Deficient Biosynthesis of N-Acetylglucosaminyl-Phosphatidylinositol, the First Intermediate of Glycosyl Phosphatidylinositol Anchor Biosynthesis, in Cell Lines Established from Patients with Paroxysmal Nocturnal Hemoglobinuria

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Summary

Paroxysmal nocturnal hemoglobinuria (PNH) is a hemolytic disorder caused by a deficiency of biosynthesis of the glycosyl phosphatidylinositol (GPI) anchor, but the biochemical defect is not completely understood. In the present study, we have analyzed affected cell lines established recently from two Japanese patients with PNH. Two lines of evidence indicate that these cells do not synthesize N-acetylglucosaminyl-phosphatidylinositol, the first intermediate in the GPI anchor biosynthesis. First, somatic cell hybridization analysis using Thy-1-deficient murine thymoma cell lines with known biochemical defects as fusion partners showed that the PNH cell lines belong to complementation class A, which is known not to synthesize N-acetylglucosaminyl-phosphatidylinositol. Second, analysis of in vitro glycolipid biosynthesis demonstrated that cell lysates of these PNH cell lines in fact did not support biosynthesis of N-acetylglucosaminyl-phosphatidylinositol. Thus, we have characterized for the first time the exact biochemical defect leading to PNH.

Materials and Methods

Cells and Cultures. GPI anchor-deficient cell lines (SS-1-, TK-1-, and TK-14-) and wild-type cell lines (SS-2+ and TK-4+) established from two Japanese patients with PNH (patients SS and TK) were described previously (16). Thy-1-deficient murine thymoma cell lines, BW5147(Thy-1-a), S49(Thy-1-a), AKR1(Thy-1-a), S1A(Thy-1-b), T1M(Thy-1-c), AKR1(Thy-1-d), BW5147(Thy-
1e), EL-4(Thy-1-f), and S49(Thy-1-h), were described previously (17–21). A Japanese Burkitt’s lymphoma cell line, P32/Ishida (22), was a gift from Dr. T. Seya (Center for Adult Diseases, Osaka, Japan) and a GPI anchor–deficient cell line, P32-2D2, derived from P32/Ishida was established in our laboratory by ethylmethanesulfonate treatment followed by flow-cytometric sorting of CD59-negative cells and cloning by limiting dilution. All cell lines were cultured in DMEM containing 10% FCS.

Complementation Analysis with Somatic Cell Fusion and Immunofluorescence Staining. Cells (1–5 × 10^6 cells each) were washed with FCS-free DMEM containing 30 mM Hepes (pH 7.4) and fused with 50% polyethylene glycol 4000 in the Hepes-buffered, FCS-free DMEM. Fused cells were cultured for 1–3 d and stained for surface expression of CD59. For this, biotinylated anti-CD59 mAb 5H8 (a gift from Drs. M. Tomita and Y. Sugita, Showa University, Tokyo, Japan) was used in combination with PE-conjugated streptavidin (Biomeda, Foster City, CA). Complementation of surface expression of CD59 was assessed by observing large heterokaryons under a fluorescent microscope. The complementation analysis with heterokaryons was used to avoid problems with chromosome segregation in interspecific hybrids.

Biosynthesis of the GPI Anchor Intermediates. Cell lysates were prepared as described previously (23). The lysates (10^6 cell equivalent) were incubated with 2 μCi of UDP-6-[3H]GlcNAc (American Radiolabeled Chemicals, St. Louis, MO) for 15 min at 37°C in the presence of tunicamycin (23). n-Butanol soluble lipid fraction was subjected to thin-layer chromatography on silica gel with a solvent consisting of chloroform, methanol, and 1 M NH₄OH (10:10:3) (23). The gel was treated for fluorography. Aliquots of the lipid fraction were treated with phosphatidylinositol-specific phospholipase C (PI-PLC) and HNO₂, respectively, to confirm presence of PI and nonacetylated glucosamine (23). The lysates were also incubated with GDP-2-[3H]mannose for 90 min at 37°C as described previously (24). In vivo labeling of cells with [3H]mannose was done as described (24).

Results

Determination of the Complementation Class of the Cell Lines Established from Patients with PNH. The SS-1− cells were fused with Thy-1-deficient murine thymoma cells of complementation classes A, B, C, D, E, F, and H, and heterokaryons were assessed for surface expression of CDS9 (Fig. 1 and Table 1). Surface expression of CDS9 was complemented on fusion with classes B, C, D, E, F, and H, but not with class A, indicating that SS-1− cells belong to complementation class A. Further analysis with two other murine class A thymoma cell lines confirmed this result (Table 1). A similar study performed with another PNH cell line demonstrated that TK-14− cells also belong to class A (Table 1). Fusion between SS-1− and TK-14− cells did not complement surface expression of CDS9, confirming that these PNH cell lines that were established from different patients belong to

![Figure 1](image_url)
Table 1. Complementation Analysis between GPI Anchor-deficient Cell Lines

| Fusion partners | Class | SS-1- | TK-14- | P32-2D2 |
|-----------------|-------|-------|--------|---------|
| BW5147 (Thy-1-a) | A     | +     | +      | ND      |
| S1A (Thy-1-b)   | B     | +     | +      | ND      |
| T1M1 (Thy-1-c)  | C     | +     | +      | ND      |
| AKR1 (Thy-1-d)  | D     | +     | +      | ND      |
| BW5147.3 (Thy-1-e) | E | +     | +      | ND      |
| EL-4 (Thy-1-f)  | F     | +     | +      | ND      |
| S49.1 (Thy-1-h) | H     | +     | +      | ND      |
| AKR1 (Thy-1-a)  | A     | +     | +      | ND      |
| S49 (Thy-1-a)   | A     | +     | +      | ND      |

SS-1-, TK-14-, and P32-2D2 cells were fused with cell lines listed and assessed for surface expression of CD59.
* Deficiency not complemented.
† Deficiency complemented.

Figure 2. Biosynthesis of glucosamine-containing glycolipids. Cell lysates of cell lines established from patients with PNH were incubated with UDP-6-[3H]GlcNAc at 37°C for 15 min. The lipid fraction was subjected to thin-layer chromatography and fluorographic analysis. Lane 1, TK-1-; lane 2, TK-4+; lane 3, TK-14-; lane 4, SS-2+; lane 5, SS-1-.

Discussion
The above results demonstrated that the two PNH cell lines belong to complementation class A. We, therefore, analyzed biosynthesis of the GPI anchor intermediates in SS-1- and TK14-. Since previous studies on class A murine thymoma cells indicated that they do not synthesize GlcNAc-PI, the first intermediate of the biosynthetic pathway, we first focused on the early steps of the biosynthesis. Incubations of the radiolabeled donor of GlcNAc, UDP-6-[3H]GlcNAc, with cell lysates of GPI anchor-sufficient cell lines, SS-2+ and TK-4+, established from the same patients as SS-1- and TK-14- resulted in synthesis of GlcNAc-PI and its subsequent deacetylation to glucosaminyl-phosphatidylinositol (GlcN-PI) (Fig. 2, lanes 2 and 4). The identities of these spots were confirmed by assessing their sensitivities to PI-PLC and HNO2 (data not shown). In contrast, cell lysates of deficient cell lines, SS-1- and TK-14-, did not support biosynthesis of GlcNAc-PI (Fig. 2, lanes 1, 3, and 5), being consistent with the above complementation analyses and previous reports on the biosynthetic defect of murine class A cells.

Being consistent with these results, both in vitro and in vivo analyses of mannolipid biosynthesis demonstrated that these deficient cell lines are able to synthesize dolichophosphate-mannose, a donor of mannose, but are not able to synthesize significant amounts of mannose-containing intermediates in GPI anchor biosynthesis (data not shown).
is deficient in these affected cell lines established from two Japanese patients.

It seems reasonable to assume that a defect in any one of the genes in the biosynthetic pathway of the GPI anchor causes a similar clinical disorder. Previous analysis of the GPI anchor biosynthesis in the affected neutrophils from several American patients with PNH indicated that biosynthesis of GlcNAc-PI and its subsequent deacetylation are normal (23), but that biosynthesis of mature GPI anchor is markedly reduced or virtually negative in affected neutrophils (4), suggesting the presence of an abnormality in the later reaction sequence. However, more recent analysis showed that deficient T cell lines obtained from one of these American patients and from other patients with PNH do not synthesize GlcNAc-

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