The PIG-Anchorin Defect in NK Lymphocytes of PNH Patients

Paroxysmal nocturnal hemoglobinuria (PNH) is clinically characterized by intravascular hemolysis, hemoglobinuria, iron deficiency anemia, and venous thrombosis. Pathophysiologically the disease has now been generally accepted as an acquired defect of phosphatidylinositol glycan (PIG)-anchored molecules on the cell surface of bone marrow-derived cells. This defect is functionally characterized by an abnormal susceptibility to complement-mediated lysis and has been described on erythrocytes, granulocytes, monocytes, and platelets. In contrast, contradictory data exist so far on the involvement of lymphocytes and natural killer (NK) cells. Using monoclonal antibodies (MoAbs) against newly defined PIG-linked surface structures such as CD48, CD55, and CD59, which are homogeneously expressed on lymphocytes of normal donors, we analyzed lymphocytes and their subpopulations in nine PNH patients by two-color immunofluorescence. Our results showed that CD3+ T cells as well as CD16+ NK cells are at least partially involved in the deficient PIG-molecule surface expression. To more clearly define the defect in PNH, we generated NK clones from a PNH patient. Phenotypic analysis of these NK clones showed that they either were positive (n = 3) for PIG-linked surface structures such as CD48, CD55, and CD59 (eg, NKP1) or were completely negative (n = 7) for all of them (eg, NKP1). In functional tests the PIG-molecule negative clone NKP2 showed increased susceptibility to human complement compared with the PIG molecule positive clone NKP1. When analyzing the mRNA levels of the PIG-linked molecules CD55 and CD59 there was no difference at all between the two clones. We conclude from our data that NK cells as well as other lymphocyte subpopulations are involved in the PIG-linkage defect of PNH. These NK clones with differential expression of PIG-linked surface structures present for the first time ex vivo mutant cell lymphocyte lines that carry the defect leading to PIG deficiency in PNH.

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previously.\textsuperscript{27} Cells were then washed three times in HBSS and resuspended in RPMI 1640 medium containing 15% human AB serum. Granulocytes were isolated from the Ficoll gradient pellets using high molecular weight dextran as described.\textsuperscript{30} Contaminating erythrocytes in the preparations were removed by hypotonic lysis. Viability of cells determined by trypan blue exclusion was always found to be >95%.

**PNH patients.** All PNH patients were diagnosed on the basis of a positive acid hemolysis and succrose test. In addition, diagnosis could be confirmed in a hemolysis test using anti-I cold agglutinin. At least a 10-fold increase of hemolysis of patients erythrocytes compared with control erythrocytes was considered as a positive diagnostic test. In our studies nine patients ranging in age from 21 to 51 years with a different degree of hemolytic anemia were available.

**Preparation of lymphocyte-conditioned medium.** Lymphocyte-conditioned medium (LCM) was produced by stimulating peripheral blood lymphocytes (PBL) at a cell density of 1.5 \( \times 10^6 \) cells/well in culture medium containing 10% LCM. There was no change in phenotype or specificity of these clones.

**Generation and expansion of cloned NK cell lines.** Cloned NK cell lines were generated as described in detail previously. Briefly, cells were plated at 1 cell/well by limiting dilution onto a feeder layer with irradiated (5,000 cGy) allogeneic PBL and Epstein-Barr virus (EBV)-transformed B lymphoblastoid cells (Laz 509). Colonies were selected for NK activity as previously described.\textsuperscript{29,30} They were expanded by subcloning for at least four times at 1,000 cells/well in culture medium containing 10% LCM. There was no change in phenotype or specificity of these clones.

**Antibodies.** CD16 MoAb Bw209/2 was a gift from Dr Kurrie (Behringwerke, Marburg, FRG) and was available as purified IgG2a fraction. CD16 antibody CLB/FOGran1 was kindly provided by Drs T.W.J. Huizinga and A.E.G.Kr. von dem Borne (Central Laboratory of the Netherlands Red Cross Transfusion Service, Amsterdam, The Netherlands). Antibody W6/32 recognizes the pan NK antibody of the IgM isotype and was kindly given by Dr J. Ritz (Dana-Farber Cancer Center, Boston, MA). CD48 antibodies MEM43 (CD59), and DAF (CD55) have been alternately spliced transmembrane isoforms for these molecules as described for LFA-3 (CD58).\textsuperscript{26,33} By cloning PIG-linked surface structures such as CD48 we observed two distinct populations, a minor negative and a major positive population (Fig 1). This proportion of surface antigens P41 (CD48), MEM43 (CD59), and DAF (CD55) have been described as PIG-linked surface structures with broad and homogeneous expression on human PBL.\textsuperscript{23} In previous experiments we treated NK clones with a PIG-specific phospholipase C (PIPLC). Expression of conventionally anchored molecules such as CD16 on NK cells remained unchanged compared with the medium control. In contrast the PIG-linked antigens P41 (CD48), DAF (CD55), and MEM43 (CD59) were released from NK clones by this enzyme and subsequently the surface expression was diminished (data not shown). By cloning PIG-linked surface structures such as CD55 and CD59 it was demonstrated that there are no alternatively spliced transmembrane isoforms for these molecules as described for LFA-3 (CD58).\textsuperscript{26,33}

When analyzing PBL from PNH patients for expression of CD48 we observed two distinct populations, a minor negative and a major positive population (Fig 1). This proportion of PIG molecule-negative lymphocytes was also detectable for CD55 (Fig 1). In contrast, PBL of normal donors showed a homogeneous positive surface expression of these PIG-linked surface antigens.

**NK and T cells are affected in PNH patients.** Since PBL from PNH patients exhibited a negative population for the PIG-linked pan-lymphocyte antigens CD48, CD55, and CD59 PNH lymphocytes were tested in two color immunofluorescence to further characterize the lymphocyte subpopulations involved in this defect. We used the CD48 MoAb using forward and site scattering gating, lymphocytes could be analyzed separate from monocytes and granulocytes. Ten thousand cells were analyzed in each sample.

**Cytotoxicity and complement lysis assays.** All experiments were performed in triplicate using V-bottom microtiter plates with 5,000 \(^{51}Cr\)-labeled target cells/well as described in detail previously.\textsuperscript{30} The medium for cytotoxicity assay was RPMI 1640 plus 5% FCS and 1% penicillin-streptomycin. Cytotoxicity was measured following a 4-hour incubation at 37°C. Specific cytotoxicity was calculated according to standard methods. In a standard cytotoxicity assay, target cells were K562 and NK clones NKPl and NKP2 were added at various E:T ratios. For complement lysis assays, 5,000 \(^{51}Cr\)-labeled target cells were given to the plates. MoAb NKH1A was then added at a final dilution of 1:10,000 of malignant asites. As a complement source, either freshly thawed rabbit serum or human serum was added at a final dilution of 1:5. Specific lysis following 1 hour incubation at 37°C was calculated according to standard methods.

**Northern blot hybridization.** Cellular RNA was prepared by ultracentrifugation on a CsCl gradient as described previously.\textsuperscript{22} Ten micrograms of denatured DNA was then separated on a 1% agarose gel electrophoresis and subsequently bound onto nylon membrane. Filters were hybridized in 5 x SSPE, 5 x Denhardt's, 0.1% SDS, 0.1 mg/mL denatured calf thymus DNA using cDNA probes labeled with \(^3P\) to a specific activity of 10\(^7\) dpm/ng. After stringent washes, filters were autoradiographed. The cDNA for DAF (CD55) was kindly provided by J. Caras (Genentech Inc, San Francisco, CA) and the cDNA for CD59 was a kind gift from Dr H. Waldmann (MRC Unit, Cambridge, Great Britain).

**RESULTS**

**PBL of PNH patients show an additional negative population of PIG-linked CD48 and CD55.** The antigens P41 (CD48), MEM43 (CD59), and DAF (CD55) have been described as PIG-linked surface structures with broad and homogeneous expression on human PBL.\textsuperscript{23} In previous experiments we treated NK clones with a PIG-specific phospholipase C (PIPLC). Expression of conventionally anchored molecules such as CD16 on NK cells remained unchanged compared with the medium control. In contrast the PIG-linked antigens P41 (CD48), DAF (CD55), and MEM43 (CD59) were released from NK clones by this enzyme and subsequently the surface expression was diminished (data not shown). By cloning PIG-linked surface structures such as CD55 and CD59 it was demonstrated that there are no alternatively spliced transmembrane isoforms for these molecules as described for LFA-3 (CD58).\textsuperscript{26,33}
J4-57 that could be demonstrated to distinguish most clearly the positive and negative lymphocyte populations. As demonstrated in Fig 2, the majority of CD16⁺ NK cells and primarily the CD16bright⁺ subset is deficient for the PIG-linked structure CD48. This deficiency was observed for the NK cell fraction in eight of nine patients although there was one patient who was consistently observed to have NK cells totally unaffected. In all patients also a significant number of CD3⁺ T cells exhibited the defect for expression of this PIG-linked molecule. In addition CD20⁺ B cells tested only in three patients were found to be affected by the PIG-linkage defect (data not shown).

CD16 expression on PBL of PNH patients. The defect in expression of PIG-linked CD16 on granulocytes of PNH patients has been well described. We detected expression of conventionally anchored CD16 on PBL, i.e., NK cells. Therefore, NK cell CD16 was not deficient in PNH but the flow cytometric pattern in all patients was clearly distinct from normals. In addition to the characteristic CD16bright⁺ peak of NK cells a second CD16dim⁺ subpopulation was detectable (Fig 3). From two color analysis it became clear that both populations express the pan NK antigen CD56. CD16bright⁺ cells are negative for CD3 and therefore represent the characteristic NK cells of CD3⁻ CD56⁺ phenotype. In contrast, CD16dim⁺ cells are almost all expressing the T-cell receptor-associated structure CD3 (Fig 4). It is also evident from the histograms that predominantly the number of CD16bright⁺, i.e., classical NK cells, is reduced in PNH patients. This is principally demonstrated in the second PNH patient in Fig 2. Here the number of CD3⁻ CD16⁺ CD56⁺ cells is less than 5% of PBL.

Comparison of PIG molecule⁺ with PIG molecule⁻ NK clones. To more clearly characterize the defect of PIG anchoring in NK cells, cloned NK cell lines from one PNH patient were generated. The analysis of these NK clones that either were positive (N = 3) for PIG-linked surface structures such as CD48, CD55, and CD59 or were negative (N = 7) for all of them is shown in Fig 5. Expression was always in parallel and the phenotype of all clones remained stable for at least four subcloning procedures. The PIG molecule-positive phenotype was also detected on all NK clones generated so far from healthy donors. They always were positive for all three PIG-linked antigens CD48, CD55, and CD59. When testing the susceptibility of
clones to complement-mediated lysis we used the monoclonal CD56 antibody NH1A, which is of the IgM isotype and therefore able to activate human as well as rabbit complement. Using rabbit complement all clones were significantly lysed in this assay. However, human complement was not able to lyse the PIG molecule expressing clone NK1 as well as another control clone CNK6. But there was a significant lysis of the PIG molecule defective clone NK2 (Fig 6). Both types of clones exhibited a comparable degree of cytotoxic activity against the NK susceptible target K562 (data not shown). When autocytoxicity was measured the PIG-molecule deficiency of clone NK2 did not result in increased susceptibility (data not shown). Extensive cytogenetic studies of both clones did not show detectable abnormalities (data not shown).

**CD55 and CD59 mRNA levels are equal in both types of PNH clones.** In order to more clearly elucidate the level on which the defect of PIG molecule-negative cells in PNH is expressed, we measured the mRNA levels for CD55 and CD59, the latter existing only in the PIG-linked isoform. Northern blot analysis in Fig 7 demonstrates that mRNA for CD55 and CD59 is detectable at a similar degree in both types of NK clones.

**DISCUSSION**

The characteristic defect in PNH presented by the lack of PIG-anchored surface proteins has recently been well defined for bone marrow-derived cells such as erythrocytes, granulocytes, and monocytes. However, the involvement of lymphocytes and NK cells in particular was not clear from the data presented so far. In the present studies we analyzed the PIG-anchoring defect and the functional consequences in PNH for lymphocytes and NK cells. By application of MoAbs against a new PIG-linked molecule P41 (CD48), which is homogenously and brightly expressed on all lymphocytes of normal donors,23 we demonstrated a positive and a distinct negative population in PNH patients (Fig 1). Using two color immunofluorescence we were able to demonstrate that this negative population consists of CD16+ NK cells as well as CD3+ T cells. From these data it can be concluded that NK cells as well as T cells are at least partially affected in PNH. These results appear to contrast earlier reports that demonstrated that lymphocytes are not deficient for DAF expression in PNH, and that normal NK cells per se lack the DAF antigen.24 These differences might be explained by the different reagents used and the low density of DAF expression on normal NK cells in contrast to the other lymphocytes (data not shown). In contrast to the earlier studies we have

![Fig 4. Two color analysis of CD16<sup>dim</sup> and CD16<sup>bright</sup> subpopulations in three PNH patients. Contour graphs are given for coexpression of CD3 and CD66 antigens.](image)

![Fig 5. Phenotypic characterization of representative NK clones NK1 (PIG molecule<sup>+</sup>) and NK2 (PIG molecule<sup>-</sup>).](image)
used well defined MoAbs with potentially higher affinities. In addition to DAF (CD55) expression, identical results for lymphocytes were obtained with monoclonals against the PIG-linked structures CD48 and CD59.

When analyzing CD16 expression of lymphocytes, it became clear that CD16 is quite distinct from normals. Most patients exhibited two distinct populations of CD16+ cells, ie, CD16_{bright} and CD16_{dim} cells. By using two color fluorescence it could be demonstrated that the CD16_{dim} cells coexpressed CD3. Obviously this CD3+ CD16_{dim} population is relatively increased in PNH patients. In contrast, the number of CD3- CD16+ NK cells appears to be diminished. This observation may correspond to the fact that CD3+CD16_{dim} cells express PIG-linked surface structures and therefore are less susceptible to autologous complement. On the other hand, the expansion of the CD3+CD16+ population that represents less than 5% of lymphocytes from normal donors has also been observed in chronic EBV disease. These phenotypic changes may also correspond to in vivo activation due to a persistent antigenic challenge in the disease. These shifts in the NK cell subsets could be the explanation for the diminished NK activity of PBL from PNH patients described in previous studies.

To further characterize the defect in PNH, cloned NK cell lines from one PNH patient were generated. Clonal analysis showed two distinct types of NK cells in regard to the expression of previously characterized PIG-linked molecules CD48, CD55, and CD59. The clones either coexpress all these PIG-linked structures or lack all of them. These PIG-negative NK cells provided for the first time long-term mutant cell lines obtained from a PNH patient. In a large series of normal NK clones generated in this laboratory none was found to be deficient for these PIG-linked antigens (data not shown). Therefore, the defect and the functional changes in these PNH cells are likely to represent the in vivo situation of these lymphocytes.

In functional studies the susceptibility of the PIG molecule-negative clone NKP2 to human complement was highly increased compared with the positive clone NKPl and control clone CNK6 (Fig 6). In contrast, all types of clones are efficiently lysed in the presence of rabbit complement. These differences may likely be due to the lack of the previously described homologously restricted complement
regulating proteins such as DAF and homologous restriction factor (HRF) on the NKp1 clone. In contrast to the complement studies there was no difference in cytotoxic function between both types of clones. They displayed an equal cytotoxicity potential against a series of target cells. Furthermore, there was no enhanced susceptibility to cellular autolysis of the PIG-deficient clone (data not shown). This observation confirms previous studies in which it was found that complement regulating proteins such as DAF and HRF have no effect on cell-mediated cytotoxicity. This is in contrast to previous assumptions that a defect of PIG-linked surface molecules may lead to a defect in natural cytotoxicity in PNH.

Finally, Northern blot analysis of both types of PNH NK clones demonstrated that mRNA levels of PIG-linked molecule such as CD55 and CD59 are similar. These data confirm those by Carothers et al17 who recently described an identical mRNA transcription for DAF in normal and in PNH cells. DAF protein is synthesized in two forms, a glycolipid-linked membrane bound and a secreted soluble form. Normal mRNA levels were confirmed by probing with a CD59 cDNA the product of which has been demonstrated to exist only in the glycopilid form. Furthermore, it has been shown that precursor molecules are processed by a C-terminal signal peptidase and even glycosylated within the golgi apparatus in the deficient PNH cells. The defect seems to be located on the level of PIG anchor construction that occurs after C terminal peptidase action.17

In summary, from these data it is evident that lymphocyte subpopulations such as NK cells and T cells are affected by the deficiency for PIG-linked surface structures, the characteristic feature in PNH. Interestingly, in contrast to hematopoietic cells the lymphocytes show PIG molecule-negative and -positive subsets. The dominance of the defect appears stronger in cells directly derived from the marrow. The long survival and the different regional homing of lymphocytes in thymus, lymph nodes, etc may explain that they are largely not yet affected by these defects. Since it has been suggested that PNH is a disease of bone marrow-derived cells the involvement of NK cells could point towards their early origin in the bone marrow.

Furthermore, it is not clear at which level of cell differentiation the PIG defect becomes manifest. Since the molecular basis for the PIG defect in PNH is not yet known, the generation of NK clones with differential expression of PIG-linked proteins may help to define the defect that is likely to represent an enzyme involved in the biosynthesis of the PIG anchor.

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