Complement biology in health and disease

Lubbers, R.

CHAPTER 8

CARBAMYLATION REDUCES THE CAPACITY OF IGG FOR HEXAMERISATION AND COMPLEMENT ACTIVATION

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ABSTRACT

Introduction: Carbamylation is a post-translational modification that can be detected on a range of proteins, including immunoglobulin G (IgG), in several clinical conditions. Carbamylated IgG (ca-IgG was reported to lose its capacity to trigger complement activation, but the mechanism remains unclear. Since C1q binds with high affinity to hexameric IgG, we analysed whether carbamylation of IgG affects binding of C1q, hexamerisation and Complement Dependent Cytotoxicity (CDC). Synovial tissues of rheumatoid arthritis (RA) patients were analysed for presence of ca-IgG *in vivo*.

Materials and methods: Synovial tissues from RA patients were analysed for presence of ca-IgG using Mass Spectrometry (MS). Monomeric or hexameric antibodies were carbamylated *in vitro* and quality in solution was controlled. The capacity of ca-IgG to activate complement was analysed in ELISAs and cellular CDC assays.

Results and conclusions: Using MS we identified ca-IgG to be present in the joints of RA patients. Using *in vitro* carbamylated antibodies, we observed that ca-IgG lost its capacity to activate complement in both solid-phase- and CDC assays. Mixing ca-IgG with non-modified IgG did not result in effective inhibition of complement activation by ca-IgG. Carbamylation of both monomeric IgG and pre-formed hexameric IgG greatly impaired the capacity to trigger complement activation. Furthermore, upon carbamylation the pre-formed hexameric IgG, dissociated into monomeric IgG in solution, indicating that carbamylation influences both hexamerisation and C1q binding. In conclusion, ca-IgG can be detected *in vivo* and has a strongly reduced capacity to activate complement, which is in part, mediated through a reduced ability to form hexamers.

INTRODUCTION

Post-translational modifications (PTM) of proteins following biosynthesis are common in the human body, and are important in the regulation of activity, stability and folding of proteins [1, 2]. Dysregulation of PTMs has been linked to inflammatory and autoimmune conditions [1]. Besides dysregulation, PTMs can also cause the formation of neoepitopes on extracellular proteins during environmental exposure and aging which subsequently give rise to autoantibodies [3, 4]. The PTM carbamylation is the chemical conversion of a positively charged lysine into an uncharged homocitrulline. This conversion is mediated by cyanate, which is in equilibrium with urea and the availability of cyanate can be increased by inflammation through the release of myeloperoxidase (MPO) from neutrophils [5]. Carbamylation is therefore especially interesting in the context of inflammatory and autoimmune diseases. Carbamylated proteins are frequently targeted by autoantibodies in patients suffering from Rheumatoid Arthritis (RA) [6, 7]. Several proteins have reported to be carbamylated *in vivo* e.g. Albumin [8], Alpha 1 anti-trypsin [9], but interestingly also IgG [10, 11]. It has been reported that carbamylation of IgG impacts on its capacity to activate complement [10].

The complement system is a well described sequential autolytic cleavage system which can be activated via three different pathways: the classical pathway (CP), the lectin pathway and the alternative pathway. The CP is commonly activated upon binding of C1q to IgM or multiple copies of antigen-bound IgG [12]. One report described that upon carbamylation of IgG, there was a dose-dependent decrease in complement activation due to the loss of capacity to bind C1q, and hence loss of ability to initiate the CP, as measured in ELISA-based and tumour opsonisation assays [10]. Recently, it has been demonstrated that binding of C1q requires a hexameric arrangement of monomeric IgG complexes, which assemble via non-covalent Fc:Fc interactions [13, 14]. Moreover, cryo-electron microscopy analyses have shown that also the C1 complex binds to hexameric IgG1 complexes [15] and IgM [16]. This could indicate that the reduced capacity of carbamylated IgG (ca-IgG) to activate complement may directly or indirectly be caused by reduced hexamerisation. If this is the case, then carbamylation of IgG may be utilised to dampen inflammatory responses triggered by non-modified IgG. By the introduction of three mutations (E345R, E430G and S440Y) in the Fc domain, the ability of IgG1 to form hexamers was enhanced, both in solution and on the cell surface [13, 14]. This hexameric IgG is able to bind C1 in solution and activate the complement system. Here we studied the effects of IgG carbamylation on complement binding, activation and CDC in both normal monomeric IgG1 and hexameric conformations. In addition, we investigated the possibility of using ca-IgG to inhibit IgG mediated complement activation.

In this study we extend the observation that ca-IgG is present in vivo and unable to activate complement, by showing that the carbamylation impacts both on the C1q

binding to the IgG Fc, but also impairs hexamerisation of IgG which is essential for effective C1q binding and classical pathway activity.

MATERIALS & METHODS

Carbamylation

For carbamylation and subsequent experiments various (therapeutic) antibodies were used: intravenous immunoglobulin (IvIg) (Nanogram, Sanquin), alemtuzumab (Genzyme), rituximab (MabThera) and IgG1-DNP (RGY) (described below). These different antibodies were carbamylated by incubation in 0.1M potassium cyanate (KOCN) (cat#215074, Sigma-Aldrich) or incubated in phosphate buffered saline (PBS) as a control at 37°C for different periods of time. After incubation, the preparations were dialysed against PBS for 48 hours, or a buffer exchange was performed with PBS (pH 7.4).

Expression and purification of anti-DNP antibodies

The IgG1-DNP antibody consists of the variable domains of mouse mAb G2a2 against the hapten dinitrophenyl (DNP) combined with the constant domains of human IgG1 and the kappa light chain [17]. A triple mutant variant of the IgG1-DNP was produced containing three mutations (E345R, E430G and S440Y) in the Fc domain, which enhance the ability of the antibody to form hexamers both in solution and on the cell surface (designated IgG1-DNP-RGY), [13, 14]. Gene constructs for heavy and light chain were separately ordered (Thermo Fisher Scientific GeneArt, Regensburg, Germany) and cloned into a pcDNA3.3 vector (Thermo Fisher Scientific). Antibodies were expressed by transient transfection of Expi293F™ cells with equimolar amounts of heavy and light chain plasmid, using the ExpiFectamine™ 293 Transfection Kit (Thermo Fisher Scientific) according to the manufacturers guidelines. Secreted antibodies were harvested from the supernatant five days post-transfection, 0.2 µm filtered and purified on a column of protein A Sepharose (Centocor, Leiden, the Netherlands). Solution phase hexamerisation of IgG1-DNP-RGY was verified using High Pressure Size Exclusion Chromatography (HP-SEC) analysis as previously described [14].

Western Blot

10% Tris-glycine gels (Biorad Cat# 456-1033) were loaded with equal amounts of untreated IgG and ca-IgG under reducing conditions. Carbamylation was analysed using anti-carbamyl-lysine antibody (cat#STA-078, Cell Biolabs). Next, loading was visualized by stripping the western blot and re-probing for human IgG (DAKO cat# P0214).

Mass spectrometry carbamylation

Mass spectrometry (MS) was carried out as described previously [9, 18, 19]. Synovial fluid (SF) was centrifuged at 2000 rpm for five minutes, the supernatant collected and stored in aliquots at -80°C. Next, SF samples (500 µg protein) were depleted, according to the instructions of the supplier, for the top-12 most abundant serum proteins (Pierce/

Thermo). Subsequently, the depleted sample (50 µg) was subjected to filter-aided sample preparation (FASP II) [20] using ^{13}C-urea instead of regular ^{12}C-urea, in order to distinguish artificial *in vitro* ^{13}C carbamylation during the FASP procedure from genuine *in vivo* ^{12}C carbamylation events. After FASP II procedure no *in vitro* carbamylation events were observed.

The cartilage and synovium samples (after their extraction with hot sodium dodecyl sulfate (SDS) to remove adherent and easily soluble protein) were digested with trypsin using the following procedure: Samples were incubated in 100 µL Dithiothreitol (DTT) (100 mM) and NH_4HCO_3 (25 mM) for 20 min at 54°C. After five minutes centrifugation at max speed, the supernatant was saved and the pellet was incubated in 150 µL iodoacetamide (15 mM) and NH_4HCO_3 (25 mM) for 30 min at room temperature. After five minutes centrifugation at max speed, the supernatant was saved and the pellet was incubated in 200 µL 25 mM NH_4HCO_3 containing 10 µg trypsin for four hours at 37°C. The supernatants from the DTT and iodoacetamide incubation were combined and concentrated on a 30 kDa filter (Microcon, Millipore), washed three times with 100 µL 25 mM NH_4HCO_3 and incubated with 1 µg trypsin for four hours at 37°C. Finally, the supernatant containing digested protein from the pellet was added to the digest on the filter. The filter was washed once with 100 µL 0.5 M NaCl. Peptides were recovered from the filtrate and subjected to solid phase extraction on C18 cartridges (Oasis HLB Waters).

ELISA to detect carbamylation

Maxisorp plates (Nunc) were coated with 10 µg/mL IgG in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH 9.6). Plates were blocked with PBS/1% BSA and carbamylated signal was detected using rabbit anti-carbamyl-lysine antibody (cat#STA-078, Cell Biolabs) and a goat anti-rabbit Horseradish Peroxidase (HRP) secondary antibody (DAKO cat# P0448). The substrate was added using 2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid (ABTS) and the carbamylation signal was measured at an absorbance level of 415 nm using Biorad iMark Microplate Absorbance Reader.

Complement activation ELISA

Maxisorp plates (Nunc) were randomly coated with 10 µg/mL (ca-)IgG in coating buffer (0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH 9.6) In mixing experiments the final concentration was 10 µg/mL with different ratios between the ca-IgG and non-modified IgG, unless indicated otherwise. Plates were blocked with PBS/1% BSA and incubated with 1% normal human serum (NHS, pooled from 4 healthy donors) or heat-inactivated NHS as a control (diluted in GVB++; 0.1% gelatin, 5mM Veronal, 145mM NaCl, 0.025% NaN_3, 0.15mM $CaCl_2$, 0.5mM $MgCl_2$, pH 7.3). Complement binding or deposition was analysed with rabbit anti-human C1q (DAKO cat# A0136), goat anti-human C4 (Quidel cat# A305), rabbit anti-human C3c (DAKO cat# A0062) and mouse anti-human C5b9 (DAKO cat# M0777) with corresponding HRP-labelled secondary antibodies in PBS/1% BSA/0.05%

Tween. Finally the substrate was added using ABTS and absorbance measured at 415 nm using Biorad iMark Microplate Absorbance Reader.

Complement dependent cytotoxicity

CDC assays with B-lymphoma cell lines (Daudi and Wien-133) were performed using 100,000 target cells opsonized with antibody concentration series in the presence of pooled NHS (20% final concentration) as a complement source (Sanquin, The Netherlands) as previously described [21]. Cells were incubated for 45 minutes at 37℃ and killing was calculated as the percentage of propidium idodide (PI, Sigma-Aldrich Chemie B.V., Zwijndrecht, The Netherlands) positive cells determined by flow cytometry. Curves were generated using non-linear regression (sigmoidal dose-response with variable slope) analyses within GraphPad Prism software (GraphPad Software, Sand Diego, CA, USA).

Antibody binding

Antibody binding assays with B-lymphoma cell lines (Daudi and Wien-133) were performed using 100,000 target cells opsonized with antibody concentration series and incubated for 30 minutes at 4℃. Next, cells were washed and incubated for 30 minutes at 4°C with R-Phycoerythrin (PE)-conjugated goat-anti-human IgG $F(ab')_2$ (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; cat #: 109-116-098). Cells were washed and analysed by determining the mean fluorescent intensity (MFI) using flow cytometry. Binding curves were generated using non-linear regression (sigmoidal dose-response with variable slope) analyses within GraphPad Prism software (GraphPad Software, Sand Diego, CA, USA).

Complement-mediated liposomal lysis assay

Liposomes were prepared using Dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), cholesterol and DNP-cap-PE, purchased from Avanti Polar Lipids (Alabama, USA). Lipid films were composed of DMPC:DMPG:cholesterol:DNP-cap-PE (45:5:49:1 mol%). Components were dissolved in chloroform:methanol (9:1 v/v) before drying under nitrogen gas and desiccation overnight. Films were rehydrated at 37°C for 30 minutes with a self-quenching concentration of sulforhodamine B (20 mM; S1402 from Sigma Aldrich, Missouri, USA) in PBS to a final lipid concentration of 0.8 mg/mL. The sulforhododamine B-liposome mixture was sonicated for five minutes at 37°C in a water bath. Purification of liposomes was performed through size-exclusion chromatography by using a prepacked NAP-25 column (17-0852-01 GE, Healthcare, Little Chalfont, UK).

To analyse complement activity via membrane attack pore membrane attack complex (MAC) mediated dye leakage, purified liposomes were diluted 10× in PBS and mixed with NHS (10% v/v final concentration) from Complement Technologies (Tyler, TX, USA). Sulforhodamine B fluorescence was measured with an excitation wavelength

of 565 nm and emission wavelength of 585 nm using a CLARIOstar microplate reader (BMG Labtech, Offenburg, Germany). Fluorescence was measured at 21°C for 100 s before different antibodies (IgG1-DNP and IgG1-DNP-RGY, both non-modified and carbamylated) were added to final concentrations of 4.35 µg/mL.ml before assaying for a further 10 minutes. Total lysis was performed by adding 70% ethanol after assay. Experiments were performed in triplicate.

Statistics

Statistical analysis was performed using GraphPad Prism version 7.02. Statistical differences were determined using T-tests, a p-value of <0.05 was considered statistically significant. Data is either representative for multiple experiments or the mean with standard deviation (SD) is shown.

RESULTS

Identification of *in vivo* occurring ca-Ig(G) using mass spectrometry

Previously, ca-IgG was detected in synovial fluid from two RA patients [10]. Therefore, we investigated various tissue samples from RA patients. In both synovium and synovial fluid from either the knee or hip, carbamylated immunoglobulin peptides were found using MS (**Table 1**). Interestingly, the same carbamylated immunoglobulin peptides were found in independent donors, for example VGVETTkpSkQSNNkYAASSYLSLTPEQWK (k = carbamylation on Lysine p = oxidation on Proline) found in two out of 14 donors (14%). Analysis of the relative abundance of the carbamylated immunoglobulin peptides revealed that, at a reasonable coverage of the immunoglobulin proteins (on average 63%,ranging from 25% to 100%), we detected the carbamylated peptides roughly at a 1000 fold lower abundance as compared to the abundance of the protein from which the peptide is derived. Moreover, in various OA tissue samples (2x synovial fluid, 2x synovium) no carbamylated Ig peptides were found. These data indicate that carbamylation of immunoglobulins, including IgG, is occurring *in vivo* as exemplified using samples from RA patients.

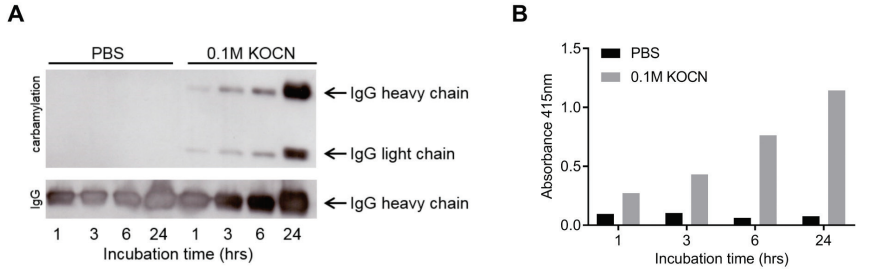
Table 1. Identification of *in vivo* occurring ca-IgG using Mass spectrometry

Diagnosis	*Sex*	*Age*	*Location*	*Tissue*	*Protein Group Accessions*	*Sequence*	*Protein Descriptions*
RA	Female	76	Knee	Synovium	A0A087X130;A0A075B6H6	H**k**VYAcEVTHQGLSSPVTK	Ig kappa chain C region
					P01857;A0A087WYE1;A0A087WYC5	KVEP**k**ScD**k**THTcppcp APELLGGPSVFLFPPKPK	Ig gamma-1 chain C region
RA	Male	56	Hip	Synovium	A0A087X130;A0A087WZW8; A0A075B6H6	H**k**VYAcEVTHQGLSSPVTK	Ig kappa chain C region
					P01876	TFTcTAAYPES**k**TPLTATLSK	Ig alpha-1 chain C region
					A0A087X130;A0A087WZW8; A0A075B6H6	VYAcEVTHQGLSSPVT**k**SFNRGEc	Ig kappa chain C region
					A0A087X130;A0A087WZW8; A0A075B6H6	ADYE**k**HKVYAcEVTHQGLSSPVTK	Ig kappa chain C region
					A0A087WYE1;A0A087WYC5	**k**VEP**k**ScDKTHTcPpcp ApELLGGPSVFLFPPKPK	Ig gamma-1 chain C region
					P01861	VD**k**RVES**k**YGppcpScp ApEFLGGPSVFLFPPKPK	Ig gamma-4 chain C region
RA	Male	49	Knee	Synovial Fluid	A0M8Q6	VGVETT**k**pS**k**QSNN**k**YAASSYLSLTPEQWK	Ig lambda-7 chain C region
RA	Male	67	Knee	Synovial Fluid	A0M8Q6	VGVETT**k**pS**k**QSNN**k**YAASSYLSLTPEQWK	Ig lambda-7 chain C region

Carbamylated immunoglobulins detected *in vivo* in samples from Rheumatoid Arthritis (RA) patients, as analysed by mass spectrometry, the carbamylated lysines are annotated (**k**) and the carbamylated lysines that are identical to those observed in the *in vitro* preparation are underlined (**k**) . k = Carbamyl, c = Carbamidomethyl, p = Oxidation.

Successful carbamylation of IgG preparations

To study the biology of carbamylation of antibodies, different IgG antibodies (alemtuzumab, rituximab, IgG1-DNP, IgG1-DNP-RGY) and IVIg were carbamylated by 0.1 M KOCN (cyanate) at 37°C for different time points (1, 3, 6 and 24 hours). Carbamylation of all antibody preparations were successful as detected by western blot and an ELISA based assay. Both the western blot and the ELISA show a time-dependent increase in carbamylation for all antibodies, as exemplified by rituximab (**Figure 1 A, B**). Moreover, the 24 hour carbamylated rituximab sample was analysed with MS to identify the carbamylated lysines (**Figure 1C)**. MS analysis showed extensive presence of carbamylation, several of the carbamylated lysines were identical to the carbamylated lysines found in the *in vivo* samples.



C

Rituximab heavy chain coverage 79%

QVQLQQPGAE LVKPGASVKM SC**k**ASGYTFT SYNMHWV**k**QT PGRGLEWIGA IYPGNGDTSY NQ**k**FK**G**K**k**ATL TAD**k**SSSTAY MQLSSLTSED SAVYYCARST
YYGGDWYFNV WGAGTTVTVS AAST**k**GPSVF PLAPSS**k**STS GGTAALGCLV K**k**DYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ
TYICNVNHKP SNTKVDKKAE P**k**SCD**k**THTC PPCPAPELLG GPSVFLFPP**k** PKDTLMISRT PEVTCVVVDV SHEDPEV**k**FN WYVDGVEVHN AKT**k**PREEQY
NSTYRVVSVL TVLHQDWLNG **k**EY**k**C**k**VSNK ALPAPIE**k**TI S**k**A**k**GQPREP QVYTLPPSRD ELT**k**NQVSLT CLVKGFYPSD IAVEWESNGQ PENNY**k**TTPP
VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG K

Rituximab light chain coverage 100%

QIVLSQSPAI LSASPGE**k**VT MTCRASSSVS YIHWFQQ**k**PG SSP**k**PWIYAT SNLASGVPVR FSGSGSGTSY SLTISRVEAE DAATYYCQQW TSNPPTFGGG
T**k**LEIKRTVA APSVFIFPPS DEQL**k**SGTAS VVCLLNNFYP REA**k**VQW**k**VD NALQSGNSQE SVTEQDS**k**DS TYSLSSTLTL S**k**ADYE**k**H**k**V YACEVTHQGL
SSPVT**k**SFNR GEC

k indicates carbamylated lysines observed in *in vitro* modified Rituximab
k indicates the carbamylated lysine positions as observed in gecarbamyleerd IgGs *in vivo*
The yellow colour highlights the coverage

Figure 1. Successful carbamylation of rituximab

The results from rituximab are shown and are representative for all carbamylated antibodies (IVIg, alemtuzumab, DNP variants). Antibodies were either incubated in PBS as a control (buffer only) or in 0.1M potassium cyanate (KOCN). (**A**) Western blot analysis of 2 µg (ca)rituximab, carbamylation was visualised using a carbamyl-lysine specific antibody. Equal loading of the preparations was shown by detection of human IgG. Incubation time is indicated in hours. (**B**) Carbamylation of rituximab as analysed by ELISA, where an equal amount of antibody (10 µg/mL) was used to coat the well. Detection was performed using a carbamyl-lysine specific antibody. (**C**) Mass spectrometry analysis of rituximab that was carbamylated for 24 hours. The carbamylated lysines are annotated (**k**), coverage is indicated with yellow highlight.

Carbamylation of IgG completely blocks the complement activating potential of IgG
Complement activation assays were performed using 10 µg/mL (ca-)IgG coated ELISA plates. Binding of C1q and deposition of C4, C3c and C5b9 was significantly decreased upon carbamylation of IVIg, rituximab and alemtuzumab, when compared to the non-modified counterpart (**Figure 2**). We did analyse whether carbamylation affected the antibody's immobilization in the ELISA plate and although we did observed some differences, these are too small to explain the observed biological effects (**Supplementary Figure 1**). Previously, it has been demonstrated that optimal complement activation via IgG is achieved when the IgGs are in a hexameric arrangement. Therefore, we wondered whether adding ca-IgG to IgG would inhibit this process by limiting the possibilities to form productive hexamers. Mixing experiments were performed to analyse whether the presence of ca-IgG indeed affects the ability of non-modified IgG to activate the complement system. Mixing ca-IgG antibodies with non-modified antibodies, while maintaining a similar end concentration, resulted in a decrease in complement activation (at the levels of C1q, C4, C3c and C5b9) (**Figure 3**), which was proportional to the decreasing concentration of coated non-modified IgG. Although ca-IgG was unable to activate complement by itself, we did not observe an inhibitory effect of ca-IgG on the complement activation induced by non-modified IgG other than the simple dose response effect of diluting the non-modified IgG. As the coated antibodies in ELISA plates are known to form a high density surface of IgGs that bind C1q without a need to form hexamers [14], the data indicate that the carbamylation on Lysine residues in the Fc impact directly on the binding of C1q.

CDC is not affected by the presence of ca-IgG
Because of the observed differences in complement binding and activation by plate-immobilized IgGs, we next explored the effect of carbamylation on antibody binding and complement dependent cytotoxicity (CDC) using tumour cell lines. IgG hexamerisation is required for optimal C1q binding and complement activation in this setting. Binding of non-modified or carbamylated rituximab and alemtuzumab was analysed using Daudi and Wien-133 cell lines, respectively. The carbamylated variants of rituximab and alemtuzumab displayed a slightly lower binding compared to their non-modified counterparts, which actually might reflect lower binding efficiency of the anti-IgG detection conjugate antibody to the ca-IgG as compared to the non-modified IgG (**Supplementary Figure 2**). Next, we investigated whether carbamylated IgG affects the ability of non-modified IgG to induce CDC. CDC assays were performed with different ratios of non-modified or carbamylated rituximab and alemtuzumab (**Figure 4**). No CDC was observed in the presence of carbamylated rituximab or alemtuzumab only, which cannot be solely attributed to the reduced binding of ca-IgG to the surface. Non-modified rituximab and alemtuzumab induced substantial CDC, while titrating in their carbamylated counterparts resulted in a decrease in killing capacity. For rituximab the decrease in killing capacity was gradual with the titrating of the carbamylated variant. Whereas for alemtuzumab there is a sharp decrease in CDC after the carbamylated

variant exceeded the 50% ratio. These results indicate that at the chosen antibody concentrations and ratios there is no dominant negative effect of ca-IgG presence on the capacity of non-modified IgG to induce CDC.

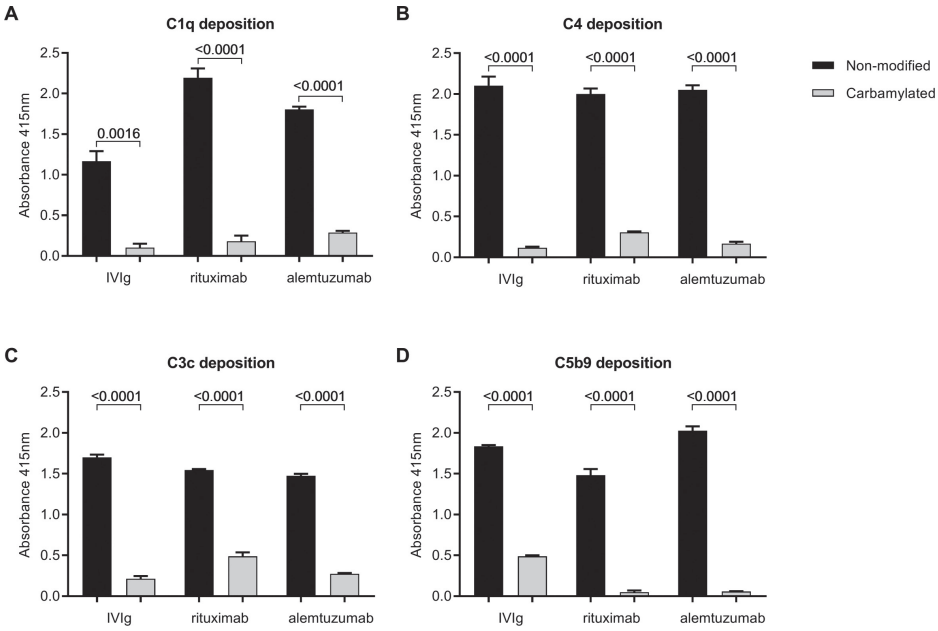


Figure 2. Complement deposition of C1q, C4, C3c and C5b9 on non-modified and carbamylated IgG

Complement deposition ELISAs were performed using 10 µg/mL plate bound non-modified or carbamylated IVIg, rituximab and alemtuzumab. C1q (**A**), C4 (**B**), C3c (**C**) and C5b9 (**D**) deposition after incubation for one hour with 1% normal human serum (NHS) was measured at an absorbance wavelength of 415 nm. Data are shown as mean with standard deviation from technical triplicate. All differences in complement deposition between non-modified antibodies and their carbamylated counterparts were significant, as analysed with T-test (all $p < 0.05$). Shown is a representative experiment performed at least twice.

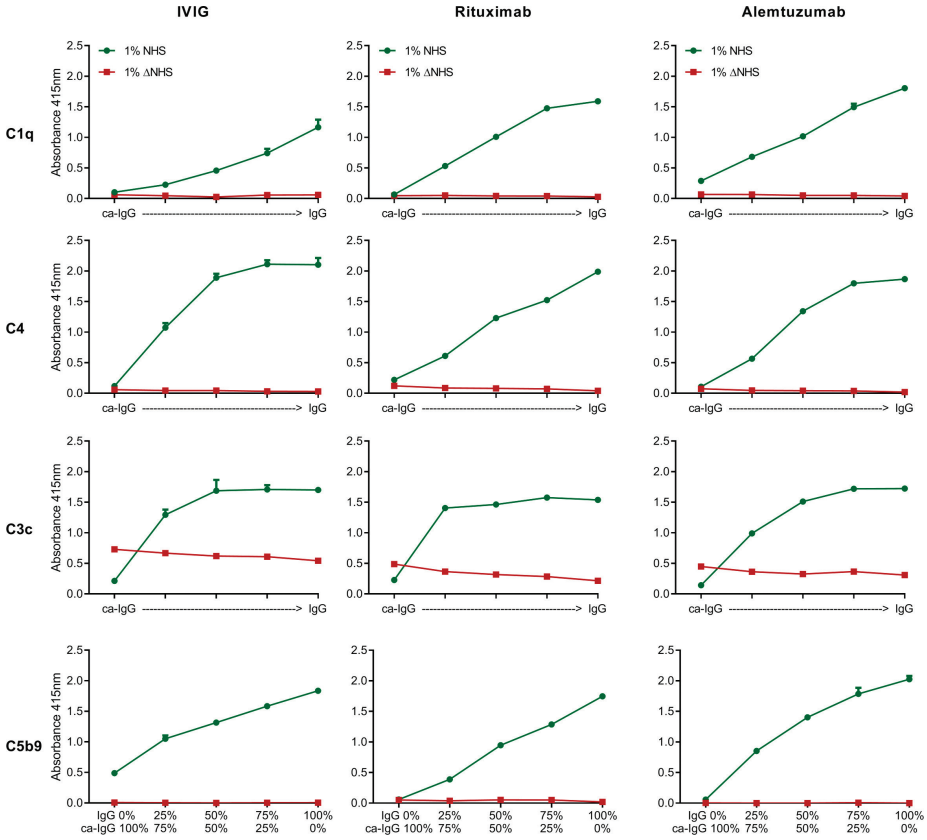


Figure 3. Deposition of different complement components is not affected by the presence of ca-IgG

Complement deposition ELISAs were performed using plate bound non-modified or carbamylated IVIg, rituximab and alemtuzumab, with a sum of 10 µg/mL coating mixed in different ratios, and for rituximab a sum of 2.5 µg/ml (C4) or 5 µg/ml (C1q, C3c, C5b9). From left to right ca-IgG vs non-modified IgG: 100%:0%, 75%:25%, 50%:50%, 25%:75%, 0%:100%. Deposition of complement components C1q, C4, C3c and C5b9 were measured at an absorbance wavelength of 415 nm. Green circles depict the complement deposition after incubation with 1% normal human serum (NHS), red squares depict complement deposition after incubation with 1% heat-inactivated NHS (ΔNHS) for one hour. Shown is a representative experiment which has been performed independently at least twice.

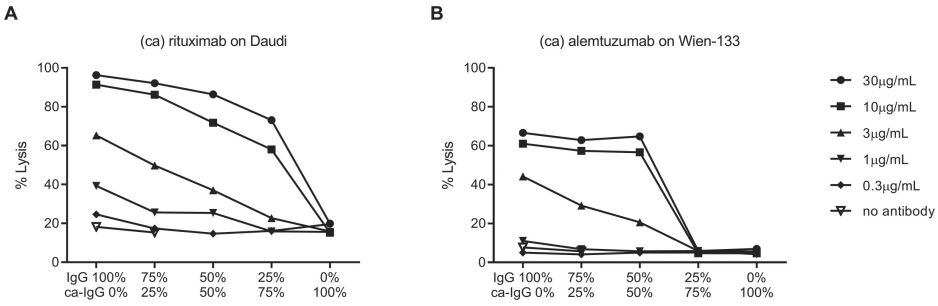


Figure 4. Complement-dependent cytotoxicity is not affected by the presence of ca-IgG
Complement dependent cytotoxicity (CDC) assays were performed with Daudi (**A**) and Wien-133 (**B**) cells opsonized with various concentrations of non-modified and/or carbamylated rituximab or alemtuzumab respectively in the presence of 20% pooled NHS. CDC assays were performed using different ratios of mixed non-modified (IgG) and carbamylated (ca-IgG) antibody variants. Ratios of ca-IgG versus non-modified IgG from left to right are: 100%:0%, 75%:25%, 50%:50%, 25%:75%, 0%:100%, each depicted for in total five different final antibody concentrations. Shown is a representative experiment which has been performed independently twice.

Monomeric and hexameric IgG

CDC activity is highly dependent of IgG1 hexamer formation, and in the CDC assay it was unclear whether ca-IgGs form hexamers in the presence of non-modified IgGs. Therefore, we explored whether the effect of reduced complement activation by ca-IgG is also present when using pre-formed ca-IgG-hexamers. DNP antibodies were produced containing three point mutations in the Fc domain (DNP-RGY), which causes the IgGs to form stable hexamers in solution [14] (**Figure 5A**). Next, these preparations were also carbamylated and the ability of IgG-RGY mutants to hexamerise in solution was analysed using size exclusion chromatography (HP-SEC). Control IgG1-DNP-RGY that primarily existed as a hexamer: an early eluting peak was observed for hexameric IgG (58.6%) and a second peak for monomeric IgG (39.4%). In solution both species are in equilibrium with a relatively high fraction forming hexameric complexes. Carbamylated IgG clearly only showed a monomeric IgG peak (98%) (**Figure 5B**) indicating that hexamerisation was abrogated. First we tested the effect of carbamylation on monomeric IgG or on hexameric IgG regarding complement activation in a liposome lysis assay, a setting in which the bound antibodies can move freely and form hexamers on the surface. In a liposome lysis assay, complement activation is measured by sulforhodamine B release from liposomes as a consequence of MAC formation [22]. In non-modified conditions, both anti-DNP antibody preparations are able to lyse liposomes (the lysis caused by DNP-RGY is higher since pre-formed hexamers enhance complement activation) [14]. Carbamylation of both these antibodies leads to a complete loss of liposome lysis (**Figure 5C**, **D**).

Next, the RGY variant of alemtuzumab was generated and carbamylated (or treated with PBS as control), and CDC assays performed. Again, carbamylation of RGY resulted in loss of CDC capacity (**Figure 5E**). Collectively, the data on the size exclusion chromatography, the liposome lysis assay and the CDC assays indicate that carbamylation removes the capacity of IgG-RGY mutants to spontaneously from hexamers in solution.

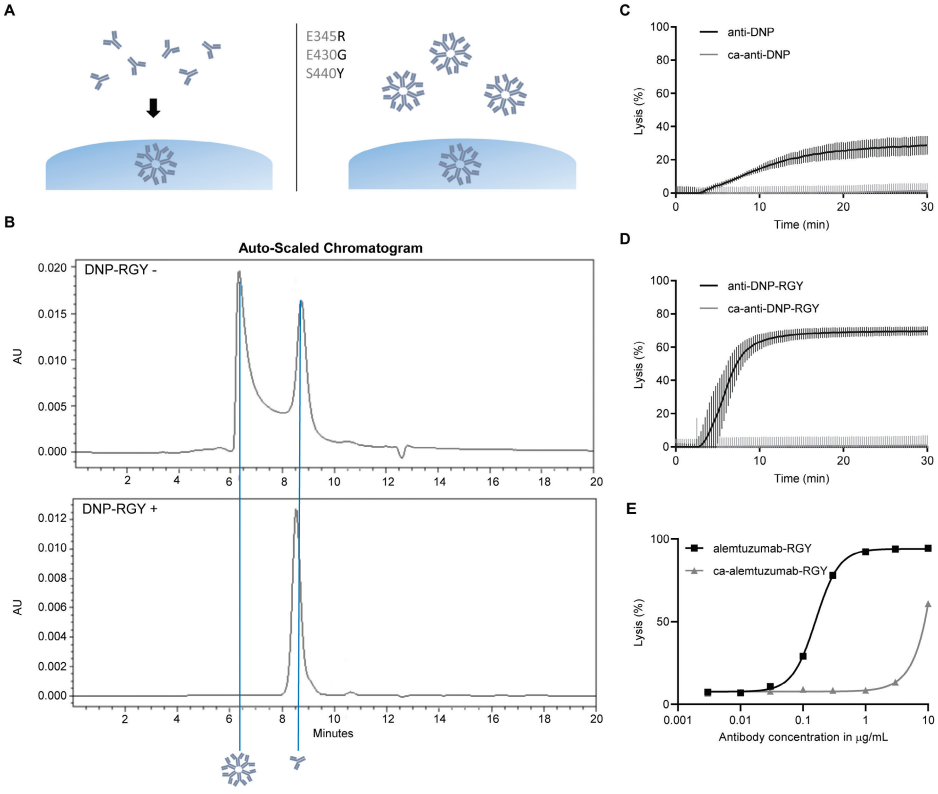


Figure 5. Monomeric and hexameric IgG both lose liposomal lysis capacity upon carbamylation
(A) Schematic representation of monomeric IgG1-DNP which forms hexamers on the cell surface (left) and IgG1-DNP-RGY which forms hexamers in both solution and on the cell surface (right). (**B**) The effect of carbamylation of IgG1-DNP-RGY on hexamerisation in solution was analysed with HP-SEC (High Pressure Size Exclusion Chromatography). (**C**, **D**) Liposomes expressing DNP were lysed in the presence of NHS and 4.35 µg/ml (carbamylated (ca))anti-DNP(RGY) antibodies. Fluorescence of sulforhodamine B is normalised to total-lysis by addition of ethanol, which is set at 100%. Data shown are the mean with SD from technical triplicate and results are representative of three independent experiments. (**E**) Alemtuzumab-RGY was carbamylated and complement dependent cytotoxicity (CDC) assays on Wien-133 were performed. Cells were opsonized with various concentrations of non-modified and/or (ca) alemtuzumab-RGY in the presence of 20% pooled NHS.

DISCUSSION

Several carbamylated proteins are known to be present *in vivo;* carbamylated albumin was identified in patients suffering from renal failure [23, 24], and carbamylated fibrinogen and A1AT were discovered in samples from RA patients. In addition, ca-IgG was present in synovial tissue of RA patients [10]. It has also been shown that carbamylation of IgG1 occurs *in vivo,* resulting in loss of C1q binding [10, 11] and in a subsequently reduced complement activation. These observations may have interesting implications. In RA anti-CarP antibodies are present that target carbamylated proteins [3, 6], possibly including ca-IgG, which in such a scenario would have rheumatoid factor-like properties. Furthermore, ca-IgG will behave differently in the inflamed joint of the patient. For several therapeutic antibodies, such as the CD20-specific antibody rituximab, the mechanism of action involves CDC. When therapeutic antibodies become compromised by carbamylation *in vivo,* their functionality may be affected, as well as their half-life. Therefore, we were interested to reproduce the previously known inhibitory effects of ca-IgG on the complement system and CDC, and specifically analyse ca-IgG in the presence of non-modified IgG and variants of IgG that form hexamers in solution.

We confirmed that carbamylation of IgG results in a decrease in complement activation, both in ELISA and in CDC assays. For plate bound assays we observed that the effect on the level of C3c deposition of ca-IgG was less pronounced, which could be related to some alternative pathway activation of the plastic wells at high serum concentrations. The binding of rituximab and alemtuzumab to cell surfaces appeared reduced upon carbamylation, however this could also be the result of reduced detection efficiency by the anti-IgG detection antibodies used, as the detection of plate bound IgG, a condition not depending on the antigen binding by the IgG, was similarly impaired. Nevertheless, when the carbamylated variants were mixed with the non-modified IgG there was no dominant inhibitory effect on complement activation. However, for alemtuzumab there is a sharp decrease in CDC once the ca-IgG exceeds the 50% ratio. This is in agreement with other published data where a less potent CDC antibody had negative effect when exceeding the 1:1 ratio [25]. These data on alemtuzumab suggest that the ability of non-modified IgG to form hexamers and consequently bind C1q was not affected by the presence of ca-IgG at ratios below 50%. To investigate whether carbamylation interferes with antibody hexamer formation (Fc-Fc interactions), we used DNP antibodies and the DNP-RGY mutant, the latter of which forms spontaneous hexamers in solution, and analysed the ability of these antibodies to lyse DNP-coated liposomes. Upon carbamylation, the IgG1-DNP-RGY was no longer able to hexamerise in solution, indicating that carbamylation negatively affects Fc:Fc interaction resulting in the loss of C1q binding by IgG.

Using MS we identified several peptides of human immunoglobulins to be carbamylated *in vivo* in the synovial fluid of RA patients. These peptides were not found in all samples analysed, which may suggest an accumulation in a specific disease condition, but may also be the result of limited sensitivity to observed these modified peptides. Although the number of peptides containing homocitrulline was limited, we did observe similar peptides in several patients. Whether or not also therapeutic antibodies may become carbamylated *in vivo* in e.g. the inflamed joint or the tumour micro-environment is currently unknown. We still expect the majority of IgG to be unmodified even in an inflammatory environment. Based on our titration experiments, used to mimic the biological scenario, it is unlikely that the *in vivo* carbamylation of IgG would have a major impact on the complement activating potential of these therapeutic antibodies. It is interesting to note that by carbamylating a therapeutic IgG preparation it is possible to completely avoid any risk of complement activation by these antibodies. This is especially relevant for antibodies that should bind to cell surfaces where they should modify receptor ligand interactions without killing or activating the cell, such as e.g. with checkpoint blockade in anti-tumour therapy.

In conclusion, the inability of ca-IgG to activate the complement system is the combined effect of both decreased binding of C1q to the modified Fc of IgG and the reduced capacity of ca-IgG to form hexamers.

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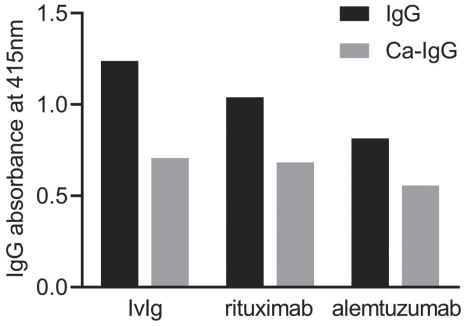
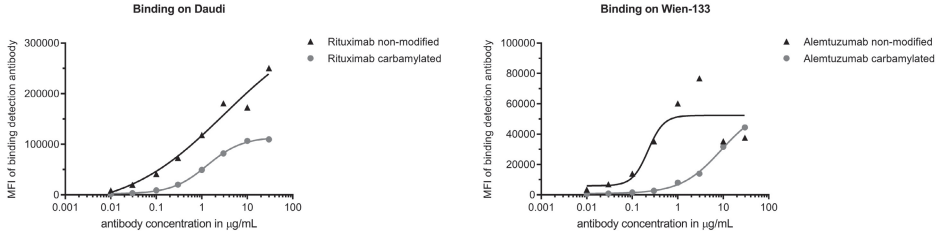
CONFLICT OF INTEREST STATEMENT

P.A.v.V and L.A.T. are listed as inventors on a patent application on the detection of anti-CarP antibodies in RA.

SUPPLEMENTARY DATA



Supplementary Figure 1. Coating of (ca-)IgG to the plate
Antibody binding to the plate of carbamylated Igs (ca-IgG) and their non-modified counterpart (IgG) was performed by directly detecting the coated antibodies with anti-human IgG-HRP labelled antibody.



Supplementary Figure 2. Cellular binding (ca-)IgG
Antibody binding was measured using flow cytometry for non-modified or carbamylated rituximab and alemtuzumab, on Daudi and Wien-133 cells respectively. Data shown are representative for two experiments.

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