

Brief Report

PURE RED-CELL APLASIA ASSOCIATED
WITH CLONAL EXPANSION
OF GRANULAR LYMPHOCYTES
EXPRESSING KILLER-CELL
INHIBITORY RECEPTORS

RUPERT HANDGRETINGER, M.D.,
ANDREAS GEISELHART, M.D., ARNAUD MORIS, M.S.,
ROGER GRAU, M.S., OLIVER TEUFFEL, M.S.,
WOLFGANG BETHGE, M.D., LOTHAR KANZ, M.D.,
AND PAUL FISCH, M.D.

KILLER-CELL inhibitory receptors inhibit cytolysis when natural killer cells encounter cells bearing HLA class I molecules.^{1,2} This phenomenon explains the preferential killing by natural killer cells of tumor cells with reduced expression of HLA class I molecules.³ This finding is the basis of the "missing self" model, in which the HLA class I allele lost by the tumor cells is not available to inhibit the killer cell's lytic machinery.

Different types of killer-cell inhibitory receptors have distinct specificity for HLA-A, B, C, and E antigens.^{1,4} In addition, some natural killer cells also possess receptors that induce lysis of cells expressing foreign HLA class I alleles.^{1,2,5} These inhibitory and activating receptors regulate cytolysis by natural killer cells of allogeneic cells that lack autologous HLA class I antigens but do express allogeneic class I alleles.¹ Such natural killer cells are thought to cause rejection of bone marrow by recipients of marrow from haploidentical donors.^{1,6} Most T-cell receptors for antigen are of the α/β type; a minority of T cells bear receptors of the γ/δ type. Normally, α/β T cells kill only cells that bear the same HLA class I molecules, a phenomenon known as major-histocompatibility-complex (MHC) restriction, whereas γ/δ are not restricted by MHC. Most of these γ/δ T cells⁷ and, rarely, cytotoxic α/β T cells⁸ also express killer-cell inhibitory receptors. Such receptors down-regulate the activating signals evoked when the T-cell receptor binds to an antigen, thereby regulating the cytotoxic activity of the T cell. We examined the clin-

ical relevance of killer-cell inhibitory receptors in an autoimmune disease.

Pure red-cell aplasia, a syndrome defined by the absence of erythroid precursors in the bone marrow, has several causes,^{9,10} including an intrinsic defect of multipotent progenitor cells and infection by human parvovirus B19, which lyses erythroid progenitor cells.¹¹ Autoantibodies against erythroid cells or erythropoietin¹² or T-cell-mediated inhibition of erythropoiesis can also cause pure red-cell aplasia.⁹ Such autoimmune mechanisms are the most likely causes of pure red-cell aplasia associated with chronic lymphocytic leukemia and thymomas.¹⁰ Some patients with pure red-cell aplasia have increased numbers of large granular lymphocytes with a natural-killer-cell or T-cell phenotype.^{10,13-18} The relation of these cells to the inhibition of erythropoiesis is unclear.

We describe a patient with pure red-cell aplasia and a clonal population of large granular lymphocytes of the γ/δ T-cell type. These cells expressed killer-cell inhibitory receptors and killed target cells in an MHC-unrestricted manner. Moreover, these cytolytic γ/δ T cells could be inhibited by signaling through killer-cell inhibitory receptors. The physiologic down-regulation of the expression of HLA class I genes in the erythropoietic lineage probably accounts for the ability of the large granular lymphocytes to lyse the patient's erythroid precursors.

CASE REPORT

A 56-year-old man presented with fatigue, dyspnea, and severe anemia. Anemia had been first noticed 18 months before admission. His medical history was otherwise notable only for major depression. There was slight hepatosplenomegaly, but no lymphadenopathy. The hemoglobin level was 5.2 g per deciliter, with a red-cell count of 1.86 million per cubic millimeter, a hematocrit of 16.5 percent, and 0.1 percent reticulocytes. The platelet count was 156,000 per cubic millimeter, and the white-cell count was 5200 per cubic millimeter, with 28 percent neutrophils, 60 percent lymphocytes, 9 percent monocytes, 1 percent eosinophils, and 2 percent basophils. Morphologically, most of the lymphocytes were large granular lymphocytes, with an absolute number of 2300 per cubic millimeter. On flow cytometry, 96 percent of the lymphocytes were T cells (CD3+); 78 percent of these T cells expressed γ/δ receptors, and 22 percent expressed α/β receptors (normally, less than 5 percent of T lymphocytes in the blood express γ/δ receptors). These γ/δ cells were positive for CD2, CD5, and CD7 and negative for CD16 and CD56 (both of which are markers of natural killer cells), CD57, and CD4, and about half expressed CD8. Less than 1 percent of the mononuclear cells were CD56+CD3- natural killer cells. B cells were virtually absent from the peripheral blood, but serum immunoglobulin levels were normal.

Bone marrow biopsy revealed slight hypercellularity and normal maturation of the myeloid lineage and megakaryocytes, but less than 1 percent of the cells were erythroid precursors (including proerythroblasts). There was no increase in blasts. There were foci of CD3+ T cells without α/β receptors. The karyotype was normal.

The Coombs' test was negative, and increased hemolysis and paroxysmal nocturnal hemoglobinuria were ruled out. The serum erythropoietin level (570 mU per milliliter) was markedly increased. Further studies revealed normal values for serum ferritin, transferrin, vitamin B₁₂, and folic acid. Tests for antibodies against

From the Departments of Pediatrics (R.H., A.G., R.G., O.T.), Immunology (A.M., P.F.), and Medicine II (W.B., L.K.), University of Tübingen, Tübingen, Germany. Address reprint requests to Dr. Handgretinger at Children's University Hospital, Hoppe-Seyler-Str. 1, 72076 Tübingen, Germany, or at kkrhand@uni-tuebingen.de.
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human parvovirus B19, cytomegalovirus, the human immunodeficiency virus, and Epstein-Barr virus were negative. Polymerase-chain-reaction assays were negative for hepatitis B and C viruses, cytomegalovirus, and human parvovirus B19.

Cultures of the patient's bone marrow cells in standard semisolid methylcellulose medium^{19,20} revealed normal numbers of erythroid and granulocytic progenitors. For every 50,000 bone marrow cells plated, there were 26 erythroid colony-forming units and 40 granulocyte-macrophage colony-forming units; the mean normal values in our laboratory (10 samples) are 23 erythroid colony-forming units (range, 10 to 30) and 52 granulocyte-macrophage colony-forming units (range, 23 to 89). The patient's serum did not inhibit the formation of erythroid or granulocyte-macrophage colonies in bone marrow cells from unrelated donors.^{9,12} The patient's HLA haplotype was A2, A32, B65, B38 (Bw4), Cw7, and Cw8.

The patient was given a diagnosis of pure red-cell aplasia with an abnormal increase in large granular lymphocytes of the γ/δ T-cell type.¹⁷ He required weekly blood transfusions. Six months of therapy with oral cyclosporine at a dose of 3 mg per kilogram of body weight per day had no effect on the anemia. Treatment with cyclophosphamide was begun.

METHODS

Analysis of Surface Phenotype and Monoclonal Antibodies

The phenotype of the patient's lymphocytes was analyzed by direct and indirect immunofluorescence on a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.) with monoclonal antibodies against human lymphocyte surface molecules (Becton Dickinson or Coulter/Immunotech, Marseilles, France) conjugated to fluorescein or phycoerythrin. The antibodies against the variable-region proteins of the γ/δ T-cell receptor were R9-12-6-2 and A13 (anti-V δ 1 antibodies) and 94 (anti-V γ 4 antibody). The antibody against monomorphic determinants of HLA class I molecules was W6/32. A monoclonal antibody against HLA-A, B, and C antigens (B1.23.2) was used to induce cytolysis.^{7,21} The monoclonal antibodies against killer-cell inhibitory receptors were EB6 (anti-p58.1 antibody), GL183 (anti-p58.2 antibody), DX9 and 5.133 (anti-p70 antibodies), XA185 (anti-CD94 antibody), and Q66 (anti-p140 antibody).

Molecular Analysis of Clonality

Rearrangements of the T-cell receptor γ chain were studied according to previously described methods.²² In brief, high-molecular-weight DNA was extracted by cell lysis, digestion by proteinase K, extraction with phenol-chloroform, and precipitation with ethanol. Then, 15 μ g of DNA, digested with restriction enzymes, was separated on 0.8 percent agarose gels and transferred to nylon membranes. The membranes were hybridized to the phosphorus-32-labeled probe M13H60,²² and the signals were quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Purification of CD34+ Cells and Erythroid Progenitors

Bone marrow was obtained from the patient and normal subjects after they had given informed consent, and mononuclear cells were purified on density gradients. CD34+ hematopoietic stem cells and glycophorin A+ erythroid progenitors were purified with magnetic beads (Miltenyi, Bergisch Gladbach, Germany).

Assays of Cytotoxic Activity

The ability of the patients' lymphocytes to lyse tumor-cell lines and B-cell lines was measured at various ratios of effector cells to target cells by means of chromium-release assays as described previously²³ and with a fluorescence-enhancing ligand — bis(acetoxy-methyl) 2,2':6',2''-terpyridine-6,6''-dicarboxylate — that is released from dying target cells.²⁴ The results of the two assays were sim-

ilar. All determinations were performed in triplicate. In some experiments effector cells or target cells were incubated with monoclonal antibodies for 30 minutes at 37°C before the addition of the target cells or effector cells, respectively. The results were confirmed in at least three independent experiments.

RESULTS

Analysis of a blood smear showed that 70 percent of the patient's mononuclear cells were large granular lymphocytes. These cells were also evident in the bone marrow. The rearranged variable-region genes of these T cells were V γ 4 and V δ 1,²⁵ as determined by an assay with monoclonal antibodies specific for the variable region (data not shown). These kinds of γ/δ T cells are undetectable in the blood of normal subjects. Analysis of the rearrangements of the T-cell-receptor gene in the large granular lymphocytes revealed clonality of the γ chain (Fig. 1A) and β chain (data not shown). The latter must have been a non-productive rearrangement, because these cells did not stain for antibodies against the α/β T-cell receptor. The γ/δ T cells expressed the killer-cell inhibitory receptors¹ p58.1 (specific for HLA-Cw2, Cw4, and Cw6), p70 (specific for the public epitope of HLA-Bw4), p140 (specific for HLA-A*0301), and CD94 (specific for HLA-E stabilized by leader peptides from many classic HLA class I molecules),⁴ but they were negative for the p58.2 killer-cell inhibitory receptor (specific for HLA-Cw1, Cw3, Cw7, and Cw8) (Fig. 1B).

The patient's lymphocytes mediated strong MHC-unrestricted cytotoxicity in vitro against two tumor cell lines with a deficiency of HLA class I molecules (Fig. 2A)²³: K562 (erythroleukemia)²⁶ and Daudi (Burkitt's lymphoma). The cytotoxicity was not restricted by MHC differences between the killer cells and target cells and did not require prior activation of the lymphocytes by interleukin-2. Daudi cells are normally resistant to lysis by natural killer cells that have not been activated in vitro; moreover, the lysis of Daudi cells by the patient's mononuclear cells was blocked by antibodies against V δ 1, the δ chain on the receptors of the patient's large granular lymphocytes (Fig. 2B). Daudi cells lack HLA class I molecules because they have a deficiency of beta-microglobulin, an essential component of the HLA class I complex. Daudi cells that expressed HLA class I antigens after transfection with the beta₂-microglobulin gene were resistant to lysis by the patient's lymphocytes (Fig. 2A).²³ The protective effect of HLA class I molecules was reversed by incubating the transfected Daudi cells with antibodies to HLA class I molecules before exposing them to the patient's lymphocytes (Fig. 2A).²¹ Moreover, antibodies against the killer-cell inhibitory receptors p58.1, CD94, and p70, but not p58.2, an inhibitory receptor that was not expressed by the clonal γ/δ T cells, decreased lysis of untransfected Daudi cells (Fig. 2B). These results indicate that the killer-cell inhibitory recep-

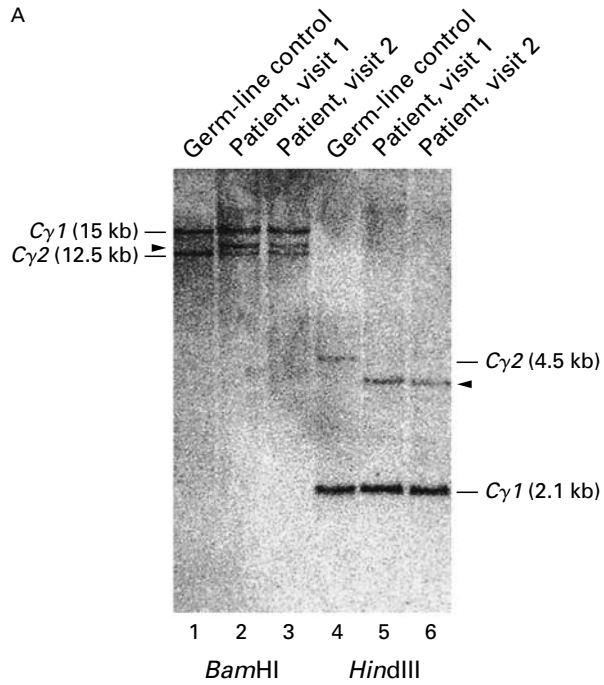
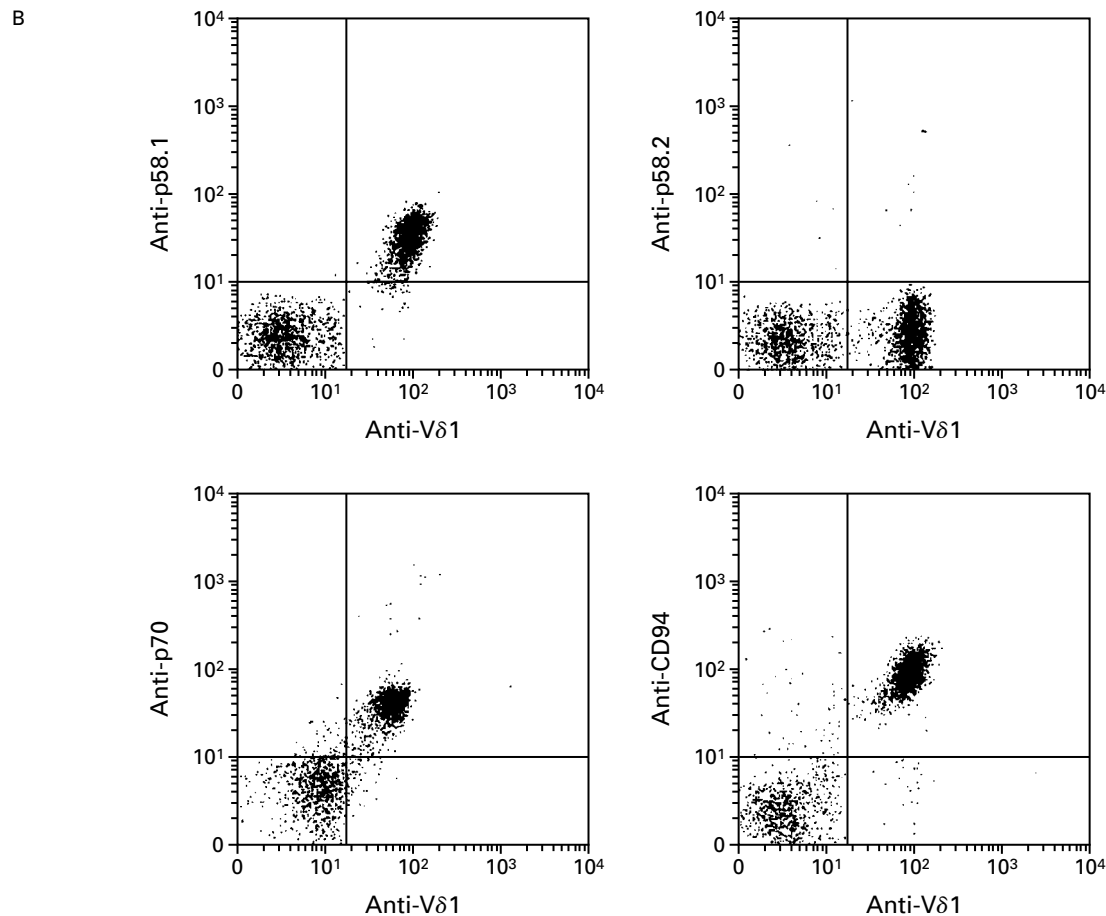


Figure 1. Clonality and Phenotype of the Patient's Lymphocytes.

Panel A shows the results of hybridization of the $C\gamma$ probe M13H60²² to DNA from the patient and from a lymphoblastoid B-cell line (the germ-line control). The DNA from the patient's lymphocytes was isolated from blood samples obtained during two clinic visits. The DNA samples were digested with the *Bam*HI or *Hind*III restriction enzymes. The sizes of the germ-line $C\gamma 1$ and $C\gamma 2$ restriction fragments are indicated for each enzyme; the arrowheads show the rearranged $C\gamma 2$ gene in the patient's DNA. In the *Hind*III digest, the band for the $C\gamma 2$ gene is weaker than that for the $C\gamma 1$ gene in the germ-line control because of a restriction-fragment-length polymorphism on one of the four germ-line $C\gamma$ genes.²² Southern blotting of the rearrangements of the T-cell-receptor β chain, as well as further analysis of the rearrangements of the T-cell-receptor γ chain by the polymerase chain reaction, confirmed the clonality of the cells. Panel B shows the results of two-color flow-cytometric analysis of the patient's cells after staining with a monoclonal antibody against $V\delta 1$ and conjugation to fluorescein and staining with monoclonal antibodies specific for the killer-cell inhibitory receptors p58.1, p58.2, p70, and CD94 after conjugation with phycoerythrin. These γ/δ T cells also expressed the killer-cell inhibitory receptor p140 (data not shown). Cells that were positive for both $V\delta 1$ and the various killer-cell inhibitory receptors appear in the upper right quadrants.



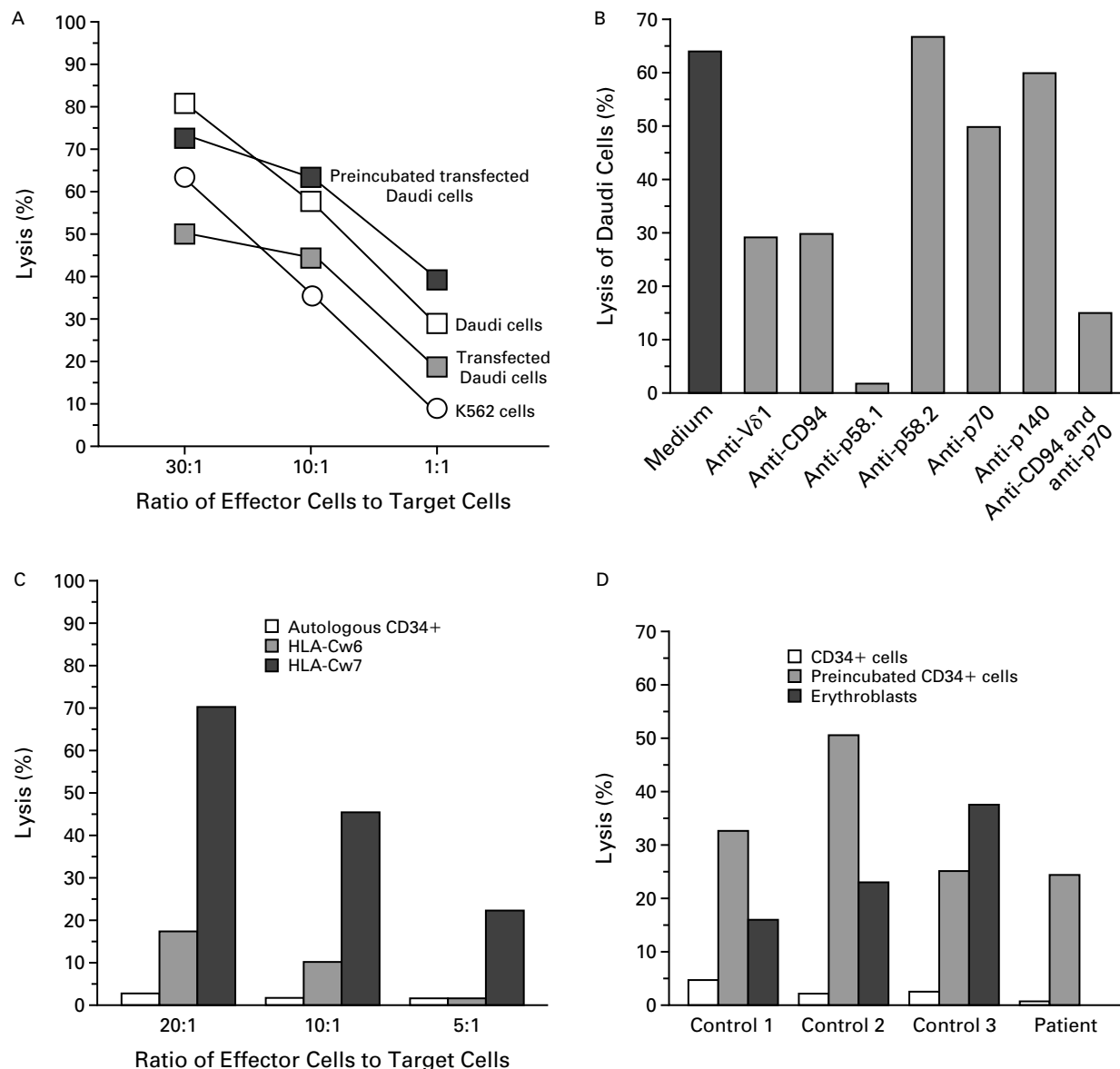


Figure 2. Cytolytic Activity of the Patient's Large Granular Lymphocytes and Function of Their Killer-Cell Inhibitory Receptors.

Panel A shows the ability of the patient's lymphocytes at three ratios of effector cells to target cells to lyse two tumor-cell lines that lack HLA class I antigens — K562 (erythroleukemia) and Daudi (Burkitt's lymphoma) — as well as Daudi cells that were transfected with HLA class I antigens. Before being exposed to the patient's lymphocytes, the transfected Daudi cells were incubated with a monoclonal antibody against HLA-A, B, and C (B1.23.2), which increased the cytolytic activity to a level similar to that of the parental Daudi cell line. Panel B shows the ability of the patient's lymphocytes to lyse untransfected Daudi cells at a ratio of effector cells to target cells of 20:1 in the absence (Medium) and in the presence of monoclonal antibodies against V δ 1; the CD94, p58.1, p58.2, p70, and p140 killer-cell inhibitory receptors; and a combination of the antibodies against CD94 and p70. In Panel C, the patient's lymphocytes did not destroy autologous CD34+ cells isolated from the bone marrow at a ratio of effector cells to target cells of 20:1 or a B-cell line deficient in class I HLA-A, B, and C antigens that was transfected with HLA-Cw6. They did, however, lyse the B-cell line when it was transfected with HLA-Cw7. In Panel D, the patient's large granular lymphocytes lysed erythroblasts (ratio of effector cells to target cells, 20:1) but not CD34+ progenitor cells. Lysis of the CD34+ progenitors could be induced by incubating these cells with monoclonal antibody against HLA-A, B, and C antigens before exposing them to the patient's lymphocytes. Erythroblasts and CD34+ cells from three unrelated control subjects and the patient's own CD34+ progenitor cells were studied as target cells. The values in all four panels are the means of triplicate determinations, with a standard deviation that was less than 3 percent of the mean.

tors of the clonal cytotoxic γ/δ T cells suppressed the cytotoxic activity of the clone.^{7,8} Negative signaling of the natural-killer-cell receptors expressed by the patient's γ/δ T cells was documented experimentally when these receptors were cross-linked by specific antibodies bound to Fc receptors expressed by Daudi cells.⁷ However, these antibodies did not induce lysis of mouse P815 cells when cross-linked by Fc receptors expressed by P815 cells (data not shown). Thus, none of the HLA class I receptors expressed by the clonal γ/δ T cells functioned as killer-cell-activating receptors.^{2,5}

A B-cell line deficient in class I HLA-A, B, and C antigens was transfected with the HLA-Cw6 gene. This line was much less susceptible to cytolysis by the patient's γ/δ T cells²⁷ than were HLA-Cw7 transfectants of this line (Fig. 2C). The inhibitory effect of the HLA-Cw6 allele was due to the presence of the p58.1 killer-cell inhibitory receptor (which binds to HLA-Cw6) on the patient's γ/δ T cells, whereas the lack of inhibition by the HLA-Cw7 allele was due to the absence of the p58.2 receptor, which binds to HLA-Cw7. As in the experiments with the transfected Daudi cells, we found that autologous and allogeneic CD34+ hematopoietic progenitor cells could be made vulnerable to lysis by the patient's γ/δ T cells by preincubation of the CD34+ cells with antibodies against HLA class I antigens (Fig. 2C and 2D).²¹ The expression of HLA class I antigens on erythroid precursors (glycophorin A+ cells) in normal bone marrow was less than that on CD34+ progenitor cells

and myeloid cells (Fig. 3). The consequence of this difference is that erythroblasts, but not CD34+ cells, isolated from the bone marrow of normal donors were sensitive to lysis by the patient's γ/δ T cells (Fig. 2D). Moreover, levels of allogeneic HLA class I antigen were considerably lower in glycophorin A+ erythroid colonies than in myeloid progenitor colonies generated *ex vivo*,²⁸ and such erythroid progenitor colonies were lysed by the patient's large granular lymphocytes to a much higher degree than were the myeloid progenitors purified from the same cultures (data not shown). These characteristics appear to be similar to those of the erythroleukemia line K562, which is sensitive to natural killer cells, does not express HLA class I antigens,²⁶ and was also lysed by the patient's γ/δ T cells (Fig. 2A).

DISCUSSION

Purified CD34+ progenitor cells from our patient were able to differentiate into the erythroid and myelomonocytic lineages *in vitro*, indicating that a stem-cell defect was not the cause of the pure red-cell aplasia. In addition, elevated serum erythropoietin levels and the absence of antibodies against erythroid precursors suggested that humoral autoimmunity did not cause the patient's disease.^{9,12} Another important finding was a clonal proliferation of large granular lymphocytes¹⁷ of the γ/δ T-cell type. These cells were highly cytotoxic *in vitro* and expressed killer-cell inhibitory receptors for HLA class I antigens. These receptors functioned as inhib-

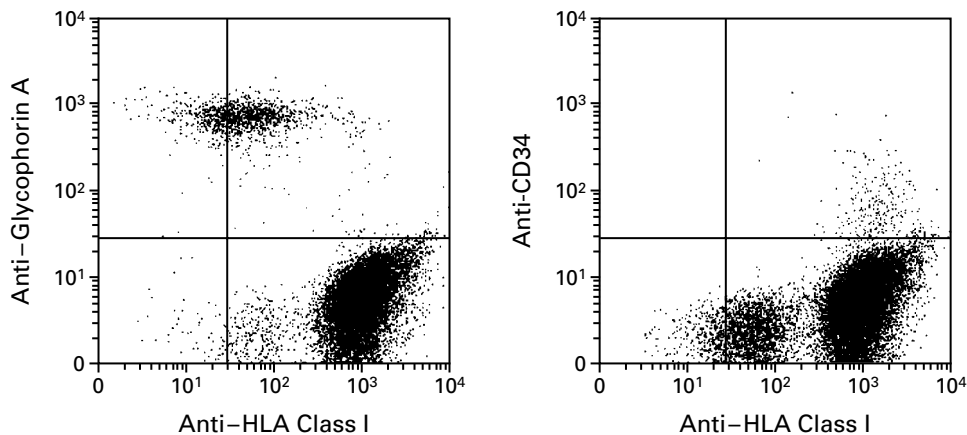


Figure 3. Expression of HLA Class I Molecules by Normal Erythroid Progenitors.

The expression of HLA class I molecules was evaluated in the following subpopulations of bone marrow cells from normal subjects: glycophorin A+ cells, representing erythroid progenitors (left-hand panel); immature CD34+ progenitors (right-hand panel); and myeloid cells (most of the remaining cells in the lower right quadrants with high expression of HLA class I molecules). Mononuclear cells were isolated on density gradients from bone marrow aspirates, followed by osmotic lysis of any remaining erythrocytes. Electronic gates were set on the blast fraction and analyzed by two-color flow cytometry after staining with an antibody against monomorphic determinants of HLA class I molecules (W6/32) that was conjugated to fluorescein and a monoclonal antibody against either glycophorin A or CD34 that was conjugated to phycoerythrin. The results shown are representative of bone marrow specimens from a total of eight normal subjects.

itory but not activating receptors, as shown by the effects of various monoclonal antibodies and transfected cells.

Many studies have documented the association of a proliferation of large granular lymphocytes with pure red-cell aplasia.^{10,13-18} In such cases, lymphocyte-mediated inhibition of erythropoiesis appears to be a major mechanism of the pathogenesis of the disease.^{9,10,13,17,18} Some patients with pure red-cell aplasia have elevated levels of large granular lymphocytes but do not fulfill the diagnostic criteria for large granular lymphocytic leukemia.¹⁷ In some of these patients, the large granular lymphocytes may be polyclonal.^{9,14,17} Our results suggest that killer-cell inhibitory receptors and reduced expression of HLA class I genes in the erythropoietic lineage account for the selective cytotoxicity of this patient's large granular lymphocytes against red-cell progenitors. Previous studies indicated that HLA-A, B, and C molecules are expressed by multipotent hematopoietic progenitor cells²⁹ and by erythroid colony-forming cells,^{19,20} whereas more mature erythroid precursors have reduced expression of HLA class I molecules. The finding that erythroid colony-forming cells were preserved but more mature glyophorin A+ precursors were absent from the bone marrow of our patient is compatible with the hypothesis that erythroid progenitors become sensitive to MHC-unrestricted lysis as the expression of HLA class I molecules is reduced.

Like natural killer cells, most cytotoxic γ/δ T cells express killer-cell inhibitory receptors, suggesting that both natural killer cells and γ/δ T cells survey the body for missing self HLA class I alleles.⁷ The finding that the large granular lymphocytes of this patient expressed the γ/δ T-cell receptor allowed us to determine clonality, and blocking of cytolysis by antibody against the γ/δ T-cell receptor in vitro identified the clonal V γ 4/V δ 1 T cells as the cytotoxic effector lymphocytes in the blood. The blocking of cytolysis does not prove that the γ/δ T-cell receptors are directly involved in antigen recognition, because the anti- γ/δ antibody may interfere with the recognition of an unidentified ligand for the γ/δ T-cell receptor, or deliver a negative signal to the γ/δ T cells.²³ Moreover, the killer cells of most patients with pure red-cell aplasia associated with increases in large granular lymphocytes do not express the γ/δ T-cell receptor.^{9,17} For these reasons, we believe that our patient's pure red-cell aplasia was not caused by the antigenic specificity of the unusual V γ 4/V δ 1 T-cell receptor.

The repertoire of killer-cell inhibitory receptors of natural killer cells differs from person to person.⁶ Natural killer cells appear to be selected to express at least one inhibitory receptor that binds to autologous HLA class I alleles; most clones have several such receptors.⁶ These inhibitory receptors normally

prevent self-reactivity of natural killer cells. In our patient, the relevant inhibitory HLA class I receptors were CD94 and p70. The p58.1 receptor was strongly inhibitory in vitro, but the natural ligand for this killer-cell inhibitory receptor (HLA-Cw2, Cw4, or Cw6) was not expressed; instead, the Cw7 and Cw8 alleles were expressed, which do not signal the killer cell through the p58.1 receptor.¹ Killer-cell inhibitory receptors are commonly expressed in the absence of a corresponding autologous HLA ligand in normal natural killer cells, probably because there is no selection against such inhibitory receptors.⁶ Thus, the inhibitory p58.1 receptor on this patient's large granular lymphocytes did not contribute directly to the pathogenesis of the pure red-cell aplasia. However, if the clonal large granular lymphocytes (which bind to the Cw7 allele) had expressed p58.2 killer-cell inhibitory receptors, this might have reduced lysis of autologous erythroid precursors and prevented a clinically apparent form of pure red-cell aplasia.

Our results imply that a clone of cytotoxic large granular lymphocytes expressing killer-cell inhibitory receptors can destroy erythroid progenitors in vivo because of the physiologic down-regulation of HLA class I antigens in the erythroid lineage. In addition to rejection of allogeneic bone marrow, a phenomenon known as "hybrid resistance,"³¹ some cases of aplastic anemia, autoimmunity induced by viral infections,^{2,30} and rheumatoid arthritis associated with increased numbers of large granular lymphocytes^{9,17} might be related to the expression of inhibitory or activating HLA class I receptors on cytotoxic effector cells.

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