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THE NATURE OF SIALIC ACIDS IN HUMAN LYMPHOCYTES

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Analysis of the sialic acids obtained by mild acid hydrolysis of B lymphocytes reveals the presence of *N*-acetylneuraminic acid and 9-*O*-acetyl-*N*-acetylneuraminic acid. For T lymphocytes only *N*-acetylneuraminic acid has been demonstrated to occur. The applied methods include quantitative colorimetry, thin-layer chromatography and combined gas-liquid chromatography-mass spectrometry.

Introduction

Sialic acids occur as constituents of many glycoconjugates [1]. It has generally been accepted that these monosaccharides play an important role in various biological processes [2]. The general name sialic acid includes a series of neuraminic acid derivatives. Two sub-groups of naturally occurring sialic acids can be distinguished, one with an acetylated and one with a glycolylated amino function at carbon-5. The hydroxyl functions at positions 4, 7, 8 and/or 9 in each sub-group can be acylated, sulphated or methylated [3–6], A species- and tissue-specific distribution of certain sialic acids has been observed [3].

For man only a limited number of sialic acids has been reported. Serum contains *N*-acetylneuraminic acid (Neu5Ac) and, in much lower concentrations, 9-*O*-L-lactyl-*N*-acetylneuraminic acid (Neu5Ac9Lac), 9-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,9Ac_2$), and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (Neu2en5Ac) as free molecules [7]. In serum glycoconjugates only Neu5Ac has been detected [7]. In saliva the same sialic acids as in serum have been found [7]. Urine contains Neu5Ac, free as well as glycosidically bound, and traces of Neu2en5Ac [7]. Brain gangliosides show the presence of Neu5Ac and of small amounts of $Neu5,9Ac_2$ [8] Although not well-characterised, glycoproteins from human bile [9] and colon [10–12] contain *N*, *O*-acylneuraminic acids. In the latter case only Neu5Ac, $Neu5,9Ac_2$ and 7,9-di-*O*-acetyl-*N*-acetylneuraminic acid ($Neu5,7,9-Ac_3$) have been identified with certainty [12]. Furthermore, evidence for the presence of *N,O*-diacetylneuraminic acid in sialyllactose from human milk and colostrum has been reported [13].

Recently, histochemical polarisation-optical investigations of human B and T lymphocytes suggested the occurrence of *N,O*-acylneuraminic acids on their cell surfaces in an orientated form [14,15]. It was therefore of interest to study the nature of the sialic acids in lymphocytes, cells which are in the limelight of biochemical and immunological interest.

Methods

Isolation of lymphocytes

Human B and T lymphocytes were isolated from the blood stream of healthy donors [16], or from tonsils [17], using the nylon wool method. The nature of the lymphocytes was determined immuno-

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logically: the adherent B cells were identified by erythrocyte-antibody-complement rosetting and the non-adherent T lymphocytes by erythrocyte rosetting [18,19].

Isolation of sialic acids

Lyophilized B or T lymphocytes (2.5×10^8 cells) were suspended in 2 ml distilled water and dialysed for 5 h at 4°C with four changes. Subsequently, the suspension was acidified with formic acid until pH 2 and heated for 1 h at 70°C, followed by dialysis against 100 ml distilled water for 24 h at 4°C with two changes. Then the lymphocyte suspension was lyophilised and the residue hydrolysed in 2 ml 0.1 M HCl for 1 h at 80°C. After dialysis for 24 h against 100 ml distilled water with two changes, the sialic acids in the combined diffusates of the two hydrolysis steps were purified by ion-exchange chromatography as described earlier [20].

Quantitative analysis procedures

Sialic acids were determined quantitatively by the periodic acid-thiobarbituric acid [21] and the orcinol-ferric chloride [22] assays. Neu5Ac was used as a standard (see also Ref. 3).

The *O*-acyl content of sialic acids was estimated with the alkaline hydroxylamine-ferric perchlorate assay [23]. Ethyl acetate was used as a standard (see also Ref. 3).

Chromatography and mass spectrometry

Sialic acids were analysed by thin-layer chromatography on 0.2 mm cellulose plastic sheets (E. Merck, Darmstadt, F.R.G) using the solvent system *n*-butanol-*n*-propanol-0.1 M HCl (1 : 2 : 1, v/v). Before use, the plates were run in 0.1 M HCl and dried under a current of air at room temperature for 30 min. Crystalline Neu5Ac and Neu5Gl were used as reference compounds. Spots were visualised using the orcinol-ferric chloride spray reagent [24].

Acylhydroxamates were analysed by thin-layer chromatography on cellulose, as described earlier [20].

Gas-liquid chromatography and combined gas-liquid chromatography-mass spectrometry of trimethylsilylated sialic acid methyl esters using 3.8% SE-30 as a stationary phase were carried out as reported previously [4,25,26].

Results and Discussion

Quantitative analysis of the isolated sialic acid materials using the orcinol-Fe^{3+}-HCl reaction showed the presence of 5.0 μmol sialic acid/10^9 B lymphocytes and 3.6 μmol sialic acid/10^9 T lymphocytes. The periodic acid-thiobarbituric acid assay gave rise to values of 3.9 and 3.5 μmol, respectively. Comparison of these data indicates a high degree of *O*-acylation in the glycerol side-chain of the sialic acids from B lymphocytes [3]. With the quantitative and qualitative acylhydroxamate methods 20–50% *O*-acetylation for the latter sialic acids were found in a series of independent experiments.

Fig. 1 shows a thin-layer chromatogram of the sialic acids isolated from T and B lymphocytes. For T lymphocytes only evidence was obtained for the presence of Neu5Ac. B Lymphocytes show the occurrence of two distinct bands with the relative R_F values of 1.00 and 1.37, corresponding with the relative R_F values of Neu5Ac and mono-*O*-acetyl-Neu5Ac, respectively.

The nature of the sialic acids was further investi-

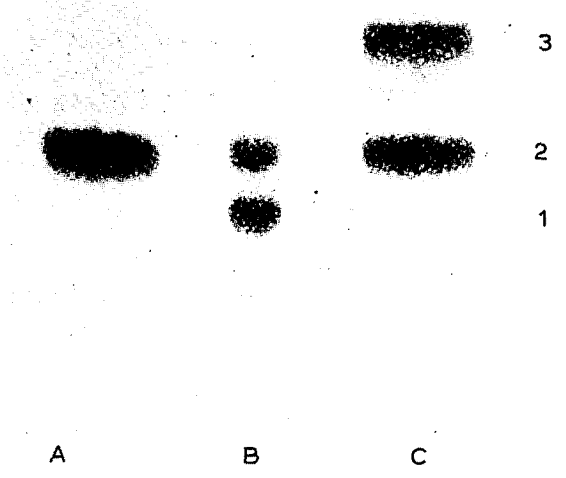
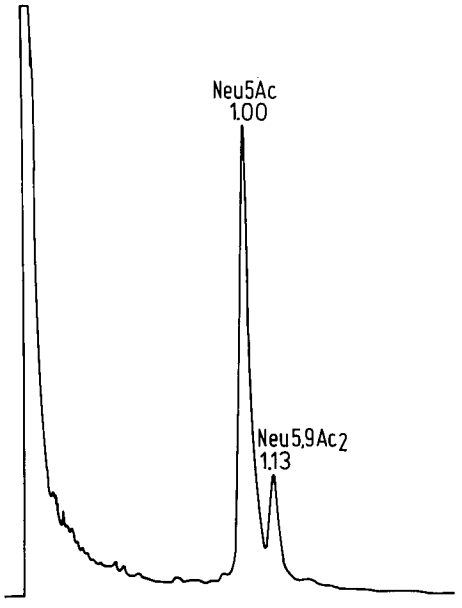


Fig. 1. Thin-layer chromatography of sialic acids from T lymphocytes (A) and B lymphocytes (C), together with some standard sialic acids (B) on cellulose. Compounds: 1, Neu5Gl; 2, Neu5Ac; 3, $Neu5,9Ac_2$.



gated by gas-liquid chromatography-mass spectrometry after esterification with diazomethane and trimethylsilylation [4]. Fig. 2 presents a gas chromatogram of the sialic acids from B lymphocytes, showing peaks at R_{Neu5Ac} 1.00 and 1.13 (the trimethylsilylated methyl ester of Neu5Ac was used as an external standard). The combined data of retention times and mass spectra of the gas-chromatographic peaks demonstrate the occurrence of Neu5Ac and $Neu5,9Ac_2$ in human B lymphocytes [4,25,26]. The mass spectrum of the $Neu5,9Ac_2$ derivative is depicted in Fig. 3 (for a mass spectrum of Neu5Ac, see Ref. 4). In T lymphocytes only Neu5Ac was

Fig. 2. Gas-liquid chromatography on 3.8% SE-30 at 220°C of the sialic acid fraction isolated from the B lymphocytes. Peaks are identified by combined gas-liquid chromatography-mass spectrometry. Derivatisation procedure: esterification with diazomethane followed by trimethylsilylation [25,26].

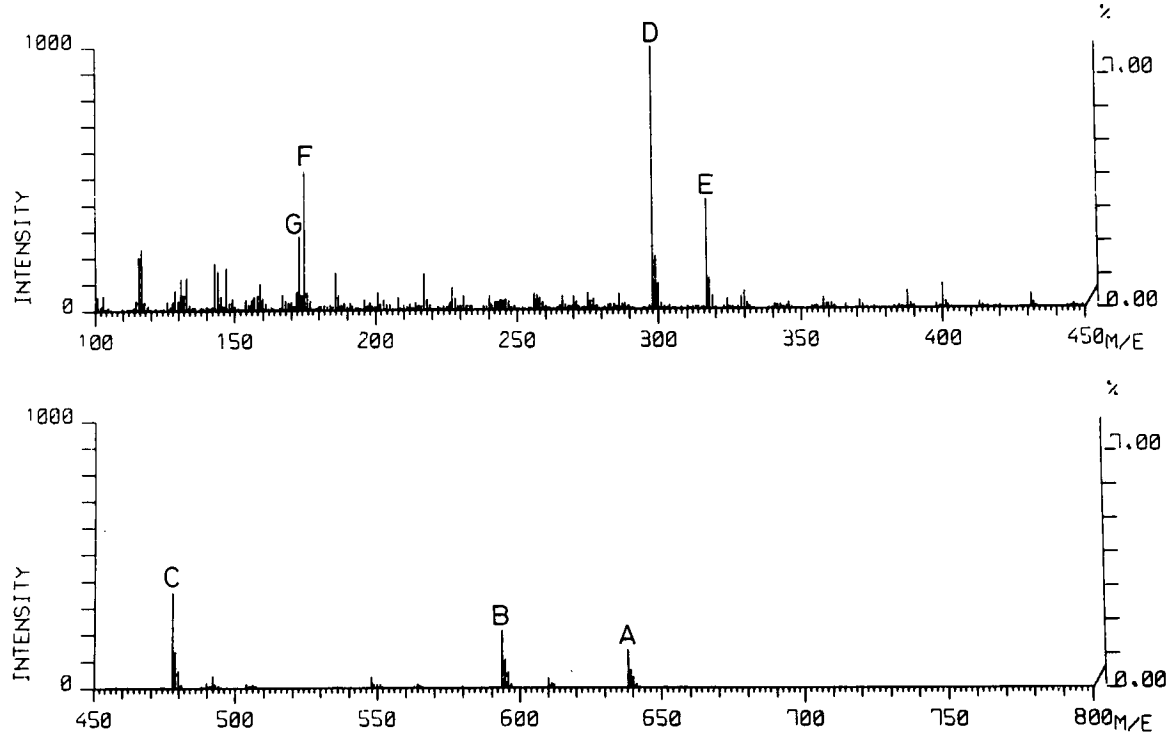


Fig. 3. Mass spectrum at 70 eV of the pertrimethylsilyl derivative of the methyl ester of $Neu5,9Ac_2$. Only m/z values over 100 are given. The specific fragment ions are indicated by A–G (see Table I) [4,25,26].

detected; in the samples investigated no *O*-acetylated sialic acids were found. The mass spectra were interpreted using previously described fragmentation rules, as summarized in Table I [4,25,26].

In conclusion, B lymphocytes contain both Neu5Ac and Neu5,9Ac_2 and T lymphocytes Neu5Ac only, as was shown in four independent experiments. Since appreciable amounts (about 50%, [3]) of the *O*-acetyl groups are hydrolysed during the isolation procedure of sialic acids, the relative amount of Neu5,9Ac_2 in native B lymphocytes can be assumed to be in the range of 70–100%. The fact that no *O*-acetylated sialic acids could be detected in the sialic acid fraction from T lymphocytes casts some doubt on the accuracy of the histochemical determination of the degree of *O*-acetylation of cell membrane sialic acids [14,15].

Little is known of the biological significance of *O*-acetylation of sialic acid side-chains. It should be mentioned that the action of sialidases on *O*-acetylated sialic acids is reduced [27]. Furthermore, *O*-acetyl groups attached to colominic acid were found to specifically influence the antigenicity of this bacterial product [28]. It is tempting to speculate that the *O*-acetylation of sialic acids may influence the hydrophobicity and conformation of glycoconjugates and thus the cell membrane architecture. Detection of *O*-acetylated sialic acids in cells of such great biological importance as lymphocytes may help to advance further elucidation of the role of *O*-acetylated sialic acids.

TABLE I

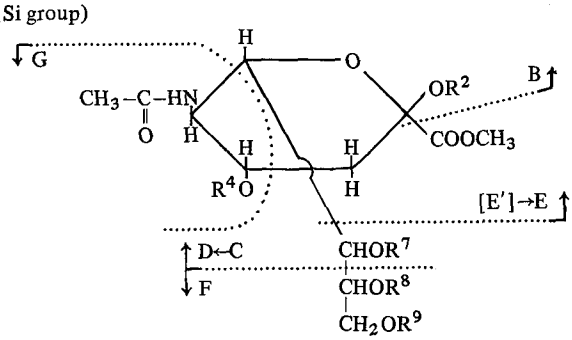
SPECIFIC FRAGMENT IONS A–G IN THE MASS SPECTRA OF THE PERTRIMETHYLSILYL DERIVATIVES OF THE METHYL ESTERS OF THE SIALIC ACIDS WITH R_{Neu5Ac} 1.00 AND 1.13 (RELATIVE TO THE TRIMETHYLSILYLATED DERIVATIVE OF THE METHYL ESTER OF Neu5Ac)

For a discussion of the applied mass spectrometric method, see Refs. 4, 25, 26.

Neu5Ac (R_{Neu5Ac} 1.00)	Neu5,9Ac_2 (R_{Neu5Ac} 1.13)	Explanation
668	638	A: M–CH_3 (from Me_3Si-group)
624	594	B: M–$COOCH_3$
478	478	C: M–$CHOR^8CH_2OR^9$
298	298	D: M–$CHOR^8CH_2OR^9$–R^2OH–R^4OH
317	317	E: M–$CHOR^7CHOR^8CH_2OR^9$–CH_3CONH_2
205	175 [a]	F: $CHOR^8CH_2OR^9$
173	173	G: $CH_3CONHCHCHOR^4$

[a] If C-8 bears an *O*-acetyl group, this fragment ion is hardly formed [4,25]. R^2, R^4, R^7–R^9 = Me_3Si- in the case of NeuAc; R^2, R^4, R^7, R^8 = Me_3Si- and R^9 = CH_3CO- in the case of Neu5,9Ac_2.

A: M minus CH_3
(from Me_3Si group)



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