PINOCYTOTIC RESPONSE OF

CIRCULATING ERYTHROCYTES TO SPECIFIC

BLOOD GROUPING ANTIBODIES

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ABSTRACT

Human blood samples from adults and newborns of blood groups O, A, and B were treated with either anti-A blood grouping serum, ferritin-conjugated anti-A serum, free ferritin, or saline *and then* prepared for electron microscopy. Morphological differences were observed between the untreated erythrocytes of infants and adults. Circulating red cells of newborns were frequently vesiculated (25.5%), whereas those of adults only occasionally showed vesicles (5.5%). On the basis of morphology and incidence, the majority of these vesiculated cells seemed to be mature erythrocytes. The introduction of anti-A serum to group A erythrocytes of infants appeared to stimulate vesicle formation, but anti-A serum did not have a similar effect on group O or B cells of infants or on group A cells of adults. Vesicles which formed in response to antiserum treatment appeared to be the result of pinocytosis. In contrast to the well dispersed ferritin along the membrane of agglutinated adult cells, the ferritin particles on the infants' cells were frequently clustered at irregular intervals. These accumulations seemed to lead to invaginations of the cell membrane, resulting in ferritin-lined intracytoplasmic vesicles. The addition of free ferritin or ferritin-conjugated antibodies of the wrong specificity to red cells did not increase vesicle formation.

A normal activity ascribed to the erythroblast is its ability to "pinocytize" protein particles. Extensive work has been published by Policard and Bessis (24, 25), Bessis and Breton-Gorius (2–4), Grasso et al. (9), Sorensen (27), and Orlic et al. (23) on the pinocytotic activity ("rhopheocytosis") of the erythroblast when in contact with reticular and hepatic cells. The observations of Bessis and his group led them to suggest that pinocytosis of endogenous ferritin might be a part of the process of iron metabolism in the producton of hemoglobin by the developing cells. Other investigators of the pinocytotic activity of erythroblasts report that rhopheocytosis in erythroblasts associated with reticular cells is the exception rather than the rule

and that ferritin attachment exists also on the free surfaces of cells (1, 14, 16, 17, 29). Jones (16) observed erythroblasts in contact with hemosiderin-laden macrophages with no evidence of rhopheocytosis. Despite the controversy concerning rhopheocytosis and the reticular cell, there is general agreement that pinocytotic activity does exist among the erythroblasts (7). Jones (16) and Grasso et al. (9) have reported decreased pinocytotic activity with the maturation of erythroblasts.

The ability of the erythrocyte to pick up particles from its immediate environment has been described only for the immature red blood cells found in the bone marrow, spleen, and liver. As

erythrocytes develop, their ability to perform pinocytosis is thought to be lost. Jones (15) observed the formation of pinocytotic vesicles in basophilic erythroblasts of rat and human liver, but this activity diminished or was absent in the polychromatic and orthochromatic erythroblasts. In the mature state, the cell is considered to be a highly specialized unit which lacks a nucleus and other cytoplasmic organelles and serves essentially as a bag of hemoglobin functioning for the transport of O_2 and CO_2 (5, 12).

According to Grasso et al. (9), "The end result is a cell type performing a highly specialized function and which is incapable of growth and reproduction." This concept of the erythrocyte as a highly specialized cell is further enhanced by earlier studies with radioiron (8) which indicated that no exchange of iron occurs between the mature erythrocyte and its surrounding plasma.

Previous reports (10, 11) on the effects of blood grouping antibodies on the red blood cells of adults and newborn infants led to some observations on pinocytosis among circulating erythrocytes. The present investigation elaborates further on those electron microscope observations and illustrates a pinocytotic response by circulating erythrocytes to treatment with specific antibodies.

MATERIALS AND METHODS

Human blood samples from eight adults and nine newborns (umbilical cord bloods) of blood groups O, A, and B were collected not more than 3 hr prior to use. The blood cells of each donor were washed three times in normal physiological saline by centrifugation and prepared as 2% suspensions. The washed erythrocyte suspensions of each blood sample were divided into four equal volumes, one of which remained untreated to serve as a control. The remaining three aliquots were treated as follows: one was mixed with free ferritin, the second was treated with unconjugated human anti-A serum (immune serum having a 1:1,000 titer), and the third was treated with anti-A serum that had been conjugated with horse spleen ferritin by the technique previously described (10). The tagging of the antibody molecule with an electron-opaque iron-protein complex makes visible in the electron microscope the site of the antigen-antibody reaction (13, 18). The conjugated and un-conjugated antisera were prepared from serum of the same donor. The treated red cells were incubated at 37°C for 30 min and then washed three times with saline, fixed in 2% osmium tetroxide, and embedded

in Epon according to the method of Lynn et al. (19). Thin sections, approximately 90 mμ in thickness, were cut on a Porter-Blum microtome with diamond knives. These sections were picked up with uncoated copper grids and observed directly without the use of heavy metal staining. We avoided staining to prevent the formation of artifacts that might be misinterpreted as ferritin particles. We employed an RCA-EMU-3F electron microscope to observe and photograph the specimens. Counts of the number of vesiculated cells per specimen and the number of vesicles per sectioned cell were made at a magnification of 8,000. A standard error of rate test was applied to the resultant data for a determination of their statistical validity.

RESULTS

A comparison of the untreated erythrocytes of the adult with the untreated umbilical cord erythrocytes by means of the electron microscope presented some interesting morphological differences. The adult red blood cell filled with an electron-opaque substance, probably representing hemoglobin and cell matrix, was fairly regular in shape (Fig. 1). The cytoplasm was free of nuclear constituents, mitochondria, and other organelles. Particles of native ferritin which one might expect to find in immature cells of the marrow were not seen in the circulating red blood cells of these normal adults. On occasions, small vesicular structures were observed, but no pinocytotic-like invaginations of the adult erythrocyte membranes were found. These vesicles were usually observed to be near the cell membrane and not throughout the cytoplasm. In contrast, the erythrocytes of newborns (Fig. 2) showed frequent vesicles within the body of the cell in addition to invaginations of the cell surface. The membranes surrounding these vesicles were usually sharply defined. Occasionally, remnants of mitochondria (evidenced by internal structure or cristae) were observed within the cytoplasm of the circulating red cells of the newborn (Fig. 4). Native ferritin or hemosiderin particles were rarely seen within the mitochondrial remnants. These thin-sectioned cells showed no nuclei or recognizable nuclear fragments. The frequencies of observed vesiculated erythrocytes in untreated preparations made from the venous blood cells of a mother and her newborn infant were compared. The counts presented in Table I show that the percentage of vesiculated cells in the mother's specimens was 5.5, whereas in her infant's specimens this percentage was considerably higher, being 25.5. The difference is
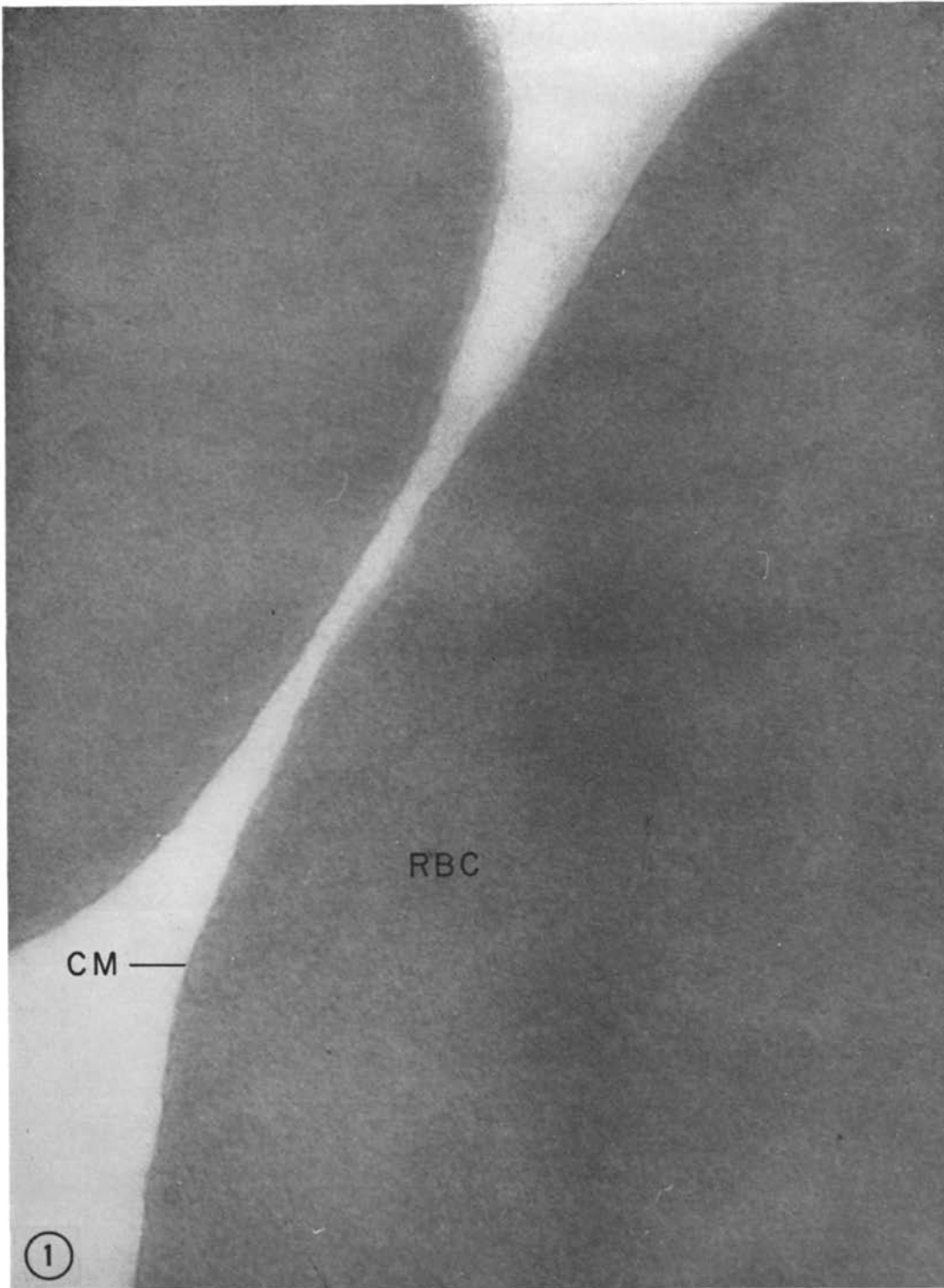
FIGURE 1   Untreated group A human erythrocytes from an adult (*RBC*). The cell contains relatively homogenous cytoplasm within a sharply defined cell membrane (*CM*). Note the absence of vesicles, native ferritin, and other cytoplasmic constituents. × 80,000.
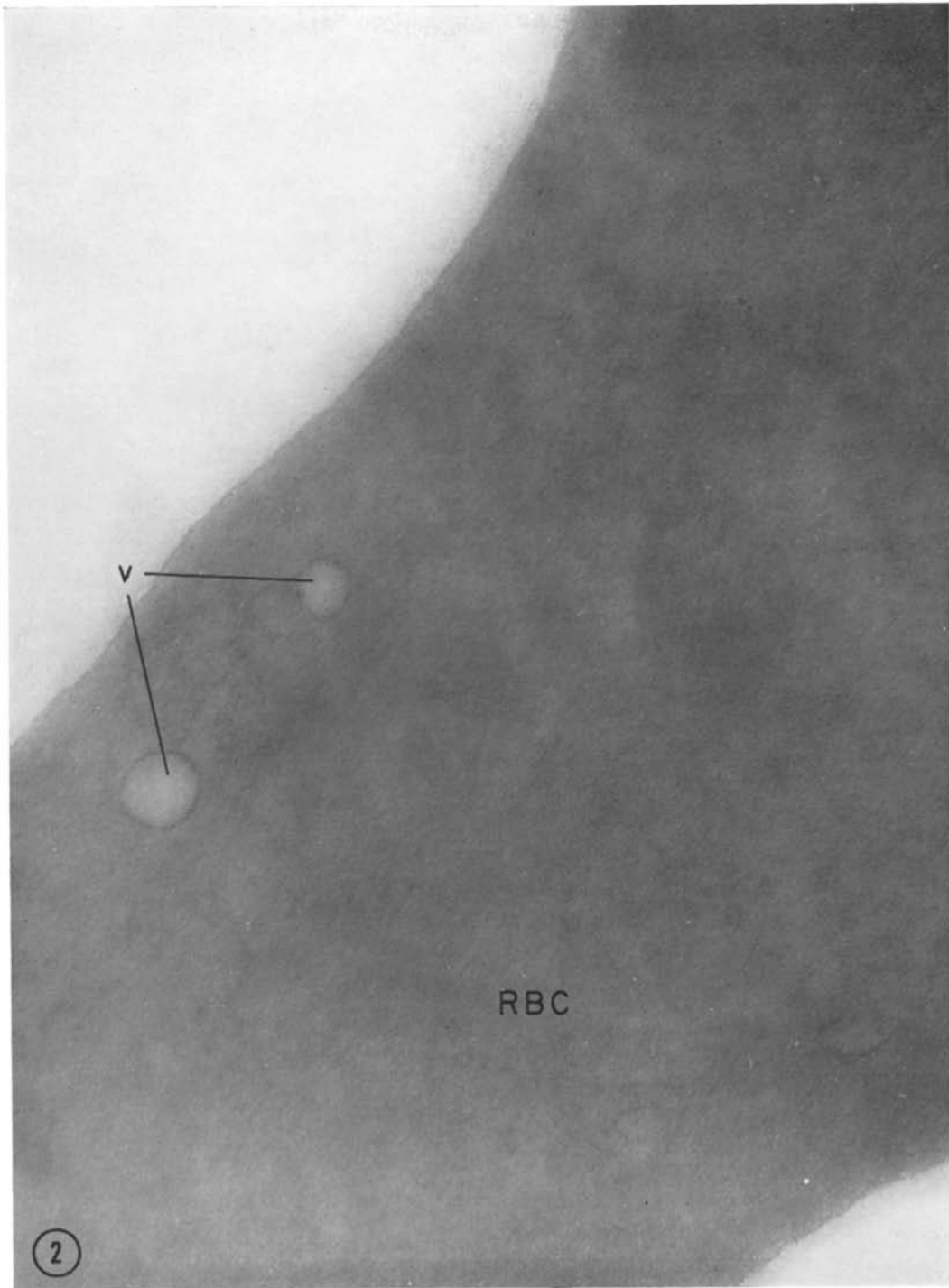
FIGURE 2 Untreated group A human erythrocyte from a newborn (*RBC*). Note the two intracytoplasmic vesicles (*V*) and the lack of native ferritin and other cytoplasmic constituents. × 80,000.

statistically valid, showing a value of p < 0.001 by the standard error of rate test.

The introduction of anti-A blood grouping serum into the system appeared to stimulate

TABLE I

*The Percentage of Erythrocytes Showing Vesicles in Sections Prepared from the Bloods of a Mother and Her Newborn Infant**

	No. of erythrocytes counted	No. of vesiculated erythrocytes	Percentage of cells vesiculated
Adult maternal blood (not antibody-treated)	1,200	66	5.5
Infant blood (not antibody-treated)	1,000	255	25.5

* Electron microscope observations made at × 8,000.

vesicle formation in the group A infant's erythrocytes, but not in the cells of its group O mother. A comparison of the effects of the anti-A serum on the erythrocytes of adult and infant bloods is presented in Table II. The occurrence of vesiculated cells in the untreated controls of both adult and infant was essentially the same as that shown in Table I. The addition of free ferritin to either the adult or infant red cell suspensions did not change the vesiculated cell counts in an appreciable manner. The addition of anti-A serum to the group O adult's cells also failed to increase vesicle formation. When the anti-A serum was added to the group A adult cells, agglutination of these erythrocytes was observed (Fig. 3), but these clumped cells did not exhibit any stimulus toward vesicle formation. However, when the anti-A serum was mixed with the group A infant erythrocyte suspensions, an increase in both the number of vesiculated cells (Table II) and the number of vesicles within cells was found (Fig. 4). The data for the specific antibody-treated A cells of infants and adults were grouped and compared to the grouped data for nonantibody-treated A cells.

TABLE II

*Effects of Blood Grouping Antibodies on the Percentage of Vesiculated Erythrocytes from Adults and Newborns**

Source	Blood group	Treatment	No. of erythrocytes counted	No. of vesiculated cells	Percentage of cells vesiculated	
Adult	O	None	300	19	6.33	No specific antibody = 5.93
Adult	O	Free ferritin	600	29	4.83	
Adult	O	Unconjugated anti-A serum	300	23	7.66	
Adult	O	Ferritin-conjugated anti-A serum	300	18	6.00	
Adult	A	Unconjugated anti-A serum	500	21	4.20	specific antibody = 3.30
Adult	A	Ferritin-conjugated anti-A serum	500	12	2.40	
Infant	A	None	400	92	23.00	No specific antibody = 25.50
Infant	A	Free ferritin	600	163	27.16	
Infant	A	Unconjugated anti-A serum	500	202	40.40	specific antibody = 39.62
Infant	A	Ferritin-conjugated serum	300	115	38.33	
		Total	4,300	694		

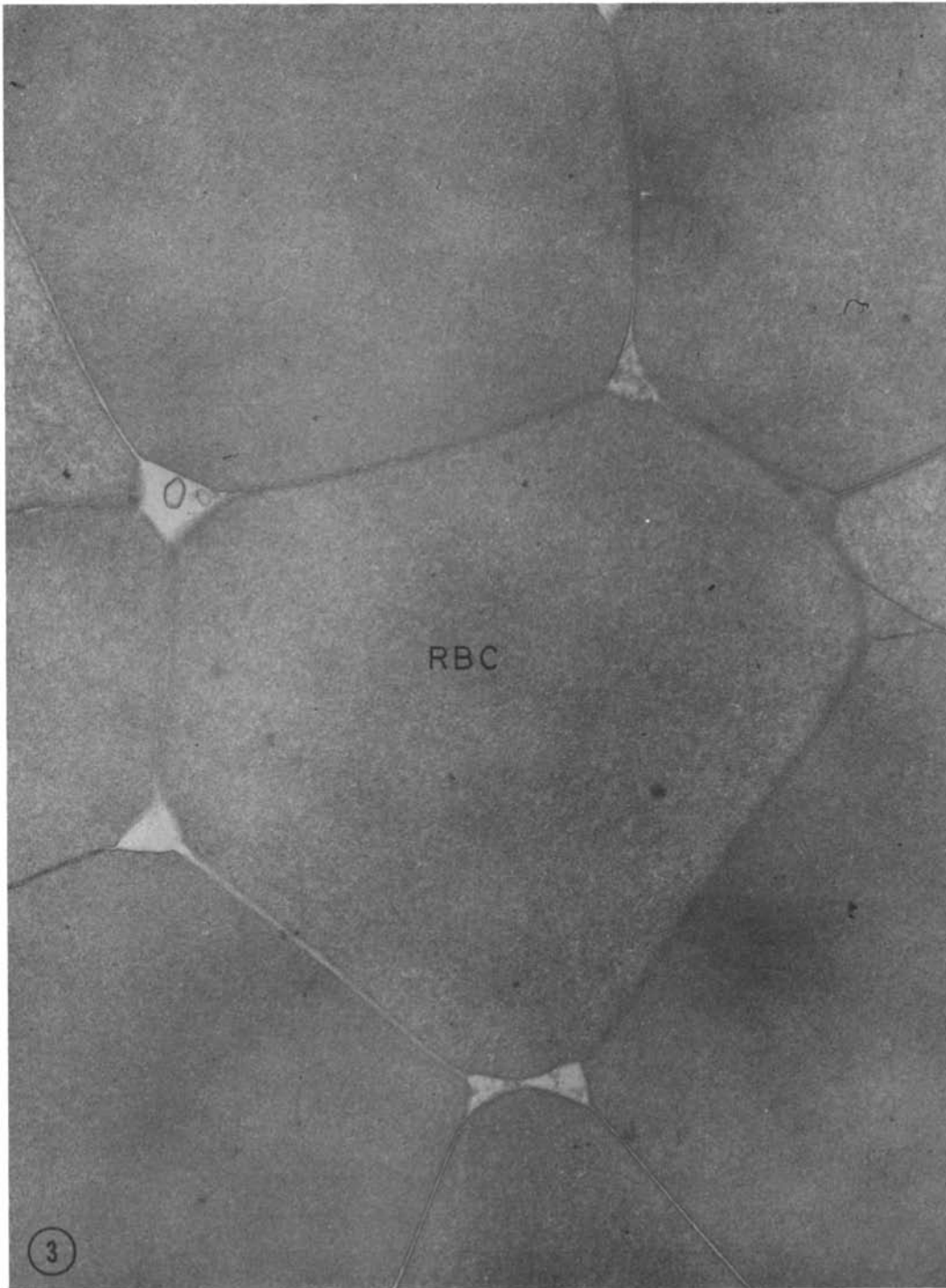The group O adult was the mother of the group A infant.
* Counts made at × 8,000.

FIGURE 3   Group A human adult erythrocytes (*RBC*) agglutinated after treatment with unconjugated anti-A serum. Native ferritin, intracytoplasmic vesicles, and other cell constituents are absent. × 20,000.
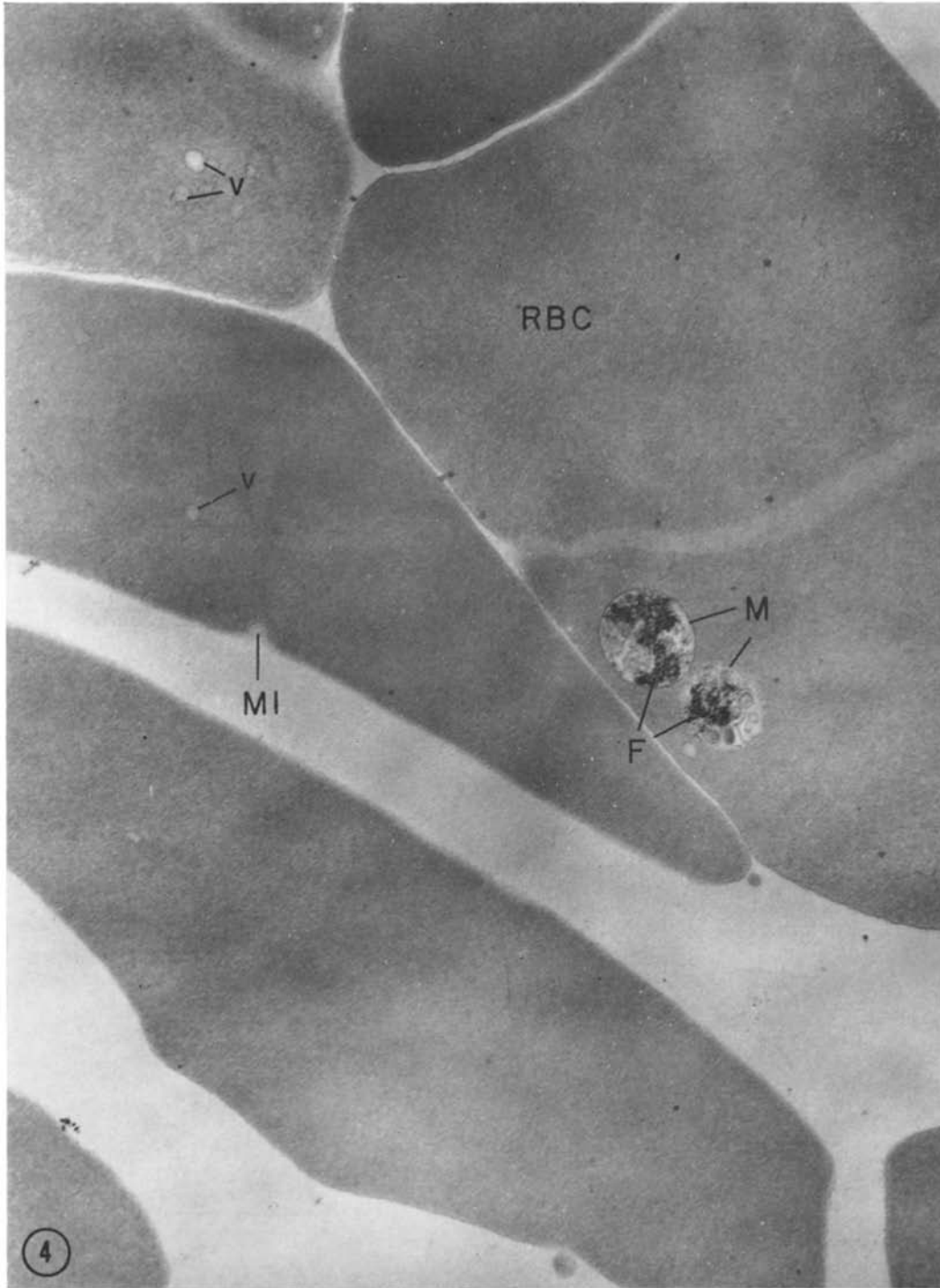
FIGURE 4   Group A human newborn erythrocytes (*RBC*) agglutinated with unconjugated anti-A serum. Native ferritin (*F*) is observed within the remnants of two mitochondria (*M*). Note the invagination (*MI*) of the cell membrane of one of the cells and the intracytoplasmic vesicles (*v*) occurring with increased frequency, as compared to similar cells untreated (Fig. 2). × 20,000.
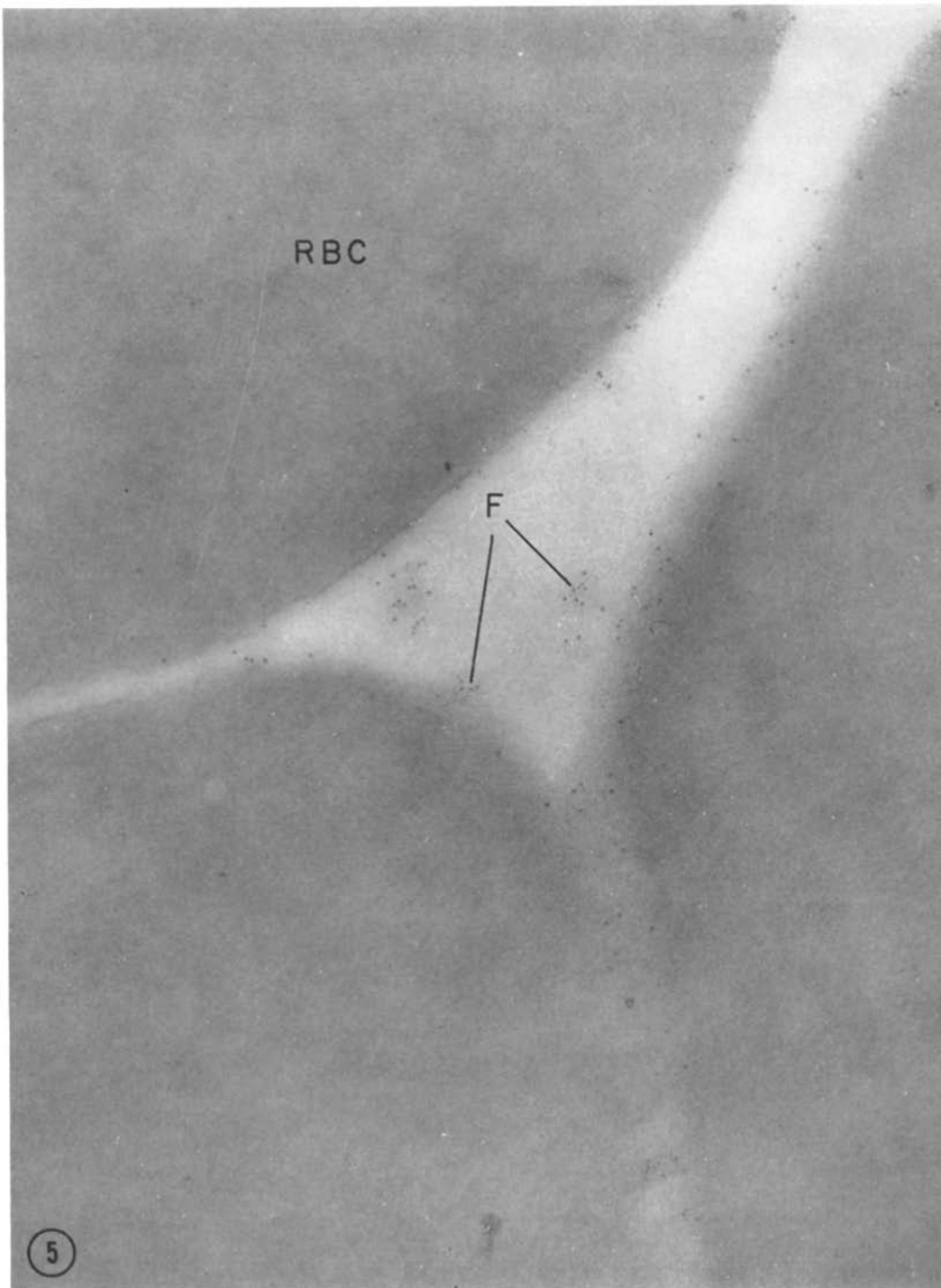
FIGURE 5 Three group A human adult erythrocytes (*RBC*) treated with ferritin-conjugated anti-A serum. Ferritin particles (*F*) are seen along the cell membranes of the sectioned cells. Note the absence of vesicles and ferritin particles within the cells. $\times$ 80,000.

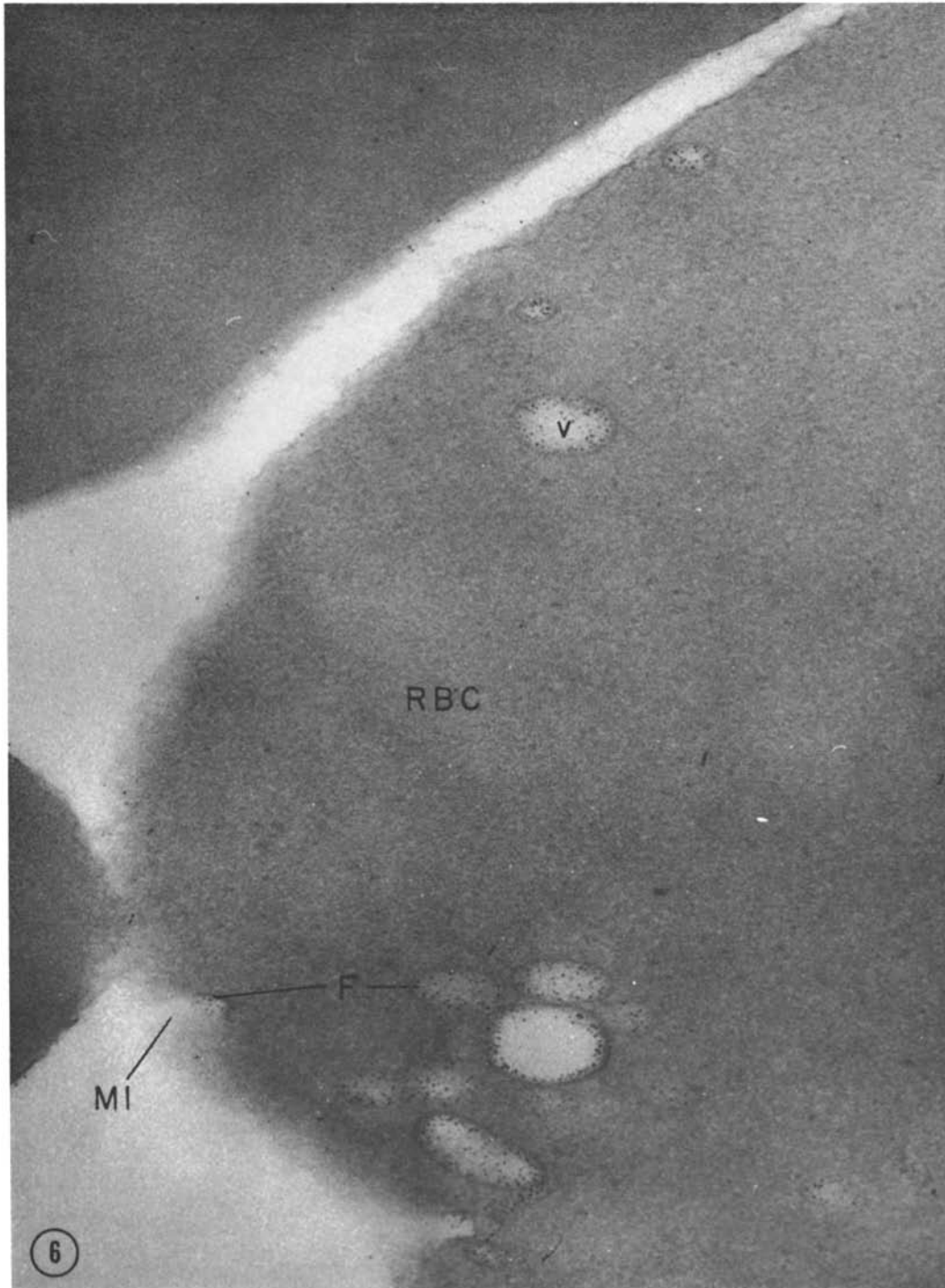FIGURE 6   Two group A human newborn erythrocytes (*RBC*) treated with ferritin-conjugated anti-A serum. Note the accumulation of ferritin particles (*F*) within the cell membrane invagination (*MI*) and lining the intracytoplasmic vesicles (*v*). Relatively few ferritin particles are observed along the cell membranes of these cells as compared with the adult cells treated in the same manner (Fig. 5). × 80,000.

These grouped data are presented in the right hand column of Table II. It can be seen that both groups of adult red cells gave essentially the same results, the percentage of vesiculated cells being 5.9 for the 1500 nonantibody-treated cells and 3.3 for the 1,000 antibody-treated cells. This small difference is not significant ($p = < 0.600$). Contrary to these observations on adult erythrocytes, the findings on 1,000 nonantibody-treated infant's erythrocytes showed that the percentage of vesiculated cells is 25.5, whereas for the antibody-treated infant's cells the percentage of vesiculation is 39.6. This percentage difference between infant's treated and nontreated cells is 14.1 and is significant ($p = < 0.001$).

When the effects of unconjugated and conju-gated anti-A sera upon group A erythrocytes of newborn infants were compared, it was found that the numbers of vesiculated cells observed were not significantly different, the percentages being 40.4 and 38.3, respectively. However, the coupling of ferritin to the anti-A serum made visible the location of the antibody. Fig. 5 shows the agglutination of three adult cells, with the ferritin-antibody conjugate surrounding the cells and indicating the site of action. No vesicles or ferritin particles are to be seen within the matrix of these cells. When this same tagged antibody was added to the infant's cells, the formation of vesicles was stimulated as seen in Fig. 6. One of the three clumped cells shows both vesicles and invaginations of the cell membrane. In contrast to the

TABLE III

*The Incidence of Observed Vesicles within Treated and Untreated Adult and Infant Erythrocytes**

| Source | Treatment | No. of vesicles per cell | | | | | | | | | | | | | | | No. of cells counted |
		0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	
No specific antibody used																	
Adult O	None	190	7	3	0	0	0	0	0	0	0	0	0	0	0	0	200
Adult O	Free ferritin	186	10	3	1	0	0	0	0	0	0	0	0	0	0	0	200
Adult O	Unconjugated anti-A serum	184	11	5	0	0	0	0	0	0	0	0	0	0	0	0	200
Adult O	Ferritin-conjugated anti-A serum	194	5	1	0	0	0	0	0	0	0	0	0	0	0	0	200
Specific anti-A serum used																	
Adult A	Unconjugated anti-A serum	191	9	0	0	0	0	0	0	0	0	0	0	0	0	0	200
Adult A	Ferritin-conjugated anti-A serum	193	7	0	0	0	0	0	0	0	0	0	0	0	0	0	200
	Total	1138	49	12	1	0	0	0	0	0	0	0	0	0	0	0	1200
No specific antibody used																	
Infant A	None	145	31	13	5	2	1	2	0	1	0	0	0	0	0	0	200
Infant A	Free ferritin	154	29	6	5	4	1	0	0	0	1	0	0	0	0	0	200
Specific anti-A serum used																	
Infant A	Unconjugated anti-A serum	123	40	23	4	2	2	1	1	0	3	0	1	0	0	0	200
Infant A	Ferritin-conjugated anti-A	130	42	12	6	1	2	1	4	0	1	0	0	0	0	1	200
	Total	552	142	54	20	9	6	4	5	1	5	0	1	0	0	1	800

* Observations made at X 8,000.

scattered ferritin along the cell membranes of agglutinated adult cells (Fig. 5), the ferritin particles along the membrane of the infant's cells are clustered at irregular intervals. The site of accumulation seems to result in an invagination (Fig. 6) which is eventually pinched off to become an intracytoplasmic vesicle. Treatment of erythrocytes from either group O or B infants with the conjugated anti-A serum did not cause agglutination, nor did pinocytosis of the conjugate occur.

The numbers of vesicles found within the erythrocytes under the various conditions of this study were tabulated by observing 200 cells for each category given in Table III. Vesicle formation in adult cells rarely exceeded 1% for the 1,200 antibody-treated and nonantibody-treated cells; in 12 cells two vesicles were observed, and in one cell three vesicles were found. However, the examination of infant's cells showed not only that the number of vesiculated cells was greater, as expected, but also that within the vesiculated cells the number of vesicles was greater, ranging from one to as many as 14 in a single section of an erythrocyte.

DISCUSSION

Although the formation of vesicles seldom occurred in circulating erythrocytes of the adult, the phenomenon was found to be common in the red cells of newborns. These erythrocytes of newborns showed frequent invaginations of the cell membrane and vesicles occurred throughout the cytoplasm, whereas erythrocytes of the adult showed no membrane invaginations in this study. The difference in the percentages of vesiculated cells between adults (5.5%) and infants (25.5%) is statistically very significant (p = < 0.001).

The relationship between pinocytotic-like invaginations of the cell membrane and the presence of vesicles was upheld by the observation that, when vesicles were situated near the cell surfaces of the infant's erythrocytes, an invagination of the membrane was frequently observed in the immediate vicinity. It appeared that the peripheral vesicles had been formed recently and were beginning to migrate away from the cell surface at the time of fixation. The possibility of misinterpreting invaginations of the sectioned membrane as vesicles, because of the angle at which the cell had been cut, was a matter of concern. However, in several of our observations, small invaginations of the membranes were constricted to such an extent that they

appeared to be forming vesicles. In addition, membrane invaginations were very shallow, whereas intracytoplasmic vesicles were seen deep within the cytoplasm of the cell (Figs. 4 and 6). The possibility of having sectioned a long irregular tubelike structure which would give the impression of an invagination with vesicles was considered to be unlikely, since in all of our observations not one such structure was seen.

This phenomenon of vesiculation was observed in circulating cells lacking nuclei, mitochondria, and the other organelles charactristic of an erythroblast, in both infants and adults. The absence of these organelles was determined by serial sections. The normal percentage of reticulocytes in the human ranges from 2.5 to 6.5 in the newborn (26) and from 0.5 to 1.5 in the adult (12). When these low incidences of reticulocytes are compared to our findings of vesicles in 25.5% of the cord blood erythrocytes of infants and in 5.5% of circulating erythrocytes of adults, it seems rather unlikely that the vesiculations observed involved only the reticulocytes. Therefore, on the basis of their morphology and incidence, the majority of these vesiculated cells appear to be mature erythrocytes.

Treatment of erythrocytes of the newborn with specific antibodies stimulated their pinocytotic activity. A statistical comparison of the antibody-treated cells with the untreated cells of the newborn showed a significant difference in the percentage of vesiculated cells (p < 0.001); a similar comparison of treated and untreated red cells of the adult showed no significant difference in this percentage (p < 0.600). Some investigators have concluded that the availability of surface membrane is the limiting factor for pinocytosis (6). If such is the case, the present findings would indicate that the erythrocytes of the newborn have the ability to form new membranes rapidly in response to antibody adsorption, but that the circulating red cells of the adult have lost this property or are nonreactive.

The stimulatory effect of antibodies on pinocytosis was visualized through the use of ferritin-conjugated antiserum. In the red cell of the infant, the area of the cell membrane that adsorbed the conjugated antibody was often the site of invagination and vesicle formation. As pinocytosis occurred, it was noted that the surface membrane labeling was reduced and that the ferritin-antibody complexes then lined the intracytoplasmic vesicles

(Fig. 6). These findings are somwhat comparable to those of Wolpert and O'Neill (28) who treated *Amoeba proteus* with a fluorescent-labeled specific antibody and observed an increase in pinocytosis. The stimulation of pinocytosis by proteins in such primitive single cell forms of life has been reported to occur also in the amoeba *Chaos chaos* by Marshall and Nachmias (20). Human platelets (other nonnucleated forms) have been reported by Movat et al. (21, 22) to incorporate or "phagocytize" particles such as antigen-antibody complexes, latex, and colloidal carbon.

In view of a normal uptake of ferritin by cells of the erythropoietic series in mammalian bone marrow, we incubated red cells from the circulation of newborns with free ferritin (unconjugated) to determine its possible effects on the cell membranes. Pinocytosis of these ferritin particles did not occur, nor was there an increase in the incidence of vesicles among these erythrocytes. That this pinocytotic process was induced merely by the environ-

mental presence of nonspecific proteins was a possibility further investigated by the treatment of erythrocytes of a group O infant with anti-A serum. This treatment caused neither clumping of the red cells nor increased vesiculation.

We treated erythrocytes of a group O infant and of a group B infant with ferritin-conjugated anti-A serum to check the possibility that these cells nonspecifically take up the ferritin-globulin conjugate. Pinocytosis of the conjugate was not observed in either case. It appears, therefore, that the increase in the number of vesicles in the erythrocytes of newborns is due to treatment with specific antibody.

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