Reaction of rheumatoid factors with IgG3 monoclonal anti-Rh(D) antibodies: more frequent reactivity to a monoclonal antibody of the Gm allotype G3m(5) in rheumatoid patients negative for G3m(5)

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SUMMARY Human monoclonal anti-Rh(D) antibodies of known IgG isotype and Gm allotype were bound to erythrocytes and then used as the target IgG antigens for rheumatoid factors (RFs) in a direct haemagglutination test. When serum samples from patients with rheumatoid arthritis (RA) were tested for RF specificity towards these IgG monoclonal anti-D antibodies the incidence and titre of reactivity towards an IgG3 monoclonal anti-D antibody was considerably greater than for a polyclonal anti-D antibody of the same Gm allotype, G3m(5). This difference was not explained by the amount of each anti-D antibody which bound to erythrocytes. Furthermore, when patients with RA were divided into groups according to their Gm phenotype, sera from a greater proportion of patients negative for the phenotype G3m(5) reacted to the G3m(5) monoclonal anti-D antibodies than sera from those patients positive for this allotype. Analysis of RF reactivities towards two IgG3 and three IgG1 monoclonal anti-D antibodies, each with different Gm allotypic epitopes, indicated, however, that individual serum samples contained RFs with a spectrum of specificities; some sera appeared to react to a single set of Gm alleles, whereas others also reacted to isotypic or iso-allotypic epitopes, or both. Our data suggest that RFs with specificity for Gm allotypes do not arise in patients who carry that particular allotype owing to tolerance induced in fetal-neonatal life. Conversely, RFs with apparent specificity for a Gm allotype formed in patients negative for that allotype may be reacting to a closely related but different epitope. Final proof requires precise specificities for each RF formed, and IgG3 monoclonal anti-D antibodies would be useful reagents for this purpose.

Key words: rheumatoid arthritis, autoantibodies, immunoglobulin allotypes.

Rheumatoid factors (RFs) are the characteristic autoantibodies in rheumatoid arthritis (RA). They can be detected early in the disease, 1 2 they are implicated in the chronic pathogenesis of rheumatoid synovitis, 3 and their specificity may be related to putative triggers of the disease process. 4

The specificity of RFs for epitopes on the Fc region of autologous IgG, which includes isotypic and Gm allotypic epitopes, has been under investigation for several decades. For example, in 1960 Harboe showed that RFs to polyclonal IgG3 anti-D antibodies are rare both in patients with RA and in normal individuals. 5 In 1970 Grubb summarised the published work on RF specificity for Gm allotypes, which also related this RF specificity to the Gm status of the donor. 6 From the few individuals studied, both Grubb and Harboe noted that anti-Gm allotype donors, whether they were rheumatoid...
patients or normal individuals, were frequently negative for that allotype.

Recently we have developed an assay for determination of RF specificities, in which monoclonal IgG anti-Rh(D) antibodies of known subclass and Gm allotype were used as antigens for RF in the direct haemagglutination test. With this assay system we showed first, that the incidence of RF in rheumatoid sera to an IgG monoclonal anti-D antibody of G3m(21) allotype is far greater than the reactivity to a polyclonal anti-D antibody of the same subclass and allotype. Secondly, and again using unseparated polyclonal RA sera, we found that the incidence of RFs reactive to monoclonal antibodies of the G3m(21) allotype was significantly greater in patients with RA negative for G3m(21) than in patients positive for this allotype. We report here that the converse also applies. We find that the incidence of RFs reactive to an IgG monoclonal anti-D antibody of the opposite allele, namely G3m(5), is significantly greater in sera from patients with RA negative for G3m(5) than in patients positive for G3m(5).

Materials and methods

Monoclonal anti-Rh(D) antibodies

The two G3m(5) monoclonal anti-D antibodies (FOG3 and GAD2) were produced using hetero-hybridomas. The four monoclonal anti-D antibodies—namely, CB6 (G3m(21)), AB5 (G1m(1,17)), IA3 (G1m(3)), and H27 (G1m(1,2,17))—were produced by Epstein-Barr virus transformed B lymphoblastic cell lines.9a

Polyclonal anti-Rh(D) sera

Two human sera (‘b’ and ‘g’) containing either G3m(5) or G3m(21) anti-D antibodies respectively were purchased from the Central Laboratory of the Netherlands, Amsterdam, as allotyping reagents for these two Gm allotypes. We were unable to demonstrate IgG1 or IgG2 anti-D antibodies (unpublished data) or IgG3 anti-D antibodies with the opposite allele (Table 1), but it is not known whether IgM, IgA, or IgG4 anti-D antibodies or antibodies of other specificities were present.

Gm allotyping

Serum samples from 107 patients with definite or classical RA (77 female, 78 seropositive for RF) were Gm allotyped with reagents purchased from the Central Laboratory of the Netherlands by the method described by Van Loghem.10 Sera which reacted strongly with antibody coated erythrocytes were separated by gel filtration, and the IgG fraction was typed.

Reactivity to monoclonal anti-D antibodies

Monoclonal anti-D supernatants were used similarly to polyclonal anti-D sera to coat erythrocytes. Briefly, group 0 R2R2 cells were stored in liquid nitrogen. After thawing and extensive washing 25 μl of packed erythrocytes was incubated at 37°C for 75 minutes with 100 μl of monoclonal anti-D supernatant (unless stated otherwise in the text) together with saline to a final volume of 225 μl. After further washing monoclonal and polyclonal antibody coated erythrocytes were stored in Alsever’s solution at 4°C for up to 10 and four days respectively. (After eight days at least 75% of monoclonal IgG and 60% of polyclonal IgG was still bound to the erythrocyte membrane.) Reactivity of sera to coated erythrocytes was measured in V well microtitre plates using 20 μl of serum dilutions, 20 μl of approximately 0-1% coated erythrocyte suspensions, and 20 μl of PFT (PBS (phosphate buffered saline)+5% fetal calf serum+0-05% Tween 20). Agglutination was read after incubation at room temperature for 30 minutes and at 4°C overnight. Unsensitised erythrocytes were included in all assays and were frequently agglutinated by low dilutions of sera. Therefore agglutination of coated erythrocytes was only considered positive at dilutions of 1:20 or greater.

Measurement of IgG bound to erythrocytes

Numbers of IgG molecules in monoclonal and polyclonal anti-D preparations which bound to erythrocytes were estimated by radioimmunoassay. Briefly, R2R2 erythrocytes were coated with various amounts of monoclonal and polyclonal anti-D preparations as listed in Table 1, using the coating procedure described above. Coated erythrocyte suspensions were made 10% v/v; then 0-1 ml of the suspensions was mixed with 0-1 ml of 125I anti-IgG, and incubated for 30 minutes at 37°C. The 125I labelled sheep antihuman IgG was used at a concentration of 20 μg/ml, at which 90% of the maximum binding of antibody to IgG is achieved. The antibody was affinity purified on a Sepharose-IgG column before iodination. The original washing procedure was modified in that after incubation and one wash in PBS the cell suspension was layered over a mixture of four parts dibutyl phthalate: one part dinonyl-n-butyl phthalate and briefly centrifuged, causing sedimentation of the cells through the oil. The tubes were then frozen and the tip containing the cell button cut off and counted. The number of IgG molecules per cell was calculated from the amount of anti-IgG bound (derived from the specific radioactivity of the labelled material),
the cell count, and Avogadro’s number. The binding ratio of seven anti-IgG molecules per IgG molecule was used in all calculations, with the assumption that the same ratio applied to polyclonal and monoclonal IgG. This seems justified as saturation of available D antigen sites with polyclonal and monoclonal anti-D antibodies gives the same amount of anti-IgG bound using this ratio. The value should be taken as approximate, however, as there is probably some variation in the binding characteristics of the anti-IgG to monoclonal and polyclonal IgG antibodies.

**RF measured by latex agglutination**
The RapiTex-RF kit (Behringwerke) was used to measure RF seropositivity for IgG coated latex particles.

**Statistical analysis**
χ² Tests were carried out with Yates’s correction. For correlation coefficients (r) Student’s t test was applied to estimate probability (p).

**Gm nomenclature**
Where reference is made to Gm phenotype, the IgG subclass of each Gm allotype has been omitted. Thus G1m(1,17); G3m(21) is expressed as Gm1,17; 21. Where patients are homozygous for G1m(1,17); G3m(21) they were incorporated with those homozygous for G1m(1,2,17); G3m(21) and they are all designated Gm1(2)(17); 21.

The G3m(5) and G3m(21) polyclonal anti-D sera are also called ‘b’ and ‘g’ respectively, after the recommended alternative numerical and alphanumeric notations for immunoglobulin allotypes: 1=a, 2=x, 3=f, 5=b₁, 11=b₀, 13=b₃, 17=z, 21=g.

**Results**

**Comparison of Reactivity to Monoclonal and Polyclonal Anti-D Antibodies**
A single serum sample from each of 107 patients with RA was tested against two G3m(5) monoclonal anti-D antibodies (FOG3 and GAD2) and a G3m(5) polyclonal anti-D serum. Serum from 31, 23, and seven patients agglutinated erythrocytes coated with FOG3, GAD2, and the polyclonal anti-D serum respectively. Thus the incidence of sera reacting with the monoclonal anti-D antibodies FOG3 and GAD2 was significantly greater than the number reacting with polyclonal anti-D serum (p<0.001 and <0.01 for FOG3 and GAD2 respectively).

**Amount of Monoclonal and Polyclonal Anti-D Bound to Erythrocytes**
To explain the low incidence of RF reactivity to polyclonal anti-D serum compared with monoclonal anti-D the number of IgG molecules in each anti-D preparation which bound to erythrocytes was estimated by a radioimmunoassay. Results in Table 1 show that the low incidence of rheumatoid sera reactive with polyclonal anti-D serum cannot be explained by a low number of polyclonal IgG molecules bound to erythrocytes. Indeed, almost twice as many IgG molecules in the polyclonal G3m(5) anti-D serum bound compared with mono-

<table>
<thead>
<tr>
<th>Anti-D reagent</th>
<th>Volume used to coat R₁R₂ cells (µl)</th>
<th>Molecules IgG bound per erythrocyte</th>
<th>Reciprocal agglutination titre of anti-allotyping sera specific for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>G3m(5)</td>
</tr>
<tr>
<td>Monoclonal G3m(5) FOG3</td>
<td>100</td>
<td>14 100</td>
<td>128</td>
</tr>
<tr>
<td>Monoclonal G3m(5) GAD2</td>
<td>50</td>
<td>6 900</td>
<td>64</td>
</tr>
<tr>
<td>Monoclonal G3m(5) GAD2</td>
<td>100</td>
<td>8 900</td>
<td>64</td>
</tr>
<tr>
<td>Polyclonal G3m(5) ‘b’</td>
<td>200</td>
<td>23 100</td>
<td>4</td>
</tr>
<tr>
<td>Monoclonal G3m(21) CB6</td>
<td>100</td>
<td>19 100</td>
<td>4</td>
</tr>
<tr>
<td>Monoclonal G3m(21) 6D10</td>
<td>200</td>
<td>19 100</td>
<td>4</td>
</tr>
<tr>
<td>Polyclonal G3m(21) ‘g’</td>
<td>100</td>
<td>19 100</td>
<td>4</td>
</tr>
</tbody>
</table>

*Repeat value obtained in a separate experiment.
NT=not tested.

Table 1 Comparison of amount of IgG in monoclonal and polyclonal anti-D preparations which bound to R₁R₂ erythrocytes
clonal Gm3(5) IgG molecules, but titres of all three anti-allotyping sera—namely, anti-Gm3(5), anti-Gm3(11), and anti-Gm3(13)—were considerably higher against the monoclonal anti-D antibody coated cells. The amount of IgG in the Gm3(21) polyclonal serum which bound was less than in the Gm3(5) polyclonal serum. When a comparison was made between cells coated with approximately 6000 molecules of polyclonal or monoclonal Gm3(21) IgG per erythrocyte, however, titres of the anti-Gm3(21) allotyping serum were fivefold greater against cells coated with monoclonal anti-D antibody.

**Relation of RF Reactivities to Patients' Gm Phenotypes**

In an attempt to explain why only 31 (29%) of 107 RA patients formed RFs which reacted with Gm3(5) monoclonal anti-D and no more than seven (6.5%) reacted with Gm3(5) polyclonal anti-D, RF activities were analysed in terms of the Gm phenotype of each patient. Patients were divided into three groups: (a) those homozygous for Gm1(2)17; 21; (b) those homozygous for Gm3; 5; and (c) heterozygous patients expressing the phenotype Gm1(2)17, 3; 21.5. Fig. 1 shows the agglutination titres of sera from each category of patient against erythrocytes coated with Gm3(5) monoclonal anti-D FOG3 and the Gm3(5) polyclonal anti-D antibody. More sera reacted and titres were higher in sera from patients who do not carry the Gm3(5) allele—namely, those patients homozygous for Gm1(2)17; 21. Table 2 provides a further analysis, in terms of the number

![Fig. 1 Comparison of agglutination titres in rheumatoid sera reacting to polyclonal and to monoclonal Gm3(5) anti-D coated erythrocytes. One hundred and seven patients with RA were divided into three groups: (a) those homozygous for Gm1(2)17; 21; (b) those homozygous for Gm3; 5 and (c) heterozygous patients, and their sera were titrated against monoclonal anti-D antibodies (FOG3) and polyclonal anti-D antibodies, both of the Gm3(5) allotype. Bar=mean titre.](http://ard.bmj.com/)

<table>
<thead>
<tr>
<th>Gm phenotype of patients with RA</th>
<th>No of patients</th>
<th>No (%) of patients with RA with reactivity to:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Monoclonal Gm3(5)</td>
</tr>
<tr>
<td>(a) Homozygous</td>
<td></td>
<td>FOG3</td>
</tr>
<tr>
<td>Gm1(2)17; 21</td>
<td>31</td>
<td>17 (55)</td>
</tr>
<tr>
<td>(b) Homozygous Gm3; 5</td>
<td>43</td>
<td>11 (26)</td>
</tr>
<tr>
<td>(c) Heterozygous Gm1(2)17, 3; 21, 5</td>
<td>33</td>
<td>3 (9)</td>
</tr>
</tbody>
</table>

*p value from χ²:*

a v b <0-05 NS <0-05 NS

a v c <0-01 <0-02 <0-05 NS

b v c NS <0-001 <0-01 NS
of patients in each category whose sera reacted to the G3m(5) and G3m(21) monoclonal and polyclonal anti-D reagents. Analysis confirmed that a significantly greater number of patients homozygous for Gm1(2)17; 21—that is, without the G3m(5) allele—reacted to G3m(5) monoclonal anti-D antibodies than patients who expressed this allele. Conversely, a significantly greater number of patients homozygous for Gm3; 5, and therefore without the G3m(21) allele, reacted to G3m(21) monoclonal anti-D antibodies than the heterozygous patients and those homozygous for Gm1(2)17; 21. The tendency was the same for reactivity to the two polyclonal anti-D sera, but the number of patients reacting to these polyclonal reagents was too few for strong statistical significance. There was no influence of Gm phenotype on RF seropositivity as measured by agglutination of IgG coated latex particles; approximately 70% of patients in each category were seropositive. An unexpected result was seen in the group of patients carrying the heterozygous Gm phenotype, where only a very small number reacted to either of the four monoclonal anti-D antibodies and none reacted to the polyclonal anti-D sera.

Relation of RFs Reacting to G3m(5) and G3m(21) Monoclonal Anti-D Antibodies

The specificity of most RFs reacting to G3m(5) monoclonal anti-D antibodies might be directed towards subclass specific epitopes on IgG3. Comparison of reactivities of 54 of the 107 RA sera, which reacted to one or more of the two G3m(5) and a G3m(21) monoclonal anti-D antibody, suggests, however, that RFs in many sera were indeed directed towards G3m(5) allelic epitopes. The correlation coefficient for reactivity to both G3m(5) and G3m(21) monoclonal anti-D antibodies (FOG3 and GAD2) was high (r=0.88, p<0.001), whereas correlation of reactivity between the G3m(5) (FOG3) and the G3m(21) (CB6) monoclonal anti-D antibodies was very low (r=0.11, p>0.1). When reactivities in sera from 30 patients with RA homozygous for Gm1(2)17; 21 were compared in the same way the correlation coefficients also indicated that RFs in some sera had specificity for G3m(5) and related alleles (Fig. 2).

Lack of 'Hidden' IgM RF Reactive with G3m(5) Monoclonal Anti-D Antibodies

A few sera from patients with each Gm phenotype were separated into their IgM and IgG components by gel filtration on S 300 Sephacryl at acid pH, 7 to test for hidden IgM RF reactive with G3m(5) monoclonal anti-D antibodies. The IgM fractions of sera from only four of 20 Gm heterozygous patients and only two of 16 patients homozygous for Gm3; 5 showed a small (two- to fourfold) increase in agglutination titre against G3m(5) anti-D (FOG3)

![Fig. 2](image_url) Correlation between reactivities to (a) two G3m(5) monoclonal anti-D antibodies (FOG3 and GAD2) and (b) one G3m(5) and one G3m(21) monoclonal anti-D (FOG3 and CB6 respectively) in sera from 30 patients with RA homozygous for Gm1(2)17; 21. Reactivities are given as agglutination titres.
Rheumatoid factor specificity for Gm allotypes

Table 3  Comparison of RF reactivities to various IgG1 and IgG3 monoclonal anti-D antibodies

<table>
<thead>
<tr>
<th>Gm phenotype of patients with RA</th>
<th>No of patients</th>
<th>No (%) of patients with RA with reactivity to monoclonal anti-D antibodies:</th>
<th>No reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>G3m(5) alone</td>
<td>G3m(21) alone</td>
</tr>
<tr>
<td>Homozygous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gm1(2) 17; 21</td>
<td>31</td>
<td>4 (13)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>Homozygous Gm3; 5</td>
<td>43</td>
<td>1 (2)</td>
<td>10 (23)</td>
</tr>
<tr>
<td>Heterozygous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gm1(2) 17; 21; 21,5</td>
<td>33</td>
<td>1 (3)</td>
<td>4 (12)</td>
</tr>
<tr>
<td>Total</td>
<td>107</td>
<td>6</td>
<td>16</td>
</tr>
</tbody>
</table>

*Reactivity to IgG1 denotes reactivity to one or more of the following monoclonal anti-D antibodies: AB5 (G1m(1,17)), IA3 (G1m(3)), and H27 (G1m(1,2,17)).
bound to erythrocytes. Indeed the concentration of G3m(5) polyclonal anti-D antibody ('b') used routinely (100 μl) almost reached saturation of the available D antigen sites, and twice this concentration only gave a small increase in the number of IgG molecules bound. The number and arrangement of Gm epitopes displayed may explain the difference. It is generally accepted that the Rh(D) peptide only allows monovalent binding of antibody. Thus both monoclonal and polyclonal antibodies should bind in a ratio of 1:1 with antigen and display their Gm epitopes in an identical fashion. Other antibodies in the polyclonal anti-D sera might mask some of the Gm epitopes displayed by polyclonal anti-D antibodies on the erythrocyte membrane, however, and prevent RFs from binding to the coated erythrocytes.

At present we cannot exclude the possibility that there are RFs which are directed towards the Gm allelic epitopes in all patients with reactivity to IgG3 monoclonal anti-D antibodies. In the few patients with reactivity solely to two monoclonal anti-D antibodies of the same allotype the reactivity is probably specific for this Gm allele. But most patients reacted either to IgG3 monoclonal anti-D antibodies of one allotype or to the monoclonal anti-D antibodies of both G3m allotypes and/or with one or more IgG1 monoclonal anti-D antibodies (Table 3). Until recently attention has largely focused on common autoantigenic epitopes for IgM RF, such as 'Ga'13 located within the Fc region of IgG1, 2, and 4, and on the presence of cross reactive idiotypes on monoclonal and polyclonal RFs.14 15 When the amount of RF with cross reactive idiotype was evaluated as a proportion of total polyclonal RF in individual patients with RA, however, it was found to represent a small fraction (<5%) of the total polyclonal RF idiotype population.16 From this result the conclusion was drawn that an individual's repertoire of RF is mostly private, quite diverse, and unique to that individual,16 and our results endorse this view (Table 3). In our previous study we attempted to identify RFs with specificity for G3m alleles by inhibition with IgG paraproteins of known isotype and allotype.7 Although inhibition studies lent support to our tentative conclusions, other experimental approaches are needed to confirm our findings, such as measurement of RF reactivities to IgG2 and IgG4 as well as to IgG1 and IgG3 epitopes, isolation from polyclonal sera of RF with specificity for single allelic epitopes, and establishment of cell lines producing RFs with these specificities.

Until exact specificities can be determined our conclusions on the role of a patient's Gm phenotype in determination of RF specificity for a particular Gm allele can only be tentative. We concluded in our previous publication that immune tolerance must prevent the appearance of clones producing antibodies to a particular G3m allelic epitope in patients who express that allele.7 This conclusion was drawn by analogy with polyclonal antiblood group A and B antibodies in normal individuals where tolerance prevents the production of natural antibodies to the B antigen in individuals with B or AB blood type, though these individuals form antibodies to Galα1-3 Gal, a similar but non-fucosylated epitope.17 Unexpectedly we found that considerably fewer heterozygous patients than homozygous patients reacted to a particular monoclonal anti-D antibody (Table 2). The reverse had been expected, that patients homozygous for G3m(5) would be more effectively tolerant than the heterozygous patients and therefore that fewer homozygous patients would react to G3m(5) monoclonal anti-D antibodies. When other reactivities were considered (Table 3), however, we concluded that heterozygous patients, who express a wider range of Gm allelic epitopes, must be tolerant to more of the complex range of RF specificities involved.

We are unable to explain satisfactorily the converse situation—namely, the apparent RF response to Gm epitopes in patients who do not carry that allele. We have already suggested transplacental or neonatal sensitisation as one of several possible explanations.7 An alternative might be somatic mutation of rearranged germline variable region immunoglobulin genes. It has been proposed that autoantibodies may arise by somatic mutation of germline genes which originally encoded antibodies that recognised foreign antigens.18 Indeed evidence in mice has shown that a somatic mutant of a mouse myeloma cell line results in the production of immunoglobulin which has changed by a single amino acid with a consequent change of antibody specificity from antiphosphocholine (an antibacterial specificity) to an autoantibody with reactivity to double stranded DNA and cardiolipin.19 Because each antigenic region of a protein consists of multiple overlapping epitopes,20 however, exact specificities of the relevant RFs are needed before we can select an interpretation of our findings.

Although IgG3 does not carry the 'Ga' or 'general' antigenic region for RFs, several studies have focused on this subclass as a target for RF specificities which may be relevant to the disease process in RA. Our results show that RFs which react with IgG3 epitopes are not as rare as was commonly believed from earlier studies with polyclonal anti-D sera.5 Indeed sera from about 50% of our patients with RA reacted with IgG monoclonal anti-D antibodies (Table 3). The importance of RFs synthesised by rheumatoid synovial cells and their
Rheumatoid factor specificity for Gm allotypes

We are grateful to Dr R Jefferis, who suggested the use of monoclonal anti-D antibodies in our study of RF specificities. We thank Dr N Hall and Dr R K Jacoby for serum samples from patients with rheumatoid arthritis and Yvonne Richards for the gift of 125I anti-IgG. We also appreciate the excellent secretarial help given by Mrs P Sanders. This work was supported by grants from the Northcott Devon Medical Foundation, the South Western Regional Health Authority, and the Wellcome Trust.

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Reaction of rheumatoid factors with IgG3 monoclonal anti-Rh(D) antibodies: more frequent reactivity to a monoclonal antibody of the Gm allotype G3m(5) in rheumatoid patients negative for G3m(5).

A H Puttick, E A Williamson, A H Merry, B M Kumpel, K M Thompson and V E Jones

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