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Specific defect in *N*-acetylglucosamine incorporation in the biosynthesis of the glycosylphosphatidylinositol anchor in cloned cell lines from patients with paroxysmal nocturnal hemoglobinuria

PETER HILLMEN*, MONICA BESSLER, PHILIP J. MASON, WINIFRED M. WATKINS, AND LUCIO LUZZATTO

Department of Haematology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London, W12 0NN United Kingdom

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ABSTRACT **Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal disorder arising in a multipotent hemopoietic stem cell. PNH manifests clinically with intravascular hemolysis resulting from an increased sensitivity of the red cells belonging to the PNH clone to complement-mediated lysis. Numerous studies have shown that surface proteins anchored to the membrane via a glycosylphosphatidylinositol (GPI) anchor (including proteins protecting the cell from complement) are deficient on the cells of the PNH clone, leading to the notion that GPI-anchor biosynthesis may be abnormal in these cells. To investigate the biochemical defect underlying PNH we have used lymphoblastoid cell lines (LCLs) with the PNH phenotype obtained by Epstein–Barr virus immortalization of lymphocytes from nine patients with PNH. By labeling cells with *myo*-[^{3}H]inositol we have found that PNH LCLs produce phosphatidylinositol normally. By contrast, PNH LCLs fail to incorporate [^{3}H]mannose into GPI anchor precursors. When cell-free extracts of PNH LCLs and normal LCLs obtained from the same patients (and expected therefore to be isogeneic except for the PNH mutation) were incubated with uridine diphospho-*N*-acetyl[^{3}H]glucosamine (UDP-[^{3}H]GlcNAc), we observed complete failure or marked reduction in the production of *N*-acetylglucosaminyl(α-1,6)phosphatidylinositol and glucosaminyl(α-1,6)phosphatidylinositol by the PNH LCLs in all cases. These findings pinpoint the block in PNH at an early stage in the biosynthesis of the GPI anchor, suggesting that the defective enzyme is UDP-GlcNAc:phosphatidylinositol-α-1,6-*N*-acetylglucosaminyltransferase. The existence of PNH type III cells and type II cells is probably explained by the transferase deficiency being total or partial, respectively.**

Most membrane-bound proteins are attached to the cell membrane by virtue of a hydrophobic amino acid domain which spans the membrane. In an alternative mode of membrane attachment, the protein is covalently linked to a glycosylphosphatidylinositol structure, or GPI anchor, whose fatty acid moiety is inserted into the lipid bilayer (1–3). GPI-linked proteins are present in a large number of organisms from simple eukaryotes to humans (3). All the GPI anchors which have been characterized in detail, from several of these species, have similar core structures (4–11) (Fig. 1).

The GPI anchor is synthesized in the rough endoplasmic reticulum of the cell, where it is then transferred *en bloc* to the newly synthesized protein (12, 13). The C terminus of the protein to be anchored has a signal peptide which is removed and replaced by the preformed GPI anchor (14–16). The mechanism of biosynthesis of the GPI anchor in trypanosomes has largely been resolved by the identification of the intermediates of the biosynthetic pathway (17). The identification of similar intermediates indicates that the GPI biosynthetic pathway in other species, including humans, is similar to that in trypanosomes.

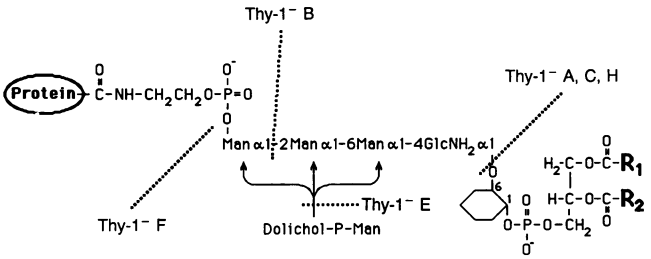

FIG. 1. GPI anchor structure. Sites of biosynthetic defects characterized in various Thy-1⁻ mouse mutant cell lines are shown by dotted lines. R_1 and R_2 represent fatty acyl groups.

Various mutant mouse thymoma cell lines have been isolated (18) which do not express GPI-linked proteins, including the Thy molecule, because they are unable to synthesize the GPI anchor. These Thy-1 mutants fall into eight different complementation groups (19), suggesting that the GPI biosynthetic pathway is complex, with at least eight different gene products involved. The sites of the metabolic blocks in these mutants have been identified (20–22).

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired hemolytic anemia due to an intrinsic red-cell defect which is associated with a thrombotic tendency (23, 24). PNH is due to the development of an abnormal clone of cells resulting from a somatic mutation occurring in a multipotent hemopoietic stem cell (25). The cells of the abnormal clone are deficient in all of the proteins which are normally attached to the cell membrane by a GPI anchor (26–33). The absence of one or more of these proteins is responsible for the increased sensitivity of PNH red cells to the action of complement (and thus for the intravascular hemolysis) (34) and for the thrombophilia (35) typically seen in PNH.

In 1973 it was first shown that in most patients the PNH red cells were 10–15 times more sensitive than normal to the action of complement (PNH type III erythrocytes), whereas in others the red cells were only 3–5 times more sensitive than normal (PNH type II erythrocytes) (36). Recently it has been shown that PNH type III red cells have a complete absence of GPI-linked proteins, whereas PNH type II red cells have only a reduction, rather than an absence, of GPI-linked proteins (37–39). This finding explains the different complement sensitivity of the two types of PNH red cells.

Recent evidence supports the view that the deficiency of the GPI-linked proteins in PNH cells is due to a block in the biosynthesis of the GPI structure, although the precise biochemical defect has remained unknown (40–42). It is possible

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Abbreviations: GPI, glycosylphosphatidylinositol; LCL, lymphoblastoid cell line; PI, phosphatidylinositol; PNH, paroxysmal nocturnal hemaglobinuria; TLCK, "N^{α}-*p*-tosyl-L-lysine chloromethyl ketone" (7-amino-1-chloro-3-tosylamido-2-heptanone).

*To whom reprint requests should be addressed.

that different steps may be affected in different patients resulting in the same phenotype and that different mutations may be expected to result in the PNH type III and type II phenotypes.

We recently reported the production of Epstein–Barr virus-immortalized lymphoblastoid cell lines (LCLs) with a PNH phenotype (deficiency of GPI-linked proteins) from patients with PNH (43). We now report the use of these PNH cell lines derived from nine different patients to localize the precise biochemical defect resulting in the PNH phenotype.

MATERIALS AND METHODS

Reagents. D-[2-^{3}H]Mannose (20 Ci/mmol; 1 Ci = 37 GBq) and *myo*-[2-^{3}H]inositol (18.8 Ci/mmol) were purchased from Amersham. Uridine diphospho-*N*-acetyl-D-[6-^{3}H]glucosamine (UDP-[^{3}H]GlcNAc; 6.1 Ci/mmol) and EN^{3}HANCE spray were purchased from DuPont-New England Nuclear. *myo*-Inositol-free RPMI 1640 medium, glucose-free RPMI 1640, tunicamycin, 7-amino-1-chloro-3-tosylamido-2-heptanone ("N^{α}-*p*-tosyl-L-lysine chloromethyl ketone," TLCK), leupeptin, sodium Hepes, adenosine 5′-triphosphate (ATP), and dithiothreitol were purchased from Sigma. Zwittergent 3-16 was purchased from Calbiochem and fetal bovine serum from Sera-Lab (Sussex, U.K.). The remaining chemicals were obtained from BDH.

Cell Lines. The production of the LCLs (normal and PNH) from the PNH patients has been described (43). We have now developed cell lines from several other patients with PNH (Table 1). Two of these PNH cell lines, both derived from patients with PNH type II erythrocytes (partially deficient in GPI-linked proteins), are partially deficient in GPI-linked proteins (Fig. 2). For brevity, PNH LCLs with a partial deficiency of GPI-linked proteins will be referred to as PNH type II LCLs, and PNH LCLs without GPI-linked proteins will be referred to as PNH type III LCLs.

Flow Cytometry. The methods for staining and analysis by flow cytometry have been described (43, 44).

Labeling with [^{3}H]Mannose. Cells (15×10^6) were washed twice with glucose-free RPMI 1640 [RPMI(Glc$^-$)] and then incubated at 37°C for 1 hr in RPMI(Glc$^-$) supplemented with 10% dialyzed fetal bovine serum and tunicamycin (3 μg/ml). The cells were then pelleted, suspended in 5 ml of RPMI(Glc$^-$) with 10% dialyzed fetal bovine serum, tunicamycin (3 μg/ml), and 62.5 μCi of [^{3}H]mannose (12.5 μCi/ml), and then incubated at 37°C in a 5% CO_2 atmosphere for 4 hr. The cells were pelleted and washed twice with RPMI(Glc$^-$). The lipids were extracted from the cell pellet with 1 ml of chloroform/methanol/water (10:10:3, vol/vol) for 1 hr, and after microcentrifugation at 13,000 rpm for 5 min the supernatant was transferred to a fresh tube. The pellet was reextracted with 1 ml of chloroform/methanol/water, and supernatants were pooled and dried under a stream of nitrogen. The residue was resuspended in 200 μl of water-saturated 1-butanol and extracted with 100 μl of butanol-saturated water. The water phase was extracted with a further 100 μl of water-saturated butanol, and the butanol phases were pooled. The pool was then extracted twice with butanol-saturated water and the butanol phase was dried under a stream of nitrogen. The residue was resuspended in 20 μl of chloroform/methanol/water and kept at −20°C until subjected to chromatography.

Table 1. Patient details

Patient no.	Age, years	Sex	Year of diagnosis	PNH red-cell type	PNH LCL	LCL GPI deficiency phenotype
5	53	M	1972	III	HH5/7P	Complete*
8	22	F	1989	III	HH8/3P	Complete
13	28	F	1985	II, III	HH13/49P	Complete
15	31	F	1991	II, III	HH15/3P	Complete
16	31	M	1980	II, III	HH16/7P	Complete
17	10	M	1991	II	HH17/42P	Partial
18	24	M	1991	II, III	HH18/8P	Partial
20	21	M	1991	II, III	HH20/9P	Complete
22	40	F	1987	III	HH22/9P	Complete

See refs. 43 and 44.
*No normal counterpart was available.

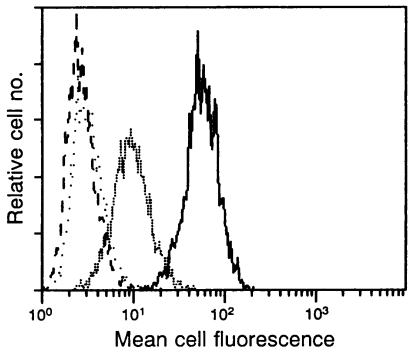

FIG. 2. Demonstration by flow cytometry of CD59 on the surface of PNH LCL cells. At least 5×10^3 cells were analyzed per sample. · · · · · ·, Normal LCL stained with a negative control antibody; ———, CD59 on a normal LCL; ·····, CD59 on a PNH type II LCL (HH18/8P); - - - -, CD59 on a PNH type III LCL (HH8/3P). HH18/8P has a reduced amount of CD59, and HH8/3P has no CD59.

Labeling with *myo*-[^{3}H]Inositol. Cells (15×10^6) were washed twice in *myo*-inositol-free RPMI 1640 [RPMI(Ins$^-$)], suspended in 15 ml of RPMI(Ins$^-$) with 10% dialyzed fetal bovine serum and 15 μCi of *myo*-[^{3}H]inositol (1 μCi/ml), and then incubated at 37°C in a 5% CO_2 atmosphere for 18 hr. The cells were washed twice in RPMI(Ins$^-$) and the cell pellet was extracted with 1 ml of chloroform/methanol/water. The lipid fraction was then purified as described above (see *Labeling with [^{3}H]Mannose*).

Labeling with UDP-[^{3}H]GlcNAc in a Cell-Free System. Cells (100×10^6) were washed twice with RPMI(Glc$^-$) and incubated in RPMI(Glc$^-$) with 10% dialyzed fetal bovine serum and tunicamycin (1 μg/ml) for 1 hr at 37°C. They were washed twice in RPMI(Glc$^-$) and then lysed by suspension of the cell pellet in 5 ml of water containing 0.1 mM TLCK and 5 μg of leupeptin and standing on ice for 25 min (lysis was confirmed microscopically). The lysate was centrifuged at $1200 \times g$ for 10 min at 4°C to remove the nuclei. The supernatant was then centrifuged at $100{,}000 \times g$ for 60 min at 4°C in a Beckman ultracentrifuge. The supernatant was discarded and the microsomal pellet was suspended in 200 μl of reaction mixture [50 mM Na Hepes, pH 7.4/25 mM KCl/5 mM $MgCl_2$/5 mM $MnCl_2$/1 mM ATP/0.5 mM dithiothreitol containing TLCK (0.1 mM), leupeptin (1 μg/ml), and tunicamycin (1 μg/ml)] and transferred to a tube containing 2 μCi of UDP-[^{3}H]GlcNAc which had been dried under a stream of nitrogen. This mixture was then incubated in a water bath at 37°C for 1 hr. The reaction was stopped by the addition of 1.5 ml of chloroform/methanol (1:1, vol/vol), mixed for 30 min, and then microcentrifuged at 13,000 rpm for 5 min. The supernatant was then dried under a stream of nitrogen and the lipid phase was purified as described above (see *Labeling with [^{3}H]Mannose*).

Thin-Layer Chromatography. The labeled products were spotted onto Silica 60 TLC plates (Merck). On all the plates the ^{3}H-labeled substrate was included as a control and, when appropriate, the [^{3}H]GlcNAc-labeled products formed with trypanosome membranes were also included on the plates (kindly provided by M. A. J. Ferguson, University of Dundee, Scotland). The TLC plates were then developed

with chloroform/methanol/water (10:10:3) to a height of 15 cm. The plates were coated three times with EN^{3}HANCE and exposed to Kodak X-Omat AR film at −70°C.

Nitrous Acid Deamination. The labeled glycolipid was dried under a stream of nitrogen and then suspended in 10 μl of 0.01% Zwittergent 3-16 (made up in 0.1 M sodium acetate/acetic acid buffer, pH 4.0) by vortex mixing for 1 min followed by sonication in a water bath for 10 min. Ten microliters of 0.5 M sodium nitrite was added and then the mixture was incubated for 1 hr at 60°C. The treatment was repeated by the addition of 10 μl of 0.01% Zwittergent 3-16 and 10 μl of 0.5 M sodium nitrite followed by incubation for 1 hr at 60°C twice. The glycolipid was partitioned in butanol/water three times. The radioactivity released into the aqueous phase was assessed by scintillation counting and the lipid phase was run on a TLC plate.

RESULTS

Incorporation of Mannose by Intact PNH Cells. Production of the complete anchor precursor was demonstrable in all normal cell lines, but it was absent in the PNH cell lines. These cell lines also failed to produce the normal mannose-containing intermediates of the GPI biosynthetic pathway [Man-GlcN-phosphatidylinositol (PI), Man_2-GlcN-PI, and Man_3-GlcN-PI], although they appeared to produce the mannose donor, dolichol-phosphate-mannose (Dol-*P*-Man; Fig. 3).

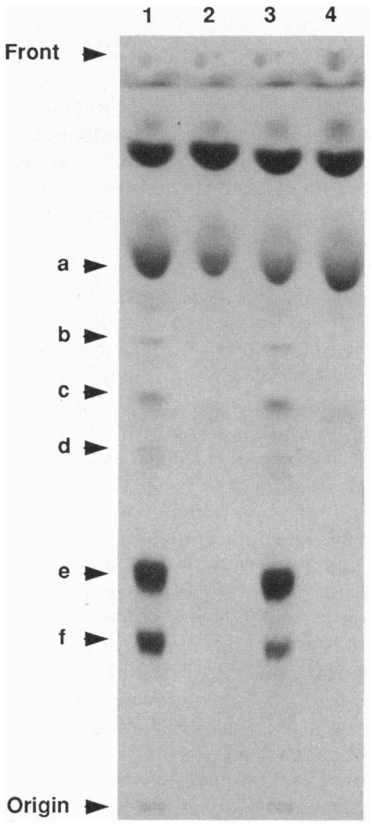

FIG. 3. Incorporation of [^{3}H]mannose into inositolphospholipid on LCL cells. The lipid fraction was fractionated, subjected to TLC, and autoradiographed. Lanes 1 and 3, normal LCLs (HH13/6N and HH17/1N, respectively); lane 2, PNH type III LCL (HH13/49P); lane 4, PNH type II LCL (HH17/42P). Presumed identity of radioactive spots: a, dolichol-phosphate-mannose; b, Man_1-PI; c, Man_2-PI; d, Man_3-PI; e and f, complete GPI structures (before and after fatty acid remodeling). Neither PNH type II or type III cells produce detectable amounts of the normal mannose-containing GPI intermediates (b–f).

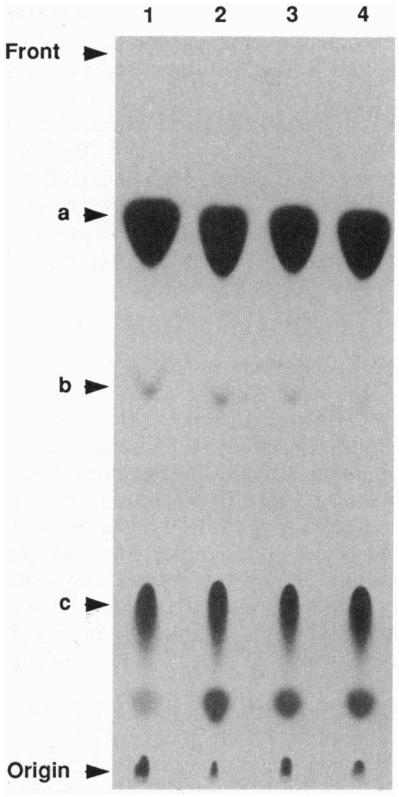

FIG. 4. Incorporation of *myo*-[^{3}H]inositol into inositolphospholipid on LCL cells. Lane 1, normal LCL from a normal subject; lane 2, PNH type III LCL (HH5/7P); lane 3, normal LCL (HH8/4N); lane 4, PNH type III LCL (HH8/3P). Identification of the spots from the literature (22): a, PI; b, lyso-PI; c, PI phosphate. PNH cells produce normal amounts of PI.

Incorporation of *myo*-Inositol by Intact PNH Cells. All 9 PNH cell lines had a pattern of *myo*-[^{3}H]inositol incorporation similar to the pattern observed in the 11 normal cell lines tested (8 from PNH patients and 3 from normal subjects) and to the pattern described in the literature (22). Thus, PNH cells are able to produce PI, an early intermediate in the GPI biosynthetic pathway (Fig. 4).

Labeling with UDP-[^{3}H]GlcNAc in Cell-Free Extracts. All normal cell lines tested (8 derived from PNH patients and 3 from normal subjects) incorporated [^{3}H]GlcNAc into two compounds which we have identified as *N*-acetylglucosaminyl(α1-6)phosphatidylinositol (GlcNAc-PI) and glucosaminyl(α1-6)phosphatidylinositol (GlcN-PI) by nitrous acid treat-

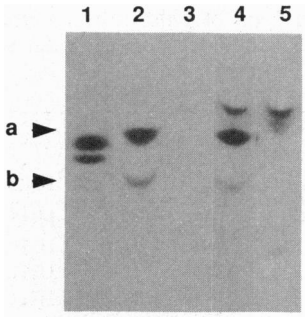

FIG. 5. Incorporation of [^{3}H]GlcNAc in cell-free extracts of LCL cells. Lane 1, trypanosome membranes (GlcNAc-PI and GlcN-PI have slightly different positions because of differing fatty acyl groups than in human GPI anchors); lanes 2 and 4, normal LCLs (HH22/1N and HH16/5N, respectively); lanes 3 and 5, PNH type III LCLs (HH22/9P and HH16/7P, respectively). a, GlcNAc-PI; b, GlcN-PI. PNH type III LCL cells incorporate no detectable GlcNAc.

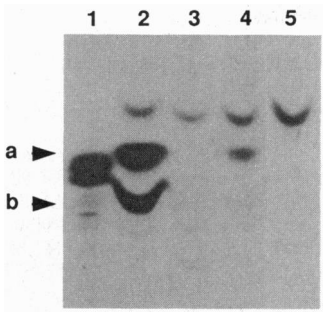

FIG. 6. Incorporation of [^{3}H]GlcNAc in cell-free extracts of LCL cells shown after prolonged exposure (35 days) of the x-ray film to the TLC plate. Lane 1, trypanosome membranes; lane 2, normal LCL (HH16/5N); lane 3, PNH type III LCL (HH16/7P); lanes 4 and 5, PNH type II LCLs (HH18/8P and HH17/42P, respectively). HH16/7P and HH17/42P incorporate no detectable GlcNAc, whereas HH18/8P incorporates greatly reduced amounts of GlcNAc.

ment of extracts from normal cell lines (see below). None of the cell lines derived from the 9 PNH patients incorporated GlcNAc normally. The 7 PNH type III LCLs showed no evidence of glucosamine incorporation even after prolonged exposure of the x-ray film to the TLC plate (resulting in increased sensitivity of detection; Figs. 5 and 6). Of the 2 PNH type II LCLs, one (HH17/12P) showed no evidence of glucosamine incorporation, just as PNH type III cell lines; the other (HH18/8P) synthesized a very small but detectable amount of GlcNAc-PI. From a titration experiment we estimated that the GlcNAc-PI synthesized was <5% of normal (Fig. 6).

Nitrous Acid Treatment of Extracts. Nitrous acid treatment of the extracts from normal cells labeled with UDP-[^{3}H]GlcNAc resulted in the release of radioactivity into the aqueous phase, as measured by scintillation counting, when compared with a control treatment (2316 cpm vs. 429 cpm).

Mixing Normal and PNH Cell-Free Extracts. The amount of [^{3}H]GlcNAc incorporated by a mixture of 5×10^6 normal cells and 95×10^6 PNH cells was similar to the amount incorporated by 100×10^6 normal cells (Fig. 7), indicating that PNH extracts do not contain an inhibitor of GlcNAc incorporation. Since the incorporation of [^{3}H]GlcNAc was undetectable with 5×10^6 normal cells alone, this experiment also indicates that PNH cell extracts can contribute apparently normal activity of GPI-anchor biosynthetic enzymes other than the deficient one.

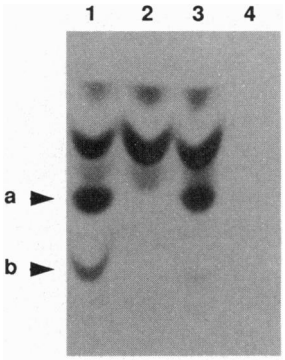

FIG. 7. Incorporation of [^{3}H]GlcNAc in cell-free extracts of LCL cells. Lane 1, 100×10^6 normal LCL cells (HH15/17N); lane 2, 100×10^6 PNH type III LCL cells (HH15/3P); lane 3, a mixture of 95×10^6 HH15/3P cells and 5×10^6 HH15/17N cells; lane 4, 5×10^6 HH15/17N cells alone. A small proportion of normal LCL cells (5%) with PNH LCL cells allows normal incorporation of GlcNAc.

DISCUSSION

In this study we have localized the defective step in the GPI biosynthetic pathway in PNH cell lines derived from nine patients with PNH. The finding that the PNH cell lines do not form the normal mannose-containing intermediates of the GPI biosynthetic pathway suggests that the defect lies at or before the addition of the first mannose residue (Fig. 1). On the other hand we have shown that *myo*-[^{3}H]inositol is incorporated normally into phosphatidylinositol by all nine PNH cell lines (Fig. 4).

UDP-[^{3}H]GlcNAc labeling of the microsomal fraction of 11 normal cell lines (3 from normal subjects and 8 from PNH patients) produced both of the predicted GPI intermediates (GlcNAc-PI and GlcN-PI), whereas none of the 9 PNH cell lines were able to incorporate detectable amounts of GlcNAc into PI. The absence of GlcNAc-PI suggests that the first step of GlcNAc incorporation is affected and that therefore the deficient enzyme is most probably a UDP-GlcNAc:phosphatidylinositol-α-1,6-*N*-acetylglucosaminyltransferase. The 7 PNH type III LCLs produced no detectable GlcNAc-PI or GlcN-PI (addition of GlcNAc to PI) even after prolonged exposure of the x-ray film to the TLC plate in order to obtain the maximum sensitivity from the assay (Figs. 5 and 6). Of the 2 PNH type III LCLs, one (HH17/12P) also showed no detectable production of GlcNAc-PI whereas the other (HH18/8P) produced <5% of the normal amount of GlcNAc-PI.

These findings are in contrast to those by Hirose *et al.* (45), who reported that the granulocytes from two patients with PNH incorporated GlcNAc and deacetylated the product normally. A possible explanation could be that granulocytes from patients with PNH are usually a mixture of mainly PNH granulocytes with a small proportion (usually 5–10%) of normal granulocytes; this small proportion of normal cells may be sufficient to provide enough of the deficient protein in a cell-free homogenate. By contrast, we have used cell lines consisting of a homogeneous cloned population of PNH cells. We have, further, performed a mixing experiment in which 95×10^6 PNH cells were mixed with 5×10^6 normal cells prior to homogenization and labeling of the microsomal fraction with UDP-[^{3}H]GlcNAc. The extract of this mixture incorporated similar amounts of glucosamine when compared with 100×10^6 normal cells, whereas no detectable incorporation was seen with 5×10^6 normal cells or 100×10^6 PNH cells, thus supporting our interpretation. This correction of the PNH biochemical abnormality in cell-free extracts is in agreement with the phenotypic correction previously reported in normal–PNH somatic cell hybrids (48).

When we consider the complexity of the GPI biosynthetic pathway, it is notable that in all nine patients the defect appears to be at the same step. This applies not only to the seven PNH type III patients but also to the two PNH type II patients. However, from the mouse thymoma mutants there is some evidence that three different gene products are involved in the transfer of GlcNAc to PI. Therefore, it is still possible that different genes involved in this one biosynthetic step are affected in different PNH patients. While this work was being conducted we learned that Takahashi *et al.* (46) had obtained similar results from LCLs derived from two patients with type III PNH and that Armstrong *et al.* (47) had similar results from T-cell lines derived from five patients with PNH type III.

In conclusion, we have demonstrated that the GPI biosynthetic abnormality in the PNH cells from nine patients with PNH results in the inability to transfer GlcNAc from UDP-GlcNAc to PI. This confirms the site of the GPI biosynthetic defect in PNH type III cells from seven patients and demonstrates that the same biosynthetic step is abnormal in PNH type II cells from two patients.

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