CLONAL ORIGIN OF ABNORMAL GRANULOCYTES IN PAROXYSMAL NOCTURNAL HEMOGLOBINURIA

To the Editor:

Two studies recently published in Blood have used the pattern of X-chromosome inactivation to test whether white blood cells of patients with paroxysmal nocturnal hemoglobinuria (PNH) and aplastic anemia (AA) belong to a single clone.1,2 The investigators' conclusions were in keeping with data previously obtained by the same approach on red blood cells.3 However, we find the interpretation of their findings rather difficult, because the PNH cells were not separated from the normal cells, which usually coexist in the patients' peripheral blood. In our view, the finding of a homogeneous (or unbalanced) pattern of X-chromosome inactivation can be regarded as conclusive proof of the clonal origin of a certain cell population only when it is demonstrated in the context of a dimorphic (or balanced) pattern in normal cells from the same lineage.

We have performed similar tests on granulocytes in five patients with PNH, but we have analyzed separately the normal and the PNH cells (see Table 1). All patients except patient 3 suffered from AA before the diagnosis of PNH. In patients 1 and 2, an unbalanced pattern is seen in both the PNH and the normal granulocytes. In patient 3 the PNH granulocytes show an unbalanced pattern, whereas normal granulocytes do not. In patients 4 and 5 the PNH granulocytes show an unbalanced pattern, and there are not enough normal granulocytes to analyze. However, in patient 4 analysis of lymphocytes (85% of which were normal) showed an unbalanced pattern; in patient 5 analysis of lymphocytes (60% of which were normal) showed a balanced pattern. Thus, for patients 3 and 5 we conclude that the PNH granulocytes are monoclonal, because the granulocytes in patient 3 and the lymphocytes in patient 5 serve as controls. For the other patients (who are similar to the majority of those in ref 1 and to some of those in ref 2), we cannot draw any unambiguous conclusion, because both the PNH and the "normal" granulocytes show an unbalanced pattern.

### Table 1. Proportion of Cells With the PNH-Phenotype and the Corresponding X-Chromosome Inactivation Pattern

<table>
<thead>
<tr>
<th>Patient</th>
<th>Red Blood Cells PNH</th>
<th>Normal</th>
<th>Granulocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>X-Chr</td>
</tr>
<tr>
<td>1. AA/PNH</td>
<td>8</td>
<td>85 U</td>
<td>15 U</td>
</tr>
<tr>
<td>2. AA/PNH</td>
<td>15</td>
<td>45 U</td>
<td>55 U</td>
</tr>
<tr>
<td>3. PNH</td>
<td>20</td>
<td>34 B</td>
<td>NA</td>
</tr>
<tr>
<td>4. AA/PNH</td>
<td>42</td>
<td>6 NA</td>
<td>96 U</td>
</tr>
<tr>
<td>5. PNH</td>
<td>95</td>
<td>4 NA</td>
<td>96 U</td>
</tr>
</tbody>
</table>

Quantitation of normal and PNH-granulocyte populations was performed by flow cytometry using monoclonal antibodies (MoAbs) to GPI-linked surface antigens (CD16, CD24, CD55, and CD59). After centrifugation over a Ficoll gradient and hypotonic lysis of the red blood cells, normal granulocytes were separated from PNH-granulocytes by staining the granulocytes with mouse MoAb to CD24 and 1 to 5 rounds of cell adhesion to immunomagnetic beads coated with goat-anti-mouse antibody until a purity of >95% PNH cell was achieved as determined by flow cytometry using anti-CD16 and anti-CD59. DNA was extracted separately from normal granulocytes (adherent to the beads) and from PNH granulocytes (nonadherent to the beads) by the sodium dodecyl sulfate proteinase-K procedure. DNA from unseparated leukocytes and from lymphocytes was used for comparison.

Restriction endonuclease digestion with *PstI* displayed the two alleles at the DXS255 locus. Methylation differences were determined by subsequently digesting the DNA sample with *MspI* or its methylation sensitive isoschizomeric enzyme *HpaII*. Southern blots were hybridized to radioactively labeled M27β-probe10 (generously provided by I. Craig, Oxford, UK). A pattern of bands that could be attributed to a mixture of cells having either X-chromosome active is referred to as balanced (B), whereas a pattern of bands that reflects one and the same X-chromosomes active in all cells is referred to as unbalanced (U).

Abbreviations: X-Chr, X-chromosome inactivation; NA, not available.
In a more recent issue of Blood the problem of clonal analysis and the relationship between PNH and AA have been further explored by Young, who recalled the possibility that some degree of marrow hypoplasia may be regularly associated with PNH. We believe that to assess what the X-chromosome inactivation approach can tell us about the origin of AA, it is necessary to consider that X-chromosome mosaicism in females pre-exists pathology. Therefore, an unbalanced pattern can arise in several different ways. (1) Specific selection of cells with one of the two X-chromosomes active: this is not unlikely to occur, especially when the bone marrow is stressed, whereby a particular X-linked allele may confer an advantage to stem cells. In this case the emerging cells will be homogeneous but not monoclonal. (2) The residual cells in AA are truly monoclonal. This in turn could be explained in two ways. (a) A stem cell has undergone a mutation (not recognizable by a distinct phenotype, as in the case of PNH) and now supports hematopoiesis. In this case, i.e., in a patient with the AA-PNH syndrome the non-PNH cells were truly monoclonal, one would have to make the non-trivial hypothesis that a second mutation is responsible for the PNH clone. (b) The bone marrow depression is so severe that only one (or very few) normal stem cells are left to support hematopoiesis. (3) Finally, we should not forget that a significant proportion of normal women show an unbalanced pattern of X-chromosome inactivation.

In conclusion, there has been evidence for some time that PNH is a clonal disorder. As for the analysis of cell populations in AA and in other disorders, we think inferences drawn from X-inactivation analysis must always be validated by comparison with the constitutional pattern of normal cells from the same patient.

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REFERENCES
Clonal origin of abnormal granulocytes in paroxysmal nocturnal hemoglobinuria [letter; comment]

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