VISUALIZATION OF ANTIGENIC SITES OF HUMAN ERYTHROCYTES WITH FERRITIN–ANTIBODY CONJUGATES

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INTRODUCTION

Blood group antigens of erythrocytes have been studied by the fluorescent antibody technique (1, 2), but their size, shape, and distribution have not been analyzed by this method. With the advent of ferritin-labeled globulins, it has been possible to identify and locate specific antigens of bacteria (3), viruses (4), tumor cells (5), erythrocytes (6, 7), and injurious molecular complexes causing tissue injury (8, 9). This report demonstrates the site and distribution of type A and type Rh_0 (D) antigens in human red cells using ferritin conjugated to immune globulins.

MATERIALS AND METHODS

The preparation of high-titered rabbit anti-A serum, raised by injection of saliva incorporated into Freund's complete adjuvant, has been previously described (2). High-titered human anti-A and anti-Rh_0 (D) sera were obtained from Ortho Research Foundation. The globulin fraction of these sera was precipitated by 50 per cent ammonium sulfate in the cold. Ferritin conjugations were carried out according to the method of Singer, using toluene 2,4-diisocyanate (10), or according to the method of Tawde and Sri Ram, using *p,p'*-difluro-*m,m'*-dinitrodiphenylsulfone (11). A 2 per cent suspension of three times washed erythrocytes was exposed to an equal volume of crude conjugate and incubated at 37°C for 30 minutes. Following three more washes, the cells were fixed in 1 per cent buffered OsO_4 (pH 7.2) and processed for thin sectioning and electron microscopic examination with a Phillips 100 instrument.

RESULTS AND DISCUSSION

Type A Erythrocytes and Ferritin-Labeled Anti-A Globulin

Type A erythrocytes were agglutinated by the conjugated specific globulins and were noted to be fairly evenly surrounded by a layer of ferritin particles (Figs. 1 and 2). Proof that these particles were ferritin was achieved by resolving the particles into individual ferritin micelles (Fig. 1, insert). These particles did not attach to similarly exposed type O or type B erythrocytes (Fig. 3). Ferritin particles frequently were clustered on the specifically labeled red cells and spaced in such a way as to suggest a pattern of antigenic sites. It was not possible to determine in these experiments whether the anatomic arrangement was artifactual or the result of using dilute solutions of labeled antibody. The number of antigenic sites on the surface of type A or B erythrocytes has been calculated to be 5×10^5 sites (12). With such a large number, the distance between antigenic sites would be small and difficult to separate clearly with the methods used here. Occasional particles of ferritin having no connection with the cell surface were noted in the matrix of the red cells (Figs. 1 and 4). It is possible that these labeled particles gained entrance to the cell through breaks in the cell membrane and had little relationship to specific binding sites.

Perhaps a brief comment should be made about the extremes in labeling of type A erythrocytes as illustrated by Figs. 1 and 2. These two micrographs demonstrate the extremes of labeling, and if the clusters of ferritin particles are counted per unit of linear surface, assuming each cluster to be an antigenic site, then the greatest variation in type A erythrocytes is 2:1. The differences in labeling type A red blood cells might also have been due to differences in the crude ferritin-globulin conjugates used in which the amount of unlabeled anti-A antibody might have blocked the ferritin conjugated globulin from binding at its specific site.

The positive labeling could not be completely blocked by prior treatment of the erythrocytes with unlabeled anti-A globulin but could be reduced. This suggested that the antigenic sites on the red cell surface were too numerous to be completely blocked by prior treatment with unlabeled antibody. Another factor which might also have contributed to the incomplete blocking reaction is a rapid exchange of ferritin-labeled antibody for unlabeled antibody. A demonstration of this latter mechanism was achieved by exposing type

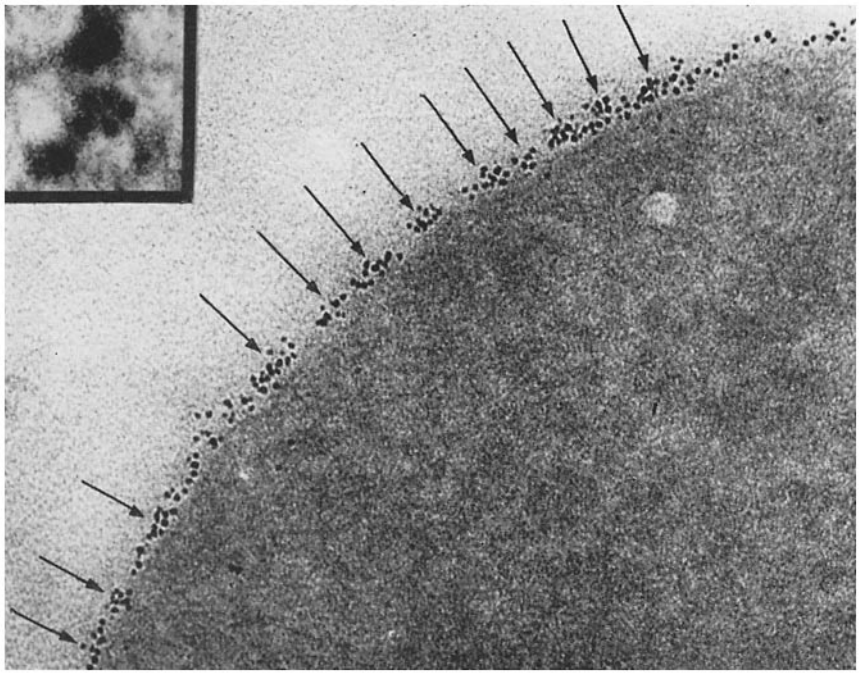

FIGURE 1 Type A red cell exposed to ferritin anti-A globulin. Extensive binding of ferritin particles to surface of erythrocyte with a suggestive pattern of discrete antigenic sites (arrows). Insert—one of the ferritin particles has been resolved to 5 of the 6 iron micelles. × 120,000; insert, × 950,000.

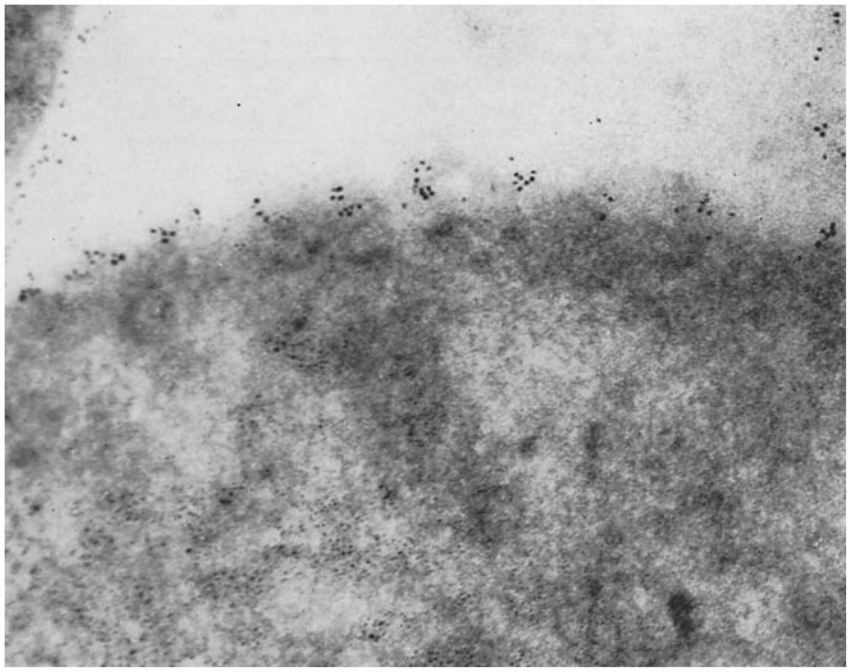

FIGURE 2 Type A red cell exposed to ferritin anti-A globulin. Less extensive binding as compared to Fig. 1. × 100,000.

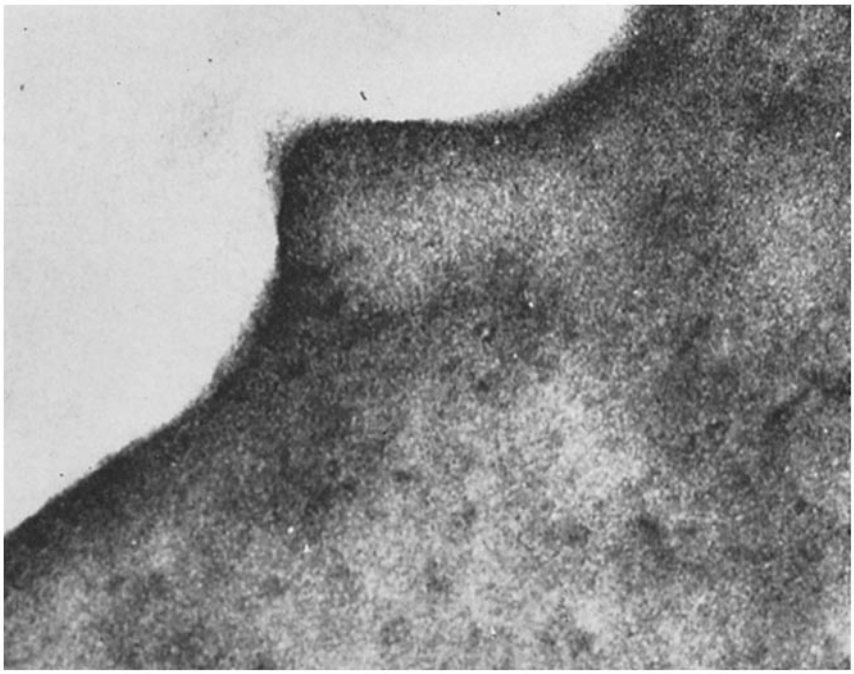

FIGURE 3 Type O red cell exposed to ferritin anti-A globulin. Compare with Fig. 1. × 77,000.

A red cells first to ferritin anti-A globulin and, after washing, to several exposures of unlabeled anti-A globulin. This resulted in a reduced number of ferritin particles attached to the surface of the cell when these cells were compared to cells which had only been exposed to ferritin anti-A globulin and which had been washed the same number of times as those cells exposed to unlabeled antibody (Figs. 4 and 5). In order to rule out non-specific attachment of ferritin-globulins to agglutinated cells, type B erythrocytes were exposed to a mixture of anti-B globulin and ferritin anti-A globulin. No labeling of the red blood cells occurred. The results are summarized in Table I.

The subgroups of group A blood have been identified on the strength of their reactions with anti-A globulin. If these differences were due to variations in the number of antigenic sites on the red cell, perhaps this method could detect them. No differences were noted, however, in the degree of labeling when A_1 cells were compared with A_2 cells. This result favors a qualitative difference in the A antigen, rather than a quantitative difference, to account for the varying reactivities of these two cell types.

TABLE I

Direct Labeling of Type A Erythrocytes

Cell Type	Globulin Solution(s)	Results
A	Ferritin anti-A	Positive
B or O	Ferritin anti-A	Negative
A	Anti-A, then Ferritin anti-A	Partial blocking
A	Ferritin anti-A, then anti-A then anti-A	Reduced labeling
B	Ferritin anti-A and anti-B	Negative

Type Rh-Positive Erythrocytes and Ferritin-Labeled Anti-Rh_0 (D) Globulin

When type O cells containing the Rh_0 (D) antigen were exposed to ferritin anti-D globulins, much less labeling occurred, and repeated attempts to increase the binding of particles by more concentrated antibody solutions yielded no better results. Each sectioned erythrocyte disclosed from

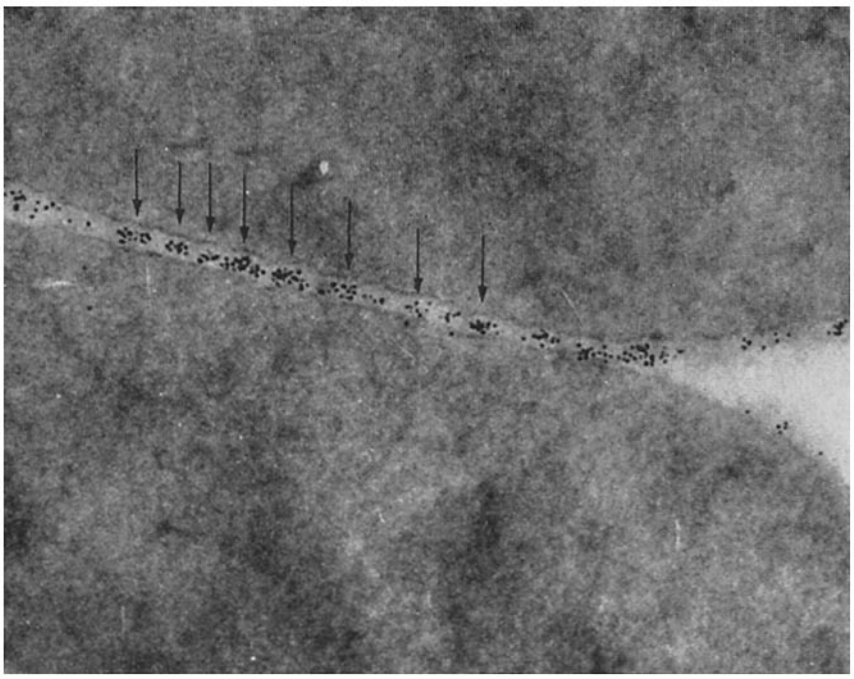

FIGURE 4 Type A cells exposed to ferritin anti-A globulin are agglutinated and display reactive sites between the two elements (arrows). × 66,000.

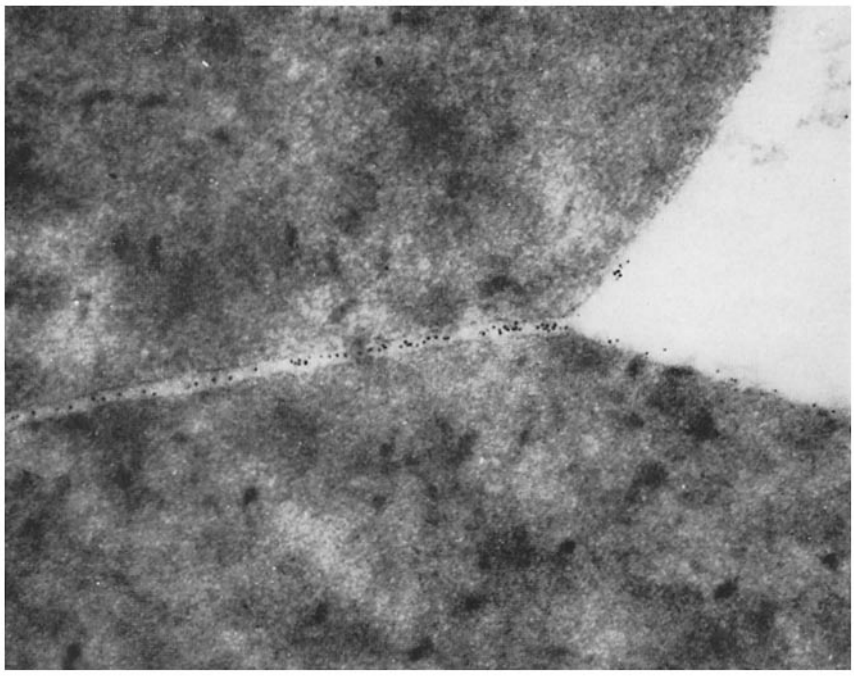

FIGURE 5 Type A cells exposed first to ferritin anti-A globulin and subsequently to unlabeled anti-A globulin. Note the reduction in the number of particles at the surface. Compare with Fig. 4. × 50,000.

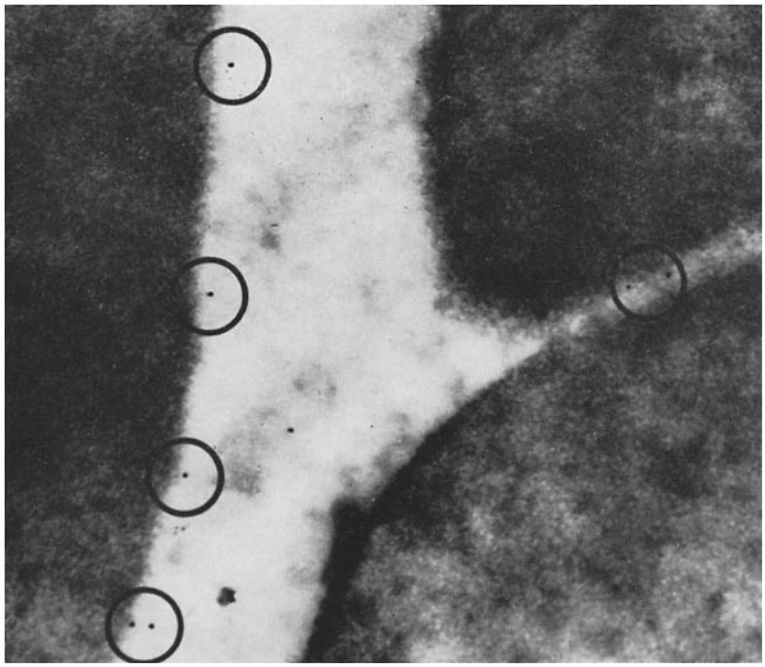

FIGURE 6 Type O $Rh_o(D)$ red cells exposed to ferritin anti-$Rh_o(D)$ globulin. Seven ferritin-globulin molecules are encircled. Compare with Fig. 1. × 60,000.

5 to 10 ferritin particles over the entire circumference (Fig. 6), each particle at a fairly constant distance from another. By assuming that 30 to 50 per cent of the globulins present were labeled with ferritin and that each section was 500 A thick, the total number of antigenic sites was minimally estimated at from 1,000 to 3,000 per cell. This was of the same order of magnitude as that calculated for Rhesus antigenic sites ranging from 5,000 to 10,000 (13, 14).

The estimation of the number of receptors on an erythrocyte has been a controversial issue. Most investigators, however, have agreed that there is a hundred fold difference between the number of A and D sites on the surface of red blood cells.

It seems likely that if minor quantitative differences existed between A_1 and A_2 cells, the ferritin-globulin technique would not be able to detect them. The smaller number of Rhesus antigenic sites will permit a more precise estimation of their location and number.

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BIBLIOGRAPHY

1. COHEN, F., ZUELZER, W. W., and EVANS, M. M., Identification of blood group antigens and minor cell populations by the fluorescent antibody method, *Blood*, 1960, **15**, 884.
2. LEE, R. E., and VAZQUEZ, J. J., Immunocytochemical evidence for transplacental passage of erythrocytes, *Lab. Invest.*, 1962, **11**, 580.
3. SMITH, C. W., METZGER, J. F., ZACKS, S. I., and KASE, A., Immune electron microscopy, *Proc. Soc. Exp. Biol. and Med.*, 1960, **104**, 336.
4. MORGAN, C., HSU, K. C., RIFKIND, R. A., KNOX, A. W., and ROSE, H. M., The application of ferritin-conjugated antibody to electron microscopic studies of influenza virus in infected

cells. I. The cellular surface, *J. Exp. Med.*, 1961, **114**, 825.

5. EASTON, J. M., GOLDBERG, B., and GREEN, H., Immune cytolysis: Electron microscopic localization of cellular antigens with ferritin-antibody conjugates, *J. Exp. Med.*, 1962, **115**, 275.
6. LEE, R. E., Electron microscopy of erythrocyte antigen sites using ferritin-labeled globulins, *Fed. Proc.*, 1963, **22**, 196.
7. HARRIS, G., Labelling of red cells with ferritin antibody complexes, *Vox Sang.*, 1964, **9**, 70.
8. ANDRES, G. A., MORGAN, C., HSU, K. C., RIFKIND, R. A., and SEEGAL, B. C., Electron microscopic studies of experimental nephritis with ferritin-conjugated antibody. The basement membrane and cisternae of visceral epithelial cells in nephritic rat glomeruli, *J. Exp. Med.*, 1962, **115**, 929.
9. ANDRES, G. A., SEEGAL, B. C., HSU, K. C., ROTHENBERG, M. S., and CHAPEAU, M. L., Electron microscopic studies of experimental nephritis with ferritin-conjugated antibody. Localization of antigen-antibody complexes in rabbit glomeruli following repeated injections of bovine serum albumin, *J. Exp. Med.*, 1963, **117**, 691.
10. SINGER, S. J , and SCHICK, A. F., The properties of specific stains for electron microscopy prepared by the conjugation of antibody molecules with ferritin, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 519.
11. SRI RAM, J., TAWDE, S. S., PIERCE, G. B., and MIDGLEY, A. R., Preparation of antibody-ferritin conjugates for immuno-electron microscopy, *J. Cell Biol.*, 1963, **17**, 673.
12. FILITTI-WURMSER, SABINE, JACQUOT-ARMAND, Y., AUBEL-LESURE, G., and WURMSER, R., Physicochemical study of human isohemagglutination, *Ann. Eugenics*, 1954, **18**, 183.
13. MASOUREDIS, S. P., Relationship between $Rh_o(D)$ genotype and the quantity of I^{131} anti-$Rh_o(D)$ bound to red cells, *J. Clin. Invest.*, 1960, **39**, 1450.
14. KABAT, E. A., Blood Group Substances, New York, Academic Press, Inc., 1956.