Affected Paroxysmal Nocturnal Hemoglobinuria T Lymphocytes Harbor a Common Defect in Assembly of N-Acetyl-D-glucosamine Inositol Phospholipid Corresponding to That in Class A Thy-1\(^{-}\) Murine Lymphoma Mutants

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Deficient expression of glycosinositol phospholipid (GPI) anchored proteins in affected paroxysmal nocturnal hemoglobinuria (PNH) cells has been traced to a defect in GPI anchor assembly. In a previous study (Schubert, J., Schmidt, R. E., and Medof, M. E. (1993) J. Biol. Chem., in press) we characterized the biosynthesis of putative Man-containing GPI anchor precursors in normal peripheral blood lymphocytes and investigated assembly of these intracellular GPI intermediates in CD48\(^{-}\) affected and CD48\(^{+}\) unaffected T and natural killer cell lines of PNH patients. We found that affected T cells from five patients exhibited a uniform defect in which dolichol-phosphoryl-Man was synthesized but no GPI manno lipids were expressed. In this study, membranes of patients' affected T cells were labeled with UDP\(^{-}\)[\(^{3}H\)]GlcNAc to evaluate earlier steps in GPI synthesis, and intact cells were fused to Thy-1\(^{-}\) murine lymphoma mutants harboring different defects in early GPI assembly to test for the presence of corresponding or complementary lesions. In all cases, affected cell membranes failed to assemble GlcNAc-inositol phospholipid, the initial precursor of GPI anchor structures, and the intact cells failed to complement class A mutants while complementing other classes. Affected polymorphonuclear leukocytes from three additional patients of different origin were then labeled with \(^{3}H\)Man and the labeling patterns found to correspond to those obtained with the T lymphocytes. Taken together the data indicate that the genetic lesion in PNH cells resides in a DNA element which: 1) encodes a product required for the synthesis of GlcNAc-inositol phospholipid, 2) corresponds to that altered in class A Thy-1\(^{-}\) murine lymphoma mutants, and 3) is commonly affected in different patients.

In paroxysmal nocturnal hemoglobinuria (PNH), somatic mutation of one or more marrow progenitors gives rise to subpopulations of circulating blood elements which fail to express glycosinositol phospholipid (GPI) anchored proteins on their surfaces (reviewed in Ref. 1). These deficiencies include decay-accelerating factor (DAF) and CD59, which render the affected cells sensitive to autologous complement-mediated injury, as well as alkaline phosphatase and Fc,RIII receptors (CD16) on polymorphonuclear leukocytes (PMN), CD14 and urokinase-type plasminogen activator receptors on monocytes, and 5' nucleotidase and CD48 on lymphocytes. The biochemical lesion responsible for the expression defect is unknown, but previous studies (2-6) have shown that it resides in the pathway providing for intracellular assembly of GPI-anchor moieties.

In a previous study (7), affected GPI-anchor defective CD48\(^{-}\) and unaffected GPI-anchor sufficient CD48\(^{+}\) T cell lines of patients were used to localize the site of interruption of the GPI assembly process. \(^{3}H\)Man labeling of the cells showed that in five different PNH patients the affected cells failed to synthesize ethanolamine (EthN)-P-ManGlcN-acetyl inositol phospholipid (PI) through EthN-P-ManGlcN-acetyl PI (8) or any earlier manno lipid intermediates (see Table I of Ref. 7 or Fig. 7 of Ref. 8 for pathway). The findings contrasted with previous observations (5, 6), in that: 1) no manno lipid GPIs were distinguished and 2) the five patients appeared to exhibit a uniform defect. Similar findings were obtained in studies of Epstein Barr virus (EBV)-transformed DAF\(^{+}\) and CD59\(^{-}\) B lymphocytes of a Japanese patient. In view of these observations, precursors of the Man-containing GPIs were investigated.

In the present study, the affected CD48\(^{-}\) T lymphocyte lines of the different patients were assayed for their abilities to assemble GlcNAc- and GlcN-PI, the first two intermediates of the GPI fabrication sequence (4, 9-12). Based on the

\(^{1}\) The abbreviations used are: PNH, paroxysmal nocturnal hemoglobinuria; GPI, glycosinositol phospholipid; DAF, decay-accelerating factor; PMN, polymorphonuclear leukocyte(s); EthN, ethanolamine-PI, inositol phospholipid; EBV, Epstein-Barr virus; TLCK, N\(^{-}\)-tosyl-L-lysine chloromethyl ketone; PEG, polyethylene glycol; PHA, phytohemagglutinin; mAb, monoclonal antibody; PTTC, fluorescein isothiocyanate; CMAW, chloroform:methanol:water; TLC, thin layer chromatography; Dol-P-Man, dolichol-phosphoryl-Man; DMEM, Dulbecco's minimal essential medium.

findings, complementation assays with Thy-1- lymphoma mutants (13) known to possess distinct genetic defects in assembly of these precursors (11, 12, 14) were performed. In common with the EBV-transformed CD59-/DAF- B cells, the CD48- T cells were found to harbor a defect in synthesis of the initial GPI anchor precursor GlcNAc-P corresponding to that in class A cells. The affected T lymphocytes from all of the patients studied exhibited a common defect, and affected PMN from three additional patients of different origin exhibited a consistent GPI mannolipid labeling pattern showing no GPI mannolipids.

MATERIALS AND METHODS

Reagents, Antibodies, and Cell Lines—UDP-[14C]GlcNAc (26.8 Ci/ mmol) was purchased from Du Pont—New England Nuclear. Dr.-[3H] Man (15 Ci/mmol) was bought from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Polyethylene glycol (PEG) (M, 1,000) was obtained from Sigma, and phytohemagglutinin (PHA) was purchased from Burroughs-Wellcome (Research Triangle Park, NC). Phosphatidylinositol-specific phospholipase C was provided by Dr. T. Rosenberg (Case Western Reserve Univ., Cleveland, OH). Murine anti-CD59 mAb IF5 provided by Dr. J. Pesando (Biomembrane Institute, St. Louis, MO) and Dr. H. Okada (Nagoya City Univ., Nagoya, Japan), and murine anti-CD48 mAb 54.57 provided by Dr. J. Pesando (Biomembrane Institute, Seattle, WA). Rat anti-Thy-1.1/1.2 M5-41 (IgG2a) was obtained from Dr. R. Hyman (Salk Institute, La Jolla, CA; Ref. 13). The mannolipid GPI anchor precursors GlcNAc-PI corresponding to the patients studied exhibited a common defect, and affected PMN from three additional patients of different origin exhibited a consistent GPI mannolipid labeling pattern showing no GPI mannolipids.

Products in butanol partitions of C:M:W extracts from each of the various samples and from PHA blasts were labeled with UDP-[3H]GlcNAc (see "Materials and Methods"). Products were chromatographed on silica gel 60 thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany) developed in C:M with 1 M NH3 (10:10:3) or C:M:W (10:10:3), respectively. Separated [3H]Man-labeled lipids on TLC plates were analyzed on an EG&G Berthold LB 285 TLC scanner.

Complementation Analyses—Cells fusions were performed as described (17). Cells (2.5 x 105 of each type) were combined, washed with t-glutamine-free Dulbecco's minimal essential medium (DMEM), and pelleted at 1,000 x g for 10 min at 20 °C. After removal of supernatant, 1 ml of 50% PEG in t-glutamine-free DMEM (previously microwaved just until boiling and returned to 20 °C) was added over 1 min with mixing. The resulting PEG/cell mixture was then digested, first with 1 ml of t-glutamine-free DMEM over 1 min and then with 5.0 ml of DMEM/10% horse serum over 3 min. The fusion mixture was centrifuged at 1,000 x g for 5 min at 20 °C, the cell pellet resuspended in 5.0 ml of DMEM/10% horse serum, and the suspended cells cultured for 24 h. Fused cells were stained with rat anti-Thy-1 mAb M5-41 and FITC-conjugated goat anti-rat Ig (Fab')2 and after paraformaldehyde fixation were plated onto microscope slides and examined using a Zeiss Axioshot microscope.

RESULTS

Comparative Abilities of Affected PNH and Control Lymphocytes to Synthesize GlcNAC- and GlcN-P—Experiments in our previous study (7) showed that following labeling with [3H]Man, CD48- PNH T cells uniformly expressed dolichol-phosphoryl-Man (Dol-P-Man) but no [3H]Man-labeled GPI products. To check for precursors of the Man-containing GPs, synthesis of GlcNAC- and GlcN-P by the affected cells was assessed. For this purpose, hypotonic lysates of CD48- T cells from patient E. W., control PHA blasts, GPI-positive murine EL-4 lymphomas, and T. brucei were prepared, and the respective membranes were incubated at 30 °C for 5 min with UDP-[14C]GlcNAC (see "Materials and Methods"). Products in butanol partitions of C:M:W extracts from each of the preparations were then compared by TLC developed in C:M:1 M NH3 permitting resolution of the two [3H]GlcNAC-labeled GPI products. As shown in Fig. 1 (panel B), membranes from EL-4 lymphomas generated peaks appropriately positioned for the two species. Consistent with their identities as GlcNAC- and GlcN-P these peaks comigrated with T. brucei standards (panel A) and were sensitive to phosphatidylinositol-specific phospholipase C digestion in control studies (not shown). As seen in panels C and D, although membranes of PHA blasts supported assembly of the two species similarly to membranes of T. brucei and EL-4 lymphoma controls, no synthesis of either product was observed with membranes of the CD48- T cell line from patient E. W.

To determine if a similar defect was related to the failed mannolipid GPI production in affected T cells of the other patients and exclude possible differences in assembly of the two intermediates between patients' T cell lines and control PHA blasts, hypotonic lysates of CD48- cells from a second patient, L. D., and of paired CD48+ and CD48- lines from a third patient, H. M., were prepared. Membranes from the various samples and from PHA blasts were labeled with UDP-[3H]GlcNAC, this time for a longer period of time (7). Products were similarly chromatographed and analyzed. As shown in Fig. 1 (panels E-H), membranes of CD48- cells from patients L. D. and H. M. exhibited the same complete defect in assembly of GlcNAC- and GlcN-P as observed with membranes from CD48- cells of patient E. W. In contrast, membranes of the paired CD48+ cells from patient H. M. effected assembly of the two precursors comparably to membranes of control PHA blasts.

Complementation Analyses between Affected PNH Cells and
analyzed by TLC developed with C:M:1 with UDP-[H]GlcNAc. Following CM:W extraction and butanol CD48- or CD48+ T cell lines were labeled at 30 °C for 5 or 15 min controls, neither product was detectable in any of the CD48- T cell peaks corresponding to GlcNAc- and GlcN-PI were observed in all lines to synthesize GlcNAc-
cells of patient H. M. were noted to contain some CD48- cells. The identities of the more polar peaks are unestablished. The following staining with anti-CD48 antibody. The control CD48' T lines. THy-1 expression. Using internal self- and cross-fusion con-
plementation between the mitogen-stimulated or transformed lymphocytes and T cell lines, additional studies were performed with CD48- T cells from patient L. D. and paired CD48- and CD48+ T cells from patient H. M., this time utilizing classes A, C, and F mutants. Control studies with PHA blasts again showed that they were able to complement Thy-1 expression by all three mutant classes. As observed with CD48- T lymphocytes from patients E. W. and E. B., CD48- cells from the two other patients, L. D. and H. M., exhibited the same specific deficit in the ability to complement Thy-1 expression by the class A line. In contrast, the paired CD48+ lymphocytes from patient H. M., grown identically to the CD48- lymphocyte line, complemented Thy-1 expression

**Fig. 1. Inability of membranes of CD48- patients' T cell lines to synthesize GlcNAc- or GlcN-PI.** Membrane preparations (2 × 10^10 cell equivalents) of the indicated control cells and of PNH CD48- or CD48+ T cell lines were labeled at 30 °C for 5 or 15 min with UDP-[H]GlcNAc. Following C:M:W extraction and butanol partitioning, [H]Man-labeled products in the organic phase were analyzed by TLC developed with C:M:1 m NH₃ (10:10:3). Although peaks corresponding to GlcNAc- and GlcN-PI were observed in all controls, neither product was detectable in any of the CD48- T cell lines. The identities of the more polar peaks are unestablished. The insets in the patient studies show flow cytometric analyses of the cells following staining with anti-CD48 antibody. The control CD48+ T cells of patient H. M. were noted to contain some CD48- cells.

**Thy-1** Murine Lymphomas—Thy-1- lymphoma mutants can be categorized into two groups (11, 12, 18), classes A, C, and H harboring defects in assembly of GlcNAc- and GlcN-PI, and classes E, B, and F possessing defects in subsequent GPI-anchor assembly steps involving Man and EthN-P incorporation into the GPI core. Complementation studies therefore were conducted in which patient and control lymphocytes were fused with different mutants belonging to the first group and control mutants belonging to the second group. The data are summarized in Table I and selected results shown in Fig. 2. In preliminary studies, control fusions of the class C mutant with self gave values of 0 and fusions of class E mutants with PHA blasts gave values >15.

**Table I**

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<tr>
<td>E</td>
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<td>47</td>
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<td>E. B.</td>
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**Fig. 2. Inability of CD48- patients' T cells to complement Thy-1 expression by class A murine lymphoma mutants.** Affected CD48- T cell or controls (2.5 × 10^6 cells) were fused with an equal number of Thy-1- lymphoma mutant cells of different classes. After overnight incubation, fusion products were examined for Thy-1 expression by fluorescent microscopy of anti-Thy-1-treated and FITC-labeled anti Ig secondarily-stained cells. Representative results for the designated fusion pairs are shown. Although CD48+ T cells complemented all classes, CD48- cells failed to complement class A cells.

*Percent of fusion products exhibiting Thy-1 expression. In this assay, 100 fusion products identified by their larger size under light microscopy were examined for Thy-1 expression under fluorescence microscopy. Under the conditions employed when cells belonging to different complementation groups are fused, some but not all of the transient fusion products exhibit complementation.

In other assays, control fusions of the class C mutant with self gave values of 0 and fusions of class E mutants with PHA blasts gave values >15.

ND, not done.
by class A cells with efficiency comparable to that observed with classes C and F cells.

To verify that the lack of complementation is specific to class A cells, additional experiments were performed with CD48-T cells from patients L. D. and E. W., this time utilizing classes A and H mutants in the former case, and classes A, H, and B mutants in the latter. Consistent with the results of the above studies, while control PHA blasts complemented Thy-1 expression by all three mutant classes, the CD48-T cells from both patients complemented Thy-1 expression by class H cells and those from patient E. W. also complemented Thy-1 expression by class B cells (not shown), but in neither case did the cells complement Thy-1 expression by class A cells.

Comparison of the Site of the Defect in Other PNH Patients—As assessed by in vivo [3H]Man labeling of intact CD48-T cell lines in our previous study (7), and the UDP-[3H]GlcNAc labeling and complementation analyses with Thy-1-murine lymphomas performed above in this study, affected cells of all of the patients analyzed appeared to exhibit a common biochemical defect. In view of this result, affected cells from three additional patients of different origin (Hungarian) were analyzed to ascertain if they shared the same defect. In the absence of available purified affected CD48 lymphocyte lines of these patients, affected DAF-/CD59-PMN were utilized. After verification that the cells >95% DAF-/CD59-, the affected PMN from the three patients and from an included healthy control (shipped and handled in an identical fashion) were labeled in vivo with [3H]Man. [3H]Man-labeled GPI products in butanol partitions of C3M-W extracts then were examined by TLC. As seen in Fig. 3, the control cells exhibited prominent peaks of ManMan(EthN-P->)ManGlcN-acyl PI (H6), EthN-P-6Man Man(EthN-P->)ManGlcN-acyl PI (H7), and EthN-P-6Man (EthN-P-6)Man(EthN-P->)ManGlcN-acyl PI (H8). In contrast, as observed in our previous study (7) with the CD48-T cell lines of all of the other patients examined, the affected PMN from each of the three patients synthesized Dol-P-Man but assembled no [3H]Man-labeled GPs.

DISCUSSION

In a previous study (7) it was found that, under conditions of stimulated GPI production, purified CD48 T cells from five PNH patients synthesized Dol-P-Man but uniformly failed to express Man-containing GPI intermediates. Here we found that the failed mannolipid production is a consequence of inability of the cells to assemble GlcNAc-PI, the first intermediate of the GPI assembly sequence. The biochemical defect underlying this deficit corresponds to that of class A Thy-1 murine lymphoma mutants. Contrary to earlier speculations, the defect among PNH patients appears to be homogeneous involving at least four and possibly extending to as many as nine patients of diverse origin.

The absence of GlcNAc-PI assembly in the affected cells localizes the underlying biochemical lesion in the cells either to the putative synthase responsible for transfer of GlcNAc from UDP to its PI acceptor, or to assembly (or presentation) of this phospholipid acceptor presumably in the ER membrane. The ability of the affected PNH cells to complement Thy-1 expression by Thy-1 mutant cells other than those belonging to complementation class A indicates that the defect is recessive. Previous findings (11, 12, 17) that three classes of Thy-1 mutants, A, C, and H, are similarly unable to synthesize GlcNAc-PI yet are able to complement each other in Thy-1 expression (13) indicate that at least three genetic loci are involved in GlcNAc-PI assembly. It is possible that the multiplicity of loci could reflect a hetero-oligomeric synthase, the requirement for a cofactor, or the need for a specific type of PI acceptor. Although some data in mamalian cells (6, 11) suggest involvement of alkylacyl- and disacyl-based- and in T. brucei (19) C18:0-containing phospholipid, the precise nature of the initial PI acceptor is not known.

The results of UDP-[3H]GlcNAc labeling of CD48 T lymphocytes in the present study contrast with earlier findings (4, 6) in which lyso- and DAF" and CD59" PMN populations of patients were found to support the in vitro assembly of GlcNAc- and GlcN-PI. Among the patients examined in this study is one (L. D.) of those previously analyzed in these earlier studies. The reason for the difference is not yet clarified. One possibility is that, despite flow cytometric characterizations, the previously studied PMN populations contained some residual unaffected cells, and that the activity of the missing factor provided by the residual cells is sufficiently high that the presence of even small amounts is able to complement the GlcNAc-PI assembly reaction. In this regard, it is noteworthy that although [3H]Man labeling of the intact PMN showed little or no polar mannolipid products (6) in accordance with our present findings, in vitro GDP-[3H]Man labeling of membrane fractions of the cells yielded TLC peaks corresponding to several of these products. The use in the present study of homogeneous lymphocyte lines or clones eliminates this potential complication.
Our finding that affected lymphocytes of all patients analyzed at both German and dominican origin exhibited a common defect was unexpected. The results are in accordance with findings for EBV-transformed DAF and CD59- B cell lines from two Japanese patients and another one of our patients. Although lymphocyte complementation studies will be required to conclusively confirm their correspondence, the additional findings in the present study that DAF- and CD59- PMN of three additional patients of different (Hungarian) origin exhibited a consistent pattern of complete absence of GPI mannolipids strongly support the proposal of a uniform or predominant defect. Among Thy-1- lymphoma lines, mutants belonging to complementation class A were also obtained most frequently and noted to arise spontaneously. Similar predominant genetic defects have been found for other conditions such as chronic myelocytic leukemia and retinoblastoma.

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