Variations in the susceptibility of erythrocyte membrane glycolipids to galactose oxidase

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1. INTRODUCTION

Treatment of cells with galactose oxidase followed by reduction with radioactive sodium-borotritide ($NaBH_4$) is widely employed for the analysis of cell surface glycoconjugates [1–3]. We have used galactose oxidase in conjunction with lectins specific for D-galactose and *N*-acetyl-D-galactosamine, to investigate the organization of glycoconjugates on human erythrocytes. In the course of these studies we have observed that erythrocytes from different individuals differ markedly in their sensitivity to the enzyme, as assessed by the decrease in agglutinability by, and binding of, soybean agglutinin (SBA). In particular, erythrocytes from certain individuals of blood type A or AB were only slightly affected by the enzyme. Since the receptors for SBA on human erythrocytes are primarily glycolipids [4,5], our data strongly suggest that there are considerable differences in the susceptibility of these glycolipids to galactose oxidase and that such differences are genetically determined.

2. MATERIALS AND METHODS

Freshly-drawn blood obtained from healthy consenting donors was collected in citrate–glucose anticoagulant; phenylmethylsulfonyl fluoride (PMSF) was then added to 0.1 mM final conc. and the blood was either used immediately or stored in the cold and used within 7–10 days. Erythrocytes were washed 3–4 times with phosphate-buffered saline (0.05 M phosphate, 0.15 M NaCl (pH 7.4) (PBS) before use. Erythrocyte ghosts were prepared by osmotic shock in 5 mM sodium-phosphate buffer (pH 8) [16], containing 0.1 M PMSF. They were dissolved in PBS containing 1% Triton X-100, 1% ethanol and 0.1 mM PMSF with 5–10 min sonication in a water bath.

SBA was purified by affinity chromatography [7]; it was iodinated by the chloramine T method in [8] and repurified by affinity chromatography. The product had 50 000–60 000 cpm/μg. Binding of [^{125}I]SBA to erythrocytes (5×10^7 cells) was performed as described for lymphocytes [9] except that separation of the labeled cells from excess lectin was by centrifugation on a 2:1 (instead of 1:1) mixture of dioctylphthalate:dibutylphthalate.

Hemagglutination tests were done by the double dilution technique in microtiter plates [10]. Polyacrylamide gel electrophoresis in sodium dodecylsulfate (SDS) was done in 8% running gels with the discontinuous buffer system in [11]. [^{125}I]SBA binding in situ was done on unstained gels by the overlay technique in [12].

Protein was determined according to [13] with the Dye Reagent from Bio-Rad Labs., using bovine serum albumin as standard.

Treatment with galactose oxidase was done on 20% suspensions of erythrocytes in PBS with varying amounts of the enzyme in the presence of 10 units/ml of catalase. After 1 h at 37°C the cells were washed sequentially with PBS, PBS containing 0.1 M D-galactose and 3 times with PBS (5–10 vol. each wash). The cells were used immediately

Abbreviations: PBS, phosphate buffered saline (pH 7.4); PMSF, phenylmethylsulfonyl fluoride; SBA, soybean agglutinin; SDS, sodium dodecylsulfate

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for agglutination or binding experiments.

Trypsinization of erythrocytes was carried out with 100 μg enzyme/ml of a 10% suspension of cells in PBS for 1 h at 37°C.

Carrier-free $Na^{125}I$ was from Radiochemical Center (Amersham). Trypsin (2 × crystallized) and galactose oxidase (50–125 units/mg) were from Sigma. To inactivate proteolytic contaminants that may be present in the latter enzyme, it was heated at 225 units/ml PBS for 30 min at 56°C before use. In the early experiments, galactose oxidase was from Kabi (Stockholm) as specified in the results. This enzyme was protease free and did not require any pretreatment. Catalase was from Worthington.

3. RESULTS AND DISCUSSION

Human erythrocytes readily undergo agglutination by SBA, with 8–16 μg/ml of the lectin giving complete agglutination in a serial dilution assay in microtiter plates. Upon treatment with galactose oxidase, we found that the cells from most individuals became much less susceptible to agglutination by the lectin (up to 16–32-fold at 20 units/ml of enzyme) (fig.1, A–C). We have designated such cells as 'sensitive'. Occasionally there were, however, donors whose erythrocytes responded poorly to oxidation by the enzyme, showing a decrease of 2–4-fold in agglutinability (fig.1, rows E–G); such cells were designated as 'resistant'. A considerable proportion of donors had cells with intermediate degrees of sensitivity. In all cases, the susceptibility to agglutination by SBA of the oxidized cells was completely restored to that of untreated cells by reduction with $NaBH_4$ (1 mg/ml, 30 min at room temperature) (fig.1, rows D, H). Such high concentrations of $NaBH_4$ had to be used since we noted that at lower concentrations the effect of galactose oxidase on the interaction of cells with SBA was

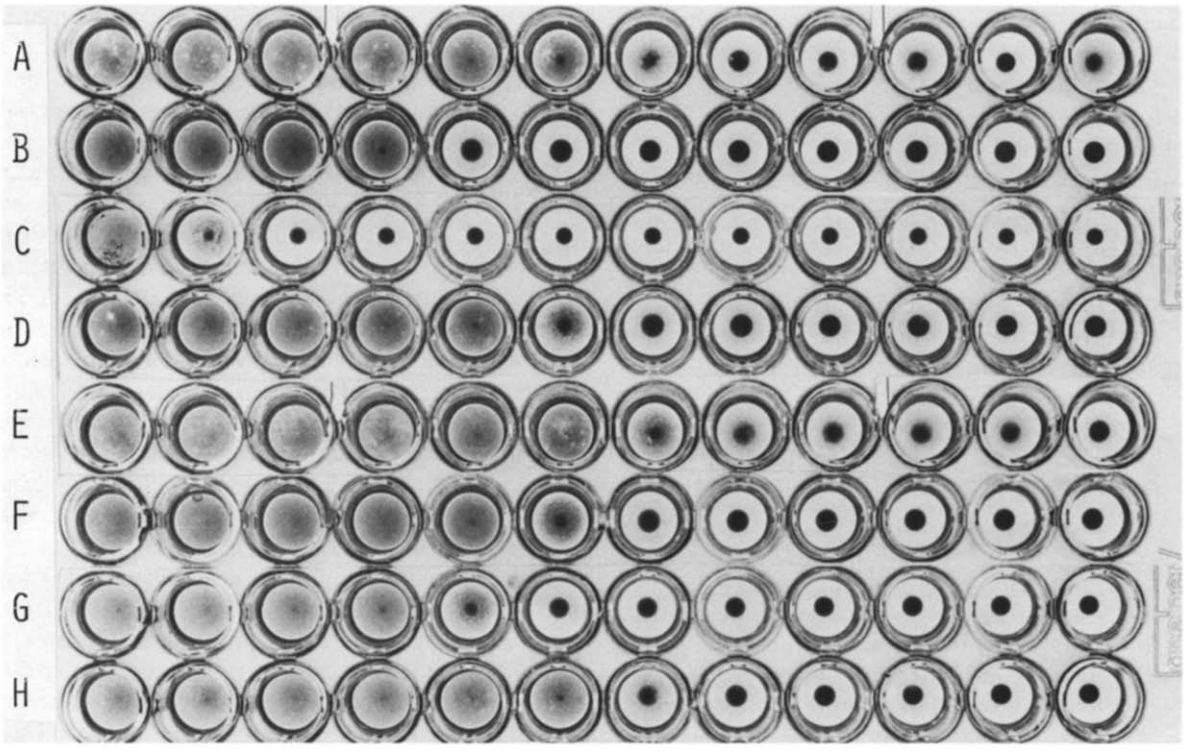

Fig.1. Effect of galactose oxidase on the agglutination of erythrocytes from two donors of blood type A. Cells in rows A–D from donor 1, D–H from donor 2: (A,E) untreated; (B,F) incubated with 2 units of galactose oxidase for 1 h at 37°C; (C,G) same with 20 units of enzyme; (D,H) cells treated as in (C) and (G), respectively, then reduced with $NaBH_4$.

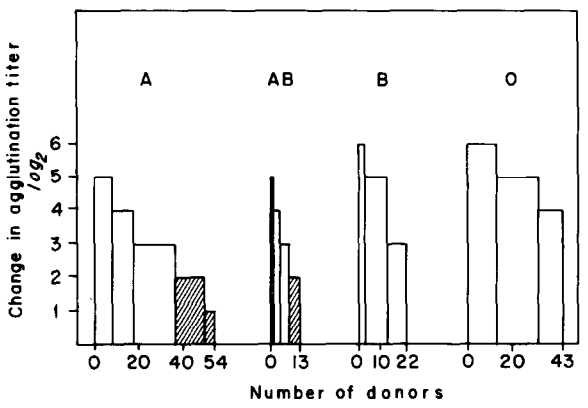

Fig.2. Distribution of cells sensitive and resistant to galactose oxidase amongst donors of different ABO blood types. The number on the ordinate gives the decrease in SBA agglutination titer upon treatment of the cells with 20 units of galactose oxidase.

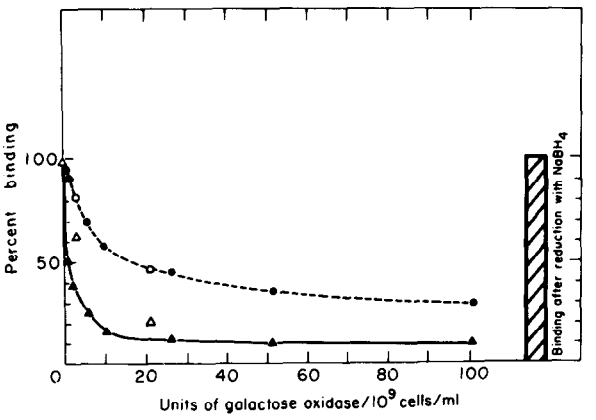

Fig.3. Effect of galactose oxidase on binding of human erythrocytes. Cells of type AB (filled symbols) or type A (empty symbols) were treated with varying amounts of galactose oxidase from Kabi or Sigma, respectively: (●,○) resistant cells; (▲,△) sensitive cells. Binding after reduction with $NaBH_4$ is given only for type AB cells.

only partially reversed. The need for high concentrations of $NaBH_4$ for the complete reduction of oxidized D-galactose and *N*-acetyl-D-galactosamine residues on erythrocytes was also reported in [14].

A survey of erythrocytes from 132 donors revealed that 16% had resistant cells (fig.2); all of these were individuals of blood type A or AB, comprising ~35% of this population. Sensitive cells, on the other hand, were found in all blood types. Sensitivity was most pronounced among certain individuals belonging to blood groups O and B, where cases were found with a 64-fold decrease in agglutinability upon treatment with galactose oxidase. No correlation with blood types A_1, A_2, Rh and P was observed. The degree of sensitivity of cells from an individual donor remained constant when fresh samples of blood were taken over a period of months up to 2 years. No difference was found in the effect of galactose oxidase on the agglutinability of sensitive and resistant cells by other D-galactose or *N*-acetyl-D-galactosamine specific lectins (from *Erythrina cristagalli*, *Ricinus communis*, *Tridacna maxima* and *Crotolaria juncea*). This may be because binding of these lectins is not affected by oxidation at C-6 of D-galactose/*N*-acetyl-D-galactosamine or because they bind to sites different from those to which SBA binds.

The decrease in agglutinability of SBA cells treated with galactose oxidase was accompanied by a decrease in binding of the lectin and was much more pronounced with sensitive than resistant cells (fig.3). Similar results were obtained with galactose oxidase from 2 sources (Sigma, used routinely, and Kabi, the production of which was discontinued some time ago). Binding (>95%) was restored by reduction of the oxidized cells with 1 mg $NaBH_4$/ml. Both sensitive and resistant untreated erythrocytes gave overlapping binding curves in the range tested (10–160 μg SBA/ml; not shown), strongly suggesting that the number of SBA binding sites is identical.

Treatment of resistant and sensitive cells with trypsin prior to oxidation with galactose oxidase resulted in a 10-fold increase in their agglutinability and only a slight increase (<20%) in the total amount of SBA bound to both cell types, but did not change the relative sensitivity of the cells to the enzyme (not shown).

The molecular nature of the SBA receptor on human erythrocytes has not been unequivocally established. In [4,5] lectin was found to bind primarily to the glycolipids of the membrane. These findings have now been confirmed by staining of gels with [^{125}I]SBA. Both with sensitive and resistant erythrocytes, the lectin binds almost exclusively at the glycolipid region (fig.4).

Our data suggest that there are genetically con-

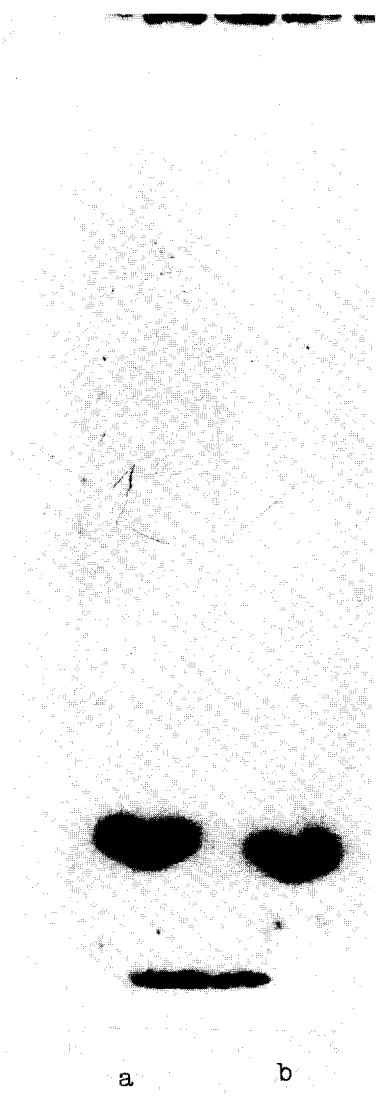

Fig.4. Autoradiograms of SDS–polyacrylamide gels of ghosts of sensitive and resistant cells stained with [^{125}I]SBA: (a) sensitive cells; (b) resistant cells.

trolled variations in the sensitivity to galactose oxidase of at least some of the erythrocyte glycolipids of different individuals. Such variations may be due to differences in composition and structure of membrane glycolipids, or due to their relative disposition in the membrane. The structure of the lipid aglycon in galactolipids [15] and the pattern of substitution of the penultimate sugar residue in galactosyl di- and trisaccharides [16] markedly affected their susceptibility to galactose oxidase. The ceramide composition may also define the orientation of the carbohydrate chain of a glycolipid in the membrane, thus affecting its accessibility to external agents [17].

One obvious candidate for the SBA receptor is globoside, since it possesses a terminal non-reducing *N*-acetyl-D-galactosamine residue for which SBA is highly specific, and is the major glycolipid of the human erythrocyte membrane (65% of total), corresponding to $\sim 10^7$ copies/cell, which is more than sufficient for the number of SBA binding sites per cell (1.2×10^6) [18]. This is also the major glycolipid substrate for galactose oxidase in the erythrocyte membrane and is more exposed on the cell surface than any other erythrocyte membrane glycolipid [1,2]. Moreover, preliminary experiments using the overlay method [19] with thin-layer chromatograms of lipid extracts of the erythrocyte membrane have demonstrated that the lectin does, indeed, bind to globoside, although binding to trihexosylceramide was also observed. Binding of SBA to the above glycolipids, primary to globoside, as well as to GM_2, has also been reported in a study on the interaction of the lectin with glycolipid components of porcine lymphocyte plasma membranes [20]. Work on the identification of the glycolipid receptor(s) of SBA in the erythrocyte membrane which is differentially affected by galactose oxidase is in progress in our laboratory.

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