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Heterogeneity of the ABH Antigenic Determinants Expressed in Human Pyloric and Duodenal Mucosae

ROSELLA MOLLICONE[1], JACQUES LE PENDU[1], JACQUES BARA[2] and RAFAEL ORIOL[1]*

[1]*Institute of Immunobiology, CNRS ER 281, Broussais Hospital, Paris 75674 Cedex 14, France*
[2]*Institute of Scientific Research of Cancer, Villejuif 94802 Cedex, France*

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We defined the chemical structure and the genetic control of the various A or B determinants expressed by pyloric and duodenal epithelial cells by indirect immunofluorescent staining using monoclonal anti-A or anti-B reagents that recognize only certain variants of A or B antigenic determinants.

Some mucous cells in pyloric and Brünner's glands express AY or BY antigens whereas other mucous cells in the same glands express only the Y antigen. Absorptive and goblet cells of the duodenal villi and Lieberkühn glands express mono- and difucosylated A or B structures, mainly of type 1. The pyloric surface epithelium expresses mono- and difucosylated, type 1 and type 2, A or B structures. In addition, A or B antigens, with a so far undefined structure are found in the pyloric surface mucosae of non-secretor individuals.

The presence of the H antigen is an absolute requirement for the synthesis of A or B antigenic determinants since the A and B glycosyltransferases use only H structures as acceptors [1]. The synthesis of these H antigens is most probably under the control of at least two structural genes, *H* and *Se* [2]. The products of these two genes are two fucosyltransferases with different kinetic properties [3-5], but each capable of *in vitro* synthesis of both type 1 and type 2 H antigens. These two enzymes are expressed in different relative amounts in different body compartments, and under certain circumstances the *H* gene product preferentially uses type 2 substrates [3-5].

Taking into account the type 1 and type 2 structures, there are four possible oligosaccharide chains with the H antigenic determinant: H type 1, H type 2, Le^b and Y (Table 1) [6], all of which may also bear the A or B antigens corresponding to the ABO phenotype of the donor. Consequently, at least four different A and four different B antigens can be expected (Table 2). In addition to the type 1 and type 2 structures mentioned above, A antigens have also been found on type 3 [7, 8], type 4 [9, 10] and on branched or unbranched oligosaccharide structures [11].

*Author for correspondence

Table 1. Structures of the non-A non-B synthetic oligosaccharides used to determine the carbohydrate specificity of the reagents.

Trivial name	Structure
Nonfucosylated	
Type 1 precursor or Le^c	Galβ1-3GlcNAcβ-O-$R_{1,2}$[a]
Type 2 precursor or I	Galβ1-4GlcNAcβ-O-$R_{1,2}$
Monofucosylated	
H type 1 or Le^d	Galβ1-3GlcNAcβ-O-$R_{1,2}$ 2 Fucα1
H type 2	Galβ1-4GlcNAcβ-O-$R_{1,2}$ 2 Fucα1
Le^a (type 1)	Galβ1-3GlcNAcβ-O-$R_{1,2}$ 4 Fucα1
X or Le^x (type 2)	Galβ1-4GlcNAcβ-O-$R_{1,2}$ 3 Fucα1
Difucosylated	
Le^b (type 1)	Galβ1-3GlcNAcβ-O-$R_{1,2}$ 2 4 Fucα1 Fucα1
Y or Le^y (type 2)	Galβ1-4GlcNAcβ-O-$R_{1,2}$ 2 3 Fucα1 Fucα1

[a] R_1 = $(CH_2)_8$-CO-NH-BSA. R_2 = $(CH_2)_8$-CO-NH-Chromosorb, Gal = galactose, GlcNAc = *N*-acetylglucosamine, GalNAc = *N*-acetylgalactosamine, Fuc = fucose.

In a previous study, two differentiation patterns were described for epithelial cells of the digestive mucosae according to the expression of type 1 (Le^a or Le^b) and type 2 (X or Y) blood group-related antigens [6]. The epithelial cells migrating from the proliferative compartment to the surface of the mucosae expressed both type 1 and type 2 antigens, whereas the cells migrating in the opposite direction, i.e. towards the deep gland area, expressed only type 2 antigens. This dissociation of the oligosaccharide structures expressed by the surface epithelium and the deep glands was present on both gastric and duodenal mucosae, in spite of the obvious structural and functional differences which exist between the two sides of the gastro-duodenal junction (Fig. 1).

Materials and Methods

Immunoadsorbents

The following synthetic, A or B blood group-related oligosaccharides, covalently bound to an insoluble matrix of Chromosorb (trade name Synsorbs®), were obtained from

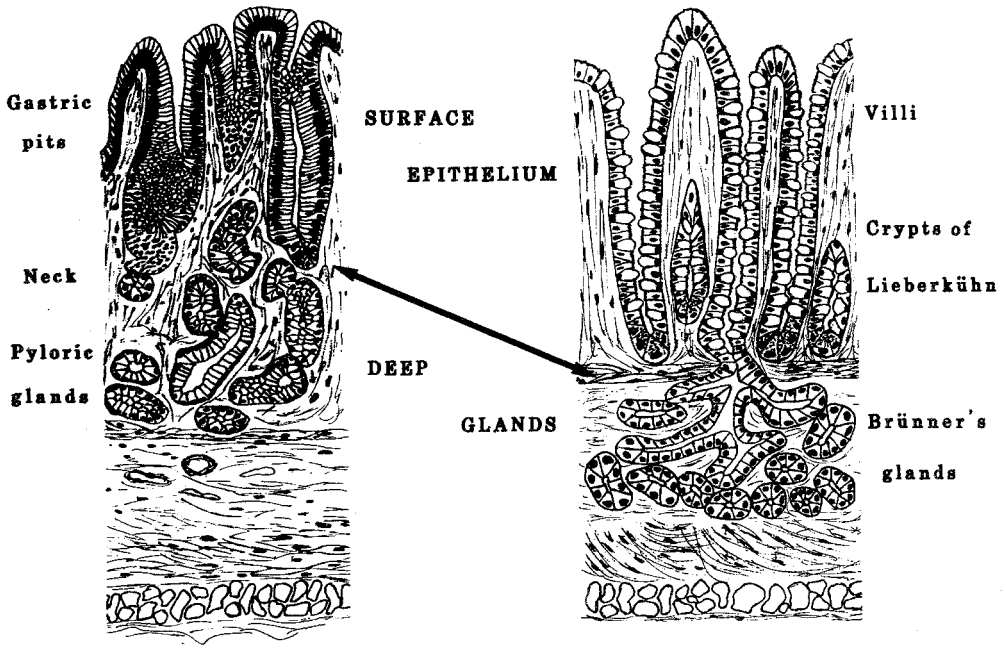

Figure 1. Diagram of the surface epithelium and deep gland areas of the pyloric and duodenal mucosae. Multiplication of epithelial cells occurs in the intermediate or proliferative compartments (arrows). From there, cells migrate upwards or downwards, differentiate and express blood group-related antigens with different structures and genetic controls. Epithelial cells migrating towards the surface epithelium express type 1 and type 2 ABH antigens under the control of the *Se* [6] and *Le* genes. Epithelial cells migrating towards the deep gland areas express type 2 ABH antigens, mainly independent of the control of the *Se* [6] and *Le* genes, and probably some type 2 antigens under the control of the *Se* and *Le* genes.

Chembiomed (University of Alberta, Edmonton, Canada): A disaccharide [D-GalNAcα1-3-D-Gal], monofucosylated A trisaccharide, monofucosylated A type 2 tetrasaccharide, difucosylated AY pentasaccharide (Table 1) and monofucosylated B trisaccharide. Polyclonal and some monoclonal antibodies were affinity purified by adsorption elution on A or B trisaccharide Synsorbs.

Radioactive Binding Tests

Direct binding of the different antibodies to synthetic oligosaccharides coupled to bovine serum albumin (BSA) was measured in a solid phase binding test using ^{125}I-labelled Protein-A [12].

The following hapten-BSA compounds were obtained from Chembiomed: A disaccharide; monofucosylated A trisaccharide; monofucosylated A type 1 tetrasaccharide; monofucosylated A type 2 tetrasaccharide; difucosylated ALe^b pentasaccharide (Table 2); monofucosylated B trisaccharide; H type 1, H type 2 and X trisaccharides; and difucosylated Y tetrasaccharide (Table 1) [13, 14].

Table 2. Structures of the A synthetic oligosaccharides used to characterize the anti-A reagents. Replacement of the terminal αGalNAc by αGal gives the B specificity to the same oligosaccharide chains, although the B tetra- and pentasaccharides have not been synthesized as yet.

Trivial name	Structure
Nonfucosylated	
A disaccharide	GalNAcα1-3Galβ-O-$R_{1,2}$[a]
Monofucosylated	
A trisaccharide	GalNAcα1-3Galβ-O-$R_{1,2}$ 2 Fucα1
A type 1	GalNAcα1-3Galβ1-3GlcNAcβ-O-$R_{1,2}$ 2 Fucα1
A type 2	GalNAcα1-3Galβ1-4GlcNAcβ-O-$R_{1,2}$ 2 Fucα1
Difucosylated	
A Le^b (type 1)	GalNAcα1-3Galβ1-3GlcNAcβ-O-R_1 2 4 Fucα1 Fucα1
A Y (type 2)	GalNAcα1-3Galβ1-4GlcNAcβ-O-R_2 2 3 Fucα1 Fucα1

[a] R_1 = $(CH_2)_8$-CO-NH-BSA, R_2 = $(CH_2)_8$-CO-NH-Chromosorb, Gal = galactose, GlcNAc = *N*-acetylglucosamine, GalNAc = *N*-acetylgalactosamine, Fuc = fucose.

Unrestricted Anti-A

An affinity purified polyclonal rabbit anti-A (124-76) was raised in A^- rabbits [15], hyperimmunized with salivary A glycoproteins emulsified in complete Freund's adjuvant; 33C13, a monoclonal antibody from mice ascites fluid was made by J. Bara; AVI-105, affinity purified monoclonal mouse anti-A antibody was obtained from Chembiomed; 6D4, a hybridoma anti-A supernatant was obtained from Celltech (Slough, UK).

Anti-A Specific for Difucosylated A Structures

A monoclonal antibody (AT1) made by Dr. Hirota at the UCLA Tissue Typing Laboratory (Los Angeles, CA, USA) was previously assumed to react with A type 1 because it reacted with A secretions but did not agglutinate red cells, which are known to have mainly type 2 epitopes [16]. However, the binding tests with synthetic antigens and immunoadsorbents were only positive with the two A difucosylated structures tested, i.e. ALe^b and AY. Consistently negative results were obtained with the non-fucosylated A disaccharide and the monofucosylated A trisaccharide and A type 1 or A type 2 tetrasaccharide structures (Table 2).

Anti-A Specific for Monofucosylated A Type 1 or A type 2 Structures

The 96-A2 monoclonal antibody, an anti-A that does not react with the AY-Synsorb or with the ALe^b-BSA antigen, was obtained from the CNTS (Paris, France). This antibody reacted strongly with the A trisaccharide and the A type 1 and A type 2 monofucosylated tetrasaccharides.

Anti-A+B Specific for Monofucosylated Type 2 Structures

A hybridoma supernatant which agglutinates A and B red cells (HI-A15) was prepared by Dr. Seitz (Kinderklinik, Hamburg University, W. Germany). This antibody reacted weakly with the A trisaccharide and strongly with the A type 2 tetrasaccharide. It did not react with the A type 1 tetrasaccharide or with the difucosylated ALe^b or AY pentasaccharides.

Anti-B Reagents

Five antibodies reacting with B-Synsorb were selected for the present study. Polyclonal goat anti-B affinity purified antibodies (AA2-137) were obtained from Chembiomed. Monoclonal mouse anti-B antibody (b-183) was obtained from Dr. Edelman (Blood Transfusion Center, St. Louis Hospital, Paris, France). Hybridoma anti-B supernatant (4B3) was obtained from Dr. Viñas (Knickerbocker, Barcelona, Spain). Affinity purified monoclonal mouse anti-B antibody (AK4-26) was obtained from Chembiomed. Monoclonal mouse anti-B (NB1) was obtained from Celltech as a hybridoma supernatant. These five anti-B reagents could not be further characterized with synthetic oligosaccharides because the mono- and difucosylated B tetra- and pentasaccharides are not yet available.

Anti-I Reagents

Two anti-I reagents were tested: human anti-I (Ma) [17], which reacts specifically with the terminal non-fucosylated trisaccharide of the type 2 precursor chains [18], and the monoclonal mouse anti-I, M18.3, with a similar anti-I (Ma) activity [19], which was produced by Dr. Edwards at the Ludwig Institute for Cancer Research (London, UK).

Lectin

Ulex europaeus lectin 1 and *Helix pomatia* lectin, both affinity purified and labelled with rhodamine isothiocyanate (TRITC), were obtained from Vector Laboratories Inc. (Burlingame, CA, USA). *Ulex europaeus* lectin 1 reacts with H type 2 and Y epitopes and does not react with H type 1, X or Le^b epitopes [13], whereas *Helix pomatia* lectin reacts with oligosaccharides containing *N*-acetylgalactosamine and *N*-acetylglucosamine units [20].

Tissue Samples

Normal digestive mucosa was taken from kidney donors [6]. Strips of 1 × 10 cm of the gastroduodenal mucosa, including the junction between both organs, were fixed in 95% ethanol, coiled into "Swiss rolls" and then embedded in paraffin as previously described [6].

Table 3. Expected ABO, Lewis and secretor genotypes of the 17 tissue donors selected for the present study as predicted from their combined ABO, Lewis and secretor phenotypes.

Combined phenotype	ALe^a	ALe^b	ALe^d	BLe^a	BLe^b	OLe^a	OLe^b
Genotype							
ABO	*A/-*	*A/-*	*A/-*	*B/-*	*B/-*	*O/O*	*O/O*
Lewis	*Le/-*	*Le/-*	*le/le*	*Le/-*	*Le/-*	*Le/-*	*Le/-*
Secretor	*se/se*	*Se/-*	*Se/-*	*se/se*	*Se/-*	*se/se*	*Se/-*
Number tested	1	6	2	1	3	1	3

ABO and Lewis blood groups of tissue donors were determined on red cells by conventional agglutination tests. Secretor status was determined by immunofluorescence with *Ulex europaeus* lectin 1 on the gastroduodenal surface epithelium [6]. Seventeen donors representing the different ABO, Lewis and secretor phenotypes shown in Table 3, were selected for the present study.

Neuraminidase Treatment

Vibrio cholera acylneuraminyl-hydrolase (EC 3.2.1.18) was obtained from Behringwerke (Marburg, W. Germany). Deparaffinated and rehydrated tissue sections were incubated in 0.1 M sodium acetate buffer pH 5.5 for 10 min. Digestion was performed with a 0.1 U/ml enzyme solution in the same buffer, for 1 h, at 37°C. After digestion, the tissue was washed with 10 mM sodium phosphate, 0.14 M sodium chloride, pH 7.2 (phosphate buffered saline, PBS) and stained for immunofluorescence.

Galactosidase Treatment

Coffee bean α-D-galactoside galacto-hydrolase (EC 3.2.1.22) was obtained from Boehringer (Mannheim, W. Germany). Twenty μl of the enzyme were diluted to 400 μl with 0.1 M sodium phosphate buffer, pH 6.1, and incubated overnight on the deparaffinated and rehydrated tissue sections at room temperature. The following day, the sections were washed with PBS and stained for immunofluorescence.

Immunofluorescence

Indirect double immunofluorescence staining was performed on deparaffinated tissue sections using the anti-A, -B, or -I reagents followed by the corresponding sheep anti-mouse, sheep anti-rabbit or rabbit anti-goat immunoglobulins which were labelled with fluorescein isothiocyanate (FITC) (Institut Pasteur, Paris, France) and the TRITC-labelled lectins as previously described [6, 21]. Stained sections were mounted with oxidized *p*-phenylenediamine in glycerol in order to diminish the spontaneous fading of the fluorescence and to visualize the nuclei [22].

Table 4. Immunofluorescent staining of pyloric surface epithelium.

Reagents		Combined ABO, Lewis and secretor phenotypes of tissue donors						
Identification	Blood group specificity	ALe^a	ALe^b	ALe^d	BLe^a	BLe^b	OLe^a	OLe^b
124-76	A type 1, A type 2, ALe^b, AY	+ +	+ +	+ + +	—	—	—	—
33C13	A type 1, A type 2, ALe^b, AY	+ + +	+ + +	+ + +	—	—	—	—
AVI-105	A type 1, A type 2, ALe^b, AY	+ + +	+ + +	+ + +	—	—	—	—
6D4	A type 1, A type 2, ALe^b, AY	+ + +	+ + +	+ + +	—	—	—	—
AT1	ALe^b, AY	—	+ + +	+/—	—	—	—	—
96-A2	A type 1, A type 2	—	+ + +	+ + +	—	—	—	—
H1-A15	A type 2, B	—	+ +/—	+ +/—	—	+ +/—	—	—
AA2-137	B	—	—	—	+ +	+ + +	—	—
b-183	B	—	—	—	+	+ + +	—	—
4B3	B	—	—	—	+ +	+ + +	—	—
AK4-26	B	—	—	—	—	+ + +	—	—
NB1	B	—	—	—	—	+ + +	—	—
Helix pomatia	?	+ + +	+ +	+ + +	+ + +	+ +	+ + +	+ + +
Ulex europaeus	H type 2, Y	—	+ + +/—	+ + +/+	—	+ + +/—	—	+ + +
M18.3	I	+ + +	+/—	+/—	+ + +	+/—	+ + +	+/—
I-Ma	I	+ +	+/—	—	+ +	+/—	+ +	+/—

Optimal dilutions of all reagents were determined by chessboard titrations. Affinity purified antibodies were used at concentrations ranging from 1 to 20 μg/ml, whole ascites fluids at dilutions from 1/50 to 1/1000 and hybridoma supernatants at 1 to 2 dilution. All dilutions were made in 1% BSA in PBS. Fluorescence was observed under a Leitz SM-LUX microscope euipped with a Ploemopak 2.3 and a lamp source of 200 Watts HBO. FITC-Labelled structures were observed with the I-2 filterblock and TRITC-labelled structures were observed with the M filterblock. Pictures were taken on Fujichrome 400 ASA film with a Leitz Photoautomat MPS 55. Double photographic exposure of the same field, with the two filterblocks, was performed to obtain pictures showing simultaneous red and green fluorescence [6, 21].

Results

Pyloric Surface Epithelium

The rabbit affinity purified anti-A (124-76) and the three monoclonal antibodies (33C13, AVI-105, 6D4), with unrestricted anti-A activity, stained membranes and cytoplasm of almost all surface epithelial cells of the pyloric mucosae from ALe^a, ALe^b and ALe^d donors (Fig. 2). The anti-A specificity of these reagents was confirmed by the negative results obtained with B and O donors (Table 4).

The three restricted anti-A (AT1, 96-A2 and H1-A15) stained the surface epithelium of ALe^b donors, but unlike the unrestricted anti-A, they did not stain the pyloric mucosae of the nonsecretor donor (ALe^a). The anti-A specific for A type 1 and A type 2 (96-A2), showed the same, almost uniform fluorescent staining of the two A secretor phenotypes, ALe^b and ALe^d (Fig. 3). The H1-A15 antibody specific for A type 2 and B had a discontinuous

fluorescent pattern with alternating positive and negative areas in ALe^b, ALe^d and BLe^b donors. The anti-A specific for the difucosylated ALe^b and AY structures (AT1) stained the tissue of the two secretor phenotypes ALe^b and ALe^d although there was a large difference between the Lewis positive (ALe^b) and the Lewis negative (ALe^d) tissues. The ALe^b donors were stained with a strong and uniform fluorescence whereas only weak and discontinuous fluroescence was obtained with the ALe^d donors.

The five anti-B reagents were positive on BLe^b and negative on A and O tissues. However, they could be separated into two groups according to their reactivity with the B non-secretor or BLe^a donors. The affinity purified polyclonal goat anti-B (AA2-137) and two monoclonal antibodies (b-183 and 4B3) gave a uniform staining of the epithelial tissue of the BLe^a donor whereas the two other monoclonal antibodies (AK4-26 and NB1) did not stain the surface epithelium of the BLe^a donor. These two fluorescent patterns of anti-B reagents corresponded with the two staining patterns of the anti-A reagents. The unrestricted anti-A reacted with ALe^a and ALe^b in a similar manner to the first three anti-B reagents whereas the last two anti-B reagents were positive on BLe^b and negative on BLe^a, as for the restricted anti-A reagents on A tissues (Table 4).

Helix pomatia lectin strongly stained (Fig. 4, red) all donors irrespective of their ABO, Lewis and secretor phenotypes. The pattern of the staining in each donor was similar to

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COLOUR PLATE. Immunofluorescence of A, B and I antigens stained with FITC labelled antibodies (green) and the lectin receptors stained with TRITC labelled *Ulex europaeus* lectin 1 and *Helix pomatia* lectin (red) on the pyloric and duodenal mucosae. Fig. 2-6, pyloric mucosae. Fig. 7-10, duodenal mucosae. Magnifications, × 100.

Figure 2. Pyloric mucosae of an ALe^d donor. Double stain with *Ulex europaeus* lectin 1 (red) and unrestricted rabbit anti-A (green) afinity purified antibodies (124-76). The pyloric glands show the "Harlequin" pattern with alternate red and green cells or glands.

Figure 3. Serial section of the pyloric mucosae of the same ALe^d donor as in Fig. 2. Double stain with *Ulex europaeus* lectin 1 (red) and anti-A (green) monoclonal reagent (96-A2) specific for monofucosylated A type 1 and A type 2 structures. The pyloric glands are only stained red, whereas the surface epithelium presents both red and green stains like in Fig. 2.

Figure 4. Pyloric mucosae of a BLe^a donor. Double stain with *Helix pomatia* lectin (red) and the anti-B (green) goat polyclonal reagent (AA2-137). Superimposition of FITC and TRITC fluorescence on the same cells gives a bright yellow-orange fluorescence.

Figure 5. Single stain with the anti-A (green) unrestricted monoclonal antibody (33C13) of both surface epithelium and pyloric glands of an ALe^a donor.

Figure 6. Single stain with the anti-B (green) monoclonal antibody (AK4-26) of the surface epithelium of a BLe^b donor. Pyloric glands are not stained with this anti-B, which reacts like the restricted anti-A on tissues from A donors (see Fig. 3).

Figure 7. Duodenal mucosae of an ALe^d donor. Double stain with *Helix pomatia* lectin (red) and the anti-A (green) rabbit polyclonal reagent (124-76). Superimposition of the green and red stains gives the bright yellow-orange fluorescence of some cells, whereas other cells are only red and green.

Figure 8. Duodenal mucosae of a BLe^b donor. Double stain with *Ulex europaeus* lectin 1 (red) and the anti-B (green) goat polyclonal reagent (AA2-137).

Figure 9. Brünner's glands of an ALe^b donor. Double stain with *Ulex europaeus* lectin 1 (red) and the anti-A (green) unrestricted monoclonal antibody (33C13). "Harlequin" pattern with alternate red and gren cells or glands.

Figure 10. Brünner's glands of a BLe^a donor. Double stain with *Ulex europaeus* lectin 1 (red) and the anti-I (green) monoclonal antibody (M18.3). The green stain illustrates the punctiform cytoplasmic fluorescence typical of the two anti-I (MA) reagents.

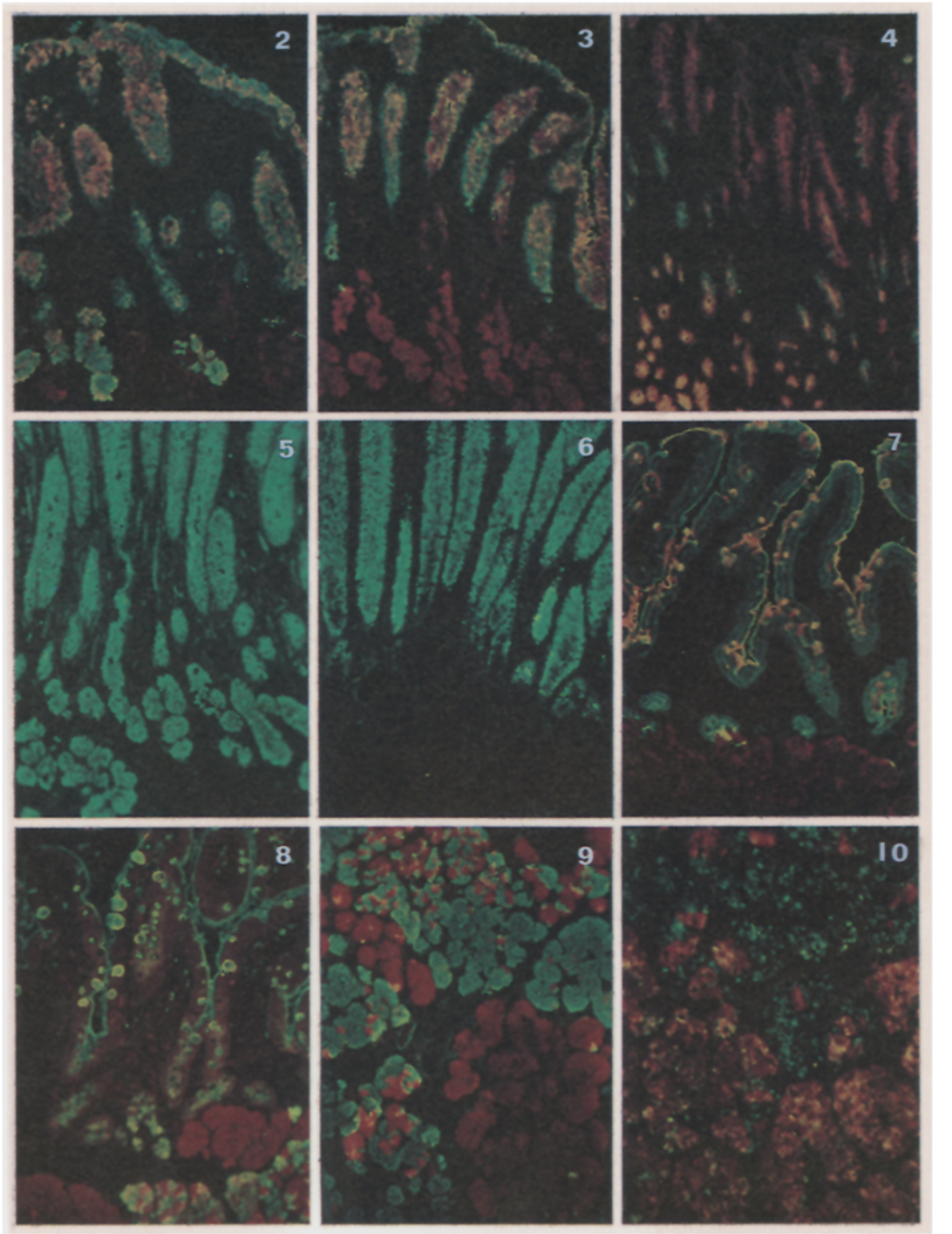
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Table 5. Immunofluorescent staining of pyloric glands.

Reagents		Combined ABO, Lewis and secretor phenotypes of tissue donors						
Identification	Blood group specificity	ALe^a	ALe^b	ALe^d	BLe^a	BLe^b	OLe^a	OLe^b
124-76	A type 1, A type 2, ALe^b, AY	++/—	++/—	+++/—	—	—	—	—
33C13	A type 1, A type 2, ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
AVI-105	A type 1, A type 2, ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
6D4	A type 1, A type 2, ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
AT1	ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
96-A2	A type 1, A type 2	—	—	—	—	—	—	—
H1-A15	A type 2, B	—	—	—	—	—	—	—
AA2-137	B	—	—	—	+++/—	+++/—	—	—
b-183	B	—	—	—	+++/—	+++/—	—	—
4B3	B	—	—	—	+++/—	+++/—	—	—
AK4-26	B	—	—	—	—	—	—	—
NB1	B	—	—	—	—	—	—	—
Helix pomatia	?	+++	+++	+++	+++	+++	+++	+++
Ulex europaeus	H type 2, Y	+++/+	+++/+	+++/++	+++/+	+++/+	+++	+++
M18.3	I	—	—	—	—	—	—	—
I-Ma	I	—	—	—	—	—	—	—

that given by the unrestricted anti-A on the A tissues. As previously reported [6], *Ulex europaeus* lectin 1 did not stain nonsecretor surface epithelium and gave a strong and uniform fluorescence on OLe^b (Table 4). The fluorescent pattern of the same lectin on tissues from ALe^b and BLe^b donors was irregular with positive and negative areas.

The two anti-I reagents gave strong but punctiform fluorescence in the cytoplasm of epithelial cells from non-secretor donors (ALe^a, BLe^a and OLe^a) and only weak, irregular, patchy fluorescence in secretor donors (ALe^b, ALe^d, BLe^b and OLe^b) (Table 4).

Pyloric Glands

Three distinct positive clusters are shown in Table 5. The four unrestricted anti-A reagents (Fig. 5), plus the anti-A (AT1) specific for the difucosylated ALe^b and AY structures, were positive on glands of A donors. The two anti-A antibodies specific for the monofucosylated A type 1 and/or A type 2 (96-A2 and H1-A15) did not stain the pyloric glands at all, irrespective of the ABO blood group of the tissue donor. The anti-B reagents behaved in a similar way. The first three anti-B (AA2-137, b-183 and 4B3) gave a positive cluster, even with BLe^a and BLe^b donors, in a similar manner to the unrestricted anti-A on A tissue, whereas the two other anti-B (AK4-26 and NB1) were negative (Fig. 6).

The fluorescent pattern of the positive reactions, in pyloric glands, was similar for the A and B antigens. With both antigens, the corresponding antibodies gave a strong but discontinuous fluorescence with alternate clusters of positive and negative glands and occasional isolated positive cells inside a negative gland or *vice versa* (Fig. 2).

The two lectins from *Helix pomatia* and *Ulex europaeus* were positive on glands of all ABO donors. However, *Helix pomatia* gave a strong and uniform fluorescence on all

Table 6. Immunofluorescent staining of duodenal villi and crypts of Lieberkühn.

Reagents		Combined ABO, Lewis and secretor phenotypes of tissue donors						
Identification	Blood group specificity	ALe^a	ALe^b	ALe^d	BLe^a	BLe^b	OLe^a	OLe^b
124-76	A type 1, A type 2, ALe^b, AY	—	+ +	+ + +	—	—	—	—
33C13	A type 1, A type 2, ALe^b, AY	—	+ + +	+ + +	—	—	—	—
AVI-105	A type 1, A type 2, ALe^b, AY	—	+ + +	+ + +	—	—	—	—
6D4	A type 1, A type 2, ALe^b, AY	—	+ + +	+ + +	—	—	—	—
AT1	ALe^b, AY	—	+ + +	—	—	—	—	—
96-A2	A type 1, A type 2	—	+ + +/—	+ + +	—	—	—	—
H1-A15	A type 2, B	—	—	—	—	—	—	—
AA2-137	B	—	—	—	—	+ + +	—	—
b-183	B	—	—	—	—	+ + +/—	—	—
4B3	B	—	—	—	—	+ + +	—	—
AK4-26	B	—	—	—	—	+ + +/—	—	—
NB1	B	—	—	—	—	+ + +/—	—	—
Helix pomatia	?	—	+ + +	+ + +	—	+ + +	—	+ + +
Ulex europaeus	H type 2, Y	—	+ +/—	+ + +/—	—	+ +/—	—	+ + +
M18.3	I	—	—	—	—	—	—	—
I-Ma	I	—	—	—	—	—	—	—

donors irrespective of their ABO phenotype. On the other hand, *Ulex europaeus* lectin 1 gave a strong and uniform fluorescence only in O donors, intermediate fluorescence on the ALe^d (Fig. 3, red), and irregular staining with bright and pale areas on the ALe^b and BLe^b donors.

Finally the two anti-I reagents were negative on the pyloric glands irrespective of the ABO phenotype of the tissue donor.

Duodenal Surface Epithelium

In contrast with the pylorus (Table 4) the surface epithelium of the duodenum of non-secretor donors (Table 6) was not stained by any of the reagents tested, including the unrestricted anti-A or anti-B, anti-I and *Helix pomatia* lectin, all of which were positive on the gastric surface epithelium of non-secretors. Among secretors, the unrestricted anti-A reagents gave the same strong and uniform fluorescence on ALe^b and on ALe^d (Fig. 7, green), whereas the three restricted anti-A reagents gave different fluorescent patterns on Lewis positive (ALe^b) and Lewis negative (ALe^d) donors. The anti-A (AT1), specific for the difucosylated structures ALe^b and AY (Table 2), gave a strong and uniform reaction on ALe^b donors and did not stain the ALe^d donors at all. The anti-A (96-A2), specific for the monofucosylated structures A type 1 and A type 2 (Table 2), reacted with both ALe^b and ALe^d donors, although the fluorescence pattern was discontinuous on ALe^b and uniform on ALe^d donors. Finally, the anti-A specific for A type 2 and B (H1-A15) did not stain any of the A or B donors, irrespective of their secretor or Lewis phenotypes.

The remaining anti-B reagents stained the tissue of secretor donors (BLe^b) with different patterns. The polyclonal goat anti-B (AAZ-137) (Fig. 8, green) and a monoclonal antibody (4B3) gave a uniform fluorescence pattern, whereas three monoclonal antibodies (b-183, AK4-26 and NB1) gave a discontinuous pattern with positive and negative areas.

Table 7. Immunofluorescent staining of Brünner's glands.

Reagents		Combined ABO, Lewis and secretor phenotypes of tissue donors						
Identification	Blood group specificity	ALe^a	ALe^b	ALe^d	BLe^a	BLe^b	OLe^a	OLe^b
124-76	A type 1, A type 2, ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
33C13	A type 1, A type 2, ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
AVI-105	A type 1, A type 2, ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
6D4	A type 1, A type 2, ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
AT1	ALe^b, AY	+++/—	+++/—	+++/—	—	—	—	—
96-A2	A type 1, A type 2	—	—	—	—	—	—	—
H1-A15	A type 2, B	—	—	—	—	—	—	—
AA2-137	B	—	—	—	+++/—	+++/—	—	—
b-183	B	—	—	—	+++/—	+++/—	—	—
4B3	B	—	—	—	+++/—	+++/—	—	—
AK4-26	B	—	—	—	—	—	—	—
NB1	B	—	—	—	—	—	—	—
Helix pomatia	?	+++	+++	+++	+++	+++	+++	+++
Ulex europaeus	H type 2, Y	+++/+	+++/+	+++/++	+++/+	+++/+	+++	+++
M18.3	I	+++	+/—	+/—	+++	+/—	+++	+/—
I-Ma	I	++/—	—	—	+/—	—	+++	+/—

The two lectins from *Helix pomatia* (Fig. 7, red) and *Ulex europaeus* (Figs. 8 and 9, red) were positive on secretor and negative on non-secretor donors, irrespective of their ABO phenotype. *Helix pomatia* lectin strongly and uniformly stained all epithelial cells of A, B or O secretor phenotypes, whereas *Ulex europaeus* lectin 1 stained the OLe^b donors with a uniform pattern and gave a positive, but discontinuous pattern on ALe^b and BLe^b donors.

Both anti-I reagents were negative on the surface epithelium of the duodenum.

Brünner's Glands

The fluorescent pattern displayed by Brünner's glands (Table 7), was similar to the fluorescent pattern given by pyloric glands (Table 5), except that the I antigen was present on Brünner's glands and absent from the pyloric glands. The reaction of anti-I reagents with Brünner's glands was strong but punctiform in ABH non-secretors (Fig. 10, green) and weak, irregular and sometimes undetectable in ABH secretor donors. This fluorescent pattern of anti-I on Brünner's glands was almost identical to the fluorescent pattern of anti-I on the surface epithelium of the pylorus (Table 4).

Neuraminidase Treatment

The only modification induced by the neuraminidase pretreatment concerned the *Helix pomatia* lectin. This lectin was positive on all untreated gastric and duodenal epithelial cells, except for the surface epithelium of the duodenum of ABH non-secretors (Table 6). After neuraminidase treatment, this area became uniformly positive in the three A, B or O non-secretor donors. The positive reaction of the lectin in other areas (Tables 4, 5, 7) was not modified by the enzymic treatment.

α-Galactosidase Treatment

Pretreatment of deparaffinated tissue sections from B donors with α-galactosidase strongly reduced the staining by anti-B reagents and increased the staining by *Ulex europaeus* lectin 1. The *Ulex europaeus* weak or negative cells in the pyloric or Brünner's glands became positive after α-galactosidase treatment and the overall discontinuous fluorescent pattern, observed with *Ulex europaeus* on B donors, was replaced by a uniform staining pattern after the enzyme treatment. Thus *Ulex europaeus* staining of α-galactosidase-treated B tissue was similar to the labelling observed with *Ulex europaeus* on O donors (Tables 4-7).

Double Staining

Double staining with FITC-labelled anti-A or anti-B and TRITC-labelled *Ulex europaeus* lectin 1 showed that the two stains are usually complementary. Cells positive with anti-A or anti-B (green) were pale or negative with *Ulex europaeus* (red) and *vice versa*. This complementarity was particulary evident in the pyloric (Fig. 2) and Brünner's glands (Figs. 8 and 9), where there was almost no overlap between the green and red stains. We call this fluorescent pattern "Harlequin", because it resembles the multicoloured patchy costume of this character from the Italian theatre. In the gastric (Fig. 2 and 3) or duodenal surface epithelia, double-stained cells (yellow-orange) coexist with single-stained cells (green or red).

Double staining with TRITC-labelled *Helix pomatia* lectin and FITC-labelled anti-A or B reagents, showed that the presence of A or B antigens does not interfere with the expression of the antigenic determinant which is recognized by *Helix pomatia*. Consequently, the double-stained cells of the stomach (Fig. 4) or the duodenum (Fig. 7) exhibit the typical yellow-orange colour which results from the superimposition of the green and red fluorescent stains.

The anti-I reagents gave a distinctive uneven punctiform fluorescence pattern, which was different to that of all the other fluorescent stains. Double fluorescence staining with *Ulex europaeus* lectin 1 (red) and anti-I (green) (Fig. 10) illustrates this punctiform staining.

Vascular Endothelium and Erythrocytes

In addition to the epithelial staining described above, A, B or H reagents also stained the erythrocyte membranes and the vascular endothelium of all the tissues tested, though the fluorescent staining of these structures was pale in comparison with the bright fluorescence given by absorptive or goblet cells. All the ABH reagents behaved in a similar way except for the AT1 antibody (anti-A specific for ALe^b and AY) that was consistently negative on erythrocytes [16] and on vascular endothelial cells.

Discussion

Most polyclonal and some monoclonal anti-ABH antibodies do not discriminate between the different variants of A, B and H determinants and we call these antibodies,

which show broad A, B or H specificity, unrestricted reagents. Conversely, many monoclonal anti-ABH antibodies, and lectins, react preferentially with certain variants of ABH antigens and we designate them as restricted ABH reagents. Other examples of such restricted anti-A [23, 24] and anti-B [23] monoclonal antibodies have been described. These antibodies also recognized preferentially certain types of the ABH epitopes or of their difucosylated derivatives. The precise definition of the specificity of these reagents can help to identify the oligosaccharides synthesized by individual cells and to understand their genetic control.

An epitope dependent on the presence of the *Le* gene should be missing in Le^d individuals because they are homozygous for the silent allele (*le/le*). Similarly an epitope dependent on the *Se* gene should not be expressed by Le^a donors since they are homozygous for the silent allele (*se/se*) (Table 3). Therefore, given that the three phenotypes (Le^a, Le^b and Le^d) had the same positive ABH fluorescent pattern in gastric glands, we conclude that there are ABH antigens in these deep glands, the expression of which is independent of both the *Se* and *Le* genes. Furthermore, the double staining with A or B in green and *Ulex europaeus* in red (Figs. 2 and 9) gave a "Harlequin" pattern. This indicates that the expression of A or B in these deep glands must be related to a cellular differentiation process which is independent of the *Se* gene. The "Harlequin" pattern, along with the presence of cells unstained by anti-A or anti-B in normal pyloric glands, leads us to suggest that the often observed lower levels of A or B antigens which are found in certain neoplastic tissues [25, 26] could be due to a higher susceptibility of the normal A or B negative cells to the induction of malignancy.

Given that only type 2 antigens are expressed in pyloric and Brünner's glands [6], the ABH antigens expressed in these deep glands should be difucosylated AY, BY and Y structures, since the specific anti-monofucosylated A or B reagents were negative and the specific anti-difucosylated A antibody (AT1) had the same positive pattern as the unrestricted anti-A. A clear difference appeared between pyloric and Brünner's glands for the type 2 precursor antigen, I(Ma). This antigen was not detectable in the pyloric glands, but was present in Brünner's glands. This shows that all the type 2 precursor had been transformed into X or Y in the pyloric glands, whereas some untransformed type 2 precursor chains remained accessible to the anti-I reagents in Brünner's glands. Furthermore, less I antigen was found in Brünner's glands of ABH secretors (Le^b and Le^d) compared with nonsecretors (Le^a) (Table 7). This suggests that some of the type 2 precursor molecules (I) could have been transformed into H by the fucosyltransferase product of the secretor gene, in Le^b and Le^d individuals. It has previously been demonstrated that in deep glands there is more X in Le^a than in Le^b donors, and more Y in Le^b than in Le^a donors [6]. Taking this observation together with the above results suggests that, in addition to the above-described ABH antigens independent of *Se* and *Le*, the products of these *Se* and *Le* genes can transform some type 2 precursor molecules into the corresponding fucosylated structures.

In contrast to the situation in the deep glands, the expression of A or B antigens in duodenal villi and crypts of Lieberkühn is under the strict control of the *Se* gene, since Le^a donors were negative and Le^b donors were positive with both unrestricted and restricted reagents. This area is also under the control of the *Le* gene since only the Le^b donors were positive with the reagent directed against the difucosylated A structures (AT1).

Like the duodenal surface epithelium, the pyloric surface epithelium (Table 4) seems to express either monofucosylated or difucosylated A and B type 1 and type 2 antigens, all under the control of the *Se* and *Le* genes. However, this tissue also expresses A or B antigenic determinants that are different from the four above-mentioned structures and independent of the *Se* and *Le* genes. These, as yet undefined determinants are only recognized by the corresponding unrestricted anti-A or anti-B in Le^a donors and do not seem to correspond to any of the ABH chemical structures that have so far been described. The antigen precursor of these "new" A or B determinants cannot be H type 2 since this latter antigen is recognized by *Ulex europaeus* which is negative on Le^a donors. Nor can it be the H type 1 or Le^d, since this antigen is not expressed in the surface epithelium of Le^a donors [27]. Furthermore, preliminary studies with anti-A monoclonals, reacting specifically with type 3 and type 4 chains, gave negative results in the gastric surface epithelium of non-secretors (unpublished results). This suggests that the A epitope expressed in this particular area is not built on either the type 3 or type 4 chains. *Helix pomatia* lectin is the only reagent, apart from unrestricted anti-A or anti-B, which binds to the surface epithelium of Le^a donors (Table 4). This supports the hypothesis that *Helix pomatia* lectin reacts with internal *N*-acetylgalactosamine or *N*-acetylglucosamine units [20].

In conclusion, the heterogeneity of combining sites of anti-ABH reagents permits the identification of different A, B or H epitopes at different levels of the digestive mucosa. These antigens could, then, be related to particular differentiation steps of individual cells as has been proposed for the oral epithelium [28]. This type of heterogeneity has important implications with regards to cell differentiation. It would have remained undetected using the classical red cell agglutination tests since most unrestricted and restricted reagents exhibit similar agglutination properties.

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