# IMMUNOCHEMICAL STUDIES ON BLOOD GROUPS

## LI. A Comparative Study of the Reaction of $A_1$ and $A_2$ Blood Group Glycoproteins with Human Anti-A*

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The basis for the difference between $A_1$ and $A_2$ specificity has been in dispute from the moment of its discovery (1–5). An excellent review of the earlier studies may be found in the thesis of Gammelgaard, recently available in English (6). One view holds that the $A_1$ and $A_2$ antigenic determinants are chemically identical, differences being essentially quantitative and ascribable to the presence of many more determinants on an $A_1$ than on an $A_2$ erythrocyte (7–11). Based on hemagglutination-inhibition titers of salivas from $A_1$ and $A_2$ secretors, Boettcher (11) proposed that individual molecules of A substance from $A_1$ secretors have a higher ratio of A:H antigenic determinants than those from $A_2$ secretors. He also proposed that the $A_1$ gene is a duplication of the $A_2$ gene. Bar-Shany et al. (12) found only a small, although significant, difference between $A_1$ and $A_2$ saliva in absorption studies. A proportion of $A_2$ bloods contain anti-$A_1$ (13–17). Mäkelä et al. (18) recently attempted to explain why anti-$A_1$ does not agglutinate $A_2$ cells without assuming that the $A_1$ and $A_2$ determinants differ. Hypothesizing the receptor density to be higher on $A_1$ than on $A_2$ erythrocytes, Mäkelä suggests that anti-$A_1$ antibodies have a low affinity for A receptors and cannot form firm bridges between red cells using only one valence per cell. Thus they would only agglutinate $A_1$ cells on which the receptors are close together. $A_1$-specific antibodies therefore would have to be multivalent and of the $\gamma$M type and bivalent antibodies such as $\gamma$G would not distinguish between $A_1$ and $A_2$ erythrocytes.

The other view contends that there is a qualitative difference between the antigenic determinants in $A_1$ and $A_2$ individuals (19–23). Friedenreich and Worsaae (23) suggested that $A_1$ and $A_2$ are qualitatively different but related antigens. Their assumption was based mainly on absorption experiments showing that anti-A serum contained at least two kinds of antibodies, anti-A and anti-$A_1$ . $A_1$ cells reacted strongly with anti-A

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and somewhat less with anti-$A_1$ . $A_2$ cells reacted less strongly than $A_1$ with anti-A and little or not at all with anti-$A_1$ . $A_2$ cells reacted better than $A_1$ cells with anti-H, which agglutinated O erythrocytes even more intensely. Furthermore, Friedenreich and Worsaae, in a large series of absorption tests with erythrocytes, found no forms intermediate between $A_1$ and $A_2$ cells, a finding strongly favoring a qualitative difference.

Hakomori and Strycharz (24) have noted that one of three glycolipids found in $A_1$ was absent from $A_2$ erythrocytes. The recognition of two types of blood group A determinants (25–31) and the demonstration that, in soluble blood group substances, these are linked to a single galactose (30) led to the suggestion (29) that $A_1$ substances might contain both types of A determinants while only one was present in $A_2$ substances. The absence of a terminal nonreducing DGalNAc[1] on one of the two determinant chains would result in increased H specificity in $A_2$ as compared with $A_1$ . Moreover, since the type 1 and 2 determinants are identical in their terminal nonreducing trisaccharide,

$$\begin{array}{l} \alpha\underline{\underline{D}}\text{GalNAc}(1 \rightarrow 3)\beta\underline{\underline{D}}\text{Gal} - \\ \qquad\qquad\qquad\qquad\quad 2 \\ \qquad\qquad\qquad\qquad\quad \uparrow \\ \qquad\qquad\qquad\qquad\quad 1 \\ \qquad\qquad\qquad\quad \alpha\underline{\underline{L}}\text{Fuc} \end{array}$$

and differ only in whether this trisaccharide is linked $\beta$ (1 → 3) (type 1) or $\beta$ (1 → 4) (type 2) to DGNAc, any specificity differences between the two determinants should be of a minor character involving only those antibody-combining sites sufficiently large to distinguish differences involving the linkage to the DGNAc. Thus, even if $A_2$ were associated with only one of the two determinants, cross-absorption experiments would generally show complete or almost complete removal of all antibodies. Thus the subtle nature of the potential difference in specificity could have been at the basis for the belief that $A_1$ and $A_2$ only differ quantitatively.

Both the type 1 and type 2 A determinants may be more complex since each may have a second fucose linked to the DGNAc. The Le gene is responsible for the addition of an $\alpha$LFucosyl residue to carbon 4 of the DGNAc of the type 1 chain, while a new gene (29)[2] determines the addition of an $\alpha$LFucosyl residue to carbon 3 of the DGNAc of the type 2 chain. Difucosyl type 2 determinants showed lower serologic activity than type 1 or 2 monofucosyl determinants (32, 33); difucosyl type 1 determinants have not been tested. Enzymatic studies with model compounds (34, 35) and with type 2 reduced oligosaccharides from blood group H substance JS, $R_L$ 0.75 and $R_{IM5}$ 2.5[3], have shown that the second fucose linked to DGNAc in both type 1 and type 2 determinants blocks the addition of the terminal nonreducing $\alpha$ (1 → 3)-linked DGalNAc in the biosynthesis of type 1 or type 2 A determinants. A higher proportion of difucosyl A determinants in $A_2$ cells and $A_2$ substances might explain their weaker reaction with anti-A sera com-

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[1] DGal = D-galactose; DGalNAc = *N*-acetyl-D-galactosamine; DGNAc = *N*-acetyl-D-glucosamine; DGlc = D-glucose; LFuc = L-fucose.

[2] Ginsburg, V., and A. Kobata. Unpublished results.

[3] Kobata, A., V. Ginsburg, and E. A. Kabat. Unpublished results.

pared with $A_1$ cells and $A_1$ substances respectively, but this would not account for the higher H activity of $A_2$.

The present study compares the chemical and immunochemical properties of the water-soluble $A_2$ and $A_1$ glycoproteins in an effort to obtain further information about the basis for the differences between $A_1$ and $A_2$; the findings clearly indicate a specificity difference of the kind suggested (29).

## *Materials and Methods*

*Antigens.*—The purification and properties of blood group $A_1$ substances MSM, McDon, and JS from human ovarian cyst fluid have been described previously (27). Blood group A substances Hog 39B2, Hog 50, and Hog 76 were also obtained in a similar manner (36). $A_2$ substance (Cyst 14) was obtained from 700 ml of lyophilized human ovarian cyst fluid kindly provided by Dr. Harold Baer. After peptic digestion, ethanol precipitation, phenol fractionation, and dialysis of the final products, 965 mg of cyst 14 phenol insoluble and 112 mg of cyst 14 10% ppt were obtained. An $A_2$ substance prepared from human saliva (W. G. pheno

TABLE I

*Analytical Properties of Blood Group Substances*

| | N | Methylpentose (Fucose) | Hexose (Galactose) | Hexosamine | Galactosamine |
|---|---|---|---|---|---|
| | (%) | (%) | (%) | (%) | (%) |
| Cyst 14 phenol insoluble ($A_2$) | 4.5 | 20.9 | 26.5 | 26.2 | 7.6 |
| Cyst 14 10% ppt ($A_2$) | 6.2 | 14.7 | 23.5 | 23.1 | 7.0 |
| Saliva W. G. phenol insoluble ($A_2$) | 5.6 | 15.4 | 19.8 | 26.4 | 9.0 |
| MSM 10% ppt ($A_1$) | 5.0 | 15.3 | 22.5 | 32.0 | 14.6 |

insol) was also used (37). A preparation of McDon treated with mild hydrochloric acid (pH 1.6, 100°C, 2 hr) and designated McDon pH 1.6 was also used. The analytical properties are given in Table I together with data on $A_1$ substance for comparison.

Insoluble derivatives of purified $A_1$ substance (McDon and Hog A) and $A_2$ substance (cyst 14, phenol insol) were prepared by copolymerization with the *N*-carboxyanhydride of L-leucine (38). These materials are designated PL $A_1$ McDon, PL $A_1$ Hog A, and PL $A_2$ Cyst 14.

*Antisera.*—The following anti-A sera were used: 59-113 (27), Jos $D_1 + D_2$ (39), Chris (39), No. 1 $D_{33} + D_{34}$ (39), R. G. (38), P. M.$_4$ (40), 6460 (27), and S. M. (33). Antisera Ortho 679096 and Gell $D_1 + D_2$ have not been used previously. Both were from individuals immunized with commercial A substance. The purified anti-A antibodies described previously (39) were obtained by absorption on a column of insoluble A antigen and elution with DGalNAc (GalNAc eluate) and then with a blood group A-active pentasaccharide, $AR_L$ 0.52 ($AR_L$ 0.52 eluate). From these eluates $\gamma$G and $\gamma$M were separated by sucrose gradient centrifugation (39). Goat anti-$Le^b$ serum was kindly supplied by Dr. D. Marcus, Albert Einstein College of Medicine, New York (41). Eel anti-H serum was a gift from Dr. G. F. Springer, Northwestern University, Chicago, Ill.

*Oligosaccharides.*—MSS $AR_L$ 0.52 and JS $HR_L$ 0.75 are reduced oligosaccharides obtained from soluble human blood group A substance (MSS) and H substance (JS) (42). Urine A

pentasaccharide was prepared from human A secretor urine (43) and lactodifucotetraose was a gift from the late professor R. Kuhn and Dr. A. Gauhe, Max-Planck Institute, Heidelberg, West Germany.

*Immunochemical Methods.*—Hemagglutination and hemagglutination-inhibition assays were performed with the Takatsy microtitrator (44). Quantitative precipitin and quantitative assays for inhibition of precipitation were performed on a microscale using about 1–5 $\mu g$ of antibody N per determination (45). Total N in the washed precipitates was determined by the ninhydrin method (27). Hemolysis and complement fixation were performed as described (45).

## RESULTS

Anti-A sera generally agglutinate $A_2$ red blood cells to a lower titer than $A_1$ cells, but substantial individual variation can be found. Table II gives titers for several anti-A sera with $A_1$ and $A_2$ red blood cells; tests were done at the

TABLE II

*Hemagglutination Titers of Human Anti-A Sera with $A_1$ and $A_2$ Erythrocytes*

| Antisera | Titer with $A_1$ erythrocytes | Titer with $A_2$ erythrocytes |
|---|---|---|
| 59-113 | 4096 | 512 |
| Jos. $D_1 + D_2$ | 1024–4096 | 128–256 |
| Chris. $D_2$ | 1024 | 1024 |
| Gell. $D_1 + D_2$ | 128 | 32 |
| R.G. | 256 | 128 |
| Ortho 679096 | 1024 | 512 |
| No. 1 | 64 | 16 |
| P.M.$_4$ | 32 | 8 |
| SM | 8192 | 1024 |

Assay: equal volumes of antiserum dilution and washed 2% suspension of erythrocytes in saline, at room temperature for 1 hr.

same time. $A_2$ cells were generally more weakly agglutinated although some sera exhibited about the same end point.

Several anti-A sera were also analyzed by quantitative precipitin assays with soluble $A_1$ and $A_2$ substances (Fig. 1). $A_1$ (MSM) substance gave the highest amount of precipitate with all four antisera. $A_2$ (Cyst 14 phenol insol) precipitated from 20% (with anti-A P.M.$_4$) to 85% (with anti-A R.G.) of the amount precipitated with $A_1$ (MSM). $A_1$ (McDon) was somewhat less effective than $A_1$ (MSM) in the two systems that were tested. With anti-A (Chris), $A_1$ (McDon pH 1.6) precipitated even less well than $A_2$ (Cyst 14 phenol insol) while McDon (not shown in Fig. 1) precipitated about as well as $A_2$ . $A_2$ (Cyst 14 10% ppt) was included only with antisera Jos and Chris and was about as potent as $A_2$ (Cyst 14 phenol insol). Saliva $A_2$ (WG phenol insol) precipitated about as well as Cyst $A_2$ (Cyst 14 phenol insol) with serum Jos and Chris; with serum RG it was somewhat less potent. The precipitin curves with $A_2$ and $A_1$

substances were typical, reaching a maximum and then decreasing if more antigen was used; the equivalence zone was about the same for both.

Assays of precipitation with purified γG anti-A fractions eluted with GalNAc or with the $AR_L$ 0.52 pentasaccharide were also done to see whether differences would be seen with antibodies with different combining site sizes. Fig. 2 *a*, *b*, and *c* gives the results of precipitation with $A_1$ (MSM) and $A_2$ (Cyst 14 phenol

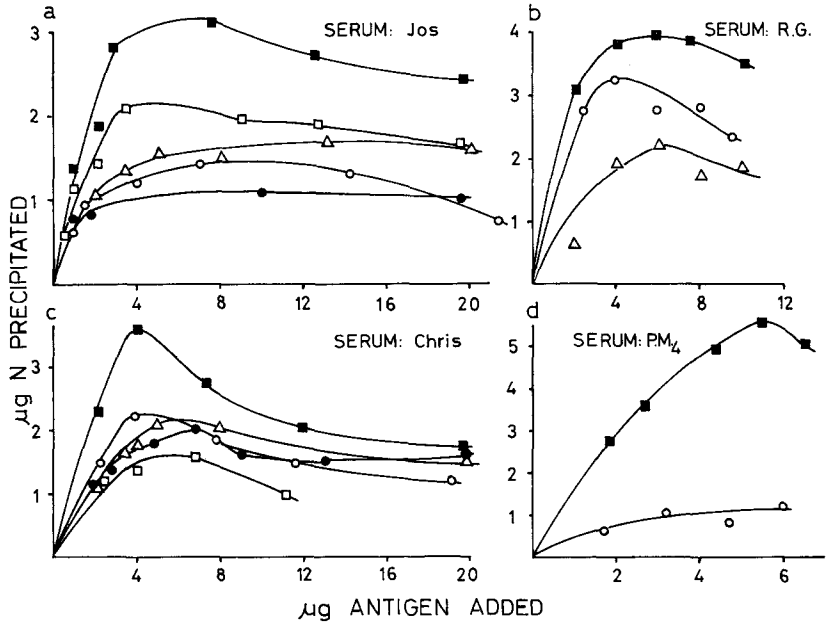


FIG. 1. Quantitative precipitin curves of anti-A sera with $A_1$ and $A_2$ substances. (*a*) 60 μl anti-A Jos; total volume, 360 μl. (*b*) 200 μl anti-A R. G; total volume, 300 μl. (*c*) 100 μl anti-A Chris; total volume, 450 μl. (*d*) 350 μl anti-A P.$M_4$.; total volume, 385 μl.

Antigens: ■ $A_1$ substance (MSM); □ $A_1$ substance (McDon with serum Jos, McDon pH 1.6 with serum Chris); ○ $A_2$ substance (cyst 14 phenol insol.); ● $A_2$ substance (cyst 14 10% ppt); △ $A_2$ substance (W. G. phenol insol.).

insol) blood group substances with both fractions of γG antibodies eluted from antisera Chris, Jos, and No. 1, respectively. Results are expressed as per cent of total N precipitated. The $AR_L$ 0.52 eluate from all three sera precipitated more efficiently with $A_1$ substance than with $A_2$ substance. The latter only precipitates about 50–70% as much antibody. The GalNAc eluate from Chris and Jos also precipitated with $A_1$ substance better than with $A_2$ substance, but with the GalNAc eluate from serum No. 1, $A_1$ and $A_2$ substance were equally effective. With Jos and No. 1 sera a larger proportion of the total N in the $AR_L$ 0.52 eluates than in the GalNAc eluates was precipitable by $A_1$ and $A_2$ sub-

stances. With Chris, however, the GalNAc eluate was as good as the $AR_L$ 0.52 eluate in precipitating with $A_1$ substance and slightly better in precipitating with $A_2$ substance.

It is clear that a much smaller proportion of the anti-A antibodies present in

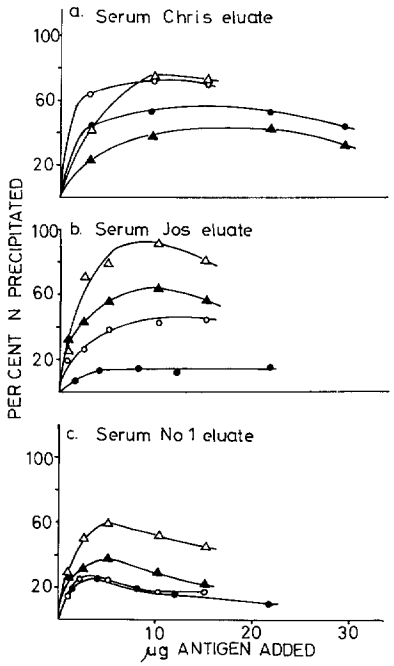


FIG. 2. Quantitative precipitin curves of purified anti-A antibodies with $A_1$ and $A_2$ substances (MSM and Cyst 14 phenol insoluble).

(*a*) Serum Chris (total volume, 230 μl): ○ 4.2 μg N γG GalNAc eluate + $A_1$ subst.; ● 4.2 μg N γG GalNAc eluate + $A_2$ subst.; △ 4.4 μg N γG $AR_L$ 0.52 eluate + $A_1$ subst.; ▲ 4.4 μg N γG $AR_L$ 0.52 eluate + $A_2$ subst. (*b*) Serum Jos (total volume, 300 μl): ○ 6.3 μg N γG GalNAc eluate + $A_1$ subst.; ● 6.3 μg Nγ G GalNAc eluate + $A_2$ subst.; △ 4.5 μg N γG $AR_L$ 0.52 eluate + $A_1$ subst.; ▲ 4.5 μg N γG $AR_L$ 0.52 eluate + $A_2$ subst. (*c*) Serum No. 1 (total volume, 300 μl): ○ 5.6 μg N γG GalNAc eluate + $A_1$ subst.; ● 5.6 μg N γG GalNAc eluate + $A_2$ subst.; △ 4.0 μg N γG $AR_L$ 0.52 eluate + $A_1$ subst.; ▲ 4.0 μg N γG $AR_L$ 0.52 eluate + $A_2$ subst.

a serum or in a purified γG fraction is precipitated by $A_2$ substance than by $A_1$ substance. To investigate this further, samples of Chris and Jos sera were absorbed with $A_2$ (Cyst 14 phenol insol) blood group substance; 30 ml of Chris and 20 ml of Jos antisera were absorbed at 37°C for 1 hr and 4°C for 1 wk with 1.99 and 2.39 mg of blood group $A_2$ substance respectively. The precipitates were centrifuged off and discarded. A second absorption was carried out with 0.29 mg of $A_2$ substance for Chris and 0.21 mg for Jos. Precipitin curves for these

absorbed sera are shown in Fig. 3 *a* and *b*. Absorption was complete since only negligible precipitation occurred with $A_2$ substance. The amount of precipitate with $A_1$ substance was less than expected from the difference in precipitating capacity observed in Fig. 1 *a* and *c*. Thus, the difference between the amount of nitrogen (N) precipitated by $A_1$ (MSM) and $A_2$ (Cyst 14 phenol insol) blood group substances is approximately 26 μg N/ml in serum Jos and 14 in serum

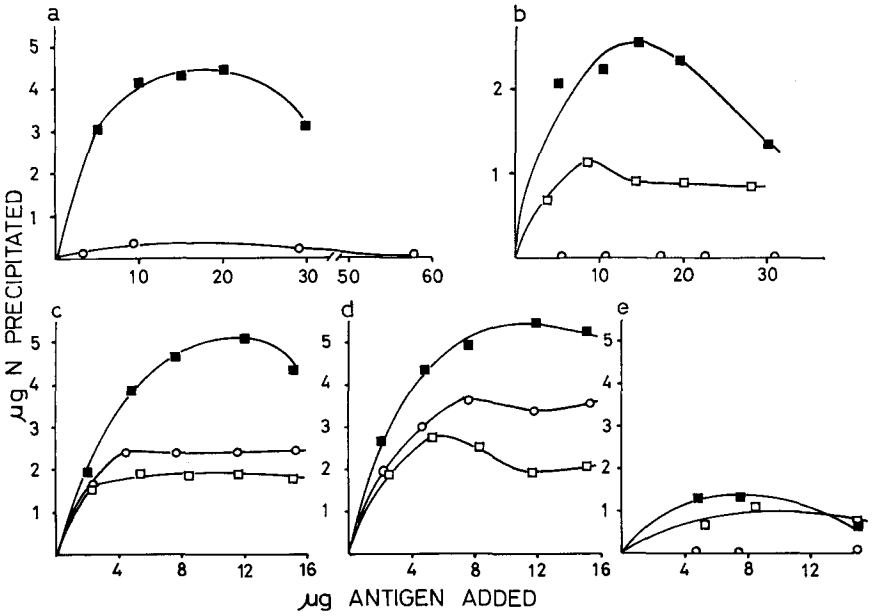


FIG. 3. Quantitative precipitin curves with absorbed anti-A sera. (*a*) 300 μl serum Jos absorbed with soluble $A_2$ substance; total volume, 465 μl. (*b*) 800 μl serum Chris absorbed with soluble $A_2$ substance; total volume, 1050 μl. (*c*) 250 μl serum Chris passed through column of PL-$A_1$ McDon; total volume, 450 μl. (*d*) 250 μl serum Chris passed through column of PL-$A_1$ Hog A; total volume, 450 μl. (*e*) 400 μl serum Chris passed through column of PL-$A_2$; total volume, 550 μl.

Antigens: ■ $A_1$-substance (MSM); □ $A_1$ substance (McDon pH 1.6); ○ $A_2$ substance (Cyst 14 phenol insoluble).

Chris but only 15 and 3.1 μg N/ml were precipitated by $A_1$ MSM after absorption.

Three 10 ml portions of antiserum Chris were passed through three columns of PL-blood group substances prepared as follows: 5 mg of each PL $A_1$ McDon, PL $A_1$ Hog A, and PL $A_2$ Cyst 14 were packed in the tapered part of three pasteur pipettes containing a plug of glass paper and a tiny amount of washed celite in the tip. The columns were packed in 0.15 M NaCl and to avoid dilution, excess fluid was thoroughly drained before serum was applied. Approximately

2 days were required for the passage of 10 ml of serum through the two $A_1$ columns and 7 days for the $A_2$ column. The effluent sera were tested for precipitating capacity (Fig. 3 *c–e*). After elution through the $A_1$ columns, the ratios of the amounts of antibody N precipitated by the three antigens used (MSM, McDon pH 1.6, and $A_2$ cyst 14) were unchanged. The effluent from the $A_2$ column, however, had completely lost its capacity to precipitate $A_2$ substance while continuing to precipitate with MSM and McDon pH 1.6 (Fig. 3 *e*) and McDon (not shown).

The ability of $A_1$ and $A_2$ substance to dissolve the precipitate obtained with absorbed antisera (Jos and Chris) and $A_1$ substance at equivalence (Fig. 3 *a* and *b*) was investigated (Fig. 4). Increasing amounts of $A_1$ (MSM) and $A_2$

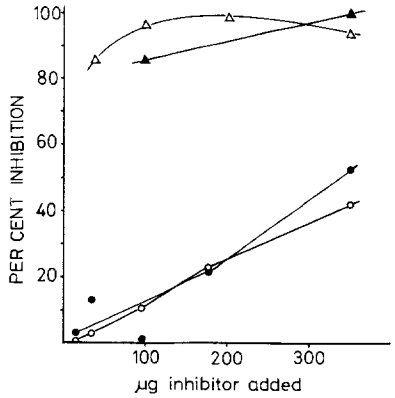


FIG. 4. Solubilization of $A_1$-anti-$A_1$-specific precipitates by increasing amounts of $A_1$ and $A_2$ substances. △ 300 μl serum Jos + 15.2 μg MSM. Inhibitor: $A_1$ substance; ▲ 800 μl serum Chris + 15.2 μg MSM. Inhibitor: $A_1$ substance; ○ 300 μl serum Jos + 15.2 μgMSM. Inhibitor: $A_2$ substance; ● 800 μl serum Chris + 15.2 μg MSM. Inhibitor: $A_2$ substance.

(cyst 14 phenol insol) substances were added to constant amounts of antiserum and $A_1$ (MSM) substance. Adding a 2-fold excess of $A_1$ substance gave almost complete inhibition. On the other hand a 23-fold excess of $A_2$ substance gave only about 50% inhibition with the two antisera.

If $A_2$ substance has fewer complete A determinants per unit weight than $A_1$ substance, one would expect higher H and $Le^b$ activity in $A_2$ substance. With goat anti-$Le^b$ (Fig. 5), $A_2$ substance precipitated twice as much and H substance (J.S.) more than three times as much specific precipitate N as $A_1$ (MSM).

The lower A activity and corresponding increase in H activity associated with $A_2$ was tested by inhibition of hemagglutination using antiserum 59–113 diluted 1/100 as anti-A and eel serum diluted 1/50 as anti-H. The results obtained with different human and hog blood group substances are summarized in Table III. Both $A_2$ substances show about 10% of the activity of the two

human $A_1$ substances tested. The H activity of $A_2$ substance was higher than that of MSM but was comparable to McDon and Hog 39B2. Hog 39B2, however, showed slightly less A activity than the two human substances.

The inhibitory capacity of $A_1$ and $A_2$ substances on agglutination of $A_1$ and $A_2$ red blood cells by anti-A was also investigated. The results, using the same number of agglutinating units of Jos and Chris antisera, are seen in Table IV. Using MSM as reference in each case, the inhibitory capacity of $A_2$ substance on the agglutination of $A_1$ cells ranged between 3 and 5%. When $A_2$ cells were used for the test, the inhibiting capacity of $A_2$ substance was between 13 and 20% of that exhibited by MSM. Thus it would seem that $A_2$ substances are somewhat better in inhibiting hemagglutination of $A_2$ than of $A_1$ cells. A similar behavior is apparent for Hog 76 but Hog 50 behaves as does the MSM.

It should be noted that with serum Jos which agglutinated $A_2$ cells to lower

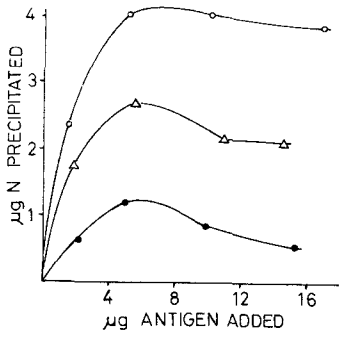


FIG. 5. Quantitative precipitation of goat anti-$Le^b$ by: ○ H substance (JS); △ $A_2$ substance (Cyst 14, 10% ppt); ● $A_1$ substance (MSM).

titer than $A_1$, it was necessary to use 10 times as much antiserum with $A_2$ than with $A_1$ cells, while with antiserum Chris which agglutinated $A_1$ and $A_2$ to the same titer, the same antiserum dilution could be used with $A_1$ and $A_2$ cells.

The ability of type 2 mono- and difucosyl A active oligosaccharides to inhibit precipitation of anti-A by $A_1$ (MSM) and by $A_2$ (cyst 14 phenol insol) substances was also studied. Six different anti-A sera were used (Fig. 6). Urine A pentasaccharide was used as a difucosyl determinant and MSS $AR_L$ 0.52 as a monofucosyl determinant. With $A_1$ substances, as reported earlier (33), the monofucosyl compound was the better inhibitor except for one antiserum (R.G.) in which the difucosyl compound was more active. Using $A_2$ substance, however, with two antisera, Jos and P.M.$_4$, the monofucosyl compounds showed no change in their relative inhibitory capacity; difucosyl compounds, however, were more effective as inhibitors than they were with $A_1$ substances. This effect was striking with Jos but relatively small with P.M.$_4$. With the other four sera,

some increase in effectiveness of both the monofucosyl and difucosyl derivatives as inhibitors was seen with $A_2$ substances as compared with $A_1$ substances. It is of special interest that with antiserum RG, for which $A_2$ cyst substances precipitated almost as well as did $A_1$ substance (Fig. 1 *b*), both the difucosyl and monofucosyl compounds give essentially complete inhibition. With antiserum Chris, with which $A_2$ substance precipitated only two-thirds as much antibody

TABLE III

*Inhibition of Anti-A and Anti-H Hemagglutination by Human $A_1$, $A_2$, and Hog Blood Group Substance Using $A_1$ and O Red Blood Cells*

| Blood group substance | Minimum concentration (in μg/ml) giving complete inhibition of agglutination with | |
|---|---|---|
| | Anti-A 59-113 (1/100) | Eel anti-H (1/50) |
| Cyst 14 10% ppt. ($A_2$) | 40 | 15 |
| Cyst 14 phenol insoluble ($A_2$) | 40 | 40 |
| MSM ($A_1$) | 3 | 500 |
| McDon ($A_1$) | 4 | 20 |
| Hog 39 B2 (A) | 10 | 40 |

TABLE IV

*Inhibition of Hemagglutination by $A_1$ and $A_2$ and Hog BGS Using $A_1$ and $A_2$ Red Blood Cells (RBC)*

| Blood group substance | $A_1$ RBC | | $A_2$ RBC | |
|---|---|---|---|---|
| | Antiserum | | Antiserum | |
| | Jos. 1/50 | Chris. 1/30 | Jos. 1/5 | Chris. 1/30 |
| Cyst 14 10% ppt ($A_2$) | 60* | 180 | 40 | 20 |
| Cyst 14 phenol insoluble ($A_2$) | 40 | 170 | 30 | 30 |
| MSM ($A_1$) | 2 | 6 | 6 | 4 |
| McDon ($A_1$) | 5 | 20 | 7 | 5 |
| Hog 76 (A) | 360 | 360 | 11 | 20 |
| Hog 50 (A) | 2 | 7 | 3 | 3 |

* Minimum concentration (ng/ml) giving complete inhibition of agglutination.

as $A_1$ substance, essentially complete inhibition was also obtained with both. With none of the other sera did the difucosyl compound show such extensive inhibition. The precipitation of anti-A Chris and anti-A RG by $A_1$ and $A_2$ substance could not be inhibited by JS $HR_L$ 0.75 or by lactodifucotetraose (Fig. 6 *d* and *f*).

The capacity of $A_2$ (cyst 14 phenol insol) substance to fix complement was investigated using purified γG antibodies from serum No. 1 (Fig. 7). An extremely low level of fixation was obtained with both the GalNAc and $AR_L$

0.52 eluates (18 and 16% respectively) using amounts of antibody that were able to fix 94 and 84% of the complement with $A_1$ substance MSM as antigen.

The lytic activity of purified antibodies was also studied and it was found that purified $\gamma$M antibodies from serum Chris were more effective in lysing $A_1$ than $A_2$ cells; the GalNAc and $AR_L$ 0.52 eluates behaved similarly (Fig. 8).

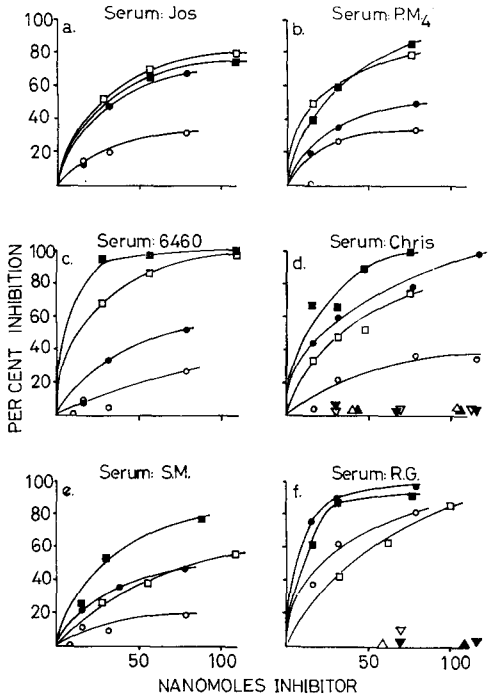


FIG. 6. Inhibition of $A_1$-anti-A and $A_2$-anti-A precipitation by mono- and difucosyl oligosaccharides. □ $A_1$-anti-A inhibited by $AR_L$ 0.52; ■ $A_2$-anti-A inhibited by $AR_L$ 0.52; ○ $A_1$-anti-A inhibited by urine A pentasaccharide; ● $A_2$-anti-A inhibited by urine A pentasaccharide; ▽ $A_1$-anti-A inhibited by $HR_L$ 0.75; ▼ $A_2$-anti-A inhibited by $HR_L$ 0.75; △ $A_1$-anti-A inhibited by lactodifucotetraose; ▲ $A_2$-anti-A inhibited by lactodifucotetraose.

All inhibitions were performed at equivalence, and with approximately the same amount of total N precipitated by $A_1$ and $A_2$ substance.

## DISCUSSION

This study has been carried out to learn whether or not A determinants differ chemically in $A_1$ and $A_2$ substances. $A_2$ substances as well as $A_2$ cells are known to react strongly with anti-H reagents indicating a larger proportion of H receptors. The results in Table III essentially confirm that $A_2$ substance has higher H activity than $A_1$ substance. The GalNAc content in the three $A_2$ preparations studied is also generally lower than that of $A_1$ substances (Table I).

This too is consistent with the presence of fewer A determinants. Greenbury et al. (22) studied the uptake of 7S and 19S rabbit anti-A by human $A_1$ and $A_2$ cells. From these experiments they calculated about $8.3 \times 10^5$ antigen sites/$A_1$ cell and about 25% as many for $A_2$ cells. Economidou et al. (46) arrived at similar values using $^{125}$I-labeled rabbit anti-A. Greenbury et al. (22) emphasize that their values are minimal estimates and do not indicate whether or not the actual numbers of receptors differ, since the values might be affected by variation in accessibility of antigenic sites or heterogeneity in physicochemical configuration in $A_2$ as compared with $A_1$. The observation (47) that both $A_1$ and $A_2$ cells

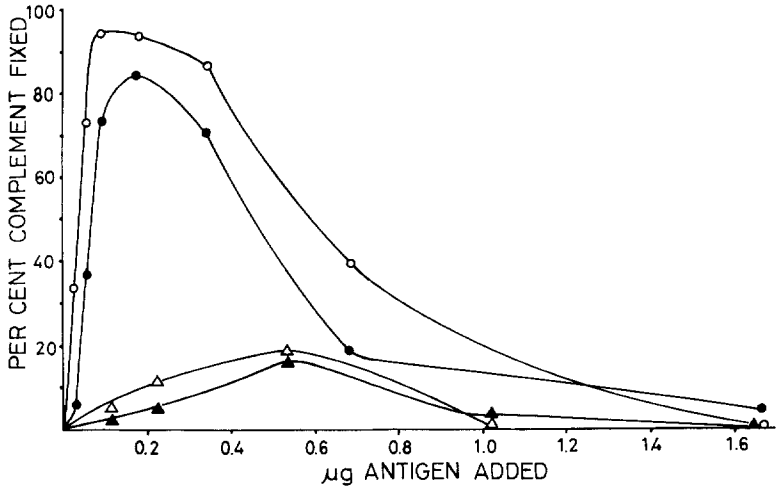


FIG. 7. Complement-fixation studies on antibodies purified from serum No. 1. ○ γG GalNAc eluate 0.302 μg AbN/ml per assay + $A_1$ substance; ● γG $AR_L$ 0.52 eluate 0.900 μg AbN/ml per assay + $A_1$ substance; △ γG GalNAc eluate 0.302 μg AbN/ml per assay + $A_2$ substance; ▲ γG $AR_L$ 0.52 eluate 0.900 μg AbN/ml per assay + $A_2$ substance.

treated with ficin each gave 4-fold increases in titer suggests that accessibility of antigenic sites is a factor; the methods used do not permit a decision as to whether $A_1$ or $A_2$ receptors are exposed to different extents. Greenbury et al. (22) also contend that $A_2$ receptors are not only fewer in number but differ qualitatively from $A_1$ receptors, combining less avidly with antibodies raised against $A_1$ cells and being less antigenic in the rabbit. It should be noted that the nature and extent of the determinants in A substance to which the rabbit reacts has never been clearly defined in comparison to human anti-A. The presence of anti-$A_1$ in $A_2$ individuals (13–17) also favors a qualitative difference. Our data deal predominantly with immunochemical differences between the water-soluble $A_1$ and $A_2$ glycoproteins. The precipitin curves presented in Figs. 1 and 2 using whole serum or purified antibodies indicate a specificity difference.

If the difference was exclusively quantitative one would expect to reach the same maximum as with $A_1$ substance by increasing the amount of antigen added. This, however, was not the case. The precipitin curves with both $A_1$ and $A_2$ substance reach equivalence in the same region and then both decrease as more antigen is added.

A specificity difference is also strongly supported by the absorption experiments (Fig. 3). Antibodies reacting with $A_2$ substance were specifically removed from an anti-A serum by absorption with soluble $A_2$ substance or by passing the serum through a column packed with polyleucyl $A_2$ substance. However, anti-A, passed through a column of polyleucyl $A_1$ substance to remove only part

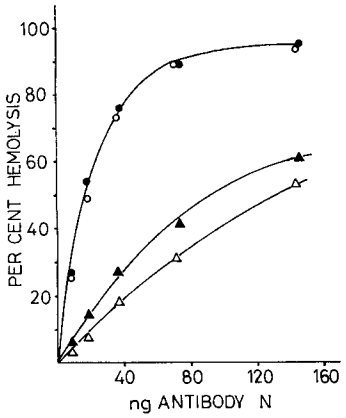


FIG. 8. Hemolytic assay of purified γM antibodies from serum Chris. 10 $CH_{50}$ units total volume 7 ml. ○ γM GalNAc eluate, 4.6 × $10^7$ $A_1$ cells; ● γM $AR_L$ 0.52 eluate, 4.6 × $10^7$ $A_1$ cells; △ γM GalNAc eluate, 4.9 × $10^7$ $A_2$ cells; ▲ γM $AR_L$ 0.52 eluate, 4.9 × $10^7$ $A_2$ cells.

of the total antibody, left the proportion of antibodies in the effluent reacting with $A_1$ and $A_2$ substances unchanged.

The findings in Fig. 2 with purified anti-A eluted by DGalNAc and by $AR_L$ 0.52 show that $A_1$ substances precipitated more antibody N than $A_2$ substances from both the DGalNAc and the $AR_L$ 0.52 eluates, except with the DGalNAc eluate from serum No. 1 which reacted as well with $A_1$ as with $A_2$ substance.

The DGalNAc eluates have been shown to be inhibited relatively more effectively by small oligosaccharides than the $AR_L$ 0.52 eluates and thus would have a larger proportion of smaller size sites (39). This type of fractionation is relatively crude and does not effect a complete separation on the basis of size of combining site. Thus it is not surprising that in the Chris and Jos eluates, the $A_1$ substance is better than the $A_2$ substance in precipitating since in the DGalNAc as well as in the $AR_L$ 0.52 eluates, a proportion of larger size sites

might remain. The exceptional eluate, the DGalNAc eluate No. 1, which reacted with both $A_1$ and $A_2$ substances to the same extent, might then be hypothesized to have been more effectively fractionated so that it essentially had only relatively small size sites and thus might not have contained anti-$A_1$ ; this would perhaps be consistent with the small proportion (20%) of the total N precipitated by $A_1$ and $A_2$ substances.

The specificity difference between $A_1$ and $A_2$ substance is also evident from the inhibition experiment presented in Fig. 4. An $A_1$ anti-$A_1$-specific precipitate could be almost completely dissolved by about a 2-fold excess of $A_1$ substance, while with $A_2$ substance as inhibitor, a 23-fold excess gave only 50% inhibition.

The hemolysis data in Fig. 8 with both $\gamma$M anti-A eluates also tend to favor a qualitative difference between $A_1$ and $A_2$ . Since one molecule of $\gamma$M hemolysin per red cell has been shown to be sufficient to cause hemolysis, the 3–4-fold reduction in the number of sites on $A_2$ ($2 \times 10^5$ sites) as compared to $A_1$ cells ($8 \times 10^5$ sites), if the determinants in both were identical, would not be expected to reduce the effectiveness of $\gamma$M anti-A causing hemolysis. The reduced susceptibility to lysis by $\gamma$M anti-A of $A_2$ cells as compared with $A_1$ cells is most reasonably explained by a structural difference between the $A_1$ and $A_2$ receptors. It should be noted that Landsteiner and Levine (48) also found $A_1$ cells to be much more easily hemolyzed than $A_2$ cells using hemolytic sera from O individuals.

The complement fixation data (Fig. 7) with the analogous $\gamma$G anti-A eluates and soluble $A_1$ and $A_2$ substances also indicate definite structural differences between them, since such a great decrease in complement-fixing capacity is not readily explainable in terms of a 4-fold difference in numbers of receptors.

If one accepts the above data as clearly establishing a qualitative difference between $A_1$ and $A_2$ substances, one must attempt to account for these differences in structural terms and also to explain the biosynthesis of the $A_1$ and $A_2$ substances. Since the inhibition data in Fig. 6 indicate that difucosyl A receptors are present in both $A_1$ and $A_2$ substances, and since they have been isolated from $A_1$ substance (42), the second fucose alone cannot establish a structural difference. The only apparent chemical basis for a structural difference would appear to be the difference in type 1 and type 2 determinants.

If anti-A were a mixture of antibodies to both type 1 and type 2 determinants, and $A_2$ substance lacked one of these determinants, one could account for the specificity difference (29). The situation is not that simple however, since the data suggest that there may really be four types of determinants in $A_1$ substance, mono- and difucosyl type 1 and mono- and difucosyl type 2. The inhibition data (Fig. 6) indicate that $A_2$ substance is selectively reacting with antibodies directed towards difucosyl A determinants and is more readily inhibited by difucosyl than by monofucosyl type 2 determinants. The increased inhibiting power of difucosyl as compared with monofucosyl type 2 deter-

minants seen in certain sera in assays with $A_1$ substance could be due essentially to inhibition of the type 2 determinants of the $A_1$ substance. Unfortunately, mono- and difucosyl type 1 determinants are not available. The inhibition results, however, clearly indicate that difucosyl type 2 A determinants are present in $A_2$ substances and no data are available indicating whether or not type 1 A determinants occur. If type 1 A determinants prove to be absent from $A_2$ substance, a satisfactory explanation for the qualitative difference will emerge. That this may actually be so is strongly suggested by the reports of Seaman et al. (49) and Crookston et al. (50, 51) who found an antibody specific for an $A_1$ $Le^b$ determinant; this antibody reacted only with $A_1$ $Le^b$ and not with $A_2$ $Le^b$, O $Le^b$, or $A_1$ $Le^a$ erythrocytes. Thus the intimate association of $A_1$ and $Le^b$ in a single determinant detected by this unusual antibody is completely consistent with the finding that $A_2$ substance shows high $Le^b$ activity (Fig. 5) and hence provides support for our inference that the $A_1$ determinant is associated with the type 1 chain. Tegoli et al. (52) have recently found an antibody reacting only with red cells that are either O, I, Le(a−b+) or $A_2$, I, Le(a−b+). The antibody does not react with $A_1$, I, Le(a−b+) red cells. This finding is also consistent with $A_2$ cells having type 2 A receptors and incomplete type 1 chains which are responsible for the H, I, and $Le^b$ activity.

One should perhaps consider the genetic basis for the structural difference between $A_1$ and $A_2$. Experiments of several workers (34, 35, and footnote 3) have shown that enzymes from $A_2$ as well as from $A_1$ individuals could transfer an $\alpha$-linked DGalNAc from uridine diphosphate (UDP)-GalNAc to the galactose in

lacto-N-fucopentaose I ($\alpha$LFuc(1 →2)$\beta$DGal(1 → 3)$\beta$DGNAc(1 → 3)$\beta$DGal(1 → 4)DGlc)
to fucosyl-lactose ($\alpha$LFuc(1 → 2)$\beta$DGal(1 → 4)DGlc)
and to $HR_L$ 0.75($\alpha$LFuc(1 → 2)$\beta$DGal(1 → 4)$\beta$DGNAc-R)
but not to lacto-N-difucohexaose I ($\alpha$LFuc(1 →2)$\beta$DGal(1 →3)[$\alpha$LFuc(1 →4)]$\beta$DGNAc(1 → 3)$\beta$DGal(1 → 4)DGlc)
lactodifucotetraose($\alpha$LFuc(1 → 2)$\beta$DGal(1 → 4)[$\alpha$LFuc(1 → 3)]DGlc)
or $HR_{IM5}$ 2.5 ($\alpha$LFuc(1 → 2)$\beta$DGal(1 → 4)[$\alpha$LFuc(1 → 3)]$\beta$DGNAc-R);

the acceptors are monofucosyl type 1 and monofucosyl type 2 compounds while the nonacceptors are difucosyl type 1 and 2 compounds. Thus the presence of a second fucose shuts off biosynthesis by the UDP-GalNAc transferase but this enzyme does not appear to differ in $A_1$ and $A_2$ individuals[3] (53). How then could a specificity difference between $A_1$ and $A_2$ arise?

One possibility which remains to be tested is that the $A_1$ and $A_2$ DGalNAc transferases differ in their ability to transfer to macromolecules although both can transfer to oligosaccharides. This essentially implies a difference in the $A_1$ and $A_2$ genes with respect to the transferases that they produce.

Since type 1 and type 2 A determinants come off as branches from a single galactose, it may well be that both determinants can react with a single antibody molecule. This, as Karush (54) has pointed out, would substantially increase the strength of the antigen-antibody bond. If only the type 2 deter-

minant were present in $A_2$ substance, with the type 1 determinant being H or $Le^b$, each anti-A molecule would only be able to react with one site of each branch. This would effect a substantial reduction in binding energy and be consistent not only with the increased H and $Le^b$ activity (Fig. 5) but also with the dramatic reduction in complement-fixing capacity (Fig. 7).

The nature of the structural difference between $A_1$ and $A_2$ will only be definitely decided when oligosaccharides from $A_2$ substances are isolated and their structures established. The isolation of type 1 A-active oligosaccharides from $A_2$ substance would be a clear-cut refutation of the hypothesis advanced. Unfortunately current methods of isolation of oligosaccharides by alkaline degradation of blood group substances result in extensive destruction of the type 1 chain so that even from $A_1$ individuals only very small amounts of type 1 oligosaccharides are obtained (42).

## SUMMARY

The immunochemical properties of purified $A_1$ and $A_2$ glycoproteins have been compared to ascertain whether their antigenic determinants differ. Quantitative precipitin and complement-fixation studies using several anti-A sera as well as purified $\gamma$G anti-A antibodies clearly showed a specificity difference. This was also supported by absorption studies: $A_2$ substance specifically removed antibodies reacting with $A_2$ substance leaving anti-$A_1$ activity. $A_1$ substance was more effective than $A_2$ substance in dissolving an $A_1$ anti-$A_1$–specific precipitate. Purified $\gamma$M anti-A hemolyzed $A_1$ cells more readily than $A_2$ cells. Inhibition studies using mono- and difucosyl type 2 A-active oligosaccharides showed that type 2 difucosyl receptors are present in $A_2$ substance. The structural basis for the specificity difference between $A_1$ and $A_2$ would appear to be that $A_2$ substances lack type 1 A determinants; this would account for the observed higher H and $Le^b$ activity in $A_2$ substances.

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