

THE PATHOPHYSIOLOGY OF ACQUIRED APLASTIC ANEMIA

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Most patients with acquired aplastic anemia can be effectively treated, either by marrow (and more recently blood cell) transplant from a histocompatible sibling donor or by immunosuppressive therapy (reviewed in 1). The most recent single center publications from large hospitals specializing in transplantation report patient survival of 80-90%, although the more general rate of success from registry data is about 65%. With intensive immunosuppression using a combination of antilymphocyte globulins and cyclosporine, 70-80% of patients show hematologic recovery. Important pathophysiologic inferences have been derived from clinical observations: the success of stem cell transfer focused attention on a hematopoietic deficit; recovery after antilymphocyte globulin and cyclosporine implied immune inhibition of marrow function.

Cytotoxic Lymphocytes and Lymphokines and Hematopoiesis, In Vitro and In Vivo

An immune mechanism in aplastic anemia was first demonstrated in hematopoietic progenitor colony assays, which showed that blood and marrow cells from aplastic patients could recover hematopoietic activity after T cell depletion and that mononuclear cells from patients suppressed normal hematopoietic colony formation in vitro (reviewed in 2). A soluble inhibitor was detected in untreated tissue culture of patients' blood and marrow and, with lectin stimulation, from normal blood. This inhibitor was later identified as γ -interferon. In vitro, interferon and also tumor necrosis factor are produced in culture coincident with the acquisition of activation markers on the cell surface of cultured lymphocytes, the receptor for interleukin-2 and later HLA-DR. Most patients show immunophenotypic evidence of cytotoxic lymphocyte activation in peripheral blood and especially marrow,⁽³⁾ increased interferon production by circulating lymphocytes, and abnormal expression of the γ -interferon gene in marrow.^(4,5) Cell clonal studies of individual cases have also implicated lymphokine secretion by specific T cell subsets.^(6,7)

Recent laboratory studies have helped characterize important features of lymphokine suppression of hematopoiesis. Both interferon and tumor necrosis factor induce Fas antigen expression on CD34+ cells, leading to increased susceptibility to apoptosis or programmed cell death.^(8,9) Fas is over-expressed on CD34+ cells from the marrow of patients with hematopoietic failure syndromes.^(10,11) Global cytotoxicity of lymphokines is reflected in standard tissue culture of adult bone marrow cells, where colony formation by both late (CD34+CD38+) and early (CD34+ CD38-) progenitor cells is inhibited by interferon and tumor necrosis factor.⁽¹²⁾ In long-term bone marrow cultures, the generation of long-term culture-initiating cells (LTC-IC, a stem cell surrogate; see below) is also severely diminished by exposure to interferon. In vitro and in patients, local effects of interferon dominate. Not only is γ -interferon mRNA expressed aberrantly in the marrow of most patients with aplastic anemia, but, in vitro, endogenous

production is far more efficient than addition of cytokines for inhibition of hematopoiesis: when normal human stromal cells were engineered to constitutively express relatively low concentrations of interferon, similar abrogation of LTC-IC generation occurred at 100-fold lower concentrations of endogenous than exogenous cytokine.⁽¹³⁾ Interferon activates diverse signal transduction pathways in target cells, and some of these pathways have been identified in CD34+ cells. Nitric oxide synthase is inducible in, and NO is toxic to, CD34+ cells.⁽¹⁴⁾ Interferon regulatory factor-1, an important transcription regulator, also is induced in CD34+ cells, and its production is at least partly responsible for inhibition of hematopoietic colony formation.⁽¹⁵⁾ These pathways may be implicated in both cell death and abnormalities of hematopoietic cell behavior, such as altered cell proliferation in response to growth factors and late clonal abnormalities due to genetic damage.

The Hematopoietic Compartment in Aplastic Anemia

By all measures, hematopoiesis is severely reduced in aplastic anemia. On light microscopy, morphologically recognizable precursor cells are largely absent on both aspirate smear and marrow biopsy. Hematopoietic cells, defined by surface membrane expression of the cytoadhesive protein CD34, are also uniformly decreased in blood and marrow.^(16,17) Lineage-committed progenitors and earlier cells capable of forming mixed colonies or blast colonies in semisolid culture medium are also markedly reduced in number. Hematopoietic stem cells can now be measured in the LTC-IC assay: total mononuclear cells or CD34+ cells are cultured on normal stroma for about 5 weeks, following which colony-forming cells are assayed in methyl cellulose. (Like true repopulating stem cells, LTC-IC are quiescent, survive in tissue culture for many weeks, demonstrate pluripotency, and have a very low frequency in marrow and blood.) LTC-IC are uniformly and extremely reduced in all severe aplastic anemia patients on presentation: their numbers are about 10-fold less than normal in both blood and marrow, and as the denominator is mononuclear cells, which are also decreased in the disease, the absolute number of LTC-IC is probably less than 1% of normal.⁽¹⁸⁾ Qualitatively, the LTC-IC compartment is also abnormal, as cells from aplastic patients show a much lower cloning efficiency, about 1, compared to normal donors' 4. LTC-IC numbers do not appear to be predictive of response to immunosuppressive therapy or of a poor outcome. Although LTC-IC do not fluctuate in number early after treatment, the average LTC-IC number is higher in patients who have responded to ATG and cyclosporine, and in about half rises into the normal range. Thus repopulation within the stem cell compartment may occur in some cases.

Hypothetically, marrow failure might result from stroma cell dysfunction. However, in aplastic anemia stroma appears to be functionally normal in vitro. Adherent cells from patients support normal CD34+ cell proliferation, while aplastic anemia CD34+ cells fail to grow on normal stroma.⁽¹⁹⁾ Growth factors are usually much increased in the circulation of marrow failure patients, and patients, cells generally show high production of hematopoietins in vitro (reviewed in 20). Only two growth factors are low: production of interleukin-1 by monocytes is deficient and stem cell factor blood levels are decreased.⁽²¹⁾ The clinical significance of these findings is uncertain, not only because of

the redundancy of the actions of multiple growth factors but because of the clinical failure of growth factor replacement with IL-1 or SCF to correct pancytopenia.^(22,23) Although growth factor treatment rarely cures aplastic anemia, the efficacy of antilymphocyte globulins, especially in comparison to monoclonal antibodies directed to T cell antigens, has been credited to their ability to promote hematopoietic growth factor production by lymphocytes.⁽²⁴⁾

Antigens and Autoimmunity in Bone Marrow Failure

Host susceptibility has been suggested by a high prevalence of HLA-DR2 among aplastic anemia patients in general, and in patients responsive to cyclosporine there has been strong linkage to a specific HLA class II haplotype.^(25,26) These results suggest an initial role for a CD4+ T cell, and lymphocytes of this class that recognize autologous marrow cells have been cloned from occasional patients.⁽⁷⁾ Transfusion-associated graft-versus-host disease, in which HLA disparity produces profound and invariably fatal aplasia, may be a good model of the immune system in acquired aplastic anemia; in this syndrome, both helper and cytotoxic lymphocytes, as well as their soluble products, are pathophysiologic.⁽²⁷⁾

Although aplastic anemia is clinically associated with specific drugs and some viral infections, the initial events that incite the aberrant immune response are poorly understood. The hepatitis/aplasia syndrome, in which severe aplastic anemia follows on an unexceptional episode of seronegative hepatitis, has not been associated with any known hepatitis virus,⁽²⁸⁾ including hepatitis C and the novel GBV-C agent.⁽²⁹⁾ Uncertainty remains as to whether viral aplastic anemia is the consequence of infection with an as yet undiscovered agent for seronegative fulminant hepatitis or an exaggerated immune response to a variety of common viruses. (Similar difficulty has been experienced in ascribing an infectious etiology to other human immune-mediated diseases such as type I diabetes mellitus, multiple sclerosis, and rheumatoid arthritis.) Molecular mimicry, antigenic spread, and altered suppressor cell function are only a few of the immunologic mechanisms that have been described in experimental models of virally incited T cell-mediated diseases in animals (reviewed in 2).

For drugs, there are long lists of incriminated agents (reviewed in 30,31), but their mechanisms of action are similarly unsatisfactorily resolved. Clearly drugs used in cancer chemotherapy—designed for cytotoxicity—regularly produce marrow aplasia, although even with these agents there may be genetic variability in the degree of hematologic toxicity among recipients. Similar to chemotherapeutic drugs, benzene produces dose-dependent marrow suppression, likely functioning as a relatively inefficient version of a chemotherapeutic agent due to variable absorption, metabolism, and activity on marrow cells. For agranulocytosis, which has a very high rate of association with drug exposure, direct effects on granulocyte production have been inferred for the phenothiazines from laboratory studies as well as the common mild leucopenia that occurs in a substantial minority of treated patients.

However, most drugs associated with blood dyscrasias do not regularly produce marrow suppression, and the frequency of severe idiosyncratic hematologic reactions is extremely low. For direct activity of a drug on the marrow, a toxic intermediate

metabolite could be generated in some individuals, due to genetic variability in a component of one of the complex enzymatic pathways leading to drug excretion. Such a toxic metabolite might be unusually or excessively generated or normally produced but not detoxified. By definition, toxic intermediate compounds are highly reactive and have a brief half-life, making them difficult to study in a clinical setting. For aplastic anemia, there is a single laboratory example implicating a toxic intermediate metabolite in a case of aplastic anemia, involving the neuroleptic carbamazepine: using an in vitro biological assay, a patient's cells were unable to convert a metabolite (generated by rat microsomes) that was toxic to his own lymphocytes, presumably through a deficiency of an epoxide hydroxylase.⁽³²⁾

In addition to direct effects of drugs or their metabolites on hematopoietic cells, a second route to marrow failure would involve immune cells. Agranulocytosis following aminopyrine is the classic example of antibody-mediated, highly selective destruction of granulocytic precursors. Plasma inhibitors, however, are variably detected in aplastic anemia and only rarely characterized as immunoglobulins, usually in the context of an established autoimmune disease like systemic lupus erythematosus. T cell-dependent mechanisms of hapten recognition have not been described for drugs implicated in aplastic anemia and only occasionally for agranulocytosis, but a role for T cells is supported by recent studies that have shown strong associations between certain HLA types and clozapine agranulocytosis in Jewish patients⁽³³⁾ and methimazole agranulocytosis in Japanese.⁽³⁴⁾

Late Clonal Disease

A high rate of relapse to pancytopenia and the late development of clonal hematologic diseases, especially paroxysmal nocturnal hemoglobinuria, myelodysplasia, and acute myelogenous leukemia, are important clinical issues in the management of patients after immunosuppressive therapy. Support of hematopoiesis from a very limited stem cell pool or, conversely, increased mitotic activity of primitive stem cells during stem cell pool regeneration might contribute to these late genetic events. Although the great majority of new patients have normal marrow cytogenetic studies, evidence of the paroxysmal nocturnal hemoglobinuria phenotype has been detected in a large proportion of aplastic anemia cases on presentation.⁽³⁵⁾

The paroxysmal nocturnal hemoglobinuria syndrome - intravascular hemolysis, venous thrombosis, and marrow failure - is characterized by deficient presentation of a class of cell surface proteins linked to the plasma membrane by a glycosphosphoinositol anchor (reviewed in 36). Although many biochemical steps are required for the synthesis of this structure, all patients suffer mutations or deletions in a single X-chromosome gene called PIG-A. The relationship of the known genetic and biochemical defects in paroxysmal nocturnal hemoglobinuria to aplastic anemia remain unknown but is more likely to be secondary to selective extrinsic pressure than to an intrinsic growth advantage for the abnormal cells. In the clinic, paroxysmal nocturnal hemoglobinuria cells appeared in the blood of some lymphoma patients treated with a monoclonal antibody directed to a glycosphosphoinositol-linked membrane protein.⁽³⁷⁾ In the laboratory, the murine PIG-A gene was disrupted in "knock-out" experiments.⁽³⁸⁾ Deficient cells formed no

hematopoietic colonies, but they also failed to undergo embryogenesis. Embryoid body formation was corrected by coculture with normal cells, and hematopoietic colony formation by the knock-out cells was normal from these chimeric pseudo-embryos. Cell to cell protein transfer of glycoposphoinositol-linked membrane proteins could be demonstrated by flow cytometry. PIG-A mutant cells may arise occasionally in normal marrow but expand under conditions of immune system attack or in a failing marrow environment.

Conclusion

In all patients with aplastic anemia, cells at all stages of hematopoietic development, from stem cells to blood elements, are severely reduced. Most aplastic anemia appears to be mediated by cells and soluble products of the immune system, which cause hematopoietic cell destruction through the Fas system and programmed cell death. Severe aplastic anemia is amenable to immunosuppressive treatments. Etiologic mechanisms are poorly understood, but identification of the virus in the hepatitis/aplasia and fulminant hepatitis syndromes should allow definition of the aberrant immune system response to certain foreign antigens. The relationship of late clonal disease - especially paroxysmal nocturnal hemoglobinuria and myelodysplasia - to autoimmune destruction of hematopoiesis and stem cell recovery may lead to novel biological insights.

References

1. Young NS, Barrett AJ: The treatment of severe acquired aplastic anemia. *Blood* 85:3367, 1995
2. Young NS: Pathophysiology II: immune suppression of hematopoiesis, in Young NS, Alter BP (eds): *Aplastic Anemia, Acquired and Inherited*, Philadelphia, W.B. Saunders, 1994, p 68
3. Maciejewski JP, Hibbs JR, Anderson S, Katevas P, Young NS: Bone marrow and peripheral blood lymphocyte phenotype in patients with bone marrow failure. *Exp Hematol* 22:1102, 1994
4. Nakao S, Yamaguchi M, Shiobara S: Interferon gamma gene expression in unstimulated bone marrow mononuclear cells predicts a response to cyclosporine therapy in aplastic anemia. *Blood* 79:2532, 1992
5. Nistico A, Young NS: g-Interferon gene expression in the bone marrow of patients with acquired aplastic anemia. *Ann Intern Med* 120:463, 1994
6. Tong J, Bacigalupo A, Piaggio G, Figari O, Sogno G, Marmont A: In vitro response of T cells from aplastic anemia patients to antilymphocyte globulin and phytohemagglutinin: Colony-stimulating activity and lymphokine production. *Exp Hematol* 19:312, 1991
7. Nakao S, Takamatsu H, Yachie A, Itoh T, Yamaguchi M, Ueda M, Shiobara S, Matsuda T: Establishment of a CD4+ T cell clone recognizing autologous hematopoietic progenitor cells from a patient with immune-mediated aplastic anemia. *Exp Hematol* 23:433, 1995

8. Maciejewski JP, Selleri C, Anderson S, Young NS: Fas antigen expression on CD34+ human marrow cells is induced by interferon-gamma and tumor necrosis factor-alpha and potentiates hematopoietic suppression in vitro. *Blood* 85:3183, 1995
9. Nagafuji K, Shibuya T, Harada M, Mizuno S-I, Takenaka K, Miyamoto T, Okamura T, Gondo H, Niho Y: Functional expression of Fas antigen (CD95) on hematopoietic progenitor cells. *Blood* 86:883, 1995
10. Maciejewski JP, Selleri C, Sato T, Anderson S, Young NS: Increased expression of Fas antigen on CD34+ cells in the marrow of patients with aplastic anemia. *Br J Haematol* 91:245, 1995
11. Philpott NJ, Scopes J, Marsh JCW, Gordon-Smith EC, Gibson FM: Increased apoptosis in aplastic anemia bone marrow progenitor cells: possible pathophysiologic significance. *Exp Hematol* 23:1642, 1995
12. Selleri C, Sato T, Anderson S, Young NS, Maciejewski JP: Interferon- γ and tumor necrosis factor- α suppress both early and late stages of hematopoiesis and induce programmed cell death. *J Cell Physiol* 165:538, 1995
13. Selleri C, Maciejewski JP, Young NS: Interferon- γ constitutively expressed in the stromal microenvironment of human bone marrow cultures mediates potent hematopoietic inhibition. *Blood* 87:4149, 1996
14. Maciejewski JP, Selleri C, Sato T, Cho HJ, Keefer LK, Nathan CF, Young NS: Nitric oxide suppression of human hematopoiesis in vitro: contribution to inhibitory action of interferon- γ and tumor necrosis factor- α . *J Clin Invest* 96:1085, 1995
15. Sato T, Selleri C, Young NS, Maciejewski JP: Hematopoietic inhibition by interferon-g is partially mediated through interferon regulatory factor-1. *Blood* 86:3373, 1995
16. Maciejewski JP, Anderson S, Katevas P, Young NS: Phenotypic and functional analysis of the bone marrow progenitor cell compartment in aplastic anemia. *Br J Haematol* 87:227, 1994
17. Scopes J, Bagnara M, Gordon-Smith EC, Ball SE, Gibson FM: Haemopoietic progenitor cells are reduced in aplastic anaemia. *Br J Haematol* 86:427, 1994
18. Maciejewski JP, Sato T, Selleri C, Anderson SA, Young NS: A consistent and severe deficit in marrow and circulating primitive hematopoietic cells (long-term culture-initiating cells) in aplastic anemia. *Blood*, in press, 1996
19. Marsh JCW, Chang J, Testa NG, Hows JM, Dexter TM: In vitro assessment of marrow 'stem cell' and stromal cell function in aplastic anaemia. *Br J Haematol* 78:258, 1991
20. Young NS: Pathophysiology I: stem cells, stroma, and growth factors, in Young NS, Alter BP: *Aplastic Anemia, Acquired and Inherited*, Philadelphia, W.B. Saunders, 1994, p 32
21. Wodnar-Filipowicz A, Yancik S, Moser Y, dalle Carbonare V, Gratwohl A, Tichelli A, Speck B, Nissen C: Levels of soluble stem cell factor in serum of patients with aplastic anemia. *Blood* 81:3259, 1993
22. Walsh CE, Liu JM, Anderson SM, Rossio JL, Nienhuis AW, Young NS: A trial of recombinant human interleukin-1 in patients with severe, refractory aplastic anemia. *Br J Haematol* 80:106, 1991

23. Nemunaitis J, Ross M, Meisenberg B, O'Reilly R, Lilleby K, Buckner CD, Appelbaum FR, Buhles W, Singer J, Peters WP: Phase I study of recombinant human interleukin-1 β (rhIL-1 β) in patients with bone marrow failure. *Bone Marrow Transplant* 14:583, 1994
24. Kawano Y, Nissen C, Gratwohl A, Speck B: Immunostimulatory effects of different antilymphocyte globulin preparations: a possible clue to their clinical effect. *Br J Haematol* 68:115, 1988
25. Nimer SD, Ireland P, Meshkinpour A, Frane M: An increased HLA DR2 frequency is seen in aplastic anemia patients. *Blood* 84:923, 1994
26. Nakao S, Takamatsu H, Chuhjo T, Ueda M, Shiobara S, Matsuda T, Kaneshige T, Mizoguchi H: Identification of a specific HLA class II haplotype strongly associated with susceptibility to cyclosporine-dependent aplastic anemia. *Blood* 84:4257, 1994
27. Anderson KC, Weinstein HJ: Transfusion-associated graft versus-host disease. *N Engl J Med* 323:315, 1990
28. Hibbs J, Rosenfeld S, Feinstone SM, Kojima S, Bacigalupo A, Locasciulli A, Tzakis AG, Alter HJ, Young NS: Hepatitis/aplasia syndrome: non A, non B, non C? *JAMA* 267:2051, 1992
29. Simons JN, Leary TP, Dawson GJ, Pilot-Matias TJ, Muerhoff AS, Schlauder GG, Desai SM, Mushahwar IK: Isolation of novel virus-like sequences associated with human hepatitis. *Nature Medicine* 1:564, 1995
30. Young NS: Drugs and chemicals, in Young NS, Alter BP (eds): *Aplastic Anemia, Acquired and Inherited*, Philadelphia, W.B. Saunders, 1994, p 100
31. Young NS: Agranulocytosis, in Young NS, Alter BP (eds): *Aplastic Anemia, Acquired and Inherited*, Philadelphia, W.B. Saunders, 1994, p 229
32. Gerson ST, Fine DG, Spielberg SP, Sensenbrenner LL: Anticonvulsant-induced aplastic anemia: increased susceptibility to toxic drug metabolites in vitro. *Blood* 61:889, 1983
33. Lieberman JA, Yunis J, Egea E, Canoso RT, Kane JM, Yunis EJ: HLA-B38, DR4, DQw3 and clozapine-induced agranulocytosis in Jewish patients with schizophrenia. *Arch Gen Psychiatry* 47:945, 1990
34. Tamai H, Sudo T, Kimura A, Mukuta T, Matsubayashi S, Kuma K, Nagataki S, Sasazuki T: Association between the DRB1*08032 histocompatibility antigen and methimazole-induced agranulocytosis in Japanese patients with Graves disease. *Ann Intern Med* 124:490, 1996
35. Schrenzenmeier H, Hertenstein B, Wagner B, Raghavachar A, Heimpel H: A pathogenetic link between aplastic anemia and paroxysmal nocturnal hemoglobinuria is suggested by a high frequency of aplastic anemia with a deficiency of phosphatidylinositol glycan proteins. *Exp Hematol* 23:81, 1995
36. Rosse WF, Ware RE: The molecular basis of paroxysmal nocturnal hemoglobinuria. *Blood* 86:3277, 1995
37. Hertenstein B, Wagner B, Bunjes D, Duncker C, Raghavachar A, Arnold R, Heimpel H, Schrenzenmeier H: Emergence of CD52-, phosphatidylinositolglycan-anchor-deficient T lymphocytes after in vivo application of Campath-1H for refractory B-cell non-Hodgkin lymphoma. *Blood* 86:1487, 1995

38. Dunn D, Yu J, Nagarjan S, Devetten M, Weichold F, Medof E, Young NS, Liu J: A knock-out model of paroxysmal nocturnal hemoglobinuria: PIG-A-hematopoiesis is reconstituted following intercellular transfer of GPI-anchored proteins. Proc Natl Acad Sci USA In press:1996