Abstract  In order to test the hypothesis that retinitis pigmentosa (RP) is associated with fatty acid abnormalities within cell membrane phospholipids, red blood cell membrane (RBC) phosphatidylethanolamine (PE) fatty acid content (% of total fatty acids) was measured using high performance liquid chromatography and capillary column gas chromatography in 155 patients from separate families with different genetic types of RP and 101 normal subjects. After controlling for the effects of age and sex, patients with all genetic forms of RP had significantly ($P < 0.001$) reduced mean RBC PE 22:603 (n-3) (docosahexaenoic acid, DHA) content, and significantly ($P < 0.001$) elevated mean RBC PE dimethyl acetal (DMA) forms of 16:0, 18:0, and 18:109 (n-9) as compared with normal subjects. RBC PE content of 22:503 (n-3) (a precursor to DHA) and 18:206 (n-6) (the major dietary essential fatty acid) were not significantly different in RP than in controls. Analysis by genetic types of RP showed that the mean RBC PE DHA percentages were significantly reduced by 24%, 14%, 30%, and 17%, respectively, in dominant, recessive, X-linked, and isolate forms of RP. The relative amounts of plasmalogens as indicated by DMA forms of 16:0 and 18:0 were significantly ($P < 0.01$) increased in dominant (by 33% and 25%), recessive (by 36% and 25%), and isolate cases (by 32% and 26%) of RP as compared with normal subjects. No such differences were seen in X-linked cases versus controls.

Our data indicate that RBC PE DHA content is decreased in all genetic types of RP patients as compared to control subjects, and that RBC PE plasmalogens are increased in dominant, recessive, and isolate forms of RP. These data raise the possibility that membrane phospholipid fatty acid abnormalities may contribute to the pathogenesis of RP.

METHODS

Sample selection

We evaluated RBC PE fatty acid content in 155 patients, ages 18–49 years, one patient per family, with the common forms of RP. These patients came from across the United States and Canada. The diagnosis of RP in

Abnormalities of plasma docosahexaenoic acid (DHA) have been observed in various forms of retinitis pigmentosa (RP) (1–5). We have previously reported significant reductions in plasma DHA content in RP patients (5). As plasma fatty acids can fluctuate because of dietary alterations, whereas red cell membrane fatty acid content is more stable in this regard, we conducted the present study to determine whether DHA content is also decreased within the red blood cell membranes of RP patients as compared to normals (6). We examined the DHA content of phosphatidylethanolamine (PE) within the red blood cell membrane (RBC) because PE is an abundant phospholipid in cell membranes and is markedly enriched in DHA especially within rod outer segment membranes in the retina, and because RBC are readily available in RP patients (7).
Laboratory analysis

Red blood cell (RBC) samples were provided to the laboratory as numbered samples in a masked fashion. RBC were lysed in cold saline and membrane lipids were extracted into isopropanol-chloroform according to the method of Rose and Oklander (10). The lipid extract was evaporated under N₂ and lipids were then reextracted by the method of Folch, Lees, and Sloane Stanley (11). High performance liquid chromatography separation of phospholipids was performed by a previously described isocratic system using a mobile phase of hexane-isopropanol-ethanol-25 mm potassium phosphate (pH 7.0)-acetic acid 376:485:100:56.2:0.3, a LiChrospher Si-100 column (25 cm × 4.0 mm, 5 µm particle size (E. Merck, Darmstadt, Germany), and a flow rate of 1 ml/min (12, 13). Detection was at 205 nm. The mobile phase was prepared as originally described, except that after mixing and filtering (to remove precipitated potassium phosphate) 75 ml water was added to the mobile phase (14). This amount of additional water resulted in elution of the PE peak free of neutral lipids and other phospholipids, within 4.0-7.5 min.

The PE fraction was collected and reextracted by the method of Folch et al. (11) to remove contaminating salts. The chloroform phase was dried and the PE was then methylated in 1 ml of 2.5% HCl in methanol (at 100°C for 4 h). This procedure converts acyl groups to methyl esters and the alkyl portion of the vinyl ether of plasmalogens to a dimethyl acetal (DMA). After methylation, the tubes were heated was added, and the methylated products were extracted into hexane. The hexane phase was then washed with NaOH and dried over NaSO₄.

The methylated products were separated by capillary gas chromatography as previously described and quantified by digital integration using a Hewlett-Packard (Avondale, PA) 3390A integrator (14). The chromatography was performed with a Shimadzu gas chromatograph Mini-2 (Columbia, MD) equipped with a flame ionization detector and a 0.20 µ SP 2330 column (50 m × 0.25 mm, Supelco, Bellefonte, PA). This method separates all fatty acids into discrete peaks.

Statistical methods

Analysis of covariance was used for the following purposes: 1) to compare RBC PE fatty acid percentages in all RP patients versus the group of normal subjects, and 2) to compare each group of RP patients by genetic type versus the group of normal subjects, after controlling for age and sex using the SAS General Linear Model (GLM) procedure (15, 16). In the first analysis, a class variable with two categories was used to distinguish affected patients from normals while controlling for age and sex. An F-test was performed to assess overall group differences after controlling for age and sex. Analysis of differences in mean fatty acid percentages of each genetic type of RP versus normals was performed with the Least Significant Difference (LSD) approach.

RESULTS

Data in all RP patients

RBC PE fatty acid contents (mole % of total fatty acids) in all RP patients and control subjects are shown in Table 1. Percentages of the dimethyl acetal (DMA) forms of 16:0, 18:0, and 18:1ω9 were all significantly higher in RP cases than in control subjects with differences of 20%, 17%, and 20%, respectively. In contrast, the percentages of 16:0, 18:0, and 18:1ω9 were all significantly reduced in RP cases versus controls with differences of 6%, 13%, and 9%, respectively. RBC PE content of 18:2ω6 and its derivative 20:3ω6 were not different from normal, but 20:4ω6 and 22:4ω6 content values were significantly increased in RP cases versus controls with differences of 6%, 13%, and 9%, respectively. RBC PE content of 18:2ω6 and its derivative 20:3ω6 were not different from normal, but 20:4ω6 and 22:4ω6 content values were significantly increased in RP cases versus controls with differences of 6%, 13%, and 9%, respectively.
TABLE 1. RBC PE fatty acids in RP patients and controls (% of total)a

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Affected (n = 155)</th>
<th>Control (n = 101)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMA 16:0 (dimethyl acetal palmitic)</td>
<td>4.77 ± 0.08</td>
<td>3.96 ± 0.13</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>DMA 16:0 (palmitic)</td>
<td>14.42 ± 0.15</td>
<td>15.31 ± 0.26</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>DMA 18:0 (dimethyl acetal stearic)</td>
<td>9.83 ± 0.12</td>
<td>8.39 ± 0.20</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>DMA 18:0 (dimethyl acetal oleic)</td>
<td>3.54 ± 0.06</td>
<td>2.78 ± 0.07</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>DMA 18:1 (stearic)</td>
<td>7.18 ± 0.12</td>
<td>8.29 ± 0.16</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>18:1 (oleic)</td>
<td>16.97 ± 0.21</td>
<td>18.60 ± 0.50</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>18:2 (linoleic)</td>
<td>5.87 ± 0.07</td>
<td>5.94 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>20:3 (dihomogamma linoleic)</td>
<td>1.00 ± 0.02</td>
<td>1.06 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>20:5 (arachidonic)</td>
<td>21.47 ± 0.15</td>
<td>19.77 ± 0.29</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>22:5 (5,8,11,14,17 eicosapentaenoic)</td>
<td>0.60 ± 0.02</td>
<td>0.66 ± 0.04</td>
<td>NS</td>
</tr>
<tr>
<td>22:6 (7,10,13,16 docosatetraenoic)</td>
<td>6.93 ± 0.08</td>
<td>6.45 ± 0.12</td>
<td>&lt;.01</td>
</tr>
<tr>
<td>22:6 (4,7,10,13,16,19 docosahexaenoic)</td>
<td>0.88 ± 0.02</td>
<td>0.88 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>22:6 (4,7,10,13,16,19 docosahexaenoic) (DHA)</td>
<td>3.72 ± 0.09</td>
<td>4.70 ± 0.18</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

aPercentages for each diagnosis total 100% after allowing for rounding.

bP-values for differences between mean fatty acid values for affected versus normal subjects after controlling for age and sex using the SAS GLM procedure.

In order to examine membrane PE fatty acid interrelationships further in RP patients versus controls, ratios of specific fatty acids to other fatty acids were calculated for RP cases and compared to controls (see Table 2). The ratios of DMA 16:0/16:0, DMA 18:0/18:0, and DMA 18:1/18:0 were all significantly higher in RP cases versus controls with differences of 26%, 33%, and 33%, respectively. The ratio of 18:2/20:4 was also calculated, and was significantly lower by 10% in RP cases versus controls. While the ratio of 20:5/22:6 was not different in RP cases versus controls, the ratio of 22:5/22:6 was significantly higher in RP cases by 29% than in controls.

**Data in RP patients by genetic type**

RBC PE fatty acid contents in patients with the various genetic forms of RP and in control subjects are shown in Table 3. RBC PE DHA content was noted to be significantly lower in all genetic types of RP than in controls. In addition, the RBC PE content of DHA was significantly lower in X-linked RP than in controls. The content of all other fatty acids in X-linked RP was similar to normal.

**TABLE 2. RBC PE fatty acid ratios in RP patients and controls**

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Affected (n = 155)</th>
<th>Control (n = 101)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMA 16:0/16:0</td>
<td>0.34 ± 0.007</td>
<td>0.27 ± 0.010</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>DMA 18:0/18:0</td>
<td>1.44 ± 0.030</td>
<td>1.06 ± 0.041</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>DMA 18:1/18:0</td>
<td>0.20 ± 0.005</td>
<td>0.15 ± 0.005</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>20:5/22:6</td>
<td>0.17 ± 0.012</td>
<td>0.14 ± 0.007</td>
<td>NS</td>
</tr>
<tr>
<td>18:2/20:4</td>
<td>0.28 ± 0.005</td>
<td>0.31 ± 0.010</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>22:5/22:6</td>
<td>0.88 ± 0.022</td>
<td>0.68 ± 0.022</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*P-values for differences between mean fatty acid values for affected versus normal subjects after controlling for age and sex using the SAS GLM procedure.
fatty acid. Dominant RP patients had a significantly lower 20:3o6 content than controls.

To further explore fatty acid interrelationships within RBC membrane PE we calculated ratios of various fatty acids as in Table 2 for all genetic types of RP (Table 4). A significantly higher ratio of 22:5o3/22:6o3 was noted in all forms of RP compared with that in control subjects. This ratio was increased by 37%, 19%, 49%, and 18%, respectively, in dominant, recessive, X-linked, and isolate cases of RP. In addition, the 20:5o3/22:6o3 ratio was significantly higher only in X-linked forms of RP versus controls.

Dominant, recessive, and isolate forms of RP had higher ratios of DMA 16:0/16:0, DMA 18:0/18:0 and DMA 18:1o9/18:1o9 as compared to controls. In autosomal dominant RP these ratios were 37%, 50%, and 40% higher, respectively, than in controls. In recessive RP cases these ratios were 37%, 54%, and 53% higher, respectively, than in controls. In isolate RP these ratios were 37%, 48%, and 53% higher, respectively, than in controls. In X-linked RP only the DMA 18:1o9/18:1o9 ratio was significantly higher than in controls after correcting for age and sex. It should be noted that the values given in all tables are prior to this

![Fig. 1. Mean ± SEM percentage of docosahexaenoic acid (22:6o3) within red blood cell phosphatidylethanolamine in various genetic types of retinitis pigmentosa and in control subjects.](image-url)
correction. These data indicate that plasmalogens as indicated by DMA 16:0, DMA 18:0, and DMA 18:1ω9 accumulate within RBC PE while the diacyl forms of these fatty acids within PE are reduced in all forms of RP except X-linked RP. In addition, the ratio of 18:2ω6/20:4ω6 was normal in X-linked RP, but was significantly lower than normal in dominant, recessive, and isolate forms of RP by 19%, 16%, and 13%, respectively, possibly due to the excess plasmalogens in these forms.

**DISCUSSION**

Photoreceptor outer segment membrane phospholipids, such as PE, contain exceptionally large amounts of long chain polyunsaturated fatty acids, especially DHA, which make up almost half of the fatty acids in outer segment PE (17). A high content of DHA within membrane phospholipids increases their fluidity (18). The high degree of fluidity of disc membranes appears to be essential for the normal functioning of rhodopsin in mammalian phototransduction (18–21). DHA is retained in the retina even with prolonged dietary deficiency of ω3 fatty acids, suggesting that it may play an important role in retinal function (22). DHA is produced in the liver and possibly in other tissues and is supplied to the developing brain and retina as a fatty acid constituent with lipoproteins or on fatty acids bound to albumin (23). Whether DHA in the brain and/or retina is partly formed in situ or is entirely derived from dietary sources and/or production from other fatty acids in the liver remains to be resolved (24). It has been documented that infant monkeys develop visual problems when they and their mothers are on diets deficient in ω3 fatty acids (25, 26). Moreover, in preterm human infants with reduced vision as assessed by electroretinograms (ERG) supplementation of formula with ω3 fatty acids resulted in improved ERG amplitudes (27–29). These data suggest that DHA is important for normal retinal function.

We and others have previously reported plasma DHA abnormalities in patients with various forms of RP (1–5). In order to examine the question of whether RP patients have a decreased DHA content in a specific phospholipid class within cell membranes, we measured DHA content within RBC membrane PE. This phospholipid class was selected because it is among the most abundant phospholipids in the rod outer segment membrane of the retina and has the highest DHA content of the various membrane phospholipids, and because of the availability of RBC (17, 20, 23). Our data are consistent with the concept that all forms of RP have significant mean decreases in the DHA content of RBC PE, even though the content of 22:5ω3, a precursor of DHA was not decreased. Mean DHA content within RBC PE was significantly reduced in dominant, recessive, X-linked, and isolate cases of RP. Moreover, in dominant, recessive, and isolate RP cases significant increases in plasmalogens reflected by increases in DHA 16:0, DHA 18:0, and DMA 18:1ω9 were noted, with reciprocal significant decreases in 16:0, 18:0, and 18:1ω9. These RBC membrane fatty acid abnormalities that we have observed help to explain the increased RBC fragility reported by others in RP patients (30, 31).

Plasma DHA levels have been found to be significantly reduced in a strain of miniature poodles with a recessively inherited disorder characterized by photoreceptor cell degeneration (32, 33). Patients with abetalipoproteinemia develop RP and have impaired absorption of all dietary fatty acids and fat-soluble vitamins due to an inability to form chylomicrons within the intestine (34, 35). Patients with Refsum’s disease accumulate an abnormal fatty acid, phytanic acid, due to a defect in the catabolism of this fatty acid, and they develop retinitis pigmentosa (36). In this disorder DHA is displaced in membranes by phytanic acid. These findings are consistent with the concept that abnormalities in fatty acid

### TABLE 4. RBC PE fatty acids in RP patients by genetic type (% of total)

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Dominant (n = 10)</th>
<th>Recessive (n = 47)</th>
<th>X-Linked (n = 50)</th>
<th>Isolate (n = 48)</th>
<th>Normal (n = 101)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMA 16:0/16:0</td>
<td>0.37 ± 0.01 †</td>
<td>0.37 ± 0.01 †</td>
<td>0.56 ± 0.01 †</td>
<td>0.37 ± 0.01 †</td>
<td>0.27 ± 0.01 †</td>
</tr>
<tr>
<td>DMA 18:0/18:0</td>
<td>1.62 ± 0.08 †</td>
<td>1.66 ± 0.03 †</td>
<td>1.03 ± 0.05 †</td>
<td>1.60 ± 0.08 †</td>
<td>1.08 ± 0.04 †</td>
</tr>
<tr>
<td>DMA 18:1ω9/18:1ω9</td>
<td>0.21 ± 0.02 †</td>
<td>0.23 ± 0.01 †</td>
<td>0.15 ± 0.01 †</td>
<td>0.28 ± 0.01 †</td>
<td>0.15 ± 0.01 †</td>
</tr>
<tr>
<td>18:2ω6/20:4ω6</td>
<td>0.16 ± 0.02</td>
<td>0.14 ± 0.01</td>
<td>0.23 ± 0.04 †</td>
<td>0.14 ± 0.01</td>
<td>0.14 ± 0.01</td>
</tr>
<tr>
<td>18:2ω6/20:4ω6</td>
<td>0.25 ± 0.01 †</td>
<td>0.26 ± 0.01 †</td>
<td>0.31 ± 0.01</td>
<td>0.27 ± 0.01 †</td>
<td>0.31 ± 0.01</td>
</tr>
<tr>
<td>22:5ω3/22:6ω3</td>
<td>0.95 ± 0.07 †</td>
<td>0.81 ± 0.05 †</td>
<td>1.01 ± 0.05 †</td>
<td>0.80 ± 0.05 †</td>
<td>0.68 ± 0.02</td>
</tr>
</tbody>
</table>

*P values for testing the null hypothesis that there are no mean differences among the five groups versus the alternative hypothesis that there are some overall differences among the five groups after controlling for age and sex using the SAS GLM procedure. For fatty acids where the overall F-statistic was significant (P < 0.05), comparisons were made between each genetic type of RP and normal controls. The P-values from the two group comparisons are indicated as † if P < 0.05 different from normal and as ‡ if P < 0.01 different from normal.
metabolism including DHA contribute to the pathogenesis of RP.

It had been thought that 22:6ω3 (DHA) was formed from 22:5ω3 by the action of a delta 4 desaturase enzyme within microsomes (6, 7, 20, 23). However, recent studies by Voss and colleagues (37) indicate that 22:5ω3 is elongated to 24:5ω3 and converted to 24:6ω3 by a delta 6 desaturase. This fatty acid, 24:6ω3, is then converted to 22:6ω3 by beta oxidation within the peroxisome. Plasmalogens are ether lipids, and the initial steps in either lipid biosynthesis also occur in peroxisomes. Plasmalogens constitute 5-20% of the phospholipids in mammalian cell membranes, and are very abundant in brain and nerve tissue (37). In myelin 80-90% of PE is in the plasmalogen form (38, 39).

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