determined as KDNα2→3Galβ1→4Glcβ1→1ceramide, (KDN)GM3 (1). Following this finding, two minor KDN-gangliosides were isolated from ovarian fluid of rainbow trout and their structures were established by chemical degradation, methylation analysis and ^{1}H NMR spectroscopy as (KDN)GD1a and (9-*O*-AcKDN)GD1a (2). In addition to these gangliosides, several other minor KDN-gangliosides have been identified. The results of these studies have suggested the general existence of KDN-gangliosides in a wider range of animal tissues and the failure to find them was apparently due to the methodology used.

To search for KDN-gangliosides, we have set out to develop sensitive probes and availability of different types of KDN-glycoconjugates has enabled us to generate monoclonal antibodies which specifically recognize KDN-glycan epitopes such as KDNα2→3Galβ1→ and KDNα2→8KDNα2→ sequences (3). With these sensitive probes (mAb.kdn3G and mAb. kdn8kdn), the rapid detection not only of major but also minor KDN-glycoconjugates should be feasible, and low relative molecular mass material extracted by chloroform/ methanol from various animal origins was tested for recognition by mAb.kdn3G and mAb.kdn8kdn. We have indeed preliminary evidence for the presence of mAb.kdn3G-positive antigens in some tissues.

Our interest in KDN-gangliosides has been extended to the synthesis of neo-KDN-gangliosides, i.e., potentially useful analogues for the corresponding gangliosides, by transferring the KDN residue(s) from CMP-KDN to a variety of Neu5Ac-gangliosides and asialo-gangliosides with the aid of the KDN-transferase activities that we recently identified (4).

(1) Yu Song, K. Kitajima, S. Inoue and Y. Inoue (1991) *J. Biol. Chem.*, **266**, 21929–21935; (2) Yu Song, K. Kitajima, S. Inoue, H. Muto, T. Kasama, S. Handa and Y. Inoue (1993) *J. Biol. Chem.* submitted; (3) Yu Song, K. Kitajima and Y. Inoue (1993) *Glycobiology*, 3, in press; (4) T. Terada, Yu Song, T. Angata, S. Kitazume, K. Kitajima, S. Inoue, F.A. Troy and Y. Inoue (1993) *In the 12th International Symposium on Glycoconjugates.*

S18.14

Detection of *N*-acetyl-9-*O*-Acetylneuraminic Acid on Human Lymphocytes

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Regulatory functions of sialic acid modifications for cellular interactions have been implicated for many years. In man, *N*-acetylneuraminic acid is the predominant species. 9-*O*-Acetylated (Neu5,9Ac_2) or 9-*O*-lactoylated (Neu5Ac9Lt) derivatives have also been found. Neu5,9Ac_2 is abundant in some tumors and considered as component of tumor-associated antigens in melanoma. In this study we wanted to investigate the expression of Neu5,9Ac_2 on human lymphocytes.

Mononuclear leukocytes were isolated from fresh buffy coat obtained from healthy donors by density gradient centrifugation. B- and T-cells were then isolated using positive selection on magnetic beads coated with specific monoclonal antibodies (anti CD-19 and CD-2, respectively). The cells were lyzed, and sialic acids released from the cell pellets by mild acid hydrolysis with formic acid (pH 2, 1 h, 70°C). Sialic acid analysis was performed after derivatization with 1,2-diamino-4,5-methylene-dioxybenzene by fluorimetric HPLC.

In total mononuclear cells, in B-cells as well as in T-cells, Neu5,9Ac_2 was detected in low quantities (5–10% of total sialic acids) together with Neu5Ac. To ensure the identification as Neu5,9Ac_2, before derivatization and analysis hydrolyzed sialic acids were treated with alkali (0.1 M NaOH, 45 min, 4°C), influenza C virus or sialate lyase (both at pH 7.4, 1 h, 37°C), or buffer alone (pH 7.4, 1 h, 37°C). Treatment with influenza C virus that hydrolyzes selectively 9-*O*-acetyl groups from sialic acids resulted in nearly complete reduction of the corresponding peak in the chromatogram; the same effect was observed after treatment with alkali that saponifies this ester group. Incubation with the lyase resulted in a significant decrease of the peak corresponding to Neu5Ac and a lower reduction of that one attributed to Neu5,9Ac_2, which fits with the known specificity of this enzyme.

In conclusions, Neu5,9Ac_2 is present on normal human B- and T-cells. Whether its occurrence is restricted to subpopulations of these cells remains to be elucidated.

S18.15

The Key Enzyme Which Regulates CMP-*N*-Acetylneuraminic Acid Hydroxylation is a Novel Protein

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We have reported that cytidine monophospho-*N*-acetylneuraminic acid (CMP-NeuAc) hydroxylation is carried out by a multi-component system which involves cytochrome b_5, an NADH-dependent cytochrome b_5 reducing factor, and CMP-NeuAc hydroxylase (the terminal enzyme)[1,2]. The CMP-NeuAc hydroxylase which regulates overall velocity of the hydroxylation reaction was purified to homogeneity from mouse liver cytosol. The purified enzyme was a single polypeptide whose molecular mass was 65 kDa on SDS-PAGE and 58 kDa on gel permeation chromatography. There is no indication of the presence of a heme prosthetic group in the enzyme. Km value of the enzyme for CMP-NeuAc is very low and the addition of CMP-NeuAc greatly stabilized the enzyme, suggesting that binding of CMP-NeuAc to the enzyme produces its conformational change. The molecular activity of CMP-NeuAc hydroxylase ($\sim 10^3$ molecule/min) is much lower than that of NADH-dependent cytochrome b_5 reductases, indicating that the hydroxylase is a rate limiting factor in the hydroxylation of CMP-NeuAc. Amino acid sequencing of peptides obtained from the purified enzyme suggests that the enzyme is a novel protein.

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2. Kawano, T. *et al.* (1993) *Glycoconjugate J.* in press.

S18.16

Enzymatic Synthesis of Neoglycoconjugates Containing KDN Residues: Use of CMP-KDN Synthetase and KCN-Transferase from Rainbow Trout Testis for Synthesis of KDN-Glycoproteins and KDN-Glycosphingolipids