Emergence of CD52\(^-\), Phosphatidylinositolglycan-Anchor-Deficient T Lymphocytes After in Vivo Application of Campath-1H for Refractory B-Cell Non-Hodgkin Lymphoma

By Bernd Hertenstein, Bettina Wagner, Donald Bunjes, Christian Duncker, Aruna Raghavachar, Renate Arnold, Hermann Heimpel, and Hubert Schrezenmeier

CD52 is a phosphatidylinositolglycan (PIG)-anchored glycoprotein (PIG-AP) expressed on normal T and B lymphocytes, monocytes, and the majority of B-cell non-Hodgkin lymphomas. We observed the emergence of CD52\(^-\) T cells in 3 patients after intravenous treatment with the humanized anti-CD52 monoclonal antibody Campath-1H for refractory B-cell lymphoma and could identify the underlaying mechanism. In addition to the absence of CD52, the PIG-AP CD48 and CD59 were not detectable on the CD52\(^-\) T cells in 2 patients. PIG-AP-deficient T-cell clones from both patients were established. Analysis of the mRNA of the PIG-A gene showed an abnormal size in the T-cell clones from 1 of these patients, suggesting that a mutation in the PIG-A gene was the cause of the expression defect of PIG-AP. An escape from an immune attack directed against PIG-AP\(^-\) hematopoiesis has been hypothesized as the cause of the occurrence of PIG-AP-deficient cells in paroxysmal nocturnal hemoglobinuria (PNH) and aplastic anemia. Our results support the hypothesis that an attack against the PIG-AP CD52 might lead to the expansion of a PIG-anchor-deficient cell population with the phenotypic and molecular characteristics of PNH cells.

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THE INTRAVENOUS application of monoclonal antibodies (MoAbs) directed against CD52 (the Campath-1 antigen) is increasingly being used in the treatment of a variety of diseases, including lymphoid malignancies\(^1\) and autoimmune disorders,\(^2\) as well as in autologous and allogeneic bone marrow transplantation.\(^3,4\) CD52 proved to be an exceptionally good target for serotherapy, probably because of its abundant expression (approximately 4.5 \(\times\) 10\(^5\) molecules per lymphocyte), its close apposition to the cell membrane, and its apparent lack of modulation. The CD52 antigen is a heavily glycosylated, phosphatidylinositolglycan (PIG)-anchored glycoprotein (PIG-AP) with a small protein sequence of only 12 amino acids and a molecular weight of 21 to 28 kDa.\(^5\) In humans it is predominantly expressed on peripheral blood lymphocytes, monocytes and macrophages. It is also present on the majority of B-cell malignancies and in some cases of acute myeloid leukemia (AML).\(^6\) An additional advantage in the clinical use of Campath-1 is the availability of various isotypes of the antibodies with certain properties.\(^7\) The rat IgM antibody Campath-1M can fix human complement and is an excellent tool for in vitro manipulations such as T-cell depletion and bone marrow purging. The IgG antibody Campath-1G has been useful in the in vivo reduction of malignant and normal T cells. A more recently developed humanized version of the antibody has reduced the problems of immunogenicity associated with the use of foreign proteins.\(^8-11\)

CD52 belongs to a group of leukocyte differentiation antigens that are attached to the cell membrane by the PIG-anchoring system. On T lymphocytes and B lymphocytes, other proteins, eg, CD48, CD52, CD55, and CD59, are expressed as PIG-AP.\(^12\) CD58 can be expressed either as a PIG-AP or as a transmembrane protein on both T and B cells. Examples of PIG-AP on other hematopoietic cells are CD55 and CD59 on erythrocytes, CD14 and CD48 on monocytes, and CD16 and CD66b on granulocytes.\(^8\)

It was recently shown that an acquired deficiency of the PIG-anchor in hematopoietic cells is the cause of paroxysmal nocturnal hemoglobinuria (PNH). This deficiency of the PIG-anchor is caused by a defect in production of N-acetylglucosaminyl-phosphatidylinositol, the first intermediate of the PIG-anchor assembly.\(^5,11\) The gene, termed PIG-A, which encodes for a protein that is essential for this particular biosynthetic step, has been cloned and sequenced.\(^12\) Abnormalities of PIG-A transcripts were found in all PNH patients studied so far.\(^13,15\) Erythrocytes, platelets, granulocytes, monocytes, and lymphocytes can be affected by the deficiency of PIG-AP in PNH patients.\(^16-19\) So far it is not clear why a single somatic mutation occurring in a hematopoietic stem cell leads to a preferential expansion of the PIG-AP-deficient PNH clone. It has been speculated that there might exist a relative growth advantage of the mutated population versus the normal hematopoiesis.\(^18,20\) An immune-mediated attack against hematopoietic progenitors that spares the PIG-AP-deficient hematopoiesis caused by the absence of PIG-AP might confer an advantage on the PIG-AP-deficient population.\(^21\) However, so far no direct evidence for a preferential expansion of PIG-AP-negative cells by an immune mechanism has been presented. We observed the emergence of CD52\(^-\) CD3\(^+\) lymphocytes in 3 patients receiving the humanized MoAb Campath 1H\(^2\) for treatment of refractory chronic lymphocytic leukemia. Further analysis showed that these cells were deficient in other PIG-AP, ie, a selection of PIG-AP-deficient cells had occurred. Thus the treatment with an MoAb directed against PIG-AP represents a model for the emergence of PIG-AP-deficient populations.

PATIENTS AND METHODS

Antibody treatment. Treatment with Campath-1H was performed in an ongoing multicenter study on the efficiency of this

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1487
antibody in the treatment of refractory B-cell non-Hodgkin lymphoma. The study was approved by the local ethics committee and the written informed consent of the patients was obtained before the start of treatment. Campath-1H was administered in increasing doses from 3 mg to 30 mg three times weekly for a total treatment period of 12 weeks.

**Immunophenotyping of peripheral blood lymphocytes.** Lymphocytes were isolated by Lymphoprep (Nycomed Pharma, Oslo, Norway) centrifugation and incubated with a panel of fluorescein isothiocyanate (FITC)-or phycoerythrin (PE)-labeled commercially available MoAbs (CD3, CD4, CD8, CD19, and CD20 [Becton Dickinson]). Analysis was performed using a FACScan flow cytometer (Becton Dickinson). CD52+ cells were determined using an FITC-labeled Campath-1 antibody (kind gift from Wellcome, Beckenham, Kent, UK). Immunophenotyping was performed before treatment and at varying intervals thereafter (Table 1).

**Immunophenotyping of PIG-A.** The expression of PIG-A was assessed by PCR. Briefly, 200 μL of EDTA-anticoagulated venous blood were added to 200 μL of phosphate-buffered saline (PBS) with 0.1% (wt/vol) bovine serum albumin (BSA) and 5% (vol/vol) horse serum. The mixture was incubated for 20 minutes with a 50 pL solution of the primary antibody. After washing, the cells were incubated with goat-anti-mouse FITC (Dako, Hamburg, Germany). Analysis was performed using a FACScan flow cytometer (Becton Dickinson). CD52+ cells were determined using an FITC-labeled Campath-1 antibody (kind gift from Wellcome, Beckenham, Kent, UK). Immunophenotyping was performed before treatment and at varying intervals thereafter (Table 1).

**Analysis of PIG-A mRNA.** Total RNA was isolated from 5 × 10⁶ cells using a modified method of Chomczynski and Sacchi²⁴ according to the order of the manufacturer (TRizol-reagent; GIBCO BRL, Eggenstein, Germany). The resulting RNA pellet was rinsed in 75% ethanol, dried, and resolved for 10 minutes at 55°C in 10 μL distilled water that was treated with diethylpyrocarbonate. A total of 8 μL of total RNA was reverse transcribed using 50 pmol of primer C (5’/AATGATATAGACIGTAGCATAAC/3’) and primer B (5’TCTACAATCTIIATCAAC/3’) with 200 U of RNase A free reverse transcriptase (Superscript™, GIBCO BRL, Germany) in 50 mmol/L Tris/HCL, pH 8.3, 75mmol/L KCl, 3 mmol/L MgCl₂, 10 mmol/L DTT, 1 mmol/L dNTP, 2.5 μg BSA in a total volume of 20 μL at 37°C for two hours, followed by a denaturing step of 5 minutes at 95°C. The coding region of PIG-A was amplified from cDNA with 10 pmol of primer A (5’/GGT-GTGCTCTAAGAACTGATGTC/3’) and primer B (5’/TCTTACAATTGCGTCTC/3’) in a total volume of 50 μL in 10 mmol/L Tris/HCl, pH 8.3, 50 mmol/L KCl, 3.5 mmol/L MgCl₂, 0.001% gelatine, 300 μmol/L dNTP, and 2.5 U Taq polymerase (Perkin Elmer Cetus, Norwalk, CT) by 35 cycles of a reaction consisting of incubation for 1 minute at 93°C, 1 minute at 64°C, and 3 minutes at 72°C, with a final incubation for 6 minutes at 72°C. For hybridization assays, PIG-A–polymerase chain reaction (PCR) products were separated on 1% agarose gels and transferred to positively charged nylon membranes (Boehringer Mannheim, Mannheim, Germany) and hybridized using standard procedures. The blots were probed with a PIG-A cDNA probe labeled with digoxigenin-11-2′-deoxyuridine-5′-triphosphate (DIG-11-dUTP; Boehringer Mannheim). The labeling reaction was performed using PCR using 100 ng of pBSI-DNA containing the entire PIG-A cDNA

### Table 1. Phenotypic Changes in Peripheral Blood Lymphocytes After Campath 1H Treatment

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<tr>
<th>Patient no. 1</th>
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<th>4 mo</th>
<th>5 mo</th>
<th>6 mo</th>
<th>9 mo</th>
<th>Patient no. 2</th>
<th>Before treatment</th>
<th>4 mo</th>
<th>5 mo</th>
<th>7 mo</th>
<th>Patient no. 3</th>
<th>Before treatment</th>
<th>2 mo</th>
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Abbreviation: NT, not tested.

* After start of treatment.
(kindly provided by Prof T. Kinoshita, University of Osaka, Osaka, Japan); 66 μmol/L of DIG-11-dUTP; 134 μmol/L of dTTP; 200 of dATP, dGTP, and dCTP; 2.5 U Taq polymerase (Perkin Elmer Cetus); and 10 pmol of primers A and B. The resulting PCR product was purified and used at a concentration of 1 ng/mL for hybridization. For visualizing of hybridization products, a DIG-DNA detection kit (Boehringer Mannheim) was used.

RESULTS

Emergence of CD52− cells after Campath-1H treatment. The results of immunophenotypic analyses of the 3 patients are summarized in Table 1. Before treatment, a monoclonal CD19/CD5− B-cell population as well as CD3+ lymphocytes and a normal CD4/CD8 ratio were present in all patients. With greater than 98% of the lymphocytes being CD52+, the baseline expression of the target antigen was normal in all 3 patients. After lymphocyte counts decreased during the antibody treatment, a slow increase in the number of lymphocytes after the end of treatment was suspicious for a reappearance of the leukemic cells in the first patient (a 52-year-old man). However, immunophenotypic evaluation did show that the lymphocytes were nearly exclusively CD3+ lymphocytes with a predominance of CD8−CD3+ cells. Monoclonal B cells were not detectable. However all CD3+ cells lacked the CD52 antigen. The CD52− T cells could persistently be shown in subsequent analyses, but CD52−CD3+ cells reemerged. In the second patient (a 54-year-old man), 3% CD19/CD5− B cells could be detected after 12 weeks of treatment. All B cells coexpressed CD52; however, only 21% of the CD3+ cells stained positive with Campath-1, whereas 79% of the CD3+ cells were CD52−. In the third patient (a 71-year-old woman), 40% of the CD3+ cells were CD52− 9 weeks after the start of Campath-IH treatment. The 3% of B cells detected at this time stained positive with Campath-1, whereas 90% of the peripheral lymphocytes were T cells, but less than 3% stained positive with Campath-1. In all 3 patients the proportion of CD52− T cells decreased again during further follow-up (Table 1).

Determination of PIG-AP and generation of PIG-AP− deficient TLC. The presence of other PIG-AP was examined in patients no. 1 and 3. In both patients CD48 and CD59 were absent on the CD52− lymphocytes. A PIG-AP− deficient population was also detectable in monocytes. A total of 72% of the monocytes of patient no. 1 and 17% of the monocytes of patient no. 3 were CD48− at 6 and 4 months after treatment, respectively. No defects in the expression of PIG-AP were detectable in granulocytes and erythrocytes. The results of immunophenotyping of PIG-AP in patient no. 3 at 4 months after the start of Campath-1H treatment and in a normal control are shown in Fig 1.

PIG-AP− deficient T-cell clones could be established from both patients. Using limiting dilution of cells sorted on the basis of CD48 expression, we established both TLCs with normal as well as TLCs with deficient PIG-AP expression. The PIG-AP− deficient clones lacked CD48, CD52, and CD59 expression. These clones had the phenotype of the helper/inducer T subset, being positive for CD2, CD3, and CD4 but negative for CD8. The PIG-AP+ clones had either the CD4+ CD8− or the CD4− CD8+ phenotype.

Analysis of PIG-A mRNA in PIG-AP− deficient TLC. To elucidate the cause of PIG-AP deficiency, samples of total RNA from healthy control donors and PIG-AP+ and PIG-AP− TLC of patients no. 1 and 3 were reverse transcribed. The coding region of PIG-A was amplified by PCR. In all TLCs without a PIG-AP deficiency we obtained three major amplification products of about 1,500, 1,200, and 800 bp. Only the largest product represents the functional full-length transcript. The amplification products from these PIG-AP− TLCs did not differ from the product obtained from lymphocytes and granulocytes of healthy donors. In contrast, in all 3 PIG-AP− TLC from patient no. 1,300 bp appeared instead. The RT-PCR of a PIG-AP+ TLC and 2 PIG-AP− TLCs of patient no. 1 are shown in Fig 2A. Both PIG-
AP` TLCs showed an abnormal size of the PIG-A mRNA. The PIG-A` TLC of patient no. 3 showed a normal size of the RT-PCR products (Fig 2B).

**DISCUSSION**

We report that the administration of a CD52 antibody for several weeks can result in the emergence of CD52` lymphocytes. CD52 is considered to be a constitutional antigen of mature lymphocytes. It did not modulate in in vitro studies using Campath-1-labeled lymphocytes. Our observation therefore was unexpected. The occurrence of CD52` cells after treatment with Campath-1 antibodies has been reported in only 2 patients. Poynton et al observed CD52` leukemic cells in relapse after autologous bone marrow transplantation for acute leukemia in 1 patient receiving a Campath-1--purged marrow graft. We reported the occurrence of a CD52`CD8`CD3` population of recipient lymphocytes in a patient receiving Campath-1G intravenously to increase the immunosuppressive potential of the conditioning regimen before allogeneic bone marrow transplantation. The early increase of these lymphocytes after transplantation was associated with graft rejection. The observation of CD52` T cells in 3 additional patients indicates that this event may be more common. CD52` T cells were also observed in 3 of 25 patients after Campath-1H treatment for rheumatoid arthritis (S. Brett, personal communication, March 1995). Because CD52` cells may be easily overlooked in routine diagnostic immunophenotyping, we cannot give an estimate of the frequency of this phenomenon in Campath-1--treated patients. Although no adverse effects of the CD52` cell population were observed in the patients reported here, the potential selection of CD52` cells by the use of Campath-1 antibodies must be considered in the increasing therapeutic use of these antibodies. The selection of cells lacking expression of the target antigen might be a mechanism of treatment failure in immunotherapy using MoAbs.

We were able to unveil the mechanism by which the CD52` population emerged. CD52 is a PIG-anchored surface molecule. This finding prompted the search for deficiency of other PIG-AP on CD52` cells. Our results show that the population selected by Campath-1H treatment was characterized by a deficiency of PIG-AP in general. PIG-AP`--deficient cells were observed in T cells and monocytes but not in granulocytes and erythrocytes. These discordant results in different hematopoietic lineages can be explained by the specificity of the Campath-1--induced selection. Because erythrocytes and granulocytes do not or only weakly express CD52, Campath-1 treatment does not confer a growth advantage of PIG-AP`--deficient cells in these lineages. In contrast, in CD52` lineages, ie, lymphocytes and monocytes, PIG-AP`--deficient cells, which also lack CD52, may have a relative advantage over their CD52` counterparts. This selective expansion of PIG-AP`--deficient cells after Capath-1 treatment in CD52` lineages only supports the assumption that this expansion was the result of a selection process. A deficiency of PIG-AP expression seems to be the basic defect in PNH. In PNH it is caused by a biosynthetic defect due to a mutation in the PIG-A gene. The fact that the size of the PIG-A mRNA was abnormal in all PIG-AP--deficient TLCs suggests that a mutation in the PIG-A gene is the cause of the expression defect at least in patient no. 1. The normal size of RT-PCR products of the PIG-A mRNA in patient no. 3 does not exclude the possibility of a PIG-A gene mutation. The majority of mutations in the PIG-A gene in PNH are point mutations, small insertions, or small deletions that do not lead to apparent abnormal size of the RT-PCR products. PIG-AP`--deficient populations have also recently been shown in patients with aplastic anemia. This observation is in keeping with the reports on the high rate of incidence of PNH in aplastic anemia patients who are long-term survivors after immunosuppression. To explain this phenomenon it has been hypothesized that somatic mutations of the PIG-linkage pathway are a common and benign event in human hematopoiesis because small percentages of PNH cells could be detected in some normal individuals. An escape from an immune attack against PIG-AP`
hemoipoiesis might be the cause for the occurrence of these cells in these diseases. The observation that in some PNH patients 2 PNH clones with different mutations are present that must have arisen independently also supports the hypothesis that there are positive selection mechanisms in favor of PIG-AP-deficient hematopoietic cells. Our results show that such a selection is indeed possible. We assume that a low number of PIG-AP-deficient cells existed in the patients before Campath-1H treatment. Interestingly, PIG-AP-deficient T lymphocytes emerged after Campath-1H treatment, but the percentage of CD52+ T cells decreased again during further follow-up. This finding indicates that the survival advantage for PIG-AP-deficient cells was caused by the selection pressure of the antibody therapy but disappeared after the end of the treatment. Because in PNH and in some patients with aplastic anemia there is a continuous expansion of PIG-AP-deficient cells, it might be speculated that either the selection pressure is continuously present or that additional events are necessary for the survival advantage of PIG-AP− cells.

Our observations demonstrate that immunotherapy directed selectively against a PIG-AP can lead to the expansion of a PIG-anchor-deficient and PIG-A gene-deficient cell population. Because these cells show the phenotypic and molecular characteristics of PNH cells, our observations support the hypothesis that the emergence of PIG-AP-deficient cells in PNH and in some patients with aplastic anemia is caused by an escape from an immune attack against PIG-AP− cells.

ACKNOWLEDGMENT

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Emergence of CD52-, phosphatidylinositolglycan-anchor-deficient T lymphocytes after in vivo application of Campath-1H for refractory B-cell non-Hodgkin lymphoma

B Hertenstein, B Wagner, D Bunjes, C Duncker, A Raghavachar, R Arnold, H Heimpel and H Schrezenmeier