Brief Definitive Report

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

By Minoru Takahashi,* Junji Takeda,* Shinichi Hirose,§ Robert Hyman,‖ Norimitsu Inoue,* Toshio Miyata,* Etsuko Ueda,‡§ Teruo Kitani,‡ M. Edward Medof,§ and Taroh Kinoshita*

*From the Departments of *Immunoregulation and ‡Internal Medicine, Research Institute for Microbial Diseases, Osaka University, Suita, Osaka 565, Japan; the §Institute of Pathology, Case Western Reserve University, Cleveland, Ohio 44106; and the ‖Department of Cancer Biology, The Salk Institute, San Diego, California 92138*

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.

Paroxysmal nocturnal hemoglobinuria (PNH) is a hematologic disorder caused by an acquired deficiency of the glycosyl phosphatidylinositol (GPI) anchor biosynthesis (1–4). Deficiencies of GPI-anchored complement regulatory proteins, decay-accelerating factor (DAF) and 20-kD homologous restriction factor (CD59), both of which inhibit complement activation on the host cell surface and thereby protect host cells from destructive action of complement, are causally related to intravascular hemolysis, a major clinical symptom (5). The deficiency of GPI-anchored proteins is found in different lineages of hematopoietic cells including lymphocytes (6–9). It is, therefore, thought that a somatic mutation has occurred in the multipotential hematopoietic stem cell, which gives rise to affected blood cells and lymphocytes. Biosynthesis of the GPI anchor involves multiple reaction steps, being represented by several complementation classes of GPI anchor–deficient mutant cell lines (10). Defects of many of them have been biochemically characterized (11–15). Cells of three different complementation classes, A, C, and H, do not synthesize an early intermediate, *N*-acetylglucosaminyl-phosphatidylinositol (GlcNAc-PI), indicating that these early complementation classes define different genes, all of which must be necessary for biosynthesis of this glycolipid. Other classes, such as B and F, have defects in the later reaction steps. We recently established affected cell lines from two Japanese patients with PNH (16). In the present study we demonstrated that those cell lines belong to the complementation class A. Analysis of glycolipid biosynthesis confirmed that those PNH cell lines in fact do not synthesize GlcNAc-PI.

Materials and Methods

Cells and Culture. 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), T1M1(Thy-1$^-$c), AKR1(Thy-1$^-$d), BW5147(Thy-

1⁻e), 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 (10^7 cell equivalent) were incubated with 2 μCi of UDP-6-[^{3}H]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 NH_4OH (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 HNO_2, respectively, to confirm presence of PI and nonacetylated glucosamine (23). The lysates were also incubated with GDP-2-[^{3}H]mannose for 90 min at 37°C as described previously (24). In vivo labeling of cells with [^{3}H]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 CD59 (Fig. 1 and Table 1). Surface expression of CD59 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 CD59, confirming that these PNH cell lines that were established from different patients belong to

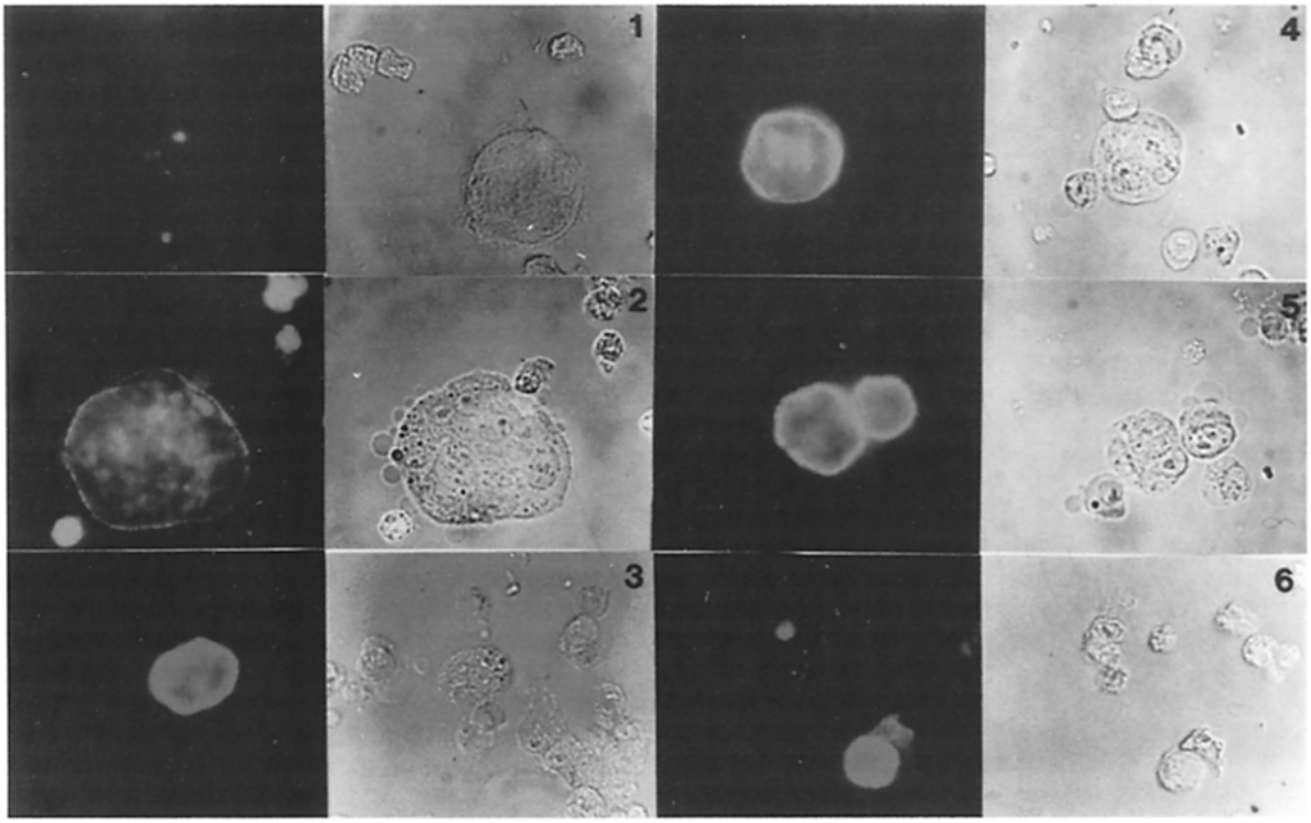

Figure 1. Complementation of deficient surface expression of CD59 on SS-1⁻ cells on fusion with class B, C, D, E, F, and H cells, but not class A cells. SS-1⁻ cells were fused with Thy-1-deficient murine thymoma cell lines of complementation class A (*1*), class B (*2*), class C (*3*), class D (*4*), class E (not shown), class F (*5*), or class H (*6*). Heterokaryons were assessed for complementation by surface staining of CD59. In each panel, fluorescent micrograph is on the left and phase-contrast micrograph is on the right.

Table 1. *Complementation Analysis between GPI Anchor–deficient Cell Lines*

Fusion partners	Class	SS-1⁻	TK-14⁻	P32-2D2
BW5147 (Thy-1⁻a)	A	−*	−	+‡
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	ND
S49 (Thy-1⁻a)	A	−	ND	ND
SS-1⁻		−	−	+
TK-14⁻		−	−	+
P32-2D2		+	+	−

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.

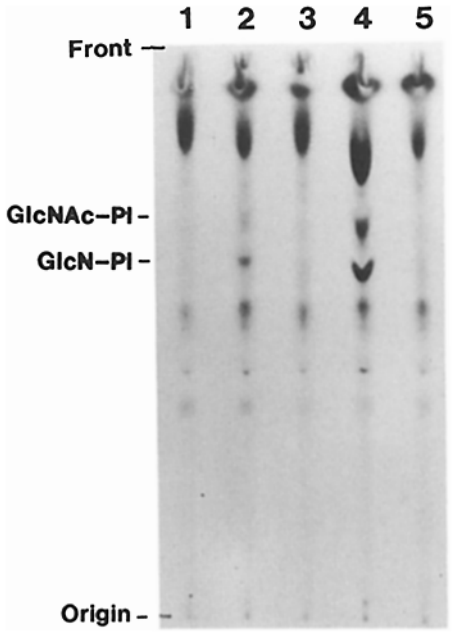

Figure 2. Biosynthesis of glucosamine-containing glycolipids. Cell lysates of cell lines established from patients with PNH were incubated with UDP-6-[³H]GlcNAc at 37°C for 15 min. The lipid fraction was subjected to thin-layer chromatography and fluorographic analysis. Lane *1*, TK-1⁻; lane *2*, TK-4⁺;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

the same class. Surface expression of CD59 was complemented when class A murine thymoma cells were fused with a GPI anchor–deficient human cell line, P32-2D2 (Table 1). This excludes a possibility that the lack of complementation between class A murine cells and the PNH cell lines was due to species incompatibility.

Deficient Biosynthesis of GlcNAc-PI in SS-1⁻ and TK14⁻ Cells. 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⁻, TK-1⁻, and TK-14⁻ cells. 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 HNO_2 (data not shown). In contrast, cell lysates of deficient cell lines, SS-1⁻, TK-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 dolichol-phosphate-mannose, a donor of mannose, but are not able to synthesize significant amounts of mannose-containing intermediates in GPI anchor biosynthesis (data not shown).

Discussion

In the present study we have characterized for the first time the exact biochemical defect of affected cells from patients with PNH. We previously established cell lines from two Japanese patients with PNH (16). The established GPI-anchored protein-deficient cell lines had the phenotype of PNH: (*a*) deficiency of surface expressions of multiple GPI-anchored proteins; (*b*) normal transcription of the DAF gene; and (*c*) intracellular biosynthesis of precursor peptides of DAF and CD59. To characterize the biochemical defect of these GPI anchor–deficient cell lines two approaches were undertaken. First, these cell lines were fused with GPI anchor–deficient mutant cell lines of known biochemical defects to assess complementation of the surface expression of GPI-anchored proteins. Second, biosynthesis of glucosamine-containing glycolipids was analyzed in an in vitro system using cell lysates and the radioactive sugar nucleotide. The complementation analysis demonstrated that both cell lines had the same defect as mutant cells of class A, which is one of the three "early mutants" that do not synthesize GlcNAc-PI (Fig. 1 and Table 1). The biosynthetic analysis provided independent and consistent evidence that GlcNAc-PI is not synthesized in these PNH cell lines (Fig. 2). It is, therefore, concluded that an early step in the biosynthesis of GPI anchor

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-PI and belong to complementation class A (Armstrong, C., J. Schubert, E. Ueda, J. J. Knez, D. M. Gelperin, S. Hirose, R. Silber, S. Hollan, R. E. Schmidt, and M. E. Medof, manuscript submitted for publication), and that deficient EBV-transformed B cells from another American patient exhibit the same defect (Ueda, E., and M. E. Medof, manuscript in preparation), suggesting that the apparent in vitro GlcNAc-PI synthesis observed with the neutrophil lysates may have occurred as a result of admixture of unaffected cells. It thus seems that the biochemical defect of the patients studied in America is the same as that of the Japanese patients studied here, and is common to most, if not all, of the patients with PNH.

We thank Keiko Kinoshita for technical assistance.

This work was supported by grants from the Ministry of Education, Science and Culture of Japan; JCR Pharmaceutical Co.; the Uehara Memorial Foundation; the Takeda Science Foundation; and the National Cancer Institute (CA-13287).

Address correspondence to Taroh Kinoshita, Department of Immunoregulation, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamada-oka, Suita, Osaka 565, Japan.

Received for publication 8 September 1992 and in revised form 3 November 1992.

References

1. Rosse, W.F. 1989. Paroxysmal nocturnal hemoglobinuria: the biochemical defects and the clinical syndrome. *Blood Rev.* 3:192.
2. Rotoli, B., and L. Luzzatto. 1989. Paroxysmal nocturnal haemoglobinuria. *Bailliere's Clin. Haematol.* 2:113.
3. Mahoney, J.F., M. Urakaze, S. Hall, R. DeGasperi, H.-M. Chang, E. Sugiyama, C.D. Warren, M. Borowitz, A. Nicholson-Weller, W.F. Rosse, and E.T.H. Yeh. 1992. Defective glycosylphosphatidylinositol anchor synthesis in paroxysmal nocturnal hemoglobinuria granulocytes. *Blood.* 79:1400.
4. Hirose, S., L. Ravi, G.M. Prince, M. Rosenfield, R. Silber, S.V. Hazra, and M.E. Medof. 1992. Synthesis of mannosyl-glucosaminyl-inositolphospholipids in normal but not paroxysmal nocturnal hemoglobinuria cells. *Proc. Natl. Acad. Sci. USA.* 89:6025.
5. Rosse, W.F. 1991. Dr. Ham's test revisited. *Blood.* 78:547.
6. Kinoshita, T., M.E. Medof, R. Silber, and V. Nussenzweig. 1985. Distribution of decay-accelerating factor in the peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. *J. Exp. Med.* 162:75.
7. Nicholson-Weller, A., D.B. Spicer, and K.F. Austen. 1985. Deficiency of the complement regulatory protein, "decay-accelerating factor," on membranes of granulocytes, monocytes, and platelets in paroxysmal nocturnal hemoglobinuria. *N. Engl. J. Med.* 312:1091.
8. Schubert, J., P. Uciechowski, P. Delany, H.J. Tischler, W. Kolanus, and R.E. Schmidt. 1990. The PIG-anchoring defect in NK lymphocytes of PNH patients. *Blood.* 76:1181.
9. Ueda, E., T. Kinoshita, J. Nojima, K. Inoue, and T. Kitani. 1989. Different membrane anchors of Fc gamma RIII (CD16) on K/NK-lymphocytes and neutrophils. Protein- vs lipid-anchor. *J. Immunol.* 143:1274.
10. Hyman, R. 1988. Somatic genetic analysis of the expression of cell surface molecules. *Trends. Genet.* 4:5.
11. Chapman, A., K. Fujimoto, and S. Kornfeld. 1980. The primary glycosylation defect in Class E Thy-1-negative mutant mouse lymphoma cells is an inability to synthesize dolichol-P-mannose. *J. Biol. Chem.* 255:4441.
12. Stevens, V.L., and C.R. Raetz. 1991. Defective glycosyl phosphatidylinositol biosynthesis in extracts of three Thy-1 negative lymphoma cell mutants. *J. Biol. Chem.* 266:10039.
13. Sugiyama, E., R. DeGasperi, M. Urakaze, H.M. Chang, L.J. Thomas, R. Hyman, C.D. Warren, and E.T. Yeh. 1991. Identification of defects in glycosylphosphatidylinositol anchor biosynthesis in the Thy-1 expression mutants. *J. Biol. Chem.* 266:12119.
14. Puoti, A., C. Desponds, C. Fankhauser, and A. Conzelmann. 1991. Characterization of glycophospholipid intermediate in the biosynthesis of glycophosphatidylinositol anchors accumulating in the Thy-1-negative lymphoma line SIA-b. *J. Biol. Chem.* 266:21051.
15. Hirose, S., R.P. Mohney, S.C. Mutka, L. Ravi, D.R. Singleton, G. Perry, A.M. Tartakoff, and M.E. Medof. 1992. Derivation and characterization of glycoinositol-phospholipid anchor-defective human K562 cell clones. *J. Biol. Chem.* 267:5272.
16. Ueda, E., J. Nishimura, T. Kitani, K. Nasu, T. Kageyama, Y.U. Kim, J. Takeda, and T. Kinoshita. 1992. Deficient surface expression of glycosylphosphatidylinositol-anchored proteins in B cell lines established from patients with paroxysmal

nocturnal hemoglobinuria. *Int. Immunol.* 4:1263.
17. Hyman, R., K. Cunningham, and V. Stallings. 1980. Evidence for a genetic basis for the class A Thy-1- defect. *Immunogenetics.* 10:261.
18. Conzelmann, A., A. Spiazzi, C. Bron, and R. Hyman. 1988. No glycolipid anchors are added to Thy-1 glycoprotein in Thy-1 negative mutant thymoma cells of four different complementation classes. *Mol. Cell. Biol.* 8:674.
19. Hyman, R., and V. Stallings. 1974. Complementation patterns of Thy-1 variants and evidence that antigen loss variants "preexist" in parental population. *J. Natl. Cancer Inst.* 52:429.
20. Evans, G.A., R. Hyman, and K. Lewis. 1987. A mutant lymphoma cell line with a defective Thy-1 glycoprotein gene. *Immunogenetics.* 25:28.
21. Trowbridge, I.S., R. Hyman, and C. Mazauskas. 1978. The synthesis and properties of T25 glycoprotein in Thy-1-negative mutant lymphoma cells. *Cell.* 14:21.
22. Hirose, M., K. Minato, K. Tobinai, T. Ise, S. Watanabe, M. Shimoyama, and T. Abe. 1983. A novel Japanese Burkitt's lymphoma cell line, p32/ishida, with a new variant chromosomal translocation(2;14). *Gann.* 74:878.
23. Hirose, S., L. Ravi, S.V. Hazra, and M.E. Medof. 1991. Assembly and deacetylation of N-acetylglucosaminyl-plasmanylinositol in normal and affected paroxysmal nocturnal hemoglobinuria cells. *Proc. Natl. Acad. Sci. USA.* 88:3762.
24. Hirose, S., G.M. Prince, D. Sevlever, L. Ravi, T.L. Rosenberry, E. Ueda, and M.E. Medof. 1992. Characterization of putative glycoinositol phospholipid anchor precursors in mammalian cells. Localization of phosphoethanolamine. *J. Biol. Chem.* 267:16968.