Carbohydrate Research, 183 (1988) 89–95
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SIALIC ACIDS IN HUMAN LYMPHOCYTES. QUALITATIVE AND QUANTITATIVE ALTERATIONS IN CANCER CASES*

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(Received November 16th, 1987; accepted for publication in revised form, March 9th, 1988)

ABSTRACT

Sialic acids, hydrolyzed from human lymphocytes, were determined, in the nanomole range, with a modified form of the periodic acid–thiobarbituric acid assay and liquid chromatography. The l.c. separations were carried out with two different systems, firstly an Aminex HPX-72 S anion-exchange resin and a 0.15M ammonium sulfate mobile phase, and secondly an amine phase (5 μm) and an acetonitrile–phosphate buffer as mobile phase. The lymphocytes of cancer-stricken persons showed an evident rise of the sialic acid content, combined with a shift of the sialic acid distribution to higher *O*-acetylated derivatives, as compared to the controls.

INTRODUCTION

The surface of many types of cells carries glycoproteins and glycolipids as constituents of receptor molecules. These glycoconjugates contain, in terminal position, neuraminic acid derivatives (sialic acids), which are supposed to have a great importance in the information function of the plasma membrane[1–3].

In the reports of the sialic acid content of lymphocytes, a group of immune cells, most of the authors[4–7] estimated a sialic acid content lower on B lymphocytes than on T lymphocytes. Kamerling *et al.*[8], however, found a higher amount of sialic acids on B lymphocytes, combined with a shift to higher *O*-acetylated derivatives. In cancer cases, it is only known[9–11] that the neuraminic acid content of the tumor cell surface is quantitatively changed, but only little is known[12] about the content of sialic acids on lymphocytes, generated by the immune response in nonlymphatic malignant diseases.

Therefore, we isolated lymphocytes from human blood samples of healthy and cancer persons to determine the total sialic acid content and to compare the

*Part of this work has been presented at the IXth International Symposium on Glycoconjugates, Lille, France, July 6–11, 1987. This work was supported by Deutsche Forschungsgemeinschaft (Fa 45/4) and Fonds der Chemischen Industrie.
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0008-6215/88/$ 03.50

distribution of the neuraminic acid derivatives on their surface. Mild acid hydrolysis of these isolated cells gave free sialic acids together with complex mixtures of carbohydrates and chemically related substances.

There are only very few efficient methods for the separation and determination of sialic acids, when only small amounts are available. Special colorimetric tests[3–15] and liquid chromatography on ion-exchange resins[16–18], or on silica phases chemically modified with covalently-bound polar groups[3,19,20] were used to analyze sialic acids in the micro- to nano-mole range[21].

We describe, herein, the use of a combination of these microanalytical methods for determining the content and distribution of sialic acids in a very low number of lymphocytes obtained from healthy donors and cancer patients.

EXPERIMENTAL

Isolation of lymphocytes. — Human lymphocytes were isolated from fresh heparinized blood samples (7–10 mL) of healthy donors (n 46), or from cancer patients (melanoma and mammary carcinoma at different stages) without chemotherapy (n 55). The lymphocytes were separated accordingly to Boyum[22], followed by centrifugation (400*g*) in a Ficoll gradient. After several washings with phosphate-buffered saline, the lymphocytes were counted by microscopy, suspended in 1 mL of distilled water, and lyophilized. The lymphocyte number of the prepared blood samples depended on the state of health of the patients and varied between 0.2 and 1.3 $\times$ 10^6 cells per mL. The yield of lymphocytes was 50–60%, or 0.1–0.7 $\times$ 10^6 cells/mL of blood. The preparations contained 0.5–7 $\times$ 10^6 cells, isolated from samples of 7–10 mL of blood.

Colorimetric determination of sialic acids. — The lyophilized lymphocyte sample was suspended in 0.5M H_2SO_4 (200 μL) and incubated for 15 min at 95°. 1.1M NaOH (200 μL) was added and the slightly alkaline mixture was kept for 1 h at room temperature to remove *O*-acetyl groups, and then lyophilized.

The sialic acid content of the lymphocytes was estimated with the periodic acid–thiobarbituric acid assay[15], adapted to lymphocyte preparations as follows. A sample of hydrolyzed lymphocytes in distilled water (100 μL) was mixed with 0.1M $NaIO_4$ in M H_2SO_4 (50 μL) and incubated for 20 min at 30°. The reaction was terminated by the addition of 6% $NaAsO_2$ in 0.5M HCl (100 μL), followed by the addition of 2% thiobarbituric acid (250 μL), adjusted to pH 8–9 with NaOH. The mixture was incubated for 30 min at 95°. The color of the developed chromophore was intensified by the addition of dimethyl sulfoxide (500 μL). The mixture was centrifuged for 3 min (8000*g*).

The sialic acid content was determined by absorption at 500 nm after calibration with *N*-acetyl-9-*O*-acetylneuraminic acid and *N*-acetylneuraminic acid (Sigma, Heidelberg, F.R.G.) under the test conditions, and with *N*-acetylneuraminic acid without hydrolysis and alkaline treatment in the range of 0.5–10 μg/100 μL.

Liquid chromatography analysis of sialic acids. — The l.c. analysis was car-

ried out with a Waters model 6000A pump (Waters Associates, Eschborn, F.R.G.), a Rheodyne 20-μL injection loop (Rheodyne, Berkeley CA, U.S.A.), a Perkin–Elmer LC75 u.v.-visible spectrophotometer (Perkin–Elmer, Norwalk, CT, U.S.A.), a Chromato-Integrator Hitachi D-2000 (Merck, Darmstadt, F.R.G.) and columns of (*a*) Aminex HPX-72 S Organic Base Analysis (300 × 7.8 mm) Micro-Guard Anion SA Refill Cartridge (Bio-Rad Labs, Richmond, CA, U.S.A.), and (*b*) LiChrosorb NH_2, 5-μm (Merck, Darmstadt, F.R.G.).

The lyophilized lymphocytes (0.5–7 × 10^6 cells of 7–10 mL of blood) were suspended in distilled water (1 mL), acidified with formic acid to pH 2, and incubated for 1 h at 70°, followed by centrifugation (8000*g*) and lyophilization. The hydrolyzed lymphocyte preparation was dissolved in bidistilled water (100–200 μL), followed by centrifugation (8000*g*) for 5 min, and decantation afterwards. An aliquot (10 μL) was injected into the column.

Column (*a*) was eluted with degassed 0.3M $(NH_4)_2SO_4$ at 1.2 mL/min, isocratic, and room temperature, and column (*b*) with 3:2 (v/v) acetonitrile Grade S (Rathburn, Walkersburn, G.B.)–15mM phosphate buffer, pH 5.2, at 1.0 mL/min isocratic, and room temperature. The sialic acids were detected at 200 nm.

The identification of the peaks was made by cochromatography with authentic sialic acids and addition of reference substances to the samples.

Mixtures of *N*-acetylneuraminic acid, *N*-acetyl-2,3-didehydro-2-deoxy-neuraminic acid (Neu5Ac2en), *N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac_2), *N*-acetylgalactosamine, and *N*-acetylglucosamine, in the range of 1–5 μg, were used for calibration. The calibration curves were obtained by relating the peak area to the amount of sample.

N-Acetyl-9-*O*-acetylneuraminic acid was isolated from bovine submandibular glands[23], and *N*-acetyl-2,3-didehydro-2-deoxyneuraminic acid was a byproduct of the isolation and could be separated by column chromatography on cellulose[23].

RESULTS AND DISCUSSION

The determination of sialic acids on lymphocytes was difficult, because it was nearly impossible to obtain large amounts of fresh blood from cancer patients. The blood samples contained ~7–10 mL of blood, which allowed a yield of 0.5–7 × 10^6 lymphocytes. In contrast to previous authors[7,8,12], who reported cell preparations in the range of 10^9 lymphocytes (from 1 L blood), we had to adapt the sialic acid determination to the range of 10^6 lymphocytes. The total lymphocyte fractions was used, because the fraction of B lymphocytes is very small (~20%) and their sialic acid content is lower than that of T lymphocytes, as shown in earlier publications[4–7] and confirmed by our own experiments.

The modified form of the periodic acid–thiobarbituric acid assay[15] is very sensitive when dimethyl sulfoxide is used to stabilize the chromophore and to intensify the color. The test was optimized for the lymphocyte samples and allowed the determination of sialic acids in the range of 0.5–10.0 μg. The hydrolysis was

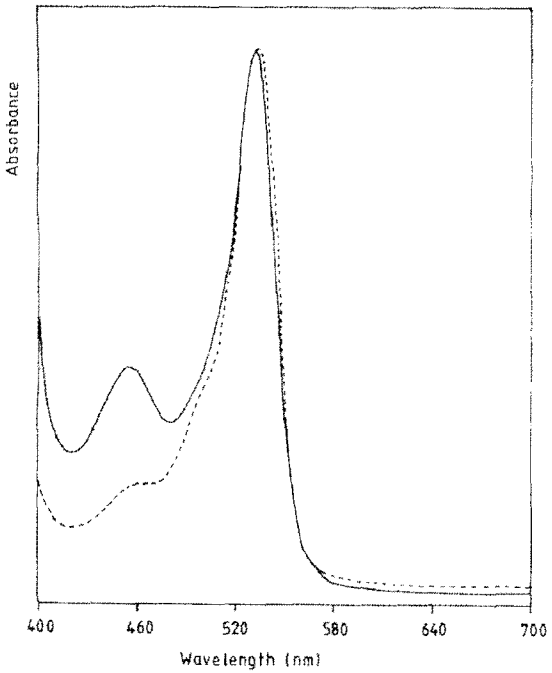

Fig. 1. Absorption spectra of the thiobarbituric acid chromophore given by the periodic acid–thiobarbituric acid assay with two lymphocyte samples: (-----) Lymphocyte sample which delivered only the thiobarbituric acid chromophore; (——) Lymphocyte sample which delivered the thiobarbituric acid chromophore together with a further chromophore around 460 nm.

carried out with 0.5M sulfuric acid at 95° for a maximum of 15 min. These were the optimal reaction conditions for obtaining the rapid liberation of sialic acids without excessive loss. By use of this method, it was impossible to distinguish between *O*- and *N*-acetylated sialic acids. The *O*-acetylated derivatives gave different reaction courses and color intensities. The *O*-acetyl groups could be eliminated with an alkaline treatment, which converted these sialic acids into compounds reactive with the periodic acid–thiobarbituric acid reagent.

The steps of the reaction, lymphocyte preparation, hydrolysis, *O*-deacetylation with a slight excess of NaOH, and determination of sialic acids were carried out

TABLE I

SIALIC ACID LEVEL AND DISTRIBUTION ON LYMPHOCYTES OF PATIENTS AND CONTROLS

Type	*Number*	*Sialic acids (μg/10⁶ cells)*			*Sialic acids (%)*	
		NeuAc (Colorim.)	*Neu5Ac (L.c.)*	*Neu5,9Ac₂ (L.c.)*	*Neu5Ac*	*Neu5,9Ac₂*
Controls	46	1.5 ±0.3	1.5 ±0.2	Traces	100	0.0
Melanomas	53	2.4 ±0.3	<0.8	2.1 ±0.2	23 ±2.9	77 ±3.7
Mammary carcinomas	21	2.6 ±0.2	<0.5	2.5 ±0.5	13 ±2.1	87 ±2.9

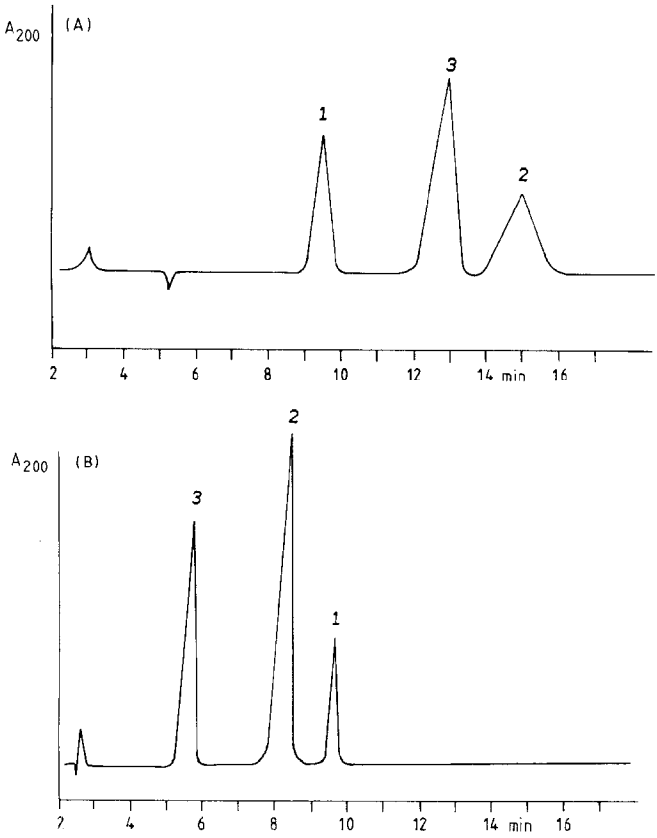

Fig. 2. Calibration of l.c. analysis: Neu5Ac (1), Neu5Acen (2), and Neu5.9Ac$_2$ (3). (A) HPX-72 S anion-exchange resin and (B) Lichrosorb NH_2 (5 μm).

in one reaction tube in order to avoid a loss of materials. The absorption spectra of the reaction mixtures showed a maximum at 535–550 nm and also, in some cases, a smaller peak at ~460 nm (Fig. 1). This second maximum had no influence on the photometric determination of the neuraminic acid chromophore. Other sugars, such as mannose, galactose, or *N*-acetylglucosamine, showed no interfering reactions under these conditions. Calibration with *N*-acetylneuraminic acid in the range of 0.5–10.0 μg gave a reproducible linear relation.

The present determinations showed that the sialic acid content of the lymphocytes of cancer-stricken persons is raised by ~60–70% as compared to the controls (Table I). In contrast to the colorimetric method used, liquid chromatography distinguished *N*- from *O*-acetylated neuraminic acid derivatives (Fig. 2). The two systems used, anion-exchange resin and amine phase, gave good reproducible results for the determination and separation of sialic acids.

The mild hydrolysis of the lymphocyte preparations with formic acid yielded ~90–95% of free sialic acids with mixtures of chemically related substances. Centrifugation, followed by lyophilization of the reaction mixtures, removed the

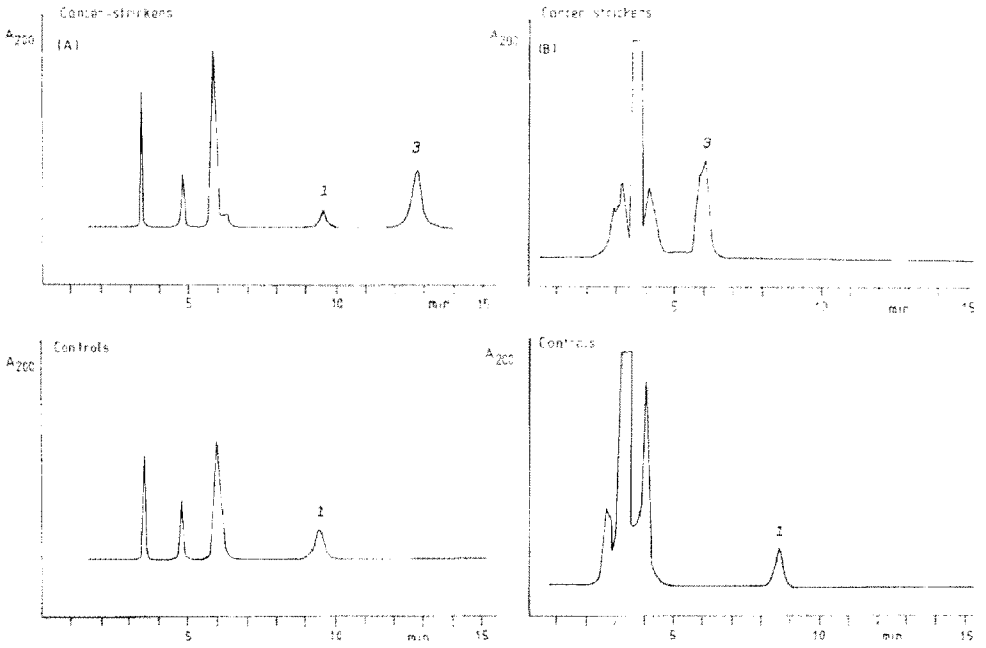

Fig. 3. L.c. analysis of sialic acids on human lymphocytes: (A) HPX-72 S anion-exhange resin, and (B) Lichrosorb NH_2 (5 μm).

denaturated cell constitutents and formic acid. Comparison with reference substances showed a very slight loss of *O*-acetyl groups, which was not taken into account in our experiments.

In accordance with the colorimetric estimations, the l.c. analysis showed a rise in the total sialic acid content at the lymphocyte surface of the cancer patients, but l.c. also showed that nearly 80% of the sialic acids were *O*-acetylated (Table I). A high amount of *N*-acetyl-9-*O*-acetylneuraminic acid was present, combined with a decrease of *N*-acetylneuraminic acid (Fig. 3a). In several cases, there was no *N*-acetylneuraminic acid detectable (Fig. 3b). It is not clear whether the high content of *N*-acetyl-9-*O*-acetylneuraminic acid was a product of *O*-acetyl-group migration from O-7 or -8 as reported by Schauer *et al.*[24].

The microanalytical measurements with lymphocytes, described herein, gave evidence that melanoma and mammary carcinoma patients show a significantly higher amount of acylneuraminic acids on their lymphocytes, with an increase of *O*-acetylated derivatives. The lymphocytes of the controls contained only *N*-acetylneuraminic acid with traces of *N*-acetyl-9-*O*-acetylneuraminic acid or other derivatives. This observation offers the potential opportunity to develop a clinical method to distinguish between cancer and noncancer patients.

ACKNOWLEDGMENTS

The authors thank Prof. Dr. Helmut Stickl and Dr. Walter Huber, Institut

für Toxikologie und Umwelthygiene, Abteilung für Impfwesen, Technische Universität, München, for making available patients material and for preparing the lymphocytes. They thank also Prof. Dr. Michael Ashworth for reading the manuscript.

REFERENCES

1 H. FAILLARD AND R. SCHAUER, in A. GOTTSCHALK (Ed.), *Glycoproteins*, New York, 1972, pp. 1240–1267.
2 A. ROSENBERG AND C.-L. SCHENGRUND (Eds.), *Biological Role of Sialic Acids*, Plenum Press, New York, 1976.
3 R. SCHAUER, *Adv. Carbohydr. Chem. Biochem.*, 40 (1982) 132–234.
4 J. N. MERISHI AND K. ZEILLER, *Brit. Med. J.*, 1 (1974) 360–362.
5 Y. REISNER, A. RAVID, AND N. SHARON, *Biochem. Biophys. Res. Commun.*, 72 (1976) 1585–1591.
6 B. A. SMITH, B. R. WARE, AND R. A. YANKEE, *J. Immunol.*, 120 (1978) 921–926.
7 R. O. KENNEDY, H. SMITH, A. CORRIGAN, AND M. CLYNES, *Eur. J. Cancer Clin. Oncol.*, 18 (1982) 437–446.
8 J. P. KAMERLING, J. MAKOVITZKY, R. SCHAUER, J. F. G. VLIEGENTHART, AND M. WEMBER, *Biochim. Biophys. Acta*, 714 (1982) 351–355.
9 G. ASHWELL AND A. G. MORELL, *Adv. Enzymol.*, 41 (1974) 99–128.
10 M. J. VALAREM, J. JOUNNEAU, AND R. BOURRILLON, *Biochem. Biophys. Res. Commun.*, 98 (1981) 7–14.
11 J. A. ALDAHEFF AND R. T. HOLZINGER, *Int. J. Biochem.*, 14 (1982) 119–126.
12 R. O'KENNEDY, H. SMITH, A. CORRIGAN, AND M. CLYNES, *Eur. J. Cancer Clin. Oncol.*, 18 (1982) 1163–1169.
13 L. SVENNERHOLM, *Biochim. Biophys. Acta*, 24 (1957) 604–611.
14 L. WARREN, *J. Biol. Chem.*, 234 (1959) 1971–1975.
15 L. SKOZA AND S. MOHOS, *Biochem. J.*, 159 (1976) 457–463.
16 P. JANDERA AND J. CHARACEK, *J. Chromatogr.*, 98 (1976) 55–104.
17 H. BAUER AND W. VOELTER, in A. HENSCHEN, K. P. HUPE, F. LOTTSPEICH, AND W. VOELTER (Eds.), *High Performance Liquid Chromatography in Biochemistry*, VCH Verlagsgesellschaft, Weinheim–Deerfield Beach, 1985.
18 V. MEYER, *Praxis der Hochdruckflüssigkeitschromatographie*, Diesterweg-Salle-Sauerländer, Frankfurt/Main–Berlin–München, 1980.
19 H. ENGELHARDT AND U. D. NEUE, *Chromatographia*, 15 (1982) 403–408.
20 P. ORTH AND H. ENGELHARDT, *Chromatographia*, 15 (1982) 91–96.
21 H. FIEDLER AND H. FAILLARD, *Chromatographia*, 20 (1985) 231–234.
22 A. BOYUM, *Scand. J. Clin. Invest.*, 21 Suppl., 97 (1968) 31–50.
23 G. REUTER, R. PFEIL, S. STOLL, R. SCHAUER, J. P. KAMERLING, C. VERSILIUS, AND J. F. G. VLIEGENTHART, *Eur. J. Biochem.*, 134 (1983) 139–143.
24 J. P. KAMERLING, R. SCHAUER, A. K. SHUKLA, S. STOLL, H. VAN HALBEEK, AND F. G. VLIEGENTHART, *Eur. J. Biochem.*, 162 (1987) 601–607.