THREE TYPES OF BLOOD GROUP I SPECIFICITY AMONG MONOCLONAL ANTI-I AUTOANTIBODIES REVEALED BY ANALOGUES OF A BRANCHED ERYTHROCYTE GLYCOLIPID

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The antigens recognized by the human cold-reactive autoantibodies anti-I and anti-i are distributed on a variety of cell types and in secretions (1–4). These antigens are known to occur on the carbohydrate chains that constitute the core structures of the blood group ABH antigens (5, 6) and certain erythrocyte gangliosides (7, 8). Recently, we reported that the straight chain glycosphingolipid lacto-N-*nor*hexaosyl ceramide:[1] Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3 Galβ1 → 4 Glcβ → Cer reacted with five out of six anti-i antibodies (7). In contrast, the branched glycosphingolipid lacto-N-*iso*-octaosyl ceramide:

$$\begin{array}{l} \mathrm{Gal}\beta 1 \rightarrow 4\mathrm{GlcNAc}\beta 1 \searrow \\ \qquad\qquad\qquad\qquad\qquad {}^{6}_{3}\mathrm{Gal}\beta 1 \rightarrow 4\mathrm{GlcNAc}\beta 1 \rightarrow 3\mathrm{Gal}\beta 1 \rightarrow 4\mathrm{Glc}\beta \rightarrow \mathrm{Cer} \\ \mathrm{Gal}\beta 1 \rightarrow 4\mathrm{GlcNAc}\beta 1 \nearrow \end{array}$$

reacted with all 5 anti-I antibodies tested but with only two out of six anti-i antibodies (9). The I and i activities of eight analogues of lacto-N-*iso*-octaosyl ceramide were compared and it was concluded that (*a*) the antigenic determinants recognized by the five anti-I antibodies tested are located on various domains within the branched lacto-N-*iso*-octaosyl structure and (*b*) the antigenic determinants recognized by four out of the six anti-i antibodies were masked by the presence of the GlcNAcβ1 → 6Gal branch at the middle galactose residue of the i-active lacto-N-*nor* hexaosyl ceramide (9).

This report is an analysis of the inhibitory activities in radioimmunoassays of lacto-N-*iso*-octaosyl ceramide and its 8 analogues (9) with 11 anti-I sera, including the 5 antisera previously tested. 10 of the anti-I antibodies were inhibited by the lacto-N-*iso*-octaosyl structure. Although none of the anti-I antibodies was identical in its reactivities with the various analogues; three main patterns of activity could be distinguished corresponding to recognition of three domains within the lacto-N-*iso*-octaosyl ceramide structure.

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[1] Nomenclature according to the Commission on Biochemical Nomenclature of the International Union of Pure and Applied Chemistry. The use of the terms *iso* and *nor* has been explained previously (7).

Materials and Methods

Erythrocyte Glycolipids. The bovine erythrocyte ganglioside (structure I, Table I) with slow migrating properties on thin-layer chromatography was isolated and its structure was determined by methylation analysis, direct probe mass spectrometry of the methylated ganglioside and of the methylated nonasaccharide liberated by endo-β-galactosidase of *Escherichia freundii*, and by sequential degradation with various exoglycosidases as described previously (9). Eight glycolipid analogues (structures II to IX) with branched and unbranched carbohydrate structures were prepared by sequential degradation of the ganglioside with various combinations of exoglycosidases (9). Anti-I cold agglutinins from patients Ma, Woj, Step, Gra, Ver, Ful, Phi, Da, Sch, Low, and Zg have been described previously (5, 10–12).

Antigenic Analysis of the Glycolipids. The reactivities of the glycolipids with the various anti-I sera were determined by radioimmunoassays as described previously (8, 13). Briefly, glycolipids complexed with cholesterol and lecithin as carrier lipids (proportions of cholesterol, lecithin and glycolipids were 2:2:1 by weight) were used as inhibitors of the binding of the anti-I sera to a radioiodinated I- or (I + i)-active glycoprotein. The results were expressed as the minimum concentration of glycolipids giving 50% inhibition of binding.

Results

Table I shows the inhibitory activities of lacto-N-*iso*-octaosyl ceramide and its analogues with the anti-I sera Ver, Ful, Phi, Da, Low, and Zg in addition to the previously tested antisera Ma, Woj, Step, Gra, and Sch. With the exception of anti-I Zg, all the anti-I sera were inhibited by structure III. The I activity of structure III was abolished after removal of the two terminal β-galactosyl residues to give lacto-N-*iso*-hexaosyl ceramide (structure VI).

A comparison of the reactions of the anti-I sera with structures I to IX indicate that none of the 11 antibodies tested was identical in the antigenic determinants that it recognized. However, three main types of reactivity could be distinguished. Anti-I sera Ma and Woj represent the first type which requires the 1 → 4, 1 → 6 chain, although the branched structure is not essential, in agreement with previous observations (6, 9, 10). Anti-I sera Step, Gra, Ver, and Ful represent the second type which requires predominantly the 1 → 4, 1 → 3 chain but, unlike the first type, the 1 → 6 branching structure is required for full expression of the I activity, except with anti-I Ver which reacts equally well with the branched and straight chain structures III and VIII, respectively. The third type of anti-I activity is represented by sera Da, Sch, Low, and Phi which require both the 1 → 4, 1 → 6 and 1 → 4, 1 → 3 branches to be present in the intact state as in structure III. Substitution of the terminal β-galactosyl residues of the two chains with α-galactose or sialic acid decreases but does not necessarily abolish the I activity as seen with structures I and II.

Discussion

From these and previous studies with purified lacto-N-*iso*-octaosyl ceramide, lacto-N-*nor*-hexaosyl ceramide and their analogues (7, 9), crucial information has been obtained on the molecular basis of I and i specificities and certain generalizations can now be made: (*a*) nonreducing terminal β-galactose is an important part of both the I and i antigenic determinants; (*b*) an intact straight chain oligosaccharide with a repeating Galβ1 → 4GlcNAcβ1 → 3 sequence expresses the majority of i and part of I antigenic determinants; (*c*) the antigenic determinants recognized by the majority of anti-I-antibodies are expressed on intact 1 → 4, 1 → 6, and/or 1 → 4, 1 → 3 branched structures.

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Table I

I Activities of a Branched Bovine Erythrocyte Ganglioside and of its Analogues as Determined by Radioimmunoassays

		Anti-I										
		Ma*	Woj*	Step*	Gra*	Ver	Ful	Phi	Da	Sch*	Low	Zg
Structure I	Galα1 → 3Galβ1 → 4GlcNAcβ1 ↘ 6,3 Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer SAα2 → 3Galβ1 → 4GlcNAcβ1 ↗	9‡	—§	8	20	20	4	30	20	>30‖	—	—
Structure II	Galα1 → 3Galβ1 → 4GlcNAcβ1 ↘ 6,3 Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer Galβ1 → 4GlcNAcβ1 ↗	17	—	7	15	8	6	20	20	7	15	—
Structure III (lacto-N-isooctaosyl ceramide)	Galβ1 → 4GlcNAcβ1 ↘ 6,3 Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer Galβ1 → 4GlcNAcβ1 ↗	30	25	3	3	3	6	12	6	7	6	—
Structure IV	Galβ1 → 4GlcNAcβ1 ↘ 6,3 Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer GlcNAcβ1 ↗	20	10	30	30	—	—	—	—	—	—	—
Structure V	GlcNAcβ1 ↘ 6,3 Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer Galβ1 → 4GlcNAcβ1 ↗	—	—	4	10	8	12	>30	—	—	—	—
Structure VI (lacto-N-iso hexaosyl ceramide)	GlcNAcβ1 ↘ 6,3 Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer GlcNAcβ1 ↗	—	—	—	—	—	—	—	—	—	—	—
Structure VII	Galβ1 → 4GlcNAcβ1 → 6Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer	9	20	—	—	—	—	>30	—	—	—	—
Structure VIII (lacto-N-*nor*-hexaosyl ceramide)	Galβ1 → 4 GlcNAcβ1 → 3Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer	—	—	30	—	3	—	—	—	—	—	—
Structure IX	GlcNAcβ1 → 6Galβ1 → 4GlcNAcβ1 → 3Galβ1 → 4Glcβ → Cer	—	—	—	—	—	—	—	—	—	—	—
Standard I-active glycoprotein¶		5	<1	2.5	<1	3	10	2	8	3	6	10

* Anti-I sera previously tested with structures I to IX (9).

‡ I activity determined by radioimmunoassays expressed as the concentration μg/ml required to give 50% inhibition of binding of ^{125}I-labeled I-active antigen to the anti-I antibodies.

§ —: not inhibitory at the highest concentration tested 30 μg/ml.

‖ >30: less than 50% inhibition at 30 μg/ml.

¶ A glycoprotein derived from human meconium was used as inhibitor of the anti-I sera except Sch and Low. With these latter antisera an I-active glycoprotein extracted from sheep gastric mucosa was used.

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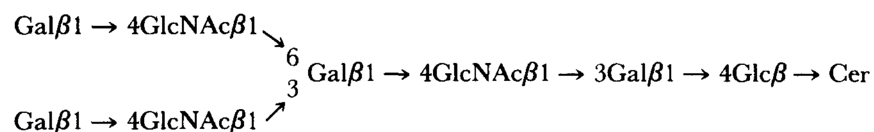
The present studies reveal three main types of I specificity at the same time as demonstrating the individuality in the fine specificity of each monoclonal anti-I antibody. The dramatic differences between anti-I and anti-i cold agglutinins of different individuals in their hemagglutinating activities with erythrocytes of various Ii phenotypes and in their precipitation reactions with various precursor-like glycoproteins are well known. These differences have been previously used to subclassify anti-I and anti-i antibodies into subtypes (10, 12). However, classification based on reactions with complex reference materials such as glycoproteins and erythrocytes are difficult to interpret, because of the extreme heterogeneity of their oligosaccharide chains. In fact, the present studies with the purified glycolipids have revealed considerable differences between the reactivities of anti-I Step and those of Sch and Low, although the three antibodies had been assigned to the same group (anti-I group 3) on the basis of their precipitation reactions with water soluble glycoproteins (10). Furthermore, among the four anti-I antibodies which require the intact branched structure, as in structure III, are antibodies previously assigned to anti-I group 3 (Sch and Low), group 4 (Gra), and group 5 (Da) (10).

The ability of several anti-I antibodies to react with their antigenic determinants in the presence of external substitutions (α-linked galactose or sialic acid) is an important consideration in the interpretation of the antigenicity of complex structures (or mixtures) and of the effects of digestion with β-galactosidase (14). Substances rich in the carbohydrate sequences found in structures II, III, IV, and V would lose much of their I activity after β-galactosidase treatment. However, substances rich in structure I would retain their activities after such treatment.

Thus far the antigenic determinants recognized by 2 of the 16 anti-I and anti-i cold agglutinins (anti-I Zg and anti-i Galli) are unknown. The specificities of the others have been shown to involve one or other kind of type 2 precursor chain (15), i.e. 1 $\rightarrow$ 4, 1 $\rightarrow$ 3 and/or 6 sequences. It is interesting to speculate whether type 1 chains (15) with 1 $\rightarrow$ 3, 1 $\rightarrow$ 3 sequence are involved in the specificities of Zg and Galli. So far, the branched or straight chain analogues of the 1 $\rightarrow$ 4, 1 $\rightarrow$ 3 sequence have been described in the precursor chains of blood group ABH-active glycolipids (16) and erythrocyte gangliosides (7, 9, 17), and among oligosaccharides isolated from human milk (18). It would be predicted that these analogues also exist as precursor chains of secreted human blood group substances, for substantial I and i activities are found on ovarian cyst glycoproteins of persons who are nonsecretors (5, 10, 19).

Summary

Blood group I activities of the purified glycosphingolipid lacto-N-*iso*-octaosyl ceramide



and 8 of its analogues have been evaluated with 11 anti-I sera including 5 anti-I sera previously tested. All but one of the antisera were inhibited by the lacto-N-*iso*-octaosyl structure. Three types of I-specificity could be distinguished although none of the anti-I sera was identical in its inhibition patterns with the nine glycosphingolipid

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analogues. The anti-I sera Ma and Woj represent the first type and require an intact Galβ1 → 4GlcNAcβ1 → 6 chain, the anti-I sera Step, Gra, Ver, and Ful represent the second type which requires Galβ1 → 4GlcNAcβ1 → 3 chain with branching, and the anti-I sera Phi, Da, Sch, and Low belong to the third type which requires both branches to be intact. Anti-I antibodies vary in their ability to react with their antigenic determinants in the presence of external substitutions with α-linked galactose or sialic acid.

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