JCI The Journal of Clinical Investigation

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Louis K. Diamond, Neva M. Abelson

*J Clin Invest.* 1945;24(1):122-126. https://doi.org/10.1172/JCI101573.

Research Article



# THE IMPORTANCE OF RH INHIBITOR SUBSTANCE IN ANTI-RH SERUMS[1]

By LOUIS K. DIAMOND and NEVA M. ABELSON

*(From the Infants' and the Children's Hospitals, the Department of Pediatrics, Harvard Medical School, and the Blood Grouping Laboratory, Boston, and from the Department of Pediatrics, University of Pennsylvania School of Medicine, Philadelphia)*

(Received for publication December 5, 1944)

In July, 1943, when attempts to pool serums preparatory to their concentration by a globulin fractionation method were begun in this laboratory, it was observed that the addition of potent anti-Rh′ serum[2] (which agglutinates $Rh_1$ cells) to equally potent anti-$Rh_0$ serum (which agglutinates $Rh_1$ and $Rh_2$ cells) produced a mixture which inexplicably was incapable of agglutinating $Rh_2$ cells. At the time, the reason for this wholly unexpected result was not appreciated. In the ensuing year, similar attempts to pool serums of different anti-Rh specificity, before concentration, led to the same unfortunate result; that is, anti-Rh agglutinin seemed lost in the process. This led to the supposition that an "inhibitor substance" occurred in some serums.

Race (2) has recently (1944) described a "partial or incomplete antibody" and Wiener (3) has independently described (1944) a "blocking antibody" in some serums which, when added to certain Rh-positive cells, is capable of preventing their agglutination by serums of specificity anti-$Rh_0$. These observations, together with those made by us during the last year and a half, afford an explanation for the behavior of these serums which, although individually of high titer (active in dilution of 128 or more), are partially inactivated when pooled. The results of such an experiment are seen in Table I. It is now clear that certain serums contain an "inhibitor substance" or "blocking antibody." It was because of the action of such inhibitor substance that mixture of serums containing individually active anti-Rh agglutinins yielded pools with diminished or undemonstrable activity.

Three of our inactive pools were recently re-examined. In one of them, there was an inhibitor substance which interfered with the action of anti-$Rh_0$, and therefore might be said to be of specificity $Rh_0$. This is the type of inhibitor substance which has already been described (2, 3). In the remaining two pools, inhibitor substances of specificity $Rh_0$, Rh′, and Rh″ were demonstrable; that is, they interfered with the action of these specific antiserums.

TABLE I

*Demonstration of inhibitor substance by mixing two types of anti-Rh serums*

| | Degree of agglutination | | | |
|---|---|---|---|---|
| Type of cell | $Rh_2$ | $Rh_1$ | Rh′ | rhrh* |
| Control No. 1: 2 drops 2 per cent cell suspension + 1 drop saline + 1 drop anti-Rh′ serum | 0 | ++++ | ++++ | 0 |
| Control No. 2: 2 drops 2 per cent cell suspension + 1 drop saline + 1 drop anti-$Rh_0$ serum | ++++ | ++++ | 0 | 0 |
| 2 drops 2 per cent cell suspension + 1 drop anti-Rh′ serum + 1 drop anti-$Rh_0$ serum | 0 | ++++ | ++++ | 0 |

* rhrh refers to the Rh-negative cell.

These inhibitor substances of specificity Rh′ and Rh″ are similar in behavior to that of specificity $Rh_0$, or to the "partial" or "blocking antibody" of Race and Wiener; they are associated with the cells after brief mixture of cells and serum, and are not dislodged after repeated washing of cells with saline.

The inhibitor substance seems to be more stable than the corresponding Rh antibody. Prolonged heating at 56° C., which inactivates anti-Rh agglutinins, weakens the activity of the inhibitor substance but slightly, and exposure to merthiolate solution over a period of time, while inactivating anti-Rh agglutinins, appears

[1] A part of the work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

[2] The nomenclature recently proposed by Wiener (1) is here employed.

TABLE II

*Effect of heating (56°C.) on serum containing inhibitor substance and anti-Rh agglutinin*

| Type of cell | Degree of agglutination | | |
|---|---|---|---|
| | $Rh_2$ | $Rh_1$ | rhrh |
| Control No. 1: 2 drops 2 per cent cell suspension + 1 drop saline + 1 drop anti-$Rh_0$ serum | +++ | +++ | 0 |
| Control No. 2: 2 drops 2 per cent cell suspension + 1 drop saline + 1 drop unheated anti-Rh′ serum | 0 | +++ | 0 |
| Control No. 3: 2 drops 2 per cent cell suspension + 1 drop unheated anti-Rh′ serum + 1 drop anti-$Rh_0$ serum | 0 | +++ | 0 |
| 2 drops 2 per cent cell suspension + 1 drop anti-Rh′ serum heated 15 hours at 56°C. + 1 drop anti-$Rh_0$ serum | 0 | 0 | 0 |

not to affect the inhibitor substance. However, these measures do not raise the titer of inhibitor substance. We have, therefore, to conclude that these procedures do not produce inhibitor substance, but rather unmask its action. This is illustrated by Tables II and III.

The observation that heating at 56° C. would inactivate or weaken anti-Rh agglutinin, leaving the inhibitor substance relatively unaffected, led to the re-examination of a number of our anti-Rh serums. Sixteen antiserums were heated at 56° C. for 15 hours and subsequently tested for their inhibiting properties by mixing them with $Rh_1$ and $Rh_2$ cells against anti-$Rh_0$, anti-Rh′, and anti-Rh″ serums. These tests are set forth in Table IV.

It is evident from these data that 13 of the serums contained inhibitor substance of specificity $Rh_0$, and 5 contained appreciable amounts of inhibitor substance of specificity $Rh_0$ and Rh′. One, Serum No. 14, appeared to contain no inhibitor substance demonstrable by this method. Serums Nos. 15 and 16 were from Rh-positive women. In one, an anti-Rh″ serum, was found a substance inhibiting the action of anti-Hr (that is, the antibody agglutinating Rh-negative cells and certain Rh-positive cells), anti-$Rh_0$, and anti-Rh′; in the other, an anti-Hr serum, there was substance inhibiting the action of anti-$Rh_0$, anti-Rh″, anti-Rh′, and anti-Hr. The inhibitor substances from these 2 serums, however, were easily washed off the cells. This raises the question as to their being true antibodies.

In addition to these 16 serums, we have had the opportunity to examine 50 other serums from pregnant women who had previously delivered, or who subsequently delivered, infants with unmistakable signs of erythroblastosis fetalis. These serums were examined without any attempt to destroy anti-Rh agglutinins and thus unmask inhibitor substance which might be present. Of the 50 serums so examined, 30 contained anti-Rh agglutinins, either alone or of such high titer as to mask any inhibitor substance

TABLE III

*Titration of inhibitor substance in heated and unheated serum*

| Dilution of anti-Rh′ serum | 1 : 0 | 1 : 2 | 1 : 4 | 1 : 8 | 1 : 16 |
|---|---|---|---|---|---|
| | Degree of agglutination | | | | |
| 2 drops 2 per cent suspension $Rh_2$ cells + 1 drop anti-Rh′ serum, unheated, + 1 drop anti-$Rh_0$ serum | 0 | 0 | 0 | 0 | + |
| 2 drops 2 per cent suspension $Rh_2$ cells + 1 drop anti-Rh′ serum, heated at 56°C. 15 hours, + 1 drop anti-$Rh_0$ serum | 0 | 0 | + | +± | ++ |
| 2 drops 2 per cent suspension $Rh_1$ cells + 1 drop anti-Rh′ serum, unheated, + 1 drop anti-$Rh_0$ serum (Control) | ++++ | ++++ | ++++ | ++++ | ++++ |
| 2 drops 2 per cent suspension $Rh_1$ cells + 1 drop anti-Rh′ serum, heated, + 1 drop anti-$Rh_0$ serum | 0 | 0 | + | ++ | ++ |
| 2 drops 2 per cent suspension $Rh_1$ cells + 1 drop saline + 1 drop anti-$Rh_0$ serum (Control) | +++ | +++ | +++ | +++ | +++ |
| 2 drops 2 per cent suspension $Rh_2$ cells + 1 drop saline + 1 drop anti-$Rh_0$ serum (Control) | ++++ | ++++ | ++++ | ++++ | ++++ |

TABLE IV

*Demonstration of inhibitor substance in various anti-Rh serums*

| Type of cell | | $Rh_2$* | | $Rh_2$ | | $Rh_1$ | | $Rh_1$ | |
|---|---|---|---|---|---|---|---|---|---|
| Type of antiserum | | Anti-$Rh_0$ | | Anti-Rh″ | | Anti-$Rh_0$ | | Anti-Rh′ | |
| | | Degree of agglutination with: | | | | | | | |
| Unknown Serum: *Specificity | | Heated, unknown serum added | Control (saline added) | Heated, unknown serum added | Control (saline added) | Heated, unknown serum added | Control (saline added) | Heated, unknown serum added | Control (saline added) |
| No. 1 | Anti-$Rh_0$′ | +++ | ++++ | +++ | ++++ | 0 | ++++ | 0 | ++++ |
| No. 2 | Anti-$Rh_0$′ | ++ | ++++ | +++ | ++++ | 0 | ++++ | ++ | ++++ |
| No. 3 | Anti-$Rh_0$ | ++ | ++++ | ++++ | ++++ | 0 | ++++ | +++ | ++++ |
| No. 4 | Anti-$Rh_0$ | +++ | ++++ | ++++ | ++++ | ++ | ++++ | ++++ | ++++ |
| No. 5 | Anti-$Rh_0$′ | 0 | ++++ | ++++ | ++++ | + | ++++ | + | ++++ |
| No. 6 | Anti-$Rh_0$ | +++ | ++++ | ++++ | ++++ | 0 | ++++ | 0 | ++++ |
| No. 7 | Anti-$Rh_0$ | ++ | ++++ | ++++ | ++++ | 0 | ++++ | 0 | ++++ |
| No. 8 | Anti-$Rh_0$ | 0 | ++++ | ++++ | ++++ | ++ | ++++ | +++ | ++++ |
| No. 9 | Anti-$Rh_0$ | + | ++++ | ++++ | ++++ | ++ | ++++ | +++ | ++++ |
| No. 10 | Anti-$Rh_0$ | 0 | ++++ | +++ | ++++ | 0 | ++++ | +++ | ++++ |
| No. 11 | Anti-Rh′ | 0 | ++++ | ++++ | ++++ | 0 | ++++ | +++ | ++++ |
| No. 12 | Anti-Rh′ | 0 | ++++ | ++++ | ++++ | 0 | ++++ | ++++ | ++++ |
| No. 13 | Anti-Rh′ | 0 | ++++ | ++++ | ++++ | 0 | ++++ | +++ | ++++ |
| No. 14 | Anti-$Rh_0$′ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| No. 15 | Anti-Rh″ | + | ++++ | ++++ | ++++ | ++ | ++++ | ++ | ++++ |
| No. 16 | Anti-Hr | 0 | ++++ | + | ++++ | + | ++++ | + | ++++ |

* "Specificity" indicates the types of agglutinins present as demonstrated in the test tube. It does not indicate "masked" agglutinins.

which may have been present; 10 contained inhibitor substance of such high titer as to mask accompanying Rh agglutinins, and 10 contained both demonstrable agglutinins and inhibitor substance.

Cells of different specificity vary in their agglutinability by different serums; for example, we have found $Rh_2$ cells to be more readily agglutinated by most of our anti-$Rh_0$ serums (the majority of which, as shown above, contain varying amounts of inhibitor substance) than $Rh_1$ cells. Conversely, Rh′ cells appear to be more easily agglutinated by anti-Rh′ serums than are $Rh_1$ cells. It is difficult to say whether this variation is due to differential sensitivity to agglutinin or to inhibitor substance. The reactions of various cells with serums containing little or no demonstrable inhibitor substance of specificity Rh′, $Rh_0$, and Rh″, respectively, and with the same serums diluted with inhibitor substance, is illustrated in Table V.

The inhibitor substance is capable of becoming

TABLE V

*Variation in action of anti-Rh agglutinin and inhibitor substance with cells of different specificity*

| Type of serum | Anti-Rh′ | | Anti-$Rh_0$′ | | Anti-Rh″ | |
|---|---|---|---|---|---|---|
| | Antiserum + saline 1 : 1 | Antiserum + inhibiting serum 1 : 1 | Antiserum + saline 1 : 1 | Antiserum + inhibiting serum 1 : 1 | Antiserum + saline 1 : 1 | Antiserum + inhibiting serum 1 : 1 |
| | Titer | Titer | Titer | Titer | Titer | Titer |
| Type of cell | | | | | | |
| $Rh_2rh$ or $Rh_2Rh_2$ | 0 | 0 | 256 | 16 | 256 | 64 |
| $Rh_1Rh_1$ | 16 | 0 | 64 | 4 | 0 | 0 |
| $Rh_1Rh_2$ | 16 | 0 | 256 | 16 | 32 | 8 |
| Rh′Rh′ | 64 | 8 | 16 | 0 | 0 | 0 |

Anti-Rh′ is the "70 per cent" serum. Anti-$Rh_0$′ is the "87 per cent" serum. Anti-Rh″ is the "30 per cent" serum.

attached to the cell at room (20° C.) and even at refrigerator (4° C.) temperatures, although incubation at 37° C. may accelerate the process. Chemically, the substance appears to behave as a protein. It is precipitated in the coagulum with heat and alcohol. Its titer is not reduced by dialysis for 24 hours in a collodion membrane, but is appreciably reduced (as is the titer of anti-Rh agglutinin) by 4 days of dialysis.

All evidence thus far points to the inhibitor substance being an antibody, as Race and Wiener have assumed. The facts that it is associated with the red cells, that it appears to behave chemically in the same way as the anti-Rh agglutinin, and that it has been found only in bloods of individuals sensitized to the Rh factor support this point of view. That the fetus is not responsible for the production of inhibitor substance is indicated by the demonstration of such substance in high titer in bloods of men and of nulliparous women sensitized by transfusion. The occurrence in certain bloods of inhibitor substance incapable of attachment to red cells, however, raises the question as to whether this "blocker" may be an intermediary which acts as an antibody only because of association with a weak agglutinin.

The possibility of using the inhibitor substance to change the course of congenital hemolytic disease of the newborn (erythroblastosis fetalis) by protecting the red blood cells against the action of anti-Rh agglutinin immediately suggested itself. The following *in vivo* experiment was carried out: A calculated amount of serum containing inhibitor substance and no demonstrable anti-Rh agglutinin by ordinary tests was given to an $Rh_1rh$ individual. One hour after administration, a few cubic centimeters of blood were removed, and the patient's cells were found to be effectively "blocked" against agglutination by an $Rh_0$ serum. The blood cells and serum upon careful examination showed no evidences of agglutination or hemolysis; nor was there hematuria, hemoglobinuria, or an abnormal amount of urobilinogen excretion. Anti-Rh agglutinins of specificity $Rh_0$ were, however, readily demonstrable in the subject's serum. *In vitro* absorption experiments with the patient's cells drawn before injection gave similar results (Tables VI and VII).

The *in vivo* injection, therefore, of this serum, containing both inhibitor substance and anti-Rh agglutinins not demonstrable by ordinary means because of the relatively higher titer of inhibitor substance present, produced a separation of inhibitor substance from at least a high proportion of the agglutinins, because the patient's serum was found to contain free agglutinins, and the patient's cells had been bound by the inhibitor substance, both actions easily demonstrable *in vitro* by tests of the patient's blood.

It seems likely that the transmission through the placenta of both inhibitor substance and anti-Rh agglutinins from the serum of a sensitized woman to the fetus will exhibit its effect upon the infant. This may explain in part some

TABLE VI

*Demonstration of* in vivo *action of inhibitor substance on $Rh_1rh$ cells*

| Dilutions of testing serum (Titer 128) | 1 : 0 | 1 : 2 | 1 : 4 | 1 : 8 | 1 : 16 | 1 : 32 |
|---|---|---|---|---|---|---|
| | Degree of agglutination | | | | | |
| 2 drops 2 per cent suspension of subject's initial blood + 1 drop anti-$Rh_0$ serum in above dilutions | ++++ | ++++ | ++++ | +++ | +++ | +++ |
| 2 drops 2 per cent suspension of subject's blood blood 1 hour after injection of serum containing inhibitor substance + 1 drop anti-$Rh_0$ serum in above dilutions | ++++ | +++ | ++ | + | 0 | 0 |
| 2 drops 2 per cent suspension of subject's blood 6 hours * after injection + 1 drop anti-$Rh_0$ serum in above dilutions | ++++ | +++ | ++ | + | 0 | 0 |

* Specimens of blood examined at hourly intervals between the first and sixth hours after injection showed the same reaction as blood drawn six hours after injection.

TABLE VII

*Differential absorption of inhibitor substance and anti-Rh agglutinins* in vivo *and* in vitro

| Type of cell | $Rh_2$ | $Rh_2$ | $Rh_2$ | $Rh_2$ | $Rh_2$ | $Rh_1$ | $Rh_1$ | $Rh_1$ | $Rh_1$ | $Rh_1$ | Rh′ | rhrh | rhrh | rhrh | rhrh | rhrh |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Subject's initial serum added to 2 per cent suspension of cells | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Subject's serum 1 hour after injection added to 2 per cent suspension of cells | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +++ | +++ | ++++ | +++ | 0 | 0 | 0 | 0 | 0 | 0 |
| Subject's serum 1 hour after injection added to 2 per cent suspension of cells + 1 drop anti-$Rh_0$ serum | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |
| Inhibitor serum absorbed with 3 volumes of subject's initial cells added to 2 per cent suspension of cells | 0 | + | ++ | + | +++ | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Inhibitor serum absorbed with 3 volumes of subject's initial cells added to 2 per cent suspension of cells + 1 drop anti-$Rh_0$ serum | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | 0 | 0 | 0 | 0 | 0 | 0 |

of the unusual features of many cases of erythroblastosis fetalis which have previously been difficult to evaluate on the basis of the action of anti-Rh agglutinin alone. This is to be discussed in a further paper.

The probable harm to the Rh-positive patient from injection of serum or plasma containing anti-Rh agglutinins undetected by ordinary tests because of the presence of inhibitor substance must be guarded against. Similarly, it is necessary to avoid the injection of incompatible cells into a sensitized recipient. It is now clear that neither the ordinary test tube method nor the "modified compatibility" test always shows the possibility of *in vivo* agglutination. Therefore, before the inhibitor substance can be put to clinical trial (if, indeed, it is of any therapeutic value), it is not only necessary to prepare the "inhibitor serum" by a method that will remove the anti-Rh agglutinin, leaving only the inhibitor substance, but also to devise better methods of demonstration of anti-Rh agglutinins which may be "masked." Such a method has now been devised and will be described in another paper.

## SUMMARY AND CONCLUSIONS

1. The presence in certain anti-Rh serums of substance interfering with the action of anti-$Rh_0$ agglutinins has been confirmed.

2. Substances interfering with the action of anti-Rh′ and anti-Rh″ agglutinins have also been observed.

3. The presence of inhibitor substance masked by anti-Rh agglutinins has been demonstrated in most of our active anti-Rh serums, and it has been shown that many inactive serums have been so called because of the presence of relatively large amounts of inhibitor substance which interferes with the demonstration of anti-Rh agglutinins.

4. Cells of different specificity have been shown to vary in their ease of agglutination with anti-Rh serums.

5. The inhibitor substance has been found to be chemically closely allied with the anti-Rh agglutinins, and the evidence thus far supports the assumption of Race and of Wiener that it is an "incomplete" or "blocking" antibody.

6. An *in vivo* test has confirmed the observation that anti-Rh agglutinins may be masked by inhibitor substance, and that cells are capable of differential absorption of these two substances, freeing agglutinins not previously demonstrable.

7. The necessity for a better method of demonstration of masked anti-Rh agglutinins has been pointed out.

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