Research paper

Complexes between C1q and C3 or C4: Novel and specific markers for classical complement pathway activation

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This paper is dedicated to Hans Wiessenberg who initiated the work on C1q-C4/C3 complexes, but unfortunately died in a car accident, before the completion of the study.

Abstract

Classical pathway activation is often assessed by measuring circulating levels of activated C4. However, this parameter does not discriminate between activation through the classical or the lectin pathway. We hypothesized that during classical pathway activation, complexes are formed between C1q and activated C4 or C3. Using ELISA, we investigated whether such complexes constitute specific markers for classical pathway activation. In vitro, C1q-C3d/C4d complexes were generated upon incubation of normal recalcified plasma with aggregated IgG or an anti-C1q mAb that activates C1 (mAb anti-C1q-130). In contrast, during incubation with C1s or trypsin, C1q-C3d/C4d complexes were not generated, which excludes an innocent bystander effect. Additionally, C1q-C3d/C4d complexes were not generated during activation of the alternative or the lectin pathway. Repeated freezing and thawing did not influence levels of C1q-C3d/C4d complexes in recalcified plasma. To measure C1q-complement complexes in plasma samples, we separated unbound complement proteins from C1q-C3d/C4d complexes in the samples prior to testing with ELISA. In samples from patients undergoing cardiopulmonary bypass surgery or suffering from rheumatoid arthritis, we found higher levels of C1q-C4 complexes than in samples from healthy

individuals. We conclude that complexes between C1q and C4 or C3 are specific markers of classical complement pathway activation.

1. Introduction

As part of the innate immune system, complement constitutes a first line of defence against many pathogens. The effector pathway of the complement system can be activated through three pathways, the classical, the lectin or the alternative pathway (Walport, 2001a,b). The first component of the classical pathway is C1. This component is composed of C1q and two pro-enzymes, the serine proteases C1r and C1s, which are associated as a Ca^{2+}-dependent tetramer, C1s-C1r-C1r-C1s (Arlaud et al., 1998; Colomb et al., 1984). Binding of C1 to activators such as immune complexes or C-reactive protein (CRP) is mediated by its recognition subunit C1q. Upon binding of C1q, conformational changes trigger the auto activation of C1r. Subsequently, active C1r converts the pro-enzyme C1s into an active protease, which in its turn activates C4 and C2 by limited proteolysis. Activated C4 and C2 form a bimolecular complex, which can function as the C3-convertase of the classical pathway (Arlaud et al., 1998; Budayova-Spano et al., 2002; Colomb et al., 1984; Thielens et al., 1999).

The lectin pathway shares some molecules with the classical pathway and is activated by binding of mannose-binding lectin (MBL), a molecule homologous to C1q, to its ligands. Among the latter are mannose or carbohydrate structures on pathogenic surfaces, which can bind MBL in a Ca^{2+}-dependent fashion. Two serine proteases, i.e., the MBL-associated serine protease (MASP)-1 and MASP-2, are activated upon binding of MBL. Subsequently activated MASPs cleave C4 and C2 to generate a C3 convertase, which is similar to that formed during classical pathway activation (Petersen et al., 2001a). Complement activation occurs in a number of immune and inflammatory diseases. Inhibition of the complement system may even be a target for therapy in some of these (Kirschfink, 2001; Makrides, 1998; Moore, 1994). Hence it may be important to monitor activation of the various complement pathways in patients. To assess activation of the classical pathway, activation of C4 has frequently been measured (Wolbink et al., 1993). However, according to current knowledge, activation of this complement protein may result from both classical and lectin pathway activation. Another limitation of measuring activated C4 is that spontaneous hydrolysis of the internal thioester in this complement protein (as well as in C3) may result in artificially high levels in a plasma sample, since the resulting iC4 and iC3 expose most of the neo-epitopes of C4b and C3b, respectively (Hack et al., 1988). As an alternative method to measure classical pathway activation specifically, levels of C1rC1s-C1-inhibitor complexes have been measured (Hack et al., 1981). The disadvantage of monitoring these C1-inhibitor complexes is, however, their fast clearance in vivo (de Smet et al., 1993).

As indicated above, C4 and also C3 contain an internal thioester that is exposed during activation of these molecules. This newly exposed thio-ester is highly reactive with amide- or hydroxyl-groups (Gadjeva et al., 1998; Tack et al., 1980; von Zabern and Gigli, 1982). The majority of the C4 or C3 molecules exposing their thio-ester will react with surrounding water molecules, but some will bind covalently to molecules in their direct neighbourhood. We hypothesized that fixation of C4 or C3 during complement activation not only occurs to the activator, but also to C1q thereby generating C1q-C4 and C1q-C3 complexes. In the present study, we have tested this hypothesis and developed an ELISA to detect complexes between C1q and activated C4 or C3. Formation of C1q-C3d/C4d complexes in vitro and in vivo was studied. Our results indicate that these complexes are highly specific for activation of the classical pathway of complement and their measurement may help to monitor classical pathway activation in vivo.

2. Materials and methods

2.1. Plasma samples

Normal human plasma was obtained by collecting blood in 10 mM EDTA (final concentration) and removing blood cells by centrifugation for 10 min at $1300 \times g$. Recalcified normal human plasma was obtained by adding a slight excess of $CaCl_2$ (12 mM) to EDTA plasma, whereafter the plasma was allowed to clot. The fibrin clot was then removed by centrifugation (10 min at $1300 \times g$) and the plasma was stored in aliquots at -70 °C until use.

EDTA plasma samples from patients undergoing cardiopulmonary bypass (CPB) or suffering from rheumatoid arthritis (RA) were collected as part of ongoing studies (Bruins et al., 1997; Molenaar et al., 2001), and stored in aliquots at -70 °C until use. The study was approved by the local institutional ethics review committee. Blood samples of CPB patients were obtained before and after induction of anaesthesia, 30 min after the start of CPB, immediately after CPB and after protamine administration. These time points are further referred to as T1-T5, respectively.

2.2. Proteins and antibodies

Trypsin was obtained from Sigma Chemical Co., St Louis, MO and C1s was from Calbiochem, Darmstadt, Germany. Aggregated human IgG (AHG) was prepared by incubating purified human IgG at a concentration of 10 mg per ml for 20 min at 63 °C (Hack et al., 1988).

Monoclonal antibody (mAb) anti-C1q-130, directed against the stem of C1q and able to activate C1 in serum, and mAb anti-C1q-85, which inhibits activation of C1q by immune complexes, have been described before (Hoekzema et al., 1988). MAb anti-C1q-2 is directed against the stem of C1q. MAb anti-C4-1, directed against an activation-dependent neo-epitope exposed on C4b/bi/c, has also been described before (Wolbink et al., 1993). MAb anti-C4-4 was obtained from a fusion of spleen cells from a mouse immunized with C4 and is directed against the C4d fragment. The use of this antibody in immunoassays has been described previously (Wolbink et al., 1993). MAb anti-C3-9 against an activation-dependent epitope on C3b/bi/c, and mAb anti-C3-19 against the C3d fragment have been published earlier (Hack et al., 1988). Isolation of C1q from human plasma was described before by Tenner et al. (1981).

2.3. Complement activation in plasma

Recalcified plasma of healthy donors was incubated for 20 min at 37 °C with various complement activators. To this end, one volume of recalcified plasma was incubated with one volume of veronal buffer supplemented with 10 mM $CaCl_2$ and 2 mM $MgCl_2$ (VB^{++}) containing AHG, mAb anti-C1q-130, trypsin or C1s at final concentrations of 0.5 mg/ml, 50 µg/ml, 0.5 mg/ml, and 20 nM, respectively. Activation with AHG and mAb anti-C1q-130 was stopped by adding two volumes of EDTA at a final concentration of 0.2 M in PBS. Trypsin-induced activation was inhibited by the addition of soy bean trypsin inhibitor (SBTI, Sigma) at a final concentration of 3 mg/ml and C1s activation was stopped by adding EDTA and benzamidine (Sigma) at final concentrations of 10 and 100 mM, respectively. Finally, the mixtures were placed on ice.

2.4. ELISAs to measure overall complement activation

Overall complement activation was measured by assessing the amount of C4b/bi/c and C3b/bi/c (abbreviated further as C4b/c and C3b/c, respectively). For the quantification of these activation products, ELISAs were used that have been described previously (Wolbink et al., 1993). Briefly, for the C4b/c ELISA, mAb anti-C4-1, diluted in 0.1 M carbonate buffer, pH 9.6 (2 µg/ml) was coated onto an ELISA plate (Nunc Maxisorp; NalgeNunc Int., Roskilde, Denmark) overnight at room temperature (RT). This and all other incubations were performed in an end volume of 100 µl. The wells were washed five times with PBS/0.02%, w/v, Tween-20 (PT) and subsequently incubated for 30 min at RT with 2%, v/v, cow milk in PBS to block residual binding sites. Hereafter, the wells were washed five times with PT again. Samples, diluted in PT containing 1%, w/v, gelatine (PTG) and 10 mM EDTA, were incubated for 1 h at 4 °C. Then the plates were washed five times with PT and incubated with biotinylated polyclonal rabbit anti-human C4c antibodies, diluted in PTG, for 1 h at RT.

After five washes with PT, the plates were incubated with 100 μl PTG supplemented with 0.1%, v/v, streptavidin-peroxidase (Amersham/Pharmacia, Uppsala, Sweden) for 30 min at RT. After five washes, the ELISA was developed with 100 μg/ml TMB in 0.11 M sodium acetate (pH 5.5) containing 0.003%, v/v, H_2O_2. Substrate conversion was stopped by addition of 100 μl H_2SO_4. Absorbance was measured at 450 nm with a Titertek multiscan.

For measurement of C3b/c levels, a similar ELISA was performed, except that the plates were coated with mAb antiC3-9 (2 μg/ml) and that bound C3b/c was detected with biotinylated polyclonal rabbit anti-human C3c antibodies.

2.5. ELISAs for C1q-C4d/C3d complexes

MAb anti-C1q-85, diluted in 0.1 M carbonate buffer (pH 9.6) to a concentration of 2 μg/ml, was coated overnight at RT onto a 96-well ELISA plate. The wells were washed five times with PT and subsequently all wells were incubated with PBS/2% cow milk for 30 min at RT to block the residual binding sites on the plate. The plate was washed five times again with PT. Samples were diluted in PTG-EDTA containing 0.5 M NaCl to prevent non-specific binding of C1q. The plate was then incubated for 1 h at 4 °C. After five times washing with PT, the plate was incubated for 60 min at RT with biotinylated mAb against C4d (anti-C4-4) or C3d (anti-C3-19) diluted in PTG, to detect C1q-C4d or C1q-C3d complexes, respectively. After five washes with PT, all wells were incubated with polymerized streptavidin-HRP (Business Unit Reagents, Sanquin Research) diluted 1 to 10,000 in PBS/2% cow milk for 30 min at RT. After five washes with PT, ELISAs were developed with TMB as described above. Absorbance at 450 nm was measured with a Titertek multiscan. Plasma incubated with mAb anti-C1q-130 was used as standard (see Results). Results obtained with samples were expressed as a percentage of the amount of complexes in the activated plasma sample.

2.6. Procedure to measure C1q-complement complexes in plasma samples

To separate C1q-C4 complexes from unbound C4, one volume of plasma was incubated with one volume of 66% saturated (final concentration 33% or 1.29 M) ammonium sulphate (Merck, Darmstadt, Germany). The ammonium sulphate had been dissolved in PBS containing 10 mM EDTA to prevent complement activation during the precipitation procedure. The mixtures were left on ice for 1 h, and then centrifuged for 30 min at $1300 \times g$ at 4 °C. Precipitates were resuspended in ELISA-buffer (high performance ELISA [HPE; Business Unit Immune reagents, Sanquin, Amsterdam, the Netherlands] buffer containing 0.5 M NaCl and 10 mM EDTA to prevent non-specific binding of C1q and in vitro complement activation, respectively). The ELISA was then performed with plates coated with mAb anti-C4-4 (5 μg/ml overnight at RT in 0.11 M sodium acetate buffer, pH 5.5). Plates were washed five times with PT prior to a 1 h incubation of the resuspended precipitates at RT. After five times washing with PT, plates were incubated for 1 h at RT with biotinylated mAb against C1q (anti-C1q-85) to detect C1q-C4 complexes. After five washes with PT, the ELISA was further performed as described above. Purified C1q, containing complexes, was used as a standard for this assay. Levels of complexes in plasma were expressed as arbitrary units, 100 au being the amount of complexes in the purified C1q sample.

2.7. SDS-PAGE analysis of C1q complexes

MAbs anti-C1q-2, anti-C3-19 and anti-C4-4 were coupled to CNBr-activated Sepharose 4B (Amersham/Pharmacia) at 2–3 mg mAb to 100 mg Sepharose. The Sepharose was suspended in PBS/0.5 M NaCl. For the immune precipitation of C1q, C1q-C3d complexes and C1q-C4d complexes, 30 μg purified C1q, diluted in PBS/0.5 M NaCl, was incubated o/n at 4 °C with 200 μl anti-C1q-2, anti-C3-19 or anti-C4-4 Sepharose suspension, respectively. After washing the Sepharose five times with PBS, bound proteins were eluted in non-reducing SDS sample buffer (2% SDS, 62.5 mM Tris–HCl pH 6.8, 10% glycerol and bromophenol blue) by incubation for 5 min at 90 °C. The Sepharose beads were removed by centrifugation for 10 min at 3000 rpm and supernatants were electrophoresed on 12% SDS gel under non-reducing conditions. After electrophoresis, proteins were transferred to a PVDF membrane. These blots were first incubated for 1 h at RT with blocking buffer (PBS/5%, w/v, milk powder/

0.5%, w/v, BSA/0.1%, w/v, Tween) and then incubated o/n at 4 °C with biotinylated mAb anti-C1q-2 or anti-C4-4, diluted in blocking buffer. After three washes with PBS/0.1% Tween the blots were incubated for 45 min at RT with polymerized streptavidin-HRP (Sanquin), diluted 1/1000 in blocking buffer. Subsequently, the blots were washed three times with PBS/0.1% Tween and two times with PBS. Proteins were visualized by chemiluminescence, using ECL (Amersham/Pharmacia).

3. Results

3.1. ELISA for C1q-C3d/C4d complexes

A differential antibody ELISA was used to detect complexes between C1q and the activated complement factors C3 and C4. Antibodies polymerized on the solid phase, potentially can activate complement and, hence, fix C3 and C4 when incubated with fresh serum or recalcified plasma. This may lead to artificially high responses in the assay. To prevent this, samples were incubated in presence of 10 mM EDTA as well as 0.5 M NaCl, to prevent in vitro complement activation. In addition, the mAb against C1q was of the IgG1 mouse isotype which activates complement poorly (Ey et al., 1980).

Recalcified plasma was activated in the fluid phase with AHG to investigate whether C1q complexes are generated during in vitro classical pathway activation. C3b/c and C4b/c ELISAs showed that complement was substantially activated, as a result of classical pathway activation by IgG aggregates. As depicted in Fig. 1A, C1q-C4d complexes were generated in recalcified plasma upon incubation with AHG for 20 min at 37 °C. Similar results were obtained for C1q-C3d complexes (Fig. 1B). When plates were coated with an irrelevant mAb, no responses were observed in the ELISA when AHG-activated recalcified plasma was tested, indicating the specificity of the assay (data not shown).

3.2. Generation of C1q complexes in recalcified plasma by various complement activators

To investigate the specificity of C1q-C4d and C1q-C3d complexes for classical pathway activation,
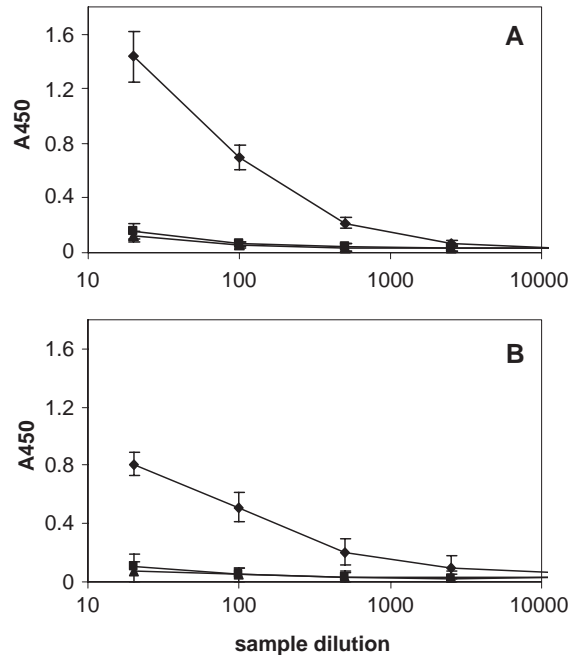

Fig. 1. Generation of C1q-C4d (A) and C1q-C3d (B) complexes in recalcified plasma incubated with aggregated human IgG (AHG). Recalcified plasma was incubated with AHG, final concentration 0.5 mg/ml (♦), for 20 min at 37 °C to activate the classical pathway of complement. C1q complexes were measured in the mixtures. As controls, plasma was incubated on ice in the presence of 0.2 M EDTA (▲) or at 37 °C with VB^{++} (■). Results represent mean and SEM (error bars) of 4 experiments.

various complement activators were tested for their potency to generate these complexes in recalcified plasma. Plasma incubated for 20 min at 37 °C with mAb anti-C1q-130, which activates C1 in serum, yielded the highest levels of either C1q-complex. Therefore, we decided to use plasma incubated with this mAb as standard for the amount of C1q complexes. Levels of C1q-C4d and C1q-C3d complexes in this activated plasma were set at 100%, and levels in other samples were related to this standard.

As already indicated above, activation with AHG resulted in the formation of C1q-C4d and C1q-C3d complexes. Conversely, recalcified plasma activated with trypsin, a protease that amongst others directly cleaves C3 and C4, hardly contained C1q-C4d complexes and only a low level of C1q-C3d complexes. The latter probably resulted from a so-called "innocent-bystander effect". Also upon incubation with C1s, which activates C4, C1q-C4d or C1q-

Table 1
Generation of C1q-complexes and activated C4 and C3 in plasma by various complement activators

	C4b/c (nM)	C1q-C4d (%)	C3b/c (nM)	C1q-C3d (%)
MAb-130 (50 μg/ml)	100	100	230	100
AHG (0.5 mg/ml)	1200	8	1200	10
C1s (20 nM)	350	0.1	200	0.16
Trypsin (0.5 mg/ml)	1500	0.2	2400	1.8
VB^{++}	22	0.05	45	0.08
Ice	8.5	0.05	9	0.05

C3d complexes did not increase in the plasma (Table 1). The lack of generation of C1q-C3d/C4d complexes was not due to insufficient activation of complement by any of the activators mentioned, since all activated plasma samples contained high levels of C3b/c and C4b/c (Table 1). Additionally, recalcified plasma incubated with mannan or zymosan, activators of the lectin and alternative pathway, respectively, did not contain C1q-C3d/C4d complexes, while these activated plasmas did contain high levels of C3b/c (data not shown). Thus, C1q-C3d/C4d complexes were apparently only generated during classical pathway activation.

3.3. C1q-C3d/C4d complexes in plasma samples of healthy individuals and RA patients

Next, we investigated the presence of C1q-C4 complexes in EDTA plasma samples obtained from healthy individuals or patients to get an impression about the value of these complexes to monitor complement activation in vivo. Unfortunately, we had problems in measuring C1q-C4 in plasma samples, since dose-response curves of plasma samples in the assay described above were not parallel with the standard. Furthermore, in gel filtration experiments significant amounts of C1q-C4 in fractions of normal EDTA plasma were detected, whereas without fractionation this plasma seemed to contain hardly any complexes. These results suggested suboptimal detection of C1q complexes due to saturation of the coating Ab with free C1q in the plasma. To improve the detection of C1q-C3d/C4d complexes in plasma, we therefore decided to purify such complexes from plasma samples with a simple one-step procedure, before testing them with ELISA. As we

anticipated, the separation of C4 from C1q-C4 was easier than that of C1q from C1q-C4 due to a larger difference in molecular size between the former pair and we adapted the ELISA by using mAb anti-C4-4 as capture Ab and mAb anti-C1q-85 as detector Ab. With purified C1q, which appeared to contain measurable amounts of C1q-C3d/C4d complexes, we established that the reversed assay measured these complexes as well as the initial assay (data not shown). Without separation of C1q-C4 from uncomplexed C4 in normal EDTA plasma, C1q-C4 complexes were detected suboptimally because of saturation of the coat with free C4 in the plasma (Fig. 2). After incubation with ammonium sulphate, C1q-C4 complexes could be measured only in the precipitate and not in the supernatant, indicating that the complexes were precipitated. Moreover, most, if not all, C1q was recovered in the precipitate, whereas most of C4 was in the supernatant (data not shown).

Using this method, we measured the amount of C1q-C4 complexes in the EDTA plasma of 21 healthy individuals and 36 RA patients, chosen randomly from a panel of patients investigated previously in another study (Molenaar et al., 2001). Levels were expressed in AU, 100 AU being the amount of complexes in the C1q preparation purified from plasma. As shown in Fig. 3, the C1q-C4 level was significantly higher in RA patients (mean 2.053 ± 0.2228) than in the healthy control group
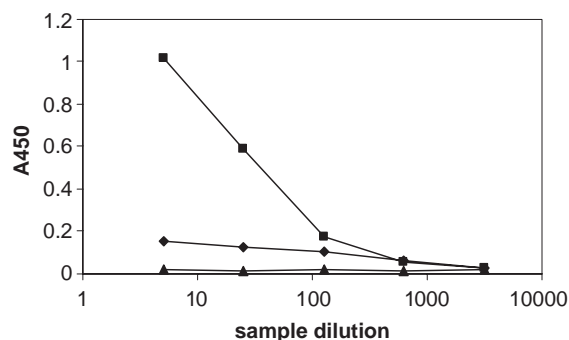

Fig. 2. C1q-C4 complexes are precipitated from normal EDTA plasma by ammonium sulphate. EDTA plasma of a healthy donor was incubated with 33% saturated ammonium sulphate, final concentration, in order to precipitate C1q-C4 complexes. Subsequently, complexes were measured in plasma before precipitation (♦), in the supernatant (▲) and in the precipitate (■). This figure is representative for 3 experiments which yielded similar results.
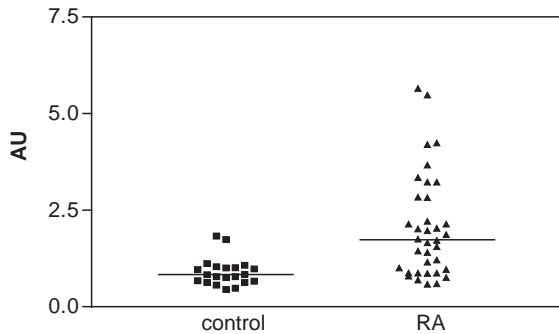
Fig. 3. C1q-C4 plasma levels in healthy individuals and RA patients. Horizontal lines indicate the median values. The mean level of C1q-C4 was significantly elevated in RA patients compared to controls, as determined by a two-tailed unpaired t-test. ($P<0.01$).

(mean 0.9002 ± 0.07735). Control experiments with irrelevant mAbs yielded no response, indicating the specificity of the assay.

3.4. C1q-C4 in plasma samples of CPB patients

We also measured C1q-C4 at various time points during CPB surgery. It has been described previously that administration of protamine to patients undergoing CPB to neutralize heparin induces activation of the classical complement pathway mainly by heparin-protamine complexes (Bruins et al., 1997). We observed that most, but not all, C4 activation indeed occurred after protamine administration. The total amount of C4 decreased after the start of CPB, because of hemodilution. To correct for this dilution total C4 was also measured in the samples, and C4 activation was calculated as a percentage of total C4 (Fig. 4A). Clear activation of C4 was observed after protamine administration. As depicted in Fig. 4B, C1q-C4 levels increased after the commencement of CPB and were most elevated after protamine administration. In the CPB patients, plasma levels of activated C4 correlated significantly with plasma concentrations of C1q-C4 (Spearman's rank correlation coefficient 0.7, $p<0.05$). This correlation was less pronounced in healthy donors (0.55, $p<0.05$) and was absent in RA patients (0.15, $p=0.3941$) as also shown by calculating Pearson's correlation coefficient (0.4, $p=0.04$, and 0.249, $p=0.14$, respectively).

To assess the stability of the complexes in plasma, we tested the effect of repeated freezing and thawing

of samples on C1q-C4 levels. Samples subjected to 5 cycles of freezing at -70 °C and thawing, contained similar levels of complexes as fresh samples. Also, upon incubation for up to 24 h at RT, plasma levels of C1q-C4 complexes were similar as those in the fresh samples. Therefore, we concluded that levels of C1q-C3d/C4d complexes in EDTA plasma samples are stable and not much influenced by handling and storage of the samples.

3.5. C1q-C3d/C4d complexes on SDS-PAGE

We observed by ELISA that purified C1q preparations contained C1q-C3d and C1q-C4d complexes. C1q-C4d complexes were immunoabsorbed from a purified C1q preparation with Sepharose-anti-C4-4 and electrophoresed on a 12% SDS-PAGE gel under non-reducing conditions as described in the Materials and Methods Section. C1q species were then visualized by immunoblotting with biotinylated mAb anti-C1q-2. Fig. 5, lane 1 (panel A) shows that C1q on 12% SDS migrated as a band with an apparent molecular mass of 55 kDa, that is at the position of
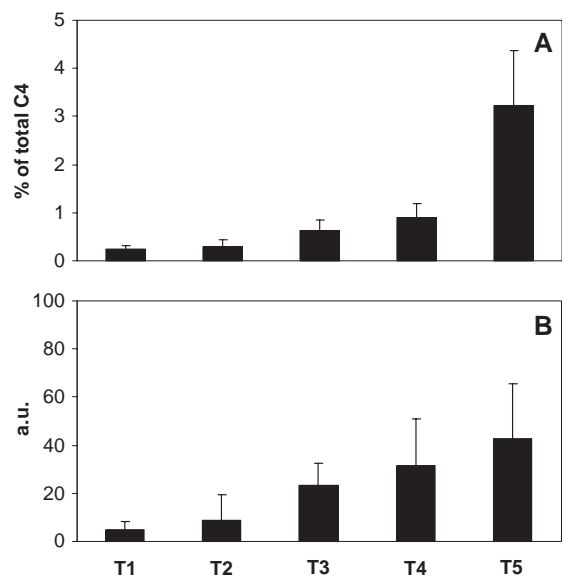

Fig. 4. Course of C4bc/C4 (A) and C1q-C4 (B) levels during CPB surgery. Data are presented as means (n=8) with standard deviations. T1: before induction of anaesthesia; T2: after induction of anaesthesia; T3: 30 min after start of CPB; T4: at the end of CPB; T5: after protamine administration.
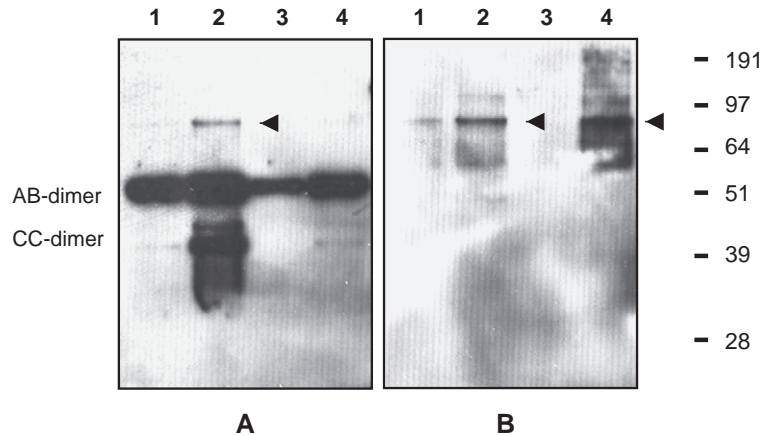
Fig. 5. Visualization of C1q complexes on SDS-PAGE. Samples were separated on 12% SDS-PAGE under non-reducing conditions and blotted. Lane 1: purified C1q; lane 2: purified C1q, immunoprecipitated with Sepharose-anti-C1q-2; lane 3: C1q, immunoprecipitated with Sepharose-anti-C3-19; lane 4: C1q, immunoprecipitated with Sepharose-anti-C4-4. Panel A was immunostained with biotinylated anti-C1q-2 and Panel B with biotinylated anti-C4-4. Antibodies were visualized by streptavidin-HRP linked ECL and following exposure to an autoradiography film. Positions of molecular weight markers (kDa) and subunits of C1q are indicated. C1q-C4d complexes are indicated by arrowheads.

the AB-dimer of normal C1q. C1q adsorbed onto anti-C1q-2-Sepharose (Fig. 5, lane 2), contained an additional band with an apparent molecular mass of 41 kDa, which corresponds to the CC-dimer of normal C1q. Note that lane 2 in Fig. 5 contains about 20 times more C1q than lane 1, which explains why the 41 kDa band was not visible in lane 1. MAb anti-C1q-2, which reacts preferentially with the AB-dimer, also reacted with low affinity with the CC-dimer. Presumably this cross-reaction reflects that this antibody is specific for the collagenous region, which is highly homologous between the A, B and C-polypeptides of C1q. After immunoabsorption with Sepharose-anti-C3-19 or -anti-C4-4 (Fig. 5), the 55 kDa band was again visible (Fig. 5, lanes 3 and 4). No bands were seen when a control mAb coupled to Sepharose was used (not shown). Thus, apparently, some C1q species in the preparation had bound C4d or C3d, respectively. In the immunoprecipitate of anti-C1q-Sepharose, an extra band was detectable at ±85 kDa, (Fig. 5, lane 2, indicated by the arrowhead). The size of this band was in the range of the supposed size of a C4d or C3d molecule attached to the AB- or CC-dimer of C1q. To further verify the nature of this band, the blot was also immunostained with biotinylated mAb anti-C4-4. Fig. 5B, lanes 2 and 4 show that the 85 kDa band, did indeed contain C4d. Note that the C1q-species immunoprecipitated by mAb anti-C3-19 did

not contain C4d (Fig. 5B, lane 3), indicating that C3d- and C4d fragments are attached to separate C1q molecules.

4. Discussion

In the present study we have described novel activation products of the classical pathway, namely complexes between the recognition unit of the classical pathway, C1q, and activated C4 or C3. In addition, we show that these complexes are specific for C1q-mediated complement activation, and are stable in vitro, which makes them suitable candidates as markers for classical pathway activation in vivo.

To measure complexes of C1q and C4 or C3, we developed an assay, in which an anti-C1q mAb was used to capture the complexes from the test sample, and anti-C4d or anti-C3d mAbs as detector antibody. To prevent in vitro fixation of C4 and C3 to the solid-phase, samples were incubated in 0.5 M NaCl and in the presence of 10 mM EDTA. Indeed, replacement of the anti-C1q mAb by an irrelevant mAb of the same subclass, completely abrogated the response in the assays, ruling out in vitro activation.

C3 and C4 both contain an intramolecular thioester which at a low but significant rate is hydrolyzed by surrounding water molecules (Gadjeva et al., 1998;

Tack et al., 1980; von Zabern and Gigli, 1982). The resulting iC3 and iC4 undergo similar conformational changes as C3b and C4b, and are generally detected in assays for C3b/c or C4b/c, respectively. Generation of iC3 or iC4, for example, during freezing and thawing of samples, may lead to artificially high levels of C3b/c and C4b/c. Interaction of the thioester with water molecules abrogates its capability to covalently link to proteins. Hence, C1q-C4 or C1q-C3 complexes are not expected to be generated as a result of spontaneous hydrolysis of the thioester. Indeed, levels of these complexes did not increase during freeze–thaw cycles of plasma. Exposure of the thioester upon activation of C3 or C4 may lead to innocent bystander fixation of proteins in the very near environment as for example has been shown for IgG (van Dam and Hack, 1987). Hence, we studied innocent bystander fixation of C3 and C4 to C1q using active C1s, trypsin, mannan and zymosan. The results of these experiments indicated that C1q-C3, but not C1q-C4 complexes, were generated at a low level during strong activation of complement by non-classical pathway activators. Thus, C1q-C4 complexes reflect classical pathway activation best.

To determine a standard for C1q-C4 in plasma, we measured these complexes in EDTA plasma obtained from healthy donors with the complex ELISA described above. Unfortunately, dose-response curves were not parallel with those of C1q purified from plasma, presumably because of saturation of the coating anti-C1q-85 mAb with uncomplexed C1q in the plasma. When activated in vitro with complement activators as AHG, plasma contained so much C1q-C4 complexes that this saturating effect was overcome, since samples could be tested at higher dilution. To improve detection of C1q-C4 complexes in plasma, we therefore decided to purify these complexes from the plasma by ammonium sulphate precipitation. In pilot experiments we assessed that it was easier to separate C4 from C1q-C4 than C1q from C1q-C4 complexes. Hence, we "reversed" the ELISA in that anti-C4-4 mAb was used for coating and anti-C1q for detection of complexes.

Complement activation in CPB patients has been described before to be classical pathway mediated (Bruins et al., 1997). Heparin-protamine complexes, formed after protamine administration to neutralize heparin, enhance classical pathway activation at least

in part via CRP. In this study, we assessed levels of C1q-C4 complexes in patients undergoing CPB surgery, as a model to demonstrate that C1q-C4 levels increase during classical pathway activation. Increased plasma concentrations of C1q-C4 were indeed found, and correlated significantly with levels of C4b/c in these patients. This indicated that C1q-C4 is a suitable parameter for classical pathway activation in pathological plasma samples. Notably, C1q-C4 complex levels were elevated in some patients during CPB surgery before C4b/c had increased. Presumably, different clearance of either activation product accounted for this. We are currently studying this.

Rheumatoid arthritis is a disease in which activation of the classical complement pathway has been described. Tradionally this activation is considered to be triggered by immune complexes (Sato et al., 1993), and may, in part, be mediated by CRP as well (Molenaar et al., 2001). Indeed levels of C1q-C4 complexes in EDTA plasma samples obtained from RA patients were higher than those in plasma samples obtained from healthy controls. Of the 36 RA patients, 32 had elevated C4b/c levels, whereas only 21 had elevated C1q-C4 complexes. This indicates that C1q-C4 detection may be less sensitive than that of C4b/c to monitor complement activation in vivo, probably because only a tiny amount, presumably 1–2% or even less, of activated C4 will bind to C1q upon activation. In spite of this limitation, measurement of C1q-C4 complex levels may be advantageous since this parameter is more specific for classical pathway activation than C4b/c, and is less sensitive for in vitro artefacts such as freezing and thawing or formation at room temperature. Notably, normal EDTA plasma samples contained detectable, basal levels of C1q-C4, indicating ongoing activation of the classical pathway under normal conditions. The trigger for this activation is currently unknown.

We activated human recalcified plasma with various complement activators and only specific activation of the classical pathway resulted in generation of C1q-C4d complexes. C1q belongs to a family of proteins which involves other complement-activating molecules such as MBL (Gadjeva et al., 2001; Petersen et al., 2001b; Vorup-Jensen et al., 2000). Hence, the results of our studies raised the question of whether similar complexes of MBL are formed during activation of the lectin pathway.

Indeed, when we incubated human recalcified plasma with soluble mannan and performed ELISAs with anti-C4d or C3d mAbs and anti-MBL mAbs, complexes were detected. These complexes were not generated in plasma during incubation with aggregated IgG. Thus, similar activation products as described here for C1q, are generated during complement activation by MBL and possibly by related molecules such as ficolin.

In our experience all C1q preparations purified from human plasma contained C1q-C4 and C1q-C3 complexes (data not shown). Absorption of these preparations with anti-C4d- or anti-C3d-Sepharose revealed that C3d and C4d had become fixed to different C1q molecules. In addition, the total amount of C1q was hardly reduced after passage of the C1q preparations over the columns, indicating that C1q-complexes constitute only a small percentage of the total C1q. The presence of C1q-C4, and also C1q-C3 complexes in purified C1q preparations may explain at least some of the biological effects ascribed to C1q such as stimulation of oxygen metabolism in phagocytes and binding to CR1 (Guan et al., 1991; Klickstein et al., 1997; Nepomuceno and Tenner, 1998; Tas et al., 1999; Tenner and Cooper, 1980, 1982). Currently we are investigating to what extent C1q-complexes may explain some of these biological effects of C1q.

A regulating effect on C1 activation has been described for nascent C3b and C4b by Ziccardi (Ziccardi, 1986), who reported that activated C3 and C4 induce feedback inhibition of C1 turnover by immune complexes by binding to the antigen moiety of immune complexes, causing the release of complement activating antibodies (Ziccardi, 1988). It is tempting to speculate that binding of C4 or C3 to C1q may also cause the release of C1q-complexes from the activator. Such a mechanism would explain the presence of increased levels of C1q complexes upon activation of the classical pathway.

In conclusion, we have described novel activation products of the classical pathway of complement, i.e., complexes between C1q and C3 or C4. We postulate that these complexes constitute suitable parameters with which it is possible to assess activation of the classical pathway in biological fluids of patients with either a chronic inflammatory condition or induced following surgical procedures such as e.g. CPB.

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