

IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS  
 LXVI. Competitive Binding Assays of A<sub>1</sub> and A<sub>2</sub> Blood Group  
 Substances with Insolubilized Anti-A Serum and Insolubilized  
 A Agglutinin from *Dolichos biflorus*\*

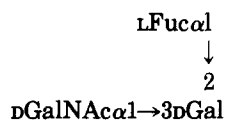
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There are conflicting views on the nature of subgroups A<sub>1</sub> and A<sub>2</sub> of blood group A. One holds that the same determinants are present on either, but that there are fewer determinants on A<sub>2</sub> than on A<sub>1</sub> erythrocytes. Soluble A<sub>2</sub> and A<sub>1</sub> substances would thus have the same kinds of determinants, but in different numbers. The A<sub>1</sub> and A<sub>2</sub> transferases are different enzymes, but the A<sub>2</sub> enzyme is less efficient than the A<sub>1</sub> transferase, but it has the same specificity (1) in adding terminal *N*-acetyl-D-galactosaminyl residues to precursor blood group H oligosaccharide side chains. Such a difference in enzymatic activity has been proposed to be responsible for the H activity of A<sub>2</sub> cells (2). A population of those anti-A<sub>1</sub> antibodies which do not agglutinate A<sub>2</sub> erythrocytes is known and has been prepared from the purified IgM fraction of anti-A sera by absorption with A<sub>2</sub> erythrocytes, but not from the IgG fractions of the same sera (3). Such anti-A antibodies have been assumed (3) to have a low affinity so that if they use only one of their ten valences per erythrocyte, they would be unable to hold two erythrocytes together. The anti-A<sub>1</sub> antibodies are hypothesized to agglutinate A<sub>1</sub> erythrocytes which have receptors that are more numerous and closer together, so that each erythrocyte could be bound by two or more of the combining sites of each antibody molecule.

The other concept, based predominantly on immunochemical studies with A<sub>1</sub> and A<sub>2</sub> glycoproteins, favors a qualitative difference (4). Absorption with insolubilized polylectyl A<sub>2</sub> substance did not remove all of the anti-A, but left anti-A<sub>1</sub>; had all determinants been present on A<sub>2</sub> as well as on A<sub>1</sub> substances, no anti-A<sub>1</sub> should have remained.

Since several different determinants on soluble blood group A substances have been found (5, 6, 7), a basis for a structural difference between A<sub>1</sub> and A<sub>2</sub> exists. All A determinants have the structure



but differ in that this trisaccharide may be linked  $\beta 1 \rightarrow 3$  or  $\beta 1 \rightarrow 4$  to dGlcNAc to give

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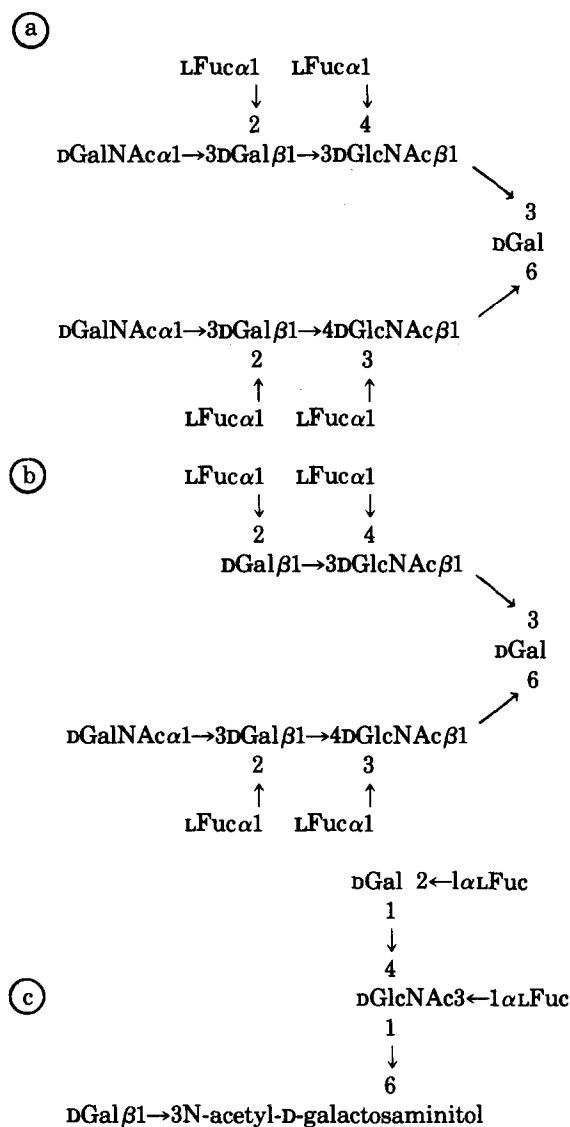


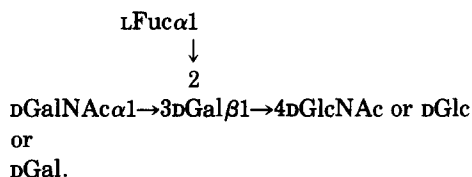
FIG. 1. (a) Structure in blood group A glycoprotein proposed for A specificity; dGalNAc, substituted  $\alpha 1 \rightarrow 3$  on the type 1 chain, is hypothesized to be  $A_1$ -specific, and on the type 2 chain,  $A_2$ -specific. (b) Structure in blood group A glycoprotein proposed for  $A_2$  specificity; the chain lacking the dGalNAc is  $HLe^b$ -specific. (c) Type 2 chain joined  $\beta 1 \rightarrow 6$  to *N*-acetylgalactosamine, compound JS  $R_{1M}, 0.91$  (14).

what have been termed (8, 9, 10) type 1 and type 2 blood group A determinants (Fig. 1). A second LFuc linked  $\alpha 1 \rightarrow 4$  or  $\alpha 1 \rightarrow 3$  to the dGlcNAc also gives two additional A determinants, and antibodies specific for the mono- or difucosyl determinants have been recognized (11). In the intact blood group substance, these oligosaccharide type 1 and type 2 determinants are linked  $\beta 1 \rightarrow 3$  and  $\beta 1 \rightarrow 6$ , respectively, to dGal.

The type 2 H determinant may be linked  $\beta 1 \rightarrow 6$  to the dGalNAc which had been cleaved from the polypeptide backbone (Fig. 1c), and by analogy to oligosaccharides isolated from blood group B substance (12, 13) it could have an additional dGalNAc $\alpha 1 \rightarrow 3$ ,

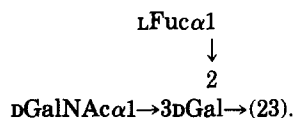
linked to the subterminal  $\text{dGal}$  of the H determinant in Fig. 1 c (14), to give a second type 2 A determinant. If, as hypothesized (4),  $A_1$  substances have both type 1 and type 2 A determinants (Fig. 1 a) whereas  $A_2$  substances lack the nonreducing  $\text{dGalNAc}$  linked  $\alpha 1 \rightarrow 3$  of the type 1 A determinant (Fig. 1 b), anti- $A_1$  would consist of antibodies specific for type 1 A determinants, and it would not be removed by insoluble  $A_2$  substance, as was found. Moreover, the absence of the terminal  $\text{dGalNAc}\alpha 1 \rightarrow 3$  on the type 1 chains would provide the many H determinants found on  $A_2$  substances, and these should be type 1 H specific.

The distinction between  $A_1$  and  $A_2$  is further complicated by the inference that erythrocytes contain only type 2 chains (15) from experiments in which an  $\text{HLe}^b$  glycoprotein purified from O erythrocytes by the procedure of Marchesi and Andrews (16) was radiolabeled with  $[^{14}\text{C}]\text{N-acetyl-D-galactosamine}$  by an enzyme from group A human milk. After alkaline borohydride degradation (17), all of the label in the reduced oligosaccharides was subsequently released by an endo- $\beta$ -galactosidase (18) which specifically cleaves the  $\beta 1 \rightarrow 4$  link in



Had type 1 chains been present and had they been labeled, they would not have been split (18); no type 1 chain was found. However, it is well established that  $\text{HLe}^b$  specificity (19, 20) requires a second fucose on the type 1 chain, and that the *N*-acetyl-galactosaminyl blood group A transferase does not act on difucosyl H determinants (21, 22). There is thus no reason to believe that the type 1  $\text{HLe}^b$  determinants were labeled.

A competitive binding assay is used in the present study to compare inhibition by  $A_1$  and  $A_2$  blood group substances of binding of tritium-labeled blood group A substance by insolubilized lectin and antiserum. Slopes of inhibition curves reflect the nature of the interaction of antigens with the combining sites of the antibodies. Structurally related or identical antigens should yield parallel inhibition lines, whereas structurally different ones will give nonparallel lines. Slopes of curves were the same with insolubilized *Dolichos biflorus*, a lectin which precipitates blood group A substances and has a specificity for terminal  $\alpha$ -linked  $\text{dGalNAc}$ , and for the trisaccharide moiety



With human anti- $A$ , differences in slope between  $A_1$  and  $A_2$  were found, indicating a qualitative difference between these substances themselves and the population of antibodies with which each reacts.

The affinities of anti- $A$  sera and anti- $A$  sera absorbed on polyleucyl- $A_2$  or polyleucyl- $A$  insolubilized blood group substances were analyzed by a modified competition assay (24, 25). The slopes of the inhibition curves are proportional to the relative affinities of the antibodies. Anti- $A_1$  antibodies not absorbed on polyleucyl- $A_2$  substance retained their high affinity for labeled blood group A substance, whereas partial absorption of the same anti- $A$  serum with polyleucyl blood group substances having  $A_1$  and  $A_2$  determinants left antibodies of much lower affinity in the supernate. This is in substantial disagreement with a key assumption of the hypothesis that the differences between  $A_1$  and  $A_2$  are only quantitative (3) which requires that the anti- $A_1$  antibodies be of low affinity.

This evidence strongly favors a structural difference in the A determinants of water soluble A<sub>1</sub> and A<sub>2</sub> glycoproteins with a distinct population of antibodies specific for each determinant, and thus raises the question of whether or not type 1 A determinants will not eventually be found on the blood group A erythrocyte.

### Materials and Methods

Blood group substance used in competitive binding assays (CBA)<sup>1</sup> was isolated from [<sup>3</sup>H]acetic anhydride-labeled hog mucin A + H (26, 27) by affinity chromatography on a *Dolichos*-Sephrose 2B column. The absorbed, A-active material was eluted with pGalNAc as previously described (26, 27). Inhibitors used in CBA included human blood group A, substances MSM 10%, MSS 10% (28), and Cyst 9 from Dr. Harold Baer (29), and human blood group A<sub>2</sub> substance Cyst 14 phenol insoluble (4). Cyst 14 fucose eluate and Cyst 14 effluent are fractions from Cyst 14 phenol insoluble separated by affinity chromatography on *Lotus*-Sephrose (30). An A<sub>2</sub> substance prepared from human saliva W. G. phenol insoluble was also available (31). Hog blood group A substance, hog gastric mucin (HGM) GalNAc eluate was also used; it reacts like human A<sub>1</sub> substances (30).

Ethyl chloroformate insolubilized (32, 33) human anti-A serum Chris D<sub>2</sub> (34) and *D. biflorus* (23) insolubilized on cyanogen bromide-activated Sepharose 2B (30, 35-37) were prepared as described earlier (26). Whole anti-A serum was acidified by adding 1.0 M sodium acetate-acetic acid buffer, pH 4.6, to a final concentration of 0.20 M (pH 4.9-5.0). Ethyl chloroformate (0.05 ml/ml serum) was added by drops with sufficient stirring to keep it in suspension. An abundant precipitate formed within 15 min; stirring was stopped and the mixture was placed in a refrigerator for 16 h. The insoluble serum was dispersed with a Kontes glass homogenizer (Kontes Co., Vineland, N. J.), centrifuged at 1,200 g at 4°C for 1 h, the precipitate washed once with cold 0.1% Na<sub>2</sub>CO<sub>3</sub>, and then repeatedly with 10 mM phosphate-buffered saline containing 0.1% NaN<sub>3</sub> until the OD<sub>280</sub> of the washings was <0.02. The final pellet was stored at 4°C until needed. The *Dolichos* lectin was coupled to Sepharose 2B in the presence of 0.1 M pGalNAc to protect the sites.

Sera were absorbed by passing samples of Chris D<sub>2</sub> or Jos D<sub>1</sub> + D<sub>2</sub> (34) through columns of polyethyl insolubilized blood group substance (4). Two 5-ml portions were passed through individual columns of PL-Cyst 14 phenol insoluble (A<sub>2</sub>) and PL-Hog A (A<sub>1</sub>). The effluent sera from the PL-BGS columns were tested for their precipitating capacity by quantitative precipitin assay (38). Samples of Christ and Jos absorbed in the previous study (4) on PL-Cyst 14 (A<sub>2</sub>), PL-Hog A (A<sub>1</sub>), and PL-McDon (A<sub>1</sub>) were available.

Two types of CBA were performed. One measured competition for insolubilized Chris, or *Dolichos*-Sephrose between labeled and unlabeled blood group substance; in the other, competition for the labeled blood group substance between insolubilized Chris and soluble antibodies was assayed. For the competition assay with unlabeled blood group substance, ≈2,000 cpm of HGM GalNAc eluate were mixed with varying amounts of unlabeled blood group substance followed by an amount of insolubilized Chris or of *Dolichos*-Sephrose sufficient to bind 50-60% of the labeled material. The tubes were mixed by rotation for 16 h at 4°C. Separation of bound from free radioactivity and preparation of the samples for counting was as previously described (26, 27). In the soluble antibody CBA, a constant amount of insolubilized Chris, sufficient to bind 50-60% of the labeled HGM GalNAc eluate, was mixed with varying amounts (measured in ng AbN/ml from a quantitative precipitin curve with MSM 10%) of antiserum containing the competing antibodies. A constant amount, ≈2,000 cpm of labeled blood group substance, was then added. The tubes were rotated in the cold for 16 h and processed as above. The total volume in both assays was 350 μl.

In both assays, the data were expressed graphically as the percent of inhibition (of binding of labeled antigen) versus nanograms of blood group substance, or of AbN added. The formula used to compute percent of inhibition was:

$$\left( 1 - \frac{\text{total cpm added} - \text{cpm in supernate with inhibitor}}{\text{total cpm added} - \text{cpm in supernate without inhibitor}} \right) \times 100.$$

The slopes of the lines in the linear portion of the sigmoid plot expressed as the change in percent of inhibition per one log unit, are computed as the difference in percent of inhibition for two points

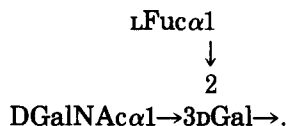
<sup>1</sup>Abbreviations used in this paper: CBA, competitive binding assay; HGM, hog gastric mucin.

one log unit apart on the linear part of the graph. All determinations were set up in duplicate, and analyses did not generally differ by more than  $\pm 5\%$ . Competitive binding data in the figures give combined results of three experiments with each substance.

Binding data presented in this manner will take the form of a sigmoid curve for a uniform set of sites. We will be discussing the apparent slopes of the lines in the linear portion of the curves and the concentration (blood group substance or antibody added) at half saturation. The concentration at half saturation reflects the apparent binding constant. The apparent slope gives an indication of interactions or heterogeneity or both.

## Results

*CBAs of A<sub>1</sub> and A<sub>2</sub> Blood Group Substances with Insolubilized Antiserum and Lectin.* Fig. 2a is the CBA with *Dolichos*-Sepharose and various A<sub>1</sub> and A<sub>2</sub> substances. A<sub>1</sub> and A<sub>2</sub> substances differ in the amount required to inhibit 50% binding; 11–12 times more A<sub>2</sub> than A<sub>1</sub> substance by weight is needed, reflecting a quantitative difference between them with respect to the number of A determinants of the structure



The two human A<sub>1</sub> substances and the HGM GalNAc eluate fall on the same line and therefore they have similar numbers of determinants. The various A<sub>2</sub> substances, WG phenol insoluble, the Cyst 14 fucose eluate, and unfractionated Cyst 14, also have the same number of determinants, but 1/11 as many as the A<sub>1</sub> substances, and they fall on another line. The slopes of the lines are similar, indicating a similar binding affinity for the *Dolichos* site of the determinants in both A<sub>1</sub> and A<sub>2</sub> substances.

Fig. 2b is a CBA using the same blood group substances with insolubilized human anti-A Chris. The A<sub>1</sub> substances are 7- to 10-fold better inhibitors of binding than are the A<sub>2</sub> substances, as reflected by the amounts needed for 50% inhibition. In contrast to the findings with *Dolichos*, individual A<sub>1</sub> substances vary in inhibiting power per unit of weight, and hence in their numbers of determinants. Quantitative precipitin data (4, 30) support these findings; MSM 10%, the best inhibitor, with 140 ng for 50% inhibition, requires 1.6  $\mu\text{g}$  of substance to precipitate 50% of the Chris *N* which it precipitated at equivalence, whereas the HGM GalNAc eluate, 190 ng for 50% inhibition, or 25% less active than MSM 10%, needs 2.0  $\mu\text{g}$  for 50% precipitation. There are no data for MSS 10%. Cyst 14 phenol insoluble, WG phenol insoluble, and Cyst 14 fucose eluate are all equal within experimental error. Cyst 14 effluent is  $\cong 2.5$  times poorer in CBA than are the other A<sub>2</sub> substances, in agreement with the finding that more than twice as much Cyst 14 effluent, as Cyst 14 phenol insoluble, is needed for 50% precipitation, 5 and 2  $\mu\text{g}$ , respectively.

As in the case of *Dolichos* assay, these differences between A<sub>1</sub> and A<sub>2</sub> are consistent with a larger number of receptors on A<sub>1</sub> substances than on A<sub>2</sub>. The slopes of the A<sub>1</sub> and A<sub>2</sub> lines differ; e.g., the ratio of amount of A<sub>1</sub> to A<sub>2</sub> substance required to inhibit a given percent of binding decreases as the percent of inhibition increases, indicating that A<sub>1</sub> substance competes relatively stronger with the labeled HGM GalNAc eluate for antibody combining sites

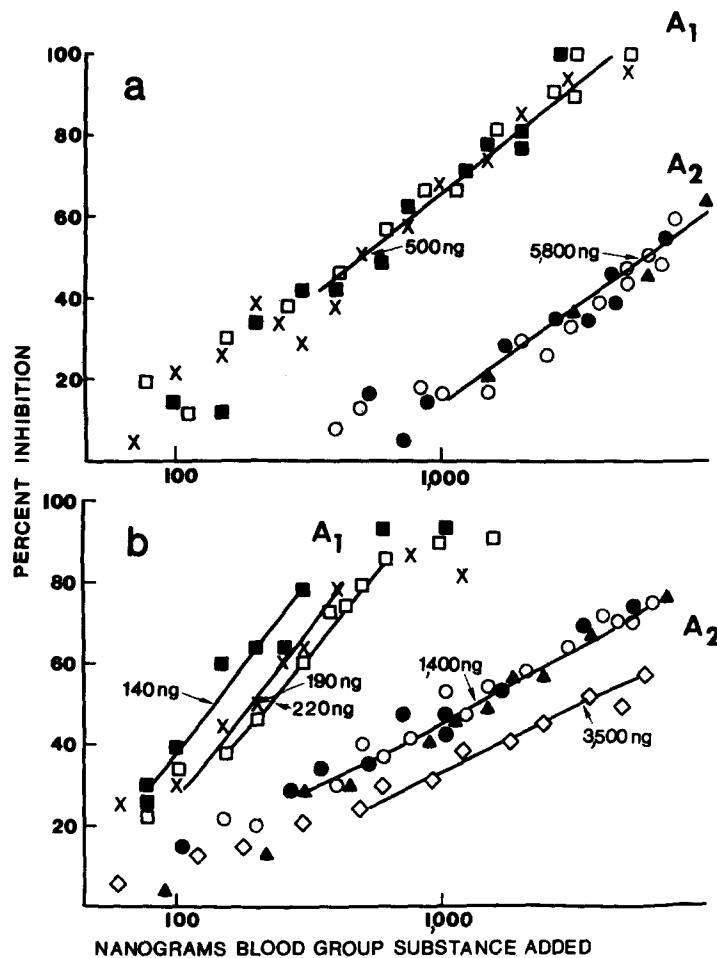


FIG. 2. Competitive binding assays of various  $A_1$  and  $A_2$  blood group substances with (a) *Dolichos*-Sepharose 2B and (b) insolubilized human anti-A Chris and [ $^3$ H]HGM GalNAc eluate. The quantity of antigen giving 50% inhibition of binding is indicated by an arrow (semilog plot).  $A_1$ : ■, MSM 10%; □, MSS 10%; ×, HogA GalNAc eluate.  $A_2$ : ○, WG phenol insoluble; ●, Cyst 14 phenol insoluble; ▲, Cyst 14 fucose eluate; ◇, Cyst 14 effluent.

than does the  $A_2$  substance. This is consistent with some qualitative differences between them, as well as with heterogeneity of the antibody populations reacting with each.

*Competition for Binding of Labeled Blood Group Substance Between Insolubilized Antiserum and Soluble Antibody.* The sera used in the soluble antibody CBA were human anti-A Chris  $D_2$  and Jos  $D_1 + D_2$ , either unabsorbed or absorbed on columns of PL-Cyst 14 ( $A_2$ ) or PL-Hog A ( $A_1$ ). The precipitating activities of these sera are shown in Fig. 3. The curves are typical, reaching a maximum and then decreasing as more antigen is added. Antibodies reacting with  $A_2$  substances were specifically removed (Figs. 3 b and 3 e) by absorption with PL-Cyst 14 ( $A_2$ ). Repeated absorption with PL-Cyst 14 ( $A_2$ ) failed to effect total removal of the antibodies reacting with the  $A_1$  substance. Absorption with

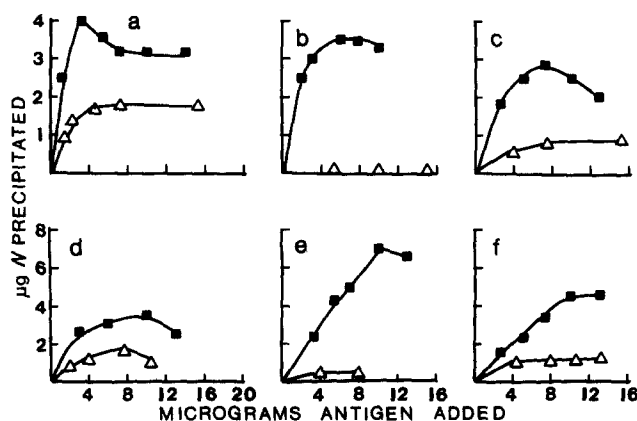


FIG. 3. Quantitative precipitin curves with absorbed and unabsorbed human anti-A serum Chris (a-c) and Jos (d-f). (a) 100  $\mu$ l serum Chris; total volume, 450  $\mu$ l. (b) 400  $\mu$ l serum Chris passed through a column of PL- $A_2$ ; total volume, 450  $\mu$ l. (c) 250  $\mu$ l serum Chris passed through a column of PL-Hog A; total volume, 450  $\mu$ l. (d) 60  $\mu$ l serum Jos; total volume, 360  $\mu$ l. (e) 300  $\mu$ l serum Jos passed through a column of PL- $A_2$ ; total volume, 450  $\mu$ l. (f) 150  $\mu$ l serum Jos passed through a column of PL-Hog A; total volume, 300  $\mu$ l. The antigens are:  $\blacksquare$ , MSM 10% ( $A_1$ ); and  $\triangle$ , WG phenol insoluble ( $A_2$ ).

PL-Hog A ( $A_1$ ), performed to remove only part of the antibodies, leaves the proportion of antibodies in the effluent reacting with  $A_1$  and  $A_2$  substance unchanged (Fig. 3c and 3f). These data agree with earlier findings (4).

Assays of the ability of the variously absorbed antisera to compete with insolubilized, unabsorbed Chris  $D_2$  for the labeled HGM GalNAc eluate are shown in Fig. 4. The slopes of the inhibition lines measure the ability of the soluble antibodies to compete with the insolubilized antiserum for A determinants. Soluble antibodies with higher affinity can compete more effectively than antibodies of lower affinities; the slope of the inhibition line is directly proportional to relative affinity. Figs. 4a and 4b show the curves for the inhibition of binding with the Chris  $D_2$  and Jos  $D_1 + D_2$  sera from Fig. 3. They were absorbed at least twice through the indicated PL-BGS column. The slope of the curve for the unabsorbed Chris is 108, and for Jos, 78; therefore, Chris has a higher relative affinity than Jos. After absorption with PL-Cyst 14  $A_2$  substance Chris and Jos have about the same affinities within the experimental error that were found for the unabsorbed serum, i.e., 97 for Chris and 73 for Jos. There is little or no change in the amounts of absorbed Chris and Jos for 50% inhibition as compared to the unabsorbed, 40 and 50 ng for Chris (Fig. 4a) and 92 and 88 ng for Jos (Fig. 4b) respectively.

Chris and Jos partially absorbed on PL-Hog A, which is like human  $A_1$  substances (4, 30), have lower binding affinities for [ $^3$ H]HGM GalNAc eluate than the unabsorbed sera. This is reflected in the lower slopes, 36 for Chris (Fig. 4a), and 31 for Jos (Fig. 4b). With both sera, about five times more AbN is needed to inhibit 50% binding as compared to the unabsorbed serum.

Figs. 4c and d show a set of inhibition curves with the samples of Chris and Jos that had been absorbed and remained from the previous study (4). One set of curves, Chris and Jos absorbed with PL-McDon ( $A_1$ ), were not done for Figs.

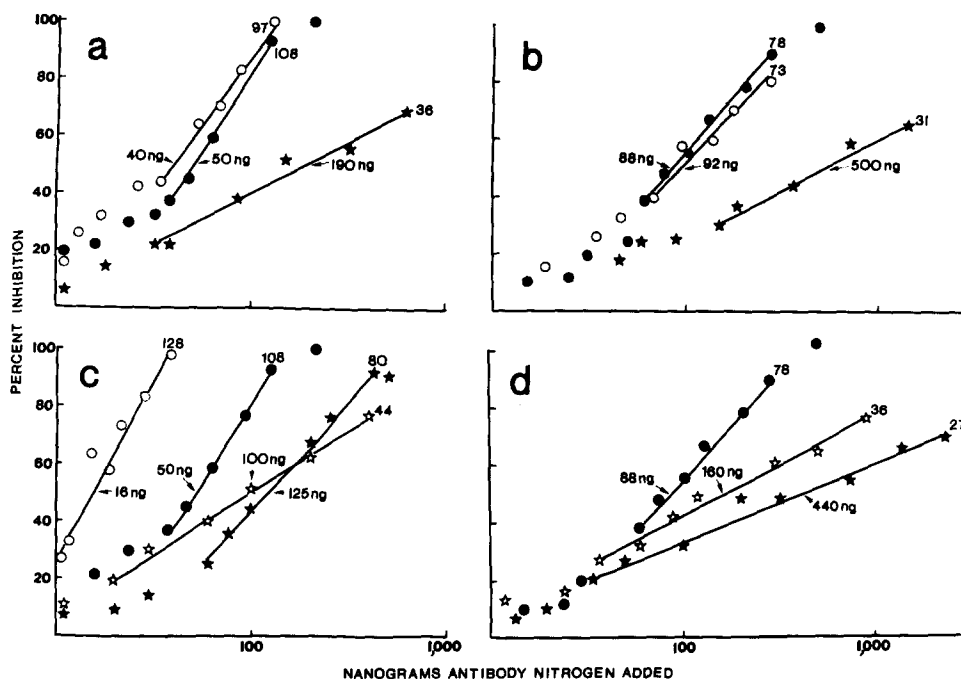


FIG. 4. Competitive binding assay with [ $^3\text{H}$ ]HGM GalNAc eluate and insolubilized human anti-A serum Chris by (a) Chris- and (b) Jos-absorbed (in this study) and (c) Chris- and (d) Jos-absorbed samples used in (4) (●), unabsorbed; (○), absorbed with PL- $A_2$ ; (★), absorbed with PL-Hog A; (☆), absorbed with PL-McDon. The quantity giving 50% inhibition of binding is indicated by an arrow and number. The number at the end of each line is the apparent slope (semilog plot).

4 a and b. The slopes with PL-McDon absorbed are similar to those of the same sera absorbed with PL-Hog A. Chris absorbed on PL-Hog A does not show as striking a change in relative affinity because the serum was passed through the column only once, and a smaller proportion of the anti-A had been removed. The slope of the Chris absorbed on PL-Cyst 14 ( $A_2$ ) is greater than that of the unabsorbed serum, indicating that its relative affinity is higher. Only 16 ng of PL-Cyst 14 absorbed Chris inhibits 50% binding. This is threefold less than that with unabsorbed Chris. Jos  $D_1 + D_2$  absorbed on PL-Cyst 14 ( $A_2$ ) is not shown, since the anti-A activity of this absorbed serum had not been determined (4). However, a soluble antibody CBA was performed adding various volumes of the absorbed serum. The slope of the inhibition line was 76 (data not shown), and thus the relative affinity is the same as that of the unabsorbed Jos, in agreement with the value in Fig. 4 b.

### Discussion

It is clear that there are more A determinants on  $A_1$  than on  $A_2$  glycoproteins or on erythrocytes (39), and this study firmly supports the views that there is also a qualitative immunochemical difference, and that  $A_1$  involves a structure not present in  $A_2$  soluble glycoproteins. The CBA inhibition curves with  $A_1$  and  $A_2$  substances and antibodies or lectin yield information not obtainable by



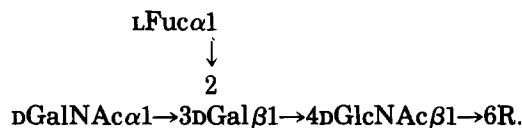
quantitative precipitin inhibition, since slopes of the inhibition lines should be parallel if the differences are exclusively quantitative.

*D. biflorus* has a homogeneous combining site specific (23) for the A trisaccharide which both A determinants, A<sub>1</sub> and A<sub>2</sub>, have in common. By CBA and quantitative precipitin assay (23) with *Dolichos*, there are quantitatively more of these terminal groups in A<sub>1</sub> than in A<sub>2</sub> saliva and cyst substances, and by CBA, the slopes are parallel as expected (Fig. 2 a). There are also about five times more *Dolichos*-reactive sites on A<sub>1</sub> than on A<sub>2</sub> erythrocytes, as determined by electron microscopy of human erythrocytes with ferritin-conjugated *Dolichos* (40).

The findings in Fig. 2 a are of interest because they show that A<sub>1</sub> and A<sub>2</sub> macromolecules which differ 11-fold in their number of reactive groups per unit of weight will give lines with the same slope in CBA, provided the receptor site (*Dolichos*) is recognizing the same structure on each determinant. Thus, variation in the number of sites per glycoprotein molecule, e.g., in their valence and despite their polydispersity, does not significantly affect the slope.

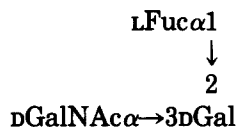
With anti-A, which is a heterogenous population of antibody molecules, however, the individual A<sub>1</sub> substances varied about twofold in number of their determinants per unit of weight. However, A<sub>2</sub> substances from two individuals, WG phenol insoluble from human saliva, and Cyst 14 phenol insoluble (A<sub>2</sub>), fell on the same curve. Cyst 14 phenol insoluble (A<sub>2</sub>) was separated on *Lotus*-Sephacrose into two portions, the nonabsorbed effluent (about one-third) and the absorbed fraction elutable by fucose (two-thirds). The finding (30) that the fucose eluate was about as potent in precipitating anti-A while the effluent had only 54% of the precipitating activity is in good agreement with experimental values in Fig. 2 b, the effluent having  $\cong 40\%$  of the activity of the original Cyst 14 phenol insoluble (A<sub>2</sub>) or the fucose eluate.

The slopes of the A<sub>1</sub> and A<sub>2</sub> inhibition curves with insolubilized anti-A serum are not parallel (Fig. 2 b), indicating a qualitative difference. This is supported by the quantitative precipitin finding that although both A<sub>1</sub> and A<sub>2</sub> substances reach equivalence in the same region and decrease as more antigen is added, the amount of AbN precipitated at equivalence by A<sub>2</sub> substance is less than that precipitated by A<sub>1</sub> substance (4). If the difference were only quantitative, one would expect to reach the same maximum with A<sub>2</sub> substance and the inhibition lines to be parallel in CBA. Thus, there is a proportion of anti-A antibodies, anti-A<sub>1</sub>, which do not precipitate with A<sub>2</sub> substance and do bind in CBA. The specificity of these antibodies could be for the A-specific trisaccharide linked  $\beta 1 \rightarrow 3$  to dGlcNAc to give a type 1 determinant. Such antibodies must have sites large enough to accommodate the whole determinant. The antibodies from Chris anti-A have been purified (34) according to size of the combining site by elution from PL-Hog A with dGalNAc followed by the AR<sub>L</sub> 0.52 pentasaccharide with the structure (5, 7)



Both IgG and IgM were eluted and subsequently separated by density gradient

centrifugation. The IgG and IgM from the GalNAc and AR<sub>L</sub> 0.52 eluates were similar in precipitating A<sub>1</sub> substance, whereas a smaller proportion of both IgG eluates was precipitated by A<sub>2</sub> substance. The IgM eluates were not tested. A<sub>2</sub> substance precipitated a larger proportion of antibodies from the IgG GalNAc eluate than from the IgG AR<sub>L</sub> 0.52 eluate (4). The GalNAc eluate would be expected to have more sites specific for the smaller trisaccharide structure



which A<sub>1</sub> and A<sub>2</sub> share, whereas the AR<sub>L</sub> 0.52 eluate would have more sites of larger size. The suggestion that a portion of the IgG anti-A is A<sub>1</sub> specific is contrary to the assumption of the quantitative theory that IgG anti-A agglutinates both A<sub>1</sub> and A<sub>2</sub> cells (3).

Only a small proportion of the absorbed Chris (31%) is eluted with AR<sub>L</sub> 0.52, and the slope difference in CBA is presumably measuring this antibody. The absorption experiments are consistent with this. Removal of A<sub>2</sub>-reactive antibodies by absorption with PL-Cyst 14 (A<sub>2</sub>) leaves an effluent which is A<sub>1</sub> specific (4), and has the same relative affinity as the unabsorbed serum (Figs. 4 a and b), or even higher (Fig. 4 c). Removal of a proportion of high-affinity antibodies by absorption on PL-Hog A or PL-McDon (A<sub>1</sub>) yields effluents of lower affinity.

The AR<sub>L</sub> 0.52 eluate from Chris was found to contain 18% IgM anti-A, whereas that from Jos had no IgM anti-A (34). This is in accord with the finding that after absorption with PL-Cyst 14 (A<sub>2</sub>), Chris had enhanced its capacity to inhibit per unit of weight as compared to the unabsorbed serum; with Jos, the PL-Cyst 14 (A<sub>2</sub>) absorbed and the unabsorbed serum had the same binding affinities.

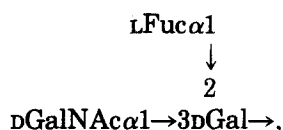
The ability to prepare anti-A<sub>1</sub>-specific antibodies by absorption with A<sub>2</sub> erythrocytes (3) or insolubilized A<sub>2</sub> glycoprotein from ovarian cyst fluid (4) casts doubt on findings that only type 2 chains are present on the glycoprotein ABH determinants of erythrocytes. Moreover, there are two important independent immunological observations which strongly support the association of A<sub>1</sub> determinants with the type 1 chain and A<sub>2</sub> determinants with the type 2 chain. One of these is the discovery of an antibody which reacted only with O<sub>1</sub>Le<sup>a</sup>-b<sup>+</sup> erythrocytes (41). Since this antibody cannot react with the type 1 A determinant on A<sub>1</sub> erythrocytes in which a dGalNAc would be substituted on the dGal of the HLe<sup>b</sup> determinant, but can react with A<sub>2</sub> or O erythrocytes which would lack the type 1 A determinant, type 1 A chains that are A<sub>1</sub> determinants must be present on the erythrocytes. The second is the demonstration of an antibody specific for an A<sub>1</sub>Le<sup>b</sup> determinant (42-44) which reacted only with A<sub>1</sub>Le<sup>b</sup> erythrocytes but not with A<sub>2</sub>Le<sup>b</sup>, OLe<sup>b</sup>, or A<sub>1</sub>Le<sup>a</sup> erythrocytes. Thus, the combining site of this antibody requires a type 1 A chain for the A<sub>1</sub> specificity on erythrocytes.

Mohn et al. (45) have recently demonstrated a qualitative difference between A<sub>1</sub> and A<sub>2</sub> erythrocytes by gel diffusion using erythrocyte stromata and antisera to human A<sub>1</sub> and A<sub>2</sub> saliva produced in rabbits which secreted an A-like substance in their saliva.

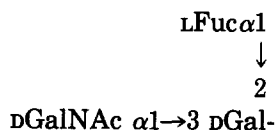
Hakomori et al. (46) demonstrated that two of four A active glycolipids isolated from A<sub>1</sub> erythrocytes were absent from A<sub>2</sub> erythrocytes but all four were built of type 2 chains, one linked  $\beta 1 \rightarrow 3$  and the other  $\beta 1 \rightarrow 6$  to the same dGal. One of the four glycolipids has an additional branch of undetermined structure. These A glycolipids, excluding the undetermined branch, all involve type 2 chains and would not account for our findings, nor for those of Mohn et al. (45).

### Summary

Competitive binding assays using <sup>3</sup>H-labeled blood group A substance and insolubilized *Dolichos biflorus* lectin or human anti-A were carried out, measuring competition by blood group A<sub>1</sub> and A<sub>2</sub> glycoproteins, and by unabsorbed anti-A sera, and with these sera absorbed with the A<sub>1</sub> and A<sub>2</sub> glycoproteins. With *Dolichos* lectin specific for



A<sub>1</sub> substances had about 11 times as many determinants as did A<sub>2</sub> substances, but the slopes of the lines in the competitive binding assays were the same. With insolubilized anti-A, A<sub>2</sub> substances gave lines of lower slopes. Although individual A<sub>1</sub> populations varied in the amounts giving 50% inhibition in the assays, as did A<sub>2</sub> substances, the slopes of the lines for the A<sub>1</sub> substances were the same and always higher than the slopes of the lines for the A<sub>2</sub> substances. Competitive binding assays with unabsorbed anti-A sera and with these sera absorbed with insoluble polyleucyl A<sub>1</sub> and A<sub>2</sub> substances showed that partial absorption of polyleucyl A<sub>1</sub> substances left antibodies of lower slope in the supernate, whereas absorption with polyleucyl A<sub>2</sub> substance left antibodies (anti-A<sub>1</sub>) having the same or an even higher slope than the unabsorbed sera. The findings indicate that human A<sub>1</sub> and A<sub>2</sub> glycoproteins differ in their determinants, and that A<sub>2</sub> specificity is determined by the type 2 chain in which the A trisaccharide



is linked  $\beta 1 \rightarrow 4$  to dGlcNAc, whereas the A<sub>1</sub> specificity is determined by the type 1 chain in which this trisaccharide is linked  $\beta 1 \rightarrow 3$  to dGlcNAc; most of the determinants in the glycoproteins have a second LFuc linked  $\alpha 1 \rightarrow 3$  and  $\alpha 1 \rightarrow 4$  to the dGlcNAc of the type 2 and type 1 chains, respectively.

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