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Inhibition of Specific Binding of Antibody to the $Rh_0(D)$ Factor of Red Cells in Antibody Excess

Abstract. *The uptake by red cells of I^{131}-labeled nonagglutinating antibody to the $Rh_0(D)$ factor was dependent on the proportion of cells to antibody during sensitization. Inhibition of antibody binding by red cells in the region of antibody excess occurred with $Rh_0(D)$ positive red cells that were both papain modified and untreated; the inhibition was independent of the Rh phenotype.*

The relation between the quantity of erythrocyte-bound antibody and the ratio of antigen to antibody at the time of reaction is of both practical and theoretical importance in immunohematology and blood grouping. The detection of blood-group antibodies depends on conditions optimum for demonstrating an antigen-antibody reaction. Occasionally in serological investigations a hemagglutinating serum fails to give visible reactions when tested with red cells until the serum is diluted. This phenomenon, which has been referred to as a prozone or zoning, is poorly understood and undoubtedly plays an important role in the hemagglutination reaction and other antigen-antibody systems in which antigenic determinants are found on large, particulate, multimolecular structures such as cells, bacteria, phage, and so forth.

This report deals with the binding of I^{131}-labeled antibody to red cells containing the $Rh_0(D)$ antigen when the amount of either antibody or antigen is constant. The $Rh_0(D)$ factor represents a genetically determined antigenic configuration on the red cell stroma which chemically is poorly defined and is present in approximately 85 percent of Caucasian blood samples (*1*). Individuals who are $Rh_0(D)$ negative, if exposed to erythrocytes containing $Rh_0(D)$ by either transfusion or pregnancy, may respond by forming an antibody to the $Rh_0(D)$ antigen.

A globulin fraction was obtained from high-titered antiserum by the ethodin (Rivanol) precipitation procedure (*2*). The globulin fraction was labeled with I^{131} by the monochloride technique (*3*), and antibody containing eluates were obtained by heat elution from $Rh_0(D)$-positive stroma which had been sensitized with the labeled globulin fraction with techniques described previously (*4*, *5*). Table 1 lists the properties of the labeled globulin and the eluate which contained the I^{131}-labeled antibody to the $Rh_0(D)$ factor. The radioactivity of sensitized red cells and antibody solutions was determined by gamma-ray spectrometry with a well-type crystal scintillation detector.

Portions of a red cell suspension (approximately 10 percent) were incubated at 37°C for 60 minutes with the eluate containing the labeled antibody to the $Rh_0(D)$ antigen at *p*H 6.5, ionic strength 0.26. After reaction, the sensitized cells were washed four times with 0.15*M*, *p*H 6.5 phosphate buffer, and the I^{131} bound to the red cells was determined. Papain-modified cells were prepared as described previously (*5*).

Figure 1 shows the results obtained when a constant volume of antibody-containing eluate (0.38 μg of nitrogen per milliliter of eluate) was added to a series of tubes containing different volumes of a 10.5-percent suspension of red cells containing $Rh_0(D)$ (type A, Rh phenotype R_1R_1). The total volume was adjusted with buffer to 4 ml for all tests. As the red cell concentration was increased, the cell-bound nitrogen increased rapidly and began to approach a plateau in the region of antigen excess. When 3 ml of a 10.5-percent cell suspension was incubated with 1 ml of eluate (0.38 μg of nitrogen), a total of 0.109 μg of nitrogen or 28.6 percent of the total I^{131} was bound to the red cells. An average of 29.9 percent of the radioactivity of this eluate preparation was specifically absorbed by $Rh_0(D)$-containing red cells when a given volume of the eluate was repeatedly absorbed with different portions of red cells. This value is in good agreement with the value of 28.6 percent (Fig. 1) obtained in marked antigen excess (approximately 10 times optimum) when a constant volume of antibody was used to sensitize progressively increasing volumes of red cells.

Figure 1 also presents the data as nitrogen bound per milliliter of packed red cells (defined by 5 minutes centrifugation at 12,000*g* or about 10^{10} red cells). The maximum I^{131} bound per milliliter of packed red blood cells occurred when 0.021 ml of packed cells was incubated with the 1 ml of the eluate containing labeled antibody to the $Rh_0(D)$ antigen and amounted to 1.45 μg of nitrogen per milliliter of packed cells. As the cell concentration was increased with a constant volume of antibody the nitrogen bound per milliliter of packed cells decreased, so that in antigen excess there was only 0.35 μg of nitrogen per milliliter of packed cells. When the red cell concentration was reduced below the concentration which gave optimum binding of antibody, 0.021 ml, there was a suggestion that the quantity of antibody bound to the red cells decreased (1.45 to 1.33 μg of nitrogen per milliliter of cells) even though under these conditions excess antibody was present.

To determine whether the decreased

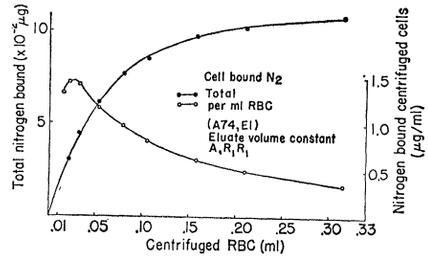

Fig. 1. Red cell binding of I^{131}-labeled antibody to the $Rh_0(D)$ red cell factor when the reaction is carried out with constant antibody concentration and varying red cell concentration (antigen). Results are shown as total nitrogen bound to all the red cells present in the reaction mixture and as nitrogen bound per unit volume of red cells (ml). The red cells were group A, and phenotypically were homozygous for the Rh factors $Rh_0(D)$, rh'(C), and rh''(e). This information is designated in shorthand form on the figure as A, R_1R_1. The reaction volume was adjusted to 4 ml for all tests by the addition of 0.15*M*, *p*H 6.5 phosphate buffer. A control suspension of $Rh_0(D)$ negative red cells bound only 0.11 percent of the I^{131} added to the cells.

Table 1. Properties of the I^{131}-labeled globulin and eluate which contained antibody to the $Rh_0(D)$ factor of red cells.

Property	Preparation A-74*	
	Globulin†	Eluate (E-I)
Antiserum donor	REZ*	
Isotope bound (%)	46.5	
Mole of iodine per mole of globulin‡	0.5	
I^{131} precipitable by trichloroacetic acid (%)	96.8	91.7
Nitrogen, μg/ml	692.	0.393
Microcuries of I^{131} per microgram of nitrogen §	2.51	
Titer original serum ‖	1024	
Titer of fraction before iodination	1024	
Titer after iodination	512	32.
Stroma pool		CHP III*
Number of units in stroma pool		38.
Total weight stroma (g)		60.
Globulin nitrogen used (mg/g of stroma)		0.30

* Personal laboratory designations which identify the globulin preparation; the initials of the serum donor and the red cell stroma pool used for preparing the eluate designated E-I. † Globulin fraction was obtained by the ethodin (Rivanol) precipitation procedure. ‡ Calculated on basis of a molecular weight of 160,000 for the total fraction iodinated. § Activity at time of iodination. ‖ Determined with the antiglobulin reaction with commercial antiserums. The original serum had an antibody to the rh′(C) red cell factor with a titer of 1 : 8 but the eluates were free of this antibody by both the antiglobulin reaction and by the failure to observe any uptake of radioactivity to red cells containing the rh′(C) factor.

binding of antibody to $Rh_0(D)$-positive red cells in antibody excess was significant, experiments were performed with constant amounts of antigen (red cells) and varying quantities of antibody. Figure 2 presents the quantity of cell-bound nitrogen for two different red cell samples—one probably homozygous for the $Rh_0(D)$ antigen (type O, R_1R_2) and the other probably heterozygous for the antigen (type O, R_1R')—as the antibody concentration is increased progressively and the concentration of red cells remains constant. Both of the red cells studied showed a progressive rise in cell-bound antibody as the amount of antibody nitrogen added to the cells was increased. The maximum cell-bound I^{131} was achieved for the $Rh_0(D)$ homozygous red cell (R_1R_2) when the eluate nitrogen concentration was 0.295 μg, and for the $Rh_0(D)$ heterozygous red cell (R_1R') when the eluate nitrogen concentration was 0.197 μg. The addition of more eluate nitrogen beyond that which produced the maximum red cell uptake resulted in a progressive decrease of cell-bound I^{131} for both red cell samples (antibody excess). In marked antibody excess there was a dramatic reduction in cell-bound I^{131}. The quantity of cell-bound I^{131} was only 20 to 30 percent of that bound at the optimum antibody concentration when the amount of antibody used was 3 to 4 times that required for optimum binding. The two cells in Fig. 2 differ significantly in the quantity of cell-bound antibody if they are compared when the antibody concentration used in the test results in maximum uptake of I^{131}. The quantity of antibody bound to the red cell heterozygous for the $Rh_0(D)$ antigen (R_1R') took up only 67 percent of the antibody bound to the cell homozygous for the $Rh_0(D)$ antigen (R_1R_2) when the proportion of cells to antibody was optimal. Figure 2 shows clearly that when the test was carried out in either antigen or antibody excess there was almost no difference in the quantity of labeled antibody bound to these two genetically different red cells which should show differences in their $Rh_0(D)$-antigen content (*6*).

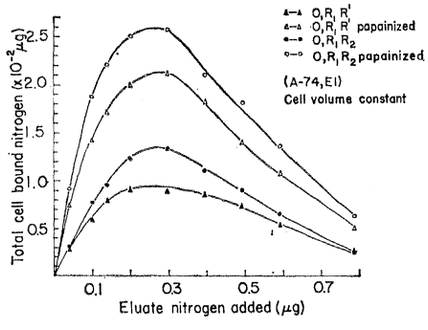

Fig. 2. Red cell binding of I^{131}-labeled antibody to the $Rh_0(D)$ red cell factor when the reaction is carried out with constant red cell concentration (antigen) and varying antibody concentration (eluate). The results are shown as total nitrogen bound to the red cells present in the reaction mixture for a type O red cell probably homozygous for the $Rh_0(D)$ antigen (R_1R_2) and for a type O red cell probably heterozygous for the $Rh_0(D)$ antigen (R_1R'). The results obtained with both untreated and papain-modified cells are presented. The reaction volume was adjusted to 2.1 ml for all tests by the addition of phosphate buffer.

Figure 2 also shows the data obtained after modification of the red cells by treatment with papain. The modified cells behave like untreated red cells when a constant volume of enzyme-treated red cells is sensitized with varying quantities of I^{131} antibody. The curves obtained with enzyme-treated cells were almost identical with those found with untreated cells except for the quantity of antibody bound. After papain modification, when the sensitization was carried out at the optimum ratio of cells to antibody, the red cell homozygous for the $Rh_0(D)$ antigen (R_1R_2) took up 1.9 times more antibody and the heterozygous cells (R_1R') took up 2.3 times more antibody.

These results show that for both untreated and enzyme-modified $Rh_0(D)$-positive red cells there is an optimum ratio of red cells (antigen) to eluate nitrogen (antibody) for maximum uptake of antibody to the red cell. When $Rh_0(D)$-positive red cells are incubated with an excess of antibody, there is a reduction in the amount of antibody bound to the red cell. The inhibition of antibody uptake by the red cell in the presence of antibody excess appears to be proportional to the quantity of antibody in excess, so that as the antibody concentration is increased there is a progressive decrease in the amount of cell-bound antibody. The inhibition of antibody binding in antibody excess occurred with all the common Rh phenotypes and appeared to be independent of the other blood-group antigens present on the red cell. This dependence of antibody binding on the ratio of cells to antibody also occurred with papain-modified cells. Even though the papain-modified cells took up about twice the amount of antibody bound to the untreated red cells, the optimum ratio of cells to eluate nitrogen was about the same for both types of cells. This suggests that the free antibody in equilibrium with the cell-bound antibody is significantly less with the papain-modified red cell. The change in equilibrium constant may be due to a reduction in the electrostatic barrier resulting from a loss of anionic groups from the red cell stroma after papain treatment (*7*).

The serological equivalent of the inhibition of antibody binding to red cells in the presence of excess antibody as observed in this study has been described as a prozone. Many prozones observed in the Rh system are due to the presence of incomplete, nonagglu-

tinating antibody to the $Rh_0(D)$ antigen in agglutinating antiserums for the $Rh_0(D)$ factor (*8*). The prozone phenomenon can be reproduced artificially by mixing saline-agglutinating with non-agglutinating-incomplete antibody to the $Rh_0(D)$ antigen (*9*). Zoning which has been observed with the agglutination of Rh-sensitized cells when serial dilutions of rheumatoid serums are used has been attributed to the agglutinating phase of the reaction and not to any effect which implicates the primary binding of antibody to the red cell (see *10*).

The results obtained in this study are not accounted for by the explanations based on serological studies which have attributed the prozone phenomenon to the secondary, agglutinating stage of the hemagglutination reaction. Only the primary binding of the non-agglutinating, incomplete antibody was followed in this study so that the inhibition observed in antibody excess was independent of any agglutination phenomena. Furthermore, the antiserum, as well as the labeled eluate, did not contain any demonstrable agglutinating activity against $Rh_0(D)$ red cells.

The phenomenon of zoning is more easily understood in precipitating antigen-antibody systems. Three zones have been delineated in a precipitating system, an antibody excess zone, equivalence zone, and an antigen excess zone. The precipitate formed in both antigen and antibody excess zones is decreased because of the formation of soluble complexes which interfere with lattice formation. As in the hemagglutination system, zoning has been associated with a secondary stage in the precipitin reaction rather than with the primary immunochemical phase, the interaction of the antibody combining site with the corresponding antigenic determinant. Zoning often occurs with horse antibodies (*11*) and has been demonstrated with precipitating rabbit antibodies. Inhibition of rabbit antibody precipitation in antibody excess has been shown (*12*) by using an antigen readily detected in low concentrations (dog intestinal phosphatase) and also by controlling the addition of antigen to antibody so that a high antibody to antigen ratio is maintained during the initial phase of the reaction (*13*).

No explanation is provided for the apparent paradox revealed by this study, whereby in antibody excess there is apparent inhibition of antibody binding to the red cell. It is conceivable that this effect may be due to the nonuniform distribution of the iodine label on the antibody molecules in the preparation. If sufficient unlabeled antibody molecules were present in antibody excess the unlabeled antibody would compete with the labeled antibody for the red cell antigen sites. Under these conditions there would be no decrease in total cell-bound antibody, but only an apparent decrease due to the displacement of the labeled antibody by the unlabeled antibody. The available data are inadequate to rule out this possibility, although it appears unlikely since zoning has been observed with more heavily labeled preparations (up to 3.3 moles of iodine per mole of γ-globulin).

Another possibility that deserves consideration is that this is a property peculiar to the antiserum obtained from this donor (REZ). Antiserums produced by other individuals will have to be tested for this property before this possibility can be evaluated.

The available evidence suggests that the paradoxical phenomenon of inhibition in antibody excess is due to some interaction between antibody molecules which interferes with the sensitization of the erythrocytes. Such an explanation is consistent with the observation that reduced antibody uptake does not occur if the excess antibody is added in two or more increments. In addition, the inhibition in antibody excess observed with papain-modified red cells suggests that zoning involves the antibody rather than the antigenic determinant in the red cell.

The immediate significance of these observations concerns the estimation of the red cell $Rh_0(D)$ antigen content. Previous studies (*6*) were based on the assumption that the maximum red cell antibody uptake would occur if the reaction were carried out in antibody excess. In view of the inhibition of antibody binding in antibody excess, previous estimates of the red cell $Rh_0(D)$ antigen content will require re-evaluation. It is also evident that the quantitative aspects of both red cell sensitization and the hemagglutination reaction require critical study.

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Simultaneous Recordings of Scalp and Epidural Somatosensory-Evoked Responses in Man

Abstract. *The calvarium and scalp markedly attenuate the amplitude, but do not alter the latency or frequency, of somatosensory responses evoked by electrical stimulation of the contralateral median nerve in man. With scalp electrodes no significant potentials are obtained that are not also present in the epidural recordings when proper averaging techniques are used. The somatosensory-evoked response recorded with scalp electrodes in man appears to be the result of brain activity.*

Electrical stimulation of mixed peripheral nerves, such as the median or ulnar, evoke an electrical response in the cerebral cortex of various vertebrates, including man. While such responses are of sufficient amplitude that they may be recorded easily and directly from the somatosensory cortex of animals, attenuation by the skull and scalp necessitates special averaging techniques for recording these potentials in intact human beings (*1*). The early components of somatosensory responses in man show the expected