RAPID COMMUNICATION

Defective Glycosylphosphatidylinositol Anchor Synthesis in Paroxysmal Nocturnal Hemoglobinuria Granulocytes

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To investigate the biosynthesis of the glycosylphosphatidylinositol (GPI) anchor in the granulocytes of paroxysmal nocturnal hemoglobinuria (PNH), the glycolipids of granulocytes from PNH patients and normal volunteers were biosynthetically labeled with [3H]mannose in the presence of tunicamycin. Extracted glycolipids were examined by thin-layer chromatography and compared with known biosynthetic intermediates. Normal granulocytes consistently showed [3H]mannose incorporation into the complete GPI core, several GPI biosynthetic intermediates, and dolichol phosphate intermediates. Normal granulocytes consistently showed [3H]mannose incorporation into the complete GPI core, several GPI biosynthetic intermediates, and dolichol phosphate intermediates.

Granulocytes from patients with PNH were unable to incorporate detectable amounts of [3H]mannose into the complete GPI core. Thus, PNH granulocytes do not synthesize detectable amounts of the complete GPI core and this defect likely accounts for the absence of GPI-linked membrane proteins on hematopoietic cells in this syndrome.

MATERIALS AND METHODS

Granulocyte isolation. Fifty milliliters of blood from normal donors or PNH patients was drawn into 5 mL of 20% EDTA and gently mixed. The blood was immediately diluted 1:1 with cold phosphate-buffered saline containing 15 mmol/L EDTA (PBS-EDTA). The procedure was repeated until the red blood cells were washed three times in PBS-EDTA and used immediately in biosynthetic labeling studies. The red blood cells were washed once in PBS-EDTA and used immediately in biosynthetic labeling studies or fixed in 1% paraformaldehyde for fluorescence-activated cell sorter (FACS) analysis.

Determination of complement sensitivity of red blood cells (RBCs). The sensitivity of the RBC of nine patients was determined by the complement lysis sensitivity (CLS) test of Rosse and Dacie. The sensitivity of the RBC of nine patients was determined by the complement lysis sensitivity (CLS) test of Rosse and Dacie.

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GPI ANCHOR BIOSYNTHESIS IN PNH

The GPI core. The glycan consists of N-glucosamine, three mannose residues, and phosphoethanolamine. In GPI core of human origin, a palmitate moiety may be attached to the inositol and a phosphoethanolamine moiety to the first mannose.

Biosynthetic labeling. Granulocytes (6 x 10⁷) or YH16.33 wild-type murine T-cell hybridoma cells (2 x 10⁷) were incubated in low-glucose RPMI (Hazleton Biologics Inc, Lenexa, KS) with L-glutamine and 10% dialyzed bovine calf serum (GIBCO Laboratories, Grand Island, NY) After preincubation with 1.0 μg/mL tunicamycin to block the formation of dolichol-linked mannose-containing oligosaccharides, [3H]mannose (Amersham Corp, Arlington Heights, IL) was added to the cultures (40 pCi/mL to granulocytes, 20 pCi/mL to YH16.33 cells) and they were continued for 45 (YH16.33 cells) or 90 minutes (granulocytes). The cells were washed in cold PBS and the labeling was stopped by extracting the cells with 1.5 mL chloroform:methanol (1:1)(C:M) followed by 1.5 mL ch1oroform:methanol:water (10:10:3)(CM:W). The CM and CM:W extracts were combined and dried under nitrogen. Dried lipids were resuspended in 1.0 mL water saturated N-butanol; 0.5 mL water was added, and the mixture vortexed for 1 minute and centrifuged for 2 minutes to separate phases. The water-saturated butanol phase was saved and the aqueous phase was reextracted with 1.0 mL water-saturated butanol followed by vortexing and centrifugation. The combined butanol phases were washed once with 1.0 mL water and then dried under nitrogen. Dried lipids were stored at -20°C.

Thin-layer chromatography (TLC). Dried lipids were resuspended in 50 μL C:M (1:1) and spotted onto silica gel 60 TLC plates and allowed to dry. The plates were developed in C:M:W (10:10:3) to a height of 19 cm and air dried.

Labeling analysis. Developed TLC plates were analyzed for [3H]mannose incorporation by detection in a linear radioisotope scanner (Berthold Corp, Hanover NH) and by exposing them to Kodak XAR film (Eastman Kodak, Rochester, NY) followed by standard developing.

RESULTS

FACS analysis. Granulocytes were isolated from patients with PNH and from normal volunteers. Characteristics of the cells from the PNH patients are shown in Table 1. Normal granulocytes were consistently positive for both CD55 and CD59. Ten patients had a relatively uniform population of granulocytes with no detectable expression of CD55 or CD59; these cells were analyzed. The granulocytes of four other patients contained residual normal cells and could not be analyzed.

Normal and PNH granulocytes were labeled with [3H]mannose as described. A wild-type murine T-cell hybridoma was labeled concurrently as a positive control. [3H]mannose-labeled glycolipids were separated by TLC along with defined standards, and autoradiographed (Fig 2). Normal granulocytes consistently showed a pattern of mannose-labeled glycolipids similar to the wild-type hybridoma (Fig 2, lanes 1 and 2). In the autoradiograph, GPI intermediates containing one to three mannose residues were not clearly resolved. The complete GPI core (labeled C) can be specifically identified in glycolipids from normal granulocytes. This moiety has the same mobility as the murine GPI core (Fig 2, lane 1) that is missing in all T mutants (mutants lacking GPI-linked proteins) and is identified by incorporation of inositol, mannose, N-acetylg glucosamine, and phosphoethanolamine. There were additional mannose-labeled moieties present in the glycolipids of the wild-type hybridoma and normal granulocytes

Table 1. Results of Analysis of GPI Core Biosynthesis and Surface Expression of GPI Proteins

<table>
<thead>
<tr>
<th>UPN*</th>
<th>% Erythrocytes by CLS†</th>
<th>% Abnormal Granulocytes by FACS Analysis‡</th>
<th>% Abnormal Granulocytes by TLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BIH</td>
<td>ND ND ND ND Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>30</td>
<td>22 78 99 Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>32</td>
<td>51 49 100 Absent</td>
<td>Absent</td>
<td>Absent</td>
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<td>115</td>
<td>64 36 95 Absent</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>160</td>
<td>86 14 99 Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>186</td>
<td>42 45 98 Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>188</td>
<td>33 33 33 100 Absent</td>
<td>Absent</td>
<td>Absent</td>
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<tr>
<td>194</td>
<td>50 43 96 Trace</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>199</td>
<td>26 74 95 Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>241</td>
<td>82 18 97 Absent</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Normal</td>
<td>&lt;1 Present</td>
<td>Present</td>
<td></td>
</tr>
</tbody>
</table>

Abbreviation: ND, not done.
*Unique patient number for Duke PNH patients; BIH, patient from Beth Israel Hospital.
†Percent of cells in each population by the CLS test of Rosse and Dacie.
‡Percent of granulocytes lacking surface expression of CD55 and CD59.
that have not been defined biochemically. The complete core and other related intermediates are undetectable in the glycolipids of 10 patients whose granulocytes (>95%) lacked detectable CD55 and CD59. The results from patient 9 are shown (Fig 2, lane 5). The glycolipids of patient 10 showed a trace amount of the complete core even though he had 96% abnormal granulocytes (Fig 2, lane 6); this patient had 43% PNH II RBCs that bear small amounts of GPI-linked proteins.8

DISCUSSION

PNH is characterized by the inability to place or maintain GPI-anchored proteins on the cell surface.1 This anchor has a core GPI structure and is found in a variety of proteins of eukaryotic organisms,7 including trypanosomes, leishmania, and mice, as well as humans. The biosynthetic pathway of the GPI core has been determined in trypanosomes.9 This consists of the sequential addition to phosphatidylinositol of N-acetyl-glucosamine (which is subsequently deacetylated),10 three residues of mannose derived from dolichol-phosphate-mannose (DPM),11 and ethanolamine phosphate. Transcribed proteins appear to be very rapidly attached to a GPI anchor precursor by transamidation of the amino group of the ethanolamine to a carboxyl group of the terminal amino acid.12 This suggests that, within the endoplasmic reticulum, there is a preformed pool of the GPI anchor precursors that is posttranslationally attached to nascent proteins.

Murine T-cell lymphoma lines that are deficient in GPI-anchored proteins have been described.3,14,15 These cell lines can be shown to have messenger RNA (mRNA) for the absent proteins and intracellular expression of nonanchored protein with no detectable surface expression.16 In these cell lines, the defects in the synthesis of the GPI anchor have been mapped to four different steps including: (1) the inability to transfer N-acetylgalactosamine to phosphatidylinositol (this step appears to be regulated by at least three genes), (2) a deficiency of the synthetase for DPM that prevents the synthesis of the mannose donor molecule (this defect has been corrected by transfection of the cells with the synthetase gene derived from yeast), (3) a defect in the addition of the third mannose and (4) in the addition of ethanolamine phosphate.3

PNH granulocytes have been shown to lack CD55 (decay accelerating factor [DAF]) on their surface17 although mRNA for the missing protein could be shown.18 These cells appear to synthesize DAF lacking the anchor and to secrete the protein into the extracellular space.19 These data suggest that the defect in PNH involves a defect in GPI anchoring but do not delineate whether this is due to a defect in biosynthesis of the anchor or to a defect in the attachment of protein to the preformed anchor.

The data in the present study strongly suggest that the abnormal PNH cells are unable to synthesize detectable amounts of preformed GPI core. This could be due to defects in several steps in the biosynthetic pathway, as is the case with the T-T hybridoma mutants previously described4 and the Thy-1 expression mutants.5 To date we have not found a patient with PNH whose abnormal granulocytes are able to make more than trace amounts of the complete GPI core (C, Fig 2), implying that the inability to attach protein to preformed anchor is not a common mechanism leading to PNH. It is possible, but unlikely, that a defect in attachment of the completed core to the protein could result in decreased synthesis or increased catabolism of the core. At present, we are unable to define specific defects in the biosynthesis of GPI core by different patients with PNH because it is difficult to resolve biosynthetic intermediates containing from one to three mannose residues in TLC plates. We are currently investigating the use of high performance liquid chromatography (HPLC), as described previously,4 to define more conclusively the precise defects.

Indirect evidence suggests that in some, if not most, patients there is only a partial defect in the biosynthesis of the GPI core. Many patients (eg, patients 186, 188, and 194, Table 1) have RBCs that are intermediate in sensitivity to complement; the GPI-anchored proteins are present on these cells in amounts markedly less than normal.7 These patients probably make limited amounts of the GPI core. In granulocytes, this limited amount of core may be preferentially attached to FcRγIII (CD16) as the GPI-anchored form of this protein is expressed on granulocytes of some PNH patients, whereas detectable CD55 (DAF) or CD59 are not.20 This discrepancy may be due to differences in the rate of transfer of the nascent proteins to a limited amount
of anchor. The mechanism for this discrimination may depend on the amino acid at the site of attachment of the anchor. Thus, a partial deficiency in GPI core production may be manifest by complete deficiency of some proteins.

Because the attachment of anchor to protein is a very rapid process (at least in trypanosomes), any newly formed anchor would rapidly be consumed, making it difficult to detect in metabolic labeling studies, (as performed in this study) particularly if quantities were limited. Thus, the inability to detect a complete GPI anchor core in these studies may not signify a complete absence of anchor production but rather a markedly decreased rate of biosynthesis. Further studies to delineate better the nature of the underlying defect(s) are currently underway.

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