The PIG-Anchor Defect in NK Lymphocytes of PNH Patients

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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 homogenously 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, NKP2). 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. © 1990 by The American Society of Hematology.

Materials and Methods

Cell preparation. Heparinized blood samples were obtained either from normal adult donors or from patients with PNH, and then mixed with an equal volume of Hank's balanced salt solution (HBSS). Mononuclear cells (MNC) were harvested from the interphase of Ficoll density centrifugation as described.
virus (EBV)-transformed B lymphoblastoid cells (Laz 509). Colore.
NKHlA (CD56) is a pan NK antibody of the IgM isotype and was
monomorphic determinant of HLA class I molecules as described.
Amsterdam, The Netherlands). Antibody W6/32 recognizes the
diagnostic test. In our studies nine patients ranging in age from 21 to
compared with control erythrocytes was considered as a positive
change in phenotype or specificity of these clones.
Preparation of lymphocyte-conditioned medium.
Lymphocyte-conditioned medium (LCM) was produced by stimulat-
ing peripheral blood lymphocytes (PBL) at a cell density of 1.5 x
10^6/mL for 3 hours with 4 ug/mL phytohemagglutinin (PHA)
(Welccome, Grossburgwedel, FRG) and 5 ng/mL phorbol myristate
acetate (PMA) (Sigma, München, FRG). Activated PBL were
washed four times to remove the mitogens and resuspended in RPMI
1640 medium supplemented with 2.5% fetal calf serum (FCS), 1%
t-glutamine, penicillin, and sodium pyruvate. After 40 hours of
culture at 37°C the supernatants were then harvested and stored at
–20°C.
Generation and expansion of cloned NK cell lines. Cloned NK
line 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). Colo-
nies were selected for NK activity as previously described.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/GoGran1 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
monomorphic determinant of HLA class I molecules as described.
NKHlA (CD56) is a pan NK antibody of the IgM isotype and was
kindly given by Dr J. Ritz (Dana-Farber Cancer Center, Boston,
MA). CD48, CD55, and CD59 MoAbs were obtained from the
Fourth International Leukocyte Typing Workshop. CD48 antibodies
used in these studies were WM68 (Dr A.J. Henniker, Westmead,
Australia) and J4-57 (Dr J. Pesando, Seattle, WA). CD55 antibo-
dies were 143-30 (Dr R. Vilella, Barcelona, Spain) and Bric-128 (Dr
D.J. Anstee, Oxford, England). The CD59 antibody used was
MEM43 (Dr V. Horejsi, Prag, Czechoslovakia). Directly
labeled MoAbs such as Leu4 (CD3) and Leu19 (CD56) were
obtained from Becton Dickinson (Heidelberg, FRG). Fluores-
coein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated
goat–anti-mouse F(ab')2, Ig antisa (GM-FITC or GM-PE, respec-
tively) were purchased from Jackson-Dianova (Hambug, FRG).
Immunofluorescence analysis. Indirect immunofluorescence was
performed with GM-FITC as described. For two color analysis
either directly FITC- or PE-labeled reagents were used. For combi-
nation of indirect and direct immunofluorescence incubation of
unlabeled antibodies was followed by GM-FITC or GM-PE as
second reagents as described in detail previously.31 In order to avoid
unspecific binding, incubation of PE- or FITC-labeled antibodies
was preceded by incubation of the cells in a 1:5 dilution of normal
mouse serum. The samples were then analyzed by flow cytometry on
a fluorescence-activated cell sorter FACScan (Becton Dickinson).
Using forward and site scattering, lymphocytes could be ana-
alyzed 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 
51Cr-labeled target cells/well as described in detail previously.30
The medium for cytotoxicity assay was RPMI 1640 plus 5% FCS
and 1% penicillin-streptomycin. Cytotoxicity was measured follow-
ing 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 NKp1 and NKp2 were added at various E:T ratios. For complement lysis assays, 5,000 
51Cr-
labeled target cells were given to the plates. MoAb NKHlA was
then added at a final dilution of 1:10,000 of malignant ascites. 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.32 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, 50% formamid,
0.1% SDS, 0.1 mg/mL denatured calf thymus DNA using cDNA probes labeled with 3P to a specific activity of 108
dpm/ng. After stringent washes, filters were autoradiographed. The
cDNA for DAF (CD55) was kindly provided by I. Caras (Genentec
Inc, San Francisco, CA) and the cDNA for CD59 was a kind gift
drift H. Waldmann (MRC Unit, Cambridge, Great Britain).
RESULTS
PBL of PNH patients show an additional negative popula-
tion 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.33 In previous experi-
ments we treated NK clones with a PIG-specific phospholi-
pase 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
alternative spliced transmembrane isoforms for these mole-
cules as described for LFA-3 (CD58).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 immuno-
fluorescence to further characterize the lymphocyte subpopu-
lations involved in this defect. We used the CD48 MoAb
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Fig 1. Phenotypic analysis of PBL of a normal donor (N) compared with a characteristic profile of a PNH patient (P). Histograms of PIG-linked surface antigens CD48 and CD55 after FACS analysis of 10,000 cells are shown.

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\textsuperscript{+} NK cells and primarily the CD16\textsuperscript{bright+} 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\textsuperscript{+} T cells exhibited the defect for expression of this PIG-linked molecule. In addition CD20\textsuperscript{+} 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.\textsuperscript{13} 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 CD16\textsuperscript{bright+} peak of NK cells a second CD16\textsuperscript{dim+} subpopulation was detectable (Fig 3). From two color analysis it became clear that both populations express the pan NK antigen CD56. CD16\textsuperscript{bright+} cells are negative for CD3 and therefore represent the characteristic NK cells of CD3\textsuperscript{−} CD56\textsuperscript{+} phenotype. In contrast, CD16\textsuperscript{dim+} 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 CD16\textsuperscript{bright+}, 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\textsuperscript{−} CD16\textsuperscript{+}CD56\textsuperscript{−} cells is less than 5% of PBL.

Comparison of PIG molecule\textsuperscript{+} with PIG molecule\textsuperscript{−} 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

Fig 2. Two color analysis of PBL of three representative PNH patients. Expression of CD56, CD16, and CD3 antigens are demonstrated versus expression of PIG-linked CD48 antigen.

Fig 3. Histograms of CD16 expression on PBL of a normal donor (N) and three PNH patients.
clones to complement-mediated lysis we used the monoclonal CD56 antibody NKH1A, 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 NKPl as well as another control clone CNK6. But there was a significant lysis of the PIG molecule defective clone NKP2 (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 NKP2 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, 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
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Fig 6. Complement-mediated lysis of PIG molecule+ clone NKPl (●), PIG molecule+ clone NKP2 (■), and control clone CNK6 (○). Lysis is demonstrated for heterologous rabbit complement compared with homologous human complement.

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, i.e., CD16bright+ and CD16dim+ cells. By using two color fluorescence it could be demonstrated that the CD16dim+ cells coexpressed CD3. Obviously this CD3+ CD16dim+ 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+ CD16dim+ 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 molecules such as CD55 and CD59 are similar. These data confirm those by Carothers et al. who recently described an identical RNA 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 protein product of which has been demonstrated to exist only in the glycolipid 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.

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 derived from the bone marrow. The long survival and the different regional homing of lymphocytes in thymus, lymph nodes, etc imply 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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