Expression of B and H antigens on red cells from a group B_{weak} individual studied by serologic and scanning electron microscopic techniques

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The proposita was classified as B_{el}, B_y, or B_m, Le(b+) by routine blood grouping and by adsorption/elution studies using anti-A and -B hyperimmune pregnancy sera. Red cells from the proposita adsorbed as much anti-B from the hyperimmune sera as did red cells from normal B individuals, but adsorbed less anti-A,B (group O serum). Saliva contained H, but not B, soluble substance. Red cells from the proposita and a normal B donor were sensitized with monoclonal A and B blood group antibodies immunolabeled with colloidal gold particles, and examined in a scanning electron microscope. B antigens were found on more than 95 percent of normal B cells, but on only 2–3 percent of red cells from the proposita. However, when the same cells were sensitized with anti-A,B that reacted strongly with B oligosaccharides other than type 2 chains, half of the labeled red cells from the proposita were labeled more strongly than any normal B cells. Our results explain why red cells from the proposita adsorb significant amounts of anti-B and anti-A,B without being agglutinated by these antibodies. The results of both adsorption/elution and immunolabeling suggest that the B antigen on her cells differs biochemically from that on normal B cells. ***Immunohematology*** 1992;8:94–99.

Weak subgroups of blood group B may be categorized according to Salmon.[1] The terms B_{el}, B_y, and B_m are applied when red cells are not agglutinated by anti-B but no anti-B is found in serum, and cells can be shown by adsorption/elution tests to bind anti-B. Secretor B_{el} and B_y individuals do not secrete B substance, while B_m individuals do. B_{el} and B_y cannot be distinguished serologically, but are thought to result from different genetic mechanisms.[1] Family studies are therefore needed to make this distinction.

The number of B antigens on erythrocytes of weak B subgroups is thought to be low. On normal B erythrocytes, the B antigen and its precursor H are expressed by monofucosylated type 2 oligosaccharide chains, as is the case with A and H on A_2 erythrocytes.[2,3] Indications exist, however, that some weak subgroups of A have a considerable number of A antigens formed by other chain types that may react poorly with anti-A.[4] The "weakness" of these subgroups may therefore be caused in part by a different biochemical composition of the A antigen and not only by a low number of antigens. Thus, it seemed worthwhile to look for similar biochemical deviations in weak B subgroups.

A general feature of carbohydrate antigens on erythrocytes is cell-to-cell variation of development.[4–7] Pronounced variations have been found for the A antigen in subgroup A_3, in which 40–50 percent of the cells appear negative for the A antigen while others are strongly positive.[4] In a preliminary study of erythrocytes of the weak subgroups A_m, A_{el}, and A_{end}, we found that A antigens were expressed on less than 5 percent of red cells, some of which were heavily labeled.[8] It seemed natural to look for similar features in weak B subgroups.

We present a study of the erythrocytes of a healthy proposita who was initially grouped as B_{el}, B_y, or B_m, Le(b+), based on categories according to Salmon.[1] In addition to extensive adsorption and elution experiments with alloantibodies from hyperimmune pregnancy sera, we performed indirect immunolabeling of the erythrocytes. The cells were sensitized with monoclonal blood group antibodies (mAbs) of biochemically characterized specificities labeled with colloidal gold particles conjugated to anti-antibodies and visualized in the backscatter electron imaging (BEI) mode of a scanning electron microscope.

The BEI mode has proved especially useful in visualizing colloidal gold probes labeling cell surface antigens.[9–12]

Materials and Methods

ABO blood group tests

A_1, A_2, A_x, and O red cells were obtained from ACD anticoagulated blood drawn from previously grouped blood donors. Red cells from the proposita were originally tested with routine ABO blood grouping reagents. They were further tested with IgM mAbs anti-B, -A,B, and -H. Serum from the proposita was tested with B cells at ambient temperature and with incubation at 4°C.

Other blood group tests

Further blood grouping of red cells from the proposita was carried out by saline technique for antigens of the Lewis system and for M and N antigens of the MNS system, by albumin technique for antigens of the Rh and Kell systems, and by antiglobulin technique for antigens of the Duffy and Kidd systems and for S and s antigens of the MNS system. Polyclonal antibodies produced and controlled in this institute were applied throughout.

Adsorption and elution

A_1 and B red cells or red cells from the proposita were used for individual adsorption of blood group O (A,B) sera previously shown to contain strong IgG anti-A (two sera) or IgG anti-B (two sera). Prior to adsorption, the cells were washed three times with saline. One volume of packed cells was mixed with two parts of serum and allowed to react for 2 hours at 4°C. The cells were then centrifuged at 4°C, serum was removed, and cells were washed six times with ice-cold saline and centrifuged at 4°C. One volume of washed, packed cells was then mixed with one volume of saline (preheated to 56°C) in vials, similarly preheated, and the mixture was shaken at 56°C for 7 minutes. Then the mixture was centrifuged in a 37°C thermostat room, and the eluate was removed immediately. Usually the eluates were tested the same day. Alternatively, 30-percent bovine serum albumin was added to give a final concentration of 6 percent, and the eluates were kept frozen until tested.

The ability of the eluate to agglutinate A_1, A_x, B, and O red cells and those from the proposita was tested by the antiglobulin technique. Two parts of eluate were mixed with one part of a 3-percent suspension of test red cells, and incubated at 37°C for 1 hour before washing and reaction with an antiglobulin reagent containing anti-IgG and -C3d.

Monoclonal antibodies

Monoclonal antibodies (mAbs) used for this study are as follows:

- Anti-B (code B 003), kindly donated by BioCarb AB, Lund, Sweden, and reacting with group B types 1 and 2 oligosaccharide chains[13]
- Anti-A,B, kindly donated by M. Hervé Broly of the Centre Régional de Transfusion Sanguine, Lille, France, and reacting strongly with group B chain types 2, 5, and 6 and group B trisaccharide[14]
- Anti-H, kindly donated by Dr. Jan Kolberg of the NIPH and found by Oriol[15] to react with H chain types 2 and 6
- Anti-A, kindly donated by M. Hervé Broly and reacting with all known A chain types[14]

Lectins

Laburnum alpinum anti-H lectin was bought from Behringwerke, Germany, and used both undiluted and at a saline dilution of 1:4.

Agglutination inhibition studies

Saliva collected from the proposita and from one group B and one group O secretor was diluted with an equal volume of saline, boiled for 10 minutes, and centrifuged. The supernatant was kept at –20°C until used in an agglutination inhibition test with mAbs anti-B, -A,B, and -H, as well as with lectin anti-H. Initial saline dilution of saliva for inhibition was 1:25.

Protease treatment

Treatment of red cells from the proposita with papain was carried out using a 30-minute incubation (Papain Merck, Darmstadt, Germany) according to the protocol applied for routine blood group serology in this laboratory.

Immunolabeling and preparation for electron microscopy[4]

All dilutions of reagents and washing of red cells were performed in 0.1 mol/L Na-cacodylate buffer in 0.1 mol/L saccharose, pH 7.2, containing 0.02% Na-azide. Briefly, red cells were prefixed in 0.1% glutaraldehyde, and washed in 0.1% glycine. The washed cells were sensitized for 30 minutes with an equal volume of mAb and then incubated overnight with goat

antimouse antibodies conjugated to 30 nm colloidal gold particles (GAM IgM G30, Janssen Life Sciences Products, Belgium) at a final concentration of 1:5. Labeling specificity was ascertained by negative control experiments with cells incubated with buffer instead of mAb, and by positive control experiments using mAb anti-A against A_2 red cells.

After labeling, the red cells were sedimented onto chips of mica, dehydrated in ethanol, and critical point dried from CO_2. The specimens were coated with a thin layer of carbon before being mounted for electron microscopy.

Electron microscopy

The red cells were examined in the BEI mode of a JSM 840 scanning electron microscope. The microscope was operated at 15 keV using a probe current of 1×10^{-10} amp and a working distance of 7–8 mm. Micrographs of 150–200 cells were recorded for each specimen at a primary magnification of 3,000 ×.

Interpretation of micrographs

A red cell was defined as labeled if at least ten gold particles were visible on its surface. Labeling strength of individual cells was scored as weak (+) or moderate/strong (++) in differential counting by comparison with a reference micrograph of immunogold-labeled A_2 cells sensitized with mAb anti-A. Differential counting was carried out independently by two of the authors on 150–200 red cells in each experiment. Similar semiquantitative evaluation of antibody binding had been carried out previously.[4,6,16]

Results

Blood grouping

The red cells from the proposita were Le(a–b+), M+N–S–s+, P_1–, ccDE, K–k+, Fy(a+b+), Jk(a+b+), and Lu(a–b+). There were no mixed-field reactions with any of the antibodies.

Adsorption and elution

From two group O sera containing immune anti-A, both A_1 and B red cells adsorbed antibodies that, after elution, agglutinated A_1 and A_2 cells strongly and B cells more weakly, but did not agglutinate red cells from the proposita or group O cells (see Table 1). By contrast, the same antibodies adsorbed by, and eluted from, red cells from the proposita agglutinated B cells more strongly than A_1 and A_2 cells, and the reaction with B cells was at least as strong as eluates from A_1 and B red cells. Only eluates from B cells agglutinated A_x cells.

From two group O sera containing immune anti-B, both A_1 and B red cells adsorbed antibodies that agglutinated A_1, A_2, and B cells moderately to strongly, but did not agglutinate red cells from the proposita or group O cells (see Table 2). Again, by contrast, the eluate from red cells from the proposita reacted quite strongly with B cells but only weakly with A_1 cells, and showed no reaction with the other cells. Only an eluate from B cells agglutinated A_x cells.

Table 1. Agglutination titers of eluates prepared from absorbing cells after incubation with two group O (A,B) sera containing strong IgG anti-A

Cells for agglutination	Absorbing cells		
	A_1	B	Proposita
A_1	512/128*	512/128	4/16
A_2	64/ND†	128/ND	4/ND
A_x	0/0	8/16	0/0
Proposita	0/0	0/0	0/0
B	32/8	16/8	32/8
O	0/0	0/0	0/0

*Indirect antiglobulin readings using two group O (A,B) sera
†Not done

Table 2. Agglutination titers of eluates prepared from absorbing cells after incubation with two group O (A,B) sera containing strong IgG anti-B

Cells for agglutination	Absorbing cells		
	A_1	B	Proposita
A_1	256/16*	64/4	1/1
A_2	16/ND†	32/ND	0/ND
A_x	0/0	4/0	0/0
Proposita	0/0	0/0	0/0
B	32/128	64/128	32/64
O	0/0	0/0	0/0

*Indirect antiglobulin readings using two group O (A,B) sera
†Not done

Inhibition of agglutination by saliva

Agglutination of normal B cells by mAb anti-B 003 diluted 1:80, and by mAb anti-A,B diluted 1:16, was inhibited completely by B secretor saliva diluted 1:50. The saliva from the proposita did not inhibit agglutination with these mAbs when used at its initial dilution of 1:25.

Agglutination of normal group O cells by mAb anti-H diluted 1:10 was inhibited completely by group O secretor saliva diluted 1:40 and by saliva from the proposita diluted 1:5, while no inhibition was

seen with group B secretor saliva. Similar results were achieved in inhibition of agglutination with lectin anti-H.

Electron microscopy of immunogold-labeled red cells

Differential counting results of immunogold-labeled group B and O cells and cells from the proposita are given in Table 3. Micrographs of immunogold-labeled group B red cells and red cells from the proposita, sensitized with mAb anti-A,B, are shown in Figure 1. Seventy-three percent of normal B cells sensitized with mAb anti-B 003 were labeled, with 3 percent showing moderate to strong (++) labeling. Only 3 percent of red cells from the proposita were labeled when sensitized with anti-B 003, and none showed ++ labeling. About 98 percent of normal B cells sensitized with mAb anti-A,B were labeled, with 10 percent showing ++ labeling. Only 2 percent of red cells from the proposita sensitized with anti-A,B were labeled, but half of them were exceptionally strongly labeled, even stronger than any of the labeled normal group B cells (see Figs. 1a and 1b). Papain treatment of red cells from the proposita did not change the labeling pattern with anti-A,B, and resulted in no labeling when mAb anti-A was used for sensitization.

Fifty-one percent of normal group O cells and 76 percent of red cells from the proposita were labeled when sensitized with mAb anti-H. Labeling strength varied considerably from cell to cell. By contrast, only about 5 percent of normal group B cells were labeled when sensitized with this mAb (micrographs are not shown here).

Table 3. Results of differential counting of immunogold-labeled red cells sensitized with various monoclonal antibodies (mAbs), with 150–200 cells counted in each experiment

Cells	mAb	% not labeled	% labeled: Weak positive	% labeled: Moderate/ strong positive
Group B	anti-B*	27	70	3
Group B	anti-A,B	2	88	10
Group B	anti-H	95	4	1
Proposita	anti-B	97	3	0
Proposita	anti-A,B	98	1	1†
Proposita	anti-H	24	62	14
Proposita‡	anti-A,B	97	2	1†
Proposita‡	anti-A	100	0	0
Group O	anti-H	49	46	5

*mAb code B 003 (see Materials and Methods section)
†Very strongly labeled cells
‡Papain-treated cells

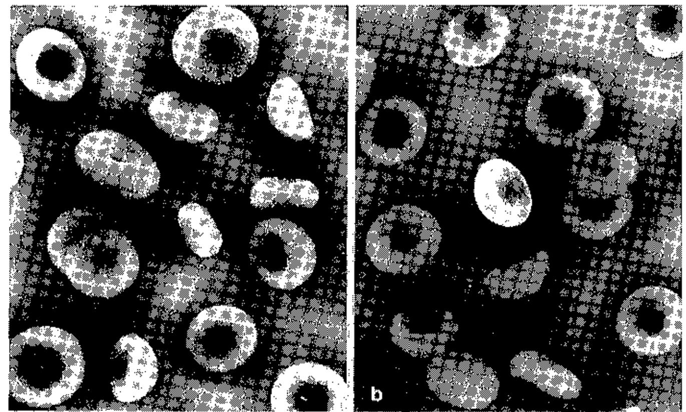

Fig. 1 Backscatter electron imaging of erythrocytes sensitized with mouse monoclonal IgM blood group antibodies and immunolabeled with 30 nm colloidal gold particles (× 4,500). (See also Table 1.)
a. Normal B cells sensitized with anti-A,B. Almost all cells are labeled, but labeling strength varies from cell to cell. No cells are very strongly labeled.
b. Red cells from the proposita sensitized with anti-A,B. A very strongly labeled cell is seen among the majority of unlabeled cells.

Discussion

The proposita fulfilled the criteria of Salmon for classification as B_{el}, B_y, or B_m.[1] As no family members were available for study, further distinction could not be made.

For this study we used hyperimmune pregnancy sera and red cells from the proposita as well as A_1 and B cells for adsorption and elution studies. Eluates from red cells of the proposita reacted about as strongly with B cells as did eluates from A_1 and B red cells. However, eluates from red cells of the proposita reacted rather weakly with A_1 and A_2 cells, in contrast to eluates from A_1 and B red cells. Only eluates from group B cells agglutinated A_x cells. The specificity of the eluates from the proposita thus appeared to deviate from eluates from A_1 and B red cells. The deviation can be explained by presuming that red cells from the proposita adsorbed relatively less anti-A,B known to be present in group O sera[17] than did A_1 and B cells. If so, the chemical structure of the B antigens on B cells from the proposita might not be similar to that of normal B cells.

Immunolabeling with monoclonal anti-B or anti-A,B showed that only about 3 percent of red cells from the proposita expressed B antigens. This finding explains why anti-B or anti-A,B reagents failed to agglutinate those cells, even though they adsorbed such antibodies from hyperimmune pregnancy sera.

With mAb anti-A,B for sensitization, 1 percent of red cells from the proposita showed an antigen density higher than on any cell from the normal group B donor (see Fig. 1b). The red cells from the proposita expressing B antigen therefore bound relatively more mAb anti-A,B than did normal group B cells, an observation that might seem at variance with the results found with adsorption and elution tests. However, the reactivity of anti-A,B with B chains other than types 1 and 2 is unknown and may well be low, while the mAb anti-A,B applied here reacts strongly with B chain types other than types 1 and 2. Our immunolabeling results may therefore be well in keeping with the hypothesis that the biochemical nature of the B antigens on red cells from the proposita differs from that of B cells from normal donors.

Unfortunately, the broad reactivity of the mAb anti-A,B used for sensitization precludes further conclusions on the nature of the B antigens on the red cells from the proposita. However, the strong binding of mAb anti-A,B cannot be due to binding to hidden A antigens,[18,19] since her papain-treated cells showed no immunolabeling when sensitized with mAb anti-A.

A reciprocal relationship is thought to exist between B and its precursor H on erythrocytes.[17] The observation that many more cells from the proposita than from normal group B donors were labeled when sensitized with mAb anti-H is therefore in accordance with B being expressed on only a few of her cells. (The percentage of labeled cells on normal group O cells was similar to our previous results with another mAb anti-H.[6]) Surprisingly, we also found that more of the red cells from the proposita were labeled than were group O cells from a normal donor, when sensitized with mAb anti-H. This observation should be interpreted with care because results of only two individuals were compared. However, Simmons and Twaitt[20] described a family in which weak B individuals had more H on their cells than O individuals. The possibility therefore exists that the H antigen on weak B cells also deviates from that on normal group O and B red cells. Studies of immunolabeled cells from the proposita should be carried out with anti-H mAbs of other fine specificities to elucidate this possibility.

As pointed out previously, cell-to-cell variation of development is a general feature of red cell carbohydrate antigens.[3–6] For the B antigen, the variation has reached an extreme degree in the proposita. In a preliminary immunogold labeling study with mAb anti-A and A subgroups A_m, A_{el}, and A_{end}, we found that immunolabeling was similarly confined to a small subpopulation of erythrocytes,[8] regardless of the secretor status of the individual, and with large amounts of antigens on a few cells. The expression of B antigens on red cells from the proposita may therefore be typical for B/A expression in several weak subgroups of both B and A.

The genetics permitting B antigens to develop on only a small minority of red cells from the proposita remain unknown. It should be emphasized that extensive blood typing outside the ABO system gave no indications that she was a chimera. As no B substance was found in her saliva, it appears that she expressed the product of the *B* gene only in a part of the bone marrow. One may conclude that expression of carbohydrate residue transferases varies not only between organs but also between parts of an organ,[3] possibly because of varying development of cell clones. Watkins[21] has proposed that a gene closely linked to the A/B locus regulates the expression of A and B antigens, but it is not known whether the activity of this gene varies between organs and clones. The genetics of the weak B and A subgroups would be further complicated if it could be confirmed that the development of H also deviates from that of normal group O, A, and B individuals.

In conclusion, our results suggest that the biochemical nature of the B antigens on the red cells of the proposita, who types as B_{el}, B_y, or B_m according to classification by Salmon,[1] deviates from that on normal B cells, and confirm that ABH antigens on red cells show major variations between both subgroups and single red cells. The highly restricted expression of B antigens on red cells of the proposita explains why these cells are not agglutinated by anti-B or anti-A,B, although they adsorb these antibodies. However, our results should be confirmed in other B_{el}, B_y, or B_m propositi before final conclusions are drawn. Similar studies should be carried out in propositi of other weak B subgroups, to provide, if possible, a more rational classification than that proposed previously.[1] Biochemical studies should focus on the nature of H, B, and A antigens in weak subgroups, on their H, B, and A forming transferases,[21] and on the nature of their *H, B,* and *A* genes,[22,23] in order to understand the genetic regulation mechanisms leading to this rather surprising pattern of blood group antigen development.

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