The Problem of Clonality in Aplastic Anemia: Dr Dameshek’s Riddle, Restated

By Neal S. Young

TWO-FIFTY YEARS AGO, William Dameshek, the founder of this journal and one of the most creative minds in American hematology, raised a provocative question: what do aplastic anemia (AA), paroxysmal nocturnal hemoglobinuria (PNH), and acute leukemia have in common? His question was prompted by three observations: (1) the frequency of development of PNH in his own patients with AA; (2) the overlap between the syndromes of aplasia and PNH; and (3) the similar high prevalence of both AA and PNH in the Orient. Dameshek speculated that PNH erythropoiesis was “ecologically advantageous,” and, furthermore, that both AA and PNH might represent different responses to marrow insults (of which he listed chemicals, ionizing radiation, and viruses). In the intervening decades since Dameshek’s editorial, the prospect of survival for patients with AA has been greatly improved; we have a much better understanding of the pathophysiology of AA at the cellular level, of PNH biochemically, and of the myelodysplasia syndromes genetically. Hematopoietic cell clonality can be measured in clinical specimens and after sophisticated manipulations in animal experiments. Provisional solutions to Dameshek’s riddle may be suggested, with some temerity, in light of these new data.

CLONAL HEMATOPOIESIS IN APLASTIC ANEMIA

Clonal hematopoiesis has been related to AA by three important clinical observations, listed here chronologically. 

PNH/aplasia syndrome. First, there are patients, whose bone marrow failure syndrome is difficult to classify, in whom there are features of classical aplasia—diminished marrow cellularity—and evidence of clonal hematopoiesis; these include especially the AA/PNH syndrome and also hypoplastic myelodysplasia. Lewis and Dacie described a positive Ham test in 7 of 46 of their AA patients, and 15 of 60 patients with PNH developed AA during their course.1 In a recently published Duke series of younger patients with PNH, the rate of AA was even higher, 58%.2 The acid or sucrose hemolysis test can be positive on presentation or during the course of otherwise typical idiopathic AA and in patients with drug3 or benzene-associated4 AA. The finding of a positive Hams test in a pancytopenic patient or marrow acellularity in a patient with a positive Hams test can present considerable diagnostic confusion. PNH, like AA, is also relatively common in the Far East, where, as in younger American patients, it is more usually associated with AA and less so with thromboses.6

Late clonal disease in AA. Second, long-term observations by European investigators of patients seemingly cured of their aplasia by antilymphocyte globulin show that many appear to evolve to clonal hematopoiesis and clonal hematologic disease. In a series of 103 patients treated by immunosuppression in Basel, 13 developed PNH and 8 developed myelodysplasia or acute leukemia; the actuarial risk of PNH was estimated at 57% at 8 years.7 Of 223 long-term survivors after immunosuppression followed-up by the European Cooperative Group for Bone Marrow Transplantation, 19 developed PNH (13% risk at 7 years), 11 had myelodysplasia, and 5 of them later manifested acute myelogenous leukemia (combined risk, 15% at 7 years).8 Myelodysplasia after aplasia may have a particularly high risk of leukemic transformation.9

Clonality on presentation. Third, as reported recently in these pages, some proportion of patients with a diagnosis of AA show evidence of clonal hematopoiesis on molecular analysis. One older woman of 7 aplastic cases in the recently published English study showed a monoclonal pattern with the M27B probe against whole blood and granulocytes, a proportion not significantly different from normal.10 In contrast, in a Dutch study of aplastic anemia using three X-linked probes (hypoxanthine phosphoribosyltransferase [HPRT], phosphoglycerate kinase [PGK], and M27β), 13 of 19 (72%) showed a monoclonal pattern, including 4 of 4 cases on presentation.11 Patients with monoclonal patterns of hematopoiesis could respond to antithymocyte globulin (ATG) and one even recovered spontaneously! (The reason for the difference in results between the two studies is not apparent; most of the Dutch cases were informative for the M27B probe alone, which for technical reasons might lead to overestimation of monoclonality, as described below.)

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Clonality for hematologists implies malignant or premalignant disease, as in the acute and chronic myelogenous leukemias, lymphoma, and myelodysplasia. The observation of clonal hematopoiesis in classical AA has suggested to some observers that AA is also fundamentally a premalignant disorder and that immunosuppressive therapy only postpones its inevitable progression. Indeed, before the dramatic improvement in survival after immunosuppression, leukemia was an unusual complication of AA, estimated at less than 1% and sufficiently rare to have warranted case reports of its occurrence after androgen therapy, marrow transplantation, and immunosuppression; less than 2% of patients with acute myelogenous leukemia have a history of aplastic anemia. However, the laboratory and clinical evidence that AA is an autoimmune disease are very strong; a majority of patients, perhaps more than 70%, respond to aggressive treatment that suppresses the immune system. Even more striking is that patients with hypoplastic myelodysplasia can respond to immunosuppressive therapy (see below). How can clonality be interpreted in the context of immunologically mediated marrow disease?

LABORATORY DETERMINATION OF CLONALITY

Current clonality techniques rely on the natural mosaicism of women for X-chromosome gene expression, due to the inactivation of most of one sex chromosome at an early stage of embryogenesis. Which X-chromosome is inactivated is randomly determined in the embryonic progenitor cell and then well preserved among its progeny that ultimately constitute an organ like the bone marrow. Fialkow first used polymorphism at the glucose-6-phosphate dehydrogenase (G6PD) allele to study blood cells from women heterozygous for enzyme types A and B, which are easily distinguished by electrophoresis. For example, Oni et al first demonstrated that PNH was a clonal disease in an African woman. Their red blood cells showed the expected mixture of A and B G6PD types, but complement lysis of the PNH erythrocytes released only G6PD type B. Similarly, in a G6PD heterozygous patient with myelodysplasia, skin and T cells showed both isozyme types while erythrocytes, platelets, granulocytes, and B cells all contained type B only, showing clonality at the level of a stem cell for hematopoietic and B lymphocytes.

An obvious limitation of G6PD expression analysis—the limited numbers of heterozygotes available—was overcome by the application of restriction enzyme digestion to X-chromosome DNA. Restriction enzymes cleave DNA into smaller fragments at specific nucleotide sequences; differences in the length of fragments of DNA can occur because some base substitutions alter the recognition sequence for an enzyme. Polymorphisms have been identified in X-chromosome genes like HPRT and PGK for which specific probes are available. To exploit polymorphisms for clonality studies, DNA is first digested with an enzyme to distinguish if the paternal and maternal X-chromosomes differ in fragment size of the gene. X-chromosome inactivation is usually accompanied by methylation of cytosine residues, and if two fragment length polymorphisms are present, the inactivated fragment can be distinguished by a second digestion with an enzyme sensitive to the presence of methyl groups such as Hpa II. In tissue of polyclonal origin, Southern analysis will show nearly equal quantities of both sizes of restriction fragments after this two-step digestion process, indicating derivation from a population of cells in which both X-chromosomes are active; if the tissue is monoclonal in origin, only a single restriction fragment length will be detected. Restriction fragment length polymorphism analysis can be applied to the majority of females, especially if combinations of probes are used: on HPRT analysis 29% of females are heterozygous; for PGK, 33%; and for two sequences called the variable copy number tandem repeat, characterized by mutiallelic variation and detected using a probe called M27B, the heterozygosity rate is greater than 80%. Restriction fragment length polymorphism analysis of myelodysplasia has demonstrated monoclonality in virtually every case.

Despite its inherent logic and simplicity, this method is prone to certain errors. Monoclonality may be missed because of contamination with uninvolved cells or tissue. Not all base substitutions are neutral in their effect on the cell, and cells expressing some alleles may face a selective disadvantage, resulting in pseudoclonality, as in HPRT deficiency, G6PD deficiency, and Wiskott-Aldrich syndrome. The pattern of methylation may be aberrant and not strictly dependent on X-chromosome inactivation, as occurs in certain tumors or with certain probes (like the pXUT23-2.1 marker or the M27B probe, which are not capable of distinguishing all active from inactive X-chromosomes). Aberrant results in the experimental tissue may become apparent on comparison with uninvolved tissue, like skin, but often the controls for putative clonally derived tissue is the same type of tissue obtained from normal donors.

Clonality of hematopoietic tissue is detected in some proportion of normal persons, perhaps a significant proportion: 6 of 42 (14%) by G6PD analysis, 3 of 81 (4%) to 15 of 65 (23%) by HPRT or PGK fragment polymorphisms; 3 of 18 (17%) by M27B analysis. Are the cells of these individuals truly monoclonal in a pathologic sense, or do they represent random chance selection of a limited number of clones, or is selection operating on X-chromosome expression? The results stress the importance of comparing assays on suspect monoclonal tissue in disease with analysis of the same tissue in normal populations and of uninvolved tissue in the same subject.

CLONALITY AS CONSEQUENCE: THE LIMITED STEM CELL POOL

Clonal succession. Regulation of the limited number of primitive stem cells is required to avoid depletion of the compartment over the life span of the animal. Kay suggested in 1965 that variation over time in the entry of primitive stem cells into mitosis could effect an ordered release of mature differentiated cells, a process termed clonal succession. The clonal succession model predicted that a very small percentage of primitive stem cells would be in mitosis at any one time, and that individual stem cells,
Some possible mechanisms of marrow failure resulting in clonal hematopoiesis. The stem cell compartment is represented as a triangle. Generational age is indicated by the narrow arrow and efflux to the committed progenitor pool by the broad arrow. The triangular model of the stem cell pool emphasizes the hierarchical nature of the compartment. The most primitive cells, shown at the apex, give rise by mitosis to the most mature stem cells, seen at the base; clones are denoted by colors. More mature cells have a greater probability of leaving the compartment to terminally differentiate because they are more numerous (a stochastic process) and because they are altered as a result of repeated cell division (a determinative process). As described in the text, clonality might be the result of a shrunken stem cell compartment with loss of a large number of stem cell clones or aborted maturation of primitive stem cells within the compartment. With immune attack on the marrow, stem cells normally lacking a ligand for cytotoxic lymphocytes might be selected, resulting in PNH or a positive Ham's test in AA. Finally, and most speculatively, under some circumstances cytotoxic lymphocyte (CTL) attack may induce genetic damage in the target cell, manifested as altered cell proliferation associated with chromosomal deletions, and the clinical diagnosis of myelodysplasia (MDS); a similar process presumably occurs after irradiation or alkylating drugs, with early stem cell death (aplasia) followed by late unveiling of accumulated mutational damage (dysplasia, leukemia).
clonality in patients with AA. In steady state normal, unstressed hematopoiesis, a larger number of stem cell clones, probably several hundred in humans, would support blood cell production. Nonetheless, these cells still would represent only a small proportion of the total primitive stem cell compartment.

**Serial transplantation and loss of self-renewal.** Another and related paradox is the difference in stem cell self-renewal inferred from observations of marrow physiology compared with experimental repopulation. Only a small proportion of stem cells needs to be active because a repopulating cell has an enormous proliferative capacity. All the experiments cited above agree that a single stem cell can support hematopoiesis in a mouse for several months. The hierarchical structure of the stem cell compartment can be demonstrated experimentally by the enrichment for primitive stem cells produced by treatment with a cycle-active drug like 5-fluorouracil,
\[5^{14,42}\] indicating that the cells of the youngest generational age have the highest proliferative and self-renewal capacity (by implication, as stem cells pass through mitoses they lose proliferative capacity and increase in probability of terminal differentiation).

Observations of intact hematopoiesis and theoretical calculations of the number and capacity of the stem cell compartment suggest that animals are more than adequately endowed with hematopoietic stem cells of very high proliferative and self-renewal capacity. Normal hematopoiesis is maintained from relatively small numbers of repopulating cells for the life-span of the animal, and there is very little evidence for aging of hematopoiesis—indeed, elderly mice make quite as good bone marrow donors as young ones.\[43,44\] Normal stem cells serially transplanted into W/W\small{\*} animals can maintain normal blood counts through several life spans,\[45\] and marrow cells from old donors repopulate at least as well as cells from young and even fetal donors.\[46\] However, hematopoietic stress profoundly affects stem cell regulation. When spleen colonies are injected into secondary and tertiary irradiated recipients, the number and size of the new spleen colonies that form are markedly reduced.\[47,48\] In the shielded limb of an irradiated animal\[49\] or after a single bone marrow transplantation, stem cell proliferative capacity declines more than over a lifetime of normal function.\[50,51\] The same phenomenon can be observed with parabiosis instead of physical removal of donor cells, and in W/W\small{\*} mice (which are only mildly anemic) as in the irradiated host, and independently of the size of the donor inoculum.\[52\] Similar permanent loss of self-renewal capacity can be observed at the stressful initiation of a long-term bone marrow culture\[53\] and after cell irradiation in vitro.\[54\] A likely mechanism of loss of repopulating cells under hematopoietic stress may be their premature recruitment into mitotic cycle, as illustrated experimentally by the devastating effect on functional reconstituting ability of marrow cells exposed to two doses of 5-fluorouracil—the first treatment recruits a large proportion of primitive stem cells into cycle, which are made susceptible to a cycle-active agent.\[55\] The discordance between stem cell function under physiologic conditions and after serial transplantation parallels the difference in clonal recruitment when stem cell number is abundant or limited. Hematopoietic stress can apparently lead to qualitative dysregulation of stem cell function in addition to and perhaps disproportionate to quantitative stem cell number loss.

Clonality in most AA patients is probably due to a reduced and dysregulated stem cell pool (Fig 1). Under conditions of hematopoietic stress, progeny of individual or very few clones can be detected when the blood is sampled. A good control for studies of clonality in bone marrow failure diseases is patients with normal marrows who are receiving cytotoxic chemotherapy. Studies of lymphoma patients after chemotherapy have been reported in abstract: 11 of 19 showed clonal hematopoiesis when studied with the M27B probe,\[56\] results analogous to studies of G6PD expression in heterozygous cats after chemotherapy.\[57\] (In another recent British study, a high rate of clonality was observed in both postchemotherapy cases [treated for hematologic disease] and normal women.\[58\])

Serial studies of marrow failure patients might be expected to show rapid clonal succession in many severely pancytopenic patients and reversion to polyclonal hematopoiesis with hematologic recovery.

**CLONALITY AS ESCAPE: PNH?**

The concept of PNH as a clonal disorder, based on the observation of G6PD type in the affected erythrocytes of a single woman,\[21\] has since been confirmed by restriction enzyme analysis of DNA in other patients, 5 of 5 cases in the recent series from England tested with the M27B probe.\[10\] The pathophysiologic basis of PNH is now understood as a defect in a class of membrane proteins that attach to the cell surface by a glycolipid anchor, a phosphatidylinositol moiety that is introduced into the lipid portion of the membrane by the fatty acids of diacyl glycerol.\[59\] Failure to express glycolipid bound proteins explains the abnormal complement sensitivity of PNH because several complement-inactivating proteins are phosphoinositol linked to the erythrocyte surface membrane.

Hematopoietic progenitor number is severely decreased in patients with cytopenias and PNH, even when the marrow is cellular.\[26,59\] Yet, normal syngeneic cells eclipse defective cells in twin transplantation.\[60\] What membrane defect could explain marrow progenitor hypoproliferation? None of the phosphoinositol-linked proteins identified so far are related to hematopoietic growth factors, their receptors, or general regulators of cell proliferation. However, several molecules important in the immune system proteins are affected by the PNH defect: the type III Fc receptor,\[61\] monocyte antigen CD14, of unknown function,\[62\] and lymphocyte function-associated antigen-3 (LFA-3), which is the ligand for the T-cell glycoprotein CD2.\[63\] LFA-3 is of special interest because it mediates adhesion of cytotoxic lymphocytes to target cells.\[64,65\]

Absence of a recognition site for cytotoxic lymphocytes would certainly provide a growth advantage in a hematopoietic system under immune system attack and allow emergence of an ordinarily handicapped type of cell (Fig 1).
Somatic mutations of the phosphoinositol-linkage pathway are probably common and relatively benign, as a small percentage of PNH cells (about 1%) can be detected in some normal individuals. Studies of fractionated erythrocytes\(^*\) and progenitor cell cultures\(^*\) from patients with AA show that complement-sensitive cells are increased in a high proportion of cases of otherwise typical AA on presentation. Rotoli and Luzzatto hypothesized that marrow failure was primary to both aplasia and PNH, the degree of selection of PNH clones in bone marrow failure determining the disease' clinical appearance as pure aplasia (no clones emerge), the mixed syndrome (some clones with limited proliferative capacity emerge), or typical PNH (multiple clones or high proliferative capacity emerge).\(^*\)

Aplastic patients with positive Ham's tests can respond to immunosuppressive therapy like antithymocyte globulin and cyclosporin.\(^*\) The relative balance among T-cell attack, LFA-3 loss, and selection of PNH clones would determine the clinical character of a fundamentally immune-mediated disease. In addition, the absence of LFA-3 on the stem cell surface would also explain the susceptibility of these cells to leukemic transformation because of loss of immune surveillance!

**CLONALITY AS ETIOLOGY: MYELODYSPLASIA?**

Clonality has been implicated as the seminal event in AA.\(^*\) But hypocellular myelodysplasia is even more problematic. How can a clone with a growth disadvantage dominate the marrow?

**Misdiagnosis?** In contrast to myelodysplasia and myelogenous leukemia, cytogenetic abnormalities are rare in acquired AA. In one large series, chromosomal abnormalities were found in only 4% of 183 cases, and those seen—deletions in chromosomes 5, monosity 7, and trisomy 8—are so frequently observed in dysplasia as to suggest misdiagnosis of hypoplastic myelodysplasia.\(^*\) Similarly, in one patient with pancytopenia and clonal hematopoiesis, as determined by G6PD analysis, multiple physical anomalies, only moderate blood count depression, and preserved erythropoiesis in the bone marrow suggest a case not typical of AA.\(^*\)

An inhibitory stem cell clone? Many molecules normally produced in the marrow, like transforming growth factor-β (TGF-β),\(^*\) inhibit,\(^*\) macrophage inflammatory protein/stem cell inhibitor,\(^*\) or macrophage colony-stimulating factor (M-CSF),\(^*\) or present there in pathologic states, like γ-interferon\(^*\) and tumor necrosis factor-α,\(^*\) can suppress hematopoiesis. These cytokines are assumed to play homeostatic regulatory roles or to suppress marrow in disease. Inhibitory activity for in vitro hematopoiesis has been reported in leukemia\(^*\) and myelodysplasia.\(^*\) However, it is not only difficult to conceive of a stem cell with a growth disadvantage and inhibitory activity dominating bone marrow, but there is no evidence that inhibitory molecules are specifically produced by hematopoietic cells or of hematopoietic cells with inhibitory activity for their neighbors' proliferation in hypocellular states.

**A common insult for aplasia and myelodysplasia?** Early marrow failure and late clonal disease do occur in two well-defined syndromes, irradiation injury and Fanconi's anemia, marked by the introduction of DNA damage in the first and inadequate repair of damaged DNA in the second. In both cases, pancytopenia due to AA is an early event and leukemia occurs much later; global stem cell destruction is the result of massive disruption of the dividing cell's genetic integrity and abnormal proliferation of a single clone is the result of accumulated mutational events.

Stem cell destruction from irradiation is an example of apoptosis. Apoptosis, or programmed cell death, is distinguished from accidental cell death by specific histologic\(^*\) and functional\(^*\) features. In necrosis, cells and their cytoplasmic organelles swell, release their contents, and elicit a local inflammatory response. In apoptosis, chromatin aggregation, cellular volume loss, and the formation of membrane-bound apoptotic bodies are characteristic, scattered cells are pyknotic, and there is no local inflammatory response. In apoptosis, cell death originates in the nucleus and can be detected in vitro by the release of ordered nucleotide fragments; DNA fragmentation precedes cell membrane lysis, the opposite sequence from cytotoxicity as a result of membrane attack by complement, antibodies, natural killer cells, or physical disruption. DNA cleavage in apoptosis is the result of endogenous endonuclease activation, and because these enzyme pathways may not be identical in all cells, cellular damage may vary in different tissues.\(^*\) Apoptosis can be triggered by a number of stimulants, including not only irradiation\(^*\) but also hematopoietic growth factor withdrawal\(^*\) and cytotoxic lymphocyte attack.\(^*\)

Apoptosis has been hypothesized as a mechanism for the destruction of intracellular viral DNA and the prevention of its release when the infected cell is disrupted.\(^*\)

On the assumption that few biologic phenomena are absolute, some cells may survive cytotoxic lymphocyte attack and have resulting residual DNA injury (Fig 1). If true, two clinical predictions could be made. First, cytotoxic lymphocyte attack should induce dysplastic as well as aplastic marrow changes. In support of this hypothesis, variable degrees of cellular atypia are not uncommon in classical AA; some patients with myelodysplasia share immunologic abnormalities with AA and some appear to respond to immunosuppressive therapy like antithymocyte globulin\(^*\) and cyclosporin\(^*\) (and our personal experience), nor does dysplasia on presentation correlate with response to ATG therapy.\(^*\) Perhaps myelodysplasia is more likely than aplasia in the older patient with a blunted immune response. Second, late clonal abnormalities in AA, some myelodysplasia and acute leukemia, would be the result of the initial cytotoxic lymphocyte insult and induced chromosomal abnormalities. In our experience and in that of others, some patients whose relapsed pancytopenia is

\(^*\) I have also observed characteristic but transient myelodysplasia in the bone marrow of a patient treated with an experimental cardiac drug (marketed as Arkin-Z in Japan) that ordinarily produces agranulocytosis at high rate (1% to 2%); the pathologic changes resolved with discontinuation of the offending medication.
associated with marrow cellularity respond well to a second course of immunosuppression. Immune attack, like radiation exposure, would thus have a dual effect on hematopoiesis, inducing both acute marrow failure and long-term genetic injury.

IMPLICATIONS FOR THERAPY

The pessimistic tone of recent comment on AA, due to the development of late clonal disease in some patients, may be premature. Immune attack on hematopoietic stem cells may be chronic and may require more intensive initial therapy or longer duration of treatment. Immune system abnormalities of lymphocyte activation and lymphokine release often persist, despite seemingly effective immunosuppressive therapy, just as progenitor number and marrow cellularity remain depressed despite adequate blood counts. Formal trials of immunosuppression of clonal diseases, like PNH and myelodysplasia, also seem warranted. Immunosuppression is far less expensive and far less traumatic than bone marrow transplantation for AA. Even ATG, a relatively crude preparation, has produced survival results equivalent to marrow replacement in most large series. When considered as an autoimmune disease, AA's therapy compares well with that of other autoimmune diseases—rheumatoid arthritis, ulcerative colitis, iritis, type I diabetes mellitus—considering the vital importance of the organ system damaged. Newer immunosuppressives and the ability to more specifically manipulate the immune response offer promise to improve immediate hematologic responses and perhaps to eliminate late clonal disease as well.

CONCLUSION

Dameshek concluded his 1967 editorial with a recognition of the need for hard clinical categorization but an appeal for the value of a "vague" approach to thinking about pathogenesis. His final statement, "That a single 'insult' to the marrow may be responsible for bringing about different kinds of abnormalities ... deserves consideration, not only from the conceptual standpoint but from the experimental approach as well," may finally be realized in both the clinic and laboratory.

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The problem of clonality in aplastic anemia: Dr Dameshek's riddle, restated [see comments]

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