standard, and the amount bound was calculated. Correction factors of 0·66 and 0·33 were used for $F(ab')_2$ on the basis of the molecular weight, respectively.

ELISA for the detection of C calculation

A method described previously was used with minor modification [12]. In brief, microwells were first reacted with 100 μl of 0·2% glutaraldehyde in 0·1 M phosphate buffer, pH 5·0 at 4°C overnight, washed with PBS, and incubated with 100 μl of dilutions of MoAbs or fragments in PBS for 60 min at 37°C. All subsequent incubations were 60 min at 37°C. After washing with PBS, wells were saturated with 150 μl of 0·5% BSA. BSA was diluted from a stock solution of 10% BSA that had been pretreated for 16 h at 4°C with 10% inhibitor cocktail. The wells were then incubated with 1% D-deficient serum in GVB^{++} containing 0·1% Tween 20 (GVB^{++}-Tw), when CP-mediated reactions were to be studied, or with 10% C1q-deficient serum in 5 mM $MgGVB^{++}$-Tw when AP activation was to be studied. As controls, incubations were also performed in the presence of 10 mM EDTA. The degree of complement activation was measured by evaluation of deposition of C3 and C4 using HRP-conjugated rabbit antibodies against C3 and C4, respectively, and was detected with the substrate 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid). The reactions were stopped by the addition of 50 μl of 2% oxalic acid. Optical densities (OD) were read in an automatic ELISA reader at 414 nm. Dilution series of aggregated human IgG (AIgG) or mouse IgG1 MoAb, which is an activator of both CP and AP pathways, were always included in each microplate as a standard of CP activation or AP activation, respectively. The capabilities of MoAbs to activate CP or AP were compared with each standard, and expressed as classical pathway units per μg (CPU/μg) or alternative pathway units per μg (APU/μg). As a control for non-specific activation of C, incubations were also performed with BSA-coated microwells. Those values which did not exceed OD values of 0·100 were subtracted as blank values from the experimental values.

RESULTS

Quantification of C activation by ELISA

In order to be able to standardize the assay for C3 and C4 deposition, we first used human AIgG or monomeric mouse IgG1 to determine CP- and AP-mediated deposition of C3 and/or C4 in either D-deficient or C1q-deficient sera. To this end ELISA wells were coated with increasing concentrations of AIgG or IgG1, washed and exposed to D-deficient or C1q-deficient serum, respectively and assessed for C3 and C4 depositions (Fig. 1). AIgG caused a dose-dependent activation

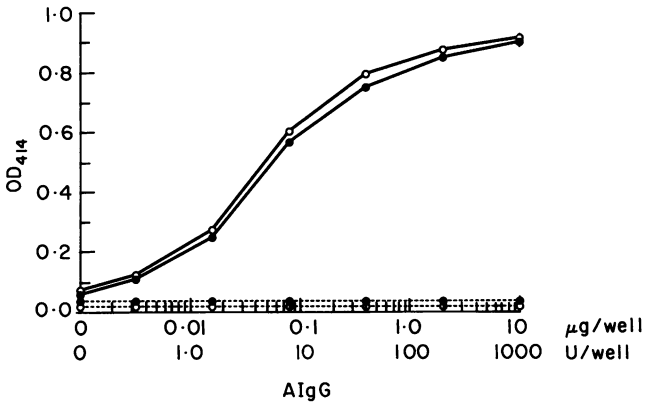

Fig. 1. Standard curve for classical pathway (CP) activation. Dilutions of aggregated human IgG (AIgG) were reacted with 1% factor D-deficient serum (D def) in GVB^{++} (——) or in EDTA-GVB (- - - -), and C3 (●) and C4 (○) depositions were detected. C4 deposition by AIgG was used as a standard for CP activation. CP activation by 1 μg AIgG was set at 100 U (CPU/μg).

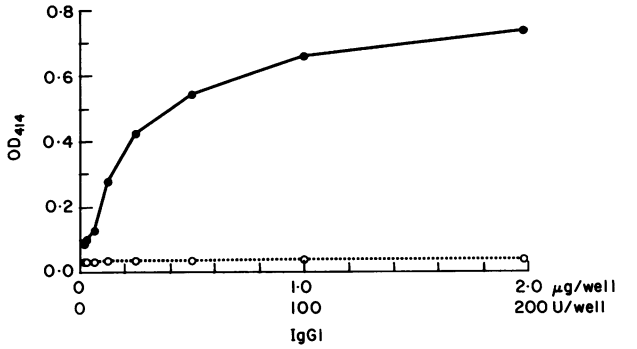

Fig. 2. Standard curve for alternative pathway (AP) activation. Dilutions of mouse IgG1 were reacted with 10% C1q-deficient plasma (C1q def) in $MgGVB^{++}$ and C3 (●) and C4 (○) depositions were detected. C3 deposition by mouse IgG1 was used as a standard for AP activation; AP activation by 1 μg mouse IgG1 was set at 100 U (APU/μg).

of complement, resulting in deposition of both C4 and C3. A plateau was reached at an input of 5–10 μg AIgG per well (Fig. 1). Arbitrarily an input of 10 μg AIgG was set at 1000 U/well of AIgG.

In C1q-deficient serum mouse IgG1 induced a dose-dependent deposition of C3, while no detectable C4 deposition was seen (Fig. 2), suggesting activation of C via the AP. An input of 2·0 μg IgG1/well was arbitrarily chosen as 200 U/well.

The capability of the various MoAbs to activate either CP or AP are expressed relative to AIgG or IgG1 in CPU or APU/μg bound MoAb. The amount of MoAb bound to the wells was calculated as described in Materials and Methods.

CP activation by mouse MoAbs

Microwells were coated with mouse MoAbs (17 IgG1, seven IgG2a, five IgG2b and three IgG3) so as to yield binding of 0·22 μg, 0·44 μg, 0·88 μg and 1·76 μg per well, and subsequently reacted with 1% D-deficient serum in GVB^{++}-Tw (Fig. 3). Six

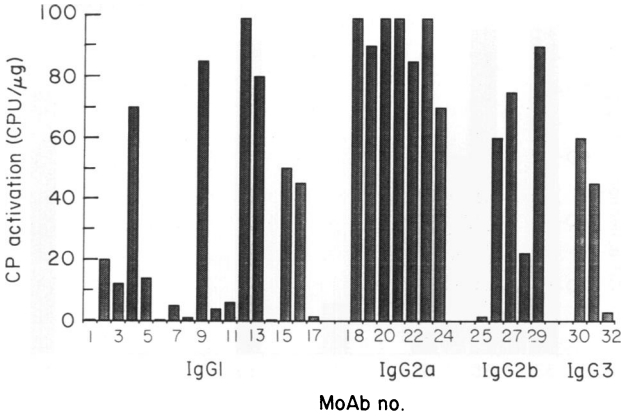

Fig. 3. Classical pathway (CP) activation by mouse MoAbs. Titres of CP-activating capabilities were expressed as CPU/μg relative to the aggregated human IgG (AIgG) standard. The numbering of each MoAb is also indicated.

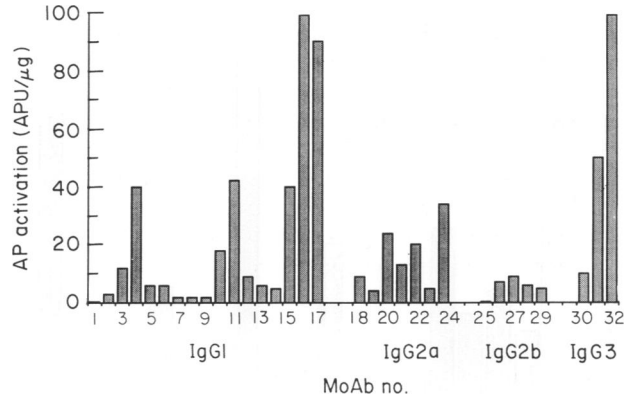

Fig. 4. Alternative pathway (AP) activation by mouse MoAbs. Titres of AP-activating capabilities were expressed as APU/μg relative to mouse IgG1 standard.

of 17 IgG1 MoAbs activated the CP at high efficiency, while five MoAbs showed no or a very limited positive reaction. Six of the MoAbs activated the CP at low to intermediate levels. All of seven IgG2a, four of five IgG2b and two of three IgG3 showed high capabilities for CP activation.

AP activation by mouse MoAbs

The capability of mouse MoAbs to activate AP was tested using 10% C1q-deficient serum in $MgGVB^{++}$ (Fig. 4). There were various levels of AP activations by mouse MoAbs. Two of 17 IgG1 showed very positive reactions and three of the MoAbs exhibited moderately high positive reactions. One MoAb showed no detectable reaction. Interestingly, some MoAbs exhibited both activation of the AP and activation of the CP. On the other hand, MoAbs 17 and 32 were strong activators of AP, but non-activators of CP. There were also some MoAbs showing reverse results, e.g. MoAbs 9, 13, 26, 28 and 29 were high activators of CP, but showed no or only slightly positive reactions with AP.

CP activation by human/mouse chimeric MoAbs

The capabilities of human/mouse chimeric MoAbs to activate CP were also tested (Fig. 5). Four of five IgG1, one of one IgG3

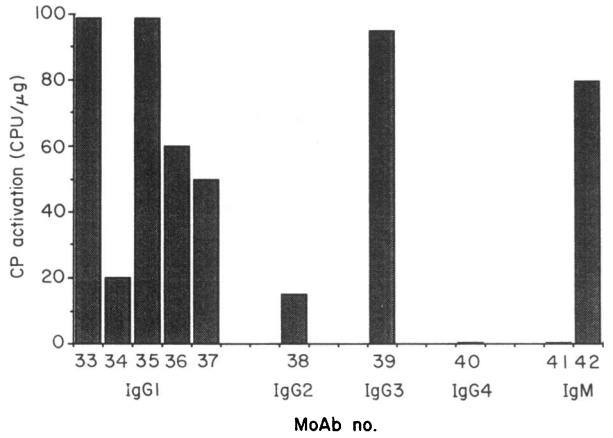

Fig. 5. Classical pathway (CP) activation by human/mouse chimeric MoAbs. Titres of CP-activating capabilities were expressed as CPU/μg relative to the AIgG standard.

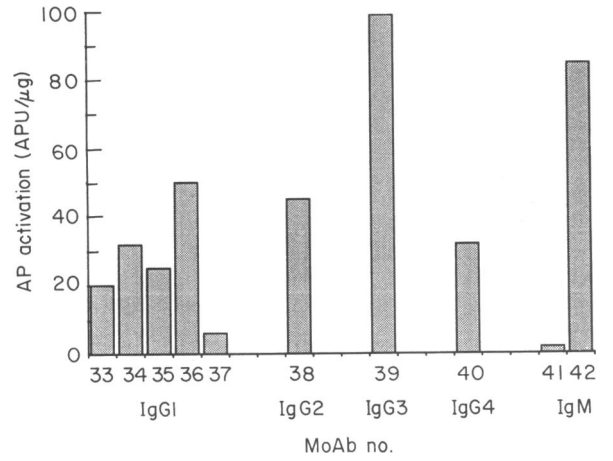

Fig. 6. Alternative pathway (AP) activation by chimeric MoAbs. Titres of AP-activating capabilities were expressed as APU/μg relative to mouse IgG1 as a standard.

and one of the IgM showed high positive reactions concerning activation of the CP. One of five IgG1, one of the IgG4 and one of two IgM tested exhibited intermediate to strong positive reactions.

AP activation by human chimeric MoAbs

Out of 10 human/chimeric MoAbs tested, one IgG3 and one of two IgM showed high positive reactions for AP activation (Fig. 6), and five IgG1, IgG2 and IgG4 exhibited low to intermediate positivities. Again, we observed that some MoAbs activated both the CP and AP.

C activation by fragments of MoAbs

$F(ab')_2$, Fab' and Fc fragments were obtained from a number of MoAbs and tested for their capabilities for C activation. As shown in Table 1, most of $F(ab')_2$ were activators of AP, but not of CP. On the other hand, two of three Fc were high activators of CP, but not of AP. Most Fab' could not activate either pathway, and only one Fab' activated the AP at a low level. Furthermore,

Table 1. C activation by $F(ab')_2$, Fab' and Fc

Fragments	MoAb no.*	Classical pathway (CPU/μg)	Alternative pathway (APU/μg)
$F(ab')_2$	1	0·7	32
	5	0·5	32
	10	0·2	6
	13	0·2	5
	23	0·2	24
	36	0·2	1
	43	0·2	20
	44	0·2	10
	45	2·6	13
Fab'	5	0·5	4
	10	0·5	12·5
	23	0·2	7
	36	0·2	1
Fc	19	99	10
	23	85	3
	46	0·2	3

* Nos 43, 44, 45 and 46 were tested only for fragments.
CPU, Classical pathway units; APU, alternative pathway units.

Table 2. C activation by MoAbs and fragments

MoAbs	Class subclass fragments	CP (CPU/μg)	AP (APU/μg)
XF8	mIgG1	0·2	0·2
	mIgG2b	1·5	0·2
	$F(ab')_2$	0·7	32
C95	mIgG1	14	6
	mIgG2a	99	13
	mIgG2b	90	5
	$F(ab')_2$	0·5	32
	Fab'	0·5	4
C4	mIgG2a	99	5
	$F(ab')_2$	0·2	24
	Fab'	0·2	7
	Fc	85	3
Mov18	hIgG1	60	50
	mIgG1	6	42
	$F(ab')_2$	0·2	1
	Fab'	0·2	1

CPU, Classical pathway units; APU, alternative pathway units.

with some MoAbs the ability of $F(ab')_2$ to activate AP was expressed only after generation of $F(ab')_2$ (XF8, C95 and C4) (Table 2). On the other hand, there was one MoAb in which $F(ab')_2$ had no ability to activate the AP, whereas the corresponding IgG had (Mov18). The results suggest that the

conversion of a MoAb to its $F(ab')_2$ fragment can alter its AP/CP activating profile and this change is not unidirectional, but can be in favour of either pathway.

DISCUSSION

To determine whether the route of complement activation by MoAbs occurs via the CP or AP, it is a necessary condition that the activation of CP or AP occurs independently. In addition, immunoglobulins in principal should be used in the form of immune complexes (IC) with specific antigens, when their C-activating capabilities are evaluated. However, only limited numbers of antibodies against defined antigens are available, together with sufficient amounts of antigen to prepare such IC. We therefore employed aggregated MoAbs on glutaraldehyde-activated microwells, instead of IC. This method has been reported previously [12], and was developed to study C activation by IgA and its fragments. The fixation by glutaraldehyde may affect the tertiary structure and the complement-fixing properties of MoAbs. On the other hand, the method permits reproducible binding of immunoglobulins to a solid phase and does seem to uncover the C-activating potential of these antibodies.

Several studies have investigated the effect of mouse MoAbs of different isotype on C activation in guinea pig or mouse serum [22–25], while there were only few investigations of the effects of activation of human C by mouse MoAbs [13]. It was reported that IC containing DNP-BSA and anti-DNP MoAbs of the IgG1, IgG2 or IgM isotype are able to activate both pathways of C in human serum, and that IgG1 also activates the CP much less efficiently than IgM or IgG2. Instead, our results also indicate that most of IgG2a MoAbs appear to be potent activators of the CP, while IgG1 is less efficient in this regard. However, we also observed that six of 17 IgG1 MoAbs are potent activators of the CP, and that there is variability in the capabilities of IgG1 to activate CP (Fig. 3). Our results also show that IgG2a and IgG2b are potent CP activators and less efficient activators of the AP. On the other hand, there were some potent AP activators in IgG1 and IgG3 (Fig. 4).

These phenomena seem to be specific for each MoAb and are not due to denaturation by glutaraldehyde or differences in amounts or density of binding to microwells. As all activators of CP or AP showed dose-dependent activation characteristics, we feel that the observed effects may be real. Furthermore, it was also observed that $F(ab')_2$ fragments generally only activate AP, whereas Fc fragments generally activate the CP. However, the results discussed above should be interpreted with caution, because the complement-activating activity that each MoAb has is a relative ability to other MoAbs, quantified using a defined but non-physiological assay system, and while it may well be that this accurately reflects the physiological situation, this remains to be unequivocally proven.

Most of $F(ab')_2$ were shown to be AP activators (Table 1), and even $F(ab')_2$ of MoAbs that could not activate the AP by themselves activate AP (Table 2). This suggests that the hinge region of immunoglobulins may play an important role in AP activation.

Of 10 human/mouse chimeric MoAbs, two of five IgG1, the only IgG3 and one of two IgM were potent CP activators, but the IgG2 and IgG4 were poor to non-CP activators. In general terms these results are in agreement with a previous report [11], but some discrepancies concerning AP activation by human IgG and IgM remain. Valim & Lachmann reported that IgM, IgG1 and IgG3 do not significantly activate the AP under any circumstances using IC of anti-NIP and NIP-BSA [11]. However, in our study a number of MoAbs from different subclasses of IgG and IgM were able to activate AP, and IgG3 especially was a potent AP activator. Our results are consistent with the report by Frank *et al.*, in which all IgG subclasses and IgM consume C3 via AP in the assay system using C4-deficient guinea pig serum [26].

Finally, we stress that MoAbs of the same isotype do not necessarily activate human complement to the same extent, although we can confirm that mouse IgG2 is a potent CP activator and human IgG4 is a non-CP activator. The present study points to the necessity to determine the complement-activating potential for each MoAb separately, and suggests that the ability of a MoAb to activate a complement in human serum is not predictable simply on the basis of its class and subclass.

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