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Research Article

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Studies of Red Cell Stromal Proteins in Tay-Sachs Disease

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ABSTRACT Hemoglobin-free red blood cell ghosts of nine patients with Tay-Sachs disease and 14 normal control subjects have been analyzed for content of total protein, hexosamines, individual amino acids, and sialic acid. Red cell ghosts from Tay-Sachs' children have been shown to contain significantly increased amounts of protein, hexosamine, threonine, and serine, and probably sialic acid, each of which was increased by approximately 25% over control values. These observations suggest that the red cell membrane in patients with Tay-Sachs disease contains a significant excess of a glycoprotein or proteins, as compared with normal, and that the metabolic defect in this disease, therefore, affects glycoproteins as well as complex lipids.

INTRODUCTION

Previous studies from this laboratory (1, 2) demonstrated a statistically significant decrease in red cell stromal sphingomyelin in patients with Tay-Sachs disease (TS) as compared with normal controls and intermediate values in their presumably heterozygous parents. It was further shown that this decrease in red blood cell (RBC) sphingomyelin could be used to detect carriers of the Tay-Sachs' trait (2). Similar changes, however, have been noted by us (1) and others (3) in Niemann-Pick disease and Gaucher's disease. In an attempt to determine whether this decrease in stromal phospholipid was secondary to a reduction of lipoprotein in the red cell membrane, we determined protein nitrogen and amino acid composition of hemoglobin-free red cell ghosts in normal subjects and in patients with Tay-Sachs disease. Instead of the expected decrease, however, increased protein content with a particular increase in threonine and serine was noted early in the studies and, therefore, in further studies hexosamines and sialic acid were also measured.

METHODS

A total of nine children with Tay-Sachs disease were studied, together with five children convalescent from non-neurological acute disease of similar age. The TS children exhibited the clinical findings of their disease. A cherry red spot was present in each and eight of them had seizures. They ranged in age from 18 to 46 months. Five were female and four were male. In addition, nine normal adults ranging in age from 18 to 42 yr (three female and six male) were examined. From each subject 8–20 ml of venous blood was drawn into centrifuge tubes containing ACD solution (USP) and the red blood cells (RBC) were separated by centrifugation and washed three times with 0.9% saline. The ghosts were prepared by lysis of RBC in hypotonic phosphate buffer (15 mOsm, pH 7.8) as described by Dodge, Mitchell, and Hanahan (4). These ghosts were shown to contain $< 1\%$ of total protein as hemoglobin (5). Before hemolysis an aliquot of the red cell suspension (hematocrit 10%) was taken for red cell counts done manually with a hemocytometer chamber. The ghosts were suspended in 10 ml of 15 mOsm phosphate buffer, pH 7.8, and stored at 4°C until analysis. In preparation for amino acid analysis, samples of each suspension were hydrolyzed for 24 hr in 4 N HCl (2 ml of 6 N acid/1 ml ghost suspension containing from 2.2 to 11.0×10^9 ghosts) in glass ampoules sealed after flushing with nitrogen. Hydrolysis was performed at 110, 113, 115, 118, and 120°C. Hydrolysis was found to be incomplete at 110°C, while at 120°C

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excessive losses of threonine and serine, with a marked increase in ammonia were noted. Therefore the data presented are based on the analysis of samples hydrolyzed at 113, 115, and 118°C. Ghosts from RBC of Tay-Sachs' children were always hydrolyzed at the same time as a group of control samples. After hydrolysis, samples were transferred with three washes of 0.1 N HCl to drying flasks and the HCl was removed on a rotary evaporator under reduced pressure with a mechanical pump. The amino acids were then taken up in 2 ml of 0.1 N HCl and samples of 0.1–0.3 ml were applied to the column for analysis on a Technicon amino acid analyser. Individual amino acids were identified by their retention times compared with known standards and quantified by triangulation (6). In each run, norleucine was added as an internal standard. Norleucine recoveries ranged from 97 to 105% compared with chromatograms of standard amino acid mixtures of known composition. All samples were analyzed in duplicate. Initially, 21.5 hr chromatograms were used. The later studies were performed with 4.5 hr chromatograms. In two patients with TS and in two normal subjects, three separate samples of blood were analyzed over a period of 1–2 yr with both types of chromatograms and reproducibility was found to be good, the coefficient of variation being less than 5% for μmole of amino acid/10^9 RBC and molar ratio of each amino acid to aspartic acid. No corrections were made for loss of threonine and serine during hydrolysis.

In order to establish the optimum conditions for the determination of total hexosamine content, three samples of ghosts were hydrolyzed at 100°C in 3 N HCl (1 ml of 6 N HCl/1 ml ghosts) for 8, 16, and 24 hr, both alone and with added standard glucosamine. Maximum yields were obtained after 16 hr of hydrolysis and this result was arbitrarily assigned a value of 100%. The relative yields at 8 and 24 hr were 75 and 59%, respectively. The hydrolysates were chromatographed on analytical grade cation exchange resin [1] on columns 10 cm long and 1 cm in diameter with 10 ml of distilled water followed by 20 ml of 2 N HCl, and then 10 ml of 2 N HCl. It was shown that 90–100% of the hexosamine was eluted with the first HCl wash and 0–10% with the second acid wash (7). Neutralization of the acid solution with NaOH or Na_2CO_3 was found to result in incomplete and variable color development with the Elson-Morgan reaction (8). Therefore the eluates were dried in vacuo, redissolved in distilled water, and dried repeatedly until all traces of HCl had disappeared, whereupon they were then made to volume in distilled water before analysis. In order to increase sensitivity, the Elson-Morgan reaction was modified by using smaller volumes of reagent (0.2 ml of acetyl acetone reagent, 2.0 ml of 95% ethanol, 0.2 ml of Ehrlich reagent, and 0.2 ml of sample) where small samples only were available for analysis. A linear response was obtained for quantities of hexosamine, from 1.0 to 30 μg by this method. The sensitivity of the standard procedure is from 40–200 μg.

[1] AG 50W-X 8, 200–400 mesh, H^+ form, obtained from Bio-Rad Laboratories, Richmond, Calif.

TABLE I

Total Proteins, Hexosamines, and N-Acetyl Neuraminic Acid (NANA) in Red Cell Ghosts of Normal Controls and Patients With Tay-Sachs (T.S.) Disease

	No.	Protein*	Hexosamines*	NANA
		mg/10^9 RBC	*μg/10^9 RBC*	*μmole/10^9 RBC*
Normals	14	0.38 ± 0.04	9.84 ± 1.4	0.046 (3)‡
T.S.	9	0.46 ± 0.07	12.58 ± 1.5	0.055 (4)
P		<0.005	<0.005	

* Values represent mean ± 1 SD.

‡ Numbers in parentheses represent number of patients in whom NANA was measured.

Total nitrogen was determined by Nesslerization after acid digestion and the result was used to calculate protein content. Lipid nitrogen was neglected, as it accounts for less than 10% of total nitrogen in RBC ghosts. *N*-acetyl-neuraminic acid (NANA) was determined by the thiobarbituric acid method (9). For measurement of lipid NANA, samples of red cell ghosts were extracted with boiling chloroform:methanol, as described by Svennerholm (10) and the gangliosides separated by column chromatography on silicic acid (10). Purity of the fractions was confirmed by thin-layer chromatography (11). Recoveries of commercial "ganglioside" [2] added to albumen before extraction averaged 71.5%. All the extracted ganglioside was recovered in the chloroform:methanol (1:4) fraction after column chromatography.

Results are expressed as mg of protein, μg of hexosamine, μmole of amino acid, and μmole of NANA per 10^9 RBC and as molar ratio of each amino acid to aspartic acid. Values are given as mean ± SD both in the text and tables. Significance of differences between the groups were tested with Student's *t* test.

RESULTS

Total protein was 0.38 ± 0.04 mg/10^9 ghosts in the normals, with no difference between children and adults, and 0.46 ± 0.07 mg in the children with Tay-Sachs disease (Table I). This difference is highly significant ($P < 0.005$). Total hexosamines were 9.84 ± 1.37 in normals compared with 12.5 ± 1.51 μg/10^9 ghosts in TS ($P < 0.005$). The normal children exhibited higher values for hexosamines than did normal adults (10.8 vs. 9.0 μg/10^9 ghosts), but the difference between normal children and TS children was still significant ($P < 0.02$). In only two instances did normal values for protein and hexosamine exceed the

[2] Pierce Chemical Co., Rockford, Ill.

TABLE II

Total and Lipid N-Acetyl Neuraminic Acid (NANA) in Red Cell Ghosts of Two Normal and Two Tay-Sachs' Subjects (TS)

Patients		Total NANA	Lipid NANA	% of total
			μmole/10⁹ ghosts	
Normals:	J.B. 1	0.043	0.00065	1.5
	2	0.052	0.0025	4.8
	3	0.044	0.0014	3.1
	E.K. 1	0.041	0.0013	3.2
	2	0.050	0.0016	3.1
TS:	P.	0.058	0.0015	2.6
	Y.	0.056	0.0017	3.1

lower limit of the TS range. Total RBC ghost NANA was measured in three normal subjects and four TS children. The mean normal value was 0.046 μmole/10^9 ghosts (range 0.043–0.052), whereas that in TS was 0.055 μmole/10^9 ghosts (range 0.049–0.058) (Table I).

It has been suggested that there is a significant increase in gangliosides in RBC in the late infantile and juvenile forms of amaurotic idiocy (12) and perhaps Tay-Sachs disease (3). In order to determine the amount of lipid NANA in red cell stroma, separate samples of the RBC ghosts of two normal subjects and from two TS patients were extracted and analyzed according to Svennerholm's method (11). It can be seen from Table II that in both normal subjects and TS patients, less than 5% of the total NANA is present in gangliosides, and there is no demonstrable increase in lipid NANA in RBC stroma in TS. At these concentrations of NANA the thiobarbituric acid assay is at its lower limits of sensitivity. However, in our hands the response is still linear at this point. It seems likely therefore that the elevated hexosamines represent glycoprotein rather than glycolipid amino sugars.

Table III shows the data on amino acids (AA) in the normals and TS expressed as μmole of AA/10^9 ghosts after hydrolysis at 113°C. Threonine and serine were found to be very significantly

TABLE III

*Amino Acid Composition of Red Cell Ghosts in 14 Normal Controls and 9 Patients with Tay-Sachs Disease (TS) After Hydrolysis at 113° C**

Amino acid	Normal	Tay-Sachs	*P*
	μmole/10⁹ ghosts		
Aspartic	0.215 ± 0.020	0.242 ± 0.029	>0.05
Threonine	0.121 ± 0.011	0.150 ± 0.022	<0.005
Serine	0.174 ± 0.014	0.213 ± 0.028	<0.005
Glutamic	0.324 ± 0.035	0.379 ± 0.017	<0.02
Proline	0.124 ± 0.012	0.153 ± 0.028	<0.05
Glycine	0.177 ± 0.020	0.209 ± 0.033	<0.05
Alanine	0.204 ± 0.021	0.246 ± 0.029	<0.01
Valine	0.160 ± 0.022	0.171 ± 0.023	>0.4
Cystine	0.037 ± 0.005	0.028 ± 0.012	>0.2
Methionine	0.059 ± 0.016	0.062 ± 0.016	>0.7
Isoleucine	0.117 ± 0.017	0.131 ± 0.018	>0.1
Leucine	0.302 ± 0.033	0.348 ± 0.041	<0.05
Tyrosine	0.061 ± 0.006	0.072 ± 0.008	<0.02
Phenylalanine	0.110 ± 0.012	0.127 ± 0.006	<0.05
Ammonia	0.415 ± 0.074	0.494 ± 0.086	>0.05
Lysine	0.135 ± 0.017	0.155 ± 0.026	>0.05
Histidine	0.064 ± 0.008	0.078 ± 0.013	<0.025
Arginine	0.139 ± 0.028	0.153 ± 0.031	>0.4

* Values represent means ± 1 SD.

TABLE IV

Micromoles of Threonine, Serine, and Alanine in Red Cell Ghosts of Normal Subjects and Children with Tay-Sachs Disease and Their Molar Ratios to Aspartic Acid

	Normal controls (14)*	Tay-Sachs (9)*	*P*
115°C hydrolysis			
Threonine, *μmole/10⁹ RBC*	0.13 ± 0.01	0.15 ± 0.01	<0.01
Serine, *μmole/10⁹ RBC*	0.18 ± 0.01	0.21 ± 0.01	<0.005
Alanine, *μmole/10⁹ RBC*	0.21 ± 0.02	0.23 ± 0.01	>0.05
Threonine/ aspartic	0.57 ± 0.02	0.63 ± 0.02	<0.005
Serine/aspartic	0.79 ± 0.04	0.87 ± 0.03	<0.005
Alanine/aspartic	0.91 ± 0.06	0.95 ± 0.04	>0.2
118°C hydrolysis			
Threonine, *μmole/10⁹ RBC*	0.11 ± 0.01	0.15 ± 0.02	<0.02
Serine, *μmole/10⁹ RBC*	0.11 ± 0.01	0.18 ± 0.02	<0.005
Alanine, *μmole/10⁹ RBC*	0.25 ± 0.03	0.26 ± 0.03	NS
Threonine/ aspartic	0.47 ± 0.05	0.55 ± 0.03	<0.025
Serine/aspartic	0.45 ± 0.06	0.66 ± 0.06	<0.005
Alanine/aspartic	1.00 ± 0.06	0.96 ± 0.01	NS

Values represent mean ± 1 SD; NS, not significant.
* Numbers in parentheses represent number of patients studied in each group.

increased in the RBC ghosts of TS as compared with normals. In only two instances were values for normal subjects higher than the lowest levels in the TS patients. Lesser increases were observed for glutamic acid, proline, glycine, and alanine. Similar differences were observed after hydrolysis at 115 and 118°C (Table IV). Aspartic acid was found to be present in similar concentration in the RBC ghosts of both normals and TS. Therefore, amino acid molar ratios were calculated with aspartic acid as the basis for comparison. The molar ratio of threonine to aspartic acid after hydrolysis at 113°C in TS was 0.61 ± 0.02 as compared with 0.56 ± 0.03 ($P < 0.005$) (Table V). The same ratio for serine was 0.88 ± 0.02 in TS and 0.81 ± 0.02 for controls ($P < 0.005$). Alanine also showed significant increase relative to aspartic acid (Table V). The molar ratio of threonine and serine to aspartic acid was significantly increased after hydrolysis at 115 and 118°C also (Table IV), whereas ratios for glutamic acid, glycine, and alanine were more variable.

If the increase in hexosamines represented increased content of glycoprotein, it would be expected that the ratio of hexosamines to threonine and serine should be similar in TS and normal ghosts, while this ratio should be elevated relative to other amino acids, as the hydroxyl groups on threonine and serine are thought to be the points of attachment of the carbohydrate side-chains in glycoproteins. This was in fact found to be generally true as shown in Table VI. For threonine and serine the probability of identity for the ratios in TS and controls was found to be > 0.4 and > 0.3, respectively; whereas, for other amino acids, P values were smaller and < 0.05 for isoleucine and aspartic acid and < 0.1 for methionine, leucine, tyrosine, phenylalanine, and arginine.

TABLE V

Molar Ratio of Each Amino Acid to Aspartic Acid After Hydrolysis of Red Cell Ghosts at 113°C

Amino acid	Normal* (14)	Tay-Sachs* (9)	P
Aspartic	—	—	—
Threonine	0.56 ± 0.03	0.61 ± 0.02	<0.005
Serine	0.81 ± 0.02	0.88 ± 0.02	<0.005
Glutamic	1.52 ± 0.08	1.57 ± 0.05	>0.1
Proline	0.58 ± 0.04	0.625 ± 0.05	>0.1
Glycine	0.83 ± 0.05	0.87 ± 0.05	>0.1
Alanine	0.95 ± 0.03	1.03 ± 0.07	<0.02
Valine	0.75 ± 0.06	0.76 ± 0.03	>0.6
Cystine	0.18 ± 0.03	0.15 ± 0.02	>0.05
Methionine	0.28 ± 0.07	0.25 ± 0.05	>0.4
Isoleucine	0.55 ± 0.04	0.55 ± 0.03	>0.9
Leucine	1.41 ± 0.06	1.44 ± 0.02	>0.1
Tyrosine	0.29 ± 0.02	0.30 ± 0.01	>0.2
Phenylalanine	0.51 ± 0.04	0.53 ± 0.02	>0.3
Ammonia	1.94 ± 0.28	2.15 ± 0.24	>0.1
Lysine	0.63 ± 0.03	0.64 ± 0.04	>0.5
Histidine	0.30 ± 0.02	0.32 ± 0.02	>0.05
Arginine	0.65 ± 0.06	0.65 ± 0.05	>0.9

* Values represent mean ± 1 SD.
Numbers in parentheses represent number of subjects studied.

TABLE VI

Ratio of Hexosamines to Each Amino Acid in Normal Subjects and Children with Tay-Sachs Disease Expressed as μg of Hexosamine/μmole of Amino Acid

Amino acid	Normal*	Tay-Sachs*	P
Aspartic	44.9 ± 4.7	51.7 ± 4.8	<0.025
Threonine	79.8 ± 10.3	84.0 ± 10.8	>0.4
Serine	55.1 ± 6.3	58.5 ± 6.6	>0.3
Glutamic	29.8 ± 4.1	32.6 ± 3.0	>0.1
Proline	78.4 ± 10.8	83.3 ± 12.3	>0.4
Glycine	54.5 ± 6.3	59.2 ± 7.1	>0.2
Alanine	47.2 ± 4.9	50.1 ± 1.8	>0.1
Valine	60.3 ± 7.2	70.5 ± 12.8	>0.1
Cystine	—	—	—
Methionine	167.8 ± 30.6	204.5 ± 45.0	>0.05
Isoleucine	82.3 ± 6.2	96.3 ± 14.0	<0.05
Leucine	31.9 ± 3.4	35.7 ± 3.6	>0.05
Tyrosine	156.4 ± 15.5	172.8 ± 18.0	>0.05
Phenylalanine	87.4 ± 8.2	98.0 ± 11.6	>0.05
Ammonia	23.0 ± 3.6	25.0 ± 4.5	>0.3
Lysine	71.6 ± 7.4	80.3 ± 10.8	>0.1
Histidine	149.9 ± 14.8	162.4 ± 24.1	>0.25
Arginine	68.9 ± 8.3	79.7 ± 12.5	>0.05

* Values represent mean ± 1 S.D.
Amino acid values are based on results of hydrolysis at 113°C.

DISCUSSION

The results reported here were unexpected. On the basis of the phospholipid changes in the red cell stroma in Tay-Sachs patients (1, 2), we had expected to find a reduced amount of lipoprotein in these red cells. The data obtained do not rule out this possibility, but certainly lend no support to such a concept. Indeed, the results indicate an increase in total protein per red cell ghost.

There are few comparable studies on red cell stromal protein in the literature. Dodge et al. (4)

obtained 0.96×10^{-13} g of nonhemoglobin nitrogen/ghost, a value corresponding to approximately 0.59 mg of protein/10^9 ghosts. This value is higher than the value in our normal subjects of 0.38 mg/10^9 ghosts. Amino acid analysis on ghosts prepared by the method of Dodge et al. (4) have been reported by Morgan and Hanahan (13) and Bakerman and Wasemiller (14). The molar ratios of serine, threonine, and aspartic acid to isoleucine observed by these authors closely resemble those noted in the present study (Table VII).

Data on lipid NANA in normal red cells have been reported by Edgar, Hooghwinkel, and Borri (12) and Hooghwinkel, Borri, and Bruyn (15, 16). These authors obtained values about twice as high as those reported in the present study, but their method of extraction was different and no attempt was made to remove any glycoprotein that may have been coextracted. It is of interest, however, that their data for total phospholipids and per cent distribution of phospholipids were similar to those reported by us earlier (1). Edgar et al. (12) reported a 1.5- to 5-fold increase in lipid NANA in four patients with juvenile amaurotic idiocy and one patient with the late infantile form. Rouser, Bauman, Nicolaides, and Heller (3) reported a possible increase in ganglioside in RBC in Tay-Sachs disease. We have found no evidence of a similar increase in lipid NANA in two patients with Tay-Sachs disease (Table II). By contrast, Eylar, Madoff, Brody, and Oncley (17) and Makela, Miettinen, and Pesola (18) obtained values in normal subjects very similar to those reported here, which was present as the N-acetyl neuramimic acid. Furthermore, Eylar et al. (17) concluded from studies with neuraminidase and changes in surface charge after incubation with this enzyme that NANA was present on the surface of the membrane in the form of glycoprotein.

TABLE VII

Molar Ratios of Some Amino Acids in the Red Cell Ghosts of Normal Subjects

Author	Hydrolysis temp.	Serine/ isoleucine	Threonine/ isoleucine	Aspartic/ isoleucine
Morgan and Hanahan (13)	105°C	1.4	1.3	2.1
Bakerman and Wasemiller (14)	110°C	1.3	1.2	1.7
Present	113°C	1.45 (1.29–1.59)	1.0 (0.76–1.13)	1.8 (1.65–1.94)

Kathan and Winzler (19) and Winzler, Harris, Pekas, Johnson, and Weber (20) have studied glycoprotein fractions isolated from human erythrocytes. They noted a high content of threonine and serine in their fractions and showed that threonine plus serine were present in amounts approximately equimolar with hexosamines and with sialic acid. This observation is in agreement with the suggested structure of a possible repeating unit in salivary gland mucin proposed by Hashimoto, Tsuiki, Nisizawa, and Pigman (21). Both this latter group and Kathan and Winzler (19) and Winzler et al. (20) found evidence that hexosamines were linked to the peptide chain at the hydroxyl groups of threonine and serine and that sialic acid was in turn linked by glycosidic linkage to the amino sugars. This conclusion is also suggested by the observations of Bhargava and Gottschalk (22).

We have not at this time isolated a glycoprotein from RBC ghosts. However, the preceding evidence indicates that red cell membranes contain glycoproteins. The data presented demonstrate statistically significant increases in total protein, threonine, serine, and hexosamine, and probably NANA, in red cell membranes from patients with Tay-Sachs disease as compared to normals. Each of these substances is increased by about 25%, and the ratio of hexosamines to threonine and to serine is similar in Tay-Sachs' red cells as in normals, whereas, the ratios of hexosamines to isoleucine and aspartic are significantly increased, suggesting that the increase of amino sugars is linked to that of threonine and serine, as would be predicted if the changes represented an accumulation of glycoprotein. These considerations, coupled with the lack of evidence in this study of any elevation of glycolipids in the red cell membrane in Tay-Sachs disease strongly suggest that there is a significant accumulation of glycoprotein in the red cell stroma in Tay-Sachs disease. It is interesting that its presence is not associated with any morphologic changes in the RBC in this disease.

Bogoch and Belval (23) have reported the presence of an abnormal protein in the brain of a child with Tay-Sachs disease and have characterized this as a glycoprotein. Our own earlier

studies (1, 2) and those of Eeg-Olofsson, Kristensson, Sourander, and Svennerholm (24) have shown that the biochemical abnormality in Tay-Sachs disease is not confined to the central nervous system. The specific biochemical defect in the Tay-Sachs disease has not yet been definitively established (25), but there is evidence that the enzyme defect may be either at the step involving the removal of *N*-acetyl-galactosamine from the Tay-Sachs' ganglioside or the addition of a galactosyl residue to this compound (26–28). However, the data of Bogoch and Belval (23) and those reported here suggest that the metabolic disorder in Tay-Sachs disease affects proteins as well as complex lipids and more specifically that a glycoprotein accumulates in the tissues of patients with this disorder.

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