A NEW ANTIBODY IN RHEUMATOID ARTHRITIS TARGETING GLYCATED IgG: IgM ANTI-IgG-AGE

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SUMMARY

Hyperglycaemia and/or oxidative stress can cause IgG to be modified by advanced glycation end products (AGE). Three patients with aggressive rheumatoid arthritis (RA) and vasculitis are described who have high titres of IgM antibodies against AGE-modified IgG (IgM anti-IgG-AGE). Diabetics and randomly selected patients with rheumatic diseases, including 50 additional RA patients, were tested for IgM and IgA anti-IgG-AGE by ELISA. AGE-modified proteins were detected using the nitroblue tetrazolium (NBT) colorimetric method. The presence of Ne (carboxymethyl) lysine, an AGE modification, was detected on IgG-AGE by immunoblotting. A total of 20/41 (49%) rheumatoid factor (RF)-positive RA patients tested had IgM anti-IgG-AGE antibodies, 4/12 (33%) RF-positive systemic lupus erythematosus (SLE) patients, 3/5 RF-positive patients with primary Sjo¨gren's syndrome (SS), and 3/5 RF-positive diabetics. All patients with RF-negative RA, SLE, SS, osteoarthritis (24), spondyloarthritis (15), adult-onset Still's disease (8), diabetes (25) and healthy controls (20) were anti-IgG-AGE negative. RF and IgM anti-IgG-AGE appeared to be a linked response. The IgM anti-IgG-AGE, along with IgG-AGE, may contribute to the pathogenesis of RA.

KEY WORDS: Advanced glycation end products, Autoantibody, Diabetes, Rheumatoid factor, Vasculitis.

RHEUMATOID arthritis (RA) is a potentially very disabling disease with both articular and systemic manifestations. Our understanding of its pathogenesis remains partial and treatment is generally not curative. Long-term outcome predictors, such as the presence of rheumatoid factor (RF) [1–5] and the QKRAA shared epitope on the HLA-DRβ chain [6–8], help clinicians decide how aggressive treatment should be. However, the picture they provide remains incomplete and the search continues for better prognostic tools.

A previously unsuspected factor which seems to correlate with clinical disease activity in RA is the level of certain circulating advanced glycation end products (AGE) [9]. Primarily studied in the diabetic population, where they appear to be implicated in tissue damage, their occurrence and clinical significance in RA had not been previously assessed. AGE result from non-enzymatic glycation of proteins [10]. Haemoglobin (glycated form haemoglobin A1c), albumin and collagen have all been identified as targets of AGE modification [10]. Increasingly recognized is the glycation of immunoglobulins ([11–15] and Newkirk, revised manuscript submitted), which has been shown to have functional implications such as decreased protein A binding and complement fixation [13], as well as decreased antigen binding ([14, 15] and Newkirk, revised manuscript submitted).

We recently identified three RA patients with severe systemic manifestations in whom we found both IgG modified with advanced glycation end products (IgG-AGE), and elevated titres of IgM and IgA antibodies directed against IgG-AGE. Subsequent analysis of diabetics and of patients with other rheumatic diseases showed that the antibody response to glycated IgG was primarily associated with RF-positive RA. The IgM anti-IgG-AGE antibodies and RF appear to be two separate and probably distinct antibody populations. We discuss the potential role of IgG-AGE and anti-IgG-AGE antibodies in the pathogenesis of RA.

PATIENTS AND METHODS

Patients and controls

IgG-AGE and IgM anti-IgG-AGE antibodies were originally identified in three patients with RA (see below for test methods). A retrospective chart review was undertaken for each patient.

In a retrospective study, sera were drawn from randomly selected patients with various rheumatic diseases from clinics at the Montreal General Hospital (Drs C. Watts, J. Esdaile and P. R. Fortin), Notre-Dame Hospital (J. P. Pelletier) and the Toronto Western Hospital (Dr R. Inman). Diseases included RA, systemic lupus erythematosus (SLE), Sjo¨gren's syndrome (SS), spondyloarthritis, adult Still's disease and osteoarthritis (OA). Diabetics, 16 Type 1 and 14 Type 2, selected for elevated haemoglobin A1c (Dr R. Gardiner, Montreal General Hospital) and 20 healthy controls, with a mean age of 32.6 ± 10.0 yr (14 female:6 male) were also selected. Information on a number of different clinical parameters, blood glucose levels and haemoglobin A1c (HbA1c; values >6.1 were considered elevated), were obtained where available from the charts.

Rheumatoid factor assay

RF was detected by nephelometry and ELISA as previously described [16]. IgG of all four subclasses (kindly provided by Prof. R. Jeffers, University of...
Birmingham) were used as target antigens in the ELISA to define RF specificity.

In vitro AGE modification of IgG and measurements of IgG-AGE

Purified monoclonal IgG of all four subclasses was AGE modified after being sterilized by passing through a 20 μm filter. The IgG was incubated in vitro with 1.5 m glucose for 3 months at 37 °C in order to modify it fully with the AGE. The amount of AGE modification on purified IgG was followed by measuring the optical density at 335 μm where the browning or pentosidine cross-links on the purified protein can be detected [17].

Nitroblue tetrazolium (NBT) colorimetric method

For this assay, which has been modified from previously published methods [18, 19], serum was fractionated into an antibody/immune complex-rich fraction and other proteins, using a polyethylene glycol precipitation (PEG) method that we have developed. One hundred microlitres of serum were incubated with 2.5% PEG 8000 for 30 min at 4 °C. The samples were centrifuged at 15 000 g for 5 min. The supernatant was collected (‘other’ proteins) and the pellet (IgG-containing immune complexes) was resuspended in 100 μl of phosphate-buffered saline (PBS). Fifty microlitres of the supernatant and resuspended pellet were added to the wells of 96-well microtitre plates, in duplicate. Standard curves were established with bovine serum albumin (BSA)-AGE (modified in vitro as above for the IgG) and IgG-AGE proteins in the range of 0–1.5 mg/ml for BSA and 0–4 mg/ml for IgG-AGE. One hundred microlitres of the NBT reagent [250 μl NBT in 0.1 M carbonate buffer (pH 10.35)] were added to each well, then incubated at 37 °C for 2 h. The plate was read in an ELISA plate reader at OD 520. The amount of AGE-modified proteins in the fractions was calculated using IgG-AGE (for the pellet) and BSA-AGE (for the supernatant) standard curves.

Immunoblots for AGE-IgG detection

Validation of the NBT assay, and further structural characterization of the AGE epitope, was done by immunoblotting. In brief, 1 μl of the immune complex fraction (as above) was resolved on 10% SDS–polyacrylamide gels, under reducing conditions, as previously described [20]. After transfer to nylon membranes [21], the membranes were blocked with 0.1% Tween 20 in PBS for 1 h at room temperature. The AGE determinant was detected using a 2 h incubation at 37 °C with the monoclonal antibody 6D12 (Wako), specific for Nε (carboxymethyl) lysine (CML) [22], diluted 1:1000 in PBS/Tween. After washing the blots, the bound 6D12 was detected after incubating for 1 h at 37 °C with HRP-anti-mouse IgG (1:30 000). A chemiluminescent ECL (Amersham) was used, with visualization on BioMax film (Kodak). To identify the components in the immune complexes, the blot was stripped according to the ECL recommendations, and reprobed with horseradish peroxidase (HRP)–anti-human IgG, IgM, or C3 (diluted 1:30 000; Jackson), followed by visualization by chemiluminescence.

The relationship between the amount of circulating IgG-AGE and the presence of IgM anti-IgG-AGE antibodies was studied by comparing the immune complexes of five randomly selected RA patients with IgM anti-IgG-AGE with those of five RA patients, eight diabetics and five healthy individuals without IgM anti-IgG-AGE. Following isolation of immune complexes (as described above), 15 μg of total protein were resolved in 10% SDS–PAGE under reducing conditions and transferred as above for immunoblotting. The relative amounts of CML as detected by the monoclonal antibody 6D12 on the heavy and light chains of the IgG were determined by laser densitometry, and then corrected for the amount of total IgG (blots were stripped and reprobed with anti-human IgG).

Measurement of antibodies to immunoglobulin-AGE

IgM anti-IgG-AGE antibodies were detected in serum or plasma, using an ELISA assay that we have established. Fully AGE-modified IgG (all four subclasses) (2 μg/ml) to which there is minimal RF binding was used to coat the wells of an EIA plate (ICN). After washing the plates, the sera or plasma, diluted 1:1000 or greater, in duplicate, were incubated in the AGE-modified Ig-coated wells for 2 h at 37 °C. After washing the plates in PBS/Tween (0.1%), the bound antibodies were detected with peroxidase-conjugated F(ab)2 fragments of anti-human IgM or IgA (Jackson), as appropriate. It was not technically possible to detect IgG anti-IgG-AGE antibodies. The titres of the anti-IgG-AGE of all classes were determined by serial dilution. In order to determine whether the anti-IgG-AGE antibodies bound to AGE modifications on other proteins, ELISA plates were coated with BSA and without AGE modifications (2 μg/ml) and the bound antibodies (IgG, IgM) were detected using appropriate HRP-conjugated second antibodies. Values were considered positive when they were above 2 S.D. of the mean of the reactivity of 20 normal individuals. To follow the reactivity over time, and keep consistent results, a serum from one normal individual (approximately the mean reactivity) was tested each time the assay was performed.

IgM anti-IgG-AGE binding specificity studies

The binding specificities of IgM anti-IgG-AGE antibodies were determined and compared to that of RFs. For this polyclonal RFs obtained from 53 RF-positive RA patients were reacted to all four subclasses of IgG (included are the 41 patients reported in Table II with an additional 12) and binding specificities were determined. Similarly, the binding specificities to fully AGE-modified IgG of all four subclasses, to which there was minimal RF reactivity (Newkirk, revised manuscript submitted), were determined in 37 RF-positive RA patients who also had IgM anti-IgG-AGE.

RESULTS

The clinical characteristics of three RA patients in whom IgG-AGE and IgM anti-IgG-AGE were initially
identified are summarized in Table I. All three patients had RF-positive destructive erosive disease, with associated vasculitis, and required corticosteroids as part of their treatment. Although none of these three individuals carried a diagnosis of diabetes mellitus, their available random serum glucose levels were increased or at the high end of normal. Unfortunately, fasting blood glucose levels were unavailable. All were found to have elevated RF titres. IgG-AGE and IgM anti-IgG-AGE were detected in these three patients (Table II). The RA patients had levels of IgG-AGE modifications similar to those of the diabetics and greater than the healthy controls. The IgM anti-IgG-AGE titres were elevated in the RA patients (titres against IgG1 were 1/160000, 1/20000 and 1/8000, respectively, for patients 1, 2 and 3; results in Table II are given in units), whereas IgM anti-IgG-AGE levels were not detected in the two representative diabetics or the two normal individuals shown. The IgM anti-IgG-AGE titres were much lower than the RF titres in these three index RA patients (data not shown). Slight differences amongst the three cases were observed in the IgM anti-IgG-AGE specificity for the subclasses of IgG; all four subclasses were recognized in cases 2 and 3, whereas case 1 recognized AGE-modified IgG1, IgG2 and IgG4 better than IgG3-AGE. IgA anti-IgG-AGE antibodies were detected in patients 1 and 2 (data not shown). Two out of the three RA patients were found to have both an IgG and an IgM anti-BSA-AGE response (Table II), but their antibodies did not detect BSA that was not AGE modified (data not shown). No anti-BSA-AGE antibody response was identified in the two normal individuals or the two diabetic patients illustrated.

Fifty-three additional RA patients were tested, retrospectively, for the presence of IgM anti-IgG-AGE (Table III). A total of 20/41 (49%) of the RF-positive patients had elevated IgM anti-IgG-AGE titres, while none of the 12 RF-negative RA patients (0%) had detectable titres. There were no significant differences in steroid use in the RF-positive vs the RF-negative groups. Additionally, in this study group, there was no correlation between the RF titre (as determined by ELISA) and the anti-IgG-AGE response. Anti-IgG-AGE antibodies were detected in 4/12 (33%) of the RF-positive SLE patients, and in 3/5 of RF-positive patients with SS, and 3/5 RF-positive diabetics. None of the RF-negative diabetics (types 1 or 2), SLE or SS patients had anti-IgG-AGE antibodies. Anti-IgG-AGE antibodies were undetectable in 20 healthy controls, in the 25 RF-negative diabetics tested, and in patients with OA, spondyloarthritis or adult-onset Still’s disease. A total of 9/35 (26%) RF-positive RA patients tested had detectable IgM anti-BSA-AGE antibodies, while 6/35 (17%) had an IgG anti-BSA-AGE response. Of the diabetics with IgM anti-IgG-AGE antibodies, two had detectable IgM-anti-BSA-AGE antibodies. No information was available on the blood glucose levels of the patient groups, other than the diabetics.

The IgA RF and anti-AGE response was also determined in some patient groups. For the patients with RA, 22/43 (51%) had IgA RFs, whereas 10/43 (23%) had IgA-anti-AGE antibodies. IgA RF was detected in 1/13 patients with spondyloarthritis. No IgA anti-IgG-AGE antibodies were detected in the spondyloarthritis group.

Immunoblots of immune complexes (IC) isolated from 1 μl of serum from the three described RA patients, using a monoclonal anti-CML antibody, revealed AGE modification of both heavy and light chains (Fig. 1). This was not observed in the IC isolated from the two diabetics or the two normal individuals. We have, however, detected CML on the IgG isolated from other diabetics (Fig. 2). Fragments of C3 were detected in the sample, along with the IgG and the IgM, indicating the presence of the IC in the RA sera (Fig. 1). The molecular weight of the heavy chain modified with CML was consistent with that of IgG and not IgM.

As can be seen in Fig. 2, after correction for the total amount of IgG in the blot, significantly more CML was detected on the IgG in the IC of RA patients with circulating anti-IgG-AGE than in IC of RA patients or normal individuals without anti-IgG-AGE (P < 0.001). All 10 RA patients tested had normal HbA1c values; one patient without anti-IgG-AGE antibodies had an elevated blood glucose value of 8.2 mmol/l (normal value < 6.5). The type 1 diabetics had similar amounts of CML present on the heavy chain regardless of the presence or absence of the anti-IgG-AGE antibodies, although the numbers are small (data not shown). CML was also detected on the light chain, and showed the same trend (higher in the RA patients with circulating anti-IgG-AGE antibodies), although with more variability (data not shown). Linear correlation analyses of the HbA1c levels of the diabetics with albumen-AGE or IgG-AGE measurements made by the NBT assay revealed a highly significant correlation (r = 0.78, P = 0.0009) for albumin-AGE, but no significant correlation was found for the IgG-AGE (r = 0.95, P = 0.75).

Binding specificity studies revealed that RF from the RA patients bound preferentially to IgG1, 2 and 4, with little binding to IgG3. Only 1/53 (2%) bound IgG3 better than IgG1, 2 and 4. In contrast, testing of 37 RA patients with circulating IgM anti-IgG-AGE (all had RF and were included in the above RF specificity studies) showed that 16/37 (43%) had preferential antibody binding to IgG3.

**DISCUSSION**

The three patients described, in whom the IgM anti-IgG-AGE antibody was initially discovered, shared several important characteristics. All had particularly aggressive RF-positive RA with vasculitis. All required continuous corticosteroid treatment. Identifying a new autoantibody in such a setting immediately raises questions as to its diagnostic, prognostic and pathogenic roles. Several points can be made from the presented data. Firstly, AGE-modified IgG are found in non-overtly diabetic RA patients. In a subsequent prospective study of 62 patients with RA, only 7% had
<table>
<thead>
<tr>
<th>Case</th>
<th>Age/sex</th>
<th>Disease duration</th>
<th>Nodules</th>
<th>Articular erosions</th>
<th>Vasculitis</th>
<th>Other RA-associated complications</th>
<th>Co-morbid illnesses</th>
<th>Treatments</th>
<th>Outcome</th>
<th>Random blood glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>56 M</td>
<td>35 yr</td>
<td>+</td>
<td>+</td>
<td>Biopsy-proven vasculitis (muscle)</td>
<td>Systemic amyloidosis, renal failure and malabsorption, Multiple septic arthritis episodes, Infected sacral nodules, with perirectal abscesses</td>
<td>SS Thyroiditis, Recurrent pancreatitis, attributed to non-steroidal anti-inflammatory drugs, Gastric ulcers</td>
<td>Surgical: Multiple joint replacements, Medical: Failed d-penicillamine, and i.m. gold, Corticosteroid dependent (oral), 2 yr monthly i.v. methyl-prednisolone boluses</td>
<td>Death at age 56 of systemic amyloidosis</td>
<td>Intermittently up (15–20 mmol/l)*</td>
</tr>
<tr>
<td>2</td>
<td>70 F</td>
<td>5 yr</td>
<td>+</td>
<td>+</td>
<td>Digital infarcts requiring amputation</td>
<td>Cervical spinal spondylolisthesis</td>
<td>Chronic obstructive pulmonary disease, Severe aortic stenosis, Osteoporosis</td>
<td>Hydroxychloroquine and azathioprine, Continuous oral corticosteroids × 5 yr</td>
<td>Deformities at &lt;1 yr of disease, Death at 70 yr of pulmonary and cardiac disease</td>
<td>11.4 mmol/l*</td>
</tr>
<tr>
<td>3</td>
<td>64 M</td>
<td>2 yr</td>
<td>+</td>
<td>+</td>
<td>Leucocytoclastic vasculitis, Digital ischaemia</td>
<td>Carpal tunnel syndrome, Pulmonary interstitial fibrosis, nodules</td>
<td>Osteoporosis</td>
<td>Oral cyclophosphamide, and oral corticosteroids</td>
<td>In remission, Functional limitation from pulmonary disease</td>
<td>7 mmol/l* HbA1C: 6.4%†</td>
</tr>
</tbody>
</table>

*Normal range for serum glucose: 3.3–6.4 mmol/l.
†HbA1c: haemoglobin A1c. Normal: <6.4%.
TABLE II
Detection of IgG-AGE and IgM anti-IgG-AGE antibodies in patients with RA and in controls

<table>
<thead>
<tr>
<th>Patient/control</th>
<th>‘Other AGE’†</th>
<th>IgG-AGE (mg/ml)‡</th>
<th>Anti-IgG1-AGE</th>
<th>Anti-IgG2-AGE</th>
<th>Anti-IgG3-AGE</th>
<th>Anti-IgG4-AGE</th>
<th>IgM anti-BSA-AGE</th>
<th>IgG anti-BSA-AGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA-1</td>
<td>0.33</td>
<td>0.1</td>
<td>2.60</td>
<td>2.30</td>
<td>0.38</td>
<td>3.0</td>
<td>2.65</td>
<td>0.42</td>
</tr>
<tr>
<td>RA-2</td>
<td>0.46</td>
<td>0.44</td>
<td>3.00</td>
<td>2.11</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
<td>0.26</td>
</tr>
<tr>
<td>RA-3a‡</td>
<td>0.42</td>
<td>0.40</td>
<td>0.70</td>
<td>0.43</td>
<td>0.54</td>
<td>0.33</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>3b</td>
<td>0.45</td>
<td>0.80</td>
<td>0.540</td>
<td>0.34</td>
<td>0.62</td>
<td>0.50</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>3c</td>
<td>0.37</td>
<td>0.38</td>
<td>0.33</td>
<td>0.17</td>
<td>0.26</td>
<td>0.17</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Normal-1</td>
<td>0.26</td>
<td>&lt;0.1</td>
<td>0.03</td>
<td>0.02</td>
<td>0.01</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Normal-2</td>
<td>0.25</td>
<td>&lt;0.1</td>
<td>0.01</td>
<td>0.03</td>
<td>0.01</td>
<td>0.02</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Diabetic-1</td>
<td>0.75</td>
<td>0.3</td>
<td>0.13</td>
<td>0.13</td>
<td>0.05</td>
<td>0.02</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Diabetic-2</td>
<td>0.68</td>
<td>0.45</td>
<td>0.11</td>
<td>0.24</td>
<td>0.06</td>
<td>0.05</td>
<td>0.01</td>
<td>NA</td>
</tr>
</tbody>
</table>

*Abnormal IgM anti-IgG-AGE value: >0.2, defined as 2 s.d. above the mean of 20 normal controls.
†NBT assay.
‡Patient 3 was tested on three different occasions: a, b and c.

TABLE III
Presence of IgM anti-IgG-AGE and antibodies against bovine serum albumin (BSA) in various rheumatic diseases

<table>
<thead>
<tr>
<th>Patient group (no. tested)</th>
<th>IgM anti-IgG-AGE (positive (%))</th>
<th>IgM anti-BSA-AGE (positive (%))</th>
<th>IgG anti-BSA-AGE (positive (%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA: RF (+) (41)</td>
<td>20 (49%)</td>
<td>9/35 (26%)</td>
<td>6/35 (17%)</td>
</tr>
<tr>
<td>RF (−) (12)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Osteoarthritis* (24)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Spondyloarthritis† (15)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>SLE: RF (+) (12)</td>
<td>4 (33%)</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>RF (−) (21)</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>SS: RF (+) (5)</td>
<td>3 (60%)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>RF (−) (6)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Adult Still's disease (8)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Normal controls (20)</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Diabetics‡:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1 RF (+) (3)</td>
<td>2 (67%)</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>RF (−) (13)</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
<tr>
<td>Type 2 RF (+) (2)</td>
<td>1/2 (50%)</td>
<td>1</td>
<td>NA</td>
</tr>
<tr>
<td>RF (−) (12)</td>
<td>0</td>
<td>0</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA, not analysed.
*Provided by Dr J. P. Pelletier, Notre-Dame Hospital, Montreal.
†Provided by Dr R. Inman, Toronto.
‡HbA1c: Type 1, 8.6 ± 2.1; Type 2, 9.1 ± 2.9.

hyperglycaemia (preliminary data, not shown). Secondly, RF-positive individuals, primarily RA patients, can mount an antibody response to IgG-AGE. Finally, this antibody response appears to be strongly linked to the production of RF.

Previous studies by Takahashi et al. [9] have shown that the AGE modification product pentosidine (which cross-links lysine and arginine) can be detected in elevated amounts in patients with RA. Approximately 50% of the RA patients in their study had elevated pentosidine levels >2 s.d. of the normal mean. Thus, this represents a significantly larger subset of patients than those with elevated blood glucose. Interestingly, they found that pentosidine correlated with clinical disease activity [9]. Of note is the association they report between RF and urinary pentosidine. Since we have not detected glycated IgM (and by association IgM RFs) in patients with RA, this link of pentosidine to RF is probably associated with the glycoxidation of the target antigen, namely IgG. These authors suggest that the increased oxidant stress in uncontrolled RA was responsible for the presence of increased serum and urinary pentosidine; however, they did not identify the proteins modified by this AGE epitope. Similarly, previous studies by Takahashi et al. [9] have shown that the AGE modification product pentosidine (which cross-links lysine and arginine) can be detected in elevated amounts in patients with RA. Approximately 50% of the RA patients in their study had elevated pentosidine levels >2 s.d. of the normal mean. Thus, this represents a significantly larger subset of patients than those with elevated blood glucose. Interestingly, they found that pentosidine correlated with clinical disease activity [9]. Of note is the association they report between RF and urinary pentosidine. Since we have not detected glycated IgM (and by association IgM RFs) in patients with RA, this link of pentosidine to RF is probably associated with the glycoxidation of the target antigen, namely IgG. These authors suggest that the increased oxidant stress in uncontrolled RA was responsible for the presence of increased serum and urinary pentosidine; however, they did not identify the proteins modified by this AGE epitope. Similarly, a recent study by Rodriguez-Garcia and co-workers [24] reports elevated levels of pentosidine in patients with RA, in the absence of hyperglycaemia; again the modified protein(s) were not identified. Reactive oxygen radicals have been identified in 90% of RA synovial fluids [24]. In blood, the reactive oxygen radicals have been found to correlate with tumour necrosis factor alpha [25]. Such radicals can contribute to the formation of advanced glycation end products [22, 26, 27].

In our study, we have identified AGE-modified IgG in RA patients, as illustrated by the detection of CML,
did find a highly significant correlation, however, between HbA1c and albumin-AGE. In the three initial cases, random blood sugars were at the upper limit of normal, or slightly above normal. The true incidence of diabetes in RA is unknown, but intermittent hyperglycaemia is generally felt to be a common occurrence.

The clinical significance of IgG-AGE in RA remains to be determined, although, as previously demonstrated, IgG glycation does impair both antigen binding and effector functions, and may render patients prone to infections ([13–15] and Newkirk, manuscript submitted). Hyperglycaemia in RA probably results from the use of corticosteroids and from the greater secretion of stress hormones, such as cortisol and epinephrine, in response to inflammation. However, this has been disputed [28]. It does appear that patients with RA have peripheral insulin resistance which probably contributes to the hyperglycaemia [29]. Interestingly, moderate dose corticosteroids may actually reverse this acutely [30]. Chronic corticosteroid use, however, is well known to predispose to the development of diabetes mellitus. Thus, it is likely that the lack of association identified between the RF/anti-IgG-AGE response and the use of corticosteroids may reflect the complicated dose effect of steroids on glucose and glucose handling.

In addition to hyperglycaemia, the amino acid composition (in particular, the number of surface lysine or arginine residues) and the circulating half-life of the protein can also affect the rate of non-enzymatic glycation [10]. The longer half-life of IgG, being ~1 month, as opposed to the 9 days for IgM, may explain why IgG-AGE, but not IgM-AGE, was detected in these patients.

The increase in IgG-AGE in patients with RA may also result from a decrease in its clearance. Soluble AGE-modified proteins are cleared by the reticuloendothelial system via specific AGE receptors, such as RAGE [31]. It is possible that IgM anti-IgG-AGE antibodies block IgG-AGE clearance by binding to IgG-AGE and thus preventing recognition via these receptors. The significant elevation in IgG-AGE in RA patients with circulating anti-IgG-AGE compared to those without (Fig. 2), despite similar glycaemic control in both groups, supports the hypothesis that anti-IgG-AGE antibodies may diminish IgG-AGE clearance.

We demonstrate that specific individuals, namely a subset of RF-positive patients, can develop antibodies to glycated IgG. We have identified these antibodies not only in serum, but also in the synovial fluid (data not shown) of RA patients who are RF positive. Because RF and IgM anti-IgG-AGE antibodies are found in the same individuals as a linked response, and because they both target IgG, it was important to ensure that these are indeed two separate antibody populations. There are five points that can be made in support of this contention. (i) Only half of the RF-positive RA patients were in fact found to have circulating IgM anti-IgG-AGE. It is likely, however, that a few of the anti-IgG-AGE-negative individuals

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**Fig. 1.**—Immunoblots of immune complexes (IC) from 1 µl sera from RA patients (lane 3 is case 1, lane 4 is case 2, and lanes 5, 6 and 7 are case 3 tested at different time points), normal controls (lanes 8 and 9), a type I diabetic with a HgbA1c of 9.5% (lane 10) and a type II diabetic with a HgbA1c of 11.7% (lane 11). Lanes 1 and 2 represent IgG2, with lane 2 being AGE-modified in vitro, and lane 12 being purified polyclonal IgG. (A) The reduced IC resolved as heavy (HC) and light chains (LC) and were probed with a monoclonal anti-Nε(carboxymethyl)lysine (CML) antibody (6D12). The molecular weight of the CML-modified HC was ~50 kDa. (B) and (C) Panel A was stripped and reprobed with anti-human Ig γ (B) and anti-human Ig μ (C). (D) Panel A was stripped and reprobed with anti-human C3.

**Fig. 2.**—Relative amounts of Nε(carboxymethyl)lysine (CML) as detected in immunoblots of the heavy chain of IgG from immune complexes, when corrected for total IgG, isolated from five normal individuals; five patients with RA who lacked circulating anti-IgG-AGE antibodies (RA-A); five RA patients with circulating anti-IgG-AGE antibodies (RA-B); eight diabetics (three with type 1 diabetes mellitus without anti-IgG-AGE antibodies, two with type 1 diabetes with anti-IgG-AGE antibodies and three with type 2 diabetes). The mean and 95% confidence intervals are plotted.
will with time develop such antibodies (as we have observed, data not shown). (ii) In separate experiments, we have shown that monoclonal RFs from patients with mixed cryoglobulinaemia do not bind to fully AGE-modified IgG, implicating specific lysine and arginine residues in the Fc as being involved in the recognition of RFs (Newkirk, revised manuscript submitted). IgM anti-IgG-AGE, in contrast, targets glycated IgG, although the precise epitopes on the IgG (Fab, Fc and/or hinge) are yet to be determined. (iii) We demonstrate the presence of IgM as well as IgG anti-AGE-modified albumin in these same patients, suggesting that the AGE modification recognized by the antibodies is probably shared by other glycated proteins as well as IgG. Monospecific RFs, by definition, do not bind to BSA either with or without AGE modifications. (iv) We found that the IgM anti-IgG-AGE preferentially recognized IgG3, whereas the RFs from the same patients recognized the subclasses IgG1, IgG2 and IgG4 better than IgG3. One of the structural differences between IgG3 and the other IgG subclasses is its long hinge region, which contains four lysine and four arginine residues, all of which could potentially be AGE modified. Additional studies are required to determine whether the hinge region of IgG3 is a site of AGE modification, and a binding site for IgM anti-IgG-AGE. (v) The precise specificities of the anti-AGE antibodies are as yet unknown; however, we suspect that the majority of the antibodies will be directed at an epitope containing CML, as has been seen for polyclonal rabbit antisera raised to other AGE-modified proteins [32]. Our data showing that there is an increase in the CML-IgG in the IC of patients with anti-IgG-AGE antibodies are consistent with this previous finding. The fact that CML is immunogenic (rabbits and mice make antibodies against it) further strengthens our finding of anti-AGE antibodies in humans. These points all provide evidence that RF and IgM anti-IgG-AGE represent two different antibody populations.

The explanation for the association between the two antibody populations may lie at the level of the immune dysregulation triggering their production. B cells are known to endocytose antigens via their surface Ig and then process it [33]. Thus, RF-positive B cells could potentially mediate the uptake of partially AGE-modified IgG (i.e. modified in sites other than the Fc), process the fragments and then through the presentation of the AGE-modified peptides via Class II MHC elicit a specific anti-IgG-AGE response by other B cells.

If both the AGE antigen and its antibody are present simultaneously, IC could result. It is possible that the deposition of IgG-AGE and anti-IgG-AGE antibody-containing complexes could contribute to the development of vasculitis. Interestingly, the older literature has questioned the relationship between corticosteroid use and the development of vasculitis in RA [34]. A causative relationship is not easily proven, as vasculitis occurs in patients more likely to be on corticosteroids because of more aggressive disease. However, if one postulates that IgM-anti-IgG-AGE is associated with more severe disease in which hyperglycaemia is more common, the presence of the anti-AGE antibody, combined with greater levels of glycated IgG, may favour IC deposition. Elevated serum glucose levels would be particularly deleterious in these patients.

Our first described patient had systemic amyloidosis, presumably of the AA type, which is associated with chronic uncontrolled inflammation and infection. Interestingly, AGE modification has been identified on β2-microglobulin amyloid deposits [35, 36]. Such modifications may also affect other classes of amyloid. It is possible that IgM anti-IgG-AGE, by binding to soluble AGE-modified amyloid, could decrease its clearance and promote systemic deposition.

We have identified a new antibody in three patients with severe RA and vasculitis, and confirmed its presence in approximately half of the additional RA patients tested. RF-negative diabetics, and patients with other RF-negative rheumatic diseases, did not mount an antibody response against glycated IgG. Because the presence of IgM anti-IgG-AGE always appears to be associated with that of RF, it may not prove to be helpful in the diagnosis of RA. However, from preliminary studies, we have found that anti-IgG-AGE antibodies correlate with greater disease activity (Lucey, manuscript submitted). Thus, it may be directly involved in tissue damage. Our study raises the issue of the potential importance of good glycaemic control in RA, and of cautious use of chronic corticosteroid therapy.

Prospective follow-up of RA patients, with testing for IgM anti-IgG-AGE and close attention to glucose levels, will help determine the prognostic value of this new autoantibody. Further information about the interaction of anti-IgG-AGE with specific IgG-AGE epitopes is, however, required. In addition, the impact of circulating anti-IgG-AGE antibodies on the clearance of IgG-AGE and other AGE-modified proteins in animal models of arthritis needs assessment.

Localizing IgG-AGE/IgM anti-IgG-AGE immune complex deposits in the endothelium and synovium of RA patients with the circulating antibody would support a direct pathogenic role.

ACKNOWLEDGEMENTS

The authors thank Katia Lepage for technical assistance; and Dr J. Esdaile, Vancouver General Hospital, University of British Columbia, Dr J. Gardiner, Dr C. Watts, Montreal General Hospital, McGill University, Montreal, Canada, Dr R. Inman, The Toronto Hospital, University of Toronto, Toronto, Canada, Dr J. P. Pelletier, Pavillon Notre-Dame, CHUM, Université de Montréal, Montreal, Canada, for providing clinical samples. We thank Dr J. Esdaile and Dr J. Rauch for critically reviewing the manuscript. MMN is a research scientist of the Arthritis Society. PRF is a research scholar of the Arthritis Society.

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