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EARLY HEMOPOIETIC PROGENITORS IN THE PERIPHERAL BLOOD OF PATIENTS WITH SEVERE APLASTIC ANEMIA (SAA) AFTER TREATMENT WITH ANTILYMPHOCYTE GLOBULIN (ALG), CYCLOSPORIN-A AND G-CSF

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ABSTRACT

Background and Objective. We previously reported that patients with acquired severe aplastic anemia (SAA) treated with antilymphocyte globulin (ALG), 6-methylprednisolone, cyclosporin A (CyA) and granulocyte colony-stimulating factor (G-CSF) can mobilize peripheral blood hemopoietic progenitors (PBHP). The aim of the present study was to assess phenotypic and functional properties of these PBHP.

Methods. We studied seven patients who underwent 43 leukopheresis (median 5) between day +30 and +80 following ALG, while in treatment with CyA and G-CSF. Mobilized peripheral blood hematopoietic progenitors were analyzed using surface markers, conventional assays for clonogenic cells (CFU-GM, BFU-E, CFU-GEMM) as well as the recently developed assay for long-term culture initiating cells (LTC-ICs).

Results. The proportion of $CD34^+$ cells ranged between 0% and 5.4% (median 0.3%), $CD34^+DR^-$ between 0% and 3.5% (median 0.1%) and $CD8^+$ cells between 3.3% and 56% (median 31%). When light density mononuclear cells (MNC) were plated *in vitro*, we could grow colony-forming units-granulo-macrophage (CFU-GM) (range 0-45/10^5 MNC; normal controls 21-200/10^5 MNC), burst-forming units-erythroid (BFU-E)(range 0-5/10^5 MNC; normal controls 0-6/10^5 MNC), multipotent colonies (CFU-GEMM)(range 0-3/10^5 MNC; normal controls 0-6/10^5 MNC) and high proliferative potential colony-forming cells (HPP-CFC) (range 0-3.4/10^5 MNC). We studied long-term culture-initiating cells (LTC-ICs) in 18 leukophereses from 4 patients; in 7/18 samples LTC-ICs were grown at low frequency (range 0.4-2/10^6 MNC) (normal controls 5-130/10^6 MNC), and in one patient in the absence of CFU-GM growth. The total yield of LTC-ICs in two patients was 7.64 and 10.5×10^2/kg of body weight.

Interpretation and Conclusions. This study suggests that cells with the phenotype and *in vitro* function of early hemopoietic progenitors are found, though in small numbers, in the peripheral blood of patients with SAA after treatment with immunosuppressants and prolonged G-CSF administration. Whether G-CSF-mobilized progenitors contribute to hemopoietic recovery in these patients remains to be determined.

Key words: severe aplastic anemia, stem cells, hematopoiesis, immunosuppressive therapy

Proposed mechanisms for the pathogenesis of acquired severe aplastic anemia (SAA) include an intrinsic abnormality of the hemopoietic stem cell,[1,2] a defective microenvironment[3] and immune suppression of bone marrow function,[4] one not necessarily being mutually exclusive of the others.[5] Whatever the pathogenesis, the result is a severe depletion of the hemopoietic progenitor cell compartment with reduced *in vitro* colony formation[6] and pancytopenia. It is more difficult to quantify the degree of depletion at the level of the stem cell, since we have been as yet unable to test for these cells *in vitro*.[7] However, a few surviving stem cells would suffice for hematologic reconstitution, as suggested by experimental data obtained in animals.[8] Indeed long-term hematologic recovery is seen in over 50% of SAA patients following immunosuppressive therapy (IS), and this figure may be closer to 80% when G-CSF is added to ALG and cyclosporin-A,[9] suggesting that most SAA patients do have residual stem cells.

The function and phenotype of these surviving hemopoietic progenitors (HP) is currently being studied; abnormal response to growth factors,[10] overexpression of the Fas antigen in bone marrow $CD34^+$ cells,[11] lack of glycosyl-phosphatidyl-inositol (GPI)-associated proteins[12] have all been described. Unfortunately, one major problem for years has been the paucity of cells available for such studies. We have recently shown that prolonged *in vivo*

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administration of G-CSF in SAA patients leads to mobilization of hemopoietic progenitors, which are then available for analysis and can actually be harvested at weekly leukophereses and cryopreserved.[13]

We therefore studied mobilized PBHP using surface markers, conventional assays for clonogenic cells (CFU-GM, BFU-E, CFU-GEMM) as well as the recently developed assay for long-term culture-initiating cells (LTC-ICs): these are primitive hemopoietic cells capable of producing clonogenic progenitors after a minimum of 5 weeks of culture in the presence of irradiated human marrow fibroblasts or engineered cell line.[14,15] Human LTC-ICs share many of the characteristics of murine LTC-ICs, which have proven *in vivo* long-term repopulating ability.[16,17]

We report here the results of these studies on PBHP from seven SAA patients who underwent 43 leukophereses following ALG, while in treatment with CyA and G-CSF.

Materials and Methods

Patients

Clinical data on the patients are presented in Table 1. Seven patients with idiopathic SAA were treated with ALG (Pasteur Merieux, Lyon, France) followed by CyA (Sandoz, Basel, CH) and G-CSF (Hoffman-La Roche LTD, Basel, CH) administration according to an EBMT protocol for SAA 9. Several leukapheresis were performed weekly using a continuous Fenwal CS 3000 Flow Cell Separator (Deerfield, USA) 30 days after ALG, while in treatment with G-CSF.

Response

Patients were classified as complete responders if they were transfusion independent with a hemoglobin level of ≥ 11 g/dL, a neutrophil count greater than 1.5×10^9/L, and a platelet count greater than 100×10^9/L; partial responders were transfusion independent with a hemoglobin level of ≥ 8 g/dL, a neutrophil count of $>0.5\times10^9$/L, and a platelet count of $>20\times10^9$/L. Persistence of transfusion dependence was taken as evidence of no response.

Normal donors

Sixteen leukaphereses from 7 normal donors treated with G-CSF (10 ug/kg/day for 5 days) were used as controls. These volunteers underwent the procedure as matched donors for their HLA-identical sibling. Informed consent was obtained from SAA patients and normal donors, and investigations were approved by our Ethical Committee.

Flow cytometry

PB cells recovered from each leukaphereses were processed with a work station Coulter Q-Prep (Coulter Corporated Hialeah, FL) in order to lyse erythrocytes and to fix white blood cells. Cell surface antigens were detected by direct immunofluorescence using a CD34 HPCA-2 for progenitor cells (Becton Dickinson, Mountainview, CA). Fluorescence was analyzed with an XL Coulter. Isotypically matched mouse Ig directly conjugated to fluorescein isothiocyanate (FITC) or phycoerythrin (PE) were used as negative controls in all experiments. Overall, 10^4 events were acquired on an FS (Forward Scatter) x SS (Side Scatter) diagram on all populations except platelets and debris. Immediately after staining, cells were sorted on a Coulter Epics 753 dual laser flow cytometer (Coulter Electronics, Hialeah, FL) equipped with a CICERO high-speed computer (Cytomation, Fort Collins, CO). FITC and PE were excited using the 488nm wavelength from a dedicated 5 W argon laser. In this staining protocol, it was still possible to obtain $CD34^+$ $HLA\text{-}DR^+$ cells, even though both HLA-DR (IgG1 isotype, Becton-Dickinson-Immunocytometry System) and CD15 were FITC conjugated (FITC-conjugated mouse antihuman-LeuM1, Becton-Dickinson).[18]

Table 1. Clinical data from patients and hematologic response to treatment.

Pts	Age/Sex		Interval DxTx days	PB counts at first IS PMN x 10^9/L	Plt	Hem.	Infect.	Resp.	Status (Fu days)
1	18	M	60	0.03	5	Y	Y	PR	AW 1006
2	22	F	703	0.1	10	N	N	no-resp	D 243
3	20	M	40	0.4	5	Y	N	PR	AW 916
4	50	F	373	0.2	10	N	N	PR	AW 852
5	18	F	49	0.09	10	Y	Y	CR	AW 973
6	17	F	24	0.1	10	Y	Y	CR	AW 566
7	33	M	30	0.3	5	N	N	PR	AW 526

DxTx, diagnosis-treatment; PR, partial response; CR, complete response; TxI, transfusion-independent; M, male; F, female; PMN, neutrophils; Plt, platelets; AW, alive and well; Fu, follow-up; D, deceased; Hem, hemorrhages; Infect., infections.

Functional assays

Light density cells from each leukapheresis were assayed for clonogenic precursors. Briefly 10^5 MNC were plated in:

A. 1.1 mL consisting of Iscove's modified Dulbecco's medium (IMDM, GIBCO BRL, Life Technologies LTD, UK) + 0.9% methylcellulose (Sigma Chemical, St.Louis, MO, USA) + 30% fetal calf serum (FCS, Gibco) + 100 ng of rhGM-CSF (Sandoz, Basel, CH);

B. 1.5 ml consisting of IMDM + 0.9%methylcellulose + 30% FCS + 1% bovine serum albumin (BSA, Sigma) + 10^{-4} M mercaptoethanol (ME, Sigma) + rhGM-CSF (10 ng) + rhIL3 (10 ng Sandoz) + rhG-CSF (10 ng) + rhEPO (4U Cilag AG, Schaffhauser, CH) + SCF (50 ng Genzyme Corporation, Cambridge, UK). After 14 days of incubation in a humidified atmosphere at 37°C in 5% CO_2, colonies were classified and counted using an inverted microscope (Zeiss, Germany). Sorted cells were cultured in the presence of PIXY and SCF as described.[18] MNC and freshly sorted cells were cultured in suspensions containing different cytokines and scored according to previously established criteria.[19]

LTC-IC assays were performed by seeding an aliquot (usually 5×10^6 MNC/flask) of light density cells over a feeder layer of irradiated (1.5 cGy) normal marrow cells. These were subcultured from adherent layers of previously established 4-week-old LTCs. LTC-ICs were maintained for 3 days at 37°C, then switched to 33°C and fed weekly by replacing half the growth medium (IMDM + 12.5% horse serum (HS, Gibco) + 12.5% FCS + 10^{-4}M ME + 10^{-6}M hydrocortisone, Sigma) containing half of the nonadherent cells with fresh growth medium. After 5 weeks, adherent cells were trypsinized and combined with the nonadherent fraction. These harvested cells were washed and assayed for clonogenic precursors in standard methylcellulose cultures (B). As previously reported,[14] the number of LTC-ICs present in the starting cell suspension was shown to be equal to the total CFC content of the 5-week-old LTC assay divided by 4.

Statistical analysis

Chi-square, Student's t-test and Mann-Whitney rank sum test were used to analyze the data.

Results

Patients

All patients completed the designed course of IS treatment and underwent leukaphereses, which

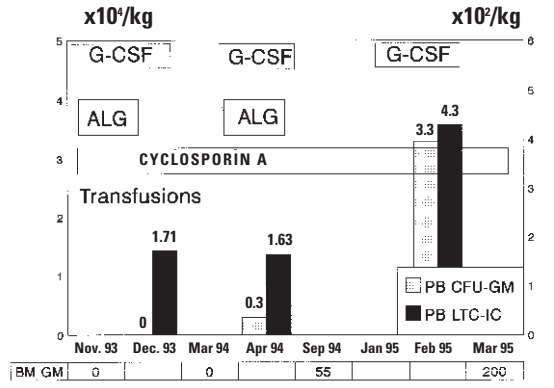

Figure 1. Diagram of patient #3 treated between November 1993 and February 1995 with antilymphocyte globulin (ALG) (2 courses), cyclosporin-A and granulocyte colony-stimulating factor (G-CSF) (3 courses). He was dependent on transfusions until March 1994. Dashed bars represent peripheral blood CFU-GM $\times 10^4$/kg; they were 0 in December 1993 (first set of leukaphereses), 0.3×10^4/kg in April 1994 (second set), and 3.3×10^4/kg in February 1995 (third set). LTC-IC (solid bars) were, respectively, 1.71, 1.63 and 4.3×10^2/kg. Bone marrow CFU-GM growth (data table, bottom line) was absent in December 1993 and March 1994; CFU-GM were $55/10^5$ mononuclear marrow cells (MNC) in September 1994 and $200/10^5$ MNC cells in March 1995.

were scheduled weekly starting on day +30 of treatment. One patient who did not become transfusion independent after a first course was treated again with a second course of ALG, CyA and G-CSF, and then a third time with CyA and G-CSF. He therefore underwent 3 courses of leukaphereses (Figure 1). Six of the seven patients are still alive at 566 to 1006 days after their first course of IS. One patient died of infection on day +243.

Flow cytometry

Flow cytometry demonstrated a higher frequency of $CD3^+$ cells (Table 2) as compared to normal controls (median 49% vs 39%; p=0.006) with an increased proportion of $CD8^+$ cells (median 31% vs 13%; p=0.004). The median CD4/CD8 ratio was 0.62 in SAA and 1.87 in normal controls (p=0.008). The proportion of $CD34^+$ and $CD34^+DR^-$ cells did not significantly differ from normal donors (p=0.4 and p=0.37). In three patients tested, $CD34^+DR^+$ cells ranged between 1.0-1.7% and $CD34^+DR^-CD15^-$ between 0-0.1%.

Committed progenitors

Samples from each apheresis were assayed for *in vitro* granulocytic, erythroid and multipotent progenitors (CFU-GM, BFU-E and CFU-GEMM). In our experiments we were able to grow mainly CFU-GM, with very low numbers of other colonies (in percentages: 25-100% CFU-GM, 0-30% BFU-E and 0-19% CFU-GEMM). Five patients showed significant increments in white blood cell (WBC) counts ($>10\times10^9$/L, Table 3) while in treatment with G-CSF; the WBC count did not correlate with the number of CFU-GM recovered (p=0.8). Table 3 outlines the median yield of PBHP collections from SAA patients. In normal controls receiving G-CSF the yields were as follows: MNC ranged between 7.7-15.4/10^8/kg; $CD34^+$ ranged between 1.5-30.5/10^6/kg and CFU-GM between 6.7-121.5/10^4/kg. In patient #3 we harvested no CFU-GM during the first course of IS, 0.3×10^4 kg CFU-GM during the second course, and 3.3×10^4 kg during the third course of G-CSF treatment (Figure 1), in spite of similar numbers of $CD34^+$ cells (Table 3).

Early progenitors

HPP-CFC were studied in 4 patients (#1, 4, 5, 6); they grew at a frequency of 0-3.4/10^5 MNC, in one case in the absence of committed progenitors.

In addition, we studied LTC-ICs in 18 leukaphereses from 4 patients: in 7/18 samples LTC-ICs were present at low frequency (range: 0.4-2/10^6 MNC) when compared with collections from G-CSF-treated normal donors (range: 5.4-130/10^6 MNC); p<0.00001) (Table 4) or with normal marrow (range: 10-238/10^6 MNC). In patient #3 we grew a significant number of LTC-ICs (1.71×10^2/kg) during the first set of leukaphereses in spite of the absence of CFU-GM (Figure 1). In the second and third set of leukaphereses we recovered 1.63×10^2/kg and 4.3×10^2/kg LTC-ICs. The total yield of LTC-ICs from patients #3 and 6 was 7.64 and 10.5×10^2/kg, respectively.

Cell sorting

Cells derived from 3 aphereses were sorted into $CD34^+DR^+$ and $CD34+DR^-CD15^-$; these were then cultured *in vitro* with different cytokine combinations. $CD34^+DR^+$ cells showed evident increments in GM, GEMM, BFU-E and HPP colony formation (Table 5). This was not the case when $CD34^+DR^-CD15^-$ cells were sorted and plated: in two patients we could grow only small numbers of CFU-GM but no other lineage restricted or HPP colonies.

Discussion

It has been always difficult to design *in vitro* colony assays in patients with SAA, mainly because of the lack of bone marrow cells to initiate such studies. We have described the presence of hemopoietic progenitors in the peripheral blood of many SAA patients after treatment with ALG-CyA and prolonged administration of rhG-CSF.[13] By performing weekly leukaphereses in such patients, one can collect large numbers of cells which can be cryopreserved and are, at the same time, available for *in vitro* assays. The aim of the present study was to assess phenotypic and functional properties of such PBHP.

As regards the phenotype, SAA PB cells contained large numbers of $CD3^+$ cells, significantly more than

Table 2. Phenotype of PB collection in SAA patients and normal donors.

	SAA (n=43) median	(range)	NPB (n=16) median	(range)	p
CD3 %	49.3	(4.5-77)	39.4	(18.4-49.8	0.006
CD %	19.4	(1.6-40.2)	25.3	(10.4-32.4)	0.4
CD8 %	31	(3.3-56.6)	13.5	(7.9-19.4)	0.004
CD4/CD8 ratio	0.62	(0.2-1.1)	1.87	(1.4-19.4)	0.008
CD34 %	0.3	(0-5.4)	0.3	(0.1-1.3)	0.4
$CD34^+DR^-$ %	0.1	(0-3.5)	0.1	(0-0.5)	0.37

SAA: severe aplastic anemia; NPB: normal peripheral blood; p: Mann-Whitney rank sum test.

Table 3. Total harvest of blood progenitors from SAA patients.

pts	Apheresis n.	max WBC counts $x10^9/L$	MNC $x10^8/kg$	CFU-GM $x10^4/kg$	CD34+ $x10^6/kg$
1	7	24	5.6	1.2	0.55
2	6	2.8	6.35	0.72	1.09
3a	5	22	8.19	0	3.5
3b	3	16.7	2.01	0.3	5.82
3c	4	12	9.13	3.3	3.13
4	5	24	7.55	2.37	4.7
5	5	15	6.94	6	0.89
6	4	22	14.1	7.74	2.39
7	4	8.9	2.22	3.59	0.99
Pts (median)	5	16.7	6.94	3.49	1.09
Controls (median)	2	38	12.8	18.3	8.7
p value		0.05	0.1	0.05	0.4

WBC: white blood cells; MNC: mononuclear cells; 3a, b and c: 3 subsequent courses of G-CSF treatment; CFU-GM: colony forming units-granulo-macrophage; p=Mann-Whitney rank sum test.

Table 4. Frequency of different type of progenitors in SAA and NPB collections.

Colonies	SAA (n=43) median	(range)	NPB (n=16) median	(range)	p
CFU-GM (x 10^5MNC)	2	(0-45)	56	(21-200)	<0.0001
BFU-E (x 10^5MNC)	0	(0-5)	1	(0-6)	0.1
CFU-GEMM (x 10^5MNC)	0	(0-3)	1	(0-6)	0.1
LTC-IC (x 10^6MNC)	0*	(0-2)	24	(5.4-130)	<0.00001

*Abbreviations. As in Table 2; BFU-E: burst-forming units-erythroid; CFU-GEMM: colony-forming units-granulo-erythroid-macrophage-megakaryocytic; LTC-IC: long-term culture-initiating cell; NPB: normal peripheral blood primed with G-CSF; *n=18.*

controls (p=0.006), with an inverted CD4/CD8 ratio. This is in keeping with reports of an excess of suppressor cells in SAA,[20,21] suggesting that the CD4/CD8 ratio is not modified by G-CSF priming. As for the phenotype of progenitors cells, the overall percentage of $CD34^+$ and $CD34^+DR^-$ cells did not significantly differ from normal donors (p=0.4 and p=0.37). This was an unexpected finding since the proportion of $CD34^+$ cells has been reported to be significantly reduced in bone marrow cell suspensions from SAA patients, especially at presentation of the disease, or in patients with very low neutrophil counts.[22] On the other hand, in this study CFU-GM formation from PB cells was significantly reduced in SAA as compared to controls, thus suggesting a poor correlation between surface phenotype and plating efficiency. We have seen a similar lack of yields at leukaphereses while on G-CSF therapy in patients with low white blood cells counts ($<1\times10^9$/L). This raises the question of surface markers and optimal culture conditions for progenitors from patients with stressed and/or suppressed hemopoiesis; the markers in use are derived from normal controls with steady state hemopoiesis, but several differences may exist in both phenotype and functional status.

Table 5. Colony formation of unfractioned and FACS sorted cells from 3 patients.

cells plated	CFU-GM	BFU-E	CFU-GEMM	HPP
Mononuclear cells (x 10^5)	14*	3	0.3	1.6
$CD34^+DR^-$ (x 10^5)	466	233	16	66
$CD34^+DR^-CD15^-$ (x 10^5)	25	0	0	0

**No. of colonies; mean values of 3 patients; cytokines = SCF (100 ng) + PIXY (10 ng) + EPO (1 U). Abbreviatiations as in Table 4.*

When SAA cells were plated *in vitro* we grew prevalently CFU-GM in numbers which were significantly lower than in normal controls, with a median 28-fold reduction. BFU-E and CFU-GEMM were seen less frequently in both SAA patients and normal controls, possibly as a result of *in vivo* triggering with G-CSF.

LTC-ICs were studied in 18 leukaphereses and scored in 7 of these, though in small numbers: they were significantly fewer than in controls (0-2 vs 5-130/10^6 MNC). In one of these patients LTC-ICs were present in the blood in the absence of peripheral blood and marrow CFU-GM, and this was also seen for HPP colonies. Therefore primitive progenitors may be present in the absence of committed progenitors, as previously suggested by hematologic reconstitution in most SAA patients in spite of no colony formation from bone marrow cells.[6]

Recently, a non cycling state of peripheral blood normal progenitors, primed with G-CSF, has been suggested[23] and this may be more evident in patients with SAA, who have a larger proportion of progenitor cells in G0.[22] In addition, we do not know whether SAA LTC-ICs are maintained *in vitro* in the same culture conditions as normal LTC-ICs

or whether their survival is limited.

When we assessed the total yield of leukapheresis from SAA patients we recovered reduced numbers of CFU-GM (p=0.05), although SAA patients underwent a median of 5 vs 2 leukapheresis performed with normal controls. It is difficult to say whether these cryopreserved cells are suitable for an autotransplant, a possibility one may wish to consider in the case of poor or absent hematologic reconstitution or in the case of evolution of the disease into overt myelodysplasia or leukemia. High-dose chemotherapy has been shown do induce complete remissions when given to SAA patients, though at a cost of prolonged periods of neutropenia;[24] the infusion of autologous cryopreserved progenitors may shorten the duration of chemotherapy-induced aplasia. The total CFU-GM content recovered from leukapheresis was in the range reported to allow hemopoietic reconstitution if infused after ablative chemo-radiotherapy, and so was the LTC-IC content in 2 out of 4 patients studied.

Another important issue may be to consider blood progenitor cell mobilization as a prediction or as a measure of clinical improvement. Indeed two complete responders (#5 and #6) mobilized high numbers of CFU-GM; one patient (#2) mobilized very few CFU-GM and showed no response. In patient #3 clonogenic progenitors appeared in the blood after the second course of IS treatment and he achieved a durable response after the third course. This suggests that the presence of committed and early progenitors in the blood during IS treatment may be predictive of clinical response, in keeping with data reported by Torok-Storb.[25] Finally, early hemopoietic progenitors mobilized with G-CSF may reseed the marrow[26] and contribute to hematologic recovery in SAA patients undergoing treatment with ALG and Cy A, as indicated by the high response rate seen with this regimen and a survival rate greater than 90%.[9] It may be possible to prove this hypothesis by using the neomycin resistance gene to mark circulating progenitors.[27]

In conclusion, this study suggests that very early progenitors are found in the peripheral blood of patients with SAA after prolonged G-CSF administration and after treatment with IS therapy. The number of such early precursors is small, and the majority of cells exhibit the features of committed progenitors. It remains to be determined whether these cells contribute to hematologic recovery and whether they can be used for autografting.

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