Anti-erythroblast autoimmunity in early myelodysplastic syndromes

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ABSTRACT

Background and Objectives

Autoimmune phenomena, mainly directed against red blood cells are described in myelodysplastic syndromes (MDS), particularly early MDS, i.e. refractory anemia (RA) and RA with ringed sideroblasts (RARS). Dysregulation of apoptosis and immunoregulatory cytokines are thought to play a role in the pathogenesis of MDS.

Design and Methods

This work was aimed to investigate anti-erythroid autoimmunity in unstimulated and mitogen-stimulated peripheral blood and bone marrow cultures of 26 patients with early MDS (RA and RARS), and to relate its presence with apoptotic markers and cytokine production. Bone marrow cytokine production in culture supernatants, and caspase-3 and nuclear factor-κB activity in cell extracts were tested by enzyme-linked immunosorbent assays.

Results

Fourteen of the 26 (53.8%) patients showed the presence of autoantibodies in bone marrow cultures, whereas none displayed a positive direct antiglobulin test in peripheral blood cultures. Incubation of culture supernatants from positive patients with autologous CD45⁻ enriched-cell suspensions showed that the autoimmune reaction was directed against autologous erythroblasts. These patients had mild signs of hemolysis and increased numbers of erythroblasts, compared with negative patients. Patients with anti-erythroblast autoimmunity displayed higher caspase-3 activity and lower tumor necrosis factor-α and interleukin-4 production than did negative patients.

Interpretation and Conclusions

Half of the patients with early MDS showed autoimmunity against erythroblasts. This evidence might support a more rationale use of steroid therapy in these patients. The lower levels of cytokines in patients with anti-erythroblast autoimmunity are consistent with the suggested hypothesis that the autoimmune phenomena observed in MDS are probably initiated and perpetuated through alterations of pro-inflammatory and/or immunoregulatory cytokine production.

Key words: bone marrow, autoimmunity, MDS, erythroblast.

Myelodysplastic syndromes (MDS) are heterogeneous clonal hematopoietic stem cell disorders, characterized by ineffective and dysplastic hematopoiesis with peripheral cytopenias and an increased probability of developing acute leukemia.[1] There is accumulating evidence that excessive apoptosis may have a role in the ineffective hematopoiesis and cytopenias observed in MDS. In addition, recent investigations have focused on the possible immune-mediated marrow failure of MDS, particularly in early MDS, i.e. refractory anemia (RA), and RA with ringed sideroblasts (RARS).[2] MDS share some of the features of aplastic anemia, a disease with an established autoimmune pathogenesis.[3] Patients with RA and RARS show a higher incidence of anti-erythrocyte allo/autoantibodies,[4-6] and manifest overt non-organ-specific autoimmune disorders.[7,8] In addition, MDS patients with cytopenia may have a hematologic response to immunosuppressive therapy with antithymocyte globulin, a therapy also effective in aplastic anemia. In MDS this therapy is associated with loss of lymphocyte-mediated inhibition of granulocyte-macrophage colony-forming units and alterations in T-cell receptor Vβ profiles.[9]

The role of immunoregulatory cytokines in autoimmunity has been extensively investigated. It has been demonstrated that Th1 cytokines [interleukin (IL)-2, interferon (IFN)-γ, and IL-12] can up-regulate T-lymphocyte-mediated autoimmunity, whereas cytokines involved in humoral immunity (IL-4, IL-6, IL-10, and IL-13), and those that inhibit Th1 immune responses [transforming growth factor (TGF)-β and IL-4 can protect from autoimmunity.[10,11] Several groups have shown increased tumor necrosis factor (TNF)-α levels in the marrow microenvironment and peripheral blood of MDS patients, these increased levels being an indicator of poor prognosis.[12-14] More recently, an overproduction of TGF-β was found in patients with advanced MDS, suggesting a pathogenic role of this potent inhibitory cytokine in MDS-associated cytopenias.[15]

We have previously described a new quantitative method for the detection of anti-red blood cell (RBC) antibodies in unstimulated and mitogen-stimulated whole blood cultures, named the mitogen-stimulated direct antiglobulin test (MS-DAT). The test was suitable for revealing cytokine modulation of anti-RBC antibody production in autoimmune hemolytic anemia (AIHA), and anti-RBC autoimmunity in AIHA in clinical remission and in DAT-negative AIHA.[16] Furthermore, we have found that one third of patients with B-chronic lymphocytic leukemia (CLL) have a positive MS-DAT, suggesting the existence of an underestimated latent autoimmunity in this disease.[17] Altogether these findings indicate that this quantitative test, through antibody production *in vitro* is able to disclose a hidden autoimmunity. The aim of this study was to investigate anti-erythroid cell autoimmunity in early MDS, both in peripheral blood (PB) and bone marrow (BM) and to relate its positivity with apoptotic markers and cytokine production.

Design and Methods

Patients

Twenty-six patients with early stage MDS (RA and RARS) (mean age±SD: 72±11 years, range 40-91; 9 women and 17 men) were studied. The control group for MS-DAT and cytokine production consisted of 23 subjects with miscellaneous non-malignant hematologic conditions. BM aspirates and PB were collected from patients and controls during diagnostic procedures after obtaining informed consent and approval from the institutional Human Research Committee. The procedures followed were in accordance with the Helsinki international ethical standards on human experimentation.

Cell purification and cultures

BM mononuclear cells from MDS patients and controls were isolated after centrifugation by Ficoll-Hypaque density gradient. Cells were plated in complete culture medium RPMI 1640 (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10 mM Hepes, 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 10% heat-inactivated fetal calf serum (FCS) (HyClone Laboratories, Logan, UT, USA), penicillin 100 units/mL and streptomycin 100 μg/mL (Gibco Laboratories). To obtain an erythroblast enriched population (CD45⁻ cells), BM aspirates were treated with *Easy Sep Human CD45 Depletion Cocktail* (Stem Cell Technologies, Vancouver, BC, Canada). Briefly, cells were resuspended to $10^7/\mu L$ in phosphate buffered saline (PBS) containing 2% FCS and 1mM EDTA. Anti-CD45 antibodies (50 μl/mL) were added to the cell suspension and incubated at room temperature for 15 min, then magnetic nanoparticles (100 μL/mL) were added. After incubation at room temperature for 10 min, CD45⁻ cells were obtained by depletion. Cytofluorimetric analysis assessed that the CD45⁻ population was 90% pure.

MS-DAT test (RBC- or BM-bound IgG quantification

To detect the presence of RBC- or BM-bound IgG, fresh heparinized blood or BM samples were diluted 1:6 with RPMI 1640 medium and either stimulated or not with 2 μg/mL phytohemoagglutinin (PHA) (Sigma) and 20 ng/mL phorbol-12-myristate-13-acetate (PMA) (Sigma) in 24-well plates and incubated for 48 hours according to previous experiments.[18] After 48 hours of culture, the MS-DAT was conducted on cellular pellets and supernatants were collected for cytokine determination by enzyme-linked immunosorbent assay (ELISA). RBC- or BM-bound IgG was quantified using a competitive solid-phase enzyme immunoassay, as previously described.[16,17] Briefly, 96-well vinyl assay plates (Costar,

Cambridge, MA, USA) were coated with 50 μL of human IgG (Sclavo, Italy) overnight at +4°C, then washed three times and blocked with 200 μL of 2% FCS-PBS for 2 hours at room temperature. The cultured cell suspension was then washed three times and incubated with peroxidase-conjugated rabbit anti-human IgG (Dako, Denmark) diluted 1:3,000 in 0.2% FCS-PBS at 37°C for 30 min. One hundred microliters of this mixture were added to the IgG-coated plates, and incubated at 37°C for 30 min. The same procedure was applied to 100 μL of 2-fold dilutions of human IgG, to create a reference curve. After five washes, 50 μL of o-phenylenediamin dihydrochloride (Sigma) were added to each well. The colorimetric reaction was measured at 450 nm after 20 min with an ELISA spectrophotometer. A reference curve (log/log plot) was constructed with optical density values and IgG antibody concentration (ng/mL), and the value of RBC- or BM-bound IgG was calculated referring to this curve.

To characterize the target cells of BM autoimmunity, both CD45$^-$ and CD45$^+$ cells derived from BM-bound IgG positive and negative patients, and CcDee RBC were incubated for 30 min at 37°C with supernatants from positive and negative patients, and with PBS. After this incubation, the test proceded as described above. To determine a cut-off for MS-DAT positive values, the mean of PHA- and PMA-stimulated cultures of 74 healthy blood donors was calculated, and the positivity for MS-DAT defined as a value exceeding the mean plus 3 SD (150 IgG ng/mL).

Enzyme-linked immunosorbent assay determinations

Cytokine production was measured in 48-hour PHA-stimulated BM culture supernatants using commercially available ELISA kits according to the manufacturers' instructions. IL-2 and TGF-β were purchased from Bender MedSystem (San Bruno, CA, USA); IL-4, IL-10, IFN-γ, and TNF-α were purchased from Sanquin (Amsterdam, The Netherlands).

Caspase-3 and NF-kB activity assays

For the determination of caspase-3 and NF-kB activity, BM total extracts were prepared from 10×10^6 cells (2×10^6/mL). The extracts were prepared using a Trans AM™ NF-κB kit (Active Motif Europe, Rixensart, Belgium) according to the manufacturer's instructions. Briefly, cells were washed once with cold PBS, and incubated for 30 min on ice in 50 μL of lysis buffer to which 0.25 μL of 1mM DTT and 0.5 μL of protease inhibitor cocktail had been added. The test-tubes were then vigorously mixed, and the homogenates were centrifuged at 4°C for 10 min at 13,000 rpm. Supernatants were stored at -80°C to be assayed for NF-κB activity with the transcription factor assay kit *TransAM™ NF-κB p65* and for caspase-3 enzymatic activity with the *Caspase-3 col-*
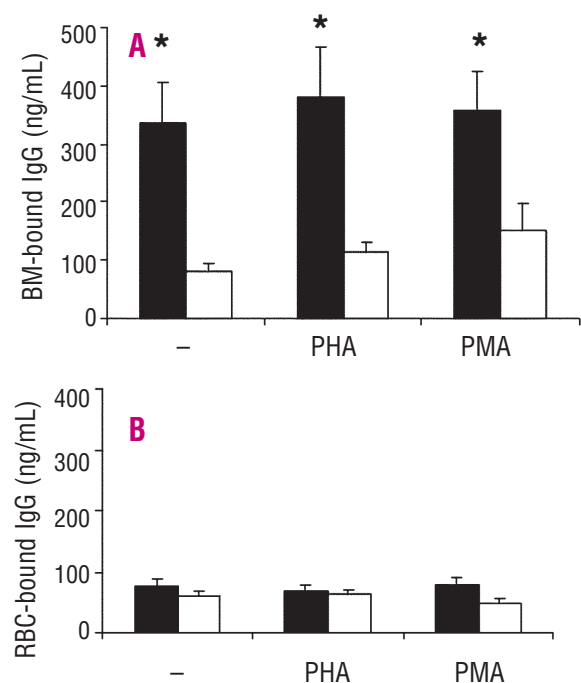

Figure 1. A. BM-bound IgG in MDS patients (∟) and controls (▫). Values are the mean ± SE of 26 patients and 23 controls with miscellaneous non-malignant hematologic conditions. *denotes statistically significant comparison, MDS patients versus controls (*p*=0.001 for unstimulated cultures, *p*=0.007 for PHA-stimulated cultures, *p*=0.02 for PMA-stimulated cultures). **B.** RBC-bound IgG in MDS patients (∟) and healthy blood donors (▫). Values are the mean ± SE of 23 patients and 74 blood donors.

orimetric assay kit (Apotech Corporation, Epalinges, Switzerland) following the manufacturers' instructions.

Statistical analysis

Continuous variables were analyzed by a two-tailed Student's t-test for paired and unpaired data, whereas differences in categorical variables were evaluated through the χ^2 test. A value of *p*<0.05 was considered statistically significant.

Results

BM-bound and RBC-bound IgG in MDS patients

Figure 1A shows the amount of BM-bound IgG in cultures from MDS patients and controls. IgG values were significantly higher in BM from MDS patients than in BM from controls in all the experimental conditions tested (*p*=0.001 for unstimulated cultures, *p*=0.007 for PHA-stimulated cultures, *p*=0.02 for PMA-stimulated cultures). In contrast, the amount of RBC-bound IgG in PB cultures from MDS patients was comparable to that in the PB cultures from controls in all the experimental conditions tested (Figure 1B). Mitogen stimulation did not

Table 1. Clinical and laboratory parameters of 26 MDS patients divided according to BM-bound IgG positivity.

	Positive	Negative	p values
No. of patients	14	12	
Gender (male/female)	9/5	8/4	n.s.**
Stage (RA/RARS)	7/7	9/3	n.s.**
IPSS (L/INT-1)	1/13	1/11	n.s.**
Therapy (0/I)	10/4	11/1	n.s.**
Hb (12-18 g/dL)	10±0.6	10.7±0.5	n.s.*
RBC (3.8-5.47 10^{12}/L)	3.2±0.2	3.3±0.2	n.s.*
MCV (81-100 fL)	95.2±2.4	92.3±7.2	n.s.*
WBC (4-9.1×10^9/L)	4.5±0.7	4.2±0.6	n.s.*
Plt (150-400×10^9/L)	167.6±21.7	146.9±23.6	n.s.*
Reticulocytes (16-84×10^9/L)	53.1±6.4	35.3±4.9	<0.05*
Unconjugated bilirubin (<0.75mg/dL)	1.0±0.2	0.6±0.05	<0.05*
LDH (230-460 U/L)	400±32	305±24	<0.05*
Haptoglobin (40-190 mg/dL)	63±14	144±23	<0.05*
Proerythroblasts (%)	2±0.6	0.77±0.3	<0.05*
Basophilic erythroblasts (%)	4.5±0.8	2.5±0.6	<0.05*
Polychromatic erythroblasts (%)	12.9±2.3	6±1.2	<0.05*
Orthochromatic erythroblasts (%)	22.2±3.3	13.6±2.5	<0.05*
Total erythroblasts (14.0-39.4 %)	40±5.4	22.8±4	<0.05*

(*) Students' t test; (**)χ^2 test, n.s.: not significant; 0: no therapy and I: steroid therapy. The reference values are given in parentheses; data for erythroblasts from Bain B, The bone marrow aspirate of healthy subjects, Br J Haematol 1996; 94:206-9.

increase the amount of IgG binding in either BM or PB cultures. Considering the cut-off value of 150 ng/mL, as defined in the Design and Methods section, 14/26 (53.8%) MDS patients showed BM-bound IgG positive values. All the MDS patients investigated had negative DAT in PB by standard techniques.

Clinical and laboratory parameters of BM-bound IgG positive and negative patients

Table 1 shows clinical and laboratory parameters of MDS patients, divided according to the presence of BM-bound IgG positive values. There was no difference in gender, MDS type, International Prognosis Scoring System (IPSS) index,[19] blood counts, or steroid therapy between groups. However, there were significant differences between the two groups in peripheral blood hemolytic parameters: BM-bound IgG positive patients had higher absolute numbers of reticulocytes (p<0.05),
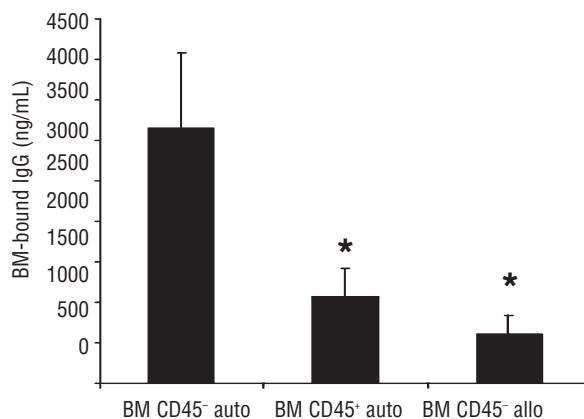

Figure 2. Target cell of BM-bound IgG positivity. Culture supernatants from five positive patients were incubated with autologous erythroblast-enriched (BM CD45⁻ auto), autologous myeloid cells (BM CD45⁺auto), and allogenic erythroblast-enriched (BM CD45⁻ allo) cell suspensions. *denotes statistically significant comparisons (p<0.05, BM CD45⁻ auto versus BM CD45⁺auto, and BM CD45⁻ auto versus BM CD45⁻ allo).*

unconjugated bilirubin levels (p<0.05), and lactate dehydrogenase levels (p<0.05), and lower haptoglobin levels (p<0.05) than did negative patients, although both groups had mean values within the normal range. Considering individual values of BM-bound IgG positive patients (Table 2), only four out of seven patients had at least three positive hemolytic markers, the other three patients had only two markers. In addition, the majority of patients (8/14) had isolated anemia, 6/14 patients also had neutropenia and only one patient had pancytopenia with non-severe thrombocytopenia. Considering BM counts, positive patients had higher percentages of proerythroblasts, basophilic, polychromatic, and orthochromatic erythroblasts than those of negative patients (p<0.05) (Table 1). There was no difference in erythroblast maturation or L/E ratio between the two groups. Considering the reference values, BM-bound IgG positive patients showed total values of erythroblasts at the upper limit of the normal range, whereas the other MDS subgroup had clearly reduced counts.

Target cells of BM-bound IgG positivity

To identify the possible BM target of the autoimmune response, we tested positive BM supernatants on CD45⁺ (myeloid cells) and CD45⁻ (non-myeloid cells). As shown in Figure 2, culture supernatants from BM-bound IgG positive patients showed greater binding to autologous erythroblast-enriched cell suspensions (CD45⁻ cells) than to autologous myeloid cells (CD45⁺) (p<0.05). In addition, the reactivity of positive BM supernatants with allogenic erythroblasts was clearly less than that with autologous cells (BM CD45⁻ allogenic cells vs BM CD45⁻ autologous cells, p<0.05). Finally, positive BM supernatants showed negligible binding on autologous and allogenic RBC (*data not shown*). The negative con-

Table 2. Individual clinical and laboratory parameters of 14 BM-bound IgG positive MDS patients.

| Patients	Gender	Stage	Hb M (14-18 g/dL) F (12-16 g/dL)	RBC (3.8-5.47×10¹²/L)	WBC (4-9.1×10⁹/L)	N (1.8-7.7×10⁹/L)	Plt (150-400×10⁹/L)	Retics (16-84×10⁹/L)	Unconj Bilirubin (<0.75 mg/dL)	LDH (230-460 U/L)	Haptoglobin (40-190 mg/dL)
1	M	RARS	8.1	2.3	6.6	3.3	101	59.0	2.8	322	24
2	M	RARS	9.5	3.6	7.1	3.7	291	37.0	0.7	345	44
3	M	RARS	8.0	2.5	2.4	0.4	135	32.0	1.6	364	34
4	F	RARS	10	3.1	2.9	1.0	292	37.0	0.9	354	30
5	F	RA	10.5	2.9	2.0	0.6	156	36.0	0.5	362	132
6	F	RA	11.5	3.8	10.8	7.6	219	86.2	0.3	341	147
7	M	RA	13.9	5.3	3.6	1.4	89	31.7	0.5	354	123
8	M	RA	5.6	1.9	1.6	0.8	54	39.0	0.8	229	54
9	F	RA	9.8	2.8	2.2	0.8	141	99.0	0.6	543	15
10	M	RA	12.3	3.8	5.2	2.1	85	68.0	0.6	388	150
11	F	RARS	10.4	3.1	5.9	3.9	198	71.8	1.5	527	15
12	M	RARS	9.8	3.1	4.5	2.7	98	31.0	1.0	569	15
13	M	RA	11.8	3.8	5.3	3.7	206	80.0	1.8	649	15
14	M	RARS	8.4	2.2	3.5	2.2	281	35.8	0.5	264	84

Reference values are given in parentheses.
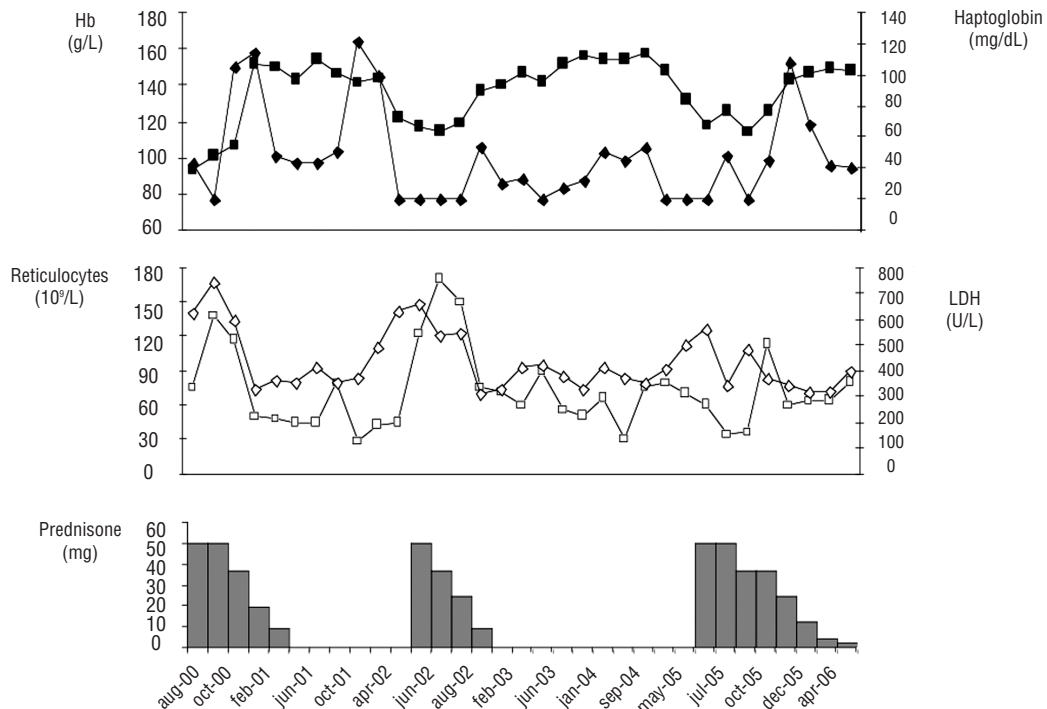
Figure 3. Clinical and laboratory follow-up (August 2000-March 2006) of a MDS patient treated with a steroid (prednisone). Hb (⌐); haptoglobin (☒); reticulocytes (◇); LDH (☒).

Table 3. BM apoptosis and cytokine production in BM-bound IgG positive and negative MDS patients.

	Positive	Negative
Caspase-3 (OD Units)	186±25*	121±19
NF-κB (OD Units)	187±42	262±61
IL-2 (pg/mL)	1719±370	1578±267
IFN-γ (pg/mL)	1073±141	1236±18
TNF-α (pg/mL)	48±22.5*	296±134
IL-4 (pg/mL)	94±24*	171±21
IL-10 (pg/mL)	365±156	968±404
TGF-β (pg/mL)	13003±3484	10769±2469

Mean± SE of 14 BM-bound IgG positive and 12 negative patients;
*p<0.05: positive vs negative patients.

trols were negative BM supernatants and PBS, which gave baseline binding (*data not shown*).

Apoptosis and cytokine production in BM cultures from MDS patients

Caspase-3 activity was significantly higher ($p<0.05$) and NF-κB activity lower, although not significantly, in patients positive for anti-erythoblast antibodies than in antibody-negative patients. Regarding mitogen-stimulated cytokine production, TNF-α ($p<0.05$) and IL-4 ($p<0.05$) levels were lower in antibody-positive patients than in antibody-negative patients (Table 3).

Effect of steroid therapy in patients with anti-erythoblast antibodies

Four out of the 14 patients with anti-erythroblast autoimmunity had received adjuvant steroid therapy. In two patients the concomitant administration of transfusions and erythropoietin makes it difficult to evaluate the efficacy of steroids alone. In one patient we observed an increase of more than 1 g/dL in hemoglobin levels, with persistence of signs of hemolysis. Tapering of the dosage was associated with a decrease of hemoglobin to the patient's baseline levels. The clearest response was observed in the fourth patient. Figure 3 shows the clinical and laboratory parameters of this 41-year old MDS patient over time. The patient had suffered from hemolytic anemia for several months, and investigations for congenital membrane and RBC enzyme defects and autoimmune tests, including RBC MS-DAT, had given negative results. The patient was considered to have a DAT-negative autoimmune hemolytic anemia and was treated with steroids, which produced clinical and hematologic responses. In April 2002, hemolytic anemia recurred and a BM investigation was performed, showing RA and positive values in the MS-DAT (389 ng/mL BM-bound IgG in unstimulated cultures, 408 ng/mL in PHA-stimulated culture and 534 ng/mL in PMA-stimulated cultures). The patient was treated again with steroids, to which he had a similar good response. In July 2005 a second relapse occurred, again controlled with steroid therapy. Repeated determinations of MS-DAT on RBC gave negative results. During the clinical follow-up the patient required no transfusion support or erythropoietin therapy.

Discussion

Both peripheral and bone marrow immune-mediated damage are thought to play roles in the pathogenesis of the red-cell cytopenias typical of early MDS.[20] In this study we investigated anti-erythroid cell autoimmunity both in peripheral blood and bone marrow and describe, for the first time, the presence of anti-erythroblast autoimmunity in half of RA and RARS patients. Only half of these patients show signs of hemolysis, although their mean values of reticulocyte number, unconjugated bilirubin and LDH concentration are higher, and haptoglobin levels lower than those of patients without evidence of autoimmunity. Consistently, the two groups have comparable hemoglobin levels and display similar clinical features of MDS. In addition, there is no difference in the erythroblast differentiation stages between patients with or without anti-erythroblast autoimmunity, indicating that there is no preferential destruction of a cell subset or blockade in red cell hematopoietic maturation. However, the amount of erythroblasts is greater in BM-bound IgG positive patients than in negative ones, suggesting that an immune-mediated destruction, if any, is not the prevalent effect. It is tempting to speculate that the autoimmune response, rather than causing mere cell lysis, probably contributes to accelerated dysplastic growth of the erythroid precursors, resulting in an increased number of erythroid precursors in patients with autoimmunity. The paradox of MDS is that despite a hypercellular or normocellular BM, the patients generally show peripheral cytopenias.[21] This might also be related to a high rate of intramedullary hematopoietic cell apoptosis induced by pro-apoptotic cytokines,[22] high Fas antigen expression on CD34+ cells[23,24] and the resultant activation of apoptosis-generated proteases (caspases).[25] We found that patients with anti-erythroblast autoimmunity have increased caspase-3 activity, suggesting a higher rate of apoptosis in the bone marrow of these patients, possibly related to their compensatory increased erythroblast proliferation. However, data are conflicting with regard to the stage of MDS at which apoptosis is most prominent, or the nature of the cell involved. Consistently with our finding, Bouscary *et al.* demonstrated that apoptosis is more evident in early stages of MDS,[26] whereas Raza *et al.*[27] suggested that apoptosis was maximal in advanced MDS and restricted to more differentiated CD34- cells.

Immunoregolatory cytokines play a key role in apoptosis and autoimmunity. In spite of the large amount of evidence of increased levels of the pro-inflammatory cytokine TNF-α in MDS,[12-14] we found that the subgroup of MDS patients with anti-erythroblast autoimmunity have reduced levels of TNF-α in their bone marrow. It has been demonstrated that TNF-α exerts a protective effect towards autoimmunity inducing apoptosis of autoreactive T lymphocytes.[11] Thus, the diminished level of TNF-α might be an additional attractive explanation for anti-erythroblast autoimmunity, although further investigation is required to prove this hypothesis. We also found that patients with anti-erythroblast autoimmunity have reduced IL-4 levels, in line with the recognized protective role of this cytokine against autoimmunity.[11] Altogether these findings are consistent with the suggested hypothesis that the autoimmune phenomena observed in MDS are probably initiated and perpetuated through alterations of pro-inflammatory and/or immunoregulatory cytokine production.[10]

The clinical and biological heterogeneity of MDS and the lack of understanding of the pathophysiology of these diseases makes the treatment strategy difficult and tentative. Here we show the clinical follow-up of a patient with severe hemolytic anemia and anti-erythroblast autoimmunity, who clearly responded to steroid therapy. The particular efficacy of steroids in MDS patients with anti-erythroblast autoimmunity should be confirmed by a controlled trial. The routine performance of BM MS-DAT in patients with MDS might be useful for demonstrating autoimmunity in bone marrow and therefore support a more rationale therapeutic choice.

Author Contributions

WB: designed the study and wrote the paper; AZ: conducted laboratory work and helped in manuscript preparation; FGI: conducted laboratory work and helped in manuscript preparation; CB: followed up patients; MC: followed up patients; AI: followed patients; AZ: supervised the study and helped in manuscript preparation.

Conflict of Interest

The authors reported no potential conflicts of interest.

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