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Antiplatelet "Hybrid" Peptides Analogous to Receptor Recognition Domains on γ and α Chains of Human Fibrinogen†

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ABSTRACT: Platelet receptor recognition domains are located on the γ and α chains of human fibrinogen. The former encompasses residues 400–411 [Kloczewiak, M., Timmons, S., Lukas, T. J., & Hawiger, J. (1984) *Biochemistry 23*, 1767], and the latter is present in two loci on the α chain (α95–97 and α572–574) [Hawiger, J., Kloczewiak, M., Bednarek, M. A., & Timmons, S. (1989) *Biochemistry* (first of three papers in this issue)]. Peptide γ400–411 (HHLGGAKQAGDV) inhibited aggregation of ADP-treated platelets mediated not only by γ-chain but also by α-chain multimers. Peptide α572–575 (RGDS) inhibited aggregation of platelets mediated by α-chain as well as γ-chain multimers. These results indicate that the platelet receptor for fibrinogen is isospecific with regard to the domain present on α and γ chains. Subsequent "checkerboard" analysis of combinations of γ400–411 and α572–575 showed that the inhibitory effect toward binding of ^{125}I-fibrinogen was additive rather than synergistic. Next, a series of "hybrid" peptides was constructed in which the α-chain sequence RGDF (α95–98) replaced the carboxy-terminal segment of γ408–411. The dodecapeptide HHLGGAKQRGDF was inhibitory with concentration, causing 50% inhibition of binding (IC_{50}) at 6 μM, 5 times more potent than γ400–411. The shorter peptides AKQRGDF and KQRGDF were also more inhibitory than γ400–411. The second series of hybrid peptides was constructed with the α-chain sequence RGDS preceding the sequence of γ400–411 or sequence RGDV following it. The hybrid peptides YRGDSQHLGGAKQAGDV and HHLGGAKQAGDVGRGDV had the same reactivity toward platelet receptors as γ400–411. Alternatively, the hybrid peptide HHLGGAKQAGDSRGDV was 3 times more potent (IC_{50} = 10 μM). Hybrid peptides mimicking the γ-chain domain represent a new class of inhibitors with enhanced potency toward the platelet receptor for fibrinogen.

The recognition of human platelet receptors by fibrinogen plays a key role in the binding of this adhesive protein to activated platelets (Hawiger, 1987). We have shown that both the γ chain and the α chain of human fibrinogen directly interact with ADP-activated human platelets (Hawiger et al., 1982). The γ-chain domain is formed by a sequence of 12 carboxy-terminal residues, γ400–411 (Kloczewiak et al., 1984, 1989). The amino acid sequence of the α chain contains two loci at residues 95–97 and 572–574 reactive with human platelet receptors (Hawiger et al., 1989). Since there is no apparent homology between the carboxy-terminal segments of the γ chain and the α chain (Doolittle, 1984), the issue arose whether γ and α chains interact with separate receptors on human platelets or different sites of the same receptor.

The human platelet receptor for fibrinogen has been identified as the glycoprotein IIb–IIIa (GPIIb–IIIa)[1] heterodimer

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[1] Abbreviations: GPIIb–IIIa, glycoproteins IIb and IIIa; IC_{50}, concentration of peptide causing 50% inhibition of binding.

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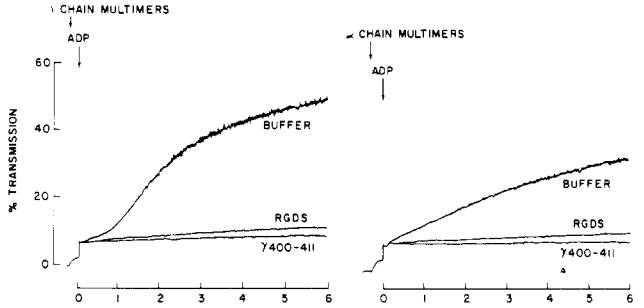

FIGURE 1: Cross-inhibition of γ-chain- and α-chain-mediated platelet aggregation by synthetic peptide analogues of sequence present in either chain. γ-Chain multimers (5 μM) or α-chain multimers (6 μM) derived from human fibrinogen were added to human platelets separated from plasma proteins and treated with 5 μM ADP. In control samples containing buffer as a diluent for peptides, aggregation was observed. In samples containing synthetic peptides γ400–411 (150 μM) or RGDS (115 μM), aggregation was blocked.

which belongs to an integrin superfamily of cell adhesion molecules (George et al., 1984; Hynes, 1987). Cross-linking experiments with fibrinogen and peptides patterned on the γ-chain domain and sequence RGDS, analogous to the "cell adhesion site" of fibronectin, showed that both GPIIb and GPIIIa subunits of the fibrinogen receptor were involved (Bennett et al., 1982; Santoro & Lawing, 1987; D'Souza et al., 1988). To examine the issue of specificity of the recognition domains on the γ chain and α chain of human fibrinogen, we undertook a series of functional experiments in which γ and α chains were directly interacting with ADP-treated platelets and synthetic peptide analogues were tested for cross-inhibition of platelet aggregation.

The next question addressed by us was what type of inhibition can be attained with a combination of free peptides representing domains on γ and α chains of human fibrinogen. Finally, we embarked on the design of "hybrid" peptides encompassing sequences from α and γ chains. Our objective was to develop peptides retaining essential features of the fibrinogen-specific γ-chain domain while endowed with higher inhibitory potency toward human platelets than dodecapeptide γ400–411.

Materials and Methods

All the materials and procedures employed in this study have been fully described in previous publications from this laboratory, including the preparation and labeling of fibrinogen (Timmons & Hawiger, 1986) as well as synthesis of peptides and their purification and analysis, the experimental system for binding ^{125}I-fibrinogen to platelets, and platelet aggregation with isolated fibrinogen chain multimers (Hawiger et al., 1982, 1989; Kloczewiak et al., 1984).

Results

Interaction of Isolated Fibrinogen Chains with ADP-Stimulated Platelets and the Inhibitory Effect of Peptides Corresponding to Recognition Domains on γ and α Chains. Purified γ and α chains of human fibrinogen prepared in multimeric forms were tested for their mediation of aggregation of ADP-stimulated human platelets separated from plasma proteins. As shown in Figure 1, aggregation tracings represent a reaction mediated by the γ chain (5 μM) and the α chain (6 μM). When a synthetic peptide analogue of the receptor recognition domain of the γ chain (γ400–411) was added to platelets prior to the γ chain, aggregation was inhibited. Unexpectedly, addition of the synthetic peptide RGDS corresponding to sequence 572–575 on the α chain resulted in a similar inhibition of γ-chain-mediated platelet aggregation, although this chain does not have a homologous sequence (Doolittle, 1984). Similarly, addition of a synthetic peptide analogue of the γ chain (γ400–411) to a system containing the α chain and ADP-treated platelets prevented their aggregation. These experiments indicate that platelet receptors interacting with γ and α chains of human fibrinogen can be blocked interchangeably by synthetic peptides representing sequences on either chain.

Table I: Checkerboard Analysis of the Inhibitory Potency of a Combination of Dodecapeptide γ400–411 and RGDS toward ^{125}I-Fibrinogen Binding to Platelets[a]

[γ400–411] (μM)	inhibitory potency (%) at [RGDS] (μM) of			
	0	5	10	15
0		30	46	58
5	2	28	54	65
15	10	55	60	72
30	40	66	65	83
60	61	73	77	93

[a] Binding of ^{125}I-fibrinogen to ADP-treated human platelets separated from plasma proteins was done in the presence of synthetic peptides γ400–411 and RGDS used solo (concentration indicated as zero for a second peptide) or in combinations of different concentrations (micromolar) of two peptides. The observed inhibition of ^{125}I-fibrinogen binding (percent) is calculated in reference to control containing diluent for synthetic peptides used.

Effect of the Combination of Synthetic Peptide Analogues of γ- and α-Chain Sequences on Binding of ^{125}I-Fibrinogen to ADP-Stimulated Platelets. Since the aggregation of human platelets mediated by γ and α chains was cross-inhibited by synthetic peptides related to both chains, it was of interest to examine whether their respective domains interact synergistically with the platelet receptor for fibrinogen (Berenbaum, 1985). A checkerboard analysis of combinations of both peptides according to the scheme illustrated in Table I was performed. The concentrations of dodecapeptide, corresponding to γ400–411, ranged from 5 to 60 μM, and tetrapeptide RGDS concentrations, corresponding to 572–575, ranged from 5 to 15 μM. The (IC_{50}) concentration causing 50% inhibition of ^{125}I-fibrinogen binding to platelets in these experiments was 35 μM for γ400–411 and 12 μM for RGDS. As shown in Table I, the inhibition of binding of ^{125}I-fibrinogen by combinations of both peptides corresponded to the sum of inhibitory activity of peptides measured individually. The expected sums of inhibition (percent) by individual peptides were plotted against the observed values obtained with combinations of peptides. They aligned along the line derived from the calculated sum of inhibitions determined for individual peptides (Figure 2). This relationship does not indicate a synergistic effect for the two peptides studied in their linear concentration range up to IC_{50} values. It is noteworthy that the inhibitory activity of peptides used at concentrations higher than the IC_{50} produced less inhibition than expected from the additive effect.

Construction of Linear Peptides Containing Sequences Present in γ400–411 and α95–98 of Human Fibrinogen. The existence of two platelet receptor recognition domains on the α chain and one on the γ chain of human fibrinogen (Hawiger et al., 1989; Kloczewiak et al., 1984) led us to construct a series of linear peptides containing these domains. The inhibition curves of these hybrid peptides are shown in Figures 3 and 4 and the results in Table II. We synthesized peptides of increased length with residues from γ400–406 followed by the sequence RGDF (α95–98). As a result, three peptides were constructed: KQRGDF, AKQRGDF, and

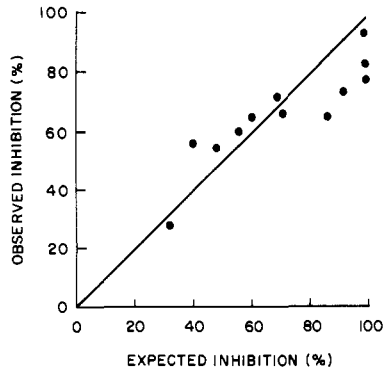

FIGURE 2: Plot of observed vs expected inhibition (closed circles) of binding of ^{125}I-fibrinogen (0.17 μM) to ADP-treated platelets separated from plasma proteins in the presence of a combination of synthetic peptides γ400–411 and RGDS. The concentrations of both peptides are indicated in Table I. The solid line represents the calculated sum of inhibitions from each peptide tested solo as indicated in Table I.

Table II: Inhibitory Potency of Synthetic Peptides Containing Sequences from γ and α Chains of Human Fibrinogen with Regard to Its Binding to ADP-Stimulated Human Platelets

peptide	IC_{50} (μM)[a]
YHHLGGAKQAGDV	30
RGDF	2
AGDF	300
KQRGDF	13
AKQRGDF	9
AKQAGDF	35
HHLGGAKQRGDF	6
HHLGGAKQAGDF	100
RGDS	10
YRGDSQHLGGAKQAGDV	30
RGDV	6
HHLGGAKQAGDSRGDV	10
HHLGGAKQAGDVGRGDV	28
CYHHLGGAKQRGDV	5
KQRGDV	12

[a] Concentration of peptide causing 50% inhibition of binding of ^{125}I-fibrinogen (0.15 μM) to human platelets (10^8/0.5 mL) stimulated with ADP (5 μM).

HHLGGAKQRGDF. Their IC_{50} values were 13, 9, and 6 μM, respectively (Figure 3). These peptides are 2–5 times more inhibitory than the dodecapeptide corresponding to the "native" sequence γ400–411 while they maintain structural features of this segment of the γ chain of human fibrinogen.

Construction of Linear Peptides Containing Sequences Present in γ400–411 and α572–575. When the sequence YRGDS was "fused" to the amino terminal of the sequence present in the γ chain (γ400–411), the synthetic peptide YRGDSQHLGGAKQAGDV produced an inhibition curve with an IC_{50} = 30 μM, identical with γ400–411. To produce a hybrid peptide containing the sequence RGD at the carboxy-terminal end, we elected the sequence RGDV in view of the significant role played by Val^{411} in receptor recognition by the γ chain (Kloczewiak et al., 1989). The inhibitory potency of the tetrapeptide RGDV (IC_{50} = 6 μM) was somewhat greater than RGDS (IC_{50} = 10 μM). The hybrid peptide HHLGGAKQAGDSRGDV produced inhibition with IC_{50} = 10 μM, which was 3 times lower than that of native γ400–411 (Figure 4). Similar potency was attained by the shorter hybrid peptide KQRGDV. Another hybrid peptide, HHLGGAKQAGDVGRGDV, had IC_{50} = 28 μM, suggesting that RGDV added to the existing sequence of γ400–411 with carboxy-terminal valine does not enhance the inhibitory potency. However, when RGDV was present as the last four residues in γ400–411, the inhibitory potency (IC_{50} = 5 μM) was 6 times higher than γ400–411.

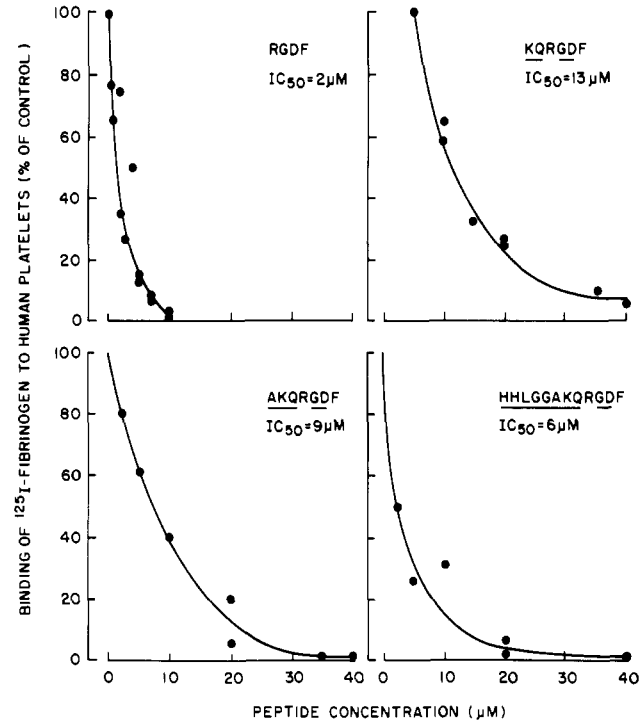

FIGURE 3: Inhibition curves for ^{125}I-fibrinogen binding to ADP-treated platelets separated from plasma proteins in the presence of the synthetic peptide RGDF (α95–98) and hybrid peptides KQRGDF, AKQRGDF, and HHLGGAKQRGDF. The parts of hybrid peptides homologous to γ400–411 are underlined.

Discussion

Our functional data in the platelet aggregation system indicate that receptor recognition domains present on the γ and α chains of fibrinogen seem to interact with the same platelet receptor, because synthetic peptides corresponding to sequences γ400–411 and α572–575 cross-inhibit the interaction of both chains. An alternative interpretation of our results would require the postulate that distinct receptors of γ-chain and α-chain specificity exist on human platelets. Such a postulate cannot be validated because synthetic peptide analogues of sequences present in γ and α chains of human fibrinogen interact with the GPIIb–IIIa complex as documented in cross-linking experiments, elution of GPIIb–IIIa from a peptide affinity column, and hydrodynamic measurement of GPIIb–IIIa (Bennett et al., 1982; Santoro & Lawing, 1987; Lam et al., 1987; Parise et al., 1987; D'Souza et al., 1988). Thus, synthetic peptides interacting with GPIIb–IIIa and blocking γ- and α-chain interaction interchangeably provide evidence for a common receptor recognized by both the γ chain and the α chain. This conclusion does not rule out the possibility of distinct structural domains specific for γ and α chains within the GPIIb–IIIa complex constituting the fibrinogen receptor. Hence, dodecapeptide γ400–411 may react with its "own" domain ("γ site") and RGDF and RGDS with their "own" domain ("α site") in the GPIIb–IIIa complex. Such a bidomainal interaction can still explain the result of cross-inhibition experiments as well as the observation made on RGDS inhibition of binding of human fibrinogen with deleted residues 572–575 in carboxy-terminal segment of the α chain (Peerschke & Galanakis, 1987; Plow et al., 1987).

Having established that the platelet receptor is isospecific with regard to fibrinogen γ and α chains, we focused on the combined reactivity of a mixture of both peptides. The range of concentrations was sufficiently wide to produce, cumula-

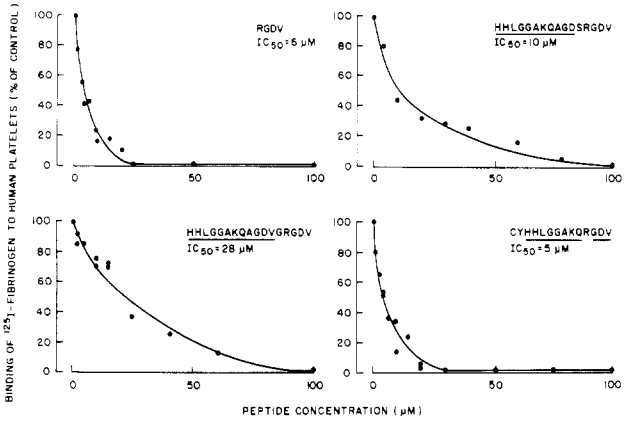

FIGURE 4: Inhibition curves for ^{125}I-fibrinogen binding to ADP-treated platelets separated from plasma proteins in the presence of synthetic peptides RGDV, HHLGGAKQAGDSRGDV, HHLGGAKQAGDVGRGDV, and CYHHLGGAKQRGDV. The parts of hybrid peptides homologous to γ400–411 are underlined.

tively, complete inhibition of binding of ^{125}I-fibrinogen to its receptor. At the lower end of this range, a combination of both peptides produced inhibition corresponding to the sum of individual peptides tested solo. At the higher end of the range, inhibition was less than expected from the additive effect, suggesting that γ400–411 and RGDS may be "cancelled out" in competition with fibrinogen and lose their grip on the fibrinogen receptor. The complexity of the experimental system containing a receptor (platelets) and a hexavalent ligand (fibrinogen), with affinity 3 orders of magnitude higher than those of the two distinct synthetic peptides tested, is not yet amenable for kinetic analysis.

Since combination of the γ and α peptides did not result in a synergistic effect, we embarked on the design of linear peptides which encompass domains from both chains, hoping to obtain a more reactive peptide that still retains fibrinogen γ-chain specificity as a ligand. Among the hybrid peptides, YRGDSQHLGGAKQAGDV did not gain inhibitory potency as compared to γ400–411. On the other hand, placing the sequence RGDV at the carboxy-terminal end of γ400–411 was more effective, especially in hybrid peptides HHLGGAKQAGDSGRGDV and HHLGGAKQRGDV which were 3 and 6 times more inhibitory than γ400–411. This gain is comparable to the level of inhibition of ^{125}I-fibrinogen binding achieved with the tetrapeptides RGDS (α572–575), RGDF (α95–98), and RGDV in these and other experiments (Gartner & Bennet, 1985; Plow et al., 1987). Increasing the number of arginine residues led Ruggeri and colleagues (Ruggeri et al., 1986) to the synthesis of a peptide of high inhibitory potency toward binding of ^{125}I-fibrinogen.

The advantage of fusing dodecapeptide γ400–411, which has no reactivity toward human and bovine vascular endothelial cells (Timmons and Hawiger, unpublished experiments), with RGD-containing peptides is the construction of hybrids of enhanced inhibitory potency yet retaining significant features of the γ-chain domain which appears to be fibrinogen specific and platelet selective (Kloczewiak et al., 1989). This is of significance because GRGDSP has broad reactivity toward integrin receptors on a variety of cells, including vascular endothelial cells, causing their detachment from the extracellular matrix (Hayman et al., 1985; Chen et al., 1987).

Taken together, we have demonstrated that the human platelet receptor for fibrinogen is isospecific with regard to the structural domains on its γ and α chains. These domains gave rise to hybrid peptides which expand a new class of platelet inhibitors acting at membrane receptors for adhesive proteins. Selectivity of these hybrid peptides toward platelet GPIIb–IIIa as compared to integrin receptors on other cells is under study.

Added in Proof

After submission of this manuscript, a paper by Bennet et al. (1988) has also described the additive rather than the synergistic inhibitory effect of a combination of tetrapeptide RGDS and decapeptide γ402–411 on binding of ^{125}I-fibrinogen to ADP-stimulated human platelets.

Acknowledgments

The assistance of Barbara Maki in the preparation of the manuscript is appreciated.

Registry No. HHLGGAKQAGDV, 89105-94-2; RGDS, 91037-65-9; YHHLGGAKQAGDV, 119336-96-8; RGDF, 110697-46-6; AGDF, 119336-97-9; KQRGDF, 119336-98-0; AKQRGDF, 119336-99-1; AKQAGDF, 119337-00-7; HHLGGAKQRGDF, 119337-01-8; HHLGGAKQAGDF, 119336-94-6; YRGDSQHLGGAKQAGDV, 119337-02-9; RGDV, 93674-99-8; HHLGGAKQAGDSRGDV, 119337-03-0; HHLGGAKQAGDVGRGDV, 119366-24-4; CYHHLGGAKQRGDV, 119337-04-1; KQRGDV, 119337-05-2.

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Gangliosides of Murine T Lymphocyte Subpopulations†

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ABSTRACT: Gangliosides from murine T lymphoblasts were analyzed by high-performance thin-layer chromatography followed by in situ neuraminidase treatment and immunostaining of the resulting asialogangliosides and compared with those from thymocytes and cloned T lymphocytes with defined functions. The ganglioside IVNeuGc/Ac-GgOse$_5$Cer (GalNAc-G$_{M1b}$), a marker for T lymphoblasts [Müthing, J., Egge, H., Kniep, B., & Mühlradt, P. F. (1987) *Eur. J. Biochem. 163*, 407–416], was found only in small amounts as the N-acetylated species in gangliosides from thymocytes and a cytolytic T cell clone. Two helper clones expressed this ganglioside like T blasts. The structures of the two major disialogangliosides from T blasts, IVNeuAc,IIINeuAc-GgOse$_4$Cer (G$_{D1\alpha}$ type) with C$_{24:0/24:1}$ and C$_{16:0}$ fatty acids, were elucidated by neuraminidase treatment and immunostaining and by fast atom bombardment mass spectrometry. Gangliosides of this type were detected in thymocytes only in minor amounts, whereas G$_{M1b}$-type gangliosides prevailed in cells from this organ. Analysis of the T lymphoblast gangliosides from six genetically unrelated mouse strains showed that terminally sialylated GgOse$_4$Cer (G$_{M1b}$), IVNeuAc-GgOse$_5$Cer (GalNAc-G$_{M1b}$), and IVNeuAc,IIINeuAc-GgOse$_4$Cer (G$_{D1\alpha}$) were conserved structures in all strains examined. We conclude that maturation or stimulation of T cells may be correlated with elongation of a common G$_{M1b}$-type precursor structure resulting in GalNAc-G$_{M1b}$ or G$_{D1\alpha}$-type gangliosides.

We and others have in the past analyzed and compared the neutral (Gruner et al., 1981; Kniep et al., 1983) as well as sialylated (Schwarting & Gajewski, 1983; Mercurio et al., 1984; Müthing et al., 1987) glycosphingolipids (GSLs)[1] of murine immune cells, in search of GSLs that might be specifically expressed by particular populations or subpopulations of immune cells. Examples of subpopulation-specific GSLs are as follows: globoside, GbOse$_4$Cer, a differentiation marker within the T cell lineage (Mühlradt et al., 1984), asialo-GM$_1$, GgOse$_4$Cer, a marker for natural killer cells (Kasai et al., 1980) and activated T cells (Stout et al., 1987), and Forssman GSL, a marker for mainly spleen macrophages (Bethke et al., 1987). Similarly, the gangliosides IVNeuGc-GgOse$_5$Cer and IVNeuAc-GgOse$_5$Cer[2] appeared specific for blasts of the T lymphocyte lineage and were not detected in B blasts (Müthing et al., 1987).

In continuation and extension of this work, we have now elucidated the structure of the major disialogangliosides from T blasts as IVNeuAc,IIINeuAc-GgOse$_4$Cer and analyzed the gangliosides from thymocytes and cloned T cells with helper and cytolytic functions for the presence of IVNeuAc-GgOse$_5$Cer, G$_{M1b}$-type gangliosides, and IVNeuAc,IIINeuAc-GgOse$_4$Cer, to be able to assign these structures to particular differentiation stages of murine T lymphocytes. We also analyzed the gangliosides from six genetically unrelated mouse strains, in order to determine whether these gangliosides are conserved structures or, like blood group specific GSLs, genetically restricted. The structures IVNeuAc-GgOse$_5$Cer and IVNeuAc,IIINeuAc-GgOse$_4$Cer were found in all strains examined and were found to be expressed mainly in mature peripheral T blasts, but only in traces in thymocytes. IV-

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[1] Abbreviations: ConA, concanavalin A; FAB-MS, fast atom bombardment mass spectrometry; GSL(s), glycosphingolipid(s); HPLC, high-pressure liquid chromatography, HPTLC, high-performance thin-layer chromatography; IL2, interleukin 2; NeuAc, *N*-acetylneuraminic acid; NeuGc, *N*-glycolylneuraminic acid; LacCer, lactosylceramide, Galβ1-4GlcCer. The designation of the following glycosphingolipids follows the IUPAC–IUB recommendations (1977): GgOse$_3$Cer or gangliotriaosylceramide or asialo-G$_{M2}$, GalNAcβ1–4Galβ1–4GlcCer; GgOse$_4$Cer or gangliotetraosylceramide or asialo-G$_{M1}$, Galβ1–3GalNAcβ1–4Galβ1–4GlcCer; GgOse$_5$Cer or gangliopentaosylceramide, GalNAcβ1–4Galβ1–3GalNAcβ1–4Galβ1–4GlcCer; II^{3}NeuAc-GgOse$_4$Cer, G$_{M1}$; IV^{3}NeuAc-GgOse$_4$Cer, G$_{M1b}$; IV^{3}NeuAc-GgOse$_5$Cer, GalNAc-G$_{M1b}$; IV^{3}NeuAc,II^{3}NeuAc-GgOse$_4$Cer, G$_{D1a}$; II^3(NeuAc)$_2$-GgOse$_4$Cer, G$_{D1b}$; IV^3(NeuAc)$_2$-GgOse$_4$Cer, "G$_{D1c}$"; IV^{3}NeuAc,-III^{6}NeuAc-GgOse$_4$Cer, G$_{D1\alpha}$; IV^{3}NeuAc,II^3(NeuAc)$_2$-GgOse$_4$Cer, G$_{T1b}$; IV^3(NeuAc)$_2$,II^3(NeuAc)$_2$-GgOse$_4$Cer, G$_{Q1b}$.

[2] The neuraminic acids of these gangliosides are in all probability α2–3-linked as established by Nakamura et al. (1987) for material from murine spleen in the case of IV^{3}NeuGc-GgOse$_5$Cer. However, in all other cases where no methylation analysis was performed, we intentionally omitted the superscripts indicating the linkage.

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