Clonal Hematopoiesis in Patients With Acquired Aplastic Anemia

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To determine whether patients with acquired aplastic anemia (AA) exhibit clonal hematopoiesis, we used restriction fragment length polymorphisms of the X-linked genes phosphoglycerate kinase (PGK), hypoxanthine phosphoribosyltransferase (HPRT), and the X-linked probe M27β. Of the 19 female patients studied, 18 (95%) patients were informative for at least one marker. Of these, eight patients (42%) were heterozygous for PGK, two (11%) for HPRT, and 16 (84%) for M27β. In 13 (72%) patients, a monoclonal pattern was found. Analysis of purified cell suspensions of four of these patients showed that both myeloid and lymphoid cells were of monoclonal origin, indicating the involvement of an early stem cell. The four patients who were studied at presentation all showed a monoclonal pattern. One of these patients showed a spontaneous recovery despite persistent clonal hematopoiesis. The presence of either clonal or polyclonal hematopoiesis did not show a correlation with the response to antithymocyte globulin (ATG) treatment. A relapse after ATG was also seen in a patient exhibiting polyclonal hematopoiesis. Conversely, a monoclonal pattern did not preclude the occurrence of a partial or complete response to ATG. Other potential markers to study clonality, including cytogenetic abnormalities or point mutations of the N-ras proto-oncogene, were not found in any of the patients. It is concluded that patients with AA may exhibit clonal hematopoiesis. The significance with respect to evolution to disorders with clonal hematopoiesis like paroxysmal nocturnal hemoglobinuria, myelodysplasia, and acute leukemia remains to be determined.

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AQUIRED APLASTIC ANEMIA (AA) is characterized by pancytopenia and hypocellularity of the bone marrow (BM). On the basis of BM cellularity and the degree of anemia, neutropenia, and thrombocytopenia, the International Aplastic Anemia Study Group has defined criteria to distinguish severe AA (SAA) and nonsevere AA. Immunosuppressive therapy with antithymocyte globulin (ATG) as a single agent or in combination with corticosteroids or androgens has greatly improved the prognosis of patients with (S)AA. The majority of patients respond to ATG treatment and become transfusion independent. Those with no or a minimal response may also survive for a prolonged period of time due to improved supportive care. Despite obtaining a clinical response, most patients show residual cytopenia or macrocytosis, suggesting that the underlying disease has not been cured.

The relation between AA and disorders that are known to exhibit clonal hematopoiesis, like paroxysmal nocturnal hemoglobinuria (PNH), myelodysplastic syndromes (MDS), and acute myelogenous leukemia (AML), has long been recognized. Tichelli et al reported the development of clonal disease after ATG treatment in 20 of 103 patients with a median follow-up of 40 months (range, 0 to 133). The probability of developing these disorders increased with longer follow-up and was 57% at 8 years. In a survey of 468 patients with SAA, a somewhat lower incidence has been reported by the Severe Aplastic Anemia Working Party of the European Cooperative Group for Bone Marrow Transplantation. In 209 long-term survivors after immunosuppressive treatment, they found an actuarial risk of developing PNH or MDS/AML of 13% and 15%, respectively, after 7 years of follow-up. Similarly, De Planque et al described the evolution of MDS/AML in 5 of 38 adult patients with a follow-up of at least 2 years after ATG treatment.

Despite the well-known association between AA and disorders with clonal hematopoiesis, there are no reports on clonality of hematopoiesis in patients with AA. In the present study, we address this issue by studying the pattern of X-chromosome inactivation using X-linked restriction fragment length polymorphisms (RFLPs) in female patients with AA. It was found that patients with AA may exhibit clonal or polyclonal hematopoiesis. No relation was observed between the presence of either a clonal or a polyclonal hematopoiesis and the response to ATG treatment.

PATIENTS AND METHODS

Patients. Nineteen female patients (median age, 29 years; range, 13 to 68 years) with an initial diagnosis of acquired AA, according to the criteria of the International Aplastic Anemia Study Group, were studied (Table 1). Sixteen patients met the criteria for SAA, and three had nonsevere AA. One patient (UPN 13) has developed PNH, in all of the other patients there were no signs of myelodysplasia or hemolysis. Cytogenetic analysis of BM cells was performed at diagnosis in 13 and during follow-up in five patients. Normal karyograms were found in 15 patients and no analyzable metaphases in two. In one patient (UPN 4) an extra ‘marker’ chromosome was found in 4 of 20 metaphases. In three patients, a relation with drugs was suspected, while in one patient SAA occurred during pregnancy. Four patients were untreated, two of these received ATG after this study, one had nonsevere AA for which ATG was not indicated, and one patient showed spontaneous recovery. The other 15 patients received a 5-day course of ATG in combination with high-dose methylprednisolone (20 mg/kg body weight intravenously [IV]). Fourteen patients were treated with equine ATG (Merieux, Lyon, France or Upjohn, Kalamazoo, MI), and one patient with rabbit ATG (RIVM, Bilthoven, The Netherlands) because of allergy to equine ATG. Four patients who were irresponsible to or relapsed after treatment with equine ATG received a second course with rabbit ATG. One patient received a second course of ATG after a relapse after ATG.

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patient received an infusion of haplo-identical BM from her brother concomitantly with ATG treatment.

Definition of response to ATG treatment. The clinical response to ATG treatment was defined as: no response (NR), continuing to fulfill the criteria of (S)AA; partial response (PR), improvement in at least one affected cell lineage; complete response (CR), achieving normal hemoglobin (Hb) concentration, neutrophil count \( \geq 1.0 \times 10^9/L \), and platelet count \( \geq 100 \times 10^9/L \).

Cell separation. Purified cell suspensions for clonal analysis were obtained from seven patients. Leukapheresis of 500 mL blood was performed and purified suspensions of polymorphonuclear cells (PMN) were obtained by Ficoll-Isopaque density gradient separation (1.077 g/cm\(^3\), 1,000g 20 minutes, 20°C). The erythrocytes were lysed by NH\(_4\)Cl. The low-density cells were further separated into fractions of lymphocytes and monocytes by counterflow centrifugation.\(^1\) Purified T cells were obtained by being rosetted with AET-pretreated sheep erythrocytes and by sedimentation of the rosetted cells through Ficoll-Isopaque.\(^2\) The purity of the PMN was determined by May-Grünewald-Giemsa staining, of the lymphoid cells by indirect immunofluorescence microscopy using the monoclonal antibodies anti-CD3 (Leu-4; Becton Dickinson, Mountain View, CA) and anti-CD19 (B4; Central Laboratory of the Dutch Red Cross, Amsterdam, The Netherlands) and fluorescein isothiocyanate (FITC)-labeled goat antimouse Ig antibodies (Nordic Immunological Laboratories, Tilburg, The Netherlands). The purity of the monocytes was determined by nonspecific esterase staining.

Southern blot analysis. High-molecular weight DNA was prepared from peripheral blood (PB) and BM cells and from the purified cell suspensions by sodium dodecyl sulfate (SDS)-pronase digestion and subsequent ethanol precipitation. Clonal analysis was performed with the probes derived from the X-linked genes phosphoglycerate kinase (PGK\(_1\)) and hypoxanthine phosphoribosyltransferase (HPRT) as described by Vogelstein et al.\(^3\) and with the M27B probe as recently described by Abrahamson et al.\(^4\) Briefly, to screen for heterozygosity for the BstXI RFLP of the PGK\(_1\) probe, 5 \(\mu\)g of DNA was digested with the restriction endonuclease \(\text{Pst I} \) and \(\text{BstXI} \) yielding two fragments of 1.05 and 0.9 kb in informative females. For clonal analysis, 12 \(\mu\)g of \(\text{Pst I} \) and \(\text{BstXI} \)-digested DNA was divided into two aliquots of which one aliquot was further digested with the methylation sensitive enzyme \(\text{Hpa II} \). Clonal analysis by the HPRT probe was performed similarly, except that the \(\text{BamHI} \) RFLP was detected by \(\text{BamHI} \) and \(\text{Pvu II} \), showing two fragments of 18 and 12 kb, respectively. The polymorphism for the M27B probe was detected by digestion with \(\text{Pst I} \). In heterozygous patients, \(\text{Pst I} \)-digested DNA was divided into three aliquots for clonal analysis. Of these, one aliquot was digested with \(\text{Hpa II} \) and one with the restriction enzyme \(\text{Msp I} \). The latter enzyme recognizes the same restriction sites as \(\text{Hpa II} \), but is not sensitive to methylation. All restriction enzymes were purchased from Promega (Madison, WI). Electrophoresis of DNA samples was performed overnight on 1.0% (PGK\(_1\), and M27B) or 0.8% (HPRT) agarose gels, followed by alkaline transfer to Hybond-N filters (Amersham International, Amersham, UK). The filters were hybridized after UV crosslinking, at 65°C overnight in a hybridization mixture consisting of 50 mmol/L Tris-HCl, pH 7.6, 10% dextrane sulphate, 1% SDS, and 100 \(\mu\)g/L denatured herring sperm DNA, and subsequently washed in 2X SSC (1X SSC: 0.15 mmol/L NaCl, 0.015 mmol/L sodium citrate, 1 mmol/L Tris-HCl, pH 7.5), 1% SDS at 68°C for 30 minutes. When necessary, additional washes in 1X, 0.5X, or 0.1X SSC, 1% SDS at 68°C were performed. Filters were exposed to X-ray films (Fuji, Tokyo, Japan) at −80°C using intensifying screens.

Probes. The probe used for analysis of the PGK\(_1\), gene was obtained by amplification with the polymerase chain reaction (PCR) using a 5' coding primer (TGT TCC GAT TCC TGC AAG CC) and a 3' antisense primer (GGA AAA TCG GCC TAG AAA CC). This process generated a 333-bp genomic fragment containing the first exon of the gene. For analysis of the HPRT gene, the plasmid pPB 1.7 was used, containing a 1.7-kb \(\text{BamHI/Pst I} \) fragment from the 5' region of the HPRT gene.\(^5\) The M27B probe (kindly provided by Dr I. Craig from Oxford, UK) is located at Xcen-p11.22 (DXS255) and recognizes multiallelic variation due to a variable number of tandem repeats (VNTR). The probes were labeled using a random primer labeling kit (Boehringer, Mannheim, Germany) and purified by elution over sephadex G50.
Detection of N-ras mutations. To detect point mutations of the N-ras protooncogene, segments containing the codons 12, 13, and 61 were amplified by PCR and subsequently hybridized with mutation-specific oligomers as described before (Van Kamp et al, submitted). Briefly, 0.2 μg DNA was added to 90 μL of PCR-buffer containing 25 pmol of each of the amplimers localized upstream and downstream of either the codons 12 and 13, or codon 61. After denaturation at 95°C, PCR was initiated by the addition of 2.5 U Taq polymerase (Cetus, Emeryville, CA) and 2.5 mmol/L of each deoxynucleotide triphosphate in 10 μL buffer. The amplification consisted of 33 cycles of denaturation (1 minute, 95°C), annealing (1.5 minutes, 55°C), and chain elongation (1.5 minutes, 72°C) using a thermocycler (Bio-med, Theres, Germany). Aliquots of 2.5 μL of the in vitro amplified DNA were spotted onto Nylon filters (Gene Screen Plus; Du Pont, Boston, MA) and hybridized to 100 pg of a γ32P-labeled oligonucleotide probe after crosslinking by illumina-crosslinking with an α254-nm UV lamp (1.6 kJ/m²). The probes, all 20-mers, represent the normal codons as well as all possible mutations (17 in total) that have been shown to activate the N-ras gene. After extensive washing, the filters were exposed to X-ray films at −80°C for 16 hours using intensifying screens.

RESULTS

Southern blot analysis was performed on DNA derived from PB and BM cells. A polymorphism for at least one of the three probes was found in 18 of 19 (95%) patients. The probes for the X-linked genes PGK, and HPRT showed a relatively low heterozygosity rate: 8 of 19 (42%) and 2 of 19 (11%), respectively. In contrast, the M27P probe was informative in 16 of 19 (84%) patients studied. The characteristics of the patients at the time of clonal analysis are given in Table 1.

In 13 of 18 patients (72%) a monoclonal pattern was found (Table 2). Purified cell suspensions of PMN, lymphocytes, and monocytes were prepared from PB cells obtained from four of these patients. A clonal pattern was found in both myeloid and lymphoid cells. The remaining five patients exhibited polyclonal hematopoiesis. Purified cell suspensions were also obtained from three of these patients, showing a polyclonal pattern in both myeloid and lymphoid cells. Five patients were analyzed with two probes (PGK, or M27β), yielding concordant results in all cases. Representative Southern blots of individuals showing a monoclonal or a polyclonal pattern, after hybridization with the PGK, or M27β probe, are presented in Figs 1 and 2, respectively.

One patient (UPN 4) showed a spontaneous recovery of a previously diagnosed SAA. Clonal analysis at diagnosis showed a monoclonal pattern in PB leukocytes of both myeloid and lymphoid origin and in BM cells. Karyotyping of BM cells performed at the same time showed the presence of an extra ‘marker’ chromosome in 4 of 20 metaphases analyzed. Despite complete recovery of hematologic parameters (Hb 9.5 mmol/L; neutrophils 1.1 × 10⁹/L; platelets 152 × 10⁹/L) and a normal karyogram, 20 months after diagnosis, a BM biopsy exhibited marked hypcellularity, and analysis of PB leukocytes and BM cells still showed clonal hematopoiesis.

All 19 female patients and nine additional male patients were screened for the presence of activating N-ras point mutations in codons 12, 13, or 61. No specific signals could be detected after PCR amplification and dot-blot hybridization with specific oligonucleotide probes, whereas the three corresponding wild-type signals were positive in all cases.

DISCUSSION

This study shows that the majority of patients (72%) with an initial diagnosis of acquired AA exhibit clonal hematopoiesis...
Monoclonality was found in cells from both myeloid and lymphoid origin, indicating the involvement of an early stem cell. The four patients who were tested before treatment all showed a clonal hematopoiesis. No relationship was observed between the existence of either a clonal or a polyclonal hematopoiesis and the response to ATG treatment. Previously, Abkowitz et al. have reported clonal hematopoiesis in a 14-year-old girl with a 2-year history of pancytopenia by showing a nonrandom distribution of glucose-6-phosphate dehydrogenase (G-6-PD) isoenzymes. In contrast to the present study, circulating lymphocytes were not of clonal origin in the latter case. This discrepancy could be due to the fact that lymphocytes are long-living cells that may originate from cells not yet involved in the disease process. All four patients with clonal hematopoiesis who were presently studied also exhibited monoclonal lymphocytopoiesis. Three of them had been treated with ATG and had a long follow-up afterwards (52 to 78 months); the other patient was not treated with ATG, but was known for 50 months with neutropenia before AA was diagnosed and clonal analysis was performed.

The probes for the X-linked genes PGK, and HPRT showed a relatively low heterozygosity rate. The recently described probe M27β, however, detects multiallelic variation due to the presence of a variable number of tandem repeats, and is highly informative. Indeed, clonal analysis could be performed in almost all (95%) of the female patients. In the five cases in which two independent probes (PGK, and M27β) were informative, the patterns observed with both probes yielded concordant results. No other markers for clonality could be applied; only one patient showed an extra marker chromosome that was not found at

![Fig 1. Southern blots of four patients showing a monoclonal (UPNs 6, 7, and 8) and a polyclonal (UPN 17) pattern in PB leukocytes (PBL), PMN, lymphocytes (LY), T cells (T), and monocytes (MO) after hybridization with the PGK, probe. Lane 1 shows the BstXI polymorphism; lane 2 shows the results of subsequent Hpa II digestion. The length of the fragments is given in kilobases.](image)

![Fig 2. Southern blots of six individuals after hybridization with the M27β probe. The Pst I polymorphism is shown in lane 2; lanes 1 and 3 show the results of subsequent Msp I and Hpa II digestion, respectively. The length of the fragments varies between 3.0 and 6.0 kb. UPNs 4, 6, 7, 9, and 10 show a monoclonal pattern (only one of the alleles is digested by Hpa II, indicating full methylation of the other allele) and UPN 15 shows a polyclonal pattern (both alleles are digested by Hpa II) in PB leukocytes. Clonality of PBL of UPNs 6 and 8 have also been shown using the PGK, probe.](image)
repeated examination after spontaneous recovery. In none of the patients could a point mutation of the N-ras proto-oncogene be detected.

In normal female tissue, the ratio of methylated maternal to methylated paternal X-chromosomes is approximately 1:1.17 It has been reported, however, that in some females an extreme lyonization can be observed with ratios exceeding 4:1.11,17 In the present study, no control DNA of unaffected tissue was analyzed. Yet, it is highly unlikely that our results represent extreme lyonization rather than true clonal hematopoiesis. Firstly, in all patients the ratio between the signal intensity of the two polymorphic bands obtained after Hpa II digestion exceeded 10:1. Secondly, the incidence of clonal disease was 72% (13 of 18). This rate is much higher than the calculated incidence of extreme lyonization, approximately 4%.11,17 Thus, our results indicate true clonal hematopoiesis in the majority of patients.

Clonal hematopoiesis in patients with AA may represent the first step in the development of a neoplastic disorder that requires additional events for the transition to MDS or AML. This finding is in accordance with the hypothesis of a multistep pathogenesis of leukemia.18 In this respect, AA may resemble MDS. In our patients, no signs of myelodysplasia were present. Also, no point mutations of the N-ras proto-oncogene, occurring in 6% to 40% of MDS patients,19 could be detected. These data support the notion that our patients were not suffering from hypoplasto myelodysplasia. Clonal hematopoiesis in patients with a PR or CR to ATG treatment may also represent a clonal remission. Similar clonal remissions have been described in patients with AML after obtaining a CR after polychemotherapy.20-22

Alternatively, a monoclonal hematopoiesis in AA may be the result of depletion of stem cells, leaving only a few residual stem cells to maintain hematopoiesis. In accordance, Abkowitz et al22 showed a nonrandom distribution of G-6-PD isoenzymes in cats after treatment with dimethyl-busulphan, suggesting that a monoclonal pattern may also reflect clonal succession from a shrunken stem cell pool. Further, in irradiated mice transplanted with genetically identical BM cells, hematopoiesis has been shown to develop from a single or a few pluripotent donor stem cells.23 Clonal hematopoiesis of donor origin has also been observed in recipients of allogeneic BM transplantations, which suggests that a small number of stem cells can maintain hematopoiesis in humans.24 Whether these patients may convert to polyclonal hematopoiesis after a longer follow-up is still unknown. Similarly, it may be possible that AA patients with a clonal hematopoiesis at diagnosis may exhibit a polyclonal hematopoiesis after obtaining a response to ATG treatment. To resolve this issue, we recently started a longitudinal study on clonality of hematopoiesis in patients with newly diagnosed AA.

The prognostic value of the existence of a clonal hematopoiesis in patients with AA has not yet been established. It is unclear whether these patients have a higher chance of developing PNH or MDS/AML than those who exhibit a polyclonal hematopoiesis. Only one of the 13 patients with a monoclonal pattern showed evolution towards PNH. In an attempt to predict which patients will show a clonal evolution, Fohlmeister et al24 have made a subdivision of AA on the basis of the presence of dysplastic features of the BM. Thus, a differentiation was made between patients with 'true' AA having a low risk and those with hypocellular MDS having a high risk of clonal evolution. Appelbaum et al25 reported cytogenetic abnormalities in 7 of 176 patients with SAA. These patients had a higher risk of developing MDS or AML than those without cytogenetic abnormalities. Although these results support the notion that patients with a clonal pattern are likely to develop PNH or MDS/AML, long-term longitudinal studies are required to resolve this issue.

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