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Modification of sialic acids by 9-*O*-acetylation is detected in human leucocytes using the lectin property of influenza C virus

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Influenza C virus spike glycoprotein HEF specifically recognizes glycoconjugates containing 9-*O*-acetyl-*N*-acetylneuraminic acid. The same protein also contains an esterase activity. Taking advantage of these two properties, influenza C virus was used as a very sensitive probe for the detection of traces of 9-*O*-acetyl-*N*-acetylneuraminic acid in human leucocytes. The binding of influenza C virus to leucocyte glycoproteins and gangliosides separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis and thin-layer chromatography, respectively, was assayed using a chromogenic esterase substrate. In this way, glycoproteins of B-lymphocytes and T-lymphocytes were found to contain 9-*O*-acetylated sialic acids. Of the various 9-*O*-acetylated gangliosides detected, one had the characteristics of 9-*O*-acetylated G_{D3}. The identification of 9-*O*-acetylated sialic acids on distinct glycoproteins and glycolipids should be helpful in assigning a physiological role to this sugar.

Key words: *O*-acetylation/gangliosides/influenza C virus/lymphocytes/sialic acids

Introduction

Sialic acids, a diverse family of 9-carbon acid amino sugars, are found on vertebrate cell surfaces mostly as terminal residues of oligosaccharides linked to either glycoproteins or glycolipids. In this exposed position, sialic acids participate in many biological and pathological processes (Schauer, 1982, 1985; Varki, 1992). *N*-Acetylneuraminic acid (Neu5Ac) is the most common sialic acid. The other members of the family are derivatives of this sugar. Many of them arise by acetylation of one or more of the hydroxyl residues (*O*-acetylation). Several observations indicate that these modifications can significantly affect the physicochemical and biological properties of the parent molecule. For example, *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac$_2$) has been shown to mediate virus attachment to cells (Rogers *et al.*, 1986; Vlasak *et al.*, 1988; Schultze *et al.*, 1990), to be developmentally regulated on chicken erythrocytes (Herrler *et al.*, 1987) and in rat and human colon (Muchmore *et al.*, 1987), to antagonize Neu5Ac in preventing activation of the alternate complement pathway (Varki and Kornfeld, 1980) and to be cleaved by sialidases more slowly than Neu5Ac (Corfield *et al.*, 1986). Studies with monoclonal antibodies which recognize the 9-*O*-acetylated form of the ganglioside G_{D3} showed that the expression of this antigen in rat and mouse is tissue specific and developmentally regulated (reviewed by Varki, 1992). In man, 9-*O*-acetylated G_{D3} was first described as a tumour-associated antigen of malignant melanoma cells (Cheresh *et al.*, 1984a,b), but has also been detected in a subpopulation of human T-lymphocytes by monoclonal antibodies which define the leucocyte differentiation antigen CDw 60 (Kniep *et al.*, 1992). Recently, another ganglioside, 9-*O*-acetylated G_{D2}, has been found in human malignant melanoma (Sjoberg *et al.*, 1992), in human neuroblastoma and some other brain tumours (Ye and Cheung, 1992). It should be noted that although 9-*O*-acetylated sialic acids have been frequently found in tumour cells, these sugars cannot be regarded as tumour specific. The analysis of purified sialic acids has shown that 9-*O*-acetylated sialic acids also occur in normal human tissues and cells (Haverkamp *et al.*, 1977; Kamerling *et al.*, 1982; Muchmore *et al.*, 1987). However, the sialoglycoconjugates bearing these residues have not been identified. The reason for this is that 9-*O*-acetylated sialic acids constitute only a minor fraction of total sialic acids in most tissues, and that many methods which are commonly used for the analysis of sialic acids and glycoconjugates are often accompanied by partial loss of the labile *O*-acetyl groups (Schauer, 1987; Varki, 1992).

Influenza C virus and some coronaviruses specifically attach with high affinity to glycoconjugates which contain Neu5,9Ac$_2$ residues (Herrler *et al.*, 1985; Rogers *et al.*, 1986; Vlasak *et al.*, 1988; Schultze *et al.*, 1990). Therefore, these viruses have been used for the detection of this type of sialic acid (Muchmore and Varki, 1987; Nishimura *et al.*, 1988; Manuguerra *et al.*, 1991; Schultze *et al.*, 1991; Zimmer *et al.*, 1992). In the case of influenza C virus, binding to Neu5,9Ac$_2$ residues is mediated by the spike glycoprotein HEF, which in addition has an acetylesterase activity (Vlasak *et al.*, 1987; Herrler *et al.*, 1988; Schauer *et al.*, 1988). We have recently shown that this viral enzyme allows the rapid and sensitive detection of receptor-bound virions using fluorigenic or chromogenic substrates (Zimmer *et al.*, 1992). In the present study, this technique was applied to show that glycoproteins and gangliosides of human leucocytes contain 9-*O*-acetylated sialic acids. The identification of such molecules should help to investigate whether Neu5,9Ac$_2$ is involved in any of the physiological functions of leucocytes, such as cell–cell recognition.

Results

Detection of 9-O-acetylated gangliosides

The thin-layer chromatography (TLC) overlay technique, originally developed for studying the interaction of glycolipids with toxins and antibodies (Magnani *et al.*, 1980, 1987), proved to be very useful for the detection of 9-*O*-acetylated gangliosides by influenza C virus. Total gangliosides from human tonsils (tonsilla palatina and tonsilla pharyngea) were

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separated by TLC and overlaid with influenza C virus. Receptor-bound virions were directly visualized by taking advantage of the viral acetylesterase activity using a chromogenic esterase substrate. As shown in Figure 1 (lane 3), various gangliosides were recognized by influenza C virus, indicating that these glycolipids contain Neu5,9Ac$_2$ residues. As a control for the specificity of the assay, gangliosides were pre-treated with ammonia in order to hydrolyse the *O*-acetyl esters. Following this saponification step, no binding of influenza C virus to the gangliosides was detectable (lane 4). When orcinol was used for the chemical detection of gangliosides, a different pattern was obtained (cf. lanes 1 and 3). Because of the relatively low sensitivity of this reagent (detection limit ~100–200 ng sialic acids), only the more abundant gangliosides were stained. Most of them were not affected by the alkaline pre-treatment (lane 2). On the other hand, the alkali-sensitive gangliosides detected by influenza C virus were not stained by orcinol, indicating that they constitute only a minor fraction.

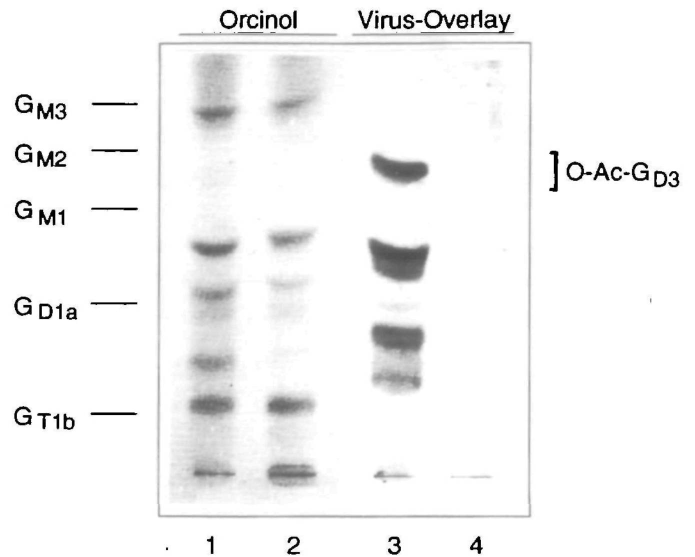

Fig. 1. Detection of 9-*O*-acetylated gangliosides in the ganglioside fraction prepared from human tonsilla pharyngea. Total tonsil gangliosides corresponding to 4 μg sialic acids/lane were separated by HPTLC; lanes 2 and 4 were exposed to ammonia vapour prior to development in the solvent system (see Materials and methods). Lanes 1 and 2 were stained by the orcinol reagent. 9-*O*-Acetylated gangliosides were detected by the influenza C virus overlay assay (lanes 3 and 4). The positions of standard gangliosides are indicated on the left, the position of 9-*O*-acetylated G_{D3} is marked on the right side.

As tonsils mainly consist of leucocytes, we wanted to know whether gangliosides detected by the virus overlay assay were derived from these cells. Therefore, a mononuclear cell fraction was prepared from tonsils, as well as from peripheral blood, by Ficoll density gradient centrifugation. Whereas the ganglioside pattern of tonsil leucocytes (not shown) was very similar to the pattern shown in Figure 1, blood leucocytes showed a less complex ganglioside profile (Figure 2): G_{M3} was detected by orcinol staining as the predominant ganglioside (lane 3), whereas a double band migrating on TLC between the standard gangliosides G_{M1} and G_{M2} showed the most intense reactivity with influenza C virus (lane 5). This double band co-migrated with the major band detected by influenza C virus in human melanoma gangliosides (lane 6), which has been previously characterized as 9-*O*-acetylated G_{D3} (Cheresh *et al.*, 1984a,b; Thurin *et al.*, 1985). The doublet has been shown to arise from fatty acid heterogeneity in the ceramide moiety (Thurin *et al.*, 1985). For further confirmation, we tested a monoclonal antibody directed against 9-*O*-acetylated G_{D3} prepared from bovine cheese whey. As shown in Figure 3, the ganglioside doublets from human melanoma cells and human blood leucocytes migrating between G_{M1} and G_{M2} were recognized by both influenza C virus and the antibody, whereas another doublet in melanoma gangliosides (lane 1) co-migrating with unsubstituted G_{D3} was only detected by influenza C virus. An *O*-acetylated ganglioside with this chromatographic behaviour has recently been characterized in human melanoma cells as

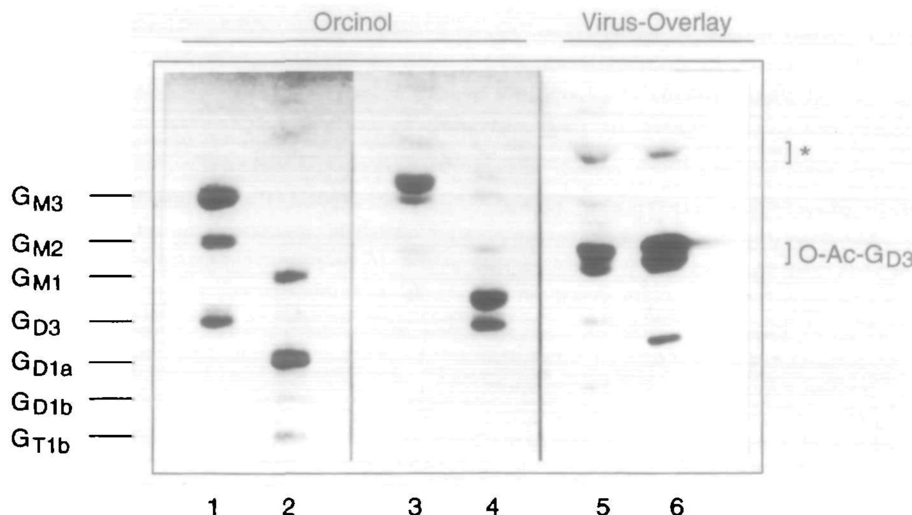

Fig. 2. Detection of 9-*O*-acetylated gangliosides in the ganglioside fraction prepared from human blood leucocytes and human malignant melanoma cells. Standard gangliosides G_{M3}, G_{M2}, G_{D3} (lane 1), G_{M1}, G_{D1a}, G_{D1b} and G_{T1b} (lane 2), total gangliosides from human blood leucocytes (lanes 3 and 5, corresponding to 2 μg sialic acids/lane) and total gangliosides from malignant melanoma cells (lanes 4 and 6, corresponding to 2 μg/lane) were separated by HPTLC. Lanes 1–4 were stained by the orcinol reagent. 9-*O*-Acetylated gangliosides were visualized by the influenza C virus overlay assay (lanes 5 and 6). The position of 9-*O*-acetylated G_{D3} is marked. The asterisk indicates bands whose staining was not due to virus binding.

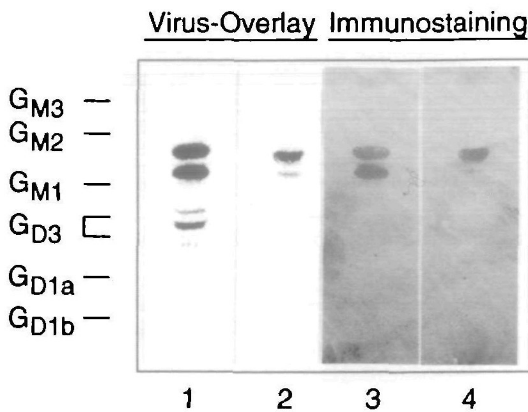

Fig. 3. Detection of 9-*O*-acetylated G_{D3}. Total gangliosides prepared from human blood leucocytes (lanes 2 and 4, corresponding to 1 μg sialic acids/lane) and human malignant melanoma cells (lanes 1 and 3, corresponding to 2 μg sialic acids/lane) were separated by HPTLC A total spectrum of 9-*O*-acetylated gangliosides was obtained by influenza C virus overlay (lanes 1 and 2), 9-*O*-acetylated G_{D3} was detected by a monoclonal antibody directed against this ganglioside (lanes 3 and 4). For details, see Materials and methods. The positions of standard gangliosides are indicated on the left.

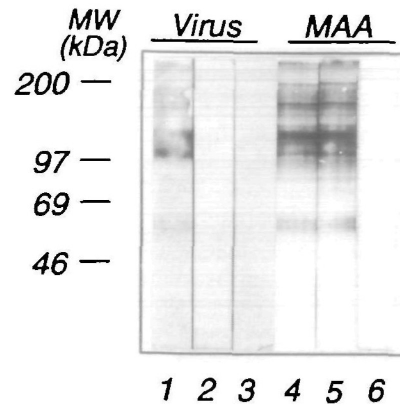

Fig. 4. Detection of 9-*O*-acetylated sialoglycoproteins from human blood leucocytes. Membrane proteins were separated by SDS–PAGE (15 μg protein/lane), transferred to nitrocellulose and probed with influenza C virus (lanes 1–3) or MAA (lanes 4–6). Prior to overlay with influenza C virus or MAA, respectively, blots were incubated with PBS (lanes 1 and 4), NaOH (lanes 2 and 5) or sialidase (lanes 3 and 6). The positions of molecular weight markers are indicated.

9-*O*-acetylated G_{D2} (Sjoberg *et al.*, 1992). An identical staining pattern was obtained when another monoclonal antibody directed against 9-*O*-acetylated G_{D3} (mAb D1.1) was used (not shown). Our findings suggest that 9-*O*-acetylated G_{D3} is not unique to malignant melanoma cells, but is also expressed by human leucocytes.

Detection of glycoproteins containing Neu5,9Ac$_2$ residues

In many cell types, the majority of sialic acids are bound to glycoproteins rather than to gangliosides. Nevertheless, it has been shown for human melanoma cells that *O*-acetylation of sialic acids may be restricted to gangliosides (Manzi *et al.*, 1990). We wondered whether this restriction is a more general phenomenon and also true for human leucocytes. In order to analyse leucocyte glycoproteins with regard to *O*-acetylation, membrane proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), immobilized on nitrocellulose and probed with influenza C virus. As shown in Figure 4 (lane 1), several glycoproteins were recognized by influenza C virus. A similar pattern was obtained when *Maackia amurensis* agglutinin (MAA) was used (lane 4), a lectin with specificity for sialic acids attached in α2,3-glycosidic linkage to galactose (Wang and Cummings, 1988; Knibbs *et al.*, 1991). Pre-treatment of blots with *Vibrio cholerae* sialidase abolished the binding of both influenza C virus and MAA (Figure 4, lanes 3 and 6). In contrast, hydrolysis of *O*-acetyl esters by sodium hydroxide did not alter the staining by MAA, but resulted in a total loss of influenza C virus binding (Figure 4, lanes 2 and 5). These findings indicate that 9-*O*-acetylation of sialic acids in leucocytes is a modification which is found on glycoproteins, as well as on gangliosides.

Neu5,9Ac$_2$ has been reported to be present in human B-lymphocytes, but to be absent in T-lymphocytes (Kamerling *et al.*, 1982). Therefore, we analysed whether glycoproteins containing 9-*O*-acetylated sialic acids are restricted to certain leucocyte subpopulations. For this purpose, B-lymphocytes ($CD19^+$) and T-lymphocytes ($CD4^+$, $CD8^+$) were isolated

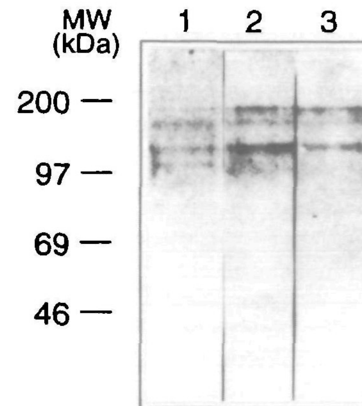

Fig. 5. Demonstration of 9-*O*-acetylated sialoglycoproteins prepared from different blood leucocyte subpopulations. $CD4^+$ cells (lane 1), $CD8^+$ cells (lane 2) and $CD19^+$ cells were separated from the non-adherent mononuclear cell fraction using immunomagnetic beads. Membrane proteins were separated by SDS–PAGE (lane 1: 20 μg protein; lanes 2 and 3: 4 μg protein), transferred to nitrocellulose and probed with influenza C virus. The positions of molecular weight markers are indicated.

from human peripheral blood leucocytes using immunomagnetic beads. The purity of all three cell populations was >96%, as verified by FACS analysis. In all cases, binding of influenza C virus to membrane proteins resulted in a very similar staining pattern (Figure 5). This suggests that 9-*O*-acetylation of sialic acids is a common feature of these subpopulations.

Discussion

Influenza C virus spike glycoprotein HEF exhibits a unique lectin-like activity by recognizing 9-*O*-acetylated sialic acids (*N*-acetyl-9-*O*-acetylneuraminic acid, *N*-glycoloyl-9-*O*-acetylneuraminic acid) in gangliosides and glycoproteins. The non-*O*-acetylated forms (*N*-acetylneuraminic acid, *N*-glycoloylneuraminic acid), the 4-*O*-acetylated forms (*N*-acetyl-4-*O*-acetylneuraminic acid, *N*-glycoloyl-4-*O*-acetylneuraminic acid) and

the 4,9-di-*O*-acetylated form (*N*-glycoloyl-4,9-di-*O*-acetylneuraminic acid) have been shown not to mediate influenza C virus binding (Rogers *et al.*, 1986; Zimmer *et al.*, 1992). We cannot completely rule out that 7-*O*-acetylated sialic acids are also recognized by influenza C virus. However, the 7-*O*-acetyl group has been shown to migrate to the more stable C9 position even under physiological conditions (Kamerling *et al.*, 1987), making it difficult to isolate and test glycoconjugates carrying exclusively 7-*O*-acetylated sialic acids. An indirect clue to the specificity of influenza C virus binding is obtained from the viral receptor-destroying enzyme, which has been characterized as a sialate 9-*O*-acetylesterase (Herrler *et al.*, 1985; Schauer *et al.*, 1988). This enzyme cleaves *O*-acetyl groups from *N*-acetyl-9-*O*-acetylneuraminic acid and *N*-glycoloyl-9-*O*-acetylneuraminic acid, but not from *N*-acetyl-7-*O*-acetylneuraminic acid and only very slowly from *N*-acetyl-4-*O*-acetylneuraminic acid. The ability of this enzyme to inactivate the receptors for influenza C virus on different cells, therefore, indicates that 9-*O*-acetylated and not 7-*O*-acetylated sialic acids serve as receptor determinants on cells. From this, we conclude that binding of influenza C virus to human leucocyte glycoproteins and gangliosides indicates the presence of 9-*O*-acetylated sialic acids on these glycoconjugates.

Previous studies on sialic acids have revealed the presence of Neu5,9Ac_2 in human leucocytes (Kamerling *et al.*, 1982; Holzhauser and Faillard, 1988; Stickl *et al.*, 1991). However, the methods used [HPLC, TLC, gas–liquid chromatography/mass spectrometry (GLC/MS)] require the previous release of sialic acids from glycosidic linkage by either enzymic or chemical hydrolysis, so that the glycoconjugates bearing this sialic acid have not been identified. In this study, we used the lectin property of influenza C virus to directly detect Neu5,9Ac_2 residues on immobilized glycoproteins and gangliosides. Whereas in a former report Neu5,9Ac_2 has only been found in the B-cell fraction (Kamerling *et al.*, 1982), our results revealed the presence of this sugar in glycoproteins of both B-cells and T-cells. A possible explanation for this discrepancy may be the release of esterases by damaged or lysed T-cells, resulting in a significant loss of *O*-acetyl groups. This could have occurred when lyophilized cells were dialysed against water for several hours prior to acid hydrolysis of sialic acids (Kamerling *et al.*, 1982).

In this study, we have described an alkali-labile ganglioside in human leucocytes migrating on TLC as a double band between gangliosides G_{M1} and G_{M2}. This ganglioside was recognized by influenza C virus, indicating the presence of Neu5,9Ac_2. Furthermore, it co-migrated with authenthic O-Ac-G_{D3} from malignant melanoma cells and was recognized by two different monoclonal antibodies directed against O-Ac-G_{D3}. These findings provide evidence for the presence of 9-*O*-acetylated G_{D3} in human leucocytes. Interestingly, this ganglioside has previously been reported to be a tumour-associated antigen in human malignant melanoma cells (Cheresh *et al.*, 1984a,b), and has been considered to be a promising candidate for immunotherapy (Ritter *et al.*, 1990). Our results indicate that this antigen is not strictly limited to malignant melanoma. This is in accordance with recent immunological data which show that O-Ac-G_{D3} is expressed by a certain subset of T-cells characterized by the differentiation antigen CDw 60 (Kniep *et al.*, 1992).

Whereas O-Ac-G_{D3} was found to be the predominant *O*-acetylated ganglioside in blood leucocytes, several so far unidentified *O*-acetylated gangliosides were detected in tonsil leucocytes, in addition to O-Ac-G_{D3}. Structural studies of different *O*-acetylated gangliosides from the nervous system (Ghidoni *et al.*, 1980; Chigorno *et al.*, 1982; Chou *et al.*, 1990; Dubois *et al.*, 1990) and melanoma cells (Thurin *et al.*, 1985; Sjoberg *et al.*, 1992) have shown that the 9-*O*-acetyl group is located on a terminal α2,8-linked sialic acid originating from the internal β1,4-linked galactose of lactosylceramide. It has been suggested that a specific *O*-acetyltransferase may act on gangliosides containing this structure (Sjoberg *et al.*, 1992). According to this hypothesis, *O*-acetylated gangliosides other than O-Ac-G_{D3} in human leucocytes are expected to also represent gangliosides of the B-series, i.e. O-Ac-G_{D2}, O-Ac-G_{D1b}, O-Ac-G_{T1b} or O-Ac-G_{Q1b}, respectively. The striking differences in the ganglioside patterns between tonsil and blood leucocytes may reflect a different cell composition and/or a different physiological state of the two cell populations. For example, B-lymphocytes represent ~50% of total lymphocytes in tonsils, but only 10–15% in peripheral blood. Moreover, tonsils, like other peripheral lymphoid tissues, are sites where mature lymphocytes respond to foreign antigens. Lymphocyte activation and differentiation may be associated with changes in glycoconjugate biosynthesis (Piller *et al.*, 1988). Interestingly, several alkali-labile gangliosides have been observed in murine thymus (Schwarting and Gajewski, 1983), although it is not known whether the alkali sensitivity is due to lactone formation or the presence of *O*-acetyl groups.

Much interest has been directed towards gangliosides in leucocytes because of their possible role in immune modulation (Miller and Esselman, 1975; Lengle *et al.*, 1979; Whisler and Yates, 1980; Ladisch *et al.*, 1983, 1984; Gonwa *et al.*, 1984; Merritt *et al.*, 1984), in cell adhesion (Crocker *et al.*, 1991; Riedl *et al.*, 1982), as lymphokine receptors (Liu *et al.*, 1982; Chu and Sharom, 1990), in modulating growth factor receptor activity (Bremer *et al.*, 1986; Hanai *et al.*, 1988; Weis and Davis, 1990), and because of their expression as differentiation markers and oncofetal antigens (Hakomori, 1981; Hakomori and Kannagi, 1983; Feizi, 1985). In most cases, 9-*O*-acetylation of gangliosides has not been taken into account, so that it is not known at present whether this modification is involved in any of these functions.

Using the virus-binding assay in a manner analogous to the Western blot technique, various leucocyte proteins were found to contain Neu5,9Ac_2 residues. The specificity of the reaction was confirmed by pre-treating the blots with sialidase or sodium hydroxide, both abolishing the virus binding. For comparison, the protein blots were also probed with the lectin MAA, which recognizes sialic acids in α2,3-glycosidic linkage to galactose (Wang and Cummings, 1988; Knibbs *et al.*, 1991). Since the glycerol side chain of sialic acid has been shown not to play a role in MAA binding (Knibbs *et al.*, 1991), it is possible that this lectin recognizes sialic acids irrespective of 9-*O*-acetylation.

In different organisms, *O*-acetylation of sialic acids has been shown to be tissue specific and developmentally regulated (Herrler *et al.*, 1987; Muchmore *et al.*, 1987; Varki, 1992). This may also be true for the human immune system. Recently, the leucocyte differentiation antigens HB-6, CDw75 and CD76 have been shown to be carbohydrate determinants generated by sialyltransferases (Kniep *et al.*, 1990; Bast *et al.*, 1992). Potential *O*-acetylation of these sialylated antigens has not been considered in these studies. On the other hand, we have identified sialoglycoproteins bearing Neu5,9Ac_2 residues, but these glycoproteins appeared to be common to CD4, CD8 and CD19 cells of the blood stream.

The knowledge about the selectin family of adhesion molecules which display lectin-like properties provides evidence for the participation of sialic acids and other carbohydrates in cell recognition (Lasky *et al.*, 1992). Recently, two other adhesion molecules, the sialoadhesin on murine tissue macrophages (Crocker *et al.*, 1991) and the B-lymphocyte adhesion molecule CD22β (Stamenkovic *et al.*, 1991), have been shown to mediate cell adhesion via sialylated ligands. In most cases, it is not known whether *O*-acetylation affects the recognition of sialic acid by these adhesion molecules. For sialoadhesin, however, it has recently been demonstrated that 9-*O*-acetyl groups prevent the binding of the lectin to mouse erythrocytes (Kelm *et al.*, 1992). Interestingly, a masking effect of 9-*O*-acetylation has also been shown for the recognition of sialic acids by most haemagglutinins of influenza A and B viruses (Higa *et al.*, 1985; Rogers *et al.*, 1986) and *Plasmodium falciparum* 175 kDa erythrocyte-binding antigen (Klotz *et al.*, 1992). Thus, although the physiological role of *O*-acetylation is still unknown, this modification may have a regulatory function in cellular interactions by masking sialic acids as recognition site or by itself representing a ligand for lectins.

Materials and methods

Virus

A mutant of influenza C virus (strain Johannesburg/1/66), differing from wild-type virus by an increased affinity for Neu5,9Ac$_2$ residues (Szepanski *et al.*, 1992) was used throughout this study. Virus was inoculated into the allantoic cavity of 8-day-old embryonated chicken eggs. After 3 days at 33°C, the allantoic fluid was harvested and clarified by low-speed centrifugation (1000 *g*, 15 min, 4°C). Aliquots were stored frozen at −80°C.

The haemagglutination assay was determined in microtitre plates. Serial 2-fold virus dilutions in phosphate-buffered saline (PBS) were prepared and to 50 µl of each dilution were added 50 µl of a 0.5% suspension of chicken erythrocytes. After 1 h at 4°C, the haemagglutination activity (HAU/ml) was determined as the reciprocal value of the highest dilution causing complete agglutination.

Cells

Mononuclear cells were prepared from the blood of healthy donors by Ficoll-Hypaque density gradient centrifugation (Boyum, 1968). The cells were washed three times to remove most of the platelets. Monocytes/macrophages were depleted from this cell population by adherence to plastic surfaces for 60 min at 37°C in the presence of human AB serum (5%). CD19$^+$, CD4$^+$ and CD8$^+$ cells were separated from the non-adherent cell fraction using immunomagnetic beads (Dynal, Hamburg) (Gaudernack *et al.*, 1986; Funderud *et al.*, 1990). The purity of these subpopulations was analysed by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies directed to CD3, CD4 and CD14, and rhodamine-conjugated monoclonal antibodies directed to CD8 and CD19 (Becton Dickinson, Heidelberg). Samples containing 3 × 10^5 cells were stained with anti-CD14 or double labelled with anti-CD4/CD8 or anti-CD3/CD19, and analysed on a FACScan cytometer (Becton Dickinson). The purity of the three cell populations was as follows: B cells (CD19 = 98.5%, CD4 = 0.1%, CD8 = 0.2%, CD3 = 0.1%, CD14 = 0%); CD4 cells (CD4 = 98.5%, CD8 = 0%, CD3 = 98.9%, CD19 = 0%, CD14 = 0.1%); CD8 cells (CD8 = 96.2%, CD4 = 0.1%, CD3 = 98.6%, CD19 = 0.1%, CD14 = 0.5%).

Tonsils (tonsilla pharyngea, tonsilla palatina) were obtained from children suffering from an inflammation and/or hyperplasia of these tissues. Mononuclear cells were isolated by mechanical disruption of tonsil tissue, followed by Ficoll-Hypaque gradient centrifugation.

The human malignant melanoma cell line SK-MEL-28 was purchased from the American Type Culture Collection (ATCC) and grown in MEM containing non-essential amino acids, sodium pyruvate (1 mM) and 10% fetal calf serum.

Analysis of gangliosides

Total gangliosides were extracted from leucocytes or melanoma cells and purified by Folch partition, gel filtration on Sephadex LH-20 (Pharmacia, Freiburg) and anion-exchange chromatography on DEAE–Sephadex A-25 (Pharmacia) using published procedures (Svennerholm and Fredman, 1980; Ledeen and Yu, 1982). Gangliosides were chromatographed on glass-backed or aluminium-backed high-performance thin-layer plates (HPTLC, silica gel 60, Merck, Darmstadt) in chloroform/methanol/0.2% aqueous CaCl$_2$, 60:40:9 (by vol.). Sialic acid-containing compounds were visualized by the orcinol/Fe^{3+}/HCl reagent (Schauer, 1978).

For the detection of 9-*O*-acetylated gangliosides, an overlay assay using influenza C virus was performed essentially as described previously (Zimmer *et al.*, 1992). Briefly, the dried chromatograms were dipped in diethylether containing 0.5% polyisobutylmethacrylate (Plexigum P28, Röhm, Darmstadt). The plates were dried, sprayed with PBS and then immersed in 1% bovine serum albumin (BSA)–PBS for 60 min at room temperature. After decanting the buffer, some drops of allantoic fluid containing influenza C virus with a haemagglutinating activity of 512 HAU/ml were added and spread over the whole chromatogram by covering it with a piece of parafilm. Virus was allowed to bind for 60 min at 4°C. The plates were washed three times with 0.1% Tween 20–PBS at 4°C, 5 min each, and then incubated with a solution containing 1 mM α-naphthyl acetate (Sigma, Deisenhofen) and 0.1% (mass/vol.) 4-chloro-2-methylbenzenediazonium salt (Fast Red TR-salt, Sigma) in PBS. After 30 min at room temperature, the reaction was stopped by dipping the plates in H$_2$O.

For immunostaining of thin-layer chromatograms, an IgM monoclonal antibody raised against 9-*O*-acetylated G$_{D3}$ from bovine cheese whey (Hanagata *et al.*, 1991) or the IgM mAb D1.1 directed against the same antigen (Cheresh *et al.*, 1984a,b) were used. After the chromatograms had been fixed by polyisobutylmethacrylate and blocked by BSA as described above, the antibodies (1:100 in PBS) were allowed to bind for 60 min at room temperature, followed by three wash steps (0.1% Tween 20–PBS) of 5 min each. The chromatograms were incubated with a peroxidase-labelled second antibody (sheep anti-mouse IgM, 1:400 in PBS) for 60 min at room temperature and washed as above. The antigen–antibody complex was detected by covering the wet plates with IBI enzigraphic sheets (Kodak, New Haven).

Analysis of glycoproteins

Cells were homogenized in ice-cold hypotonic buffer [20 mM Tris–HCl (pH 7.4)], nuclei were removed by low-speed centrifugation (500 *g*, 10 min, 4°C) and membranes were pelleted from the supernatant by ultracentrifugation (100 000 *g*, 60 min, 4°C). The pellet was suspended in 100 mM Tris–HCl (pH 6.8), up to a protein concentration of 5 mg/ml as determined according to Bradford (1976). For solubilization of membrane proteins, an equal volume of 2 × concentrated reducing SDS–sample buffer was added, and the sample was heated at 95°C for 10 min. Insoluble material was removed by centrifugation (100 000 × *g*, 30 min, 22°C). The solubilized membrane proteins were separated by SDS–PAGE (Laemmli, 1970) and then transferred to nitrocellulose by electroblotting (Kyhse-Andersen, 1984). To prevent the loss of *O*-acetyl groups, electroblotting was modified by lowering the pH values of the two buffers at the anode site from 10.4 to 9.0 (facing the anode) and 7.4 (facing the nitrocellulose), respectively. Non-specific binding sites were blocked by incubating the nitrocellulose with 1% BSA–PBS for 1 h at room temperature, followed by two wash steps with PBS, 5 min each. The blots were then overlayed with influenza C virus (512 HAU/ml) for 1 h at 4°C, and unbound virions were removed by washing the nitrocellulose three times with pre-chilled (4°C) 0.1% Tween 20–PBS, 5 min each. Bound virus was visualized by incubating the blots with esterase substrate (see the previous section on analysis of gangliosides). After 30 min at room temperature, the reaction was stopped by rinsing the blots with H$_2$O. Detection of sialoglycoproteins by digoxigenin-labelled MAA was performed as described previously (Haselbeck *et al.*, 1990) using a commercially available kit (Glycan Differentiation Kit, Boehringer, Mannheim).

Alkaline treatment

Blots were incubated with 0.1 M NaOH for 30 min at room temperature, washed three times with PBS, 5 min each. Non-specific binding sites were blocked with BSA as described above. Gangliosides were applied to TLC plates and exposed to ammonia vapour (25% ammonia) for ~12 h at room temperature. After thorough drying, the plates were developed as described above.

Sialidase treatment

After blocking by BSA, blots were incubated with 1 U/ml sialidase from *Clostridium perfringens* (Sigma) in 50 mM sodium acetate buffer (pH 5.0), overnight at 37°C. The blots were washed three times with Tween–PBS, 5 min each, and again blocked with BSA–PBS for 30 min at room temperature before incubation with virus or lectin as described above.

Abbreviations

BSA; bovine serum albumin; FITC, fluorescein isothiocyanate; HAU, haemagglutination units; HPTLC, high-performance TLC; Neu5Ac, *N*-acetylneuraminic acid; Neu5,9Ac$_2$, *N*-acetyl-9-*O*-acetylneuraminic acid; MAA, *Maackia amurensis* agglutinin; PBS, phosphate-buffered saline; TLC, thin-layer chromatography.

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