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Monoclonal Antibodies Specific for Type 3 and Type 4 Chain-based Blood Group Determinants: Relationship to the A1 and A2 Subgroups

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Two monoclonal antibodies, specific for A type 3 and A type 4 blood group determinants, are described. These antibodies recognized A1 but not A2 erythrocytes. A third monoclonal antibody showing specificity for A type 3 and A type 4, and also for H type 3 and H type 4, did not discriminate between A1 and A2 erythrocytes. On red cells these three antibodies recognized glycosphingolipids and binding to glycoproteins could not be demonstrated. On paraffin-embedded tissue sections the three antibodies labelled a supranuclear area, characteristic of the Golgi apparatus, of all cells producing A antigens. This labelling occurred irrespective of the A1, A2 status.

The results suggest that glycolipids of erythrocytes and possibly of other cell types bear the A type 3/4 determinant specific for the A1 subgroup and that A type 3/4 determinants of glycoproteins might be present in both A1 and A2 subgroups on short oligosaccharide chains which are only detectable at the level of the Golgi apparatus.

The blood group A determinant minimum structure is the well characterized trisaccharide, GalNAcα1-3[Fucα1-2]Galβ-R [1].

Four types of precursor chains have been recognized:

Type 1: Galβ1-3GlcNAcβ-R
Type 2: Galβ1-4GlcNAcβ-R
Type 3: Galβ1-3GalNAcα-R
Type 4: Galβ1-3GalNAcβ-R

Determinants based on type 1 chains are expressed in tissues of endodermal origin,

whereas determinants based on type 2 chains are expressed on various tissues of both ecto- and endodermal origin [2].

Biochemical studies have shown the existence of the type 3 or type 4 based ABH antigens in saliva [3], red cells [4-6] and kidneys [7]. More recently, studies of erythrocyte glycolipids have revealed that A type 3- and A type 4-based determinants were specific for the A1 subgroup [5, 6] and that A type 4 glycolipids were present in A1 kidneys and absent (or present in trace amounts) from A2 kidneys [8].

The nature of the difference between the A1 and A2 subgroups has been the subject of a long controversy. Some have proposed a qualitative difference between the two subgroups, i.e. the presence of A1 specific determinants [9-11]. Others have suggested that the difference is simply quantitative, with red cells of the A1 phenotype having more A determinants than red cells of the A2 phenotype, but all determinants being of the same nature [12-14].

We now wish to report the preparation and characterization of three monoclonal antibodies specific for type 3 and type 4 chain-based ABH determinants and to discuss their relationship to the A1 and A2 subgroups.

Materials and Methods

Antibodies

Two antibody specificities were found in an ascitic fluid produced in Balb/c mice by inoculation of a hybridoma cell line. The hybridoma, cloned twice by limiting dilutions, had been produced after immunization of a mouse with neuraminidase-treated O red cells. The ascitic fluid thus obtained contained an IgG_3 antibody reacting specifically with A, B or O neuraminidase-treated erythrocytes, which is not further analyzed in this study, and a second antibody called TS-1. Purification of TS-1 from 1 ml of ascitic fluid was achieved by immunoadsorption on 200 mg of the H type 3 synthetic oligosaccharide linked to an insoluble matrix of crystalline silica (Chembiomed, University of Alberta, Edmonton, Canada). The H type 3, rather than the A type 3 epitope, was used since the elution from A type 3 immunoadsorbent was not possible. However, TS-1 antibody showed no binding to H type 3 structures in radioimmunoassays (see results) due to its very weak affinity for this trisaccharide. After extensive washings with 10 mM sodium phosphate, 0.14 M sodium chloride, pH 7.2 (phosphate buffered saline, PBS), the bound antibody was desorbed with 1% ammonium hydroxide (pH 11) and immediately neutralized with KH_2PO_4. The antibody was finally dialyzed against PBS. Monoclonality of TS-1 was assessed by isoelectrofocusing. This antibody was an IgG_1 as determined by immunodiffusion.

Antibody KB-26.5 was obtained by immunization of a Balb/c mouse with commercial, animal AB substance (Ortho, Raritan, New Jersey, USA). The mouse received three injections of 100 μg of AB blood group substance in Freund complete adjuvant, each injection separated in time by three weeks. A final booster injection was given four days before the spleen was removed. The spleen cells were fused by the method of Köhler and Milstein [15], using polyethyleneglycol and the myeloma cell line MOPC21 NSI/1. Hybrids were screened by agglutination of A1 red blood cells and cloned twice by

limiting dilutions in microtitre wells. The antibody KB-26.5 was an IgG_3 kappa, as determined by immunodiffusion.

Antibody H1R8 was obtained by immunization of Balb/c mice with human erythrocytes according to the method of Stähli *et al.* [16]. The red cells were from a child suffering from acute hemolytic-uremic syndrome and typed as A1, D^+ ce/ce, MNss, Kneg, P^+, Fy(a-b+), JK(a-b+), Le(a-b+), L(a-). The immunoblasts from the mouse spleen were fused with the myeloma cell line P3x63-Ag8-U1 using polyethyleneglycol [15]. Hybrids were selected by agglutination of human A erythrocytes and by a cell binding radioimmunoassay. H1R8 was cloned twice by limiting dilutions. The antibody produced was an IgM as determined by an immunodot assay. Only culture supernatant was used for this antibody. A commercial monoclonal anti-A antibody (lot A581) was obtained from Dakopatts, Glostrup, Denmark.

Agglutinations

Erythrocytes from healthy donors were typed for their ABO and Lewis subgroups by standard agglutination techniques. Agglutinations with antibodies KB-26.5 and H1R8 were performed in saline at room temperature. Titres were determined as the last antibody dilution giving a macroscopic agglutination.

Coated Erythroctyes

Polyvinyl microtitre plates (Dynatech, Alexandria, VA, USA) were treated with poly-L-lysine P-2636 (Sigma, St Louis, MO, USA) diluted to 2 mg/100 ml with distilled water. After incubation for 2 h at 37°C, the remaining poly-L-lysine was discarded and 10^6 erythrocytes were added per well. Plates were centrifuged at 1500 rpm for 10 min and dipped into a solution of 0.25% glutaraldehyde/PBS for 30 min at room temperature. The glutaraldehyde solution was then discarded and 100 μl of 3% gelatine/PBS (w/v) was added per well and incubated for 1 h at 37°C. This solution was discarded and after addition of 100 μl of 0.5% gelatine/PBS, 0.02% Tween 20 (Merck, Darmstadt, W. Germany) (Buffer A) the plates were frozen and kept at -20°C until use.

Binding of Antibodies to Erythrocytes

50 μl of antibody, serially diluted in buffer A, were added per well and incubated at room temperature for 2 h. The plates were then washed three times with PBS, and 50 μl of purified rabbit anti-mouse immunoglobulin, prepared according to the method of Oriol *et al.* [17], diluted in buffer A, were added to each well and incubated overnight. After three washings, ^{125}I-labelled protein A [18] was added and incubated for 3 h. After three further washings, wells were sliced off and the bound radioactivity was measured in an LKB automatic gamma counter.

Binding to Artificial Antigens

Blood group related artificial antigens [19], with 15-20 hapten groups per molecule, were made using synthetic oligosaccharides covalently bound to bovine serum albumin (BSA) [20]. The structures of the artificial antigens used (R_2) are listed in Table 4. They were obtained through the kindness of Dr. R.U. Lemieux and Chembiomed Ltd.

Polystyrene tubes (L.E.F. Viry-Chatillon, France) were coated with the artificial antigens diluted to 1 μg/ml in PBS by incubating overnight at room temperature. Antibodies TS-1, KB-26.5 or H1R8 were added in two-fold serial dilutions and incubated for 3 h. Binding was measured by the sequential addition of purified rabbit anti-mouse immunoglobulins and ^{125}I-labelled protein A. Bovine serum albumin (Sigma), 3% in PBS, was used to prevent non-specific binding; washings were performed between each step with 1 ml of tap water.

Adsorptions on Immunoadsorbents

Synthetic oligosaccharides bound to a solid matrix of crystalline silica (SYNSORBS) were obtained from Chembiomed. The immunoadsorbents used (R_1) are listed in Table 4. Antibodies TS-1, KB-26.5 or H1R8, 100 μl, diluted in 1% BSA in PBS, were incubated with 20 mg of each of the immunoadsorbents. After 2 h of incubation, the supernatant was tested for activity by immunofluorescence on parotid glands from donors of A1 Le(a-b+) phenotype.

Tissue Samples

Formaldehyde-fixed and paraffin-embedded tissue sections were obtained through the kindness of Dr. C. Krainic (Institut Médicolégal, Paris, France). All tissues listed in Table 3, except for stomach and duodenum samples, had been taken from cadavers examined for forensic medicine purposes. Gastric and duodenal mucosa sections were obtained from Dr. J. Bara (IRSC, Villejuif, France). These tissues had been fixed in ethanol and paraffin-embedded [21]. ABO and Lewis blood groups of all tissue donors were determined on red cells by conventional agglutination tests. Le(a+b-) donors were considered as ABH non-secretors and Le(a-b+) donors were considered as ABH secretors. The secretor character of Le(a-b-) donors was determined by the reactivity of the anti-H lectin from *Ulex europaeus*, either in salivary glands or in the gastroduodenal surface epithelia [21].

Immunofluorescence

All reagents, diluted in 1% bovine serum albumin (BSA/PBS), were incubated for 30 min in a moist chamber. Optimal dilutions were determined by chessboard titrations. Concentrations from 5-10 μg/ml of purified monoclonal antibodies were used. Indirect labelling was performed using rabbit anti-mouse immunoglobulins (H+L) labelled with fluorescein isothiocyanate (FITC) from Cappel Laboratories (West Chester, PA, USA). When an irrelevant primary antibody was used no immunofluorescence was observed. In a three step technique, a third incubation was performed using a lectin labelled with tetramethylrhodamine isothiocyanate (TRITC). TRITC-labelled lectin 1 from *Ulex europaeus* was obtained from Vector Laboratories (Burlingame, CA, USA). TRITC-labelled *Helix pomatia* lectin was obtained from IBF (Villeneuve la Garenne, France). Between each step, washings were performed by incubation for 10 min in PBS. After the last washing, cover slips were mounted with one drop of oxidized *p*-phenylenediamine in glycerol [22]. The excess mounting medium was removed with filter paper and the slides were examined with a Leitz SM-LUX microscope equipped with a Ploemopak 2.3

Table 1. Agglutination scores[a] given by antibodies KB-26.5 and H1R8 with erythrocytes of various ABO and Lewis phenotypes.

Red cell phenotype	Number tested	Average score ± SD	
		KB-26.5	H1R8
A1 Le(a—b+)	20	57 ± 6	26 ± 3
A1 Le(a+b—)	15	56 ± 6	30 ± 3
A1 Le(a—b—)	15	54 ± 5	27 ± 3
A1B Le(a—b+)	10	51 ± 4	26 ± 3
A1B Le(a+b—)	8	50 ± 6	26 ± 3
A1B Le(a—b—)	5	55 ± 6	30 ± 3
A2 Le(a—b+)	15	1 ± 3	19 ± 5
A2 Le(a+b—)	8	1 ± 2	28 ± 2
A2 Le(a—b—)	8	8 ± 14	25 ± 11
A2B Le(a—b+)	8	1 ± 2	1 ± 2
A2B Le(a+b—)	4	0 ± 0	N.D.
O	4	0 ± 0	0 ± 0
B	3	0 ± 0	0 ± 0

[a] Agglutination scores were calculated by addition of the points for each tube of two-fold serial dilutions in saline (+++ = 10; ++ = 8; + = 5; (±) = 2; — = 0). The first dilution for KB-26.5 was ascitic fluid 1:20 and for H1R8 culture supernatant 1:4.
N.D. not determined.

Table 2. Radioimmunoassay titres given by the three anti-A antibodies with erythrocytes[a].

Phenotype	Number tested	Titres		
		KB-26.5	TS-1	H1R8
A1	11	95000 ± 23000	300 ± 55	1000 ± 500
A2	10	2000 ± 600	<40	520 ± 275
O	7	<200	<40	<2
B	3	<200	<40	<2
A1/A2 titre ratio		≈50	>7	≈2

[a] Subdivision into the various Lewis phenotypes did not alter the data. Radioimmunoassays, on coated erythrocytes, were performed as described in Material and Methods. Titres were taken to be the last antibody dilution which gave a binding two times greater than the background level.

and a lamp source of 200 W HBO. The I-2 filter-block was used to detect the green fluorescence of FITC-conjugates and the yellow fluorescence of nuclei. The M filter-block was used to detect the red fluorescence of TRITC conjugates. Photographs were taken on a Photoautomat MPS 55 with Fujichrome 400 ASA film.

Binding of Antibodies to Blood Group Glycolipids on Thin-layer Plates

Non-acid glycosphingolipid fractions from erythrocyte membranes of single, serologically well characterized blood donors were prepared as described [23]. The glycosphingolipid fractions were applied, in duplicate, to a high-performance thin-layer plate (HPTLC, Merck Si 60 on aluminium sheets) and developed in an appropriate solvent mixture. After chromatographic development, the thin-layer plate was divided into two sections. One section was treated with anisaldehyde [23]. The antigenic reactivity of the separated glycolipids was tested on the remaining section, using a modification [24] of the method of Magnani *et al.* [25]. The plates were treated with primary monoclonal antibody (KB-26.5, TS-1, H1R8, A581) followed by appropriate ^{125}I-labelled secondary antibody. Plates were dried and autoradiographed.

Results

Agglutinations

The three antibodies, KB-26.5, TS-1 and H1R8, were tested for their capacity to agglutinate human red cells of various ABO and Lewis phenotypes. Antibody TS-1 could only cause a faint agglutination of A1 erythrocytes after papain treatment (data not shown). Antibody KB-26.5 agglutinated specifically the A1 and A1B red cells, whereas antibody H1R8 agglutinated A1, A1B and A2 red cells but not A2B red cells. These agglutination patterns were not influenced by the Lewis and secretor status of the red cell donor as shown in Table 1.

Binding to Erythrocytes

The binding of the three anti-A antibodies on red cells was quantified by a radioimmunoassay. The results are depicted in Table 2. It appears that antibody KB-26.5 binds very strongly to A1 red cells but only weakly to A2 red cells. Specific binding of antibody TS-1 could only be detected on A1 red cells. Antibody H1R8 binds to both A1 and A2 subgroups with almost equal efficiency. None of the three antibodies bound to red cells of O or B blood group.

Tissue Distribution

The expression of the antigens recognized by antibodies KB-26.5, TS-1 and H1R8 has been studied by indirect immunofluorescence. An account of the results obtained with the various tissues tested is given in Table 3. On these paraffin-embedded sections, no staining was visible on vascular endothelium or on red cells.

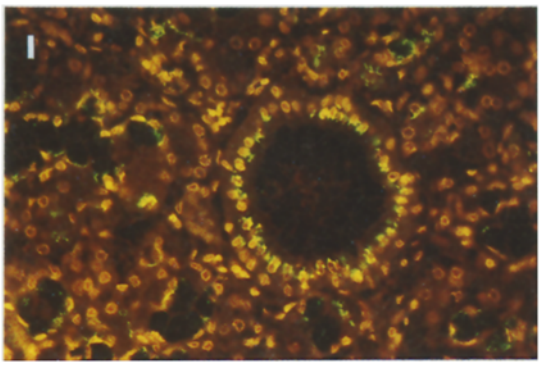
With KB-26.5 and TS-1, staining could only be observed in tissues of individuals of blood-group A. Tissues from five blood group O secretor individuals and two blood group B secretor individuals were negative. It appeared that all cell types able to express the A antigen could also express the determinants recognized by KB-26.5, TS-1 and H1R8. However, an important difference was apparent when the staining pattern is compared with that elicited by standard anti-A antibodies [2]. Indeed, staining by KB-26.5, TS-1 and

Table 3. Overview of the immunofluorescence staining given by TS-1, KB-26.5 and H1R8 antibodies on paraffin embedded tissues from A1 secretor individuals.

Tissues	Staining
Salivary glands	+
Oesophagus mucosa	+
Stomach mucosa	+
Small intestine mucosa	+
Large intestine mucosa	
proximal	+
distal	—
Pancreas	
exocrine	+
islets of Langerhans	—
Liver	
hepatocytes	—
biliary ducts	+
gall bladder	+
Skin	
epidermis	—
sweat glands	+
Lung	
alveolar epithelium	—
bronchial epithelium	+
Kidney	
glomeruli	—
proximal convoluted tubules	—
distal convoluted tubules	+
collecting tubules	+
Reproductive system	
testis	—
epididymis	+
ovary	—
oviduct	+
Nervous system	
brain	—
spinal cord	—
posterior root ganglia	+
Adrenal gland	—
Heart	—
Skeletal muscle	—
Connective tissue	—
Spleen and lymph nodes	—
Vascular endothelium	—
Erythrocytes	—

H1R8 was mostly limited to a supranuclear area corresponding to the location of the Golgi apparatus.

In the parotid gland of nine A1 secretor individuals, both Lewis positive and Lewis negative, a strong supranuclear staining in duct and mucous acinar cells was noticed. A much weaker staining of the mucous itself could also be observed. Not all duct cells nor mucous acinar cells were labelled. This pattern was essentially the same in the sub-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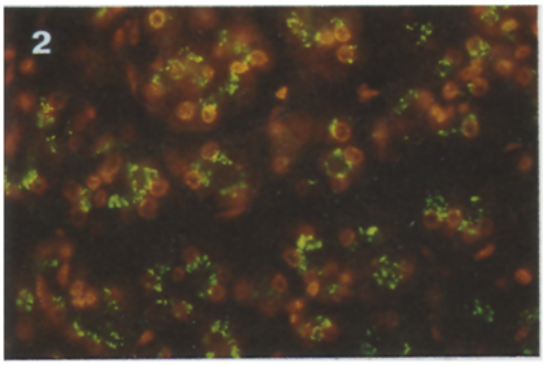
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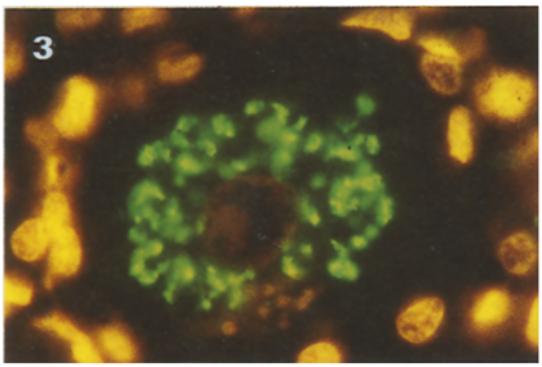
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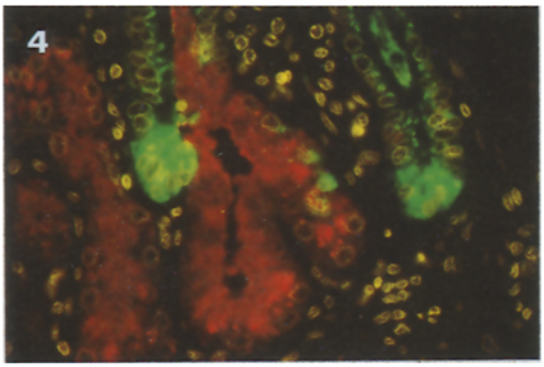
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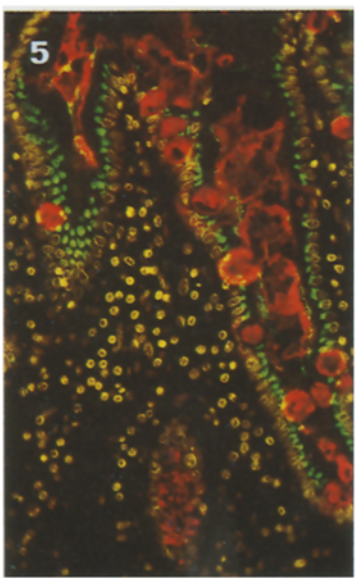
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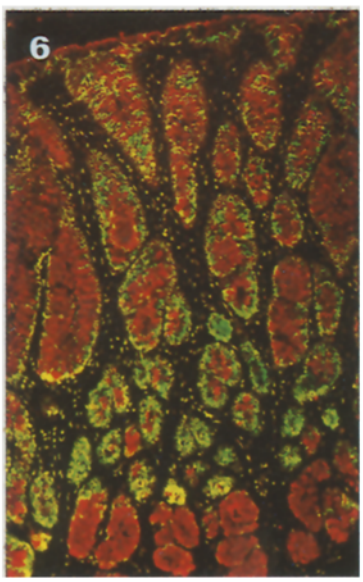
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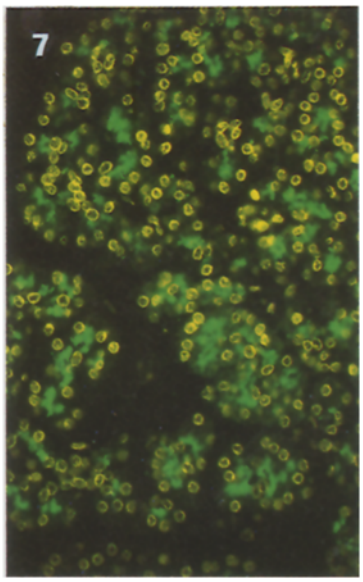
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maxillary gland. For these cells, a double labelling with the lectin *Helix pomatia* revealed that those groups of acinar cells which gave a supranuclar staining with KB-26.5 and TS-1, secreted mucous which was labelled by the lectin. This showed that all cells, producing the A antigen recognized by the lectin, were also producing the determinant revealed by KB-26.5 and TS-1. The staining given by these two antibodies was essentially the same. When salivary glands from three A2 secretors were tested, the same pattern was visible. The supranuclear area of some duct and mucous acinar cells was strongly stained with these two antibodies. The mucous staining could also be observed although it appeared even weaker than on A1 secretors.

Antibody H1R8, stained the tissues of subjects from A, B or O blood groups although the staining was stronger in tissues from blood group A individuals. The same supranuclear labelling which was observed for KB-26.5 and TS-1 was apparent, but when present, the mucous secretion of blood group A individuals was also stained. This last observation is consistent with the fact that this antibody was significantly inhibited by salivas from blood group A people.

Illustrations of the tissue distribution of the antigens recognized by KB-26.5, TS-1 and H1R8 are given in Figs. 1-7. Fig. 1 shows the staining given by antibody TS-1 on the parotid gland of an A1 Le(a-b+) individual. It shows the strong supranuclear staining of the salivary duct cells and of some acinar cells. It also shows the weak staining of the mucous. Fig. 2 shows the staining of the acinar pancreatic cells from an A1 Le(a-b+) donor with antibody TS-1. Only a supranuclear area is labelled. Fig. 3 depicts the staining given by the same antibody on a dorsal root ganglion neuron from an A Le(a+b-) donor. The labelled area corresponds to the Golgi apparatus of these cells. No labelling at all is detectable in the cytoplasm or at the cell membrane. Fig. 4 shows the staining

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COLOUR PLATE. Immunofluorescent staining of fixed paraffin-embedded tissue sections. Nuclei of all cells are stained yellow-brown by the oxidized *p*-phenylenediamine which was used as the mounting medium [22].

Figure 1. Parotid gland of an A1 Le(a-b+) donor stained with antibody TS-1. Supranuclear spots in cells of the large striated duct; some smaller ducts and some acinar cells are strongly positive (yellow-green). Mucous secretion of some acinar cells have only a faint fluorescence (green). (× 300).

Figure 2. Exocrine pancreas of an A1 Le(a-b+) donor stained with antibody TS-1. Acinar cells show positive supranuclear spots (green). (× 300).

Figure 3. Primary sensory neurone of a dorsal root ganglion from an A Le(a+b-) donor stained with TS-1 antibody. Note the intracytoplasmic vesicles (green) with the shape and distribution expected for the Golgi apparatus. (× 1200).

Figure 4. Deep area of the crypts of Lieberkühn of an A1 Le(a-b+) donor double stained with the KB-26.5 antibody (green) and the *Ulex europaeus* lectin 1 (red). The Paneth cells and supranuclear spots of columnar cells are positive with KB-26.5, whereas extra-cellular mucins and mucous-secreting cells are positive with *Ulex europaeus* lectin 1. (× 480).

Figure 5. Villi of the duodenal mucosa of an A1 Le(a-b-) secretor donor, double-stained, as in Fig. 4. Positive supranuclear spots (green) are visible with KB-26.5 in all columnar cells, whereas goblet cells and extra-cellular mucus are stained (red) with the *Ulex europaeus* lectin 1. (× 300).

Figure 6. Pyloric mucosa of the stomach of an A1 Le(a-b-) secretor donor, double-stained, as in Fig. 4. Some mucous cells are positive with KB-26.5 (green). Other mucous cells, as well as extracellular mucous, are positive with the *Ulex europaeus* lectin 1 (red). Cells stained with both reagents show a clear dissociation of the two dyes. KB-26.5 predominates in the paranuclear area and *Ulex europaeus* predominates in the mucous secretion. (× 120).

Figure 7. Pancreas of an O Le(a-b+) donor stained with the H1R8 antibody (green). (× 300).

Table 4. Binding of antibodies TS-1, KB-26.5 and H1R8 to immobilized synthetic oligosaccharides[a].

Trivial name	Structure[b]	Adsorption[c]. KB-26.5	TS-1	H1R8
Le^c or type 1 precursor	Galβ1-3GlcNAcβ-*O*-$R_{1,2}$	—	—	—
Type 2 precursor	Galβ1-4GlcNAcβ-*O*-$R_{1,2}$	—	—	—
T or type 3 precursor	Galβ1-3GalNAcα-*O*-R_1	—	—	—
Lewis disaccharide	Fucα1-4GlcNAcβ-*O*-$R_{1,2}$	—	—	—
Le^a	Galβ1-3GlcNAcβ-*O*-$R_{1,2}$ 4 Fucα1	—	—	—
X or Le^x	Galβ1-4GlcNAcβ-*O*-$R_{1,2}$ 3 Fucα1	—	—	—
Le^b	Galβ1-3GlcNAcβ-*O*-$R_{1,2}$ 2 4 Fucα1 Fucα1	—	—	—
Y or Le^y	Galβ1-4GlcNAcβ-*O*-$R_{1,2}$ 2 3 Fucα1 Fucα1	—	—	—
H type 1	Galβ1-3GlcNAcβ-*O*-$R_{1,2}$ 2 Fucα1	—	—	—
H type 2	Galβ1-4GlcNAcβ-*O*-$R_{1,2}$ 2 Fucα1	—	—	—
H type 3	Galβ1-3GalNAcα-*O*-$R_{1,2}$ 2 Fucα1	±	±	+
H type 4	Galβ1-3GalNAcβ-*O*-$R_{1,2}$ 2 Fucα1	±	±	+
A monosaccharide	GalNAcα-*O*-R_1	—	—	—
A disaccharide	GalNAcα1-3Galβ-*O*-$R_{1,2}$	—	—	—
A trisaccharide	GalNAcα1-3Galβ-*O*-$R_{1,2}$ 2 Fucα1	—	—	—
A type 1	GalNAcα1-3Galβ1-3GlcNAcβ-*O*-R_2 2 Fucα1	0	0	0
A type 2	GalNAcα1-3Galβ1-4GlcNAcβ-*O*-$R_{1,2}$ 2 Fucα1	—	—	—
A type 3	GalNAcα1-3Galβ1-3GalNAcα-*O*-$R_{1,2}$ 2 Fucα1	+	+	+
A type 4	GalNAcα1-3Galβ1-3GalNAcβ-*O*-$R_{1,2}$ 2 Fucα1	+	+	+
A Le^b	GalNAcα1-3Galβ1-3GlcNAcβ-*O*-R_2 2 4 Fucα1 Fucα1	0	0	0
A Y	GalNAcα1-3Galβ1-4GlcNAcβ-*O*-R_1 2 3 Fucα1 Fucα1	—	—	—

B trisaccharide	Galα1-3Galβ-*O*-$R_{1,2}$ 2 Fucα1	—	—	—
B type 3	Galα1-3Galβ1-3GalNAcα-*O*-R_1 2 Fucα1	±	±	+
I pentasaccharide	Galβ1-4GlcNAcβ1 6 Galβ-*O*-R_1 3 Galβ1-4GlcNAcβ1	—	—	—
Forssman disaccharide	GalNAcα1-3GalNAcβ-*O*-R_1	—	—	—

[a] Antibodies were incubated with the immobilized oligosaccharides for 30 min at room temperature before being assayed by immunofluorescence on sections of parotid gland from an A1 Le(a-b+) individual.

[b] R_1 = $(CH_2)_8$-CO-NH-Chromosorb (crystalline silica). R_2 = $(CH_2)_8$-CO-NH-Bovine serum albumin. Gal = galactose, GlcNAc = *N*-acetylglucosamine, Fuc = fucose, GalNAc = *N*-acetylgalactosamine.

[c] + = Complete disappearance of fluorescent staining after adsorption; ± = Partial disappearance of staining; — = not done.

given by antibody KB-26.5 and *Ulex europaeus* on the deep area of the crypts of Lieberkühn of an A Le(a-b+) donor. A supranuclear area of the columnar cells is labelled by KB-26.5, while *Ulex europaeus* labels the mucous. KB-26.5 also labels the cytoplasm of the Paneth cells. These are the only cells displaying this labelling pattern with the three anti type 3/4 antibodies. Fig. 5 shows the villi of duodenal mucosa from an A Le(a-b-) secretor individual stained by KB-26.5 and the lectin 1 from *Ulex europaeus*. All columnar cells show a typical supranuclear labelling with KB-26.5, while the goblet cells and the mucous itself are strongly labelled by the lectin. Fig. 6 illustrates the double labelling obtained with KB-26.5 and the lectin 1 from *Ulex europaeus* in the pyloric mucosa from an A Le(a-b-) person. The punctiform green staining given by KB-26.5 is mostly restricted to a supranuclear area whereas the red staining given by the lectin appears also on the extracellular mucous. Finally, the labelling given by antibody H1R8 in the exocrine pancreas of an O Le(a-b+) donor is shown in Fig. 7. Here also the labelling is restricted to a supranuclear area although it might be different from that shown in Fig. 2.

Binding to Synthetic Oligosaccharides

In order to define the sugar specificity of antibodies KB-26.5, TS-1 and H1R8, the antibodies were adsorbed on synthetic oligosaccharides linked to silica *via* an aliphatic spacer. After adsorption, the supernatants were tested for their capacity to stain the parotid gland from an A1 secretor individual. The results are shown in Table 4. Out of 23 oligosaccharides tested, only five were able to remove some antibody activity. The most effective, which completely absorbed all three antibodies, were the tetrasaccharides termed A type 3 and A type 4. The closely related structures B type 3, H type 3 and H type 4 greatly decreased the immunofluorescence staining of KB-26.5 and TS-1 and completely removed that of H1R8.

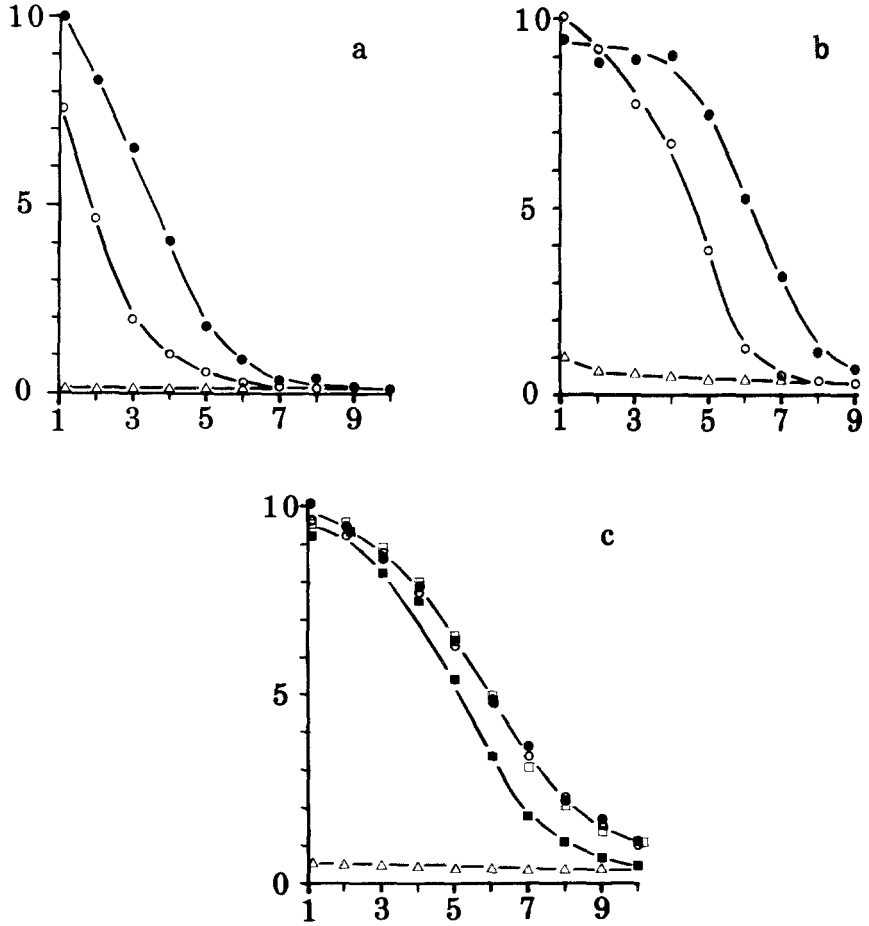

Figure 8. Binding of the three monoclonal antibodies to the artificial hapten-BSA antigens (R_2) listed in Table 4. The amount of antibody bound was detected with ^{125}I-labelled protein A. The results are plotted as cpm × 10^{-3} (ordinates) against the concentration of the antibodies expressed as the $\log_2$ of the antibody dilution. a. TS-1 antibody, the first dilution tested was 1/10; b. KB-26.5 antibody, the first dilution was 1/3200; c. H1R8 antibody, the first dilutin was 1/20.
With TS-1 and KB-26.5, only A type 3 (●) and A type 4 (○) antigens gave significant binding, all the other antigens tested were at background level (△). H1R8 had significant binding to A type 3 (●), A type 4 (○), H type 3 (□), H type 4 (■). All the remaining antigens tested were at background level in the system (△).

A more quantitative estimate of the binding to these oligosaccharides was obtained by a radioimmunoassay. Antibodies KB-26.5 and TS-1 bound specifically to the A type 3 and A type 4 tetrasaccharides. No significant binding could be observed to any of the other oligosaccharides tested. Antibody H1R8 behave differently. It was strongly bound to A type 3, A type 4, H type 3 and H type 4. None of the other oligosaccharides tested gave a significant binding. Therefore, out of 25 related oligosaccharides tested, only the type 3 and type 4 ABH-based compounds reacted with these antibodies (Fig. 8). Antibody H1R8 showed the broadest spectrum as it reacted equally well with the A, H and B compounds. Antibodies KB-26.5 and TS-1 were specific for the A compounds.

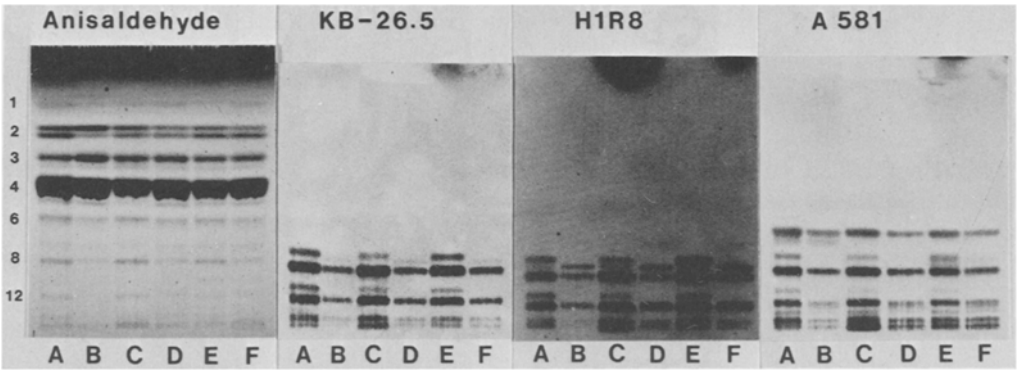

Figure 9. Thin-layer chromatograms of total non-acid glycosphingolipids from erythrocyte membranes of individual human donors. The blood group phenotype of the different donors are: lane A, A1 Le(a-b-) secretor; lane B, A2 Le(a-b-) secretor; lane C; A1 Le(a+b-) nonsecretor; lane D, A2 Le(a+b-) nonsecretor; lane E, A1 Le(a-b+) secretor; and lane F, A2 Le(a-b+) secretor. The thin-layer plates were developed in chloroform/methanol/water, 60/35/8 by vol. Detection was achieved with a chemical reagent, anisaldehyde (left), and by autoradiography. Autoradiography involved binding different monoclonal antibodies (KB-26.5, H1R8 and A581) to the plate followed by treatment with ^{125}I-labelled anti-mouse immunoglobulins (three chromatograms on the right). The amount of substance applied for each lane was 20 μg. The numbers to the far left indicate the approximate number of sugar residues in each glycolipid band.

Binding to Glycolipids

Total non-acid glycosphingolipid fractions from A1 and A2 erythrocyte membranes of different Lewis and secretor phenotypes were prepared. The binding of the monoclonal antibodies KB-26.5, TS-1, H1R8 and A581 to specific glycolipids in these fractions was revealed by the antibody overlay technique on thin-layer chromatograms [24, 25]. Fig. 9 shows that KB-26.5 binds to slow-moving glycolipids from both A1 and A2 individuals. Nevertheless, there appears to be some qualitative differences between the two A subgroups. Some glycolipids from A1 individuals are revealed by KB-26.5 (Fig. 9) and TS-1 (not shown) and do not appear in the fractions from A2 individuals. Antibody H1R8 gives the same binding pattern with glycolipids from A1 red cells, but with glycolipids from A2 red cells it strongly stains a band which seems to be absent in A1 individuals. This band is only faintly revealed by KB-26.5 in glycolipids from A2 individuals (Fig. 9).

By comparison, the glycolipid pattern revealed by a standard monoclonal anti-A (A581) is quite different (Fig. 9). This mouse monoclonal anti-A antibody has been shown to react with all blood group A glycolipids tested (4-12 sugar components, type 1, 2 and 4 or globo-series carbohydrate chains, mono- and di-fucosyl structures) and does not cross-react with the Forssman pentaglycosylceramide nor a number of other glycolipids tested (G. Hansson, N. Strömberg, unpublished results). Both quantitative and qualitative differences between A1 and A2 individuals are revealed as was the case for KB-26.5. However, in addition, this standard anti-A also recognizes a faster moving glycosphingolipid which migrates as a blood group A hexaglycosylceramide with a type 2 chain structure. This structure is recognized in both A1 and A2 individuals, with some

quantitative differences, but is not recognized by KB-26.5, TS-1 or H1R8. None of the antibodies differentiates between the different Lewis and secretor phenotypes in their binding patterns.

Using purified glycosphingolipids [7] and the antibody overlay technique, KB-26.5, TS-1 and H1R8 were found to react with globo-A (A type 4), but not with type 1 or 2 chain blood group A hexaglycosylceramides. Furthermore, H1R8 reacted weakly with globo-H (H type 4) and a blood group A difucosyl heptaglycosylceramide with a type 2 carbohydrate chain. This last reactivity is surprising since the antibody did not react with a synthetic AY pentasaccharide (with a similar terminal epitope) linked to a solid support (Table 4).

Discussion

The three antibodies KB-26.5, TS-1 and H1R8 showed blood-group A specificity, as defined by agglutination, but KB-26.5 and TS-1 recognized only A1 red blood cells whereas H1R8 recognized red cells from both A1 and A2 subgroups. Based on experiments with synthetic oligosaccharides, it appears that these three antibodies were specific for type 3 and 4 based blood group antigens. However, a major difference was revealed between KB-26.5 and TS-1 on the one hand and H1R8 on the other hand. Indeed, both KB-26.5 and TS-1 were specific for A type 3 and A type 4 structures whereas H1R8 recognized equally well A type 3, A type 4, H type 3 and H type 4. The fact that the blood group A1 specificity of KB-26.5 and TS-1 is not simply due to a difference in amount or distribution on the cell surface of similar A determinants is shown by the binding of both antibodies to slow moving glycolipids specific for A1 erythrocytes. Nevertheless, glycolipids common to both A1 and A2 erythrocytes were recognized by these two antibodies. Antibody H1R8 recognizes the same glycosphingolipids from A1 red cells, but it also strongly recognizes a glycosphingolipid from A2 red cells. In view of the carbohydrate specificity of this antibody, one can predict that this glycolipid bears a terminal H type 3 structure. Antibodies with similar specificities were recently described [26, 27].

Attempts to reveal binding of antibodies KB-26.5 and TS-1 to erythrocyte glycoproteins, which had been electrophoretically separated and transferred to nitrocellulose, failed. This was in sharp contrast to the binding of unrestricted polyclonal and monoclonal anti-A antibodies. Those stained most erythrocyte glycoproteins including band 3 and bands 4-5 (data not shown). Furthermore, in paraffin-embedded sections, erythrocytes were not labelled by KB-26.5, TS-1 and H1R8. It is very likely that the processing of the tissues through organic solvents necessary for paraffin embedding removes most of the membrane glycolipids. Very recently, Clausen *et al.* [6] characterized a repetitive A structure terminated by an A type 3 determinant which is carried by glycolipids of A1 red cells and not of A2 red cells. These authors failed to demonstrate the presence of this determinant on glycoproteins, in perfect agreement with our own results. Nevertheless, Takasaki *et al.* [4] reported the existence of A type 3/4 oligosaccharides on glycopeptides isolated from human erythrocytes. Some of these oligosaccharides were sialylated. The presence of a sialic acid residue could have explained the lack of reactivity of our antibodies with erythrocyte glycoproteins. However, treatment of tissue sections with various neuraminidases did not reveal staining of erythrocytes (data not shown). Thus, it is possible that the determinants which KB-26.5, TS-1 and H1R8 recognize on erythrocytes are carried only by glycolipids.

In nucleated cells, the restricted A determinants, recognized by these three antibodies, were mainly localized on a supranuclear structure of all cells which were able to synthesize the A blood group substance. The localization of these strongly positive structures always correlated with the expected position of the Golgi apparatus. This leads us to believe that it is the Golgi apparatus itself that is recognized, although electron microscopy studies should be performed to confirm this proposition. For these nucleated cells, as for erythrocytes, the possibility exists that the lack of staining of the cell membrane is due to a restriction of the expression of A type 3/4 determinants to glycolipids, which would have been extracted during the fixation and embedding of the tissue. Nevertheless, it is noteworthy that the staining of these supranuclear structures is not notably influenced by the A1 or A2 phenotype.

In salivary glands, stomach and duodenum, the mucins were only weakly stained by antibodies KB-26.5 and TS-1. This is consistent with the very weak inhibition given by salivas in radioimmunoassays (data not shown). A1 salivas were a little more inhibitory than A2 salivas. Similarly, in parotid glands from A1 individuals, the mucous was stained more intensively than in parotid glands from A2 individuals, this latter being extremely faint. Antibody H1R8 stained the mucous from blood group A individuals more strongly, and was inhibited by salivas, although both staining and inhibition were much weaker than those obtained with standard anti-A [28, 29].

A or H antigens based on type 3 or 4 chains have been described in salivary mucins [3] as well as in stomach mucins [30]. Nevertheless, in both cases the type 3/4 precursor was directly linked to the peptide moiety. Type 1 or type 2 precursors were found on longer oligosaccharide chains carried by the same mucins. It is thus possible that the weak reactivity of antibodies KB-26.5, TS-1 and H1R8 with glycoproteins is due to a restricted accessibility of sterically-hindered type 3/4 based determinants. One can further speculate that the strong supranuclear reactivity of our three antibodies might be possible because, at the level of the Golgi apparatus, the long oligosaccharide chains of the glycoproteins would not yet have been synthesized; only the short 3/4 determinants present would thus be accessible to the antibodies. This fits well with the observation that antibody H1R8 was not A-specific on tissues although it was A-specific on red cells. A supranuclear staining could be observed on tissues from O and B individuals. This would be due to the reactivity of this antibody with the short H type 3/4 chains at the level of the Golgi apparatus.

Clausen *et al.* [31] have shown that the A1 *N*-acetylgalactosaminyltransferase is much more effective than the A2 enzyme in terminating the repetitive A type 3 structure, which they characterized on A1 erythrocyte glycolipids [6]. On these glycolipids, possibly due to steric hindrance by the internal fucose, the A2 enzyme is expected to leave unchanged a terminal H type 3 determinant that is recognized by antibodies such as H1R8. On the other hand, the A1 and A2 glycosyltransferases should be able to use, with approximately equal efficiency, H type 3 as an acceptor, if this trisaccharide is directly linked to a protein moiety. This stems from the fact that anti-A type 3/4 specific antibodies are inhibited almost equally well by A1 and A2 salivas and also bind equally well to the Golgi apparatus of cells from A1 and A2 individuals. Therefore, at the level of the secretory glycoproteins, the only major difference between the A1 and A2 subgroups would be due, as previously described [28, 29], to the competition of the A enzymes with the Lewis and possibly the X fucosyltransferases for their common H acceptor substrates.

In conclusion, we wish to remark that, while the tissue distribution and genetic control of type 1 and type 2 based "blood-group" related antigens is quite well understood [21, 32-34], very little is known about type 3/4 based antigens. The results described above provide evidence that these structures can be expressed in both the compartment under the control of the secretor gene and the compartment independent of the secretor gene, which is known to be under control of the H gene in red cells. To our knowledge, "blood-group" related determinants have not been previously described with a reactivity restricted to the Golgi apparatus. Hopefully, these antibodies will be useful to study the subcellular biosynthesis of these carbohydrate structures. It will also be important to see whether their reactivity might change during ontogenesis or in pathological situations such as cancers.

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