Acquired Aplastic Anemia and Paroxysmal Nocturnal Hemoglobinuria: Studies on Clonality


We used X-chromosome methylation patterns to study clonality in aplastic anemia (AA) and paroxysmal nocturnal hemoglobinuria (PNH). AA is usually not considered to be a clonal stem cell disorder, although this has not been directly investigated. PNH is generally assumed to be a clonal disorder, although there is contradictory evidence. Methylation analysis was performed on DNA from separated granulocytes and mononuclear cells, using the M27 beta and hypoxanthine phosphoribosyl transferase (HPRT) probes. Six of seven AA patients showed a polyclonal pattern of X inactivation. In contrast, five of five PNH patients showed a monoclonal pattern. These results imply that at least 80% of the cell population derives from a single stem cell. Because this high proportion of PNH cells might be considered surprising, three patients were studied for membrane expression of decay accelerating factor (DAF). In support of the DNA data, more than 95% of the granulocytes were DAF-ve in all three cases. We conclude that AA is predominantly a polyclonal disorder, whereas PNH is a clonal stem cell disorder. Our data support a model in which a single PNH stem cell has a growth advantage over other remaining stem cells and eventually dominates hematopoiesis.

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sensitivity to complement lysis in large mature erythroid bursts, but not in small immature bursts. This has been interpreted as evidence of the PNH defect being acquired during erythrocyte cell maturation.

In this report, we have directly investigated the clonality of the granulocyte and lymphocyte populations in a series of PNH patients using the techniques of Southern blotting with probes for polymorphic regions of the X chromosome. These have been compared with a series of remission aplastics who have not (yet) developed PNH. We show here that granulocytes of PNH patients are predominantly clonal, whereas in the majority of AA patients they are polyclonal. The implications for the etiology of AA and PNH are discussed.

MATERIAL AND METHODS

Patients. Female patients with AA and/or PNH referred to St. George’s Hospital, London were included in the study. Clinical details are shown in Table 1. AA patients had duration of disease between 9 months and 9 years. All patients had acidified serum lysis tests (Ham’s test) performed at approximately 6-month intervals. Patients had been treated with antilymphocyte globulin, steroids, or androgens at various times. Two patients with longstanding AA had developed PNH 9 and 24 months before this study. Since this study was completed, the RBC and WBC counts of the most recently diagnosed AA/PNH patient have improved, but with increasing clinical evidence for PNH. Other PNH patients had longstanding disease of between 9 and 20 years.

DNA extraction and Southern blotting. Twenty to thirty milliliters of peripheral blood was collected in EDTA-anticoagulated tubes according to a protocol approved by the Institutional Ethics Committee. Density gradient centrifugation in Ficoll-Paque (Pharmacia, Upsala, Sweden) was used to separate mononuclear cells from granulocytes. Granulocytes were separated from erythrocytes by sedimentation in 5% dextran. High molecular weight DNA was prepared from granulocytes and mononuclear cells according to standard methods. Ten micrograms of DNA was digested with restriction endonuclease PstI (GIBCO/BRL, Uxbridge, UK), ethanol precipitated, aliquoted in two portions, and one aliquot further digested with HpaII (NBL, Cromlington, UK) in fivefold excess for between 4 and 12 hours. Digests were run in pairs on a 0.8% agarose gel, followed by Southern blotting onto Hybond N+ membrane (Amersham International, UK) according to the manufacturer’s recommendations. The M27 beta probe (kindly provided by N. Fraser, Y. Boyd, and I. Craig, Oxford, UK) was radiolabeled by the random primer method (MULTIPRIME labeling system, Amersham International, UK), and after hybridization and washing the membranes were exposed to x-ray film for 2 to 10 days. After HpaII digestion, all the bands migrate slightly faster, but this can be compared with other lanes on the gel. Three-band patterns due to partial digestion with HpaII were classified as uninformative, since they may resolve into either a polyclonal or monoclonal pattern on further digestion (data not shown). One patient (no. 11) was studied using the BamHI polymorphism of the HPRT gene as described by Vogelstein et al.

Flow cytometry. To prevent selective enrichment for cells that are less susceptible to lysis than the abnormal cells, 3.5% dextran alone was used to separate leukocytes from the bulk of erythrocytes in whole blood. Monoclonal antibodies (MoAbs) BRIC 110 and BRIC 216 (International Blood Group Reference Laboratory, Elstree, UK) and anti-DAF (a kind gift of Professor V. Nussenzweig, New York, NY), all IgG1 antibodies, were used to label the CD55 DAF molecule on leukocytes in the indirect immunofluorescence method.

Briefly, 50 µL of 20 × 10^6/mL leukocytes were added to saturating amounts of primary MoAb or isotype-matched irrelevant mouse immunoglobulin and incubated for 30 minutes at 4°C on ice. After two washes in washing solution (0.01 mol/L phosphate-buffered saline, pH 7.3, containing 5% fetal calf serum and 0.2% sodium azide), 100 µL of 1 in 20 rabbit anti-mouse F(ab’)2 fluorescein isothiocyanate (Dakopatts A/S, Copenhagen, Denmark) was added and incubated under the same conditions as above. Cells were then washed three times in washing solution and analyzed in an EPICS V flow cytometer (Coulter Electronics, Hialeah, FL) fitted with an argon ion laser tuned to 488 nm. Trypan blue viability before and after labeling was greater than 98%. Granulocytes, monocytes, and lymphocytes were distinguished on the basis of cell size, measured by forward angle light scatter, and granularity, determined by right-angle light scatter.

RESULTS

DNA analysis of clonality. The M27 beta probe detects a highly polymorphic region on the X chromosome, caused by an adjacent variable number tandem repeated (VNTR) sequence. Flanking HpaII sites have been reported as

<table>
<thead>
<tr>
<th>Table 1. Patient Data</th>
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<tr>
<td>Patient No.</td>
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<tr>
<td>1</td>
</tr>
<tr>
<td><strong>Diagnosis</strong></td>
</tr>
<tr>
<td><strong>Age (yr)</strong></td>
</tr>
<tr>
<td><strong>Duration of disease (yr)</strong></td>
</tr>
<tr>
<td><strong>Count at onset</strong></td>
</tr>
<tr>
<td>Hb (g/dL)</td>
</tr>
<tr>
<td>WBC (×10^6/L)</td>
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<tr>
<td>Granulocytes (%)</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
</tr>
<tr>
<td>Plts (×10^11/L)</td>
</tr>
<tr>
<td><strong>Count of sample used in this study</strong></td>
</tr>
<tr>
<td>Hb (g/dL)</td>
</tr>
<tr>
<td>WBC (×10^6/L)</td>
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<td>Plts (×10^11/L)</td>
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Abbreviations: Hb, hemoglobin; Plts, platelets.
unmethylated on inactive X chromosomes, but methylated, and therefore resistant to HpaII digestion, on active X chromosomes. This has enabled the use of M27 beta to study X-chromosome inactivation patterns, and thus determine clonality.

Granulocyte and mononuclear cells of female AA and PNH patients were separated by density gradient centrifugation. DNA was extracted, digested with PstI alone or PstI and HpaII, and examined by Southern blotting using the M27 beta probe. All cases in this study were distinguishably heterozygous, so that a PstI digest gave two bands on Southern blot analysis. On further digestion with HpaII, the two-band pattern resolved into a four-band pattern indicative of polyclonality, or into a two-band pattern, with one of the bands decreased in size, indicative of monoclonality.

Results are shown in Fig 1 and summarized in Table 2. In five of six AA patients examined, we saw a polyclonal pattern in granulocyte and lymphocyte DNA, and only one AA patient gave evidence of monoclonality. This patient did not have a positive Ham's test or any indication of malignant disease. The monoclonal pattern was seen in whole blood and in granulocyte DNA.

Four of four PNH patients examined showed a monoclonal pattern with their granulocyte DNA. A fifth patient, whose granulocyte DNA failed to digest, showed a monoclonal pattern with her lymphocyte DNA. While one patient exhibited a monoclonal pattern in both granulocyte and lymphocyte DNA, another showed a polyclonal pattern in the lymphocytes despite having a monoclonal pattern with the granulocytes. This patient presented with a posi-

Table 2. Clonality Results

<table>
<thead>
<tr>
<th>Granulocyte status</th>
<th>AA (Patient No.)</th>
<th>PNH (Patient No.)</th>
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<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6</td>
<td>7 8 9 10 11</td>
</tr>
<tr>
<td>No. with monoclonal granulocytes</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>No. with polyclonal granulocytes</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
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Abbreviations: PC, polyclonal; MC, monoclonal; FD, DNA failed to digest with one or both enzymes.
tive Ham's test only 9 months before the study without clinical evidence of PNH. AA had been diagnosed 7 years before the Ham's test became positive.

The difference between the AA and the PNH groups was statistically significant (Table 2), even when only including patients with granulocyte data available.

**Surface antibody studies.** The method described above will not detect a clonal population in the presence of polyclonal cells, but requires the majority (>80%) of the cells to belong to the clone. There is considerable evidence that PNH cells do not completely displace normal hematopoiesis: for instance, the proportion of complement-sensitive RBCs does not usually exceed 60%. Our consistent finding of clonality might therefore seem surprising. To examine this further, repeat samples were obtained from three patients and studied for surface antigen expression using anti-decay accelerating factor (DAF) antibodies. The results of a fluorescence-activated cell sorter (FACS) analysis are shown in Fig 2. In all three cases, the proportion of DAF negative granulocytes was greater than 95%, which is fully consistent with the DNA analysis. However, in one patient, a small number of DAF positive monocytes and lymphocytes were detected.

**DISCUSSION**

AA is a disorder of the hematopoietic system, the nature of which is not well understood. There appears to be a defect of hematopoietic stem cell proliferation that is not corrected in vitro by culture on a normal stroma. In contrast, AA stroma is able to support normal hematopoiesis in the majority of cases (F. Gibson, J. Scopes, E.C. Gordon-Smith, submitted). However, there may be some heterogeneity. Hematopoietic failure is not usually complete; spontaneous or therapy-induced recovery may occur, although hematological indices usually remain low or in the low normal range. It was not previously known whether recovered hematopoiesis derives from a single preserved stem cell or represents general recovery of many clones. To test this directly, we examined clonality in the hematopoietic cells of AA patients using the M27 beta probe. In five of six patients with AA, there was clear evidence of polyclonal hematopoiesis. In the remaining patient with AA, we found uniform inactivation of one X chromosome, indicating monoclonality. A DNA methylation pattern indicative of monoclonality has been observed in a small proportion of hematologically normal individuals. This may be due to a chance bias in lyonisation in a small number of stem cells, or to selective lyonisation, or possibly represents real monoclonality. If the monoclonality is genuine, it could be due to a single normal stem cell that has survived a generally damaging event that has ablated the other stem cells. Alternatively, clonal AA might represent a different disease from other AA cases, and involve a clonally abnormal stem cell.

In contrast to AA, we were able to demonstrate uniform inactivation of one X chromosome, indicating monoclonality, in five of five PNH patients. The technique used will not detect a minority clone in the presence of polyclonal cells. Thus, our results imply that 90% or more of the granulocytes in these patients derive from a single stem cell. This might be considered surprising, as most PNH patients appear to retain a significant proportion of normal hematopoiesis; for instance, the proportion of complement-sensitive RBCs rarely exceeds 60%. To investigate this further, we studied DAF expression on WBCs from three PNH patients. In all three, greater than 95% of granulocytes were DAF negative (Fig 2), which is fully consistent with the DNA results. This is a higher percentage than has usually been observed (see Kinoshita et al). We have seen higher percentages of DAF positive cells in a few experiments, but only when there was significant loss of cell viability in vitro. We believe that this represented in vitro enrichment for DAF positive cells, and our current procedures were designed to maintain cell viabilities greater than 95%.

In one PNH patient (no. 8), we observed polyclonality in the lymphocytes despite monoclonality in the granulocytes. This patient was diagnosed with AA 7 years ago; a routine check only 9 months ago showed a positive Ham's test without clinical evidence for PNH symptoms. She could accordingly have a PNH clone, which is still (because of short duration) obscured by normal polyclonal lymphocytes. Since the completion of the study, we have observed an improvement in her WBC and RBC counts in parallel with increasing clinical manifestation of PNH, which supports this interpretation. However, there was evidence for DAF +ve lymphocytes and monocytes in a second PNH patient with much longer duration of disease, and lack of involvement of lymphocytes has previously been reported in some, but not other cases of PNH.

What, then, of the arguments against clonality in PNH based on membrane protein expression or sensitivity of cells to complement lysis (as laid out in the introduction)? It is necessary to differentiate between the primary PNH defect and the cell surface phenotype of PNH cells. If the primary defect is viewed as the propensity of cells to lose inositol lipid-anchored membrane proteins, the variable degree of the actual loss of surface proteins could depend on other factors, including the cell lineage, maturity of cells, type of particular protein, and other environmental factors.

Our results show clearly that PNH is a clonal stem cell disease. This is consistent with a model in which the PNH clone has a proliferative advantage and/or suppressor effect on the normal stem cells in a comparable way to the myelodysplastic and myeloproliferative syndromes. Our results are consistent with the view that the PNH clone becomes predominant in hematopoietic cell replication, but that the residual normal hematopoiesis is magnified in the longer lived cell lineages due to differential cell survival. We cannot exclude the possibility that our five patients had, by chance, a more dominant PNH clone than usual, but this was not reflected in the clinical expression or in the Ham's test. Since recovery from AA in the absence of PNH is predominantly polyclonal, our findings do not support the idea that the PNH clone is just passively revealed due to the failure of the other stem cells during AA (see Rotoli and Luzzatto). Because recovery from AA is predominantly polyclonal, the disease must presumably be due to a suppressive effect...
Fig 2. DAF expression on cell populations distinguished by flow cytometry. RBC-depleted total WBCs were incubated with anti-DAF antibody, or an irrelevant mouse antibody, followed by fluoresceinated antimouse Ig antibody. Granulocytes, monocytes, and lymphocytes were distinguished by cell size and light scatter. The distribution of DAF expression is shown for each cell type. (—) Anti-DAF antibody; (---) irrelevant mouse antibody.

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Acquired aplastic anemia and paroxysmal nocturnal hemoglobinuria: studies on clonality [see comments]

KM Josten, JA Tooze, C Borthwick-Clarke, EC Gordon-Smith and TR Rutherford