Neutral Glycosphingolipids in Hairy Cell Leukemia†

William M. F. Lee,* John C. Klock,‡ and Bruce A. Macher

ABSTRACT: The neutral glycosphingolipids of hairy cells from a patient with hairy cell leukemia were chemically analyzed by thin-layer and gas–liquid chromatography, mass spectrometry, combined gas chromatography–mass spectrometry, and glycosidase treatment. These cells were found to have compounds containing one to four sugars with the following structures:

Glcβ1→1Cer

Galβ1→4Glcβ1→1Cer

Galα1→4Galβ1→4Glcβ1→1Cer

GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1Cer

These compounds belong to the globo series of neutral glycosphingolipids and are similar to those found in human lymphocytes and chronic lymphocytic leukemia cells. They differ from the neutral glycosphingolipids found in human neutrophils and chronic myelogenous leukemia cells which are of the lactoneo and gala type. Neutral glycosphingolipids may be useful in classifying leukemias of uncertain origin.

Hairy cell leukemia is a distinct type of human leukemia with characteristic clinical and pathological features. Patients with this disease frequently have peripheral pancytopenia, splenomegaly, and the presence of hairy cells in the peripheral blood and bone marrow. The cellular origin of hairy cells has been the subject of much controversy; certain characteristics of these cells suggest that they are derived from monocyte precursors (Fu et al., 1974; Rosenszajn et al., 1976; Jaffee et al., 1974), and other charcteristics suggest a lymphoid origin (Golde et al., 1977; Rieber et al., 1979; Gordon et al., 1978). No clear resolution of this problem is available at this time although the weight of evidence favors a lymphoid origin.

The glycosphingolipid composition of human leukocytes and some of their leukemic counterparts has been elucidated in the past few years. Human tonsillar lymphocytes were found to contain globo neutral glycosphingolipids (Stein & Marcus, 1977), whereas human neutrophils isolated from the urine of a patient with a urinary tract infection were found to contain neolacto compounds (Wherrett, 1973). More recently, in other laboratories as well as our own, the neutral glycosphingolipids in human leukocytes have been studied in a more definitive and comprehensive manner. Human pooled peripheral blood lymphocytes (Lee et al., 1981), B cells, T cells (Schwarting, 1980), and chronic lymphocytic leukemia cells (Lee et al., 1981; Schwarting, 1980) were found to contain globo compounds as their predominant, if not exclusive, type of neutral glycosphingolipid. Human blood neutrophils and chronic myelogenous leukemia cells were found to contain neolacto compounds (Macher & Klock, 1980; Klock et al., 1981). Thus, normal and leukemic lymphoid cells differ from normal and leukemic myeloid cells in their neutral glycosphingolipid composition, and these compounds are potentially useful in classifying leukemic disorders of uncertain origin. These findings would be highly useful in characterizing hairy cell leukemia except for the fact that very few patients have high numbers of circulating hairy cells (Golomb et al., 1978). Thus, it is difficult to obtain sufficient numbers of hairy cells for chemical glycosphingolipid analysis. In addition, hairy cells in the blood are difficult to separate from peripheral blood lymphocytes by the usual techniques employed (W. M. F. Lee, unpublished observation), and the purity of hairy cell preparations from most patients is inadequate for definitive analysis. However, we recently have had the rare opportunity of obtaining large amounts of pure hairy cells from a patient with hairy cell leukemia and have chemically analyzed the neutral glycosphingolipids present in these cells to determine which compounds are present in these unusual cells.

Experimental Procedures

(A) Isolation of Hairy Cells. Hairy cells were obtained from a patient with hairy cell leukemia by leukapheresis with an IBM 2997 cell separator (Hester et al., 1979). Our patient fulfilled the diagnostic criteria of hairy cell leukemia. The patient is a 37-year-old female who initially presented with pancytopenia and splenomegaly. Hairy cells were present in her peripheral blood, and her bone marrow was characteristic of hairy cell leukemia. Both peripheral blood and bone marrow cells stained positively for tartrate-resistant acid phosphatase (Golomb et al., 1978). After splenectomy, her blood cell counts improved temporarily, but severe pancytopenia soon recurred along with the appearance of large numbers of hairy cells in the peripheral blood. As part of the treatment for this development, she underwent leukapheresis to lower her leukemic cell burden. At the time of leukapheresis, her complete blood count showed a leukocyte count of 68 000/mm^3, of which 95% were hairy cells. Her platelet count was 84 000/mm^3, and her hematocrit was 24%.

The hairy cells were purified from the leukapheresis product by using the method we have employed for isolating peripheral blood lymphocytes and chronic lymphocytic leukemia cells (Lee et al., 1981). A 40-mL sample of the lymphapheresis product was layered over 7.5 mL of Ficoll-Hypaque in a 50-mL polypropylene centrifuge tube and centrifuged at 800*g* for 20 min at room temperature. The mononuclear cell layer was

† From the Department of Medicine, Cancer Research Institute, University of California, San Francisco, San Francisco, California 94143. *Received March 3, 1981.* This work was supported by a grant from the Leukemia Research Foundation and by Cancer Research Funds from the University of California.

* Correspondence should be addressed to this author at the Cancer Research Institute, University of California, Moffitt Hospital M-1282, San Francisco. W.M.F.L. is the recipient of a postdoctoral fellowship from the National Institutes of Health and Giannini—Bank of America.

‡ J.C.K. is a scholar of The Leukemia Society of America, Inc.

0006-2960/81/0420-6505$01.25/0

virtually a pure preparation of hairy cells. A Wright-stained smear of this preparation contained over 98% hairy cells; the remaining leukocytes were lymphocytes and monocytes. The ratio of the number of platelets to the number of hairy cells was less than 1:2. We obtained a total of 60×10^{10} hairy cells from this patient for our studies.

(*B*) *Extraction and Purification of Hairy Cell Neutral Glycosphingolipids.* Because detailed procedures for the purification and structural analysis of neutral glycosphingolipids from human leukocytes have been presented in our earlier studies (Macher & Klock, 1980; Lee et al., 1981), only a brief summary is given here. Glycosphingolipids were extracted from the hairy cells at room temperature with 20 volumes of each of the following solvent mixtures: chloroform/methanol, 2:1, 1:1, and 1:2 (v/v) (30 min for each extraction). Neutral lipids and glycosphingolipids were separated from gangliosides and phospholipids by ion-exchange column chromatography on DEAE-Sephadex A25 (Pharmacia, Piscataway, NJ) in the acetate form (Ledeen et al., 1973). Nonglycosphingolipid contaminants were removed, and an enrichment of the individual neutral glycosphingolipids was achieved by silicic acid column chromatography (Bio-Sil, Bio-Rad, Richmond, CA) using solvent mixtures of increasing polarity (from 100% chloroform to 100% methanol). Final purification of each neutral glycosphingolipid was by preparative thin-layer chromatography (TLC)[1] using silica gel 60 high-performance plates (EM Laboratories, Inc., Cincinnati, OH) in solvent system A (chloroform/methanol/H_2O, 60:35:8 v/v). Glycosphingolipids were visualized by a brief exposure to iodine which was then sublimated, and the individual bands were scraped from the plate and eluted (chloroform/methanol/H_2O, 50:50:10 v/v). A small aliquot of each glycosphingolipid species was rechromatographed in solvent system A and visualized with orcinol reagent to demonstrate homogeneity. Carbohydrate-positive spots visualized with orcinol reagent were quantitated densitometrically with a dual-beam recording microdensitometer (MK III CS, Joyce, Loebe and Co., Ltd., England).

(*C*) *Carbohydrate Compositional Analysis.* (Trimethylsilyl)methyl glycoside derivatives were prepared as described by Macher et al. (1979). Standard compounds (neolactotetraosylceramide from human neutrophils and globotetraosylceramide from human erythrocytes) were derivatized and analyzed at the same time. A Bendix 2500 gas–liquid chromatograph (Ronceverte, WV) equipped with glass columns was used for the analysis. (Trimethylsilyl)methyl glycosides were separated on 3% SE-30 (Supelco Co., Bellefonte, PA) temperature programmed from 140 to 240 °C at 3 °C/min.

(*D*) *Methylation Analysis.* Partially methylated alditol acetate derivatives of glycolipids were prepared as previously described (Macher et al., 1979) and analyzed with an HP-5985S combined gas–liquid mass spectrometer under the following conditions: column packing, 3% OV-225; column temperature, linear program from 150 to 250 °C at 5 °C/min; separator temperature, 275 °C; ion source temperature, 200 °C; electron impact ionization, 70 eV; electron energy, 300 μA.

(*E*) *Direct Probe Mass Spectrometry.* Glycosphingolipids were permethylated (Hakomori, 1964), and samples were subjected to electron impact/desorption analysis with a Varian MAT CH-5 DF mass spectrometer under the following conditions: emission current, 300 μA; electron energy, 70 eV; acceleration voltage, 3 kV; ion source temperature, 160 °C; emitter wire current, programmed from 0 to 35 mA.

[1] Abbreviations used: GL1, monohexosylceramide; GL2, dihexosylceramide; GL3, trihexosylceramide; GL4, tetrahexosylceramide; TLC, thin-layer chromatography; Cer, ceramide.

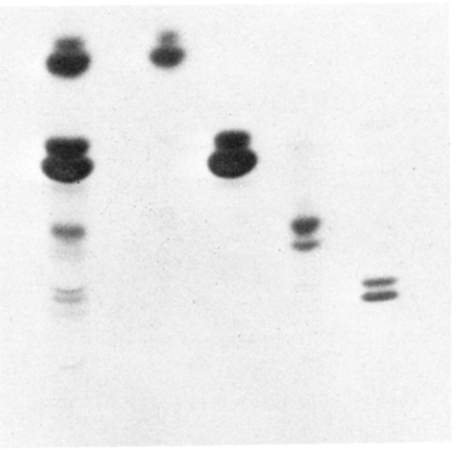

FIGURE 1: Thin-layer chromatogram of hairy cell neutral glycosphingolipids. The separation is on a plate of silica gel 60 (HPTLC) in the solvent chloroform/methanol/water (60:35:8 v/v), and the glycolipids were visualized with orcinol reagent spray. HCL, neutral glycosphingolipids from hairy cell leukemia; GL1–GL4, neutral glycosphingolipids from human erythrocytes (glucosylceramide, lactosylceramide, globotriaosylceramide, and globotetraosylceramide, sequentially).

(*F*) *Enzymatic Degradation.* Purified preparations of hairy cell glycosphingolipids were incubated with various exoglycosidases to determine the sequence and anomerity of each monosaccharide residue. Ficin α-galactosidase, jack bean β-galactosidase, and β-hexosaminidase were kindly supplied by Dr. Y.-T. Li, Tulane University School of Medicine, New Orleans, LA. The conditions for incubation were similar to those described by Li & Li (1977). Reaction mixtures contained 50 mM sodium citrate buffer, pH 4.0 (100 μL), sodium taurodeoxycholate (100 μg), purified glycosphingolipid (20–50 μg), and the glycosidase (1.0 unit, β-hexosaminidase; 0.2 unit, β-galactosidase; or 0.05 unit, α-galactosidase) in a total volume of 150 μL. The reaction mixtures were incubated at 37 °C for 16–40 h, and then 5 volumes of chloroform/methanol (2:1 v/v) were added. The glycosphingolipids in the lower phase were separated by thin-layer chromatography in solvent A. Carbohydrate-positive spots were visualized with orcinol reagent.

Results

Isolation of Neutral Glycosphingolipids. The total yield of neutral glycosphingolipids from the hairy cells was approximately 1 mg per 10^{10} cells. The neutral glycosphingolipids showed four major components appearing as singlets or doublets, with TLC mobilities corresponding to those of mono-, di-, tri-, and tetraosylceramide standards from human erythrocytes; these were labeled GL1 through GL4 sequentially (Figure 1). Hairy cell GL1 and GL2 accounted for about 90% of the total neutral glycosphingolipids. The GL1:GL2 ratio was 2:3. In all subsequent analyses, doublets for the individual glycosphingolipid species were analyzed together.

Carbohydrate Composition. The carbohydrate compositions of hairy cell GL1–GL4 as determined by as chromatography are given in Table I. *N*-Acetylgalactosamine was the only amino sugar found, and this was present only in the GL4 species. Hairy cell GL1 contained only glucose, GL2 contained approximately equimolar amounts of glucose and galactose,

Table I: Carbohydrate Composition of Neutral Glycosphingolipids from Hairy Cells

fraction	molar ratios[a] glucose	galactose	*N*-acetyl-galactosamine
GL1	1.0		
GL2	1.0	1.1	
GL3	1.0	2.3	
GL4	1.0	2.2	0.7

[a] The ratios are expressed relative to glucose.

Table II: Determination of Glycosidic Linkage Positions in Neutral Glycosphingolipids from Hairy Cells

methylated sugar	molar ratio of fractions[a] GL1	GL2	GL3	GL4
2,3,4,6-tetra-*O*-methylglucitol	1.0			
2,3,4,6-tetra-*O*-methylgalactitol		1.0	1.0	
2,3,6-tri-*O*-methylglucitol		1.0	0.9	1.0
2,3,6-tri-*O*-methylgalactitol			1.0	1.0
2,4,6-tri-*O*-methylgalactitol				0.8
3,4,6-tri-*O*-methyl-*N*-methylacetamidogalactitol				0.6

[a] The ratios are expressed relative to 2,3,6-tri-*O*-methylglucitol, except for fraction GL1 where 2,3,4,6-tetra-*O*-methylglucitol is used for comparison.

GL3 contained approximately twice the amount of galactose as glucose, and GL4 contained glucose, galactose, and *N*-acetylgalactosamine in the ratio 1.0:2.2:0.7. The low molar ratio of *N*-acetylgalactosamine is in keeping with the general experience that the recovery of amino sugars is less than that of neutral sugars (Chambers & Clamp, 1971).

Mass Spectrometry and Methylation Analysis. Figure 2 shows simplified direct probe spectra for the permethylated glycosphingolipids containing $C_{24:0}$ fatty acid isolated from hairy cells, along with proposed structures and indications of some cleavage sites within the molecules. In the analysis shown in Figure 2, information on the type of carbohydrates (hexose or hexosamine), fatty acids, and sphingosine base, and the sequence of the sugars, is obtained. The sequence for GL3 of hexose-O-hexose-O-hexose and for GL4 of hexosamine-O-hexose-O-hexose is consistent with globo structures. The simplified spectra do not show the rearrangement ions (*m/z* 294, 312, and 364) for sphingosine or the ions for the other major fatty acids ($C_{16:0}$, $C_{24:1}$) at *m/z* 294 and 404 and corresponding ceramides at *m/z* 548 and 658.

In order to determine the glycosidic linkage positions between carbohydrate residues, permethylated alditol acetates were prepared from the purified glycosphingolipids. The resulting permethylated alditol acetates were separated by gas–liquid chromatography, and the mass spectrum of each was obtained. The spectra indicated that hairy cell glycosphingolipids had carbohydrates with the following glycosidic linkages (Table II): GL1, 1-linked glucose; GL2, 1-linked galactose and 1,4-linked glucose; GL3, 1-linked galactose, 1,4-linked glucose, and 1,4-linked galactose; GL4, 1-linked *N*-acetylgalactosamine, 1,3-linked galactose, 1,4-linked galactose, and 1,4-linked glucose.

Glycosidase Hydrolysis. The anomerity of the glycosidic linkages for each glycosphingolipid was established by hydrolysis with highly purified plant glycosidases (data not shown). Hairy cell GL2 was cleaved to GL1 and a monosaccharide by β-galactosidase, showing that it contained a terminal β-linked galactose residue. The GL3 component had a terminal α-linked galactose, and, after removal of this sugar, another galactose residue could be released by β-galactosidase.

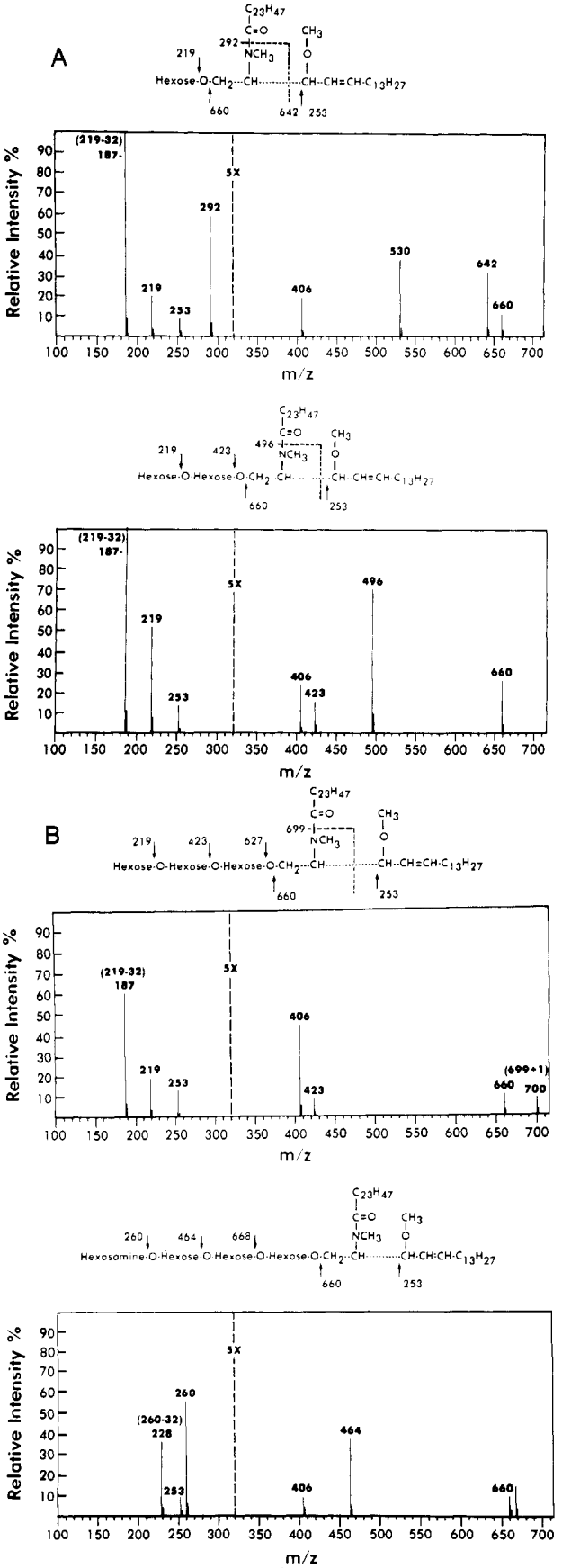

FIGURE 2: Simplified mass spectra of the intact permethylated glycosphingolipids GL1–GL4 from hairy cells. Conditions were as described under Experimental Procedures. Proposed structures and indications of some cleavage sites within the molecules giving rise to the peaks are shown above the respective mass spectra. The simplified spectra show permethylated glycosphingolipids containing $C_{24:0}$ fatty acid (*m/z* 406) and its corresponding ceramide containing $C_{18:1}$ sphingosine base (*m/z* 660).

Therefore, GL3 had a nonreducing terminal disaccharide structure of Gal$\alpha\rightarrow$Gal$\beta\rightarrow$. By treating the hairy cell GL4 sequentially with β-hexosaminidase, α-galactosidase, and β-galactosidase, it was established that the nonreducing terminal trisaccharide of this compound was GalNAc$\beta\rightarrow$Gal$\alpha\rightarrow$-Gal$\beta\rightarrow$.

Discussion

Hairy cell leukemia is a neoplastic disease whose cellular origin is unknown. Some lines of evidence have supported a monocytic origin for hairy cell leukemia, whereas others have supported a lymphoid origin. Support for a monocyte origin of hairy cells comes from the ability of these cells to ingest latex particles (Fu et al., 1974), their expression of a nonspecific esterase enzyme (Rosenszajn et al., 1976), and their having strong receptors for the Fc portion of IgG molecules (Jaffee et al., 1974). A lymphocytic origin for hairy cells is suggested by their ability to synthesize intracytoplasmic immunoglobulins of restricted light chain type (Golde et al., 1977) and, when cultured, to secrete newly synthesized immunoglobulins into the culture supernatant (Golde et al., 1977; Rieber et al., 1979; Gordon et al., 1978), frequently with an excess of free light chains (Gordon et al., 1978). These apparently conflicting findings are not fully resolved and have led to speculations that hairy cells are "hybrid" cells or are derived from some not well-characterized subset of lymphocytes.

We studied the neutral glycosphingolipids present in hairy cells to determine the type of compounds in these unusual cells. Our analyses show that these cells contain neutral glycosphingolipids having one to four sugars with the following structures:

$$\text{Glc}\beta1\rightarrow1\text{Cer}$$

$$\text{Gal}\beta1\rightarrow4\text{Glc}\beta1\rightarrow1\text{Cer}$$

$$\text{Gal}\alpha1\rightarrow4\text{Gal}\beta1\rightarrow4\text{Glc}\beta1\rightarrow1\text{Cer}$$

$$\text{GalNAc}\beta1\rightarrow3\text{Gal}\alpha1\rightarrow4\text{Gal}\beta1\rightarrow4\text{Glc}\beta1\rightarrow1\text{Cer}$$

The structures of these compounds belong to the globo series and are identical with the structures of the neutral glycosphingolipids containing one to four sugars found in human lymphoid cells (Lee et al., 1981). We found no evidence of compounds of the lactoneo series in these hairy cells. Since chronic leukemic cells appear to retain the type of neutral glycosphingolipids found in their normal leukocyte counterpart, we believe that a myeloid origin for hairy cell leukemia is unlikely. However, we do not know the glycosphingolipid composition of monocytes and, therefore, cannot comment upon a monocytic vs. a lymphocytic origin for hairy cells. However, analysis of other complex carbohydrates (glycoproteins) from hairy cells suggests that these cells have a pattern similar to that of lymphoid cells and different from that of monocytic cells (Andersson, 1980).[2] Finally, it is known that hairy cells from different patients may differ in certain characteristics, suggesting that heterogeneity exists in this disorder. Our analysis was performed on the cells from a single patient, which limits our ability to generalize the results presented here. Unfortunately, only the very rare patient with hairy cell leukemia yields sufficient numbers of pure hairy cells to permit detailed studies such as ours. This means that studies of glycosphingolipids found in hairy cells from different patients will have to be carried out with more sensitive but less definitive techniques.

These considerations are leading us to study the glycosphingolipids present in monocytic cells to see whether they contain myeloid, lymphoid, or yet another pattern of glycosphingolipids. In addition, we are using high-performance liquid chromatography to study the glycosphingolipids of hairy cells from additional patients to see if there are differences in their glycosphingolipid content.

[2] Dr. Andersson may be contacted at the Transplantation Laboratory, University of Helsinki, Helsinki, Finland.

Acknowledgments

We thank Dr. Harold Mielke of the Presbyterian Hospital, Pacific Medical Center, San Francisco, CA, for providing the hairy cells used in our analysis and Dr. Yu-Teh Li for providing the glycosidases. We also thank Betty Baltzer of the Michigan State University—National Institutes of Health Mass Spectrometry Facility for assistance with the mass spectrometric analyses and Jim Harris for typing this manuscript.

References

Andersson, L. C. (1980) presented at the Third Annual Bristol-Myers Symposium on Cancer Research, Stanford University Medical Center, Nov 20–21, 1980.

Chambers, R. E., & Clamp, J. R. (1971) *Biochem. J. 125*, 1009.

Fu, S. M., Winchester, R. J., Rai, K. R., & Kunkel, H. G. (1974) *Scand. J. Immunol. 3*, 847.

Golde, D. W., Stevens, R. H., Quan, S. G., & Saxon, A. (1977) *Br. J. Haematol. 35*, 359.

Golomb, H. M., Catovsky, D., & Golde, D. W. (1978) *Ann. Intern. Med. 89*, 677.

Gordon, J., Smith, J. L., & Roath, S. (1978) *Br. J. Haematol. 39*, 150.

Hakomori, S. I. (1964) *J. Biochem. (Tokyo) 55*, 205.

Hester, J. P., Kellogg, R. M., Mulzet, A. P., Kruges, V. R., McCredie, K. B., & Freireich, E. J. (1979) *Blood 54*, 254.

Jaffee, E. S., Shevach, E. M., Frank, M. M., & Green, I. (1974) *Am. J. Med. 57*, 108.

Klock, J. C., D'Angona, J. L., & Macher, B. A. (1981) *J. Lipid Res. 22*, 1079.

Ledeen, R. W., Yu, R. K., & Eng, L.-F. (1973) *J. Neurochem. 121*, 829.

Lee, W. M. F., Klock, J. C., & Macher, B. A. (1981) *Biochemistry 20*, 3810.

Li, Y.-T., & Li, S.-C. (1977) in *The Glycoconjugates* (Horowitz, M. I., & Pigman, W., Eds.) Vol. 1, p 51, Academic Press, New York.

Macher, B. A., & Klock, J. C. (1980) *J. Biol. Chem. 255*, 2092.

Macher, B. A., Pacuszka, T., Mullin, B. R., Sweeley, C. C., Brady, R. O., & Fishman, P. H. (1979) *Biochim. Biophys. Acta 588*, 35.

Rieber, E. P., Hadam, M. R., Linke, R. P., Saal, J. G., Reithmüller, G., Heyden, H. W., & Waller, H. D. (1979) *Br. J. Haematol. 42*, 175.

Rosenszajn, L. A., Gutman, A., Radnay, J., David, E. B., & Shoham, D. (1976) *Am. J. Clin. Pathol. 66*, 432.

Schwarting, G. A. (1980) *Biochem. J. 189*, 407.

Stein, K. E., & Marcus, D. M. (1977) *Biochemistry 16*, 5285.

Wherrett, J. R. (1973) *Biochim. Biophys. Acta 326*, 63.