Serology of Acquired Hemolytic Anemias

By W. Weiner and G. H. Vos

If a serum contains a gamma globulin which will react with (agglutinate, hemolyze or sensitize) certain genetically well-defined red blood cells but not others, it is said to possess an antibody and the antibody is said to be specific for a certain property of the red cells. Thus the specificity of an antibody depends on this selective reactivity. This is often difficult to demonstrate if the range of activity is widespread and if there are only a very few cells available which will lack the property against which the antibody reacts. A good many blood grouping laboratories have in their freezers, sera containing antibodies against "Public Antigens" which up to now remained unclassified because they react with all available red cells except those of the patient or donor. Nobody, however, will dispute the antibody nature of these reagents or call them "unspecific." It is remarkable that this point of view is not applied to the antibodies found in acquired hemolytic anemia (AHA). Some of these antibodies have been demonstrated to have narrow blood group specificity, but nevertheless some workers designate the majority of them as "unspecific" and even doubt that they are antibodies. If these "unspecific" agents could be shown to be "specific," that is, not to react with certain genetically well-defined red cells, more workers might be persuaded to regard them as true antibodies. We, therefore, thought that a useful purpose might be served by investigating hemolytic anemias with the use of a large cell panel which included some rare and very rare cells.

Material and Methods

As has been pointed out previously, these investigations should be performed with eluates obtained from the red cells rather than with sera, as the latter often do not contain all the antibodies one can find in the eluates. We used eluates which had been prepared in the past and stored for varying periods at -25 C. or we prepared fresh eluates from frozen cells also stored for varying periods. In some cases, cells from the patient or donor had been laid down in glycerol and frozen. These were reconstituted and then eluted. The eluates were prepared either by the alcohol method or the ether method and eluates which were derived from aliquots of the same blood samples gave identical results. An eluate was included if it still worked, if it was available in sufficient quantity for the tests to be performed and if it did not show specificity against any of the well known antigens (and was thus "pan-agglutinating"). The eluates were tested against a large panel of test cells with common rhesus genotypes and the following selected rare samples: Cde/Cde, cde/cde, CdE/cde, CDE/cde, cde/cde KK, one D-/D-- blood, one Dc-/Dc- sample and the unusual blood described by Vos et al.: The cell panel from the rhesus antigen point of view thus consisted of: "normal" cells (not deleted:nl), "partially" deleted cells (pdl) and "fully" deleted (dl) cells. The rare types of red cells had been kept in glycerol and were of approximately the same age. They were reconstituted by
the dialysis method. The more common cells were obtained from laboratory staff or donors and were used not later than 24 hours after collection. In Birmingham, papain was used according to the method previously described; in Perth, bromelin was used preferentially. The tests were performed with the usual positive and negative control. Indirect Coombs tests were done in many instances to confirm doubtful results but generally the enzyme method proved to be somewhat more sensitive. None of the eluates produced agglutination of saline-suspended cells. Most eluates agglutinated cells suspended in bovine albumin but the reactions were much better with enzyme-treated cells and the latter method was, therefore, generally used.

Through the courtesy of Dr. Levine, we were also able to test the red cells with guinea-pig "anti-D-like" (Rh LW) antibody. Dr. Wallace was kind enough to send us samples of blood from "Mrs. G." whose cells, though of the genotype CDe/cde, lack the "D-like" antigen and whose serum contains "anti-I)-like" (Rh LW) antibody. We were thus able to test for the presence of the "D-like" (Rh LW) antigen and to compare some eluates with the "anti-D-like" (Rh LW) antibody.

Absorptions and Re-elutions

Absorptions were done in the following way: 1 volume of packed red cells (usually unmodified) was incubated with 1 volume of eluate at 37 C. for 2 hours. The supernatant was removed and kept. The red cells were then (without washing) incubated for another 2 hours with 3-4 volumes of the same fresh eluate and were then eluted.

Titrations

Titrations were done using doubling dilutions of the eluates in saline and enzyme-treated cells. Sixty-six eluates were found to be available in large enough quantities for more detailed investigation. Six which showed "blood-group specificity" were excluded. Of the 60 which remained, 10 had been obtained from "normal" donors who were accidentally discovered to have "coated" red cells. Details of these "normal" donors will be described elsewhere. The remaining 50 eluates had been obtained from patients suffering from typical autoimmune hemolytic anemia (warm type) of varying severity.

Results

It should be stated briefly that none of the eluates investigated showed specificity with regard to any of the "public antigens" we could test. Cells lacking "public antigens" (like Vell, Ge, U and other "minus-minus" phenotypes) were as strongly agglutinated by the eluates as unselected red cells. Some eluates failed to react with --- red cells (see below) and this suggested that they might contain an "anti-D-like" (Rh LW) antibody. This possibility was excluded because (1) the eluates could not be converted into specific "anti-D-like" (Rh LW) reagents by absorption on, and re-elution off, Rhesus negative red cells and when eluted had the same specificity (or apparent lack of it) as before; (2) Rh positive red cells fully coated with eluates were strongly agglutinated by the specific guinea-pig "anti-D-like" (Rh LW) antibody; (3) Mrs. G's serum behaved identically, indicating that the agglutination by the guinea-pig serum was not due to an anti-species antibody; (4) Mrs. G's cells which lack the "D-like" antigen were agglutinated as strongly as unselected cells by all eluates including those which did not agglutinate the --- cells; (5) re-elution of these eluates from Mrs. G's cells produced no evidence of specific "anti-D-like" (Rh LW) activity in the re-eluate.

The pattern of reactivity of the antibodies present in the 60 eluates again suggested that they are related to the system of Rhesus antibodies. Like the
latter, most eluates will give reactions with albumin-suspended cells, enzyme-treated cells and react in the indirect Coombs test. There were only two eluates (one from a patient and the other from a donor) which reacted poorly with enzyme-treated red cells, but gave excellent titers when tested with the indirect Coombs test. These differences are, however, also occasionally noted in the reactivity pattern of Rh antigena. It is also important to note that eluates possessing "specific activity" almost always react with an antigen within the Rh system (e.g. e, c, etc.). Lastly we observed that some eluates can be used in the same way as some anti-D sera in the investigation for the Gm factors.\textsuperscript{11} It was natural that we considered it of importance to investigate the eluates having regard to specificity within the Rh system in the first instance. Though narrow blood group specificity could again be excluded using the large cell panel, the inclusion of red cells showing "partial" or "full" deletion of known Rh antigens clearly indicated that most of them were "specific." In a previous communication\textsuperscript{7} it was mentioned that when the "partially deleted cells (D--/D--) were used, together with other "normal" red cells, the eluates fell into three groups which were called Class 1, Class 2 and Class 3, depending on whether they reacted as strongly with D--/D-- cells as with "normal" cells (Class 1), weaker with the former (Class 2) or not at all with them (Class 3). This classification was confirmed in this investigation but the inclusion of the "completely" deleted ---/--- cells necessitated a further subdivision. Thus some Class 1 eluates reacted equally strongly with ---/--- cells (1a), others reacted weaker (1b), and the rest showed no reactivity with them (1c). Class 2 eluates could be subdivided in the same manner (2a, 2b, 2c) but Class 3 eluates gave only one pattern of reactivity, i.e., they did not react with the deleted (---/---) cells at all. Table 1 shows the reactivity of the 60 eluates.

There was no correlation between the class and subdivision of the eluates with the ABO groups of Rhesus genotypes of the patients or donors who produced the antibodies. Both ABO groups and genotypes showed the expected distribution.

Amongst the patients, there were 28 men and 24 women. Of the "normal"
Table 2.—Distribution of the Various Classes of Eluates between Male and Female, Patients and Donors

<table>
<thead>
<tr>
<th></th>
<th>Class 1</th>
<th>Class 2</th>
<th>Class 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
<td>c</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Patients</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Donors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It would appear that more women than men develop an antibody which will react with the ---I--- cell, but the differences shown in the table are statistically not significant.

Table 2 also shows that of a total of 60 eluates investigated, 32 failed to react against the ---I--- cell and an additional 11 eluates showed less activity for these cells than against "normal" cells. Thus a total of 43 (71 per cent) eluates did not react or reacted weakly against ---I--- red cells when compared with the reactivity against "normal" red cells. This pattern of reactivity suggested a mixture of antibodies in the "reacting" eluates and absorption and re-elution experiments confirmed this impression.

A few typical examples only will be given. Eluate B.A.B. gave the following reactions: ++++ against normal cells, ++++ against the D--/D-- cell and +++ against the ---I--- cell. When this eluate was absorbed with ---I--- cells, the antibody against ---I--- was taken out but the reactivity against normal cells and D--/D-- was almost identical with the pre-absorption activity. A further absorption with D--/D-- left an antibody in the supernatant which reacted exclusively with "normal" cells. Re-elution of these cells produced reagents which had the expected reactivity (table 3).

Table 4 shows a similar absorption experiment on eluate L.A. Eluate B.A.B. came from a normal donor (table 3) and eluate L.A. from a very ill child (table 4).

Non-reactivity of certain eluates against D--/D-- and/or ---I--- red cells might have been thought to be the result of qualitative rather than quantitative factors. We could, however, demonstrate that the classes of the eluates obtained were independent of the titers of the eluates against "normal" cells. Examples of scores of different eluates against different red cells are shown in table 5. It can be seen that the scores (or titers) against normal cells certainly have no influence on the class or subdivision of the eluate. In Class 3, for instance, scores range from 25 to 85 and in other classes conditions were similar.

We found three eluates which behaved differently from the rest. Two Class 1a eluates could be fully absorbed by ---I--- cells. On re-eluting the latter, the re-eluate had again full reactivity against all cells of our panel. The third...
eluate (Class 1c) was similarly absorbable by $D_{--}/D_{--}$ cells which on re-eluting gave off a reagent fully active against the whole cell panel (with exception of the $---/---$ cell). It is possibly worth mentioning that these three eluates (incidentally obtained from "normal" donors) were also fully absorbable by "normal" cells.

**DISCUSSION**

From our results we came to realize that the eluates of cells of patients with AHA and from "normal" donors contained three different types of antibodies.

The antigens which we think are reacting with these antibodies we have tried to show in Table 6.

Our results suggest that the eluates may contain anti-nl, anti-pdl, and anti-dl, singly or in combination. Thus Class 1a eluates will contain anti-nl, anti-pdl and anti-dl in approximately equal strength. However, an eluate

**Table 3.—Absorption Experiments**

<table>
<thead>
<tr>
<th>Eluate B.A.B.</th>
<th>cdE/cdE</th>
<th>D_{--}/D_{--}</th>
<th>Cde/Cde</th>
<th>cde/cde</th>
<th>---/---</th>
<th>CDe/cDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1. Original eluate</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>No. 2. Eluate absorbed $---/---$</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>No. 3. Eluate absorbed $D_{--}/D_{--}$</td>
<td>3</td>
<td>–</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>No. 4. No. 2 absorbed $D_{--}/D_{--}$</td>
<td>2</td>
<td>–</td>
<td>2</td>
<td>2</td>
<td>–</td>
<td>2</td>
</tr>
<tr>
<td>No. 5. Eluate re-eluted off $---/---$</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>No. 6. Eluate re-eluted off $D_{--}/D_{--}$</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

The figures indicate degree of agglutination (0–4) of papainized cells by the eluates.

**Table 4.—Absorption Experiments**

<table>
<thead>
<tr>
<th>Eluate L.A.</th>
<th>cdE/cdE</th>
<th>D_{--}/D_{--}</th>
<th>Cde/Cde</th>
<th>cde/cde</th>
<th>---/---</th>
<th>CDe/cDE</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1. Original eluate</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>No. 2. Eluate absorbed $---/---$</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>No. 3. Eluate absorbed $D_{--}/D_{--}$</td>
<td>4</td>
<td>–</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>No. 4. No. 2 absorbed $D_{--}/D_{--}$</td>
<td>4</td>
<td>–</td>
<td>3</td>
<td>3</td>
<td>–</td>
<td>3</td>
</tr>
<tr>
<td>No. 5. Eluate re-eluted off $---/---$</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

The figures indicate degree of agglutination (0–4) of papainized cells by the eluates.

**Table 5.—Scores of Eluates against Papainized Cells**

| Class 1a eluate | 30 | 30 | 30 |
| Class 1b eluate | 40 | 40 | 34 |
| Class 2c eluate | 85 | 56 | – |
| Class 2c eluate | 59 | 20 | – |
| Class 3 eluate | 85 | – | – |
| Class 3 eluate | 45 | – | – |
containing only anti-dl will also be classed as 1a until absorption experiments have proved it to contain this antibody only. We encountered two such eluates which were described above. Similarly an eluate containing anti-nl and anti-pdl in equal strength will be classed as 1c. Again we found one eluate which also had to be classified as 1c but this contained anti-pdl only. The Class 2 eluates we found to contain at least two antibodies, the anti-pdl being of lower titer than the anti-nl. Class 3 eluates obviously contain anti-nl only. We were not surprised to find that one person forms one antibody whereas another may form two or three against related antigens. This is common experience in people developing antibodies within the rhesus system (e.g., anti-D plus anti-C and anti-C). It appears that all "normal" cells contain the antigen we designated as nl. This may be produced by a gene on the E locus as it is absent from the D--/D-- and the Dc-/Dc- cells. On the other hand, it may be a "composite" antigen whose production depends on the presence of a full complement of "normal" genes. Its absence from the partially deleted cells could be explained by either hypothesis. The anti-pdl antibody may react with an antigen produced by a gene near the D locus, nearer in fact than the C gene as both D--/D-- and Dc-/Dc- carry it. It is not a part of the D antigen as it can be found on D negative cells in the same strength as on D positive ones. It is also different from the "D-like" antigen and, as we have shown, occurs on all "normal" cells. As to the dl antigen, this has, as far as our present experience goes, a universal distribution and could, if this expression had any serologic meaning, be called "unspecific." Our investigation has thus demonstrated that the "fully" deleted cell is not the "last word" in deletion and that it still carries an antigen capable of reacting with an antibody. The antigen occurs, of course, also on the partially deleted (D--/D--) cells.

It is not clear which factors determine the type of antibody which is formed in particular patients. The severity of the illness has apparently no bearing on this question as all types of eluates were found in all types of patients right down to "normal" donors. Similarly the genotype of the patient or donor has no bearing on the development of a particular class of antibody, nor has sex. At present it is impossible to explain why some people form an antibody, e.g., against the c or e antigens and others against the antigens described in this paper, though all patients or donors who could be investigated carried the latter antigens on their cells. It would have been impossible to be certain about this for when the eluates were first obtained, the cells were of course heavily coated and unsuitable for investigation with these "blocking" antibodies. Some of the donors and a few patients followed for years became "Coombs negative" and their cells were found to be agglutinated.
by all classes of eluates. They showed normal reactivity. Clearly reactivity was also found when the cells of the patients or donors were investigated with Rhesus antisera. In other words, we could find no evidence that any of the antigens were missing, less well-developed or unusual.

It has previously been found that the antibodies in these eluates are unsuitable for the discovery of heterozygous deleted red cells as they are incapable of giving definite double dose effects. \(^3\) (Class 3 eluates would, of course, be suitable for the detection of the homozygous D--/D-- cell). Thus cDE/D-- red cells gave in our hands the same score as cDE/cde or cDE/cDE bloods when titrated against an eluate of Class 3 (or a suitably absorbed eluate of Class 1 or 2). Apart from the unusual blood described by Henningson\(^3\) which is in all likelihood a heterozygous --- red cell, no other sample of this nature has been described and we were thus not able to use a blood sample of this genetic constitution in our investigation.

The investigation of the antibodies contained in the eluates of our patients and "normal" donors has thus demonstrated three antigens within the Rh system, antigens which seem to be additional to the ones recognized by the usually applied Rh antisera. They have demonstrated that the "fully deleted cell" still carries an antigen. Is this antigen the "ground substance" of the Rh system? We think not, for if this antigen was "ground-substance" one would expect that it would have been used up in the "normal" cells by the "normal" antigens and, on the other hand, the antigen present on the "fully deleted cell" may represent a "part" of the complex Rh system necessary for the expression of other Rh antigens. We think, therefore, that the antibody in the eluate (anti-DI) indicates indeed an antigen which is, as far as we can tell at present, universally distributed. It is, of course, conceivable that blood samples exist where every recognizable Rh antigen has been shed (if this would be compatible with life) and these cells should then not show any reactivity with the eluates of cells from patients of AHA.

### SUMMARY

The investigation of 60 "pan-agglutinating, unspecific" eluates has disclosed distinct blood group specificity within the rhesus system in at least 70 per cent. This has increased our confidence in their true antibody nature. Their investigation has also contributed to our understanding of the makeup of this complex system of Rhesus antigens and antibodies.

### SUMMARIO IN INTERLINGUA

Le investigation de 60 eluatos "pan-agglutinante e nonspecific" ha revelate in al minus 70 pro cento del casos un distincte specificitate de gruppo de sanguine intra le systema rhesus. Iste facto ha reinfortiate nostre confidentia in lor genuin proprietates anticorpore. Lor investigation ha etiam contribuite a nostre comprehension del complexe systema rhesus de antigenos e anticorpore.

### ACKNOWLEDGMENTS

This investigation could not have been done had it not been for the great help we received with blood specimens and discussions from Dr. R. R. Race and Dr. R. Sanger, and
SEROLOGY OF ACQUIRED HEMOLYTIC ANEMIAS

the many rare and very rare cells from Dr. T. E. Cleghorn. Dr. M. Shapiro sent the very rare blood of Mrs. Shabalala and we are greatly indebted to Dr. J. Wallace for blood samples from "Mrs. G." Generously, as usual, Dr. P. Levine has supplied us with the "anti-D-like" (Rh LW) antibody and we are greatly indebted for his help. One of us (G. H. V.) would like to acknowledge the kindness of Dr. G. A. Kelsall for permission to collaborate in this joint investigation and we wish to acknowledge the assistance of the King Edward Memorial Hospital for Women for their financial help to obtain and dispatch the rare blood from "Mrs. E. N." We also wish to thank sincerely Dr. J. Morrison for all the help he gave.

REFERENCES


G. H. Vos, Senior Technical Officer, King Edward Memorial Hospital for Women, Subiaco, Western Australia.
Serology of Acquired Hemolytic Anemias

W. WEINER and G. H. VOS