Increased resistance of PIG-A− bone marrow progenitors to tumor necrosis factor α and interferon γ: possible implications for the in vivo dominance of paroxysmal nocturnal hemoglobinuria clones

Background and Objectives. Paroxysmal nocturnal hemoglobinuria (PNH) is a clonal disorder due to a PIG-A gene mutation, resulting in deficient expression of GPI–anchored-proteins. Both immune–mediated suppression of hematopoiesis and cytokine alterations have been reported in aplastic anemia, a disease closely related to PNH whereas no data are available on PNH itself. The aim of this study was to investigate the effect of exogenous cytokines on clonogenic activity in PNH.

Design and Methods. We evaluated burst-forming units–erythroid (BFU-E) and colony-forming units–granulocyte-macrophage (CFU-GM) in bone marrow mononuclear cells (BMMC) from 5 PNH patients and 5 controls, alone or in the presence of transforming-growth–factor (TGF–)−β, interferon (IFN−)−γ, tumor necrosis factor (TNF–)−α, and specific antibodies. Molecular analysis of the PIG–A gene was performed by polymerase chain reaction (PCR) and direct sequencing on every single colony.

Results. Patients’ cells showed less clonogenic activity than did control cells. In PNH, addition of TGF–β inhibited both BFU-E and CFU-GM; IFN−γ and TNF−α inhibited BFU-E alone. In patients cytokines modulated normal and mutated clones differently: TGF–β reduced the number of PIG−A− and PIG−A+ colony-forming–cells (CFC), whereas TNF−α and IFN−γ reduced PIG−A− CFC only. BMMC from patients showed higher TGF–β production than did BMMC from controls.

Interpretation and Conclusions. TGF–β could contribute to the genesis of the unfavorable bone marrow microenvironment but does not seem to play a role in the in vivo dominance of PIG−A− deficient cells. Mutated clones were more resistant to the inhibitory effects of IFN−γ and TNF−α, suggesting that PNH cells may have a growth advantage in an unfavorable microenvironment.

Key words: PNH, cytokines, bone marrow, CFU.
(IL)-2 has been reported, leading to bone marrow damage. Few data are available on cytokine production in PNH, except for the report of a marrow-inhibiting soluble factor produced by PNH lymphocytes and of normal plasma levels of TNF-α, IL-3, stem cell factor (SCF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IFN-γ.

The aim of this paper was to study the bone marrow microenvironment in which the PNH clone could arise, with particular regard to the role of IFN-γ and TNF-α, which are overexpressed in aplastic anemia, and transforming growth factor (TGF)-β which was found to be increased in a PNH-clone that emerged after campath-1H therapy. To this purpose, we investigated formation of colony-forming unit-granulocyte-macrophage (CFU-GM) and burst-forming units erythroid (BFU-E) in basal conditions, in the presence of exogenous cytokines and after neutralization of endogenous cytokines by antibodies. In the same experimental conditions we studied cytokine modulation of the PNH clone by molecular analysis of the PIG-A gene on every single colony. Finally we evaluated the production of IFN-γ, TNF-α and TGF-β by peripheral blood (PB) and bone marrow mononuclear cells (BMMC) from PNH patients and controls.

**Design and Methods**

**Reagents**

Recombinant human (rh)IFN-γ, rhTNF-α and rhTGF-β were purchased from Biosource International (Camarillo, CA, USA), anti-IFN-γ and anti-TGF-β antibodies from Sigma Chemicals (St. Louis, MO, USA), and anti-TNF-α from Biosource. Phytohaemagglutinin (PHA) was purchased from Sigma Chemicals.

**Patients**

BM aspirates were collected from 5 PNH patients (4 males, 1 female; mean age 40.6±11.1 years) during diagnostic procedures and from 5 age and sex matched healthy volunteers, after obtaining informed consent and approval from the institutional Human Research Committee. The patients belong to a group of 20 PNH patients regularly followed by our Department who did not undergo BM aspirate.

Table 1 reports the clinical, hematologic and molecular data of the 5 PNH patients.

**Peripheral blood and BMMC cytokine production**

Fresh heparinized peripheral blood samples from PNH patients were diluted 1:6 with RPMI 1640 medium (Gibco laboratories, Grand Island, NY USA) and then either left unstimulated or stimulated for 48 hr with PHA. BMMC were isolated after centrifugation of heparinized marrow on a Ficoll-Hypaque density gradient (Axis-Shield, Norway) and cultured at the concentration of 10^5/mL in RPMI-1640 medium for 48 hr alone or in the presence of PHA. Supernatants were collected and assayed for IFN-γ, TNF-α and TGF-β by commercially available ELISA, according to the manufacturer’s instructions (R&D systems, Minneapolis, MN, USA), as previously described.

**Methylcellulose colony-forming assay**

BMMC (10^5 cells) from PNH patients and controls were plated on methylcellulose medium, MethoCult GF-H4434 (Stemcell Technologies) either alone or in the presence of the following cytokines: IFN-γ, TNF-α and TGF-β, at concentrations of 20 ng/mL, according to previous experience, or antibodies: anti-IFN-γ, anti-TNF-α and anti-TGF-β, at concentrations of 10 µg/mL. In some experiments with BMMC from PNH patients antibodies were used at the concentration of 100 µg/mL. Cytokines and antibodies were added at the beginning of the culture and subsequently every 48 hours. After 14 days of culture BFU-E, CFU-GM and colony-forming unit granulocyte-erythroid-megakaryocyte (CFU-GEMM) were counted using an inverted microscope.

**Molecular analysis of colonies**

Every single colony was picked up for molecular analysis of PIG-A gene, washed in PBS and suspending in 25 µL of 10 ng/mL lysis buffer containing Proteinase K (50 µg/mL), Tween-20 (0.5%) and NP-40 (0.5%); cell lysate (5–10 µL) was used for the PCR reaction and

**Table 1. Clinical, hematologic and molecular data of the 5 PNH patients.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Age at onset</th>
<th>PNH type at onset</th>
<th>Hb g/dL</th>
<th>Retics. 10^9/L</th>
<th>WBC 10^9/L</th>
<th>Plt. 10^9/L</th>
<th>PIG-A gene mutation</th>
<th>Granulocytes CD55/59 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>31</td>
<td>Hemolytic</td>
<td>6.3</td>
<td>137</td>
<td>6.6</td>
<td>91</td>
<td>Del T774</td>
<td>85/90</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>34</td>
<td>Hemolytic</td>
<td>8.9</td>
<td>85</td>
<td>4.5</td>
<td>174</td>
<td>Del C423</td>
<td>63/59</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>43</td>
<td>Hemolytic</td>
<td>10.6</td>
<td>149</td>
<td>4</td>
<td>92</td>
<td>Del 166-167 CT</td>
<td>55/54</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>37</td>
<td>AA/PNH</td>
<td>9.1</td>
<td>3</td>
<td>4.3</td>
<td>22</td>
<td>Del C337</td>
<td>41/23</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>19</td>
<td>Hemolytic</td>
<td>10.7</td>
<td>118</td>
<td>5.2</td>
<td>89</td>
<td>A383G</td>
<td>75/73</td>
</tr>
</tbody>
</table>
direct sequencing, searching for the mutation previously identified in each patient (Table 1). The primers used for PCR amplification were prepared according to Iida et al.18 with minor modifications. Amplified DNA was automatically sequenced (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Warrington, UK) using the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Warrington, UK).

Statistical analysis
Results were analyzed by two-tailed Students’ t-tests for paired or unpaired data. p<0.05 was considered statistically significant.

Results

Clonogenic activity in PNH patients and controls
Table 2 shows the number of CFU-GM, BFU-E, CFU-GEMM, and CFC of PNH patients and controls, either in medium alone or in the presence of exogenous cytokines (TNF-α, IFN-γ and TGF-β). The baseline clonogenic activity of PNH patients was lower than that of controls, the reduction being significant for the number of CFC and BFU-E colonies (p=0.03 and p=0.02, respectively). Addition of TNF-α, IFN-γ and TGF-β significantly reduced CFC formation in patients (p=0.03, p=0.01, p=0.001, respectively), the inhibitory effect being more evident in the presence of TGF-β. In controls, only TNF-α and TGF-β significantly reduced CFC formation (p=0.04 and p=0.02, respectively).

Considering the different progenitors, in patients TNF-α and IFN-γ significantly inhibited BFU-E (p=0.03 and p=0.02, respectively), and TGF-β significantly inhibited both BFU-E and CFU-GM (p=0.004 and p=0.05, respectively). In controls only TNF-α and TGF-β were able to reduce BFU-E significantly (p=0.04 and p=0.01, respectively). Considering the lower number of PNH colonies, we also calculated ∆ percentages of inhibition, according to the following formula: [(n. of colonies in the presence of the cytokine - n. of colonies in medium)/n. of colonies in medium]*100. The degree of the inhibitory effect of the cytokines investigated on CFC was comparable between patients and controls.

Effect of cytokines on PIG-A- and PIG-A+ clones
In PNH patients the effects of TNF-α, IFN-γ and TGF-β were investigated on PIG-A mutated and unmutated CFC. Cytokines modulated PIG-A- and PIG-A+ clones differently. Addition of TGF-β significantly reduced only the number of PIG-A- and PIG-A+ CFC (p=0.03 for both), whereas TNF-α and IFN-γ significantly reduced the number of PIG-A+ CFC (p=0.04 and p=0.05, respectively) (Table 3).

Considering ∆ percentages of inhibition, calculated according to the formula [(n. of colonies in the presence of the cytokine - n. of colonies in medium)/n. of colonies in medium]*100, the lower sensitivity of mutated PNH clones to the inhibitory effect of TNF-α and IFN-γ was confirmed, whereas PIG-A+ CFC were more affected and behaved as CFC from controls (Table 1 and Table 3).

Production of TGF-β, TNF-α and IFN-γ by BMMC and peripheral blood from PNH patients and controls
Table 4 shows cytokine production by unstimulated and PHA-stimulated BMMC and peripheral blood from

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**Table 2. Number of CFU-GM, BFU-E, CFU-GEMM, and CFC in PNH patients and controls, in medium alone or in the presence of exogenous cytokines.**

<table>
<thead>
<tr>
<th></th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Medium</td>
<td>TNF-α</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>11.2±3.7</td>
<td>5.2±1.2</td>
</tr>
<tr>
<td>BFU-E</td>
<td>18.8±3.1†</td>
<td>5.2±2.2*</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>1.4±0.7</td>
<td>0.4±0.2</td>
</tr>
<tr>
<td>CFC</td>
<td>31.4±3.7†</td>
<td>10.8±3.1*</td>
</tr>
<tr>
<td>CFU-GM</td>
<td>20.2±8.8</td>
<td>9.0±3.4</td>
</tr>
<tr>
<td>BFU-E</td>
<td>61.7±18.3</td>
<td>19.5±8.3*</td>
</tr>
<tr>
<td>CFU-GEMM</td>
<td>2.0±1.0</td>
<td>1.5±1.0</td>
</tr>
<tr>
<td>CFC</td>
<td>84.3±25.9</td>
<td>30.0±11.0*(-64)</td>
</tr>
</tbody>
</table>

Values are expressed as number of colonies mean±SE of 5 PNH patients and 5 controls. *p<0.05, †p<0.01 cultures in the presence of cytokines vs medium alone; ‡ p<0.05 patients vs controls. ∆ percentage of inhibition are shown in brackets.
PNH patients and controls. Both spontaneous and PHA-stimulated TGF-β production by BMMC was higher in PNH patients than in controls, the former significantly so (p=0.02). In contrast, TGF-β production by peripheral blood cultures was comparable between patients and controls. TNF-α production by BMMC and peripheral blood was lower in patients than in controls, although not significantly so, both in basal conditions and after mitogen stimulation. Spontaneous and PHA-induced IFN-γ production was reduced in BMMC from patients whereas it was increased in peripheral blood cultures, although not significantly.

Effect of anti-TGF-β antibody on clonogenic activity in PNH patients and controls

Given the high TGF-β production by BMMC of PNH patients and the particularly strong inhibitory activity of TGF-β on colony formation, we investigated the effect of anti-TGF-β neutralizing antibody on the number of BFU-E and CFC (Figure 1). In controls, addition of anti-TGF-β antibody increased the number of BFU-E and CFC, the latter significantly (p=0.01). In contrast, in PNH patients anti-TGF-β antibody was ineffective, both at the standard concentration in the experiments shown and at a ten fold higher concentration (data not shown). Similar experiments were performed with the addition of anti-TNF-α and anti-IFN-γ; these two anti-ciliate antibodies did not have significant effects on cells from either patients or controls (data not shown).

Discussion

Several hypotheses have been considered to explain the physiopathologic mechanism of bone marrow failure in PNH, particularly an intrinsic deficiency of the stem cell and/or an extrinsic immune-mediated damage and an unfavorable bone marrow microenvironment. Moreover, there is an ongoing debate on whether the emergence of the PNH clone derives mainly from a primary deficiency in normal cells or from a growth advantage of mutated progenitors.21,22

Table 3. Effect of cytokines on unmutated and mutated CFC in PNH patients.

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>TNF-α</th>
<th>IFN-γ</th>
<th>TGF-β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unmutated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22.4±6.3</td>
<td>5.4±2.4*</td>
<td>9.4±2.4*</td>
<td>1.6±0.4*</td>
</tr>
<tr>
<td>PIG-A -76%</td>
<td>(-76%)</td>
<td>(-58%)</td>
<td>(-93%)</td>
<td></td>
</tr>
<tr>
<td>Mutated</td>
<td>9±2.9</td>
<td>5.4±2.6</td>
<td>5.2±1.4</td>
<td>0.2±0.1*</td>
</tr>
<tr>
<td>PIG-A -40%</td>
<td>(-40%)</td>
<td>(-42%)</td>
<td>(-100%)</td>
<td></td>
</tr>
</tbody>
</table>

Values are the mean±SE of 5 patients. *Denotes statistically significant comparisons (p<0.05) between cultures in the absence or in the presence of the different cytokines investigated. ∆ percentage of inhibition are shown in brackets.

Figure 1. Effect of anti-TGF-β antibody on BFU-E and CFC. Cells from the bone marrow of PNH patients and controls were cultured in the absence (□) and in the presence of anti-TGF-β antibody (■). BFU-E and CFC colonies were counted after 14 days. Values are the mean±SE of 5 patients. *denotes a statistically significant difference (p=0.01) between cultures in the absence or in the presence of anti-TGF-β antibody.

Table 4. Cytokine production in bone marrow mononuclear cells from PNH patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>TGF-β</th>
<th>TNF-α</th>
<th>IFN-γ</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unstimulated</td>
<td>PHA</td>
<td>Unstimulated</td>
</tr>
<tr>
<td>Patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>3145±241*</td>
<td>2267±378</td>
<td>4.1±2.4</td>
</tr>
<tr>
<td>Peripheral Blood</td>
<td>3886±1335</td>
<td>6149±1527</td>
<td>2.6±2.0</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bone Marrow</td>
<td>1594±486</td>
<td>1361±391</td>
<td>13.8±6.6</td>
</tr>
<tr>
<td>Peripheral Blood</td>
<td>4235±645</td>
<td>7978±945</td>
<td>5.3±3.1</td>
</tr>
</tbody>
</table>

Values are given in pg/mL, mean±SE of 5 PNH patients and 5 controls. *p<0.02, patients vs controls for unstimulated bone marrow cultures.
TNF-α and IFN-γ resistance of PIG-A progenitors

Chen et al. demonstrated that GPI-deficient CD34 cells derived from PNH bone marrow behaved as did CD34 cells from normal donors, while those with a normal phenotype obtained from PNH patients showed little growth. Moreover, PIG-A progenitor cells from PNH patients showed increased expression of Fas and consequently greater susceptibility to apoptosis than did mutated bone marrow progenitors. This failure of PIG-A progenitor cells does not exclude that additional factors could play a role in the relative growth advantage of GPI-deficient cells.

In particular, it can be hypothesized that mutated progenitors might be spared from extrinsic immune-mediated damage. In aplastic anemia, a disease closely related to PNH, both immune suppression of hematopoiesis and alterations of regulatory cytokines have been reported.

Thus, we investigated whether regulatory cytokines could be involved in immune-mediated bone marrow damage also in PNH. Among cytokines, we first focused on TGF-β, which is a well known inhibitory factor both in animal models and in human diseases, and which was found to be increased in a PNH clone that emerged after campath-1H therapy.

Our results showed that BMPC from PNH patients produced increased amounts of TGF-β and that addition of this cytokine was able to inhibit bone marrow clonogenic activity in a similar manner in patients and controls. Considering PIG-A and PIG-A progenitors of PNH patients, TGF-β does not act selectively on the two populations, showing non-specific inhibition of both. These data are in line with the findings of Nishimura who showed that TGF-β suppressed the growth of BFU-E bursts from both CD59-positive and CD59-negative BMPC. Thus, TGF-β could contribute to the genesis of the bone marrow failure, due to its inhibitory effect and increased expression in PNH, but it does not seem to play a role in the in vivo dominance of the PIG-A mutated clones.

It is known that TNF-α and IFN-γ are cytokines involved in cytotoxic immune responses and programmed cell death. Selleri et al. demonstrated that addition of IFN-γ and TNF-α triggered apoptosis of total bone marrow and CD34+ cells in normal subjects, and speculated that increased TNF-α and IFN-γ in vivo may result in a broad depletion of progenitor cell pools.

Our results show that addition of TNF-α and IFN-γ reduced clonogenic activity both in PNH patients and controls, and that IFN-γ production by PBMC was increased in PNH patients. Both these cytokines could contribute to produce extrinsic damage to bone marrow precursors in PNH. However, at variance with TGF-β, we found that PIG-A mutated clones were more resistant to the inhibitory effect of IFN-γ and TNF-α, suggesting the existence of a relative growth advantage of the mutated clone in an unfavorable microenvironment.

A higher level of IFN-γ in the bone marrow than in the PB, and a higher level in PNH patients than in controls would have better explained the increased resistance to IFN-γ and the relative growth advantage of the PNH clone. The limited number of cases, the wide dispersion of data, and the low yield of colonies obtained do, however, suggest caution in the interpretation of these findings. In any case the increased resistance of PIG-A mutated clones to IFN-γ and TNF-α is in line with the already demonstrated increased resistance to apoptosis of CD34+/CD59 compared to CD34+CD59 bone marrow progenitors from PNH patients.

The different susceptibility of PIG-A mutated and unmutated clones to IFN-γ and TNF-α is unlikely to depend on abnormal IFN-γ and TNF-α receptor expression and function in GPI-deficient cells. In fact, TNF-α and IFN-γ bind transmembrane receptors, which do not involve the GPI anchor. Accordingly, Kulikarni and Bessler demonstrated in a murine model of PNH, that PIG-A and PIG A-hematopoietic progenitors cells have a similar ability to respond to inhibitory cytokines, including TNF-α and IFN-γ. Further experiments aimed at evaluating TNF-α receptor expression on PIG-A and PIG A bone marrow cells would clarify the function of TNF-α receptors in humans.

Our data, although obtained in a small number of patients, show that TNF-α and IFN-γ modulate PIG-A and PIG A bone marrow progenitors differently. The effect of these cytokines on the bone marrow of PNH patients, and in particular the lower susceptibility of PIG A progenitor cells, might be involved in the in vivo expansion of the PNH clone.

All authors contributed equally to the conception, design, analysis, and interpretation of data. All authors equally contributed to drafting the article and revising it critically, giving final approval of the version to be published. The authors reported no potential conflicts of interest.

This work was supported by research grants from the Istituto Superiore di Sanità, Programma Nazionale sulle Cellule Staminali, convenzione n. CS76, and R.C. 99 160/03 from IRCCS Ospedale Maggiore di Milano.

Manuscript received January 19, 2004. Accepted April 6, 2004.
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