

Carbamylation of immunoglobulin abrogates activation of the classical complement pathway

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Post-translational modifications of proteins significantly affect their structure and function. The carbamylation of positively charged lysine residues to form neutral homoitrulline occurs primarily under inflammatory conditions through myeloperoxidase-dependent cyanate (CNO⁻) formation. We analyzed the pattern of human IgG₁ carbamylation under inflammatory conditions and the effects that this modification has on the ability of antibodies to trigger complement activation via the classical pathway. We found that the lysine residues of IgG₁ are rapidly modified after brief exposure to CNO⁻. Interestingly, modifications were not random, but instead limited to only few lysines within the hinge area and the N-terminal fragment of the CH2 domain. A complement activation assay combined with mass spectrometry analysis revealed a highly significant inverse correlation between carbamylation of several key lysine residues within the hinge region and N-terminus of the CH2 domain and the proper binding of C1q to human IgG₁ followed by subsequent complement activation. This severely hindered complement-dependent cytotoxicity of therapeutic IgG₁. The reaction can apparently occur *in vivo*, as we found carbamylated antibodies in synovial fluid from rheumatoid arthritis patients. Taken together, our data suggest that carbamylation has a profound impact on the complement-activating ability of IgG₁ and reveals a pivotal role for previously uncharacterized lysine residues in this process.

Keywords: Carbamylation · IgG₁ · Complement · Inflammation · Rheumatoid arthritis



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Introduction

Nearly all newly translated proteins undergo post-translational modifications that affect a range of their functions [1, 2]. Recently,

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the chemical conversion of lysine into uncharged homocitrulline (i.e., carbamylation) within the polypeptide chain has raised considerable interest in the context of inflammatory and autoimmune diseases [3, 4]. In vivo carbamylation is mainly dependent on the activity of myeloperoxidase (MPO), an enzyme located in the azurophilic granules of neutrophils in the context of $\text{H}_2\text{O}_2/\text{SCN}^-$ generated by activated neutrophils. Thus, the amount of carbamylated residues is relatively high in the inflammatory milieu. Loss of charge due to carbamylation may induce conformational changes and subsequently result in the loss of protein function [5]. In fact, carbamylation has been reported to abrogate the activity of matrix metalloproteinase 2, tissue inhibitor of metalloproteinase 2, and insulin [6–8]. Moreover, homocitrulline residues may act as neoepitopes leading to production of autoantibodies, which have predictive value in the pathogenesis of rheumatoid arthritis (RA) [9] and juvenile idiopathic arthritis [10].

Complement is a pivotal component of the innate immune system and plays a key role in antibody-dependent and -independent immunity [11]. In addition, activated complement factors modulate function of adaptive immunity [12]. Over 30 soluble proteins form the complement system and provide a defence against bacteria, viruses, and tumor cells and are essential for the clearance of antigens and immune complexes [13]. The biological activity of complement factors is triggered via three independent pathways: classical, alternative, and lectin, which converge at the level of C3 cleavage. The classical pathway is initiated by the binding of C1q, the first component of the classical complement pathway, to antigen-bound IgG (or IgM), which subsequently triggers complement dependent cytotoxicity (CDC) [14]. Moreover, this event induces recruitment of effector cells, which in turn mediates antibody-dependent cellular cytotoxicity (ADCC) [15]. In addition to IgG and IgM, C1q can also recognize a wide array of host and pathogen proteins (e.g. LPS, bacterial porins, apoptotic cells, and cartilage molecules) [14, 16, 17]. The binding of C1q to human IgG₁ is relatively well characterized, and most of the binding sites have been mapped to the C-terminal portion of the constant heavy chain (CH2) domains of the Fc fragment of the molecule. Although the affinity of soluble IgG for C1q is very weak, upon immune complex formation the strength of binding increases over 1 000-fold [18].

Molecular modeling studies based on the crystal structure of a globular portion of C1q [16, 19] and mutation analysis of human IgG₁ [20] suggested that the Lys³²², Pro³²⁹, and Pro³³¹ residues in the hinge region of the Fc fragment are important for interaction between IgG₁ and C1q, which then results in complement activation. Further studies revealed that Glu³³³ and Lys³²⁶ also play pivotal roles in the activity of IgG₁ molecules [21]. Nevertheless, there is no doubt that the main C1q binding site is located in close proximity to Lys³²², Pro³²⁹, and Pro³³¹ [22] in agreement with the results of crystal structure studies, suggesting a critical role for the hinge region of human IgG₁ in C1q binding [16]. Interestingly, to date more than 25 therapeutic antibodies available in the market belong to the IgG class and the clinical efficacy of nearly all of them relies either on the complement-dependent cytotoxicity (CDC) or Ab-dependent cellular

cytotoxicity ADCC mechanism [23]. In addition to suppressing effector functions any carbamylation located on the effector Fc portion of the therapeutic antibody might have profound impact on pharmacokinetics and pharmacodynamics.

We report here the inhibitory effect of lysine carbamylation of human IgG₁ on complement activation. Although the transformation of lysine into homocitrulline is a chemical process, these modifications occurred in a very specific pattern dependent on the availability of the polar residues to cyanate ions; only 5 of 26 lysine residues in the heavy chain of human IgG₁ underwent substantial carbamylation. This modification hindered the ability of IgG₁ to bind C1q and initiate complement activation, thus providing insight into the IgG₁–C1q interaction. Furthermore, we showed that carbamylation selectively inhibited complement-dependent cytotoxicity in rituximab (RTX) without affecting antibody-dependent cellular cytotoxicity of RTX. Finally, we detected modified immunoglobulins in the synovial fluid of RA patients, a finding that suggests that IgG carbamylation occurs in vivo and may hinder complement activation.

Results

Lysine residues in human IgG₁ heavy chain undergo rapid carbamylation

The unique domain architecture at the hinge and within the Fc fragment is crucial for activation of the classical complement pathway and interaction with the Fc receptor on immune cells. Immunoglobulins present in the inflammatory environment are exposed to thiocyanate and MPO released by activated neutrophils. Therefore, we evaluated the process of carbamylation of human IgG₁ and the potential downstream effects of this modification on immunoglobulin function. To this end, IgG₁ was incubated with 0.1M potassium cyanate (KCNO) for up to 24 h at 37°C and the carbamylation of Lys residues was assessed by western blotting using antibodies designed to detect homocitrulline residues. The amount of homocitrulline increased significantly within the first 3 h. Prolonged incubation (6 and 12 h) resulted in only a moderate increase in band density (Fig. 1A). Using a colorimetric method we estimated the total amount of formed homocitrulline residues on IgG₁ treated with 0.1M KCNO. The number of modifications increased rapidly within first 3 h of incubation reaching 80 nmol/mg of protein. Further time points showed only moderate increase of the amount of the homocitrulline within the molecule reaching 95–130 nmol/mg after 24 h incubation. That indicates that the majority of lysines susceptible to carbamylation are modified very rapidly in presence of KCNO (Fig. 1B).

Gingipain K (Kgp) of *Porphyromonas gingivalis*, a Lys-Xaa peptide bond-specific protease, specifically cleaves the Lys²²²–Thr²²³ peptide bond in the IgG₁ hinge region, generating Fc and Fab fragments [24]. Since the conversion of lysine to homocitrulline prevents this cleavage, we utilized Kgp to examine carbamylation at K222 in IgG₁. Significant cleavage of control IgG₁ was observed as early as 5 min after incubation with Kgp, whereas modified

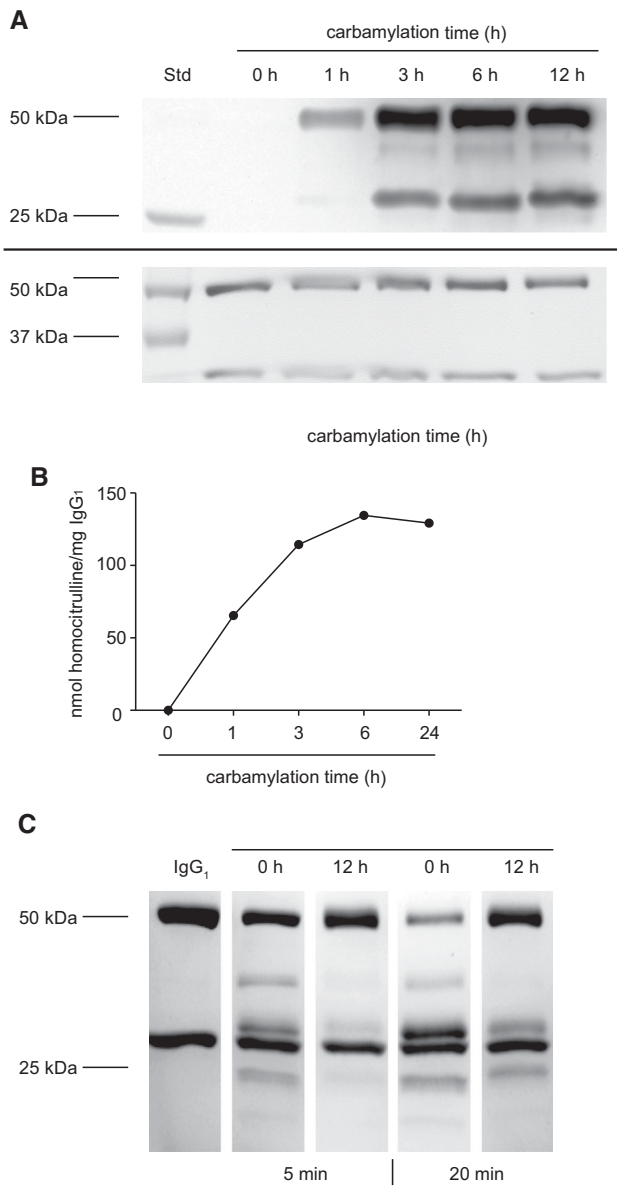


Figure 1. Human IgG₁ undergoes rapid carbamylation, which protects it from Kgp cleavage. (A) Western blot analysis of 2 µg of IgG₁ carbamylated with KCNO in a time-dependent manner. The level of carbamylation was detected with homocitrulline-specific antibodies (upper panel) together with loading controls (Coomassie staining; lower panel). Data are representative of three independent experiments. (B) The time-dependent effect of KCNO incubation on carbamylation of IgG₁. Carbamylated IgG₁ was degraded with proteinase K and samples were incubated with urea nitrogen reagent and 3% w/v 2,3-butanedione monoxime. The amount of homocitrulline/mg IgG₁ was quantified by measurement of the absorbance at 530 nm. Each point represents the mean of duplicate samples and data are from a single experiment representative of four independent experiments using IgG₁ and RTX. (C) 2.25 µg of carbamylated and control IgG₁ were incubated for the indicated times with 10 nM Kgp and SDS-PAGE was performed to assess the cleavage of IgG. All samples were separated on 4–15% gradient gels under reducing conditions. Data shown are representative of three independent experiments.

forms of IgG₁ were not susceptible to the cleavage. After 20 min of incubation with 10 nM Kgp, the majority of the heavy chain of control IgG₁ was cleaved, in contrast to carbamylated IgG₁, which remained mostly intact (Fig. 1C). These findings indicate that the heavy chain of human IgG₁ is rapidly carbamylated in the presence of cyanate and suggest that K222 in the hinge region is an important target for modification.

Analysis of the *in vitro* and *in vivo* IgG₁ carbamylation by mass spectrometry

To determine the pattern and extent of IgG₁ carbamylation, we employed mass spectrometry. As expected, we detected a time-dependent increase in the amount of carbamylation [25, 26]. Intriguingly, the modifications were not random and only a few of the 26 available lysines within the IgG₁ heavy chain underwent modification (Fig. 2). After 1 h of incubation with KCNO, we observed only two lysine residues that were efficiently modified with spectral counts above 10 (Fig. 2B and C). These residues were identified as K326 and K334, located at the N-terminus of the polypeptide chain folding into the CH₂ domain (Fig. 2B). Consequently, the degree of modification of these two residues increased with time of incubation, suggesting that they are exposed and easily accessible for modification. After 6- and 12-h incubations, in addition to increased modification of K326 and K334 (Fig. 2A), our analysis revealed a significant amount of carbamylation of K322 on the CH₂ domain and K222 and K246 within the hinge region and in its proximity, respectively. This fully corroborates our findings from the Kgp cleavage experiment. Increasing the time of exposure of IgG₁ to cyanate up to 24 h did not change the overall pattern of modification. Carbamylation of K322, K326, and K334 remained comparable to that observed at the 6- and 12-h time points. By contrast, carbamylation of hinge region lysine K222 and neighboring K246 progressed to very high levels after 24 h.

Carbamylation is fuelled by inflammatory conditions that engage excessive numbers of activated neutrophils, a condition, which occurs in the synovial fluid of RA patients. Therefore, we examined synovial fluid samples for the presence of modified IgGs. Total IgG was purified from synovial fluid samples from two patients and subjected to mass spectrometry. In both samples, carbamylation of K246 and K334 on IgG₁ was detected (Supporting Information Table 1). This is consistent with our *in vitro* findings, which show that these lysines are susceptible to carbamylation.

Collectively, these results provide strong evidence that although carbamylation is a nonenzymatic reaction, it has a significant level of specificity and affects only a small number of lysine residues within the IgG₁ chain, regardless of exposure time. Furthermore, the presence of carbamylated proteins in synovial fluid confirms that this process occurs *in vivo*.

IgG₁ carbamylation inhibits complement activation

Efficient binding of C1q to the Fc portion of IgG₁ is a pivotal step in complement activation. This prompted us to investigate the

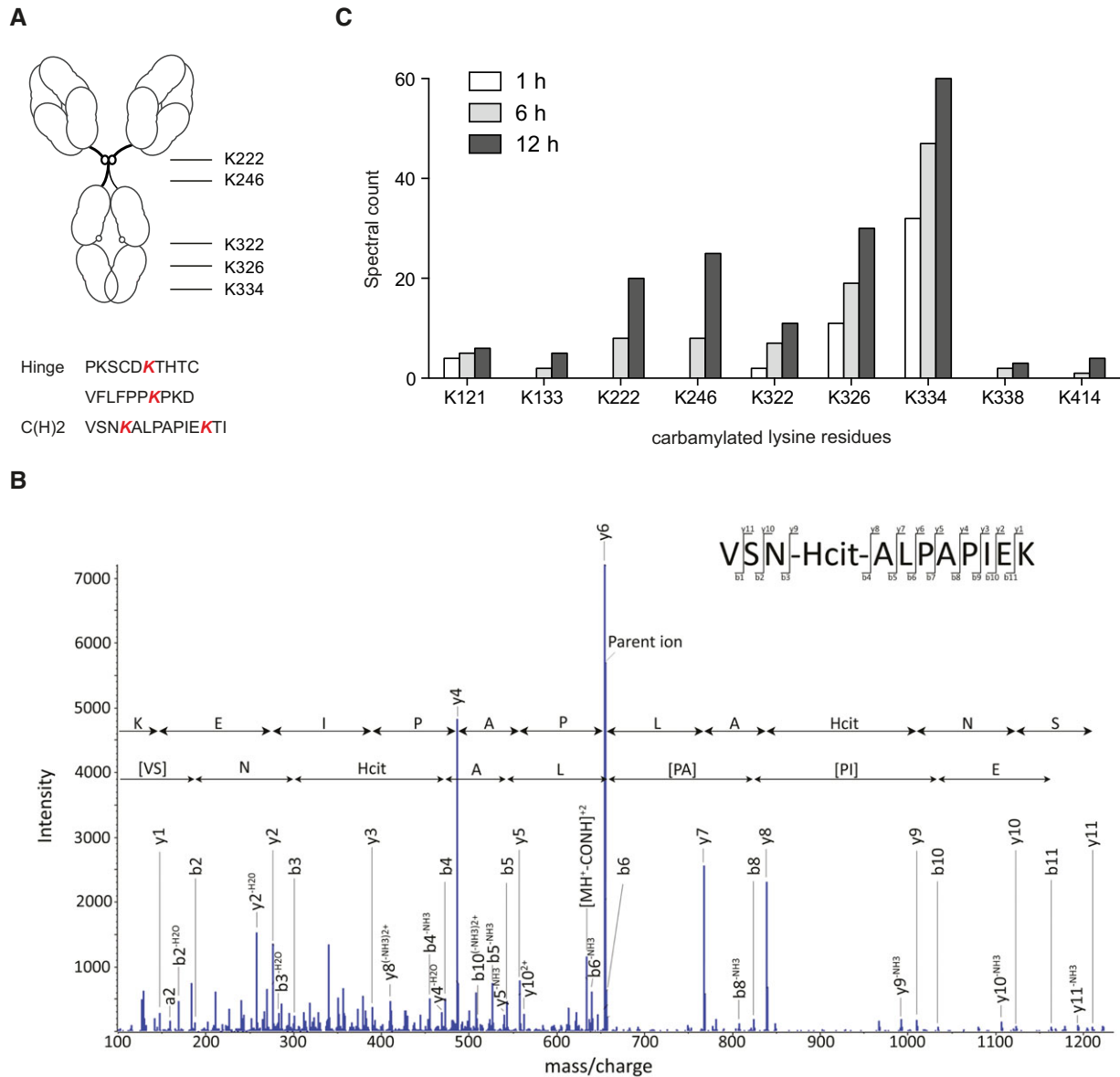


Figure 2. Carbamylation of CH2 and the hinge region sites of IgG1. (A) Schematic representation of the IgG₁ sites observed to be preferentially carbamylated in our experiments. The positions of the highly modified lysine residues are indicated in red. (B) An example of the tandem mass spectrometry assignment of carbamylation of the CH2 site K326. The MS/MS spectrum displays the VSN-Hcit-ALPAPIEK peptide fully sequenced from the C-terminus (y-ions) and nearly fully sequenced from the N-terminus (b-ions). In the spectrum, additional evidence of ions formed by collision-induced neutral loss of water, ammonia, and isocyanic acid ([MH⁺-CONH]²⁺) are marked; the latter is a characteristic feature of carbamylation. Results shown are representative of 50 MS/MS spectra assigned to the VSN-Hcit-ALPAPIEK modification. (C) Mass spectrometric evaluation of the time-dependent carbamylation of detected putative carbamylation sites in IgG₁ performed using spectral counting at the indicated time points. Each bar represents the spectral count of a single carbamylation experiment and is representative of two independent experiments.

capacity of carbamylated IgG₁ to trigger this event. Our results revealed that carbamylation has a profound impact on the ability of C1q to bind IgG₁ (Fig. 3A–C). After 3 h incubation, we detected a nearly 60% decrease in the deposition of C1q on modified immunoglobulins (Fig. 3A, $p < 0.05$). Longer carbamylation times (6, 9 or 12 h) resulted in complete abrogation of the ability of IgG₁ to bind C1q. For these time points, C1q deposition

was approximately equal to that of the negative control (Fig. 3A). In addition, we observed decreased deposition of the C4b fragment, a component of C3 convertase in the classical pathway of complement activation (Fig. 3B). Carbamylated IgG₁ (6 h) triggered significantly less formation of C4b in response to 1% normal human serum (NHS) than intact IgG₁. Subsequently, deposition of opsonin C3b was also significantly abrogated (9 h and 12 h

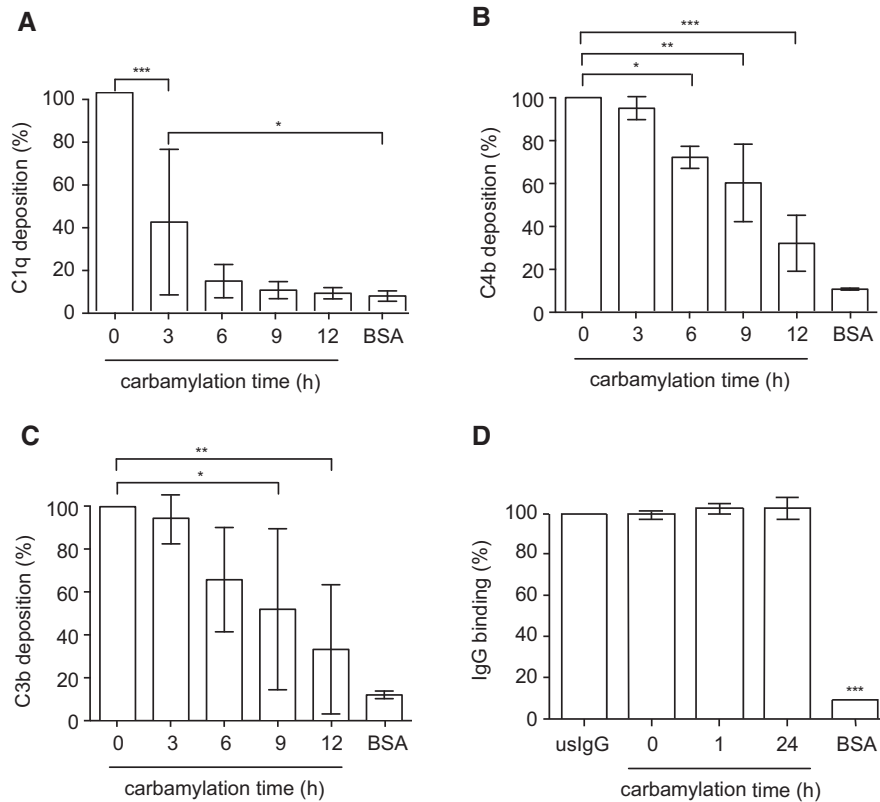


Figure 3. Carbamylation of IgG₁ inhibits binding of C1q and subsequent deposition of C4b and C3b. (A–C) The deposition of (A) C1q, (B) C4b, and (C) C3b on carbamylated IgG₁. Plates were coated with native and carbamylated heat-aggregated human myeloma IgG₁ and incubated with 1% NHS in GVB⁺⁺. Deposited complement proteins were detected with specific antibodies by using an ELISA-based method. The data are shown as the means ± SD of duplicates pooled from at least three independent experiments, where deposition of complement products induced by native IgG₁ was considered to be 100%. (D) Detection of heat-aggregated native and carbamylated human myeloma IgG₁. Plates were coated with the immunoglobulins at a concentration of 10 μg/mL. The immunoglobulins were detected by using rabbit anti-human IgGs in an ELISA-based assay. Native IgG₁ worked as a positive control to ensure proper binding to the plate and was considered to show 100% binding. The data shown are derived from duplicated samples pooled from four independent experiments, where the plates were coated with 1.25–20 μg/mL of each IgG. Data are expressed as means ± SD. The statistical significance was evaluated by one-way ANOVA followed by Tukey's multiple comparisons post-test: **p* < 0.05; ***p* < 0.01; ****p* < 0.001.

p < 0.05 and *p* < 0.01, respectively, Fig. 3C). This significant difference was clearly not a result of impaired plate-binding capacity of IgG₁ after carbamylation since there was no difference in coating with carbamylated IgG and control IgG (Fig. 3D).

IgG₁ carbamylation abolishes CDC but does not affect ADCC

To further confirm our findings we investigated the capacity of carbamylated RTX, a CD20 specific chimeric mAb with a human Fc fragment of the IgG₁ subtype, to bind C1q and subsequently exert CDC on Raji cells. The ability of carbamylated RTX to lyse cells was decreased by 25% already after 30 min carbamylation and completely abolished after 3 h incubation with 0.1M KCNO (Fig. 4A).

In contrast to CDC, carbamylation did not affect the ability of RTX to induce ADCC, which depends on an antigen binding by Fab and interaction of Fc fragment of IgG with CD16a (FCγRIIIa) on natural killer (NK) cells. Even after 3 h carbamylation, when nearly all available lysines were carbamylated, there was no significant difference in ADCC induced cell death caused by carbamylated and control untreated antibodies (Fig. 4B). Taken together these results confirm that carbamylation of IgG specifically targets the region of Fc, which is responsible for C1q binding and complement activation but has no impact on antibodies ability to recognize antigens and interact with CD16a.

Discussion

IgG is the most abundant circulating immunoglobulin isotype and represents close to 75% of the total serum immunoglobulin content. It plays a key role in the inflammatory environment by recognizing invading pathogens via its Fab fragment, and once in complex with the antigen, it triggers complement activation via its Fc domain. Downstream products of complement cascade activation function as chemoattractants, opsonins, and proinflammatory mediators and cause direct cell lysis through formation of the membrane attack complex [27]. Antigen-bound IgG does also mediate cytotoxic effects through interaction with FcγR present on effector cells, such as macrophages, neutrophils or NK cells [28]. Any modifications of the Fc fragment can have a profound effect on the ability of immunoglobulin to execute its effector functions. It was shown previously that some post-translational modifications of IgG, such as glycosylation [29, 30] and oxidation [31] significantly influence the capacity of IgG to efficiently bind C1q. Here, we analyzed the pattern of IgG₁ carbamylation occurring in vitro and in vivo and studied the effect of this modification on the ability of IgG₁ to trigger the classical complement pathway and induce CDC and ADCC.

Appreciation of the role of carbamylation in autoimmune and inflammatory diseases was significantly increased by the discovery of autoantibodies recognizing carbamylated peptides in patients with both RA [9] and juvenile idiopathic arthritis [10]. Although carbamylation has initially been studied in the context of uremia,

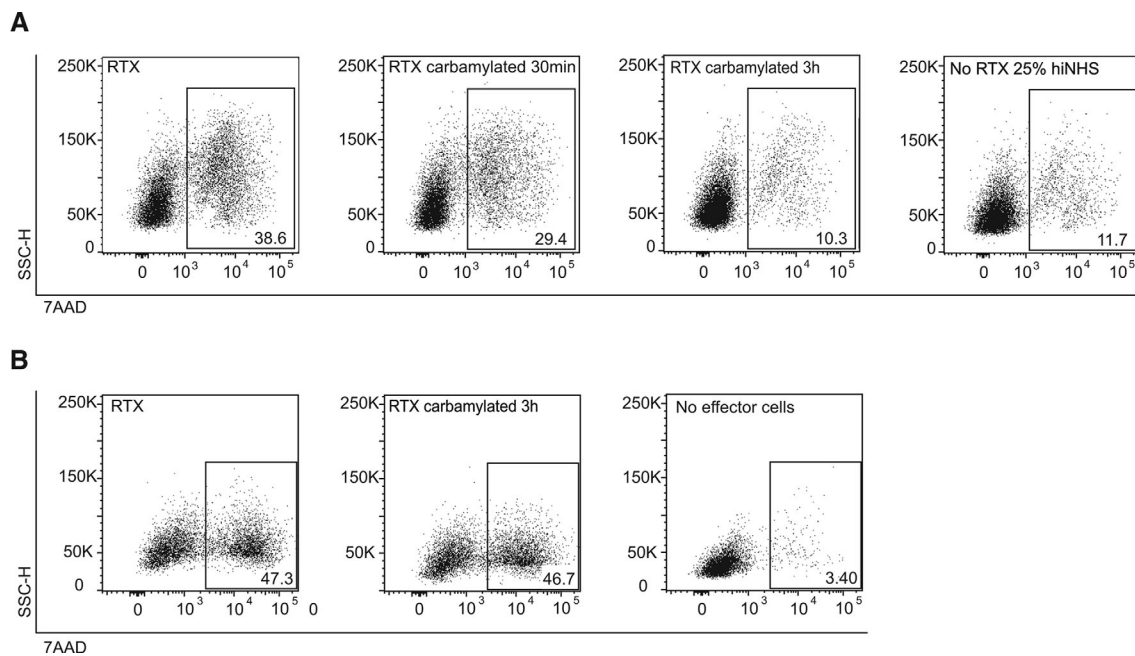


Figure 4. Carbamylation of RTX affects CDC but not ADCC. (A) Flow cytometry was performed to assess 7AAD expression from two independent CDC assays displaying Raji cells incubated with 25% NHS or hiNHS and 10 $\mu\text{g}/\text{mL}$ native or modified RTX for the duration of 12 h. Plots are representative of two independent assays. (B) Flow cytometry was used to assess 7AAD expression from ADCC assay with NK:Raji cell ratio of 11:1 and 10 $\mu\text{g}/\text{mL}$ native or modified RTX following 15 h incubation. Results are representative of three separate assays with NK:Raji ratio ranging from 6.5:1 to 11:1.

as urea exists in equilibrium with cyanate anions (OCN^-) [6, 32], it was recently shown to be effectively mediated by MPO produced by activated neutrophils [3]. Therefore, protein carbamylation *in vivo* may be mainly driven by inflammation. In chronic inflammatory diseases such as periodontitis, in which MPO levels are significantly elevated, the amount of carbamylation might be significantly higher, especially in smokers in whom high SCN^- levels are detected as well as in uremic patients with high level of urea in the blood [33, 34].

In the current study, we found that selected lysine residues in IgG₁ were rapidly modified after exposure to KCNO. Interestingly, these modifications were specifically limited to Lys residues within the hinge region and N-terminal fragment of the CH2 domain. Formation of neutral homocitrulline from positively charged lysine in these two regions of the heavy chain had a profound impact on the capacity of IgG₁ to bind C1q [21] thus demonstrating the importance of K322 (shown in blue in Fig. 5) in binding of C1q to IgG. We observed a dramatic decrease in C1q binding and subsequently CDC already after short time (30 min to 3 h) exposure to KCNO, at which time only two Lys residues (K326 and K334; shown in red in Fig. 5) other than the previously mentioned K322 were significantly carbamylated. Therefore, we propose that in addition to K322, K326 and K334 are crucial for effective binding of C1q, and that modification of these residues abrogates the ability of IgG₁ to effectively activate the complement cascade effectively. Previous crystallization studies also suggested the importance of the hinge region in C1q binding [16] and antigen recognition, but this was not verified by experimental data. In our study, however, exposure to cyanate for more than 3 h rendered IgG₁ incapable

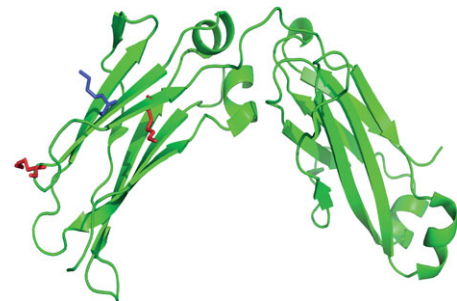


Figure 5. Three-dimensional structural model of the Fc fragment of IgG₁. Lysine in position 322 is represented in blue. Carbamylated lysine residues in position 326 and 334 are shown in red.

of complement activation, with the level of C1q deposition and CDC activation equivalent or nearly equivalent, respectively, to that of the negative control. These findings closely correlated with the carbamylation of K222 and K246, emphasizing the importance of these hinge residues in C1q binding. Interestingly, a previous study showed a significant and unexplained decrease in C1q binding to IgG after immunoglobulins were exposed to neutrophils, a finding that failed to be mimicked *in vitro* by oxidative agents (HOCl and H_2O_2) [35]. We propose that this loss of function by IgG₁ was due to carbamylation of immunoglobulin molecules via the MPO/ H_2O_2 / SCN^- system generated by activated neutrophils.

Downstream complement activation products such as C3b and C4b play a major role in the clearance of immune complexes from the circulation [36]. Binding of C3b and C4b to complement

receptor 1 (CR1), which is present on multiple cell types (erythrocytes, phagocytes, neutrophils, mesangial cells, and lymphocytes) [37] facilitates the removal of the circulating immune complexes [38]. Therefore, an increased level of immune complexes may result from improper complement activation and decreased C3b/C4b opsonisation. Decreased immune complex clearance is implicated in the pathogenesis of various autoimmune diseases, such as systemic lupus erythematosus [39]. Importantly, using the synovial fluid of RA patients we found evidence that carbamylation of IgG occurs in vivo that might affect their ability to activate the complement pathway and remove immune complexes from the synovial fluid. Interestingly, clearance of immune complexes in dialyzed uremic patients is also significantly decreased [40], which correlates closely with the observation of increased levels of carbamylation in that patient group. In keeping with this, we fully confirmed the presence of carbamylated IgG in the sera of dialyzed patients (data not shown) but did not analyze them for complement activation. Nevertheless, there is a little doubt that impairment of antibacterial activity in people suffering from renal failure contributes to increased susceptibility to infections. This contention is supported by multiple reports of high infection burden in patients with end-stage renal disease, in which around 80% are unrelated to the dialysis procedure (for review see [41]). The most frequent bacterial isolates in these cases were *S. aureus*, *P. aeruginosa*, *E. coli*, *Klebsiella* species, all sensitive to the bactericidal activity of complement. Taking into consideration all the data, it is tempting to speculate that carbamylation of IgG with the downstream inhibition of complement could play a role in higher susceptibility to infections in uremic patients.

Biologics are rapidly gaining the ground in the therapy of cancers [42, 43] and autoimmune diseases [44–46]. Their therapeutic effect often relies on exerting CDC or ADCC [23]. NK cells are primary mediators of ADCC activation by binding of the Fc part of antigen-loaded antibodies to surface-expressed FC γ RIIIa [15]. Previously, the binding site for FC γ RIIIa was mapped to amino acids present on the hinge proximal end of the CH2 domain and the CH2–CH3 interface [47]. Site directed mutagenesis substituting K322 [48], K334 and K338 [47] to Ala was shown to affect the FC γ RIIIa binding and hindered ADCC activation. Nevertheless, our finding here using RTX clearly showed that carbamylation of K322 and K334 did not alter NK-cell mediated activation of ADCC. In striking contrast, however, CDC of RTX was totally abolished by carbamylation. This indicates that different regions of Fc fragments of antibodies are involved in complement activation and signalling via interaction with FC γ RIIIa.

It is appreciated that the high level of cyanate in serum of uremic patients can rapidly carbamylate therapeutic antibodies and reduce their effectiveness. Here we showed the presence of carbamylated IgG₁ in inflamed synovial fluid. This finding together with susceptibility of RTX to carbamylation strongly argues that inflammation-driven modification of antibodies may reduce effectiveness of biologics used to treat inflammatory diseases.

Taken together, in this study we presented evidence that lysine residues within the IgG₁ chain undergo carbamylation at spe-

cific positions in vitro and in vivo. We identified the involved residues by mass spectrometry and demonstrated their essential role in effective activation of the classical pathway of complement. Since protein carbamylation is clearly associated with inflammatory reactions it is important to understand the pathophysiological effects of such a modification, not only relating to biological activities of IgG, but also to other proteins. Results of these studies may shed a new light on the pathogenesis of chronic inflammatory and autoimmune diseases and lead to novel diagnostic and therapeutic approaches.

Materials and methods

Carbamylation of IgG₁ and Rituximab

Human myeloma IgG₁ (Calbiochem, Darmstadt, Germany) and Rituximab (Mabthera, Roche) were carbamylated by incubation with 0.1 M KCNO in 0.1 M Tris buffer (pH 7.4) at 37°C for 30 min–24 h. KCNO was removed by ultrafiltration using Amicon Ultra-2 filters (50 k, Millipore, Darmstadt Germany).

Western blot

Carbamylated IgG₁ samples were separated on 4–15% Tris-HCl gels (Bio-Rad) and transferred to PVDF membranes (Bio-Rad). Membranes were blocked with 5% w/v skim milk in TTBS (20 mM Tris (pH 7.5), 150 mM NaCl, and 0.05% v/v Tween-20). Rabbit anti-carbamyl-lysine polyclonal antibody (1/1 000, Cell Biolabs) was used as the primary antibody with a HRP-conjugated goat anti-rabbit HRP conjugated antibody (1/20 000, Jackson Laboratories) as the secondary.

Quantifications of homocitrulline on IgG₁

Carbamylated and native IgG₁ (0.45 mg/mL) were digested for 2 h at 37°C with 4 μ g Proteinase K (Sigma-Aldrich, Oslo, Norway) in a final volume of 30 μ L PBS containing 1% w/v SDS. Thereafter, 105 μ L nitrogen reagent (0.83 M sulfuric acid, 1.13 M orthophosphoric acid, 0.55 mM thiosemicarbazide, and 2.6 mM cadmium sulfate) and 21 μ L 3% 2,3-butanedione monoxime (Sigma-Aldrich) were added to the reaction mixture. The samples were incubated at 97°C for 30 min on a dry heater block, cooled for 5 min and centrifuged 10 000 \times g for 10 min at room temperature. The absorbance of the supernatant was measured at 530 nm. A standard curve ranging from 0 to 800 μ M was created using homocitrulline (N6-carbamoyl-dl-lysine, Sigma-Aldrich).

Complement activation C1q, C4b, and C3b

Carbamylated and native IgG₁ were diluted with PBS and aggregated by incubation at 65°C for 20 min. Duplicate wells of MaxiSorp microtitre plates (Fisher Scientific) were coated overnight at 4°C with aggregated human myeloma IgGs₁ diluted in 75 mM sodium carbonate (pH 9.6) to a final concentration of 10 µg/mL. Additional wells were coated with BSA as a negative control. After washing with 50 mM Tris (pH 8.0), 150 mM NaCl, and 0.1% v/v Tween-20, wells were blocked with 1% v/v BSA diluted in PBS and incubated for 2 h at room temperature. Complement activation was achieved by incubation with 50 µL NHS, pooled from healthy volunteers and diluted in GVB⁺⁺ (5 mM veronal buffer (pH 7.3), 140 mM NaCl, 0.1% v/v gelatin, 1 mM MgCl₂ and 0.15 mM CaCl₂) to a final concentration of 0.5, 1, 2, 4, 6, 8 or 10%. After 45 min (C1q) or 20 min (C3b and C4b), the wells were washed, and bound complement proteins were detected after 1 h incubation with anti-C1q, anti-C4c (diluted 1/4 000), and anti-C3b (diluted 1/2 000) antibodies (Dako) followed by a secondary horseradish peroxidase-conjugated swine anti-rabbit antibody (Dako). After washing, the plates were developed with OPD tablets (Dako) and the absorbance was measured at 490 nm using a plate reader (Varian). To ensure proper binding of the immunoglobulins to the plate, Maxisorp plates were coated overnight with serial dilution (1.25–20 µg/mL) of each aggregated IgG₁ as above. Duplicate wells were coated with aggregated native IgG₁ as a positive control and BSA was used as a negative control. Bound IgG was detected with rabbit anti-human IgG (diluted 1/2 000) (Dako). After 1 h incubation at room temperature, the plate was washed and incubated for another hour with swine anti-rabbit IgGs conjugated to horseradish peroxidase (diluted 1/2 000) (Dako). The plate was developed with OPD tablets and the absorbance was measured at 490 nm.

Cell cytotoxicity by CDC

Raji cells were cultured in complete RPMI medium containing UltraGlutamine (Life technologies), 10% v/v FCS (PAA, Pasching, Austria), 100 units/mL penicillin, and 100 µg/mL streptomycin (Sigma-Aldrich). For CDC assay 4×10^5 cells/mL were incubated in complete RPMI, without FCS, containing 10 µg/mL native or modified RTX and 25% pooled NHS or Heat inactivated normal human serum (hiNHS) at 37°C for 12 h. Following incubation cells were stained with 7-amino-actinomycin D (7AAD) for discrimination between live and dead cells. Cells were collected on a BD LSRFortessa and analyzed using FlowJo ver. X.0.7 (Tree Star Inc.).

Cell cytotoxicity by ADCC

NK cells were purified using the EasySep human NK cell enrichment kit (Stemcell) according to the manufacturer's instructions. Isolated cells were analyzed for purity through staining with PE

conjugated anti-CD56 (N901, Beckman Coulter) and flow cytometric analysis displayed a purity of 89% (data not shown). NK cells were cryopreserved in RPMI containing 25% v/v FCS and 10% v/v DMSO and stored short for term in –70°C until use. Following defrosting cells were allowed to rest in complete RPMI media for 24 h. ADCC assay was performed as follows: 2×10^5 Raji cells/mL were cultured in complete RPMI media together with NK in ratios up to 11:1 (NK cells:Raji cells). Cultures were incubated for 5, 12 or 15 h in 37°C and stained with 7AAD to discriminate between viable and dead cells. Raji cells were discriminated from NK cells by gating of FSC and SSC (Supporting Information Fig. 1), which was confirmed through staining of Raji cells with CFSE prior to coculture.

Preparation of IgG from synovial fluid

Immunoglobulins from synovial fluid were purified using HiTrap Protein A HP columns (GE Healthcare). Briefly, samples of synovial fluid from two patients with rheumatic inflammatory joint disease diluted 1:4 in PBS were loaded onto 1 mL columns. Bound immunoglobulins were thereafter eluted with 0.1 M sodium citrate (pH 3.0). The study was approved by the Committee of Ethics at the University of Bergen (nr. 242.06). All patients gave their informed consent.

Mass spectrometry

Immunoglobulin samples were brought to a final concentration of 0.1 M NH₄HCO₄ and disulfide bridges were reduced with 10 mM DTT for 45 min at 56°C followed by cysteine alkylation with 55 mM iodoacetamide at room temperature for 30 min. Residual iodoacetamide was quenched with 20 mM DTT before trypsin digestion using 0.02 mass equivalents of Sequencing Grade Modified Trypsin (Promega) overnight at 37°C in 50 mM NH₄HCO₃. The digested samples were acidified by the addition of formic acid to 5% v/v and peptides were purified using Porous R2 (C8-C18) 50 µm beads (PerSeptive Biosystems) packed as microcolumns in 20 µL GELoader tips (Eppendorf). Eluted peptides were dried to a volume of a few microliters using a vacuum evaporator and then diluted in 5% formic acid prior to performing mass spectrometry. Tandem mass spectra were recorded with a TripleTOF 5600 (AB Sciex) quadrupole time-of-flight mass spectrometer equipped with a Nanospray[®] III ion source (AB Sciex) and interfaced with an Easy-nLC II HPLC system (Thermo Scientific). Up to 0.7 µg peptide sample per analysis was loaded onto a 13 cm in-house packed fritted emitter fused silica column packed with 3 µm Reprosil-Pur C18-AQ (Dr. Maisch GmbH) reverse phase material in a PicoFrit[™] (New Objective) fritted fused silica emitter (75 µm i.d., 10 µm tip). Peptides were eluted using a 30 or 60 min gradient from 5 to 35% organic phase (90% acetonitrile in 0.1% formic acid in water) at a constant flow rate of 250 nL/min. MS was performed using unattended data-dependent acquisition with 50 scans per 2.8 s duty cycle, a survey scan accumulation time of

250 ms, MS/MS accumulation times of 100 ms and a peptide fragmentation threshold set to 150 arbitrary intensity units. Calibration was performed every fifth sample, ensuring mass accuracies below 10 ppm. The generated raw data were converted into Mascot generic format (mgf) peaklists and searched using the Mascot search engine (v. 2.3.02, Matrix Science) against the Swiss-Prot database (human, 20,248 sequences, downloaded February 2013) with the following restrictions: peptide mass tolerance 30 ppm, fragment mass tolerance 0.1 Da, two allowed missed cleavages (semispecific trypsin) and a minimum MudPIT peptide score of 20. In addition, carbamidomethylation of cysteines, carbamylation of lysines, and oxidation of methionines were allowed as variable modifications. Assigned immunoglobulin lysine carbamylations were manually reviewed to rule out false assignments.

Limited proteolysis of IgG₁ by Gingipain K

Carbamylated and native IgG₁ at a final concentration of 15 μM was incubated with 10 nM Gingipain K (Kgp) in 0.1 M Tris (pH 8), 1 mM EDTA and 2 mM L-cysteine. The samples were incubated for 1 h at 37°C and cleavage of IgG₁ was analyzed by SDS-PAGE using 4–15% gradient SDS-PAGE gels (Bio-Rad). Protein bands were visualized by Coomassie bio-safe staining (Bio-Rad) staining.

Statistical analysis

Statistical differences were determined using one-way ANOVA with Tukey's post-test for multiple comparisons. All statistical analyses were performed using GraphPad Prism, version 6.0b for Mac (GraphPad), and a *p* value of <0.05 was considered statistically significant.

Acknowledgments: This work was funded by grants from the European Commission (FP7-HEALTH-2010–261460 “Gums&Joints,” FP1717-HEALTH-306029 “TRIGGER”) and Marie Curie ITN (RAPID No. 290246). JP is supported by grants from the National Institutes of Health (Grant DE 09761 and DE 022597 USA), National Science Center (2011/01/B/NZ6/00268, Kraków, Poland) and Polish Ministry of Science and Higher Education: projects 137/7.PR-EU/2011/2 and K/PMW/000095. AB is supported by Swedish Research Council (K2012-66X-14928-09-5), foundations of Österlund, Kock, King Gustav V's 80th Anniversary, Swedish Rheumatism Association and grant for clinical research (ALF). The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a beneficiary of structural funds from the European Union (POIG.02.01.00-12-064/08).

Conflict of Interest: The authors declare no financial or commercial conflict of interest

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Abbreviations: ADCC: antibody-dependent cellular cytotoxicity · CDC: complement-dependent cytotoxicity · CH2: constant heavy chain 2 · KCNO: potassium cyanate · Kgp: Gingipain K · MPO: myeloperoxidase · NHS: normal human serum · RA: rheumatoid arthritis · RTX: rituximab · 7AAD: 7-amino-actinomycin D

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Received: 26/5/2014
 Revised: 22/7/2014
 Accepted: 12/8/2014
 Accepted article online: 13/8/2014