Current concepts in the pathophysiology and treatment of aplastic anemia

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Aplastic anemia, an unusual hematologic disease, is the paradigm of the human bone marrow failure syndromes. Almost universally fatal just a few decades ago, aplastic anemia can now be cured or ameliorated by stem-cell transplantation or immunosuppressive drug therapy. The pathophysiology is immune mediated in most cases, with activated type 1 cytotoxic T cells implicated. The molecular basis of the aberrant immune response and deficiencies in hematopoietic cells is now being defined genetically; examples are telomere repair gene mutations in the target cells and dysregulated T-cell activation pathways. Immunosuppression with antithymocyte globulins and cyclosporine is effective at restoring blood-cell production in the majority of patients, but relapse and especially evolution of clonal hematologic diseases remain problematic. Allogeneic stem-cell transplant from histocompatible sibling donors is curative in the great majority of young patients with severe aplastic anemia; the major challenges are extending the benefits of transplantation to patients who are older or who lack family donors. Recent results with alternative sources of stem cells and a variety of conditioning regimens to achieve their engraftment have been promising, with survival in small pediatric case series rivaling conventional transplantation results. (Blood. 2006;108:2509-2519)

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Introduction

More than 25 years have passed since our first, highly speculative review of aplastic anemia; in that fortunately obscure publication, pathophysiology was addressed tentatively and immunosuppressive therapies hardly at all. The article did reflect both the dismal prospects for patients with the severe form of marrow failure and the formidable practical difficulties of experimentation in a rare disorder in which the cells of interest had disappeared. Aplastic anemia was considered heterogenous in origin and virtually impossible to study systematically. At the bedside, the clinical emphasis was the identification of a putative causal factor—exposure to benzene or a culpable pharmaceutical—to allow classification in an otherwise doomed patient. As progenitor assays were developed, diverse factors could be held theoretically responsible for failure to form colonies in tissue culture, ranging from quantitative and qualitative defects in stem cells and blocks in differentiation to a lack of stroma support or inadequate cytokine production, or the effects of a chemical poison.

In the intervening decades, our understanding of aplastic anemia has cohered around a unified immune mechanism of hematopoietic-cell destruction, which was inferred from but also has informed effective immunosuppressive therapies for the disease (Figure 1). Technical advances in cell biology, flow cytometry, molecular biology, and immunology have provided methods to measure numbers and function of very limited numbers of cells. As a result, we have a more unified and rational view of aplastic anemia's pathophysiology; the disease is understood in its relation to other related marrow failure syndromes; and in many important respects an unusual blood syndrome can model more common autoimmune diseases of other organ systems (Figure 2). Particularly satisfying is that aplastic anemia is now amenable to cure or amelioration in most patients, based both on high-quality clinical trials and mechanistic insights from the experimental laboratory.

Our intention in this circumscribed review is to emphasize the most current aspects of aplastic anemia. As a complement, the reader is referred to monographs, textbook chapters, and other recent reviews.[1-6]

Etiologies

Clinical associations

Since Ehrlich's description of the first case of aplastic anemia in a pregnant woman,[126] precipitating factors have been sought from the individual patient's history. An enormous literature, dating from the beginning of the 20th century, described chemical- and drug-induced disease, stimulated by observations of the effects of benzene on blood counts, of dipyrone's association with agranulocytosis, and a seeming epidemic of aplastic anemia after the introduction of chloramphenicol.

These associations are worth reassessment in the context of the immune hypothesis of marrow failure. Pregnancy appears a real association, as deduced more from the documented improvement of blood counts with its termination than from formal epidemiologic study.[7] The unusual syndrome of eosinophilic fasciitis also is strongly linked to aplastic anemia. Five to 10% of cases of aplastic anemia follow an episode of seronegative hepatitis, in which immune activation is inferred from the pattern of T-cell activation, cytokine production, and HLA association.[8] Despite intensive efforts, including sophisticated molecular and immunologic approaches and animal inoculations, an infectious agent has not been

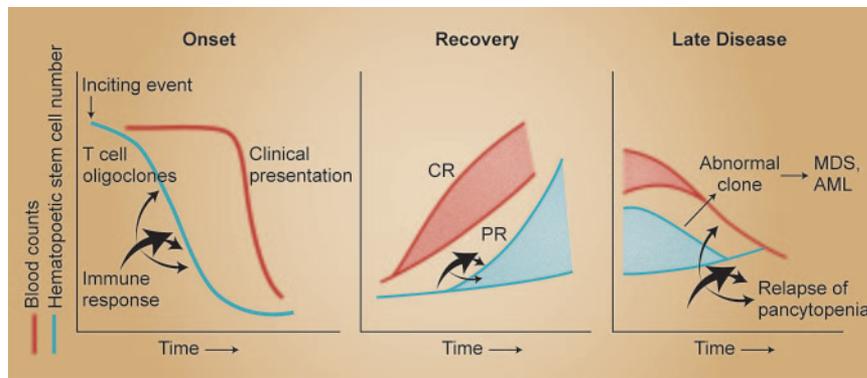© 2006 by The American Society of Hematology

Figure 1. Pathophysiology of acquired aplastic anemia. The figure stresses the crucial and related roles of the hematopoietic stem-cell compartment as a target for the immune response. An inciting event, such as a virus or medical drug, provokes an aberrant immune response, triggering oligoclonal expansion of cytotoxic T cells that destroy hematopoietic stem cells (left panel, Onset). Bone marrow transplantation or immunosuppressive therapy leads to complete response (CR) or partial response (PR) by eradicating or suppressing pathogenic T-cell clones (middle panel, Recovery). Relapse occurs with recurrence of the immune response, and the immunologically stressed and depleted stem-cell compartment also allows selection of abnormal hematopoietic clones that manifest as paroxysmal nocturnal hemoglobinuria, myelodysplasia (MDS), and occasionally acute myelogenous leukemia (AML) (right panel, Late Disease).

identified. Benzene, or more correctly its metabolites, is a marrow toxin in animals and humans, but in the West benzene exposure as an etiology of aplastic anemia is now rare. Ancient case reports and series leave doubt as to whether marrow failure in benzene workers was not often myelodysplasia rather than aplastic anemia. Additionally, benzene also has effects on immune function.[9]

Of greatest practical import is the relationship of medical drug use to aplastic anemia—unpredictable marrow failure in this setting is devastating to the patient and physician and has serious legal ramifications for pharmaceutical drug development.[10] The study of idiosyncratic drug reactions, by definition extremely rare, is difficult. That genetic differences in drug metabolism, especially in detoxification of reactive intermediate compounds, underlie susceptibility is best supported by one study of a single individual exposed to carbamazepine, published more than 20 years ago.[11] Overrepresentation of deletions in the drug-metabolizing glutathione-S-transferase genes (*GSTM1, GSTT1,* which would increase concentrations of toxic drug intermediates) has been observed in some series.[12,13] Astonishingly, no satisfactory mechanism has been developed for the most notorious pharmaceutical, chloramphenicol, or for other heavily inculpated agents such as penicillamine or gold. Many drugs on "black lists" also more commonly cause mild marrow suppression, and possibly regular but only modest destruction of marrow cells is a prerequisite for a much more infrequent immune response to an exposed neoantigen. A parallel mechanism is supported by the clinical observation of

little difference between patients with idiopathic aplastic anemia and those with an assumed drug etiology, in demographics, response to therapy, or survival.[14] In contrast, very few chemotherapeutic agents, despite being designed as cell poisons and administered in milligram or gram quantities, directly result in irreversible marrow destruction without obvious effects on other organs. Claims of permanent aplastic anemia after idiosyncratic exposure to minuscule quantities of chloramphenicol, for example (as in ophthalmic solutions), more likely reflect observation and reporting biases than a mechanism of extreme sensitivity to a hidden metabolite.

Epidemiology

Two more reliable approaches to identifying etiology are epidemiology and laboratory identification of antigens. Unfortunately, neither has yielded conclusive results. Two large, controlled, population-based studies have been conducted, the International Aplastic Anemia and Agranulocytosis Study in Europe and Israel in the 1980s[15] and the recently completed Thai NHLBI Aplastic Anemia Study in Bangkok and a northeast rural region.[16] The incidence of aplastic anemia in the West is 2/million and about 2- to 3-fold higher in Asia. Benzene and pesticides, while significantly associated, accounted for only a small number of cases in both studies, and medical drugs have a negligible role in Asia. In rural Thailand, exposure to nonbottled water, as well as to certain animals (ducks and geese), to animal fertilizer, and also to pesticides, suggested an infectious etiology.

Autoantigens

A few putative antigens have been teased from screening antibodies in patients' sera against a peptide library (by expression of genes in fetal liver or leukemic-cell lines). Kinectin, a widely expressed protein, bound to antibodies from about 40% of aplastic patients.[17] Another antigen that bound to antibodies, in a smaller minority of marrow failure patients, was diazepam-binding related protein-1, an enzyme essential in the oxidation of unsaturated fatty acids and broadly distributed in tissues.[18] The relevance of these autoantibodies to a cellular pathophysiology of aplastic anemia is unclear. For kinectin, reactive cytotoxic T cells could be generated in vitro and inhibited human hematopoietic colony formation, but antikinectin T cells were not found in patients.[17] For diazepam-binding related protein-1, a putative T-cell epitope derived from this protein could stimulate cytotoxic T cells obtained from one patient, and T-cell precursors with peptide-binding activity were present in 2 cases.
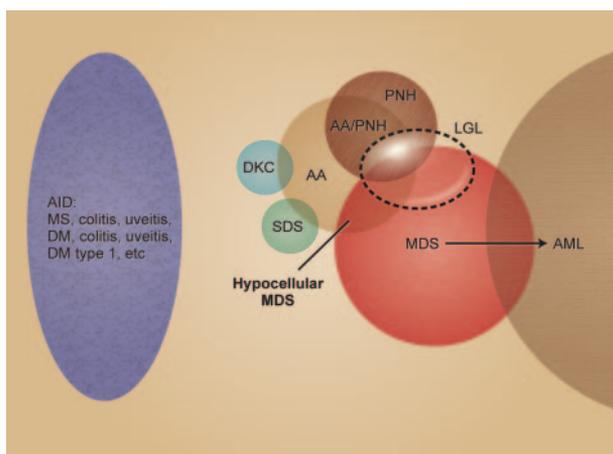

Figure 2. Venn diagram of the clinical and pathophysiologic relationships among the bone marrow failure syndromes, leukemia, and autoimmune diseases. Overlapping circles indicate difficulties in diagnostic discrimination and shared underlying mechanisms.

Pathophysiology

In most cases, aplastic anemia is an immune-mediated disease. Cellular and molecular pathways have been mapped in some detail for both effector (T lymphocyte) and target (hematopoietic stem and progenitor) cells (Figure 3). Exposure to specific environmental precipitants, diverse host genetic risk factors, and individual differences in the characteristics of the immune response likely account for the disease's infrequency, variations in its clinical behavior, and patterns of responsiveness to treatment.

### Immune-mediated T-cell destruction of marrow

An immune mechanism was inferred decades ago from the recovery of hematopoiesis in patients who failed to engraft after stem-cell transplantation, when renewal of autologous blood-cell production was credited to the conditioning regimen. Also suggestive was that the majority of syngeneic transplantations in which bone marrow was infused without conditioning failed.[19] The responsiveness of aplastic anemia to immunosuppressive therapies remains the best evidence of an underlying immune pathophysiology: the majority of patients show hematologic improvement after only transient T-cell depletion by antithymocyte globulins (ATGs); relapse also usually responds to ATG; and dependence of adequate blood counts on administration of very low doses of cyclosporine is not infrequent. As immunosuppression has intensified, from early attempts with corticosteroids to aggressive strategies such as high-dose cyclophosphamide, and the proportion of responders has risen, the willingness to ascribe an immunologic mechanism also increased. Indeed, little distinguishes responders to immunologic therapy from refractory patients (other than age, as children show higher rates of recovery and survival). A nonimmune pathophysiology has been inferred from a failure to respond to immunosuppression, but refractoriness to therapy is also consistent with very severe stem-cell depletion, a "spent" immune response, or immunologic mechanisms not susceptible to current therapies.

In early laboratory experiments, removal of lymphocytes from aplastic bone marrows improved colony numbers in tissue culture, and their addition to normal marrow inhibited hematopoiesis in vitro (reviewed in Young[20]). The effector cells were identified by immunophenotyping as activated cytotoxic T cells expressing Th1 cytokines, especially γ-interferon. CD8 cells containing intracellular interferon may now be measured directly in the circulation,[21] and oligoclonal expansion of CD8+CD28− cells, defined by (1) flow cytometric analysis for T-cell receptor (TCR) Vβ subfamilies; (2) spectratyping to detect skewing of CDR3 length; and (3) sequencing of the CDR3 region to establish a molecular clonotype.[22] In general, patients at presentation demonstrate oligoclonal expansions of a few Vβ subfamilies, which diminish or disappear with successful therapy; original clones re-emerge with relapse, sometimes accompanied by new clones, consistent with spreading of the immune response. Very occasionally, a large clone persists in remission, perhaps evidence of T-cell tolerance.

The impact of T-cell attack on marrow can be modeled in vitro and in vivo. γ-Interferon (and tumor necrosis factor-α) in increasing doses reduce numbers of human hematopoietic progenitors assayed in vitro; the cytokines efficiently induce apoptosis in CD34 target cells, at least partially through the Fas-dependent pathway of cell death.[23] In long-term culture of human bone marrow, in which stromal cells were engineered to constitutively express γ-interferon, the output of long-term culture-initiating cells (LTCI-ICs) was markedly diminished, despite low concentrations of the cytokine in the media, consistent with local amplification of toxicity in the marrow milieu.[24] Immune-mediated marrow failure has been modeled in the mouse: infusion of parental lymph node cells into F1 hybrid donors caused pancytopenia, profound marrow aplasia, and death.[25] Not only a murine version of ATG and cyclosporine but also monoclonal antibodies to γ-interferon and tumor necrosis factor abrogated hematologic disease, rescuing animals. A powerful "innocent bystander" effect, in which activated cytotoxic T cells kill genetically identical targets, was present in secondary transplantation experiments.[26] In a minor histocompatibility antigen-discordant model, marrow destruction resulted from activity of an expanded H60 antigen–specific T-cell clone.[27]

Figure 3. Immune destruction of hematopoiesis. Antigens are presented to T lymphocytes by antigen-presenting cells (APCs), which trigger T cells to activate and proliferate. T-bet, a transcription factor, binds to the interferon-γ (INF-γ) promoter region and induces gene expression. SAP binds to Fyn and modulates SLAM activity on IFN-γ expression, diminishing gene transcription. Patients with aplastic anemia show constitutive T-bet expression and low SAP levels. IFN-γ and TNF-α up-regulate other T cells' cellular receptors and also the Fas receptor. Increased production of interleukin-2 leads to polyclonal expansion of T cells. Activation of Fas receptor by the Fas ligand leads to apoptosis of target cells. Some effects of IFN-γ are mediated through interferon regulatory factor 1 (IRF-1), which inhibits the transcription of cellular genes and entry into the cell cycle. IFN-γ is a potent inducer of many cellular genes, including inducible nitric oxide synthase (NOS), and production of the toxic gas nitric oxide (NO) may further diffuse toxic effects. These events ultimately lead to reduced cell cycling and cell death by apoptosis.
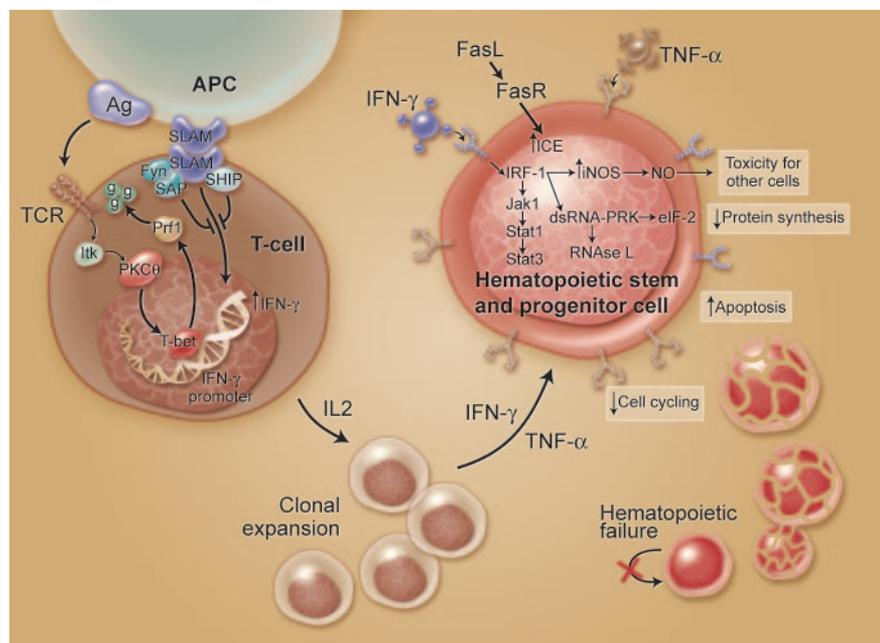
Why T cells are activated in aplastic anemia is unclear. HLA-DR2 is overrepresented among patients, suggesting a role for antigen recognition, and its presence is predictive of a better response to cyclosporine.[28,29] Polymorphisms in cytokine genes, associated with an increased immune response, also are more prevalent: a nucleotide polymorphism in the tumor necrosis factor-α *(TNF2)* promoter at −308,[30,31] homozygosity for a variable number of dinucleotide repeats in the gene encoding γ-interferon,[32] and polymorphisms in the interleukin 6 gene.[33] Constitutive expression of T-bet, a transcriptional regulator that is critical to Th1 polarization, occurs in a majority of aplastic anemia patients.[34] Mutations in *PRF1,* the gene for perforin, are responsible for some cases of familial hemophagocytosis; mutations in *SAP,* a gene encoding for a small modulator protein that inhibits γ-interferon production, underlie X-linked lymphoproliferation, a fatal illness associated with an aberrant immune response to herpesviruses and aplastic anemia. Perforin is overexpressed in aplastic marrow.[35] We have detected heterozygous mutations in *PRF1* in 5 adults with severe aplasia and hemophagocytosis of the marrow, and SAP protein levels are markedly diminished in a majority of acquired aplastic anemia cases (E. Solomou, unpublished data, June 2006). These alterations in nucleotide sequence and in gene regulation suggest a genetic basis for aberrant T-cell activation in bone marrow failure. Genome-wide transcriptional analysis of T cells from aplastic anemia patients has implicated components of innate immunity in aplastic anemia, including Toll-like receptors and natural killer cells,[36] for which there is some preliminary experimental support.[37,38]

Hematopoiesis

Immune attack leads to marrow failure. "Anhematopoiesis" was inferred from the empty appearance of the marrow at autopsy by the earliest observers of the disease. The pallor of the modern biopsy core or empty spicules of an aspirate, few or no CD34 cells on flow cytometry, and minimal numbers of colonies derived from committed progenitors in semisolid media all reflect the severe reduction in hematopoietic cells that defines the disease. Stem-cell "surrogate"—really correlative—assays, LTC-ICs,[39] or cobblestone-forming cells,[40] which measure a primitive infrequent and quiescent multipotential progenitor cell, also show marked deficiency, and from the product of the low percentage of marrow cellularity and the scant numbers of LTC-ICs per mononuclear cell, suggest that only a small percentage of residual early hematopoietic cells remains in severely affected patients at presentation. Qualititative features of these few cells, as measured, for example, by poor colony formation per CD34 cell or inadequate response to hematopoietic growth factors, are harder to interpret, although recent genetic studies have suggested explanatory mechanisms (see next paragraph). The reduced number and function of the marrow is secondary to cell destruction, and apoptosis is prevalent among the few remaining elements.[41,42] Microarray of the scant CD34 cells from marrow failure patients revealed a transcriptome in which genes involved in apoptosis, cell death, and immune regulation were up-regulated[43]; this transcriptional signature was reproduced in normal CD34 cells exposed to γ-interferon.[44]

One peculiar feature of white blood cells in aplastic anemia is short telomeres.[45,46] Telomere shortening was initially most easily blamed on stem-cell exhaustion. However, the discovery, first by linkage analysis in large pedigrees, that the X-linked form of dyskeratosis congenita was due to mutations in *DKC1* and subsequently purposeful identification of mutations in *TERC* in some autosomal dominant patients with this constitutional marrow failure syndrome indicated a genetic basis for telomere deficiency. Central to the repair machinery is an RNA template, encoded by *TERC,* on which telomerase, a reverse transcriptase encoded by *TERT,* elongates the nucleotide repeat structure; other proteins, including the *DKC1* gene product dyskerin, are associated with the telomere repair complex. Systematic surveys of DNA disclosed first *TERC*[47,48] and later *TERT* mutations[49] in some patients with apparently acquired aplastic anemia, including older adults. Family members who share the mutation, despite normal or near-normal blood counts, have hypocellular marrows, reduced CD34-cell counts and poor hematopoietic colony formation, increased hematopoietic growth factor levels, and of course short telomeres; however, their clinical presentation is much later than in typical dyskeratosis congenita, and they lack typical physical anomalies.[47,49] Chromosomes are also protected by several proteins that bind directly to telomeres, and polymorphisms in their genes *(TERF1, TERF2)* are also more or less prevalent in aplastic anemia compared with healthy controls.[50] A few of our patients also have heterozygous mutations in the Shwachman-Bodian-Diamond syndrome *(SBDS)* gene. Almost all children with this form of constitutional aplastic anemia are compound heterozygotes for mutations in *SBDS,* and their white cells have extremely short telomeres[51]; however, the *SBDS* gene product has not been directly linked to the telomere repair complex or to telomere binding. A parsimonious inference from all these data is that inherited mutations in genes that repair or protect telomeres are genetic risk factors in acquired aplastic anemia, probably because they confer a quantitatively reduced hematopoietic stem-cell compartment that may also be qualitatively inadequate to sustain immune-mediated damage.[52]

Telomeres are short in one third to one half of aplastic anemia patients,[45,46] but mutations have been identified in only less than 10% of cases. The most interesting explanation is involvement of other genes, including genes for other members of the large repair complex, telomere binding proteins, still obscure components of the alternative repair system, and some DNA helicases. Alternatively, telomere shortening may be secondary to stem-cell replication.

Clonal evolution

Clinically, aplastic anemia may coexist or appear to evolve to other hematologic diseases that are characterized by proliferation of distinctive cell clones, as in paroxysmal nocturnal hemoglobinuria (PNH) or myelodysplasia (MDS; Figure 2). The mechanisms linking immune-mediated and premalignant pathophysiologies are not elucidated in marrow failure or in parallel circumstances (chronic hepatitis and hepatocellular carcinoma, ulcerative colitis and colon cancer, and many others). The presence of tiny clones at the time of diagnosis of aplastic anemia, detected using extremely sensitive assays—phenotypic (flow cytometry for PNH) or cytogenetic (fluorescent in situ hybridization for MDS)—also creates the problems of disease classification and patient diagnosis.

PNH. Fifty percent or more of patients at presentation with pancytopenia have expanded populations of PNH cells, easily detected by flow cytometry due to the absence of glycosylphosphatidylinositol-linked membrane proteins, the result of somatic *PIG-A* gene mutations.[53] Most clones are small and do not lead to clinical manifestations of hemolysis or thrombosis, but classic PNH can be dominated by marrow failure (the "aplastic anemia/PNH syndrome"), and all PNH patients show evidence of underlying hematopoietic deficiency. The global absence of a large number of cell-surface proteins in PNH has been hypothesized to allow "escape" and survival of a pre-existing mutant clone. Association

of an expanded PNH clone with HLA-DR2[29] and with autoantibodies,[18] and as a predictor of responsiveness to immunosuppressive therapies,[54] suggests that the escape is from immune attack. However, there is little concrete experimental evidence of reproducible differences in either differential immune responsiveness or susceptibility of PNH clones compared with phenotypically normal target-cell populations.[55,56] In contrast to cells of normal phenotype, the marrow PNH clone retains its proliferative capacity in tissue culture and does not overexpress Fas[57]; comparison by microarray shows that residual cells of normal phenotype in the PNH bone marrow up-regulate the same apoptosis and cell-death genes as do CD34 cells in aplastic marrow, while the PIG-A$^-$ clone appears transcriptionally similar to CD34 cells from healthy donors.[58] Despite a few provocative studies,[37,55,59] no satisfying mechanism to explain clonal escape has convincing empiric support (see Young[53] for review).

MDS. Stereotypical patterns of aneuploidy develop in a minority of patients over time: monosomy 7 or trisomy 8 is most characteristic.[60] Trisomy 8 is MDS with many immune abnormalities that resemble aplastic anemia. Patients respond to immunosuppressive therapies, and oligoclonal T-cell expansions are usually present.[61] T-cell oligoclones appear to recognize the aneuploid cells, and specifically WT1 antigen that they express at high levels,[62] but the target cells are not killed due to up-regulation of antiapoptosis genes, including *c-myc, survivin,* and *CDK1.*[63] For trisomy 8, abnormal cells targeted by the immune system appear to be selected for their capacity to survive cytotoxic lymphocyte attack.

Monosomy 7 is also a frequent cytogenetic abnormality in aplastic anemia but has a poorer prognosis, with patients usually succumbing to refractory cytopenias or evolving to acute leukemia.[64] Emergence of monosomy 7 has been linked to exogenous use of G-CSF in aplastic anemia,[65] as occurs in treated severe congenital neutropenia. Laboratory studies of marrow from aplastic anemia and myelodysplasia patients suggest that monosomy 7 clones expand in an abnormal cytokine milieu: high G-CSF concentrations lead to selection of cells that bear a short isoform of the G-CSF receptor that signals proliferation but not differentiation.[66]

Treatment

Immunosuppression

Antithymocyte globulin (ATG) and cyclosporine (combined or intensive immunosuppression). For aplastic anemia that is severe, as defined by peripheral-blood counts, definitive therapies are immunosuppression or stem-cell transplantation; immunosuppressive therapies are most widely used because of lack of histocompatible sibling donors, patient age, and the immediate cost of transplantation. Even in responding patients, blood counts often remain below normal but adequate to avoid transfusion and to prevent infection. Most specialists use an ATG-based regimen in combination with cyclosporine, based on the outcomes of relatively large studies performed in the 1990s[67] (Table 1). The larger experience is with ATG produced in horses, although a rabbit ATG, recently approved for use in the United States, is more potent by weight and in the treatment of graft rejection after solid organ transplantations (a current NIH trial is directly comparing these 2 ATGs as first therapy in severe aplastic anemia). ATG is cytolytic: lymphocyte numbers consistently decline during the first few days of infusion and then return to pretreatment levels in a week or 2. ATGs are produced by immunizing animals against human thymocytes, not lymphocytes, and the mix of antibody specificities plus direct experimentation suggests that ATG may be immunomodulatory as well as lymphocytotoxic, perhaps producing a state of tolerance by preferential depletion of activated T cells. The toxicity of ATG is allergic, related to administration of a heterologous protein, and there is little added infection risk beyond the neutropenia intrinsic to the disease. ATG doses and regimens are empiric and traditional. By administration of a larger dose over fewer days, immune complex formation and consequent serum sickness are minimized, as patients usually do not produce their own antibodies to the foreign protein until a week or 10 days after exposure.

Cyclosporine's selective effect on T-cell function is due to direct inhibition on the expression of nuclear regulatory proteins, resulting in reduced T-cell proliferation and activation. While severe aplastic anemia can respond to cyclosporine alone, it is less effective than either ATG alone or ATG plus cyclosporine.[73,74] As with ATG, doses and length of treatment have not been formally established. Cyclosporine has many side effects, but most are manageable by dose reduction; permanent kidney damage is unusual with monitoring (to maintain blood levels at nadir of about 200 ng/mL). Maintenance of blood counts may be achieved with very low doses of cyclosporine, such that drug levels in blood are undetectable and toxicity is minimal, even with years of treatment.

Outcomes of combined immunosuppressive therapy. Reported hematologic response rates vary, at least in part due to lack of consensus on parameters (transfusion independence, absolute or relative improvement in blood counts) and defined landmarks. In our experience, improvement of blood counts so that the criteria for severity are no longer met highly correlates with termination of transfusions, freedom from neutropenic infection, and better survival. By this standard, about 60% of patients are responders at 3 or 6 months after initiation of horse ATG.[70] Comparable figures for hematologic response rates have come from Europe[69] and Japan.[71] Responders have much better survival prospects than do nonresponders. Long-term prognosis is predicted by the robustness of the early blood count response (defined as either platelets or reticulocytes $> 50 \times 10^9$/L [50 000/µL] 3 months after treatment): about

Table 1. Intensive immunosuppression (ATG plus cyclosporine) for severe aplastic anemia

Study	N	Median age, y	Response, %	Relapse, %	Clonal evolution, %	Survival, %
German[68]	84	32	65	19	8	58 at 11 y
EGMBT[69]	100	16	77	12	11	87 at 5 y
NIH[70]	122	35	61	35	11	55 at 7 y
Japan*[71]	119	9	68	22	6	88 at 3 y
NIH†[72]	104	30	62	37	9	80 at 4 y

Only studies of more than 20 enrolled patients are tabulated. Responses to immunosuppressive therapy are usually partial; blood counts may not become normal but transfusions are no longer required and the neutrophil count is adequate to prevent infection. Relapse is usually responsive to further immunosuppressive therapies. Clonal evolution is to dysplastic bone marrow changes and/or cytogenetic abnormalities. For details, see "Immunosuppression."

*With androgens and ± G-CSF.

†With mycophenolate mofetil.

50% of patients who are treated with horse ATG have a robust response and almost all of them will survive long term. Outcomes of immunosuppressive therapy are related to patient age: 5-year survival of more than 90% of children has been reported in recent German,[75] Japanese,[71] and Chinese[76] trials, compared with about 50% survival for adults older than 60 years in the collective European experience.[77]

Relapse, defined conservatively as a requirement for additional immunosuppression and not necessarily recurrent pancytopenia, is not uncommon, occurring in 30% to 40% of responding patients. Relapse defined by renewed need for transfusion was estimated at 12% of European patients at 3 years, but prolonged cyclosporine dependency among all patients was common.[69] Reinstitution of cyclosporine usually reverses declining blood counts, but when required, a second round of horse[78] or rabbit[79] ATG is usually effective. In our experience, relapse does not confer a poor prognosis, but it is obviously inconvenient and may not always be remediable. Molecular analysis of the T-cell response in aplastic anemia, discussed in "Pathophysiology," suggests that the major reason for relapse is incomplete eradication of pathogenic clones by ATG.

More serious than relapse is evolution of aplastic anemia to another clonal hematologic disease, PNH, myelodysplasia, and leukemia. Small PNH clones present at diagnosis usually remain stable over time but may expand sufficiently to produce symptomatic hemolysis. For myelodysplasia and leukemia, the cumulative long-term rate of clonal evolution is about 15%[70,80]; evolution is not inevitable in aplastic anemia, and some cytogenetic abnormalities may be transient or, as with trisomy 8, responsive to immunosuppressive treatments. As discussed in "Clonal evolution," emergence of monosomy 7 may be favored in severely neutropenic patients who require chronic G-CSF therapy.[66]

Improving on ATG and cyclosporine. *Growth factors.* Historically, intensification of immunosuppression has increased response rates. However, attempts to improve on ATG plus cyclosporine have been frustratingly disappointing. Megadoses of methylprednisolone only added toxicities. Small pilots of GM-CSF[81] and much larger, randomized studies of G-CSF[71,82] as routine additions to ATG and cyclosporine have been negative to date; improved neutrophil counts did not translate into a higher rate of recovery or even less infection. A very large ongoing European study of G-CSF should definitively answer efficacy and safety concerns.

Other immunosuppressive drugs. As the addition of cyclosporine clearly improved outcomes compared with the use of ATG alone, other immunosuppressive drugs might be predicted, based on their mode of action, animal studies, and experience in other human diseases and with organ transplantation, to be effective. Mycophenolate mofetil is a tolerizing agent, as it selectively depletes activated cells by inhibition of inosine monophosphate dehydrogenase, a critical enzyme of the purine salvage pathway, therefore blocking activated lymphocyte proliferation. Nonetheless, its addition to ATG and cyclosporine in an NIH trial of 104 patients did not change hematologic response (about 62%), relapse (37%), or evolution rates; at best, there was a modest sparing of cyclosporine usage.[72] The 4-year survival rate for all treated patients was 80%—almost certainly due to better supportive care rather than any new drug effect.

Sirolimus, which blocks the serine-threonine kinase known as mammalian target of rapamycin is synergistic with cyclosporine in tissue culture and in clinical transplantation. Again, when tested in a randomized protocol in combination with ATG and cyclosporine

in aplastic anemia, results were not superior to these agents only (P.S., unpublished data, January 2006).

Cyclophosphamide. As with ATG, recovery of blood counts can occur after a failed bone marrow transplantation preceded by conditioning with cyclophosphamide. High-dose cyclophosphamide was used intermittently by investigators at Johns Hopkins University (Baltimore, MD) in the 1980s during periods in which ATG apparently was not available to them; in their most recent update, of 38 previously untreated patients, the response rate was 74% and survival estimated at 86%.[83,84] In contrast, an NIH randomized study was halted early due to the development of fungal infections and a much higher death rate in the cyclophosphamide arm,[85] and both relapse and cytogenetic evolution were observed.[86] The major toxicity of high-dose cyclophosphamide, prolonged neutropenia with concomitant susceptibility to infection, is now addressed by the Baltimore investigators by routine antimicrobial prophylaxis and prolonged G-CSF administration. Cyclophosphamide therapy does not eradicate PNH clones, and relapses now have been observed in Baltimore.[84] In the absence of another randomized trial, comparison of data from a small, single-center pilot with historical and more general results is problematic; it is especially difficult to exclude biased patient selection, both explicit (such as exclusion of those unlikely to respond or with a generally poor prognosis or older patients) and implicit (inability to treat uninsured individuals or foreign citizens).

Management of refractory aplastic anemia. There is no established algorithm for the management of patients who have failed to respond to ATG.[67] Transplantation from an alternative donor is offered by many centers to children who have failed a single course of immunosuppression and to adults after 2 rounds of ATG therapy (see "Other stem-cell sources"). Response rates to second ATG have ranged from 22% to 64%.[78] In an Italian study of rabbit ATG as second therapy, 23 (77%) of 30 improved[87]; in the NIH experience, the proportion responding was closer to 30%.[79] Cyclophosphamide also has been administered in this setting, with a response rate of about 50% reported.[83] A third course of immunosuppression may benefit only patients who showed some response to a previous treatment.[88] Response to retreatment correlates to a better survival compared with refractory patients.[79] The improved survival of patients who are refractory to immunosuppression, due to better supportive care, complicates the decision to undertake high-risk transplantation (see "Other stem-cell sources"). We have tested alemtuzumab, a humanized monoclonal antibody specific for CD52, an antigen present on all lymphocytes; alemtuzumab induces profound immunosuppression by lymphocytotoxicity and has been effective in lymphoproliferative diseases, graft-versus-host disease (GVHD), and autoimmune disorders. To date, 4 of 8 patients who were refractory to treatment with horse ATG have responded to alemtuzumab, and toxicity has been modest (P.S., unpublished data, January 2006); we are now testing alemtuzumab in a randomized comparison with both horse and rabbit ATG in severe aplastic anemia at presentation.

Treatment of moderate pancytopenia. Clinically, the course of moderate aplastic anemia is variable: some patients progress to severe disease, others remain stable and may not require intervention; regular transfusions may not be required.[89] Very few clinical trials have specifically addressed moderate disease. Immunosuppression can reverse moderate pancytopenia and alleviate transfusion requirements; ATG and cyclosporine are more effective in combination,[73] but in practice are often used sequentially. Daclizumab, a humanized monoclonal antibody to the interleukin-2 receptor,

improved blood counts and relieved transfusion requirements in 6 of 16 evaluable patients; the outpatient regimen had little toxicity.[90]

When there is residual hematopoietic function, androgens may be effective (although male hormones have failed most rigorous trials in severe aplastic anemia). Some moderate aplastic anemia likely results from telomere gene mutations and stem-cell exhaustion. In vitro, androgens increase telomerase activity in human lymphocytes and CD34 cells, acting through the estradiol receptor,[91] and this activity may provide a mechanism of action for their effects on marrow function.

Hematopoietic stem-cell transplantation

Allogeneic HLA-matched sibling donor transplantation. Hematopoietic and immune system cells are replaced by stem-cell transplantation; conditioning with cyclophosphamide is not myeloablative but is sufficiently immunosuppressive to prevent and to eliminate residual host marrow by a graft-versus-marrow effect.

Allogeneic transplant from a matched sibling donor cures the great majority of patients (Table 2): the most recent cohort reported to the IBMTR showed 77% 5-year survival,[107] and in children, and patients undergoing transplantation who are minimally transfused, survival of 80% to 90% may be routinely achieved. Graft rejection, a historic problem in the application of transplantation to aplastic anemia (most dramatically manifest in rejection of unprepared syngeneic stem cells), is now not frequent in patients who undergo transplantation early and with a modest transfusion burden, likely a benefit of less immunogenic blood products (leukocyte-depleted erythrocytes, for example) from fewer donors (platelets collected by cytopheresis). Conditioning regimens that do not include irradiation now regularly achieve engraftment and avoid many of irradiation's long-term complications, especially late cancers. In a recent series of 81 patients who were prepared by cyclophosphamide plus ATG, sustained engraftment was achieved by 96%, and 3 of the 4 patients who initially rejected the transplant successfully underwent a retransplantation; 88% of the patients survived long term.[104] The combination of cyclophosphamide plus fludarabine, with or without ATG, has achieved high rates of graft acceptance and survival even in heavily transfused patients who received a transplant of mobilized peripheral-blood stem cells, months after proving refractory to immunosuppressive drugs.[106,108]

Graft-versus-host disease remains a serious problem for older patients, even with routine cyclosporine prophylaxis. In the IBMTR, rates of severe GVHD doubled in adults compared with children (15%-20% for recipients ≤ 20 years of age to 40%-45% for > 20 years of age).[107] In Seattle, chronic graft-versus-host disease developed in 41% of patients who had survived more than 2 years after transplantation, tripling the risk of death and often requiring years of immunosuppressive therapy.[109] Even with resolution, chronic GVHD remains a risk factor for late complications such as growth and endocrine system effects, pulmonary disease, cataracts, neurologic dysfunction, and secondary malignancy. Addition of ATG[105] and more recently its substitution by alemtuzumab[110] may reduce the frequency and severity of acute GVHD, a predictor of chronic GVHD.

Matched unrelated donor transplant. A matched sibling donor is available in only 20% to 30% of cases. As the outcome in aplastic patients who have failed a single round of ATG has been poor, alternative sources of hematopoietic stem cells have been sought, usually from now very large donor registries (Table 3). Outcomes of 318 alternative donor transplants performed from 1988 to 1998 recently have been summarized for the European registry[92]: for matched unrelated donors, the rejection rate was 15% and for grades II to IV GVHD, 48%, and 5-year survival was estimated at 39%. From diverse registry data (collected through EBMT, IBMTR, and the National Marrow Donor Program),[92,107,121] the mortality rate is about twice that observed in matched sibling transplants; even with predominantly younger patients as recipients, age is probably the most powerful influence on survival, but also important are the closeness of the class I HLA match and the length of time from diagnosis. A retrospective analysis from the Japan Marrow Donor Program suggested that patients with the most favorable characteristics and conditioned with a minimal dose of radiation might anticipate survival comparable with matched sibling transplants.[113]

Prospective trials have enrolled fewer patients but have better results (perhaps due to superior protocols, but both careful patient selection and publication bias are likely important). In contrast to allogeneic sibling transplants, transplants from unrelated donors still require irradiation to ensure engraftment, due both to source of the donor cells and the transfusion status of

Table 2. Allogeneic sibling transplantation for severe aplastic anemia

Institution/study	Years of study	N	Age, y (median in y)	Graft rejection/failure, %	Acute GVHD,* %	Chronic GVHD, %	Actuarial survival, %
IBMTR[92]	1988-1992	471	20 (1-51)	16	19	32	66 at 5 y
Vienna[93]	1982-1996	20	25 (17-37)	0	26	53	95 at 15 y
EBMT[94]	1991-1998	71	19 (4-46)	3	30	35	86 at 5 y
Seoul[95]	1990-1999	22	22 (14-43)	5	10	33	95 at 5 y
Seoul[96]	1990-2001	64	28 (14-43)	18	31	19	79 at 6 y
Hamburg[97]	1990-2001	21	25 (7-43)	5	5	5	86 at 5 y
Paris[98]	1994-2001	33	20 (8-42)	6	0	42	94 at 5 y
Sao Paulo[99]	1993-2001	81	24 (3-53)	22	37	39	56 at 5 y
Taipei[100]	1985-2001	79	22 (4-43)	8	7	35	74 at 5 y
Tunis[101]	1998-2001	31	19 (4-39)	16	11	3	86 at 2 y
Seoul[102]	1995-2001	113	28 (16-50)	15	11	12	89 at 6 y
London[103]	1989-2003	33	17 (4-46)	24	14	4	81 at 5 y
Seattle[104,105]	1988-2004	94	26 (2-59)	4	24	26	88 at 6 y
Mexico City[106]	2000-2005	23	25 (4-65)	26	17	26	88 at 4 y

In contrast to Table 1, response rates are not provided because, in surviving patients who do not experience primary graft rejection or secondary graft failure, full hematologic recovery with donor hematopoiesis is anticipated. Only studies reporting at least 20 patients are tabulated.

GVHD indicates graft-versus-host disease; IBMTR, International Blood and Marrow Transplant Registry; and EBMT, European Group for Bone Marrow Transplant.

*Results are generally for grades II to IV and patients at risk.

Table 3. Alternative donor stem-cell transplantation for severe aplastic anemia

Study	Year of publication	N	Donor source, N	Conditioning	Age, y, median (range)	Acute GVHD,* %	Chronic GVHD, %	Survival, %
Nagoya[111]	2001	15	MUD: 11; MMUD: 4	Cy/ATG/TBI	11 (3-19)	33	13	100 at 4 y
Great Britain[112]	2001	8	MUD: 7; MMUD: 1	Cy/CP/TBI	7 (0-10)	25	0	100 at 3 y
Japan Marrow Donor Program[113]	2002	154	MUD: 79; MMUD: 75	Cy ± TBI or LFI; Cy/ATG ± TBI or LFI	17 (1-46)	29	30	56 at 5 y
Memphis[114]	2004	9	MUD: 4; MMUD: 5	High CD34+ cell dose, TCD, Cy/ATG/TLI or TBI ± thiotepa	11 (6-16)	0	0	89 at 4 y
Gyeonggi-do[115]	2004	5	MUD	Cy/Flu/ATG	13 (7-18)	0	0	80 at 2 y
Guangzhou[116]	2004	6	UCB	Cy/ATG	26 (22-37)	0	33	66 at 2 y
Genoa[117]	2005	38	MUD: 33; MMRD: 5	Cy/Flu/ATG	14 (3-37)	11	24	73 at 2 y
Philadelphia[118]	2005	12	MUD: 4; MMUD: 8	Partial TCD, TBI + Cy/Ara-C or Cy/TT or ATG	9 (1-20)	33	25	75 at 4 y
Seoul[119]	2005	13	MUD: 12; MMUD: 1	Cy/ATG	22 (15-34)	31	62	75 at 3 y
IBMTR[92]	2006	318	MUD: 181; MMRD: 86; MMUD: 51	Various	16 (1-55)	48 for MUD	29 for MUD	39 at 5 y for MUD
Seattle[120]	2006	87	MUD: 62; MMUD: 25	Cy/ATG/TBI	19 (1-53)	70 for MUD	52 for MUD	61 at 5 y for MUD

Only studies reporting at least 5 patients are tabulated. GVHD, graft-versus-host disease; IBMTR, International Blood and Marrow Transplant Registry; MUD, matched unrelated donor; MMUD, mismatched unrelated donor; Cy, cyclophosphamide; ATG, antithymocyte globulin; TBI, total body irradiation; CP, alemtuzumab; LFI, limited field irradiation; TCD, T-cell depletion; TLI, total lymphoid irradiation; Flu, fludarabine; UCB, umbilical cord blood; MMRD, mismatched related donor; and TT, thiotepa.

*GVHD results are generally for grades II to IV and patients at risk.

the recipient. In a recent multicenter study, 62 patients with severe aplastic anemia who were refractory to immunosuppressive therapy underwent matched unrelated stem-cell transplantation following conditioning with cyclophosphamide, ATG, and total body irradiation; graft failure occurred in 2%, acute grades II to IV GVHD was observed in 70%, chronic GVHD was observed in 52%, and overall survival was 61%. Twenty five patients who lacked an HLA-identical donor received an HLA-nonidentical stem-cell graft: 88% showed sustained engraftment, and overall survival was 44%.[120] The interval from diagnosis to transplantation in this study did not impact survival.

A European protocol substituted irradiation with fludarabine for unrelated and mismatched family donors: 73% were estimated to survive 2 years; while GVHD rates were relatively low, perhaps due to absence of radiation damage, graft rejection occurred in about one third of the older children and younger adults.[117] Children's Hospital of Milwaukee pioneered a rigorous conditioning regimen of cytosine arabinoside, cyclophosphamide, and total body irradiation, which produced long-term survival of about 50% with very little GVHD.[122] Other single-institution protocols have used a diversity of strategies to improve graft acceptance and reduce GVHD: T-cell depletion, CD34-cell purification, alemtuzumab, chemotherapy and monoclonal antibodies in combination; while almost exclusively enrolling small numbers of children and still preliminary, survival and morbidity may rival results of conventional sibling transplants. In current practice, unrelated transplant is offered for children who have failed a single course of immunosuppression and to adults who are refractory to multiple courses of ATG and alternative therapies such as androgens. Studies with longer follow-up of larger numbers of patients are crucial to establish the optimal conditioning regimen and to define which patients will benefit and especially how early unrelated transplantation should be performed.

Other stem-cell sources. HLA mismatching is better tolerated for umbilical cord transplantations, making them in theory widely applicable. Published data for this procedure in marrow failure is limited, and almost all recipients have been small children, due to the small numbers of stem cells contained in a single cord-blood sample. In a report from the National Marrow Donor Program, engraftment occurred in less than half of 19 recipients, and almost all the patients died from transplant-related causes or survived due to autologous reconstitution or a second transplantation.[123] Surprisingly, 5 of 6 Chinese adult aplastic anemia patients successfully engrafted, and 4 survived.[116] Advocates of this approach are using pooled donations to increase stem-cell numbers.

Family members are almost always available to the patient as a stem-cell source, but survival after haploidentical transplantation has been poor. More recently, engraftment was achieved in 3 children using the St Jude protocol, but one ultimately rejected and mixed chimerism in the others required further immunosuppression and donor lymphocyte infusions.[124]

Conclusions and prospects

The treatment of severe aplastic anemia, whether by allogeneic stem-cell transplantation or immunosuppression, has improved dramatically over the last 25 years, and long-term survival of more than 75% of patients can be anticipated with either therapy.[125] For transplantation, the immediate challenge is the extension of stem-cell replacement to all patients, regardless of age, with a histocompatible sibling, and to others who lack a family donor using alternative stem-cell sources. The ability to achieve engraftment under these difficult circumstances may require conditioning regimens in which complications, particularly second malignancies, may not be apparent for many years. More optimistically, donor selection based on high-resolution histocompatibility typing may improve outcomes. The success of umbilical cord-blood transplantations, with their low risk of GVHD, may be enhanced by larger pools and histocompatibility matching. For immunosuppression, many new drugs and biologics have yet to be tested in aplastic anemia. Again, the costs of intensification need to be balanced against the benefits of higher hematologic response rates and lower rates of relapse and

evolution. Repeated courses of immunosuppression offer the possibility of blood-count restitution to 75% to more than 90% of patients, based on the range of published hematologic recovery rates with initial horse ATG followed by rabbit ATG, alemtuzumab, or cyclophosphamide. If residual stem-cell numbers are limiting, ex vivo expansion of hematopoiesis may be possible, as for example using Hox box proteins. Quantitative and practical measurements of oligoclonal T-cell activity and of hematopoietic stem-cell number and function would allow laboratory testing to guide treatment decisions. Ultimately, definition of genetic risk factors, affecting hematopoietic-cell function and the immune response, will clarify how agents in the environment initiate and perpetuate the marrow destruction of aplastic anemia.

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