The IgG Subclasses of Red Cell Antibodies and Relationship to Monocyte Binding

By Neil Abramson and Peter H. Schur

Purified IgG1, IgG2, IgG3, and IgG4 myeloma proteins were coupled to red blood cells with chromic chloride either individually or in various combinations. Only red blood cells coated with IgG1 or IgG3 formed rosettes; red cells coated with IgG4 appeared to predominate in this reaction. IgG2, IgG4, and IgA myeloma proteins coupled to red blood cells did not appear to inhibit the IgG1 and/or IgG3 on the red cells from forming rosettes. The IgG subclass specificity of a number of red cell isobodies was determined. The majority were IgG1 and IgG3, as previously noted. IgG2 was observed among some of these antibodies, namely in anti-D, anti-CD, anti-CDE, anti-s, and anti-Jk4. Although rosette formation was strongly associated with the relative amounts of IgG1 and IgG3, particularly the latter, on red cells, a number of exceptions were noted.

The mechanisms by which red blood cells are destroyed in immunohemolytic anemias are at present poorly understood. However, a model to explore these mechanisms has been developed: Red blood cells are coated in vitro with IgG antibodies and are then bound circumferentially about a monocyte to form a "rosette". The red blood cells become deformed and resemble cells that have been observed in vivo in some patients with immunohemolytic anemias. Subsequent studies utilizing individual myeloma proteins coupled to red blood cells have shown that the immunoglobulin receptor of the monocyte was specific for two of the four subclasses, IgG1 and IgG3. These studies suggested that red blood cells coated in vivo with IgG1 and/or IgG3 antibodies would be associated with hemolysis. This association has been indicated in one case where red cells were coated with IgG3, while red cells coated with IgG4 in another case were not associated with hemolysis. However, most IgG antibodies have been shown to consist of a mixture of two or more of the IgG subclasses, although antibodies consisting of only one subclass have been reported. Anti-Rh antibodies have been shown to consist primarily of IgG1 and IgG3.

This study was, therefore, undertaken (1) to investigate the interaction between the IgG subclasses and the subsequent effect of this interaction on
the binding of red cells to monocytes, (2) to describe the IgG subclass(es) of a
number of red cell antibodies with different specificities, and (3) to attempt to
correlate the IgG subclass of these red cell antibodies with the ability of these
sensitized blood cells to react with monocytes.

MATERIALS AND METHODS

Proteins

Proteins of IgG1, IgG2, IgG3, and IgG4 were isolated by starch block electrophoresis
from sera of patients with multiple myeloma.11 Purity was confirmed by immunoelectro-
phoresis12 against anti-whole sera and by micro-Ouchterlony and radial immuno-
diffusion,14 using antisera to the immunoglobulins including the other IgG subclasses.
Antisera were produced in rabbits and monkeys by direct immunization14,15 or by
immunizing partially tolerant animals.16 The antisera were made monospecific as necessary
by absorption with purified Bence Jones proteins and IgG subclasses that were often
coupled to solid immunoadsorbents.9 Monospecificity was judged by immunoelectrophoresis,
micro-Ouchterlony, and radial immunodiffusion. The 75 fraction of the absorbed antisera
was then isolated by gel filtration chromatography (Sephadex C-200).

Red cell isoantibodies were obtained either from patients previously immunized or from
another source (by Dr. C. A. Alper or purchased commercially). IgG fractions of isoanti-
obodies were isolated by DEAE-cellulose with 0.007 M phosphate buffer, pH 6.3.

Cells

White cells were obtained from heparinized peripheral blood by dextran sedimentation,
light centrifugation, and resuspension of the cell button in Hanks' balanced salt solution.17
Blood was always drawn from the same donor. All combinations of subclasses used in
experiments on subclass interaction were tested on the same white cell concentrate to
minimize variation in the monocyte count. Red cells were obtained from human blood by
defibrination, or were anticoagulated with heparin and were trace radiolabeled with
sodium 51chromate (50-150 μCi/ml of whole blood) according to standard techniques.18

Purified proteins were coupled to red cells using chromic chloride19 as follows: A
mixture was made of one part red cells (defibrinated blood washed six times with saline)
at a 50% hematocrit, five parts of a protein solution (1 g/100 ml of saline), and two
parts of chromic chloride (Cr3+) in saline (5 μmole Cr3+/ml of packed red cells for the
coupling of mixed IgG or IgG subclasses). The Cr3+ solution was added last. After 4 min
at room temperature, the reaction was terminated by four saline washes. For isoantibodies,
an equal volume of cells and antibody was incubated for 1 hr at 37°C.

Identification of Attached Proteins

Attachment of proteins to red cells was confirmed by a standard type Coombs' test
utilizing the purified anti-IgG subclass antibody. Antisera were heated at 56°C for 30 min
and absorbed twice with an equal volume of well-washed, packed, normal ABO red cells
at 37°C for 1 hr and again at 4°C overnight. The absorbed antisera possessed no aggluti-
nating or lytic activity for normal red cells. Coombs' tests were performed by mixing
one drop of 2% red cell suspension (washed with 100 volumes phosphate-buffered saline
or plain saline for chromic chloride-treated cells) and one drop antisera and centrifuging
for 45 sec. The cell button was gently suspended, and agglutination was judged according
to previously described criteria.4 Dilutions of antisera were made in phosphate-buffered
saline, and the agglutination titer reported was the reciprocal of the greatest dilution of
antisera causing agglutination. Tests were performed in triplicate.

Incubation Technique

White cell suspensions layered into vinyl cups resulted in monolayers, and red cells
were incubated over them in duplicate for 2 hr at room temperature. Four washes with Hanks’ solution removed unattached red cells. Rosette formation or red cell binding was quantitated by release of $^{51}$chromium-labeled hemoglobin after water lysis.

RESULTS

Table 1 shows the specificity of the IgG subclass antisera as performed with Coombs’ tests. IgG1 antisera (anti-IgG1) reacted with red cells coated with IgG1 at 1:20 dilution and cross-reacted weakly with IgG2- and IgG3-coated cells only when undiluted. IgG2 and IgG3 antisera were specific. IgG4 antisera detected IgG4-coated red cells but, when employed undiluted, weakly agglutinated IgG2-coated red cells.

Figure 1 represents the mean radioactivity released from red cells bound to leukocyte monolayer and the data points of each experiment performed in duplicate. Quantitation of the amount of the subclasses bound to red cells by chromic chloride was not obtained; however, equal concentrations of subclasses were employed, and attachment was confirmed semiquantitatively with purified subclass Coombs’ sera. Cells coated with IgG1 and IgG3 were avidly bound to the leukocyte monolayer, but cells coated with IgG2 and IgG4 were not. Binding appeared slightly more avid with IgG3-coated cells as compared to IgG1.

Figure 1 also shows the effect of combining two or more subclasses on red cells. When one subclass is placed on cells, a precise ratio of red cells and chromic chloride must be achieved in order to couple an adequate amount of protein and to avoid nonspecific agglutination that may occur with excess chromic chloride. Consequently, in remaining experiments the quantity of subclass protein varied depending on the number of proteins coupled. Thus, when two subclasses were coupled, each was employed at one-half concentration; when coupling three subclasses, each was employed at one-third concentration, and when coupling four subclasses, each was employed at one-fourth concentration. An IgA myeloma mixture was used as control to compare the effect of reducing the protein content of each IgG subclass. IgA-coated red cells would not bind to mononuclear cells.

Combining IgG1 and IgG3 failed to result in greater red cell binding than IgG1 or IgG3 combined with other subclasses. IgG4 combined with IgG3 (in three of five experiments) or with IgG1 resulted in less binding when compared

<table>
<thead>
<tr>
<th>Table 1. Specificity of Anti-IgG Subclass Antisera</th>
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<tr>
<td>Subclass Titer</td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td>RBC coated with IgG1</td>
</tr>
<tr>
<td>RBC coated with IgG2</td>
</tr>
<tr>
<td>RBC coated with IgG3</td>
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<tr>
<td>RBC coated with IgG4</td>
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</tbody>
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Purified IgG subclasses are coupled to red cells using chromic chloride. Titer represents the greatest dilution of anti-IgG subclass antisera that resulted in agglutination.
Fig. 1. IgG subclass interaction. Mean radioactivity represents $^{51}$Cr-labeled hemoglobin released from red cells bound to monolayers. Each datum point refers to an experiment performed in duplicate.

to other combinations of IgG1 or IgG5; however, the differences were not statistically significant. When three subclasses were combined on red cells, the greatest uptake occurred with IgG1, IgG2, and IgG3. Substitution of IgG4 appeared to decrease uptake, but the IgA controls did not confirm this impression. Addition of four subclasses further decreased red cell binding.

The IgG subclass characteristics of various red cell isoantibodies were studied using the IgG subclass Coombs’ sera. Isoantibodies were used as untreated serum, except in the case of weak antibody activity when a concentrate was prepared by DEAE-cellulose chromatography. Table 2 shows the results. The titer represents the reciprocal of the greatest dilution of Coombs’ sera that resulted in the agglutination of red cells coated with protein.

**Anti-D, Anti-CD**

Seven samples of anti-D were tested. IgG1 and IgG3 predominated in most instances. IgG2 was present occasionally in low titer. In testing two samples of anti-CD and one of anti-c, IgG1 and IgG3 again predominated.

Leukocyte monolayers tested the ability of these antibody-coated red cells to be detected by mononuclear cells. Rosette formation was quantified by measuring the uptake of radiolabeled red cells per white cell monolayer, with anti-D (Ham)-coated red cells used arbitrarily as 100% uptake. Anti-D (Ham) was used at the same concentration and ratio to red cells and monolayer as noted in previous publications. 4,17,19 The amount of rosette formation appeared proportional to quantities of IgG3 and IgG1 present on red cells. As seen with (S.A.), (Nis), and (Hel), when lesser quantities of IgG1 and IgG3
were present on red cells, then rosette formation was significantly decreased. Anti-CD (Pol) and anti-CD (Ges) gave greater quantities of bound IgG₃ as compared to anti-D (Ham) and increased rosette formation.

Table 2. Subclass Specificity and Rosette Formation of Isoantibodies

<table>
<thead>
<tr>
<th>Antibody Specificity</th>
<th>Subclass Titer</th>
<th>Rosette Formation</th>
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<tbody>
<tr>
<td></td>
<td>IgG₁</td>
<td>IgG₂</td>
</tr>
<tr>
<td>Anti-D (Ham)</td>
<td>80</td>
<td>160</td>
</tr>
<tr>
<td>Anti-D (Bre)</td>
<td>20</td>
<td>160</td>
</tr>
<tr>
<td>Anti-D (S.A.)</td>
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<td>10</td>
</tr>
<tr>
<td>Anti-D (Nis)</td>
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<td>0</td>
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<tr>
<td>Anti-D (Hel)</td>
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<td>20</td>
</tr>
<tr>
<td>Anti-D (ARH-1-3)</td>
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<td>20</td>
</tr>
<tr>
<td>Anti-D (ARH-1)</td>
<td>160</td>
<td>10</td>
</tr>
<tr>
<td>Anti-CD (Ges)</td>
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<td>Anti-CD (Ges) 1:2 dil</td>
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</tr>
<tr>
<td>Anti-CD (Ges) 1:4 dil</td>
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<tr>
<td>Anti-CD (Pol)</td>
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<tr>
<td>Anti-CD (Pol) 1:2 dil</td>
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<tr>
<td>Anti-CDE (Mur)</td>
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<td>640</td>
</tr>
<tr>
<td>Anti-D (Ham) + Anti-CDE (Mur)</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>Anti-D (Ham) + Anti-CDE (Mur)</td>
<td>40</td>
<td>320</td>
</tr>
<tr>
<td>Anti-CD (Ges) + Anti-CDE (Mur)</td>
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<td>Anti-c (ARHu-9)</td>
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</tr>
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<td>Anti-a (BGL)</td>
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<td>Anti-a (C338-8)</td>
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<tr>
<td>Anti-K (CKI-7)</td>
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</tr>
<tr>
<td>Anti-Kp⁺ (CK 4-6)</td>
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<td>0</td>
</tr>
<tr>
<td>Anti-Kp⁺ (BGL)</td>
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<td>0</td>
</tr>
<tr>
<td>Anti-Jk⁺ (CKA-16)</td>
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<td>20</td>
</tr>
</tbody>
</table>

* DEAE concentrate.

Subclass titer represents the greatest dilution causing agglutination using anti-IgG subclass antisera in Coombs' tests. Rosette formation was measured as amount of red cell binding to monolayers (using ⁸¹Cr-labeled red cells). Anti-D (Ham) was arbitrarily designated as 100% uptake. Letters and numbers in parentheses represent a coding system.
Anti-CDE, Anti-c

One sample of anti-CDE deposited IgG2 and IgG3 onto red cells. As estimated by IgG subclass Coombs' sera, IgG2 was present in greater quantity. Little or no red cell binding to monolayers occurred with anti-CDE (Mur). Addition of anti-D (Ham) or anti-CD (Ges) to anti-CDE (Mur) increased, though slightly, the quantities of bound IgG1 and IgG3 as compared to anti-CDE alone. The quantities of bound IgG2 remained essentially the same. Those mixtures significantly increased red cell binding to leukocyte monolayers. As determined by special tests using a red cell panel, the anti-E portion of anti-CDE functioned as an agglutinin. One anti-c contained IgG1 and IgG3 but produced minimal rosette formation.

Anti-s

In two samples of anti-s tested, IgG3 was found attached to red cells in both and minimal quantities of IgG2 were found in one. Greater quantities of IgG3 readily demonstrated rosette formation.

Anti-K

Three samples of anti-K had small quantities of IgG1. Rosette formation was minimal.

Anti-Kp

With anti-Kp, small quantities of IgG1 were found with two samples and IgG3 with one. Rosette formation occurred with IgG1 and IgG3 present on red cells but failed to occur with IgG1 alone.

Anti-Jkα

Of three samples of anti-Jkα studied, two were weak antibodies and resulted in no deposition of IgG subclasses even with IgG concentrates. The third showed minimal quantities of bound IgG2. No rosette formation was noted.

DISCUSSION

The present studies have explored the possible significance of the IgG subclasses of red blood cell antibodies in mediating hemolytic reactions. The model used to study this problem has been that of rosette formation between monocytes and sensitized red blood cells. Previous evidence has suggested that this phenomenon can serve as a model of the pathophysiologic events observed in vivo.1-3 This and other studies have now clearly shown that IgG1 and IgG3, but not IgG2 or IgG4, proteins when coupled to red blood cells will subsequently react with monocytes to form rosettes.4-5 In addition, the present studies confirm the observations of Huber et al.5 that rosette formation by IgG1 or IgG3 could not be inhibited by IgG2 or IgG4 proteins. IgG3 appeared to have more activity than IgG1, but the addition of IgG1 and IgG3 to red cells failed to further
increase binding to the monolayer. These data suggest that IgG1 and IgG3 compete for the same receptor on the mononuclear cell surface, which is consistent with the report that IgG1 blocked the uptake of red cells coated with IgG3.5

The quantities employed in the experiments of two IgG subclasses were one-half that used for attachment of proteins individually; hence IgA, which has no receptor on mononuclear cells, was used in combination with the subclasses as a control. It is unclear why IgG3-IgA-coated red cells had avid binding to monocytes. Since coupling of proteins to red cells with chromic chloride is nonimmunologic, the subclass interaction studied in this manner may be, in part, artificial and not applicable to the mononuclear cell receptor for the Fc fragment. However, it is presumed that chromic chloride results in attachment of IgG in such a manner that the Fc is free for monocyte interaction, because rosette formation of red cells coated with IgG in this way is blocked by the free Fc fragment. Although quantities of IgA were used to replace the subclasses on a mole basis, it is not known how IgA is coupled by chromic chloride and whether it affected the bound IgG subclasses in some way so as to alter monocyte binding. Because IgG2 or IgG4 bound to red cells in combination with IgG3 or IgG1 had little, if any, effect on rosette formation, except when expressed in terms of the IgG3-IgA control, it is presumed that there was no demonstrable subclass interaction or inhibition.

Less binding to leukocyte monolayers was observed as the number of IgG subclasses was admixed and subsequently combined to red cells. The mixture was not in physiologic ratio but at equal protein concentration. The observed decrease in red cell binding appeared related to lesser quantities of attached IgG1 and IgG3 once again, rather than to any specific blocking effect or interaction of all subclasses.

These studies, however instructive, represent an artificial system. Therefore, it was decided to study the IgG subclasses of a number of red cell isoantibodies and to determine the ability of these antibodies to cause rosette formation. Natvig et al., in a survey of anti-Rh antibodies noted that they were all IgG1 and/or IgG3.10 21 Frame et al., in a survey of 35 anti-D antibodies, five anti-c, one anti-e, 11 anti-K, one anti-Fy*, and one anti-Jk* antibodies, confirmed these findings but noted in addition that four of the anti-D were also in part IgG4 proteins.22 Gelfand et al. observed a case of acquired hemolytic anemia with erythrophagocytosis and rosette formation in a fresh buffy coat where IgGs, but not IgG1, IgG2, or IgG4, was detected on the red blood cells.6 Gergely et al. noted a case with a strongly positive antiglobulin test without increased red cell destruction where the eluted antibody was IgG4.7 The present studies confirm these observations that most red cell autoantibodies (tested) were IgG1 and IgG3. In addition, using Coombs' specific antisera, we were able to detect IgG2, plus IgG1 and IgG3, in a number of red cell isoantibodies, including anti-D, anti-CD, anti-CDE, and ant-s but not in those anti-c, anti-K, and anti-Kp* tested. The one anti-Jk* tested appeared to be entirely IgG2. One anti-CD also had some detectable IgG4, in addition to IgG1, IgG2, and IgG3.
Numerous studies of antibodies indicate that many of them are found predominantly in one subclass or another. If they are found primarily in the IgG1 and less so in the IgG2 subclass, then this is what one might expect based on the normal serum distribution of these immunoglobulin subclasses (IgG1, 64%-70%; IgG2, 23%-28%; IgG3, 4%-7%; and IgG4, 3%-4% of total IgG). However, when antibodies are present in only one or two subclasses, or if IgG3, IgG4, or IgG2 are overrepresented proportional to their serum concentration, this then suggests marked, genetic restriction of the antibody response to certain antigens. Similar observations have been made in rabbits in response to bacterial polysaccharides. Whether different types of antigenic sites, such as those with carbohydrates, determine the subclass of the immune response, even to some degree, is an intriguing possibility that is certainly suggested by results from different laboratories. In addition, as more is learned about the biological role of each IgG subclass, a better understanding can be developed of the significance of finding antibodies, or immunoglobulins, within a restricted number of these subclasses.

The correlation between IgG subclass of these isoantibodies and their ability to form rosettes with monocytes was also investigated. It appeared that there was a good association between rosette formation and the presence and relative amounts on red cells of IgG1 and IgG4, particularly the latter, for those anti-D, anti-CD, anti-s, and anti-Kb antibodies studied. Where relatively large amounts of IgG1 and IgG4 were present, there was a considerable amount of rosette formation (anti-D, anti-CD, anti-s). When these antibodies were serially diluted (anti-CD), or when less antibody of IgG1 and/or IgG4 was present (some anti-D, anti-K), then this was associated with less rosette formation. However, a few exceptions to this pattern were seen, namely one example each of an anti-CDE, anti-c, and anti-Jk*. These were not associated with rosette formation. It seemed reasonable not to expect rosette formation with the anti-Jk* since it appeared to consist only of IgG2, a subclass that does not mediate rosette formation.

The results with anti-CDE are most intriguing. This anti-Rh antibody differed from others because of significant quantities of bound IgG4 and inability to mediate rosette formation. Whether IgG2 isoantibodies blocked rosette formation by the IgG1 and IgG4 molecules also present on the cell was investigated by adding anti-D, known to be high in IgG1 and IgG4, to the test system. This resulted in somewhat increased amounts of detectable IgG1 and IgG4 on red cells, as well as increased rosette formation. These limited studies, therefore, suggested that IgG2 isoantibodies, when present in sufficient amounts, might partially block rosette formation by IgG1 and IgG4. This blocking effect could not be reproduced when IgG3 myeloma proteins were mixed with purified IgG1 and IgG3 and coupled to red cells with chromic chloride. More studies are obviously needed to resolve some of these problems.

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