Specific Antibodies to IgG4 Hinge can Exacerbate Chronic Antibody-mediated Inflammation and Can Be Found in RA Patients during All Stages of Disease

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ABSTRACT

Background
In rheumatoid arthritis (RA), autoantibodies are developed to neo–epitopes that are formed under inflammatory conditions by posttranslational modifications. Antibodies themselves can also be subjected to modifications such as cleavage by inflammatory associated proteases. The hinge of F(ab’)2 fragments can serve as neo–epitope and so called anti–hinge antibodies (AHA) can be found in RA patients. AHA might modulate the function of antibodies by forming complexes with them.

The purpose of this study was to investigate the presence of AHA in different stages of RA and to study their function.

Methods
AHA were detected with radioimmunoassay (RIA) in different cohorts: healthy controls, blood donors who later developed RA, arthralgia patients, early RA patients and established RA patients. Specificity of the AHA was analyzed with inhibition assays and the complement activating ability was studied with a C4b deposition assay.

Results
Antibodies to F(ab’)2 of IgG1, IgG2, and IgG4 were detected in established RA patients. Antibodies to IgG4 hinge (AH4A) were the most specific. These were detected in 0.9% and 1.3% of two healthy control populations, 3.8% of pre–RA blood donors, 13% of arthralgia patients, 19% of early RA patients and 16% of established RA patients. AHA were specific for the hinge, no anti–idiotype or anti–allotype antibodies were found. AH4A were able to restore C4b deposition of IgG4 F(ab’)2 fragments, which were unable to deposit C4b on their own. In arthralgia and early RA patients AH4A were associated with rheumatoid factor (RF) and anti–citrullinated protein antibodies (ACPA).

Conclusion
AH4A are present in RA patients and have a low sensitivity but high specificity. Furthermore, they can be detected before RA diagnosis and are associated with the presence of IgM–RF and APCA. They may play a role in the vicious circle of epitope spreading that maintains the perpetual inflammation in RA.
INTRODUCTION

Some chronic inflammatory diseases such as rheumatoid arthritis (RA) are characterized by the formation of autoantibodies. In RA rheumatoid factors (RF) and anti citrullinated protein antibody (ACPA) are thought to play a pathogenic role. RF recognize epitopes located on the Fc part of IgG and are often IgM but can also be of the IgG class.\textsuperscript{1-3} Due to their low affinity they preferentially bind IgG containing immune complexes.\textsuperscript{4,5}

ACPA recognize citrullinated proteins. Citrullination is a posttranscriptional modification that is mediated by peptidyl arginine deiminase (PAD) and that is more abundant in inflammatory conditions. Citrullination is not the only posttranscriptional modification that results in the formation of autoantibodies. Anti–carbamylated protein antibodies are another family of autoantibodies found in RA patients.\textsuperscript{6} Carbamylation is a process in which lysines are converted into homocitrullines under the influence of cyanate. Cyanate can be formed from thiocyanate under influence of myeloperoxidase and thus carbamylation will be also more abundant in inflammatory conditions.

As such, inflammation in itself seems to result in the formation of autoantibodies. These antibodies may form complement fixating immune complexes and drive inflammation further, causing more tissue destruction. In the tissue debris more proteins may be modified causing formation of new autoantibodies. This cycle can repeat itself over and over, thereby inducing chronic inflammation. The trigger of this inflammation probably takes place years before inflammation becomes clinically apparent, because already in a pre–clinical disease phase, low grade inflammation is present and antibodies to modified proteins can be found.\textsuperscript{7,8}

Proteins can also be modified by cleavage. Proteases such as elastase or cathepsin–G are associated with inflammation.\textsuperscript{9} Not only matrix proteins, but also antibodies themselves can be subjected to their actions. These proteases are known to be able to cleave IgG into Fab or F(ab’)2 fragments.\textsuperscript{10} The hinge region of these antibody fragments may serve as a neo–epitope and elicit an antibody response, thereby contributing to inflammation. Antibodies to IgG digested by pepsin, but also by a wide range of different endogenous proteases, have been reported in many studies.\textsuperscript{10-17} In recent years it was firmly established that these antibodies recognize epitopes in the hinge region.\textsuperscript{9,18-20} Therefore, these antibodies are nowadays referred to as ‘anti–hinge antibodies’ (AHA).
AHA have also been described in RA patients.\textsuperscript{12,21} However, reported incidences of AHA vary widely, probably reflecting sensitivity as well as selectivity of the assays that were used. Previously, we developed an antigen-binding test for F(ab’)2 that only detects high-affinity AHA.\textsuperscript{22} We noticed that high-affinity AHA could be detected in some healthy individuals as well as a subset of RA patients. Strikingly, further examination of these responses indicated that these antibody responses are essentially specific to F(ab’)2 fragments of a particular IgG subclass.

In this respect, it is noteworthy that the second most prominent IgG subclass of ACPA is IgG4,\textsuperscript{23,24} a subclass associated with chronic antigenic immune stimulation, and dampening of effector mechanisms of the immune system.\textsuperscript{25-27} IgG4 ACPA will not activate complement and not trigger Fc gamma receptors.\textsuperscript{28,29} F(ab’)2 of IgG4 ACPA in complex with anti-IgG4 hinge antibodies (AH4A) on the other hand may be potent triggers of inflammatory processes. Therefore, we wondered if antibodies that bind to the hinge of IgG4 might be formed in conditions of antibody-mediated chronic inflammation such as RA.

Here we investigated AHA responses to IgG1, 2, and 4 in healthy individuals as well as individuals with different phases of RA. We demonstrate that in a subset of RA patients specific AH4A can be detected that may contribute to inflammation and are associated with formation of ACPA and RF.

**MATERIALS AND METHODS**

**Materials**

Therapeutic antibody formulations used are: IgG1: infliximab (Remicade; Schering-Plough), adalimumab (Humira, Abbott), IgG2: panitumumab (Vectibix, Amgen), IgG4: natalizumab (Tysabri, Biogen Idec and Elan Pharmaceuticals, Inc). Other IgG1 and IgG4 antibodies were prepared recombinantly using the FreeStyleTM 293 expression system from Invitrogen as described before.\textsuperscript{30} Intravenous immunoglobulin (IVIG) was obtained from Sanquin, Amsterdam, The Netherlands.

**Study population**

For the present study, different patient cohorts available at Reade and Sanquin were used. The first cohort consisted of 79 RA patients who donated blood prior to RA diagnosis and who were recruited as described previously.\textsuperscript{8} Of each patient the last available serum sample prior to onset of symptoms was selected. Median (IQR) time
to RA diagnosis of these samples was 1.0 (0.6–3.9) years. For each serum sample, a serum sample from a healthy blood donor was selected that was matched for sex, age, date of collection and storage conditions, as a control. The second cohort consisted of 237 arthralgia patients with a positive test for aCCP and/or IgM–RF who were recruited and followed prospectively for the development of arthritis as described before. Median (IQR) follow-up was 47 (30–60) months. The third cohort, the early arthritis cohort (EAC), consisted of DMARD naïve patients with peripheral arthritis in two or more joints and symptom duration of less than three years. A random selection of 138 patients fulfilling the 1987 ACR RA criteria were used for the present analysis. In the fourth cohort, 111 patients with RA according to the 1987 ACR RA criteria with a disease activity score in 28 joints (DAS28) of >3.2 and failure to respond to at least two disease-modifying antirheumatic drugs (including methotrexate (MTX)) at maximal or tolerable dosage who were prescribed etanercept were included. In all cases, sera were obtained prior to treatment with etanercept or any other anti–TNF biological. A cohort of 111 healthy volunteers that were frequently boosted with tetanus toxoid was used as a control group.

**Pepsin digestion**

F(ab′)2 fragments of monoclonal antibodies were prepared as follows. Ten milligrams of IgG were digested with pepsin (1:100 w/w) at pH 3.5 by overnight incubation at 37 °C. The reaction was stopped by adding 1M Tris until pH was 7.5. After dialysis against PBS (10 mM sodium phosphate pH 7.4; 140 mM sodium chloride) the resulting F(ab′)2 fragments were purified using a protein A sepharose column (GE Healthcare, Uppsala, Sweden) to remove traces of undigested material.

**Detection of antibodies using radioimmunoassay**

Antibodies to F(ab′)2 fragments were measured with radioimmunoassay (RIA) essentially as described previously. One microliter of serum diluted in PBS/0.3% bovine serum albumin (BSA) and 0.02% Tween (PA buffer) was incubated with 1 mg protein A Sepharose (GE healthcare, Chalfont St. Giles, UK) or CaptureSelect anti–human Fc agarose (BAC, Naarden, The Netherlands) in 800 μl of total volume. After overnight incubation, samples were washed and radioactive labeled F(ab′)2 fragments of the respective monoclonal antibody was added, with or without inhibitor as indicated in the text. After overnight incubation, unbound radiolabel was washed out and Sepharose–bound radioactivity was measured. A lower limit of
detection was based on mean + 3 SD in presence of 10 μg/test IVIG F(ab’)2, measured in 100 healthy donors.

**C4b deposition assay**

Microtiterplates (Nunc MaxiSorp) were coated overnight at 4°C with 20 μg/ml IgG or F(ab’)2 PBS. All incubations were performed in a final volume of 100 μl; a serum containing no anti–hinge antibodies and no rheumatoid factor was added to each well (1:100) and served as source of complement. Sera were heat–inactivated by incubation at 56 °C for 30 minutes. Heat inactivated serum samples were diluted 1:10, followed by twofold dilutions in Veronal buffered saline, pH 7.4 supplemented with 10 mM CaCl₂, 2 mM MgCl₂ and 0.02% Tween–20 and incubated for 1 hour at RT. Subsequently, plates were washed five times with PBS/0.02% tween–20. To detect C4 deposition, plates were washed for 1 hour at RT with biotinylated anti–C4–10 (0.25 μg/ml), diluted in high performance ELISA buffer (HPE; Business Unit reagents, Sanquin, Amsterdam, the Netherlands). After five washes with PBS/0.02% tween–20, plates were incubated with streptavidin–peroxidase (Amersham/Pharmacia, Uppsala, Sweden) (1:1000 in HPE) for 30 minutes at RT. After five washes with PBS/0.02% tween–20, the ELISA was developed with 100 μg/ml tetramethylbenzidine in 0.11 M sodium acetate (pH 5.5) containing 0.003% v/v H₂O₂. Substrate conversion was stopped by addition of 100 μl H₂SO₄. Absorbance was measured at 450 nm with a Titertek multiscan.

**ACPA and RF assays**

ACCP and IgM–RF levels were determined at baseline by second–generation aCCP ELISA (Axis Shield, Dundee, United Kingdom) and in–house ELISA, respectively, as described previously. The cut–off level for aCCP positivity was set at 5 arbitrary units/ml (AU/ml), according to the manufacturer’s instructions. The cut–off level for IgM–RF positivity was set at 30 IU/ml determined on the basis of the analysis of receiver operating characteristic (ROC) curves.

**Statistical analysis**

Data evaluation and statistical analysis were performed with SPSS version 17.0 software (SPSS Inc., Chicago, USA). Categorical data were analyzed by Chi–square test.
RESULTS

Antibodies to the hinge of IgG4 are detected in a subset of patients with established RA

We investigated the incidence of AHA using an F(ab’2) antigen binding test that was previously developed. Antibodies to F(ab’2) of IgG1, IgG2, and IgG4 were measured in a panel of 111 healthy donors as well as a panel of 111 RA patients using both protein A Sepharose and CaptureSelect anti-human Fc agarose (Figure 1). The latter method detects all IgG subclasses whereas the former does not detect IgG3. As expected, with both methods, more sera were found positive in the RA group. Furthermore, the incidence in both groups was larger if IgG3 antibodies were also detected, in line with a previous study. Few sera tested positive for IgG2 F(ab’2) antibodies, indicating that antibodies to IgG1 F(ab’2) do not cross-react with IgG2 F(ab’2). Analogously, only 1 healthy donor tested positive for antibodies to IgG4 F(ab’2). Interestingly, using IgG4 F(ab’2), 18 out of 111 RA patients tested positive vs 1 out of 111 healthy donors, if tested with CaptureSelect anti-human Fc agarose. In other words, a subset of RA patients, but not healthy individuals, produces anti-IgG4 hinge antibodies (AH4A), of which a large proportion consists of IgG3. (Baseline characteristics of RA patients as well as of the other cohorts are tabulated in Table 1).

Table 1 Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>preRA Blood donors</th>
<th>Arthralgia</th>
<th>Early RA</th>
<th>Established RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients</td>
<td>n = 79</td>
<td>n = 237</td>
<td>n = 138</td>
<td>N = 111</td>
</tr>
<tr>
<td>Age in years, mean ± SD</td>
<td>53 ± 11*</td>
<td>47 ± 11</td>
<td>54 ± 14</td>
<td>53 ± 12</td>
</tr>
<tr>
<td>Female sex</td>
<td>49 (62%)</td>
<td>173 (73%)</td>
<td>93 (67%)</td>
<td>88 (79%)</td>
</tr>
<tr>
<td>Disease duration in months, median (IQR)</td>
<td>12 (9–36)</td>
<td>3 (2–6)</td>
<td>65 (29–166)</td>
<td></td>
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</tbody>
</table>

* age at RA diagnosis  SD = standard deviation; IQR = interquartile range

Table 2 Prevalence of anti–hinge antibodies

<table>
<thead>
<tr>
<th></th>
<th>Tetanus</th>
<th>Healthy Blood donors</th>
<th>Pre Blood donors</th>
<th>Arthralgia</th>
<th>Early RA</th>
<th>Established RA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>111</td>
<td>79</td>
<td>79</td>
<td>237</td>
<td>138</td>
<td>111</td>
</tr>
<tr>
<td>Positive patients (%)</td>
<td>1 (0.9)</td>
<td>1 (1.3)</td>
<td>3 (3.8)</td>
<td>30 (13)</td>
<td>26 (19)</td>
<td>18 (16)</td>
</tr>
</tbody>
</table>
Figure 1. Anti–hinge antibodies in patients with established RA and in healthy controls. Sera were incubated with A) protein A Sepharose, or B) Capture Select anti–human Fc agarose, and binding of radiolabeled F(ab’)2 fragments of IgG1, IgG2 or IgG4 was measured. Protein A binds IgG1,2 and 4, whereas Capture Select anti–human Fc binds all human subclasses. The larger number of positive sera in B therefore indicate that IgG3 antibodies are the dominant subclass of anti–hinge antibodies. Detection of anti–IgG4 F(ab’)2 was essentially restricted to RA patients. Cut–off is indicated with dotted line.
The anti–hinge antibody response is largely restricted to a specific subclass

Specificity of the AHA was further investigated by inhibition experiments. To demonstrate the specificity of these antibodies to exposed hinge, sera containing AHA were incubated with radiolabeled F(ab’)2 in the presence of intact monoclonal IgG from which the F(ab’)2 was derived (Figure 2A). No inhibition was observed with any of the sera, confirming that only AHA are detected, and no anti–idiotype or anti–allotype antibodies. Also, reactivities of RA sera to F(ab’)2 fragments of two different IgG1 or IgG4 antibodies correlated well (Figure 3A, B), further demonstrating that the idiotype of the F(ab’)2 fragments did not play a role in the binding. Subclass specificity of the AHA was also investigated. Binding of anti–IgG1 antibodies to radiolabeled IgG1 F(ab’)2 could be inhibited by unlabeled IgG1...
F(ab’)2, but not or only slightly by a 10,000 fold excess of IgG4 F(ab’)2, and vice versa (Figure 2B). Thus, AHA to IgG1 or IgG4 are subclass specific. To investigate a possible relationship between antibody responses to IgG1 or IgG4 hinge, correlations between both types of antibody response were examined. There was no correlation between reactivities to IgG1 vs IgG4 F(ab’)2 fragments (Figure C, D), suggesting that the AHA to different subclasses may form independently. In fact, in many sera only antibodies to either IgG1 or IgG4 F(ab’)2 fragments could be detected, which shows that the AHA response can be restricted to a specific subclass.

Figure 3. Anti-hinge antibody responses are restricted. Correlation between reactivity of sera to A) two different IgG1 F(ab’)2 fragments, B) two different IgG4 F(ab’) fragments, and C,D) IgG1 and IgG4 F(ab’) fragments. Correlation of reactivities is good for different F(ab’)2 fragments of the same subclass demonstrating that the specificity of the F(ab’)2 does not play a role in the binding. No correlation is observed between reactivities to IgG1 and IgG4 F(ab’)2 fragments, and sera may contain only anti-IgG1 hinge or anti-IgG4 hinge antibodies.
Figure 4. Complement activation by IgG4 antibody fragments in the presence of anti–hinge antibodies. A) IgG1 or IgG4 was coated onto a microtiter plate and incubated with a serum containing no rheumatoid factors or anti–hinge antibodies. Complement activation via the classical route was monitored by measuring C4b deposition. B,C) IgG1 or IgG4 F(ab')2 fragments were coated and heat–inactivated sera containing anti–IgG1 hinge (anti–G1hg⁺), anti–IgG4 hinge (anti–G4hgh⁺), or neither were incubated, together with a negative serum (1:100) that served as source of complement. The sera containing anti–IgG4 hinge antibodies result in C4b deposition if incubated with the IgG4 F(ab')2 coat.
Complement activation by IgG4 antibodies as F(ab’)2 in complex with anti-IgG4 hinge antibodies

IgG4 antibodies cannot activate complement (Figure 4A). We reasoned that conditions that result in the formation of IgG4 F(ab’)2 fragments can lead to AH4A formation and effectively transform the IgG4 antibodies into complexes that do activate complement. To test this hypothesis, IgG4 F(ab’)2 fragments were coated onto a microtiter plate and incubated with sera containing AH4A. Complement was added in the form of a serum containing no RF and AHA, and C4b deposition was measured to monitor complement activation. IgG4 F(ab’)2 fragments alone did not result in complement activation, but in the presence of AH4A C4b deposition was observed (Figure 4C). Anti-IgG1 hinge antibodies were ineffective in this system, but were able to activate complement in case IgG1 F(ab’)2 fragments were used as coat (Figure 4B).

Anti-IgG4 hinge antibodies can be detected before diagnosis of RA

To study the presence of AH4A in RA and the development of these antibodies over time, we determined the presence of these antibodies in several cohorts covering the range from increased risk for RA to established RA (pre-clinical RA cohorts such as healthy blood donors that developed RA and ACPA and/or IgM rheumatoid factor [IgM-RF] [seropositive] arthralgia patients, and early and established RA cohorts) (Table 2). Furthermore, their possible association with other autoantibodies was investigated.

Of both healthy control groups, one patient (0.9% and 1.3% respectively) was positive for AH4A. Of the pre-RA blood donors, 3 donors (3.8%) were positive for AH4A. These three donors were also positive for both aCCP and RF. In the arthralgia cohort 30 patients (13%) tested positive for AH4A. Eighty-eight patients (37%) developed arthritis after a median (IQR) follow-up of 15 (6-27) months. Seventy-nine of these patients (90%) could be classified as having RA according to the 2010 ACR/EULAR criteria. There was no significant association of the presence of AH4A with the development of arthritis, although more AH4A could be detected in the group that developed arthritis than in the group that did not (15% versus 11%, p = 0.45). However, AH4A were significantly associated with the presence of ACPA and ACPA plus RF. Of the 30 AHA positive patients, 2 (6.7%) were ACPA negative and RF positive, 11 (37%) were ACPA positive and RF negative and 17 (57%) were ACPA and RF positive (p< 0.001).
In the EAC cohort, 26 patients (19%) tested positive for AH4A. The association between AH4A and ACPA and RF was also found in the EAC patients. Seventeen patients (65%) of the AH4A positive patients were also aCCP positive, whereas 45 patients (40%) of the AH4A negative patients was aCCP positive (p=0.02). For IgM–RF the association was even stronger. Twenty four AH4A positive patients (92%) were positive for IgM–RF, whereas 64 AH4A negative patients (57%) were positive for IgM–RF (p=0.001).

In the established RA cohort, 18 patients (16%) were AHA positive. In this cohort only a trend towards more IgM–RF positive patients (78% vs 58%, p=0.116) in the AH4A positive group could be observed. For aCCP, no association with AH4A was seen in the established RA cohort.

**DISCUSSION**

In this paper, we characterized antibodies directed against the hinge of degraded antibodies (AHA) in RA patients. We found that AHA can be directed towards different subclasses of IgG, antibodies to the hinge of IgG1 and IgG4 being the most prominent. The low frequency of anti–IgG2 hinge antibodies fits with a recent study in which less anti–IgG2 hinge reactivity was measured in a serum pool compared to anti–IgG1 hinge. This can be explained by the relative resistance of IgG2 to proteolysis. The reactivity towards IgG1 and IgG4 was very specific, since no cross reactivity could be observed and antibodies to IgG1 hinge did not correlate with antibodies to IgG4 hinge (AH4A).

Interestingly, AH4A were the most specific for RA. Since IgG4 antibodies cannot activate complement, we wondered whether the degradation of IgG4 into IgG4 F(ab')2 fragments with subsequent AH4A formation could counteract this inability. Indeed AH4A in complex with IgG4 F(ab')2 fragments were able to activate complement. This indicates that AH4A can antagonize the anti-inflammatory properties of IgG4. Since IgG4 is known to be formed in chronic inflammation supposedly by perpetual antigenic stimulation, AH4A may play role in perpetuating disease. In inflammatory conditions, all kinds of proteases become activated and can degrade the proteins present in the inflamed tissue. Antibodies themselves are also subjected to degradation and apparently can be targeted by newly formed antibodies that can activate complement, further stimulating inflammation. As such, a vicious circle is created. The role of IgG4 and AH4A in perpetuating chronic disease is further supported by the finding that IgG4 is preferentially decreased by TNF blocking therapy. By reducing inflammation, TNF
blocking therapy may decrease antigenic load, thereby decreasing IgG4 production and slowing the progression of the circle.

This circle may be initiated even before the diagnosis of RA, since we found that AH4A cannot only be detected in patients with established RA, they can also be detected before RA is diagnosed, albeit in low numbers. We observed that in pre-clinical RA and early RA, the presence of these antibodies is associated with the presence of other antibodies and that the percentage of AHA positive patients increases in the different subsequent phases of the disease. These results also suggest that the inflammatory process facilitates the formation of these antibodies, further supporting the hypothesis that these antibodies can contribute to the pathogenesis of chronic disease.

We did not observe an association between AHA and other antibodies in our established RA cohort, although a trend was seen towards more IgM–RF positive patients in the AHA positive subgroup. This may indicate that during the progression of RA these associations become less apparent, or that certain antibodies are influenced more by treatment than others. It is known, for instance that IgM–RF reflect disease activity more pronounced than APCA. The same could be true for AHA, since these antibodies also seem dependent on inflammatory activity for their formation.

In conclusion, AH4A can be formed in inflammatory conditions, such as RA. In line with IgG4, they become more prevalent during progression of disease. They can add to inflammation by activating complement when in association with IgG4. They are associated with the presence of IgM–RF and APCA, which further supports their role in inflammation. These findings indicate that AH4A play a role in the vicious circle of epitope spreading that maintains the perpetual inflammation in RA.
REFERENCES


