Species differences in the expression of carbohydrate
differentiation antigens on mammalian blood cells
revealed by immunofluorescence with monoclonal antibodies

Susan J. THORPE and Ten FEIZI
Applied Immunochemistry Research Group, Clinical Research
Centre, Watford Road, Harrow, Middlesex HA1 3UJ
(Received 19 July 1984)

Following recent observations using monoclonal antibodies that carbohydrate structures behave as
differentiation antigens of man and mouse, we have
made a preliminary survey of the expression of 8
monoclonal antibody-defined carbohydrate antigens on
blood cell smears of man, baboon, mouse, rat, rabbit,
pig, and dog. There are considerable species differ-
ences in the patterns of antigen expression. However,
certain generalizations can be made as follows: the i
and I antigens, associated with linear and branched
carbohydrate chains consisting of repeating N-acetyl-
lactosamine sequences (Galβ1-4GlcNAc, termed Type-2
backbone sequences) are widely distributed among
granulocytes and lymphocytes of all the species studied,
and on erythrocytes, monocytes, and platelets of some
of them. Substantial amounts of Type-1 backbone
sequences (Galβ1-3GlcNAc) may occur on rabbit
lymphocytes. The N-acetylneuraminic acid-containing
antigens, Pr₂ and Gd, are also expressed to varying
degrees on blood cells. On the other hand, antigens
based on mono- and difucosylated N-acetyllactosamine,
termed SSEA-1 (or X-hapten) and C14 (or Y-hapten)
are predominantly granulocyte/monocyte-associated
antigens. The former antigen is expressed in overt
form only on untreated human granulocytes but occurs
in cryptic state, masked by sialic acid, on human
monocytes, and on the granulocytes and monocytes of
baboon, rabbit, and dog but not on those of mouse, rat,
and pig. The latter antigen is expressed on human
granulocytes and on neuraminidase-treated monocytes
and granulocytes of dog. Lymphocytes of dog are
unusual in their expression of C14 antigen, in cryptic
state, masked by sialic acid residues. Although the
physiological roles of these various carbohydrate
structures, in vivo, are not yet known, they seem
excellent candidates as determinants of species and
cell-type differences in susceptibilities to infective
agents.
Studies with monoclonal antibodies have shown that a number of developmentally-regulated and differentiation antigens of man and mouse are carbohydrate structures related to the major blood group antigens (reviewed in refs. 1 & 2). The first to be characterized were the i and I antigens: they occur as distinctive markers of human foetal and adult erythrocytes, respectively, and have also been found to behave as developmentally regulated antigens during early mouse embryogenesis (3,4). These antigens are recognized by monoclonal antibodies, anti-i and anti-I, which occur in the serum of patients suffering from cold agglutinin disease and they are expressed on linear and branched carbohydrate chains, respectively, consisting of repeating N-acetyllactosamine (Galβ1-4GlcNAc) sequences (Fig. 1). These are also termed Type-2 backbone structures (5,6), for they occur commonly as precursor chains which may be further glycosylated with the monosaccharides that form the blood-group H, A, and B antigens and a number of other antigens shown in Fig. 1. An antigenically distinct backbone sequence based on the Type-1 (Galβ1-3GlcNAc) isomer is the determinant recognized by a human Waldenstrom macroglobulin 'IgMW00' (7) (Fig. 1). This structure has also been shown (8) to be recognized by the mouse hybridoma-derived antibody FC 10.2 raised against an undifferentiated human teratocarcinoma cell line (9). The hybridoma-defined stage-specific embryonic antigen of mouse, SSEA-1, which appears at the 8-cell stage of mouse embryogenesis (10), is also a carbohydrate structure and consists of 3-fucosyl-N-acetyllactosamine [α1→3 fucosylated Type-2 chains (refs. 11,12; Fig.1)]. This structure was previously termed X-hapten (13). There are species differences

![Chemical structures](image)

Fig. 1. Carbohydrate structures recognized by the monoclonal antibodies used in this study.
in the expression of this antigen: whereas in the adult mouse SSEA-1 is expressed only in restricted areas of the brain and genito-urinary tract (10,14), in man this antigen is more widely distributed (reviewed in ref. 15). Studies with the hybridoma-derived antibodies VEP8 and VEP9 (15), VIM-D5 (16,17), and a number of other antibodies (18) which also recognize 3-fucosyl-N-acetyllactosamine have shown that among cells of human peripheral blood, this structure ranks as a 'myeloid-specific' antigen. This antigen is not expressed on granulocytes of the mouse (14,19). The related difucosylated Type-2 structure (Fig. 1) recognized by the hybridoma-derived antibody C14 (20) occurs as an antigen of human granulocytes (20). This structure was previously termed Y-hapten (13). Two human monoclonal autoantibodies designated Pr2 and Gd which recognize N-acetylneuraminic acid α2\+3 or α2\+6 linked to galactose and α2\+3 linked to repeating Type-2 backbone structures respectively [(21); Fig. 1], also detect marked changes during cellular differentiation in the mouse (Pennington J, Uemura K, and Feizi T, unpublished observations). Thus there is a growing list of differentiation antigens that are saccharide structures which are known to occur on a variety of glycoproteins (2,15) and glycolipids (22,21,23).

In the present studies we have used 9 monoclonal anti-carbohydrate antibodies to examine differences in the expression of the antigens they recognize on blood cell smears of seven mammalian species, man, baboon, mouse, rat, rabbit, pig, and dog.

Materials and Methods

Monoclonal antibodies

Human naturally occurring monoclonal antibodies. Anti-I autoantibody Ma is directed at the Type-2 branch-point sequence (Fig. 1) which occurs along oligosaccharide backbones of glycoproteins and glycolipids (Structure 1) and at the core regions of O-glycosidically linked oligosaccharides (Structure 2, [24,6]). Anti-I autoantibody Step recognizes long-chain, branched Type-2 sequences (24,25) such as Structure 3. Anti-i Den recognizes the linear Type-2 sequence (26) and may require a sequence longer than the hexasaccharide (Fig. 1) for immunofluorescence, since it fails to bind to glycolipids with shorter structures on thin-layer chromatograms (25). IgM WOO is a Waldenstrom macroglobulin (7) which recognizes the same Type-1-based Structure 7 as the hybridoma-derived antibody FC 10.2 (8). Anti-Pr2 (LTH) reacts with N-acetyl neuraminic acid joined by α2\+3 or α2\+6 linkage to galactose residues, as in Structure 8, and anti-Gd (Kn) shows reactions with N-acetyl neuraminic acid α2\+3 linked preferably to long chain Type-2 sequences, as in Structure 9 (21).

Mouse hybridoma antibodies

Anti-SSEA-1 (10-12), C14 (20), and FC 10.2 (8) antibodies have been described previously and the determinants they recognize are shown in Fig. 1. All these anti-carbohydrate antibodies are of IgM class. The human antibodies were used as plasma and the hybridoma-derived monoclonal antibodies as culture supernatants, except anti-SSEA-1 which was ascites.
Preparation of leucocyte-enriched peripheral blood

The blood cells of seven species were examined: human, baboon, mouse (CBA), rat (Sprague-Dawley), rabbit (New Zealand White), pig, and dog (Beagle). Heparinized whole blood was mixed 1:1 with 0.01 M phosphate-buffered saline pH 7.3 (PBS) containing 3% (w/v) gelatin, and allowed to stand at 37°C for 30-60 min until most of the erythrocytes had sedimented. The leucocyte-enriched supernatant was removed and washed three times with PBS containing 1% (w/v) bovine serum albumin (BSA).

Immunofluorescence

Cytocentrifuge smears of leucocyte-enriched peripheral blood (prepared using approx. 25,000 cells/slide) were air-dried and fixed for 12 min in acetone at 4°C. The smears were incubated at 4°C with the monoclonal antibody-containing human sera followed by fluorescein-labelled rabbit anti-human IgM (Dako Immunoglobulins, Mercia Brocades Ltd., Weybridge, Surrey) or the mouse monoclonal antibodies followed by rabbit anti-mouse Ig (Nordic Immunological Laboratories, Maidenhead, Berks.). Each incubation was for 30 min, and unbound antibodies were removed after each incubation by washing the slides in two changes of PBS. Normal human serum or mouse serum or culture medium containing an irrelevant hybridoma supernatant were used as negative controls. The smears were fixed in PBS containing 4% (w/v) formaldehyde and mounted in buffered glycerol [PBS:glycerol, 1:9 (v/v)] containing 1,4-diazobicyclo(2,2,2)octane (Sigma Chemical Company, Poole, Dorset) at a concentration of 25 g/l, pH 8.6 (27). Some smears were counterstained with haematoxylin after immunofluorescence labelling to aid determination of cell type. Smears were viewed with a Zeiss u.v. epifluorescence microscope and the intensity of immunofluorescence of granulocytes, monocytes, lymphocytes, erythrocytes, and platelets clearly detectable above control staining was graded from weak to very strong. Immunofluorescence is described in the results section as being on cells. However, with the nucleated blood cells, positive immunofluorescence was noted both intracellularly and in association with surface membranes. At least 200 granulocytes, lymphocytes, erythrocytes, and platelets were scored per slide, and 10-20 cells showing the appearance of monocytes were scored similarly. Fluorescence and phase-contrast photomicrographs were taken using Ilford HP5 and Pan F films respectively.

Neuraminidase treatment of smears

After fixing in acetone, the smears were incubated with neuraminidase from Vibrio cholerae (200 mU/mL: Behringwerke, West Germany) for 1 h at 37°C and washed with PBS before immunofluorescence staining as described above.

Results

I antigen expressed on branched Type-2 oligosaccharide backbones

The I antigenic determinants recognized by anti-I(Ma) and anti-I(Step), associated with branched Type-2 backbone sequences (Fig. 1) and best known as human erythrocyte antigens (28), gave a weak
or moderate immunofluorescence of human erythrocytes and intense immunofluorescence of rabbit erythrocytes in accordance with earlier observations on the strong I antigen content of these latter cells (29, Figs. 2 and 4). On the baboon erythrocytes, only the I(Step)

---

**Fig. 2. Distribution and intensity of immunofluorescence of seven carbohydrate determinants on blood cell smears of man, baboon, mouse, rat, rabbit, pig, and dog. Symbols for immunofluorescence staining: black, very strong; dark speckled, moderately strong; lightly speckled, weak; +, appearance of staining after neuraminidase treatment; small arrow, increase in intensity of staining after neuraminidase treatment; large arrow, increase in intensity of staining and of the number of cells stained after neuraminidase treatment.**
determinant was detected and on pig erythrocytes only the I(Ma) determinant. However, neuraminidase treatment revealed I(Step) activity on pig erythrocytes and I(Ma) on those of the dog (Fig. 2).

Both I determinants were expressed to varying degrees on sub-populations of the lymphocytes of the seven species studied (additional reactivities were revealed in several instances after neuraminidase treatment) and on the majority of granulocytes of the five non-primate species studied. On human and baboon granulocytes, both determinants occur in the masked state and could be revealed by neuraminidase treatment (Figs. 2 and 4). Thus, there is evidence for the presence of branched Type-2 backbone structures on the granulocytes and lymphocytes of all seven species studied. These antigens were also detected amongst their monocytes [except I(Ma) in the mouse and I(Step) in baboon, mouse, and rat] as indicated in Fig. 3 (see also Fig. 4). Among platelets the I(Ma) determinant was detected in overt or cryptic state in all seven animal species studied: the platelets of baboon and mouse contained, in addition, cryptic I(Step) determinants.

\textit{i} antigen expressed on linear Type-II oligosaccharide backbones

The i antigen associated with linear Type-2 sequences which are hexasaccharides or longer oligosaccharides \cite{26,25}, and best known as an antigen of human foetal erythrocytes \cite{30}, was not detected by immunofluorescence on adult human erythrocytes but was expressed on a high proportion of baboon erythrocytes in accordance with a previous report \cite{31}. A sub-population of dog erythrocytes and, after neuraminidase treatment, rat erythrocytes expressed the i(Den) antigenic determinant.

The i(Den) determinant, which is known to be a prominent antigen of human lymphocytes \cite{32,33}, was moderately or strongly expressed on a considerable proportion of lymphocytes of all seven species studied and on the majority of their granulocytes except in those of

![Table of carbohydrate determinants among monocytes of the seven mammalian species studied. Symbols, as in Fig. 1.](image-url)
Fig. 4. I(Ma) immunofluorescence (A-D) and corresponding phase-contrast micrographs (A'-D') of human (A-C) and rabbit (D) blood cells. Of the human cells shown in A, only the monocyte (m), and erythrocytes (e) are stained. However, after neuraminidase treatment (B & C), the I(Ma) determinant is revealed on human granulocytes (g) and platelets (p), and the expression of this antigen on monocytes (m) and erythrocytes (e) is increased. Also shown in B is a lymphocyte (l) showing strong immunofluorescence with this antibody. In D, rabbit erythrocytes, granulocytes, and a monocyte (m) are strongly stained and a lymphocyte (l) is weakly stained. Magnification X 620.
the pig (Fig. 2). This determinant was detected with certainty on monocytes of the baboon and rat, and, after neuraminidase treatment on those of man and mouse (Fig. 3). Among platelets only those of the baboon expressed the i(Den).

_IgM WOO and FC 10.2 determinants expressed on Type-1 oligosaccharide backbones_

Type-1 chains recognized by IgM WOO and FC10.2 could not be detected with certainty on untreated blood cells of any of the species tested, although there was some weak staining of rabbit erythrocytes with IgM WOO but not FC10.2. However, after neuraminidase treatment, 85% of rabbit lymphocytes stained intensely with both monoclonal antibodies indicating that Type-1 chains may exist on these cells and are normally masked by sialic acid.

_SSEA-1 and C14 determinants expressed on fucosylated Type-2 chains_

Among the seven species tested, human granulocytes were the only blood cells on which the monofucosylated (SSEA-1) and difucosylated (C14) Type-2 chain determinants were detected in overt form (Figs. 2 & 5). However, neuraminidase treatment revealed the SSEA-1 determinant on human monocytes (Fig. 5) as well as on the granulocytes and monocytes of the baboon, the rabbit, and the dog (Fig. 5) and the C14 determinant in a proportion of dog granulocytes, monocytes, and lymphocytes.

_Pr2 and Gd antigens expressed on N-acetylneuraminic acid-containing oligosaccharides_

By immunofluorescence only the Pr2 determinant was detected on human erythrocytes although anti-Gd strongly agglutinates these erythrocytes (34). However, a high proportion of mouse erythrocytes were stained by this antibody. The majority of pig and dog erythrocytes and a sub-population of rabbit erythrocytes also expressed Pr2. Dog erythrocytes expressed Gd in accordance with previous knowledge (35).

The majority of human platelets and granulocytes of man, mouse, and dog as well as a sub-population of human, mouse, and rat lymphocytes expressed both antigens. Small sub-populations of rabbit and rat granulocytes and lymphocytes of rabbit and pig also expressed one or both of these antigens as shown in Fig. 2.

Discussion

This preliminary survey shows the ease of obtaining structural information on glycoconjugates of diverse cell populations with the aid of well characterized monoclonal antibodies, and should lead to more detailed studies of the blood cells of individual members of a species, and more sensitive assays using cytofluorimetry and additional anti-carbohydrate antibodies as they become available.

From the observations summarized in Figs. 2 and 3, it can be inferred that both linear and branched backbone structures of the i and I antigen type are widely distributed among blood cells of the 7 animal species tested, although the pattern of I and i antigen
Fig. 5. SSEA-1 immunofluorescence (A–F) and corresponding phase-contrast (A'–F') micrographs of human (A–C) and rabbit (D–F) blood cells before (A & D) and after (B, C, E, F) neuraminidase treatment. Of the untreated cells in A and D only the human granulocytes are stained. However, after neuraminidase treatment, SSEA-1 is revealed on human monocytes (m) in B and C and rabbit granulocytes and monocytes (m) in E and F. Magnification X 620.
expression in each type of blood cell was different for each animal studied. The I and i antigens are in part masked by the presence of sialic acid residues. It is well established (24) that other substituents, for example, fucose residues α1-2 linked to the peripheral galactose residues, also result in masking of the li antigens. Therefore additional antigenically cryptic backbones of I and i type may well be present.

Type-I chains occur on human erythrocyte membranes associated with Lewis^a and Lewis^b antigens (36) and in cryptic state, masked by sialic acid (37); although they are readily detectable by haemagglutination, they could not be detected by immunofluorescence on native or neuraminidase-treated human erythrocytes. However, there was marked immunofluorescence of neuraminidase-treated rabbit lymphocytes with IgM WOO and FC10.2 compatible with the presence of Type-1 chains on these cells. These observations now require biochemical confirmation.

Of the four peripheral-region-associated antigens studied, the two N-acetylneuraminic acid-containing antigens Pr_2 and Gd were also rather widely distributed among blood cells. The total amount of sialylated carbohydrate structures would be underestimated with the Pr_2 and Gd antibodies which fail to react with oligosaccharides containing the N-glycolyl form of neuraminic acid (21).

In contrast to the wide distribution of the I, i, Pr_2, and Gd determinants, the mono- and difucosylated Type-2-chain antigens, SSEA-1(X-hapten) and C14 (Y-hapten) were notable in being predominantly restricted to granulocytes and monocytes. SSEA-1 was overtly expressed only on human granulocytes, but occurred in cryptic state (masked by sialic acid residues) on monocytes of man and on both granulocytes and monocytes of the baboon, rabbit, and dog (those of the dog also contained cryptic C14 antigen), but not on those of the mouse, rat, and rabbit. Lymphocytes of dog were unusual in expressing the C14 antigenic determinant after neuraminidase treatment. The expression of the C14 determinant on human granulocytes was not affected by the secretor gene, for this antigen was detected on the granulocytes of several individuals irrespective of their secretor status (unpublished observations).

Whereas human autoantibodies anti-I, i, Pr_2, and Gd were incidentally found to be directed against differentiation antigens of man and mouse, the hybridoma-derived antibodies were raised with the aim of identifying distinctive antigenic markers, the inference being that such markers would represent molecules with important functions in the cells expressing them. It now appears that a proportion of these antigens are saccharide structures closely related to the blood group antigens (2,38) and are associated with a variety of glycoproteins and glycolipids. Thus, there is a need to seek with renewed interest the physiological roles of these structures. On account of their occurrence as prominent structures of the cell surface, they (and the various substituents they may carry) seem likely candidates as recognition structures (33,38); however there is very little information regarding the roles of individual structures on cell surfaces as specific receptors for endogenous ligands. One observation has been the preferential reaction in vitro of II-active substances compared with those of ABH activities, with the β-galactoside-binding lectin of animal tissues (39).
Whether there is a physiological effect of such an interaction in vivo is not yet known. As for the 3-fucosyl-N-acetyllactosamine sequence, it is difficult to envisage a common role for it and related structures on cells of myelomonocytic lineage in several species (excluding mouse, rat, and pig) and on the 8 cell stage mouse embryo (10,11) and on non-metastatic variants of B16 mouse melanoma cells (40). On the other hand the close association of li-active carbohydrate chains with receptors for the mitogenic lectin Con A on human lymphocytes (33), the occurrence of blood group chains on the receptor for epidermal growth factor (EGF) (41), and the reported stimulation of DNA synthesis in fibroblasts (42) after treatment with an anti-EGF receptor antibody (TL5) which has blood group A specificity (43) suggest that perturbation of these carbohydrate chains may somehow influence cell growth.

Contrasting with the speculative role of these saccharide structures as receptors for endogenous ligands are observations on their roles as receptors for infective agents. Carbohydrate sequences of li antigen type with sialic acid α2→3 linked to their terminal galactose residues have been shown to serve as receptors for the human pathogen Mycoplasma pneumoniae (44) and possibly for Sendai virus (44,45). Structures related to these may also be the cellular receptors for α-toxin of Staphylococcus aureus (46), while parts of the corresponding asialo-structures may be receptors for Streptococcus pneumoniae (47). One important prediction from the present observations on the differing antigenicities, and by inference the differing saccharide structures among blood cells of various animal species, would be that they would differ in their susceptibility to a variety of infective agents.

Acknowledgements

S.J.T. is supported by the Cancer Research Campaign. The authors are most grateful to Professor R.W. Baldwin, Drs. D. Solter, R.A.J. Mcllhinney, M. Crookston, W. Pruzanski, and D. Roelcke for their gifts of monoclonal antibodies and to Mrs. Sally Schwarz for the preparation of the manuscript.

References

35. Roelcke D, Pruzanski W, Ebert W, Romer W, Fischer E, Lenhard V