merman, T. S. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5708.
Santoro, S. A., & Lawing, W. J., Jr. (1987) *Cell 48*, 867.
Timmons, S., & Hawiger, J. (1978) *Thromb. Res. 12*, 297.
Timmons, S., & Hawiger, J. (1986) *Trans. Assoc. Am. Physicians 99*, 226.
Timmons, S., Kloczewiak, M., & Hawiger, J. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4935.

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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NeuAc-GgOse$_5$Cer was found in helper T cell clones, not in a cytolytic clone. It is discussed that these structures represent another example for a GSL differentiation marker for T lymphocytes.

Materials and Methods

Animals. Female CBA/J and AKR inbred mice 6–8 weeks of age were purchased from Gl. Bomholtgard Ltd. (Ry, Denmark) and female DBA/2, C57/BL6, and BALB/c mice from the Zentralinstitut für Versuchstierzucht (Hannover, FRG). A breeding nucleus of WHT/HT mice was a generous gift from P. L. Russel (Grey Laboratory, Northwood, Middlesex, U.K.).

Murine Spleen T Lymphoblasts, Cell Culture, and Mitogen Stimulation. Spleen lymphocytes were isolated and cultivated as previously described (Müthing et al., 1987). Briefly, splenocytes, at a concentration of 5×10^6 cells/mL, were cultured in RPMI 1640 medium with 5% fetal calf serum in 45-mL volumes in 75-cm^2 tissue culture flasks (Nunc, Wiesbaden, FRG) in a humidified atmosphere of 10% CO_2 in air at 37 °C. They were stimulated for 40–48 h with 2 μg/mL of the mitogen concanavalin A (ConA, Pharmacia Fine Chemicals). Resulting T blasts were separated from nonproliferating and dead cells by centrifugation on a one-step gradient on Lymphoprep (Nyegard, Oslo; 1.077 g/L) and further cultivated in two parts RPMI 1640 medium and one part conditioned medium from the primary culture, as a source of the T cell growth factor interleukin 2 (IL2). Alternatively, stimulated T cells were propagated with HPLC-purified human IL2 (Kniep et al., 1984). The T cells were grown to densities from 5×10^5 up to 3.0×10^6 cells/mL. Larger amounts of cells were propagated in bioreactors in 1- or 2-L volumes (Müthing & Mühlradt, 1984). After harvesting, the cells were suspended in chloroform/methanol (2/1 v/v) and stored at −20 °C.

Metabolic Labeling of Cells. T blasts, propagated as above in the presence of IL2, were labeled for 20–30 h with 1 μCi/mL D-[1-^{14}C]glucosamine hydrochloride (56.8 Ci/mol; Amersham Buchler, Braunschweig, FRG) and 1 μCi/mL D-[1-^{14}C]galactose (55.7 Ci/mol; Amersham Buchler). Labeled cells were harvested as above, washed twice with 0.85% NaCl, 1 mM Gal, and 1 mM GlcN, suspended in chloroform/methanol (2/1 v/v), and stored at −20 °C.

Preparation of CBA/J Thymocytes. A single cell suspension of CBA/J thymocytes was prepared by passing the thymuses through a 47-μm mesh nylon sieve. The cells were counted, washed twice with Hank's balanced salt solution, suspended in chloroform/methanol (2/1 v/v), and stored at −20 °C.

T Cell Clones from C57/BL6 Mice. Allospecific cloned cells were derived from a C57/BL6 anti-DBA/2 mixed lymphocyte culture by limiting dilution technique (0.3 cells/well) (Weiss et al., 1986). The cells were cultured in 24-well culture plates (Costar) in Medium 199 (Gibco) supplemented with 10% fetal calf serum and 20 units/mL IL2 from a ConA-stimulated rat spleen cell supernatant. Cloned cells were maintained by weekly passaging 5×10^4 cells/well and were restimulated with 5×10^6 irradiated (30 Gy) DBA/2 spleen cells. Seven days after the last restimulation, feeder cells were removed by Ficoll (Pharmacia) centrifugation, and labeling with [^{14}C]glucosamine and [^{14}C]galactose was performed as described above for T blasts.

Isolation of Gangliosides. Gangliosides were isolated as previously described (Müthing et al., 1987). Briefly, chloroform/methanol (2/1 v/v) suspended cells were subsequently extracted with chloroform/methanol (1/1 v/v) and chloroform/methanol (1/2 v/v). The evaporated extract was dissolved in chloroform/methanol/water (30/60/8 v/v/v) and chromatographed as described (Yu & Ledeen, 1972) except that DEAE-Sepharose CL-6B (Pharmacia Fine Chem) was used instead of DEAE-Sephadex. Gangliosides were eluted with chloroform/methanol/0.8 M sodium acetate (30/60/8 v/v/v) and desalted by use of Sep-Pak C_{18} cartridges (Millipore, Milford, MA) as described by Williams and McCluer (1980). Separation of gangliosides into mono- and disialogangliosides was performed by chromatography on DEAE-Sepharose with an ammonium acetate gradient system (Momoi et al., 1976). Radioactivity was determined in a Packard 2450 liquid scintillation spectrometer (Packard Instrument Co.).

Analytical and Preparative Thin-Layer Chromatography. High-performance thin-layer chromatography plates (HPTLC plates, size 10 cm × 10 cm, thickness 0.24 mm, E. Merck, Darmstadt, FRG) were used for analytical and preparative purposes. Two solvent systems were used for the separation of gangliosides (all ratios are v/v): I, chloroform/methanol/water (120/85/20) containing 2 mM $CaCl_2$; II, chloroform/methanol/2.5 N NH_4OH (120/70/16). Neutral GSLs were separated in III, chloroform/methanol/water (120/70/17) containing 2 mM $CaCl_2$. Radioactive GSLs were located by autoradiography on Hyperfilm-^{3}H (Amersham Buchler, Braunschweig, FRG). The isolation and purification of gangliosides by preparative HPTLC were done as previously described (Müthing et al., 1987).

Preparation of Asialogangliosides. One to ten micrograms of gangliosides were hydrolyzed in 1 mL of 1 N acetic acid for 45 min at 100 °C. After neutralization by adding 1.67 mL of 0.6 N NaOH in methanol, 3.33 mL chloroform was added, and the sample was partitioned according to Folch et al. (1957). The lower phase, containing the asialogangliosides, was washed twice with Folch's theoretical upper phase and dried in a stream of N_2.

Alkali Treatment. One to ten micrograms of gangliosides were dissolved in 1 mL of 0.5 N NaOH in methanol and kept overnight at room temperature. The solution was then neutralized with 0.5 mL of 1 N acetic acid. After evaporation of the solvent in a stream of N_2, the gangliosides were taken up in 0.1 M KCl and desalted with a Sep-Pak C_{18} cartridge (Williams & McCluer, 1980).

Neuraminidase Treatment of Gangliosides on HPTLC Plates. Neuraminidase treatment was performed as described (Müthing & Mühlradt, 1988b). Briefly, gangliosides were separated on HPTLC plates, and the silica gel was fixed by chromatography in poly(isobutyl methacrylate) (Plexigum P28, Röhm, Darmstadt, FRG) saturated hexane. The plates were cut in strips of 1.5 cm × 10 cm, 5 cm × 10 cm, or 10 cm × 10 cm to be used with 5, 20, or 40 mL of enzyme solution, respectively. The strips were incubated with 2.5 milliunits/mL *Vibrio cholerae* neuraminidase (EC 3.2.1.18, Behring, Marburg, FRG) for 2 h at RT or with 10 milliunits/mL *Arthrobacter ureafaciens* neuraminidase from Boehringer (Mannheim, FRG) for 18 h at RT. Buffers used were 0.05 N sodium acetate and 9 mM $CaCl_2$ (pH 5.5) for *V. cholerae* neuraminidase and 0.1 N sodium acetate (pH 4.8) for the *A. ureafaciens* enzyme. Where mentioned, incubation with the latter enzyme was done in the presence of 0.5 mg/mL sodium taurodeoxycholate (Sigma, München, FRG) according to Hirabayashi et al. (1986).

Enzyme-Linked Immunostaining Procedure for GSLs on HPTLC Plates. The detailed procedure is described elsewhere (Bethke et al., 1986). Briefly, the plates were soaked for 15

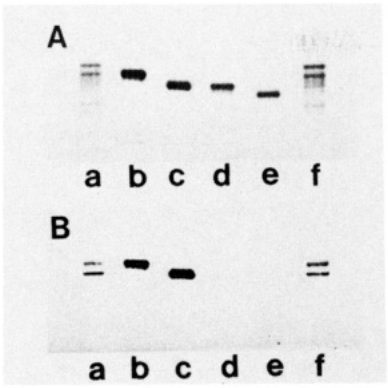


FIGURE 1: Different susceptibility of IVNeuAc-$GgOse_5Cer$ and IV-NeuGc-$GgOse_5Cer$ to *A. ureafaciens* neuraminidase in the presence of sodium taurodeoxycholate. (A) Autoradiography of HPTLC-purified monosialogangliosides with the $GgOse_5Cer$ backbone. (B) Detection of $GgOse_5Cer$ on the same HPTLC plate with anti-$GgOse_3Cer$ antibody after neuraminidase treatment. Lanes a and f: 0.5 μg (500 cpm) and 1.0 μg (1000 cpm) of CBA/J T blast gangliosides. Lanes b and c: IVNeuAc-$GgOse_5Cer$ (C_{24} fatty acid and C_{16} fatty acid, respectively). Lanes d and e: IVNeuGc-$GgOse_5Cer$ (C_{24} fatty acid and C_{16} fatty acid, respectively), 1000 cpm (approximately 1 μg) of each.

min in solution A (phosphate-buffered saline supplemented with 1% bovine serum albumin) to block unspecific binding sites. Monoclonal IgM antibody against $GgOse_3Cer$ (asialo-G_{M2}; Young et al., 1979) was produced with the hybridoma clone 2D4 (TIB 185), obtained from the American Type Culture Collection (ATCC, Bethesda, MD). The culture supernatant was diluted 1:10 with solution A for overlaying the plates. Rabbit antiserum against HPLC-purified $GgOse_4Cer$ (asialo-G_{M1}) was produced according to the method of Kasai et al. (1980) and used at a 1:500 dilution in solution A. Goat anti-mouse IgM antiserum (Sigma, München, FRG) and goat anti-rabbit IgG antiserum (Medac, Hamburg, FRG), both labeled with alkaline phosphatase, were used as second antibodies. The plates were then washed twice with 0.1 M glycine, 1 mM $ZnCl_2$, and 1 mM $MgCl_2$, pH 10.4, before the bound antibodies were visualized by development with 0.05% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (Sigma) in this buffer.

Mass Spectrometric Analysis of Purified Gangliosides. Fast atom bombardment mass spectrometry (FAB-MS) of the native and permethylated gangliosides were done as described by Egge and Peter-Katalinić (1987).

Results

Immunological Detection of IVNeuAc-$GgOse_5Cer$ in Complex Ganglioside Mixtures. It was important to clarify at what stage of T cell development the T cell surface markers IV-NeuAc-$GgOse_5Cer$ and IVNeuGc-$GgOse_5Cer$ are first expressed, whether they are restricted to functionally defined T subpopulations, and whether such structures are a common murine T cell marker or are genetically restricted to CBA/J mice. Because isolation and complete structural characterization of these gangliosides appeared not always feasible, we developed a screening system for these gangliosides. CBA/J splenic T lymphoblast gangliosides and authentic IVNeuAc-$GgOse_5Cer$ and IVNeuGc-$GgOse_5Cer$, isolated and characterized by FAB-MS, partial acid hydrolysis, and immunostaining as described (Müthing et al., 1987), were chromatographed on a HPTLC plate (see Figure 1). The plate was incubated with *A. ureafaciens* neuraminidase in the presence of sodium taurodeoxycholate. $GgOse_5Cer$ could then be detected by immunostaining with a monoclonal antibody against

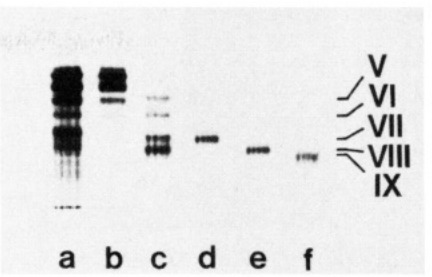


FIGURE 2: Autoradiography of ^{14}C-labeled disialogangliosides from CBA/J splenic T lymphoblasts. Lane a: Starting material (whole gangliosides fraction, 5000 cpm). Lanes b and c: Separated mono- and disialogangliosides (2000 cpm each). The disialogangliosides were numbered from V to IX. The isolated GSLs VII, VIII, and IX (500 cpm each) are shown in lanes d, e, and f, respectively. Exposure time: 140 h.

$GgOse_3Cer$ (Müthing et al., 1987) (Figure 1B). Interestingly, only IVNeuAc-$GgOse_5Cer$ with both C_{24} (Figure 1B, lane b) and C_{16} fatty acids (Figure 1B, lane c), but not the corresponding *N*-glycolyl-substituted gangliosides, was degraded to $GgOse_5Cer$. None of the four GSLs were susceptible to the enzyme in the absence of detergent.

Disialogangliosides from Spleen T Lymphoblasts. Disialogangliosides from murine T lymphocytes have not yet been characterized. To facilitate detection, the GSLs of ConA-stimulated spleen T lymphocytes were metabolically labeled with [^{14}C]Gal and [^{14}C]GlcN. Figure 2 shows the total gangliosides (lane a) in comparison to the separated mono- (lane b) and disialogangliosides (lane c). We have previously reported on the separation and structural characterization of the monosialogangliosides (Müthing et al., 1987). We now purified the disialogangliosides by similar methods. Three different disialogangliosides were isolated by preparative HPTLC, chromatographing in the range between human G_{D1a} and G_{D1b} (Figure 2, lanes d–f, VII–IX). Interestingly, all disialogangliosides (V–IX) changed their chromatographic behavior after extraction from the adsorbent. Former single bands gave rise to several additional ones migrating faster than the starting products (not shown). Whereas the low-polarity derivatives of gangliosides VII–IX could be converted back into the starting products by mild alkali treatment, gangliosides V and VI, and their derivatives, were transformed into the gangliosides VII and VIII, respectively, by this procedure. Compounds V and VI may be the O-acetylated or lactone forms of VII and VIII. Not enough material was available to further characterize these compounds.

In order to obtain information about the asialoganglioside backbone and the positions of the neuraminic acids, the disialogangliosides VII–IX were neuraminidase treated and immunostained on the HPTLC plate (Müthing & Mühlradt, 1988b). In contrast to disialogangliosides sialylated in position II of the $GgOse_4Cer$ chain, such as G_{D1a} and G_{D1b}, the compounds VII–IX were degraded by *V. cholerae* neuraminidase to $GgOse_4Cer$ which could be identified by immunostaining with a highly specific polyclonal antibody against $GgOse_4Cer$ [these data not shown, but see Figure 5A, lane b (Müthing & Mühlradt, 1988b)]. According to these findings disialogangliosides VII–IX could be either of the $G_{D1\alpha}$ type (for ganglioside structures see Table I) or $IV^3(NeuAc)_2$-$GgOse_4Cer$, described by Bartoszewicz et al. (1986) in murine thymoma and designated as "G_{D1c}". Sufficient amounts of the gangliosides VII and VIII were available for subsequent FAB-MS analysis to test these possibilities.

FAB-MS of the Native and Permethylated Disialogangliosides VII and VIII. Molecular-mass-related ions of

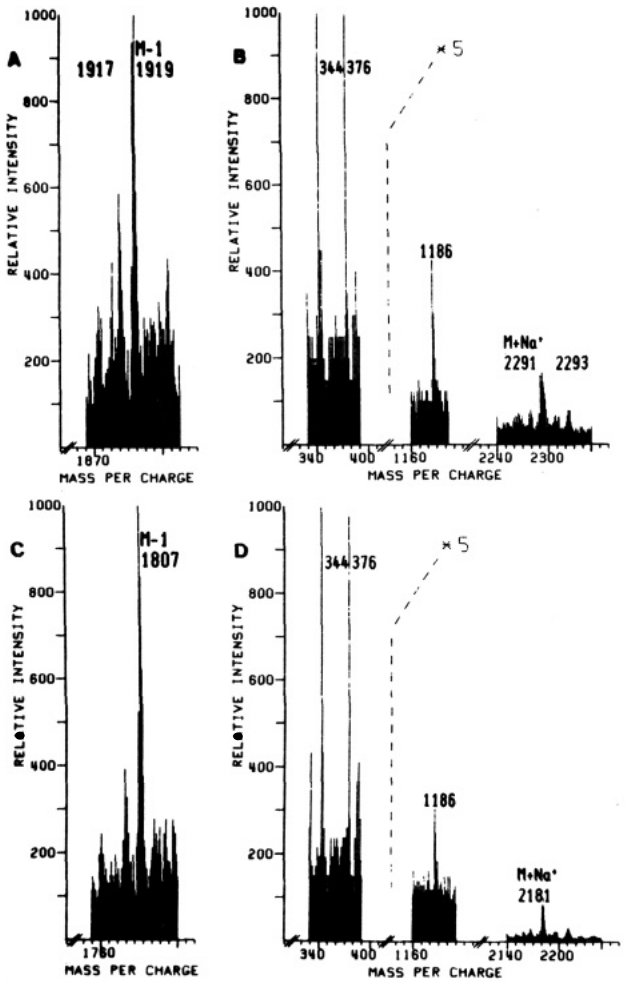
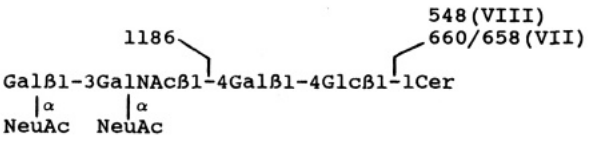


FIGURE 3: Partial fast atom bombardment mass spectra of disialogangliosides VII and VIII. The molecular ions of the native disialogangliosides VII (A) and VIII (C), run in negative ion mode, are shown beside relevant fragments and molecular ions of the permethylated disialogangliosides VII (B) and VIII (D), run in positive ion mode, under standard conditions (Egge & Peter-Katalinić, 1987).

the native disialogangliosides VII and VIII were observed at m/z 1919/1917 and 1807, and FAB-MS of the permethylated disialogangliosides gave pseudomolecular ion $(M + Na)^+$ peaks of 2293/2291 and 2181, respectively, corresponding to a disialylated $GgOse_4Cer$ carrying $C_{24:0/24:1}$ (VII) and $C_{16:0}$ fatty acids (VIII) (Figure 3). Both permethylated compounds gave rise to the fragment at m/z 1186, characteristic for a terminal tetrasaccharide $HexHexNAcNeuAc_2^+$. The disialo fragment $NeuAcNeuAc^+$ at m/z 737, typical for G_{D1b} and G_{Q1b} (Egge et al., 1985), was not detected, but instead the monosialo fragment $NeuAc^+$ at m/z 344 and 376, indicating that these *V. cholerae* neuraminidase sensitive disialogangliosides represent the $G_{D1\alpha}$-type IVNeuAc,IIINeuAc-$GgOse_4Cer$:



Gangliosides of Murine Thymocytes. Gangliosides from thymocytes were tested as described above for the presence of IVNeuAc-$GgOse_5Cer$ in comparison with splenic T lymphoblasts (both from CBA/J). As shown in Figure 4A (lane b), gangliosides from 1×10^7 splenic T lymphoblasts were sufficient to detect the double band of IVNeuAc-$GgOse_5Cer$ with C_{24} (upper) and C_{16} (lower band) fatty acid, whereas

Table I: Structures of Gangliosides

G_{M1}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer 3|α 2|α NeuAc
G_{M1b}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer 3|α 2|α NeuAc
G_{D1a}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer 3|α 3|α 2|α 2|α NeuAc NeuAc
G_{D1b}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer 3|α 2|α NeuAc8-2αNeuAc
"G_{D1c}"	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer 3|α 2|α NeuAc8-2αNeuAc
$G_{D1\alpha}$	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer 3|α 6|α 2|α 2|α NeuAc NeuAc
G_{T1b}	Galß1-3GalNAcß1-4Galß1-4Glcß1-1Cer 3|α 3|α 2|α 2|α NeuAc NeuAc8-2αNeuAc

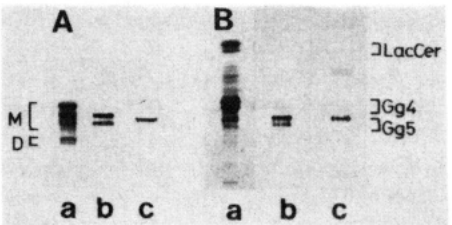


FIGURE 4: Detection of the $GgOse_5Cer$ backbone in gangliosides from thymocytes and T lymphoblasts from spleen. (A) *A. ureafaciens* neuraminidase treatment of gangliosides followed by immunostaining with anti-$GgOse_3Cer$ antibody. Lane a: Reference autoradiography of splenic T lymphoblast gangliosides (5000 cpm). M = monosialogangliosides; D = disialogangliosides. Lane b: Gangliosides from 1×10^7 splenic T lymphoblasts. Lane c: Gangliosides from 1×10^9 (!) thymocytes. After chromatography the silica gel was fixed, and enzyme treatment was performed as described under Materials and Methods. $GgOse_5Cer$ bands were detected by immunostaining with cross-reacting anti-$GgOse_3Cer$ antibody. (B) Immunostaining with anti-$GgOse_3Cer$ antibody of asialogangliosides obtained by mild acid hydrolysis of gangliosides from CBA/J thymocytes and splenic T lymphoblasts. Lane a: Reference autoradiography of splenic T lymphoblast asialogangliosides (5000 cpm). LacCer = lactosylceramide; Gg4 = $GgOse_4Cer$; Gg5 = $GgOse_5Cer$. Asialogangliosides from (lane b) 1×10^7 splenic T lymphoblasts and (lane c) 1×10^9 (!) thymocytes were chromatographed. After silica gel fixation $GgOse_5Cer$ was detected by immunostaining with anti-$GgOse_3Cer$ antibody.

gangliosides from 1×10^9 (!) thymocytes (Figure 4A, lane c) had to be applied to obtain a comparable stain. As opposed to two bands of IVNeuAc-$GgOse_5Cer$ from spleen T blasts, only one was detectable in the thymocyte fraction. This was confirmed by mild acid hydrolysis and subsequent immunostaining of both ganglioside mixtures (Figure 4B). Since the enzymatically and chemically prepared $GgOse_5Cer$ derived from the same number of thymocytes gave similar staining intensities (compare lanes c of panels A and B, Figure 4), we conclude that no neuraminidase-resistant N-glycolylated

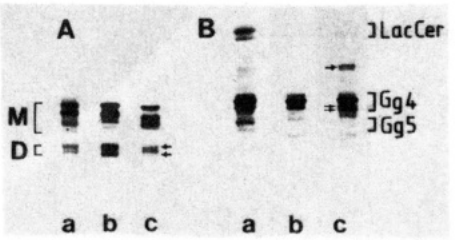


FIGURE 5: Detection of GM_{1b}- and $G_{D1\alpha}$-type species in gangliosides from thymocytes and splenic T blasts. (A) *V. cholerae* neuraminidase treatment followed by immunostaining of $GgOse_4Cer$ with specific antibodies. Lane a: reference autoradiography of splenic T lymphoblast gangliosides (5000 cpm). M = monosialogangliosides; D = disialogangliosides. Gangliosides of 5×10^6 splenic T lymphoblasts (lane b) and of 2×10^8 thymocytes (lane c). After chromatography the silica gel was fixed, enzyme treatment was done as described under Materials and Methods, and $GgOse_4Cer$ bands were detected by immunostaining with specific anti-$GgOse_4Cer$ antibodies. (B) Asialogangliosides of thymocytes and splenic T lymphoblasts, immunostained with specific anti-$GgOse_4Cer$ antibodies. Lane a: Reference autoradiography of splenic T lymphoblast asialogangliosides. LacCer = lactosylceramide; Gg4 = $GgOse_4Cer$; Gg5 = $GgOse_5Cer$. Asialogangliosides of 2.5×10^6 splenic T lymphoblasts (lane b) and of 1×10^8 thymocytes (lane c). After chromatography the silica gel was fixed, and $GgOse_4Cer$ bands were detected by immunostaining with anti-$GgOse_4Cer$ antibodies.

ganglioside with the $GgOse_5Cer$ backbone had escaped detection.

Next, gangliosides from CBA/J thymocytes and splenic T lymphocytes were assayed for the presence of terminally sialylated gangliosides with the $GgOse_4Cer$ backbone (Müthing & Mühlradt, 1988b). Gangliosides were chromatographed on a HPTLC plate and treated with *V. cholerae* neuraminidase. The degradation products were in this case immunostained with an anti-$GgOse_4Cer$ antibody. The results are shown in Figure 5. Whereas G_{M1b}-type gangliosides were detected in the monosialoganglioside fraction of splenic T lymphoblasts as well as thymocytes, both cell types differed considerably in the disialoganglioside range. An appreciable portion of the gangliosides from splenic T lymphoblasts were terminally sialylated disialogangliosides of the $G_{D1\alpha}$ type (as described in this paper), but there was only a minor band of material present in thymocytes (indicated by arrows in Figure 5A, lane c), which chromatographed like $G_{D1\alpha}$ and reacted with anti-$GgOse_4Cer$ antibody after neuraminidase treatment on the plate, although gangliosides from 40 times as many thymocytes were applied. The asialogangliosides from thymus (Figure 5B, lane c) consisted besides of a minor, fast migrating unknown GSL cross-reacting with the anti-$GgOse_4Cer$ antibody mainly of $GgOse_4Cer$. It is therefore likely that the material chromatographing in the disialoganglioside range is indeed $G_{D1\alpha}$ rather than an extended monosialoganglioside with a $GgOse_4Cer$ epitope.

Gangliosides of Murine T Cell Clones. T lymphocytes can be subdivided into functionally and antigenically distinct subpopulations: those of the helper/amplifier and those of the cytolytic/suppressor type. It was of interest to compare the ganglioside compositions of cloned T cells representing such different subpopulations. The characteristics of three T cell clones from C57/BL6 mice, two of the helper type, I-2 and II-8, and one cytolytic clone, II-3, are summarized in Table II.

The ^{14}C-labeled gangliosides from the two helper clones (Figure 6A, lanes b anc c) and the cytolytic T cell clone (Figure 6A, lane d) differed in their chromatographic patterns as detected by autoradiography. To allow better interpretation of these patterns, gangliosides from identical cell numbers of

Table II: Characteristics of C57/BL6 T Cell Clones

functions	T cell clone		
	helper		cytolytic
	I-2	II-8	II-3
surface markers			
Thy 1.2	+	+	+
Lyt 1.2	+	+	–
Lyt 2.2	–	–	+
lymphokine secretion[a]			
interleukin 2	+	+	–
interferon γ	+	+	+
cytolytic activity	–	–	+
antigen specificity	H-2^d	Mls^a	H-2 D^d

[a] Stimulated by concanavalin A.

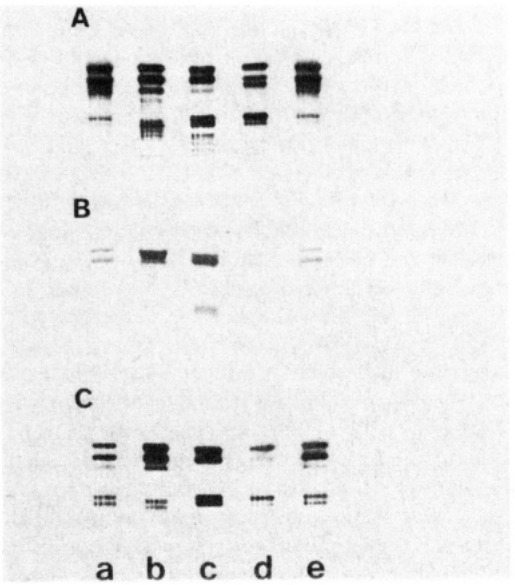


FIGURE 6: Gangliosides of three different C57/BL6 T cell clones. Lanes a and e: CBA/J splenic T lymphoblasts. Lane b: Clone I-2 (helper). Lane c: Clone II-8 (helper). Lane d: Clone II-3 (cytolytic). (A) Autoradiography of ^{14}C-labeled gangliosides (3000 cpm per lane). Exposure time: 330 h. (B) *A. ureafaciens* neuraminidase treatment of gangliosides, followed by immunostaining with the anti-$GgOse_3Cer$ antibody. Gangliosides from 1×10^7 T lymphoblasts and T cells of each clone were chromatographed. (C) *V. cholerae* neuraminidase treatment of gangliosides followed by immunostaining of $GgOse_4Cer$ with specific antibodies. Gangliosides from 2×10^6 T lymphoblasts and T cells of each clone were chromatographed.

cloned T cell subsets and primary splenic T lymphoblasts were screened for the presence of IVNeuAc-$GgOse_5Cer$ as described above. Both T helper clones, I-2 and II-8, expressed the IVNeuAc-$GgOse_5Cer$ structure as shown in Figure 6B. Two bands are discernible, probably representing two gangliosides with identical carbohydrates but different (C_{16} and C_{24}) fatty acids. The cytolytic clone II-3 lacked these gangliosides almost entirely except for traces of the upper band (Figure 6B, lane d). These data were confirmed by immunostaining of the asialogangliosides obtained by mild acid hydrolysis to exclude the possible presence of N-glycolylated $GgOse_5Cer$ which would not have been susceptible to neuraminidase action and consequently escaped detection (data not shown). In the gangliosides from helper cells two faint additional bands occurred in the disialoganglioside range (Figure 6B, lanes b and c), suggesting also disialogangliosides with the $GgOse_5Cer$ backbone.

Terminally sialylated gangliosides with the $GgOse_4Cer$ backbone were screened for by combination of *V. cholerae* neuraminidase treatment and immunostaining with specific

antibodies. Figure 6C shows the analysis of the three T cell clones in comparison to splenic T lymphoblasts. Again, gangliosides from identical cell numbers were applied. On principle, helper T cells appeared to express more sialylated $GgOse_4Cer$ than the cytolytic T cells.

Gangliosides from Spleen T Lymphoblasts of Genetically Different Inbred Mouse Strains. To exclude genetic restriction of the described gangliosides, especially of IVNeuAc-$GgOse_5Cer$, to particular strains of mice, we examined the spleen T cell gangliosides of genetically unrelated inbred mouse strains. The autoradiography patterns of the ^{14}C-labeled gangliosides from CBA/J, DBA/2, C57/BL6, BALB/c, AKR, and WHT/HT mice were identical (not shown). When gangliosides from 1×10^7 cells of each strain were investigated for IVNeuAc-$GgOse_5Cer$, this ganglioside was detected in all strains, although it was more strongly expressed in DBA/2, C57/BL6, BALB/c, and AKR mice than in CBA/J and WHT/HT mice (data not shown). IVNeuAc-$GgOse_5Cer$ with C_{16} fatty acid was the main component, whereas IVNeuAc-$GgOse_5Cer$ with C_{24} fatty acid represented a minor band.

The detection of terminally sialylated gangliosides with the $GgOse_4Cer$ backbone in the GSLs of the six inbred strains has been recently published (Müthing & Mühlradt, 1988a). The patterns are basically similar, although quantitative differences could be seen in the monosialoganglioside range and additional qualitative differences in the disialoganglioside range.

Discussion

In a previous paper (Müthing et al., 1987) we characterized the monosialogangliosides from murine spleen T lymphoblasts. IVNeuAc- and IVNeuGc-$GgOse_4Cer$ and the elongation products IVNeuAc- and IVNeuGc-$GgOse_5Cer$ (in the terminology of some authors designated as G_{M1b} and GalNAc-G_{M1b}) were found to be the main monosialogangliosides from these cells. In the present study we describe the T cell disialogangliosides and extend our studies looking for the expression of GSL markers on thymocytes and cloned T cells with defined functions. We also compared T blasts from different mouse strains to detect a possible genetic restriction of ganglioside expression.

The major disialogangliosides were of the $G_{D1\alpha}$ type, IV-NeuAc,IIINeuAc-$GgOse_4Cer$, with different fatty acid moieties ($C_{16:0}$ and $C_{24:0/24:1}$). Two other minor gangliosides were alkali labile. We think that they are either the lactones or the O-acetylated forms of the two main T cell disialogangliosides, because they were converted to these by mild alkaline hydrolysis. This may be the first example of the occurrence of IVNeuAc,IIINeuAc-$GgOse_4Cer$ in untransformed murine cells. However, $G_{D1\alpha}$ has previously been demonstrated in the murine T lymphoma Eb (Murayama et al., 1986), and in murine Friend erythroleukemic cells (Rokukawa et al., 1988).

Biochemical characterization of gangliosides from subpopulations of immune cells is often not feasible for lack of sufficient material. We developed therefore an immunological screening method for the detection of the IVNeuAc-$GgOse_5Cer$ structure in complex ganglioside mixtures. The test is based on desialylation of IVNeuAc-$GgOse_5Cer$ by *A. ureafaciens* neuraminidase plus taurodeoxycholate—which is essential for the reaction—to be followed by immunologic detection of the $GgOse_5Cer$ backbone. The *N*-glycolyl derivative was absolutely resistant to this enzyme. A general low susceptibility of *N*-glycolyl derivatives toward a number of neuraminidases has been previously observed by Corfield et al. (1981). A similar test was used for demonstrating the presence of terminally sialylated gangliosides with the $GgOse_4Cer$ backbone (Müthing & Mühlradt, 1988b).

IVNeuGc/Ac-$GgOse_5Cer$, when within the hemopoietic system, is likely to be restricted to the T lymphocyte lineage (Müthing et al., 1987). The structure of these compounds was isolated in sufficient amounts from whole spleen to be thoroughly characterized as IV^{3}NeuGc-$GgOse_5Cer$ (Nakamura et al., 1987). This compound (only in the N-acetylated form) was also demonstrated in minor amounts in human Tay-Sachs brain gangliosides (Itoh et al., 1981), and in the murine tumor MDAY-D2 (Schwartz et al., 1985; Lafertē et al., 1987). By immunostaining gangliosides from defined cell numbers, we showed that IVNeuAc-$GgOse_5Cer$ is only a minor GSL on thymocytes in comparison to splenic T lymphoblasts: to obtain immunobands with identical color intensities, gangliosides from 100 times as many thymocytes than spleen T blasts were required. Thymocytes are heterogeneous, consisting of about 90% immature and only 5–10% mature T cells. We think therefore that only cells within this minor, immunocompetent population of thymocytes may carry the IVNeuAc-$GgOse_5Cer$ structure. Our ganglioside analysis of cloned T cells suggests a restriction of this GSL to helper T cells, but since only three clones have been examined so far, these results are still preliminary.

IVNeuAc-$GgOse_5Cer$ with C_{24} and C_{16} fatty acid was found on splenic T lymphoblasts of all examined mouse strains including the WHT strain which is partially deficient in ganglioside biosynthesis (Hashimoto et al., 1983). These results are in accordance with the findings of Nakamura et al. (1988), who demonstrated that the genetic polymorphism of ganglioside expression in mice is restricted to the liver cells and erythrocytes of these animals.

Terminally sialylated $GgOse_4Cer$ (G_{M1b}) was detected on mouse thymocytes (Schwarting & Gajewski, 1983) as well as in mouse spleen extracts (Nakamura et al., 1984). Results from our immunostaining procedure suggest subtle differences with regard to terminally sialylated $GgOse_4Cer$ (G_{M1b}) between T lymphoblasts, thymocytes, and T cell clones. Interestingly, the $G_{D1\alpha}$-type ganglioside is strongly expressed on stimulated splenic T blasts but appears only in traces on thymocytes (shown for the CBA/J strain).

In summary, we conclude from our results that stimulation or maturation of T cells may be correlated with the elongation of the common G_{M1b} precursor structure, the main ganglioside on thymocytes. This may be extended on the one hand by a GalNAc transferase resulting in GalNAc-G_{M1b} or on the other hand by a sialyltransferase to yield the $G_{D1\alpha}$-type ganglioside.

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Registry No. G_{M1b}, 105417-47-8; G_{M1}, 37758-47-7; G_{D1c}, 118537-16-9.

References

Bartoszewicz, Z., Kościelak, J., & Pacuszka, T. (1986) *Carbohydr. Res. 151*, 77–88.

Bethke, U., Müthing, J., Schauder, B., Conradt, P., & Mühlradt, P. F. (1986) *J. Immunol. Methods 89*, 111–116.

Bethke, U., Kniep, B., & Mühlradt, P. F. (1987) *J. Immunol. 138*, 4329–4335.

Corfield, A. P., Veh, R. W., Wember, M., Michalski, J.-C., & Schauer, R. (1981) *Biochem. J. 197*, 293–299.

Egge, H., & Peter-Katalinić, J. (1987) *Mass Spectrom. Rev. 6*, 331–393.

Egge, H., Peter-Katalinić, J., Reuter, G., Schauer, R., Ghidoni, R., Sonnino, S., & Tettamanti, G. (1985) *Chem. Phys. Lipids 37*, 127–141.
Folch, J., Lees, M., & Stanley, G. H. S. (1957) *J. Biol. Chem. 226*, 497–509.
Gruner, K. R., van Eijk, R. V. W., & Mühlradt, P. F. (1981) *Biochemistry 20*, 4518–4522.
Hashimoto, Y., Otsuka, H., Sudo, K., Suzuki, K., Suzuki, A., & Yamakawa, T. (1983) *J. Biochem. 93*, 895–901.
Hirabayashi, Y., Koketsu, K., Higashi, H., Suzuki, Y., Matsumoto, M., Sugimoto, M., & Ogawa, T. (1986) *Biochim. Biophys. Acta 876*, 178–182.
Itoh, T., Li, Y.-T., & Yu, R. K. (1981) *J. Biol. Chem. 256*, 165–169.
IUPAC–IUB Commission on Biochemical Nomenclature (1977) *Eur. J. Biochem. 79*, 11–21.
Kasai, M., Iwamori, M., Nagai, Y., Okumura, K., & Tada, T. (1980) *Eur. J. Immunol. 10*, 175–180.
Kniep, B., Hünig, T. R., Fitch, F. W., Heuer, J., Kölsch, E., & Mühlradt, P. F. (1983) *Biochemistry 22*, 251–255.
Kniep, E. M., Kniep, B., Grote, W., Conradt, H. S., Monner, D. A., & Mühlradt, P. F. (1984) *Eur. J. Biochem. 143*, 199–203.
Lafertē, S., Fukuda, M. N., Fukuda, M., Dell, A., & Dennis, J. W. (1987) *Cancer Res. 47*, 150–159.
Mercurio, A. M., Schwarting, G. A., & Robbins, P. W. (1984) *J. Exp. Med. 160*, 1114–1125.
Momoi, T., Ando, S., & Nagai, Y. (1976) *Biochim. Biophys. Acta 441*, 488–497.
Mühlradt, P. F., Bethke, U., Monner, D. A., & Petzoldt, K. (1984) *Eur. J. Immunol. 14*, 852–858.
Murayama, K., Levery, S. B., Schirrmacher, V., & Hakomori, S. I. (1986) *Cancer Res. 46*, 1395–1402.
Müthing, J., & Mühlradt, P. F. (1984) *Hoppe-Seyler's Z. Physiol. Chem. 365*, 1036–1037.
Müthing, J., & Mühlradt, P. F. (1988a) in *Sialic Acids 1988, Proceedings of the Japanese–German Symposium on Sialic Acids* (Schauer, R., & Yamakawa, T., Eds.) pp 248–249, Kieler Verlag Wissenschaft + Bildung, Kiel.
Müthing, J., & Mühlradt, P. F. (1988b) *Anal. Biochem. 173*, 10–17.
Müthing, J., Egge, H., Kniep, B., & Mühlradt, P. F. (1987) *Eur. J. Biochem. 163*, 407–416.
Nakamura, K., Hashimoto, Y., Suzuki, M., Suzuki, A., & Yamakawa, T. (1984) *J. Biochem. 96*, 949–957.
Nakamura, K., Suzuki, M., Inagaki, F., Yamakawa, T., & Suzuki, A. (1987) *J. Biochem. 101*, 825–835.
Nakamura, K., Hashimoto, Y., Yamakawa, T., & Suzuki, A. (1988) *J. Biochem. 103*, 201–208.
Rokukawa, C., Nakamura, K., & Handa, S. (1988) *J. Biochem. 103*, 36–42.
Schwarting, G. A., & Gajewski, A. (1983) *J. Biol. Chem. 258*, 5893–5898.
Schwartz, R., Kniep, B., Müthing, J., & Mühlradt, P. F. (1985) *Int. J. Cancer 36*, 601–607.
Stout, R. D., Schwarting, G. A., & Suttles, J. (1987) *J. Immunol. 139*, 2123–2129.
Weiss, J., Schwinzer, B., Kirchner, H., Gemsa, D., & Resch, K. (1986) *Immunobiology 171*, 234–251.
Williams, M. A., & McCluer, R. H. (1980) *J. Neurochem. 35*, 266–269.
Young, W. W., MacDonald, E. M. S., Nowinski, R. C., & Hakomori, S.-I. (1979) *J. Exp. Med. 150*, 1008–1019.
Yu, R. K., & Ledeen, R. W. (1972) *J. Lipid Res. 13*, 680–686.

Transformed Glucocorticoid Receptors Consist of Multiple Subspecies with Differing Capacities To Bind DNA–Cellulose†

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ABSTRACT: The glucocorticoid receptor can be transformed into a DNA-binding protein by a process that is both hormone and temperature dependent. We have used a modification of the conventional method of anion-exchange chromatography to separate and analyze a variety of receptor subspecies that result from this transition. One receptor form (peak A) was found to have a capacity to bind DNA–cellulose which was significantly greater than that of the other species. Under conditions of mild heating (15 °C), the relative abundance of peak A in the receptor population and the rate of receptor transformation were both increased as a result of incubating samples with alkaline phosphatase. The mechanism appears to involve the conversion of the more "acidic" forms into that of peak A. The results indicate that receptor transformation is a multistep process which may be promoted by the removal of phosphate from either the receptor or a receptor-bound regulatory factor.

The glucocorticoid receptor is a hormone-dependent regulatory protein possessing the ability to enhance transcription of individual genes through its interaction with specific DNA sequences (Ringold, 1985; Yamamoto, 1985; Majors & Varmus, 1983; Chandler et al., 1983; Payvar et al., 1983). The capacity to bind hormone, transform into a DNA-binding protein, translocate to the nucleus, and eventually undergo productive interactions within a circumscribed set of genes could all potentially be controlled by modifications of the receptor's structure. For instance, there is considerable evidence that receptor sulfhydryl groups may play an important role in determining both steroid- and DNA-binding capacities (Granberg & Ballard, 1977; Sando et al., 1979; Bodwell et al., 1984a,b; Tienrungroj et al., 1987). Covalent modification at these sites by sulfhydryl oxidizing reagents causes a loss

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