




Carbamylation/citrullination of IgG Fc in bronchiectasis, established RA with bronchiectasis and RA smokers: a potential risk factor for disease

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ABSTRACT Bronchiectasis (BR) and smoking are risk factors for rheumatoid arthritis (RA) development. The mechanisms by which smoking and BR trigger RA are unknown, but are associated with concurrent rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibody (anti-CCP) positivity. Anti-carbamylated protein antibodies (anti-CarP) have also been observed in BR patients and can be induced by smoking. Given that RF only has one antigen, immunoglobulin G (IgG) we have suggested that post-translational modifications to the Fc region of the heavy chain of IgG (IgGH) are a potential explanation for the clustering of the RA-associated autoantibodies in RA.

Protein analysis was undertaken on 22 individuals. Four of the individuals had a diagnosis of BR at the time of protein analysis and subsequently developed RA up to 18 months following blood sampling. Four smoking RA patients and 4 patients with both BR and RA and 10 healthy controls were also studied.

We identified modified arginines (Arg) frequently in the variable region and CH3 domains of IgG in patients and control subjects alike, but only observed carbamylated Lys and/or citrullinated Arg modifications in the RF binding site of the IgG CH2 domain of 5/12 (41.7%) patients investigated (1 BR, 2 RA and 2 BRRA), but in no control subjects (0/10, 0%) $p=0.02$.

This is the first report of citrullination and carbamylation at the RF binding site of IgG in RA. These results point towards the concept of a universal antigen in RA, an antigen that is post-translationally modified at the Fc region of IgGH.



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Introduction

Bronchiectasis (BR) is a very strong risk factor for rheumatoid arthritis (RA). WALKER [1], as long ago as the 1960s, demonstrated a striking association between BR and RA. In this study, BR was observed to be 10-fold more prevalent in RA than in appropriate control subjects. The increased incidence of BR in RA in this study could not be explained by the greater susceptibility to infection by patients with RA, because the symptoms of bronchiectasis preceded those of arthritis in the majority of the cases. We recently studied 122 BR patients prospectively, and four individuals developed RA over an 18-month period [2]. In this study, the presence of both rheumatoid factor (RF) and anti-citrullinated peptide antibody (ACPA) in the BR patients' sera was significantly greater than in healthy controls and predicted the development of RA. Interestingly, a significant association between the two RA autoantibodies was observed. We noted that 4/31 (13%) RF-positive BR patients were strongly ACPA-positive, versus 0/91 (0%) RF-negative BR patients (p -value=0.0005) [2]. These results demonstrate a marked clustering of RF and ACPA in BR patients who developed RA. Recently, anti-carbamylated protein antibodies (anti-CarP) were described as a third autoantibody system in RA [3] associated with disease activity [4]. Again, these RA-specific autoantibodies are more frequent in BR (3/80, 3.8%) than in healthy controls (0/36, 0%) [5].

A pathological characteristic shared between BR and RA is the presence of tertiary lymphoid structures. Bronchial associated lymphoid tissue (BALT) was first described in BR [6], and similar lymphoid tissue is present in the synovium of RA patients [7]. Smoking, another risk factor for RA development [8], associates with BALT development, and the presence of BALT is associated with both RF and ACPA in established RA with pulmonary disease [9].

Mucosal lung biopsies of early RA (<1 year) observed lymphocyte infiltration more frequently in ACPA-positive patients (9/18, 50%) than in ACPA-negative patients (1/6, 17%) [10]. This suggests that BALT development potentially initiates RA and is not necessarily a secondary consequence of disease activity in established RA.

Given this evidence, we have recently suggested that the lung is a potential site for RA development [11]. Furthermore, we have suggested that potential antigenicity of the heavy chain of immunoglobulin G (IgGH) is triggered by B-cell activation in the lung: this process involves post-translational modifications (PTMs) to IgGH. These PTMs of IgGH are the conversion of the amino acid arginine to citrulline (citrullination) and the conversion of the amino acid lysine to homocitrulline (carbamylation), orchestrating a local (lung) and distant (joint) immune response involving the production of RF, ACPA and anti-CarP, triggering RA [12]. In support of this hypothesis, carbamylated IgG has been observed in RA synovial fluid [13] and also citrullinated IgG in the RA joint [14, 15]. Recently it has been demonstrated that cigarette smoke can induce carbamylation of vimentin [16]. Similarly, the carbamylation of vimentin has been noted in the lungs of individuals with chronic obstructive airways disease, irrespective of their smoking history, suggesting that airways inflammation can give rise to carbamylation rather than smoking per se [17].

Accordingly, we have investigated whether the Fc region of IgGH is citrullinated and/or carbamylated in RA in a proof of concept study. We studied a small cohort of RA smokers and patients with both RA and BR, as we considered these patients to be at particularly high risk for the presence of citrullinated and/or carbamylated IgG given the above. Secondly, we investigated a small cohort of BR patients who had subsequently developed RA to determine if any of these patients' sera contained citrullinated and/or carbamylated IgG. In this pilot study we have observed, for the first time, carbamylation and/or citrullination at the Fc region of IgGH. This was observed in RA patients with BR and RA patients who smoked. Intriguingly, the only individual to have both citrullination and carbamylation present at the Fc region of their IgGH was a BR individual who subsequently developed RA within 18 months of blood sampling. Although observed in a small cohort of patients, the presence of citrullination or carbamylation at the Fc region of IgGH was significantly associated with RA and/or BR rather than in controls. We feel that these findings are of importance and could point towards the origin of RF and ACPA in some individuals with RA.

Methods

Sera from patients and controls were selected from our BRACRA (Bronchiectasis, Asthma, Control, Rheumatoid Arthritis) study. The overall study design, including RF and ACPA assays and approval, has been reported previously [2]. Cut-offs for RF and cyclic citrullinated peptide (CCP) positivity were $130 \text{ U}\cdot\text{mL}^{-1}$ and $600 \text{ U}\cdot\text{mL}^{-1}$, respectively. Importantly, in this study all the BR patients were examined by a trained rheumatologist prior to blood sampling to exclude any signs or symptoms that could be attributed to RA.

All the RA patients fulfilled the American College of Rheumatology (ACR) 2010 classification criteria for RA [18]. Samples of sera from four BR individuals were taken prior to their subsequent development of

RA (ACPA+ve and RF+ve prior to developing RA), four RA smokers without overt lung disease, four BRRRA never smokers and 10 control subjects (five never smokers and five current/ever smokers) and were investigated for evidence of citrullinated and carbamylated IgGH (figure 1a).

One microlitre of serum from selected samples was separated on SDS-PAGE gels. Individual 5 mm citrullinated protein bands identified by immunoblotting with rabbit anti-human citrulline (Abcam ab100932) antibody, in the region of 37–50 kDa, were excised from Coomassie-stained gels. The proteins were digested, fractionated and analysed using an LTQ-Orbitrap Velos mass spectrometer. Tandem mass spectra were acquired in the data-dependent acquisition mode. Search criteria included carbamidomethylation of cysteine (+57 Da) as a fixed modification, citrullination (+0.98 Da) at Arg and

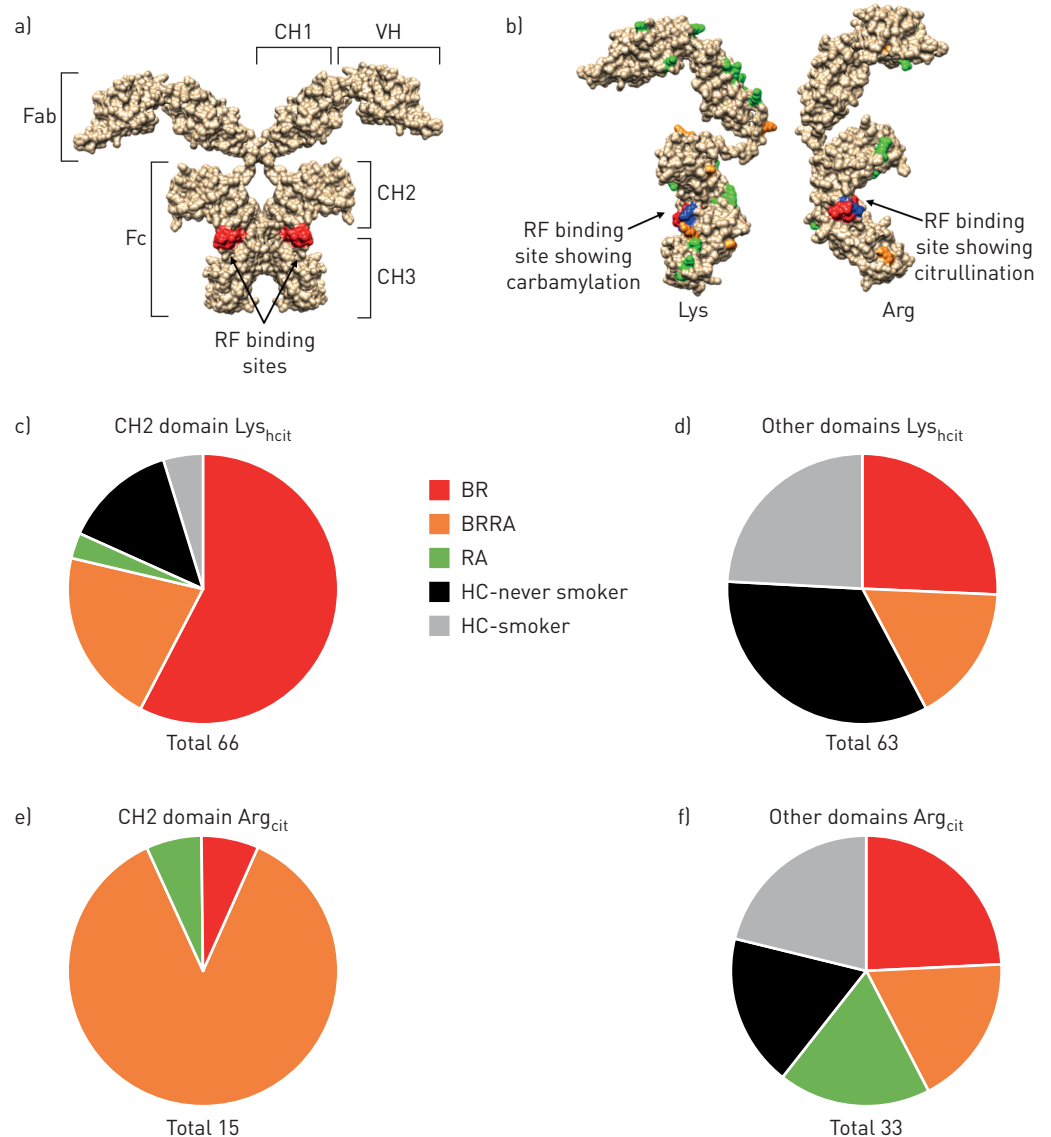


FIGURE 1 Detection of lysine carbamylation and arginine citrullination in IgGH from bronchiectasis and rheumatoid arthritis patients. **a)** Schematic model of IgG dimer showing RF binding sites (red). **b)** IgGH fragment showing post-translational modification of Lys (blue: left model) or Arg (blue: right model), detected at the RF binding site (red: both models) in individual patients. Green: other unmodified Lys (left model) or Arg (right model). Orange: other modified Lys (left model) or Arg (right model). **c)** Proportion of Lys_{hcrit} peptides (n=66) found in the CH2 domains of individual cohorts of patients and control subjects. **d)** Proportion of Lys_{hcrit} peptides (n=63) found in the IgG VR, CH1 and CH3 domains of individual cohorts of patients and control subjects. **e)** Proportion of Arg_{cit} peptides (n=15) found in the CH2 domains of individual cohorts of patients and control subjects. **f)** Proportion of Arg_{cit} peptides (n=33) found in the IgG VR, CH1 and CH3 domains of individual cohorts of patients and control subjects. RF: rheumatoid factor; HC: healthy control; BR: bronchiectasis; RA: rheumatoid arthritis.

carbamylation (+43 Da) at Lys, and were included as variable modifications in two separate searches. Only peptides where citrullination at Arg and carbamylation at Lys were ranked “1” in their respective searches (indicating that those residues were the most likely sites of modification) were considered. This identified IgGH as one of the most abundant proteins identified as being citrullinated and carbamylated (supplementary tables S1 and S2). We confirmed the presence of citrullination and carbamylation (rabbit anti-carbamyl lysine; Abcam ab175132) in the heavy chains of affinity-purified IgG *via* fast protein liquid chromatography from our test subjects, followed by immunoblotting of purified IgG from individual subjects using the antibodies cited above.

Results

We examined the degree of carbamylated Lys (hCit) and citrullinated Arg (Cit) residues in IgG from patient and control subject sera (table 1), especially the CH2 domain, which contains the RF binding peptides (figure 1a). Using mass spectrometry we found 8/34 Lys carbamylated and 3/12 Arg citrullinated (figure 1b) in the IgGH region. We identified modified arginines frequently in the variable region and CH3 domains of IgG in patients and control subjects alike, but only observed carbamylated Lys and/or citrullinated Arg modifications (figure 1a and b) in the RF binding site of IgG CH2 domain of 5/12 (41.7%) patients investigated (1 BR, 2 RA and 2 BRRA), but in no control subjects (0/10, 0%) (table 1). Although these numbers are small, statistical significance was reached at 0.02. Furthermore, the degree of carbamylation of the CH2 domain varied between cohorts according to BR>BRRA>healthy control (HC)>RA (figure 1c). By contrast, the degree of carbamylation in other IgGH domains was very similar for all cohorts (figure 1d). To a lesser extent, citrullination of the RF binding site in the CH2 domain was observed in a single BRRA patient (BRRA ID#7) (figure 1e). Citrullination in other areas of the IgGH region showed no discernible difference across disease cohorts (figure 1f).

The presence of citrullination and carbamylation IgGH and/or IgGL was tested by immunoblotting the sera with anti-citrulline and anti-carbamyl-lysine antibodies (figure 2a and c). The presence of citrullination was confined only to the 50 kDa IgGH of all subjects (figure 2b). Similarly, the IgGH in all subjects were carbamylated, but we also observed some carbamylation in some patient and control IgGL (figure 2d).

Discussion

This study demonstrates that the Fc region of IgG is citrullinated and carbamylated, and these PTMs can occur prior to the development of RA. We suggest our findings extend the previous findings of carbamylated and citrullinated IgG in the synovial fluid of RA patients [13, 15, 19]. Citrullinated IgG was

TABLE 1 Demographics and serology of individual subjects in study

ID	Demographics				Anti-CCP		RF	
	Cohort	Age years	Sex	Smoker	Actual result	ACR anti-CCP interpretation	Actual result IU·mL ⁻¹	ACR RF interpretation
1	BR [#]	72	F	Never	522	High positive	56.2 ^{¶,+}	High positive
2	BR [#]	67	F	Ex	43	High positive	16	Low positive
3	BR [#]	77	M	Never	340	High positive	22	Low positive
4	BR [#]	71	F	Ex	92	High positive	130	High positive
5	BRRA	75	F	Never	88	High positive	27.5 ⁺	Low positive
6	BRRA	81	F	Never	600	High positive	130	High positive
7	BRRA	60	F	Never	229	High positive	130 [¶]	High positive
8	BRRA	66	F	Never	340	High positive	130	High positive
9	RA	52	F	Current	197	High positive	74.6 ⁺	High positive
10	RA	38	F	Current	6.9	Negative	78.1 [¶]	High positive
11	RA	44	F	Current	340	High positive	130	High positive
12	RA	62	F	Current	1.5	Negative	8.4	Negative
13–17	Control	68 {33.5} [§]	F	Never	1	Negative	<7.0	Negative
18–22	Control	53 {24} [§]	F	Current/ ever	1	Negative	7.8	Negative

ID: identifier; CCP: cyclic citrullinated peptide; RF: rheumatoid factor; ACR: American College of Rheumatology; BR: bronchiectasis; RA: rheumatoid arthritis; F: female; M: male. [#]: bronchiectasis patients who went on to develop RA 12–18 months post-sampling; [¶]: citrullination of arginine at RF binding site; ⁺: carbamylation of lysine at RF binding site; [§]: median (interquartile range).

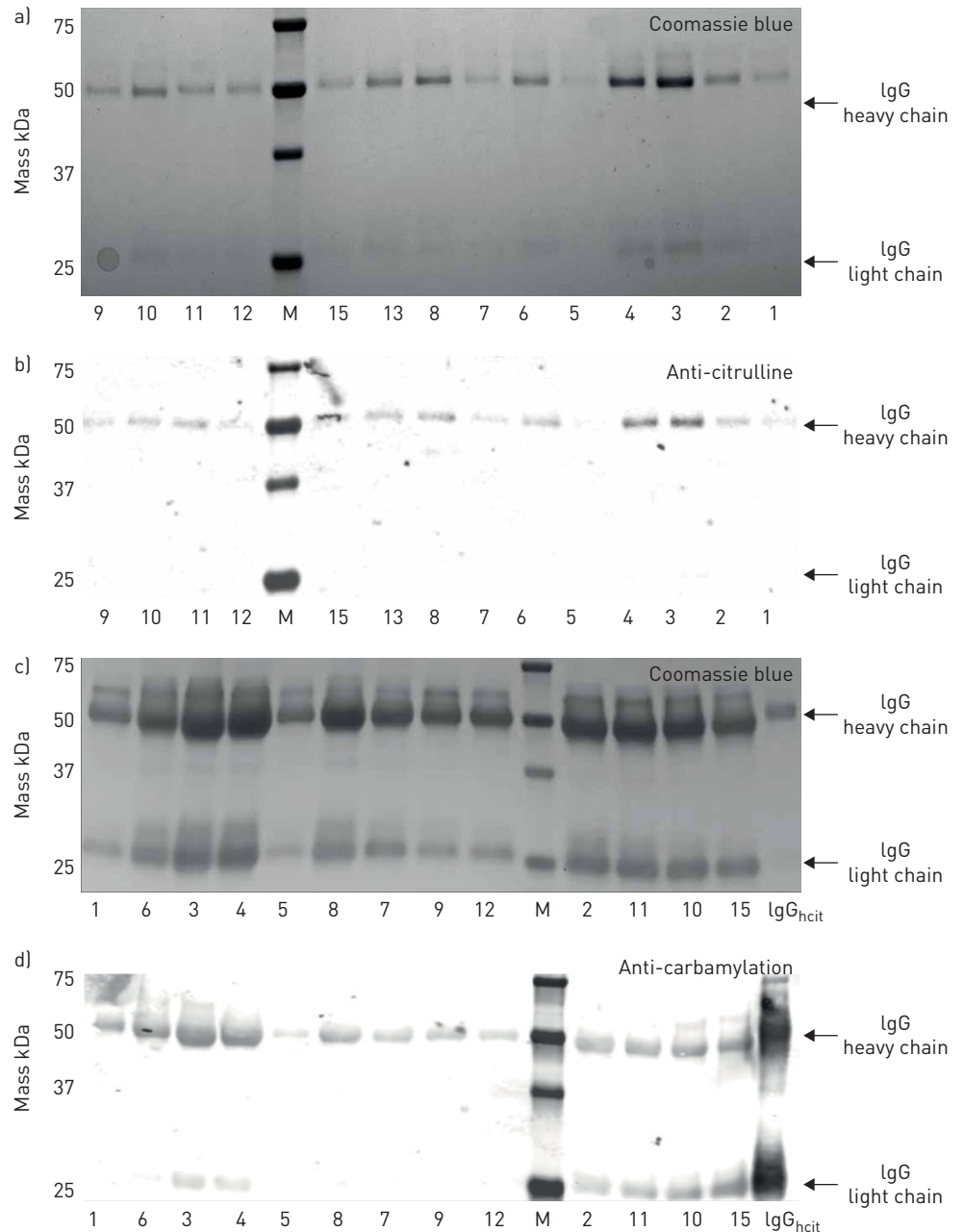


FIGURE 2 SDS-PAGE and immunoblots of purified IgG for citrullination and carbamylation. a) Representative Coomassie-blue-stained isolated total IgG from patients and control sera used to blot for citrulline. b) Immunoblot of IgGs probed with anti-citrulline. c) Representative Coomassie-blue-stained isolated total IgG from patients and control sera used to blot for carbamylation. d) Immunoblot of IgGs probed with anti-carbamylation. Numerals correspond to patient identification codes in table 1.

observed in healthy subjects and RA patients. However, specific citrullination and carbamylation of the RF binding site in the IgG CH2 domain was restricted to patients with seropositive RA (4/8) with/without apparent lung disease and a patient with BR who subsequently developed seropositive RA (1/4). This suggests that PTMs of the IgG RF binding site may arise as a result of B-cell activation in the bronchiectatic lung or as a result of smoking-associated RA BAL. Either way, the lung is a potential initiating site of autoimmunity prior to RA symptoms [11]. One important consideration to note is that one patient (RA ID#11) tested CCP-ve/RF+ve, although mass spectrometry identified a citrulline residue in the CH2 region. One explanation for this could be that the CCP assay is reliant on a cross-reaction with a synthetic peptide (unrelated to IgG), with a previous study highlighting a false-positive rate as high as 31% [20]. Given that RF is an antibody against the Fc region of IgG [21] and ACPA is an antibody against

a citrullinated peptide, an antibody generated against the specific PTM noted at IgGHFc in this study could explain our previous findings of the clustering of ACPA and RF in BR [2] and also explain the interesting finding of bispecific antibodies against cyclic citrullinated peptide and IgG in RA [19]. We feel that the finding of citrullination and carbamylation of the Fc region of IgG may be one of the explanations for the clustering of RF isotypes with anti-CCP2 and anti-carbamylated protein antibodies [22].

This study is limited by the small sample size and only represents a snapshot in time of IgGH in the sera of patients and healthy controls. However, an antibody response is a legacy of exposure to a particular antigen. The development of an antibody against the PTM IgGHFc region may be of value as a specific biomarker of RA development in high-risk groups such as BR and heavy smokers.

PTM IgGH is not exclusive to the BR lung; it may arise in smoking-associated BALT and the joint. Interestingly, PTM IgGH was observed in 2 heavy-smoking RA individuals without BR. The importance of PTM of IgGHFc is not only in the development of a neo-antigen, but in a fundamental change to the polarity of the Fc region and a potential enhanced interaction with the Fc receptor. Arginine citrullination and lysine carbamylation at the Fc region changes the electrical charge from positive to neutral. This may alter binding and/or signalling with the FcγRIIB1 of B-cells and FcγRIIB receptors of follicular dendritic cells, stimulating the development of autoimmune disease *via* tertiary lymphoid tissue development. PTM IgGHFc may enhance the ligand activity of IgGH by permitting binding to specific Fc receptors containing positively charged amino acids at critical sites of the Fc receptor (reviewed in [23]). For example, a single Arg₄₃₅ instead of His, which is found in all other IgG subclasses [24], reduces the half-life of IgG3 to 7 days, compared to 21 days in IgG1, 2 and 4. This arises as a consequence of IgG3 binding to the Fc IgG-salvage receptor being inhibited in the presence of IgG1 due to intracellular competition between IgG1 and IgG3. We suggest that the fundamental change in amino acid or charge of the Fc region of IgG could potentiate an inflammatory response that results in the development of tertiary lymphoid tissue. In further, larger studies, it will need to be determined whether smokers and individual with BR have PTM of IgGHFc and whether this confers a risk for RA development. In a well-characterised cohort of BRRAs patients [25], we demonstrated that in 31/53 patients (58%) BR preceded RA; however, the sequence of disease development occurred the other way round in 22/53 (42%) of RABR patients. Further studies are needed to confirm if PTMs to the Fc region of IgGH are present in the sera of individuals who have tertiary lymphoid tissue and whether this potentiates a risk for RA development in BR and vice versa.

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