Red cell aplasia and autoimmune hemolytic anemia following immunosuppression with alemtuzumab, mycophenolate, and daclizumab in pancreas transplant recipients

Milena Elimelakh, Vanessa Dayton, Katharine S. Park, Angelika C. Gruessner, David Sutherland, Robert B. Howe, Mark T. Reding, Ted Eastlund, Jo-Anne van Burik, Timothy P. Singleton, Rainer W. Gruessner, Nigel S. Key

From the Departments of Medicine, Surgery, and Laboratory Medicine and Pathology, University of Minnesota Medical School, Minneapolis, MN, USA.

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Correspondence:
Milena Elimelakh, MD, MMC 480, Mayo Building 420 Delaware St S.E., Minneapolis, MN 55455, USA.
E-mail: elime002@umn.edu

ABSTRACT

Background and Objectives

Acquired red cell aplasia (RCA) is a rare disorder and can be either idiopathic or associated with certain diseases, pregnancy, or drugs. In exceptionally rare cases, it has been reported to co-exist with other autoimmune cytopenias. We report a high incidence of RCA and autoimmune hemolytic anemia (AIHA) in pancreas transplant recipients on alemtuzumab-based maintenance therapy.

Design and Methods

Between February 2003 and July 2005, 357 pancreas transplant recipients were treated with immunosuppressive regimens containing the lymphocyte-depleting antibody alemtuzumab, the T-cell activation inhibitor daclizumab, and the anti-metabolite mycophenolate mofetil (MMF). We retrospectively reviewed medical records, blood bank data and bone marrow biopsy specimens of patients with a Transplant Information Services database diagnosis of RCA and AIHA from February 2003 to November 2005.

Results

Severe RCA, AIHA, and idiopathic thrombocytopenic purpura (ITP) occurred independently or in combination, in 20 out of 357 (5.6%) pancreas transplant recipients, 12 to 24 months following the initiation of the aforementioned immunosuppressive regimens. Severe opportunistic infections developed late in 14/20 (70%) of these patients. Atypical morphologic features, including variable dysgranulopoiesis, variable megakaryocytic hyperplasia with normal or low peripheral platelet counts, and atypical lymphoid aggregates were found in bone marrow trephine sections of 11 patients in whom the diagnosis of RCA was made.

Interpretation and Conclusions

We hypothesize that the combination of alemtuzumab, daclizumab and MMF can result in immune dysregulation thereby permitting autoantibody formation. Because the use of these three immune suppressants is becoming increasingly common, it is important to recognize the severe hematologic complications that can arise.

Key words: red cell aplasia, AIHA, ITP, MMF, alemtuzumab, daclizumab.

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Acquired red cell aplasia (RCA) and autoimmune hemolytic anemia (AIHA) are not commonly seen following solid organ transplantation. RCA is a relatively uncommon condition characterized by progressive anemia and severe reticulocytopenia with preservation of platelet and leukocyte counts. Bone marrow findings include complete or near complete absence of erythroid precursors, with erythroblasts comprising <0.5 to 5% of nucleated hematopoietic precursors. In most cases, the marrow is cellular, with unaffected myelopoiesis, lymphopoeisis and megakaryocytopoeisis.[1] In solid organ transplant recipients RCA occurs most commonly due to Parvovirus B19 infection.[2] In addition, many immunosuppressive agents routinely used in organ transplantation (mycophenolate mofetil [MMF], antithymocyte globulin, tacrolimus, and azathioprine) have been occasionally associated with RCA.[3-8]

Despite improved early kidney graft survival rates and better immunosuppressive protocols since the mid-1990s, chronic allograft nephropathy is the most common cause of long-term kidney graft failure. Histologic findings of calcineurin inhibitor-induced nephrotoxicity, implicated in late graft injury, were almost universally seen at 10 years following transplantation in kidney graft biopsies of patients with type 1 diabetes, as described by Nankivell *et al.*[9] To avoid such drug toxicity and to eliminate the deleterious side effects of long-term glucocorticoid use, we instituted a protocol change at the University of Minnesota Medical Center-Fairview (UMMC-F) in the year 2003 to implement a novel calcineurin inhibitor- and steroid-free immunosuppressive regimen in pancreas transplant recipients; this regimen contained alemtuzumab, MMF, and (in some patients) daclizumab.[10] The combination of alemtuzumab and MMF produced similar patient survival rates, with 53% of alemtuzumab recipients remaining solely on MMF at 1 year, as compared to those of the historical regimen of thymoglobulin (induction) and tacrolimus/MMF (maintenance), a rare finding for the success of non-calcineurin inhibitor maintenance monotherapy in solid organ transplantation. On the other hand, we observed lower graft survival rates, a higher rate of graft loss from rejection (in the pancreas transplant alone patients), a higher incidence of reversible rejection episodes (in both pancreas-after-kidney and simultaneous pancreas-kidney transplant patients), and a higher incidence of systemic infections (33% in the alemtuzumab treated group versus 14% in the control group; $p<0.0001$) at 1 year following transplantation. At an average median follow-up of 12.8 months, the incidence of post-transplant lymphoproliferative disorder was not significantly different (1% vs. 2%; $p>0.59$) between the alemtuzumab and control groups, respectively (*unpublished data*).

In addition, we observed a high incidence of RCA and AIHA in these transplant recipients. These hematologic complications tended to be of delayed onset and were associated with abnormalities in granulopoiesis and thrombocytopoiesis. We report the clinical and hematologic findings in this cohort of patients and speculate about possible pathophysiologic mechanisms.

Design and Methods

The results presented in this paper are part of a pilot study for a prospective randomized study that was approved by the University of Minnesota Institutional Review Board. The procedures followed for this study were in accordance with the ethical standards of the responsible committee on human experimentation and with the Helsinki Declaration. Patients signed an informed consent. Between February 2003 and July 2005, an alemtuzumab-based immunosuppressive protocol was used in the treatment of 357 pancreas transplant recipients. The details of this protocol are shown in Table 1. As a result of the recognition of the first cases of RCA and AIHA in 2005, we searched the Transplant Information Services database from February 2003 to November 2005 for the diagnoses of RCA and AIHA and retrospectively reviewed the clinical characteristics and laboratory data of patients with these hematologic complications.

Definitions of hematologic complications

RCA was defined as anemia with reticulocytopenia (absolute reticulocyte count <25,000/mm^3) with < 5% erythroid precursors in the bone marrow aspirate.[1] We defined recovery from RCA as a non-transfusional increase in hemoglobin (Hb) by >2 g/dL or a clear improvement in the reticulocyte count. AIHA was defined as clinically significant anemia in the presence of a positive direct antiglobulin test (DAT), with an increase in serum lactate dehydrogenase (LDH), and a decline in serum haptoglobin concentration to a subnormal level. Notably, reticulocyte counts were variable, being elevated in patients with isolated AIHA, but markedly reduced in those considered to have hemolysis in association with aregenerative anemia. Recovery from AIHA was defined as a non-transfusional increase in Hb of > 2 g/dL, accompanied by a decrease in LDH, with or without resolution of the positive DAT. Idiopathic thrombocytopenic purpura (ITP) was defined according to the American Society of Hematology consensus statement on the diagnosis and treatment of ITP.[11]

Immunosuppressive regimens

In the induction (administered within 6 weeks of transplantation), induction and maintenance (administered following transplantation and continued long-term) and conversion (calcineurin inhibitor withdrawal) regimens, the lymphocyte depleting antibody alemtuzumab (CamPath™; Genzyme, Cambridge, MA, USA) and the anti-metabolite MMF (CellCept™; Roche Pharmaceuticals, Nutley, NJ, USA) were used. When the MMF dose was limited by neutropenia, daclizumab (Zenapax™; Hoffman-La Roche Inc, Nutley, NJ, USA) was added. In addition, to deplete CD52 negative T cells, one dose of rabbit anti-thymocyte globulin (Thymoglobulin™; Genzyme, Boston, MA, USA) was given within the first week of initiation of both induction and maintenance regimens (Table 1). For anti-microbial and Candida prophylaxis, piperacillin/tazobactam was given for 3 days and

flucanozole for 7 days post-operatively. For viral and *Pneumocystis* pneumonia prophylaxis, daily oral valgancyclovir and single strength trimethoprim/sulfamethoxazole tablets, respectively, were given for one year.

Laboratory studies

Hematopathology

The peripheral blood and bone marrow smears, biopsy sections, and immunohistochemical stains were evaluated for morphologic features of dysgranulopoiesis, megakaryocytic hyperplasia, and atypical lymphoid aggregates. Of the patients with a database diagnosis of RCA, all 14 had bone marrow biopsy specimens available for review. Eight of these patients had had their bone marrow biopsies performed at UMMC-F, while the other six had had their bone marrow biopsies performed at outside institutions. The bone marrow biopsies and aspirate smears were processed and stained according to local laboratory protocols.

Immunohematology

DAT was not routinely performed but rather done when judged to be clinically indicated by the treating physician, or when antibodies were found during a pre-transfusion evaluation. Standard blood bank techniques were used for ABO and Rhesus group typing and for the DAT. Plasma testing for red cell antibodies was performed using a gel technique (ID-Micro Typing System™; Pompano Beach, FL, USA). DAT was performed using broad-spectrum, polyspecific anti-IgG and anti-C3d (Immucor, Norcross, Georgia, USA). If positive, the presence of IgG and C3d on the patient's red cells was demonstrated by testing with monospecific reagents, anti-IgG and anti-C3d. If the DAT was positive with IgG, the IgG was eluted from the red cells using glycine (Gamma EluKit II; Gamma Biologicals, Houston, TX, USA). Antibody was removed from red cells by multiple adsorptions with dithiotreitol-treated and papain-treated patient's red cells or, in cases in which the patient had been recently transfused, by alloadsorption with dithiotreitol-treated and papain-treated allogeneic red cells.[12]

Table 1. Immunosuppressive regimens.

Induction and maintenance therapy		
Alemtuzumab	Induction	30 mg intraoperatively, POD 2, 14, 42
(If ALC ≥200/mm^3)	CNI withdrawal Maintenance	30 mg days 0, 2, 4 30 mg IV monthly (maximum 8 doses)
Thymoglobulin	Induction CNI withdrawal	1.25 mg/kg IV POD 4 1.25 mg/kg IV day 3
Mycophenolate mofetil (MMF)	Induction/ Maintenance/ CNI withdrawal	≥2 g/day starting immediately post-transplant or the day CNI is discontinued
Daclizumab (add if MMF dose is limited to <2 g/day by ANC ≤200/mm^3)	Induction/ Maintenance/ CNI Withdrawal	2 mg/kg IV loading dose then 1 mg/kg IV every other month
Rejection therapy		
If ALC ≥200/mm^3	Alemtuzumab Thymoglobulin	30 mg IV on days 0, 2, 4 1.25 mg/kg IV on day 3
If ALC < 200/mm^3 or Alemtuzumab given in the last month	Thymoglobulin or Muromonab-CD3	1.25 mg/kg IV for 7 days first dose of 5 mg IV followed by 2.5 mg IV daily for 5-7 days

CNI: calcineurin inhibitor; POD: post-operative day; ALC: absolute lymphocyte count; ANC: absolute neutrophil count; IV: intravenous. Muromonab CD3 (Orthoclone OKT3; Ortho Biotech, Bridgewater, NJ, USA).

Results

Patients

The 357 pancreas transplants recipients received induction only (n=65), induction and maintenance with (n=156) or conversion to (n=108) alemtuzumab and MMF. Twenty-eight patients were treated for rejection. In 121 of 357 patients (34%), daclizumab was added. Of the 357 patients, 106 were pancreas transplant alone (PTA) recipients, 142 were pancreas after kidney (PAK) recipients, 94 were simultaneous pancreas-kidney (SPK) recipients, and 15 were kidney after pancreas (KAP) recipients. In total, 23 diagnoses of either RCA or AIHA were identified between September 2004 and November 2005. Three patients diagnosed with RCA were excluded from the study after further bone marrow biopsy review, two patients because they had >5% erythroid precursors and the third because the marrow aspirate was dilute and inadequate for diagnosis. Therefore, in accordance with our definitions, 20 patients developed AIHA or RCA. Of these, nine had AIHA, four had isolated RCA, and seven had RCA with an associated hemolytic component. Two of the 20 patients (one with RCA and one with AIHA) were also diagnosed with ITP.

Clinical course

Of the 20 patients who developed AIHA or RCA, one received induction only, ten induction and maintenance, and eight were converted to the alemtuzumab and MMF regimen. One patient (#19) was given alemtuzumab for the treatment of rejection, but also received MMF and daclizumab as part of his therapy. Eleven patients were recipients of a pancreas following a previous kidney transplant, three were simultaneous pancreas and kidney recipients, three were recipients of a pancreas transplant, and two were recipients of a kidney transplant after previously receiving a pancreas transplant. One patient had received a previous heart transplant. These 20 patients received between four and 11 doses of alemtuzumab prior to the diagnosis of AIHA or RCA. AIHA or RCA was first diagnosed a median of 9 months (range 2 to 15 months) after administration of the last dose of alemtuzumab. Nineteen of the 20 patients also received three to 15 doses of daclizumab. In some, AIHA or RCA developed up to 14 months following the last dose of daclizumab, and in others AIHA or RCA developed while taking the drug. The hematologic diagnosis in the only patient who never received daclizumab was AIHA. Seventeen patients had been immunosuppressed using some other therapy prior to initiation of the new regimen.

Fifteen of them had undergone a previous solid organ transplant. Four patients died. Three had been converted to the

Table 2. Patients' characteristics.

Pt no	*Sex, Age*	*Regimen, Transplant type*	*Diagnosis*	*Latency to diagnosis, in months*	*Cumulative alemtuzumab dose , in mg*	*DAT, RBC agglutinin*	*Treatment modalities*	*Outcome*
1	M, 61	M , PAK	RCA	20	330			Died of myocardial ischemia
2	M, 43	M, PAK	RCA with hemolysis	22	210	IgG	MMF discontinued	Recovered from RCA, hemolysis developed in 3 months
3	M, 56	M, PAK	RCA	16	180	IgG, C3d	MMF discontinued	Recovered from RCA
4	M, 36	C, PAK	RCA with hemolysis	14	240	IgG, C3d	Splenectomy, rituximab, vincristine, plasmapheresis	Died of RCA- associated hemolysis
5	M, 42	M, PAK	RCA with hemolysis, ITP	18	180	IgG, C3d	MMF discontinued, IVIG	Recovered from RCA, ITP developed in 2 months, hemolysis developed in 4 months
6	M, 34	M, PTA	RCA with hemolysis	16	330	IgG, C3d	MMF dose reduced, splenectomy, rituximab, IVIG, CSA, steroids	Recovered from RCA and hemolysis
7	M, 50	C, PAK	RCA with hemolysis	24	180	IgG, C3d	MMF discontinued	Died of sepsis, DLBCL
8	F, 43	M, PAK	RCA	15	240		MMF discontinued	Recovered from RCA
9	F, 55	C, PTA	RCA with hemolysis	12	240	IgG, C3d	MMF discontinued	Recovered from RCA, hemolysis developed in 5 months
10	M, 59	C, SPK	RCA	13	150		MMF dose reduced	Recovered from RCA
11	F, 51	C, KAP	RCA with hemolysis	13	300	IgG, C3d	Steroids	Recovered from RCA and hemolysis
12	F, 23	M, PTA	AIHA	14	210	IgG, C3d	Steroids, rituximab, IVIG, plasmapheresis	Active AIHA, on treatment
13	M, 44	M, PAK	AIHA	19	120	IgG, C3d	Steroids, MMF discontinued	Active AIHA, on treatment
14	M, 38	M, SPK	AIHA	13	270	IgG, C3d	Steroids, MMF discontinued	Active AIHA, on treatment
15	M, 52	C, KAP	AIHA	15	120	IgG, C3d	Steroids	Recovered from AIHA
16	M, 41	C, PAK	AIHA AIHA	19	150	IgG, C3d	IVIG, steroids Steroids, rituximab,	Active AIHA, on treatment AIHA relapsed 5 months following splenectomy.
17	F, 41	I, PAK	ITP	22	210	IgG, C3d	IVIG, splenectomy	ITP resolved with steroids
18	F, 50	M, PAH	AIHA	23	300	IgG, C3d	Steroids, IVIG	Active AIHA, on treatment
19	M, 32	R, SPK	AIHA	23	180	IgG, C3d	Steroids	Recovered from AIHA
20	M, 57	C, PAK	AIHA	13	210	IgG, C3d	Steroids, IVIG, rituximab, plasmapheresis	Died of myocardial infarction, with AIHA

Pt no: patient number; M: male; F, female; I: induction only; M, induction and maintenance: C, conversion; R: rejection; PTA: pancreas transplant alone; SPK: simultaneous pancreas and kidney transplant; PAK: pancreas after kidney transplant; RBC: red blood cell; IgG: immunoglobulin G; IVIG: intravenous immunoglobulin; C3d: complement C3d; DLBCL: diffuse large B cell lymphoma.

alemtuzumab, MMF and daclizumab protocol, while one had received the protocol for induction and maintenance. One patient (#4) died of severe hemolysis associated with aregenerative anemia, with a Hb level of 2.1 g/dL at the time of death. One patient (#20) died of myocardial infarction secondary to AIHA, with a Hb of 3.7g/dL, and a reticulocyte count of 0.1% immediately prior to death. Bone marrow biopsy was not obtained in time for a formal diagnosis of RCA to be established. A third patient (#1) died of myocardial ischemia, and a fourth (#7) died of *E. coli* sepsis with diffuse large B-cell lymphoma (Table 2).

Red cell aplasia

Eleven of these 20 patients were diagnosed as having RCA 12-24 months after starting induction and maintenance or converting to the new combination of immunosuppressive medications. No evidence of active human parvovirus B19 infection was found by testing for human parvovirus B19 antibodies or DNA by polymerase chain reaction in the ten patients in whom it had been evaluated. Clinical and laboratory (positive DAT accompanied by decrease in haptoglobin and elevation of LDH levels) evidence of hemolysis in four patients was documented at the time of RCA diagnosis, and in another three patients in 3 to 5 months following the diagnosis of RCA. Additionally, patient #3 had a positive DAT and Hb of 7 g/dL at the time the diagnosis of RCA, without a concomitant reduction in haptoglobin concentration. In patient #2, a positive DAT was documented 5 months prior to the diagnosis of RCA. Six patients received erythropoietin (started prior to the diagnosis of RCA and maintained through the recovery phase in three). Serum erythropoietin levels ranged from 285 to 2722 mU/mL (mean 1021 mU/mL) when measured in six patients at the time of the diagnosis of RCA. Testing for anti-erythropoietin antibodies was not performed. None of the patients diagnosed with RCA was a carrier of HLA-DR2 or its serologic split, HLA-DR15, alleles, previously reported to be overrepresented in myelodysplastic syndrome and aplastic anemia.[13]

Autoimmune hemolytic anemia

The remaining nine of the 20 patients were diagnosed with AIHA in the absence of RCA. In these individuals, AIHA was

diagnosed 13 to 23 months after the initiation of alemtuzumab and MMF. In fact, the most of these patients were diagnosed recently, and are still actively undergoing treatment. The AIHA in all of the patients was of the warm type. No cases of cold AIHA were observed. Of the 16 patients with AIHA, the red cells of 15 patients were coated with IgG and C3d. In one patient only IgG was present. None of the patients had only C3d on their red cells.

Idiopathic thrombocytopenic purpura

Two of these 20 patients were also diagnosed as having ITP, one at the time of the diagnosis of AIHA, and the other 2 months following RCA and 2 months prior to the onset of hemolysis, with nadir platelet count below 10,000/μL. Bone marrow biopsy confirmed the presence of increased megakaryocytes in one of these patients. Testing for antiplatelet antibodies was negative in both patients.

Infectious complications

Fourteen of the 20 (70%) patients developed late infections. The causative micro-organism included cytomegalovirus (n=10), BK virus (n=4), *herpes simplex virus* (n=1), *Zygomycetes* (n=1), *Blastomycetes* (n=1), *Aspergillus* (n=2), and *Mycobacteria* (n= 2). Serum levels of one or more immunoglobulin groups were markedly reduced in all six patients in whom they were measured. One patient (#7) was incidentally discovered to have a liver mass simultaneously with the diagnosis of RCA. On biopsy, this mass was found to be a diffuse large B-cell lymphoma. Despite the marked immunosuppression, 13 of the 20 patients experienced at least one acute episode of allograft rejection. Patient #17, who was profoundly leukopenic, had agranulocytosis on bone marrow biopsy at the time of her diagnosis of AIHA and ITP.

Blood and bone marrow findings

The absolute lymphocyte count in the peripheral blood at the time of bone marrow biopsy ranged from 204 to 2720/μL. In the bone marrow aspirate smears, the percentage of erythroid precursors ranged from 0 to 4.6%, with a mean of 1.8%. When present, erythropoiesis was markedly left-shifted, with very few erythroid precursors beyond the basophilic normoblast stage. Giant proerythroblasts, characteristic of RCA associated with parvovirus B19 infection, were not observed. Near absence of marrow erythropoiesis was confirmed by performing paraffin section immunostaining for hemoglobin A (Figure 1). Some degree of dysgranulopoiesis, histologically benign lymphoid aggregates and normal to increased numbers of megakaryocytes were seen in the 11 bone marrow biopsy samples available for review from patients with RCA. The most consistent feature of dysgranulopoiesis seen in all patients' specimens was loss of normal nuclear chromatin condensation with neutrophil maturation, resulting in mature neutrophils showing chromatin features resembling those of mature monocytes as well as atypical nuclear hyperlobation in some cells, as illustrated in Figure 2. In addition to increased megakaryocyte numbers, four of the 11 cases showed frequent loose clusters of megakaryocytes (Figure 3). Cytogenetic analysis was performed in seven RCA cases, and showed a normal karyotype. One patient (#5) had a follow-up bone marrow biopsy after recovery from RCA showing normal trilineage hematopoiesis.

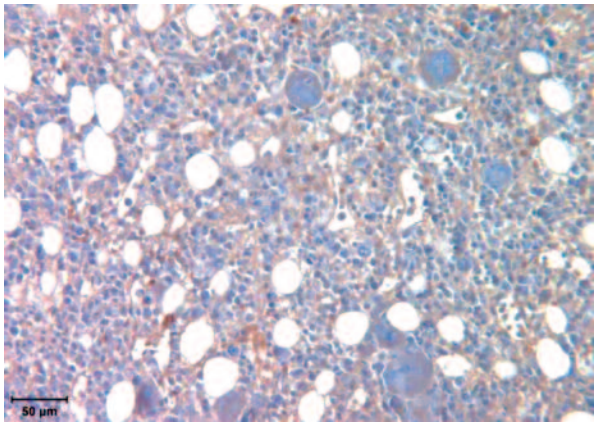

Figure 1. Paraffin section immunostaining for hemoglobin A highlights the absence of erythroid precursors (patient #8; 200×).

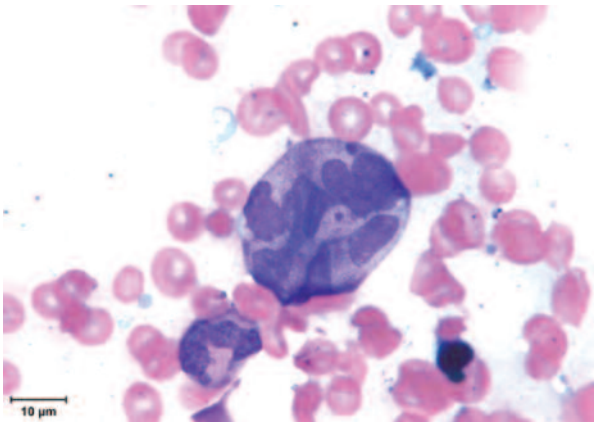

Figure 2. A dysplastic neutrophil in peripheral blood showing decreased nuclear chromatin condensation and atypical nuclear hyperlobation (patient # 4; Wrights Giemsa stain; 1000×).

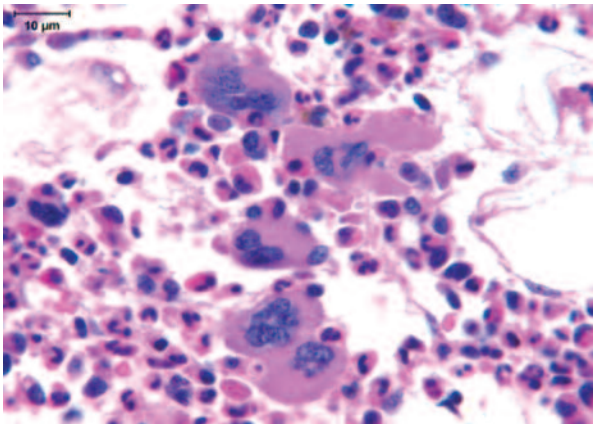

Figure 3. Clusters of megakaryocytes on a bone marrow trephine biopsy section (patient #2, hematoxylin and eosin stain; 1000×).

Discussion

We report the occurrence of severe RCA or AIHA in 5.6% of patients following immunosuppression with alemtuzumab, daclizumab and MMF after pancreas transplantation. This is a preliminary report of the prevalence of RCA and AIHA in these patients as more of them may develop similar complications in the future.

From an etiologic perspective, RCA may be either primary (autoimmune, preleukemic, idiopathic) or secondary, when it is found in association with thymoma, underlying malignancy, immune disease, infection, pregnancy and drugs.[14] Immune mechanisms - both cellular and humoral - for the inhibition of erythropoiesis have been implicated in both primary and secondary forms of RCA. The presence of an immunoglobulin G (IgG) inhibitor of erythropoiesis in the sera of patients with primary RCA has been demonstrated in several experimental *in vitro* and *in vivo* models.[1,15-16] On the other hand, inhibition of erythroid colony forming units (CFU-E) and erythroid bursts (BFU-E) colony formation has been documented in the presence of E-rosettes, without addition of sera, suggesting a T-lymphocyte-mediated suppression of erythroid differentiation.[17] An immune pathogenesis has also been suggested for drug-induced RCA, in which an IgG inhibitor to CFU-E and BFU-E was detected in the sera of RCA patients treated with diphenylhydantoin and rifampicin.[18,19]

Furthermore, RCA due to the presence of neutralizing IgG antibodies to the protein component of exogenous recombinant human erythropoietin has been well described in patients with anemia of chronic renal failure.[20] Remarkably, in renal transplant recipients, anemia secondary to relative endogenous erythropoietin deficiency has been described in the presence of normal graft function, and in the setting of acute and chronic allograft rejection.[21] Although erythropoietin antibody testing was not performed in our cohort of patients, elevation of serum erythropoietin levels appropriate for the degree of anemia was documented in six of our RCA patients. This argues against a significant contribution of decreased endogenous erythropoietin production to the development of aregenerative anemia in our patients.

Very rarely, RCA has been previously reported to co-exist with other autoimmune hematologic conditions, such as AIHA and ITP.[22] In our patients, the antibody eluted from the red cells was a typical pan-agglutinating autoantibody. One possibility is that a single antibody was directed against both erythroid precursors and mature red cells in our patients, as has been previously reported in other rare cases.[1,23] On the other hand, not all cases of hemolytic anemia following solid organ transplantation are due to autoantibodies, and specific testing for autoantibodies is needed. A high prevalence (20%) of unexplained hemolysis within 1 year of ABO-matched lung transplantation was reported by Riechsteiner *et al.*, but the DAT, when done, was negative in all patients.[24] Alloimmune hemolysis is common among minor ABO-incompatible solid organ transplant recipients, with an incidence ranging from 9% in kidney transplant to 70% in heart-lung transplant recipients. In that setting, hemolysis is usually acute and short-lived, with the last positive DAT detected at a median of 5.5 weeks post-operatively in one study.[25] In contrast, in our cohort of patients we observed Coombs' positive hemolytic anemia that appeared to be quite delayed in onset. It is remarkable that 16 of the 17 (94%) patients with a positive DAT had both complement and immunoglobulin detected on their red cells, a proportion that is substantially higher than the estimated 31-64% of patients with both complement and antibodies on the red cells in idiopathic AIHA.[26] The presence of both complement and antibody on the red cell membrane may explain the severity of hemolysis as evident by treatment refractoriness and even death due to AIHA in our cohort. The presence of complement-fixing immunoglobulin on the erythrocyte membrane has been suggested to potentiate the degree of hemolysis by others and has been demonstrated to accelerate immune clearance of red blood cells in guinea pigs, as reported by Schriber and Frank.[26,27]

Paradoxically, both alemtuzumab and MMF have been used successfully in the treatment of RCA and AIHA, respectively.[22,28,29] In our cohort of patients, RCA resolved with MMF discontinuation or dose reduction in 8 of 11 patients. This does not necessarily imply that MMF played a direct dose-related causative role in the development of RCA, despite the report of a previous case of parvovirus B-associated RCA in a renal transplant recipient that responded to discontinuation of MMF.[2] MMF is an inhibitor of guanosine triphosphate synthesis and causes selective inhibition of B-and T- cell lymphocyte proliferation. The lack of a consistent association between RCA and MMF, despite extensive use of MMF as a single immunosuppressive agent over a number of years, indicates that the pathogenesis is likely to be more complex. We hypothesize that the benefit of MMF dose reduction resulted from a net reduction or qualitative alteration of the global immunosuppression status. However, just as RCA has been reported to occur with the use of MMF, development of autoimmune phenomena including RCA, autoimmune neutropenia, Graves' disease, ITP and erythroderma have been noted with the use of alemtuzumab, and, more recently, daclizumab, respectively.[22,28,30]

Alemtuzumab is a humanized monoclonal immunoglobulin directed against CD52 antigen expressed on T-and B-cell lymphocytes, monocytes, macrophages, NK cells, and a subpopulation of granulocytes, but not on hematologic precursors. It is approved in the United States for the treatment of fludarabine-refractory chronic lymphocytic leukemia (CLL). It has also been used in the treatment of other hematologic malignancies, autoimmune cytopenias, other autoimmune conditions such as multiple sclerosis and rheumatoid arthritis,[31,32] and in solid organ and bone marrow transplantation. RCA, AIHA, and fatal ITP have been reported as complications of alemtuzumab in the treatment of

CLL.[33-35] In addition, there is a report of one case of AIHA in a kidney transplant recipient (who did not suffer from CLL) after induction with alemtuzumab.[36] In our study, two patients developed ITP after undergoing induction and maintenance with alemtuzumab and MMF. Similarly, ITP occurred in three of 334 patients with multiple sclerosis being treated with alemtuzumab, and this resulted in the suspension of a phase II trial at the 1 year analysis. Although we have administered alemtuzumab monthly for maintenance and observed ITP in patients receiving cumulative doses of 180 to 210 mg of alemtuzumab, ITP developed in one patient with multiple sclerosis 25 months after receiving a lower dose regimen involving 12 mg intravenously daily for 5 days with three 12 mg doses repeated 12 and 24 months later. The remaining two patients with multiple sclerosis who have developed ITP 19 and 23 months after initiation of alemtuzumab received a high dose regimen of 24 mg daily for 5 days followed by three daily doses of 24 mg intravenously 12 months later.[37] In another study, 15 (27%) of 57 of patients with multiple sclerosis treated with alemtuzumab developed new onset Graves' disease 5 to 21 months after receiving a 5-day course (100 mg total dose) of alemtuzumab.[31] Ten of the 15 cases were asymptomatic but were detected by routine testing for thyroid stimulation hormone levels.

DAT screening in our study was not routinely performed in recipients of alemtuzumab, and therefore the incidence and the timing of the onset of a positive DAT in the entire cohort of patients receiving alemtuzumab and MMF maintenance is not known. However, in one patient, IgG was detected on the red cells 5 months prior to the onset of overt clinical hemolysis. Alemtuzumab's association with autoimmune hemolytic phenomena has also been reported by others. Willis *et al*. reported a positive DAT in two patients treated with alemtuzumab (10 mg daily) for autoimmune cytopenias; these findings were observed 9 and 15 months after the initial administration. Additionally, anticardiolipin antibody and antinuclear antibody, and IgG anticardiolipin antibody, antinuclear antibody, and thyroid microsomal antibody developed in these two patients, respectively.[22] We therefore speculate that the number of patients we observed with clinically overt DAT$^+$ hemolytic anemia represents the *tip of the iceberg*, and that many more recipients of this protocol had sub-clinical/compensated AIHA. A prospective controlled study is needed in order to determine more accurately the prevalence of AIHA and RCA with the use of these drugs.

Daclizumab, a humanized monoclonal immunoglobulin that binds to the alpha chain (Tac/CD25) of the interleukin-2 receptor expressed on the surface of activated T cells, is used extensively in solid organ transplantation and is becoming increasingly popular in the treatment of hematologic malignancies and autoimmune disorders.[38,39] Furthermore, a group of investigators at the National Heart, Lung, and Blood Institute (National Institute of Health, Bethesda, MD, USA) recently found daclizumab to be efficacious when used to treat 15 patients with idiopathic pure RCA, producing a response in 6 (40%) of the patients.[40] On the other hand, they have also reported daclizumab-associated erythroderma in five (two with pure RCA) of 88 patients with aplastic anemia and pure RCA.[30] Two of these five patients also developed an inflammatory arthritis. CD4$^+$CD25$^+$ T-cell counts were expanded 2-10 fold, leading the authors to speculate that the immune phenomena may be linked to a relative depletion of CD4$^+$CD25$^+$ T-regulatory cells, thereby permitting a relative over-expansion of CD4$^+$CD25$^+$ effector cells. Analogously, organ-specific autoimmune disease develops in mice depleted of CD4$^+$CD25$^+$ T regulatory cells.[41] It is also notable that 19 of the 20 patients in our study received multiple doses of daclizumab. Therefore it is possible that a combination of alemtuzumab, daclizumab and MMF may have produced a similar T lymphocyte subset misbalance, resulting in the disruption of immunologic self-tolerance. In support of this possibility, alemtuzumab is known to cause prolonged, severe lymphopenia, affect reconstitution of T lymphocytes and suppress the T-cell response to phytohaemagglutinin, a T-cell mitogen.[42,43] When used as pulse therapy in multiple sclerosis, alemtuzumab produces severe T-cell depletion lasting a median of 60 months for CD4 lymphocytes, and 30 months for CD8 lymphocytes, with CD4 and CD8 values at 18 months of only 30-40% of pretreatment values.[31,42] In our entire patient cohort, and, in particular in the group that developed hematologic complications, the profound degree of immunosuppression is evident from the striking rate of opportunistic infections. We speculate that in our patient cohort a combination of a T-cell depleting agent and a T-cell proliferation antagonist profoundly and disproportionably depleted T-cell subsets and impaired T regulatory cell activation. In summary, we have observed a high incidence of late occurring, severe autoimmune cytopenias, including RCA, AIHA and ITP with the use of alemtuzumab, daclizumab and MMF. Immune dysregulation and the development of autoimmunity may be implicated in the pathogenesis of these disorders.

Authors' Contributions

ME: designed research, performed research (major part), analyzed data and wrote the manuscript; VD, KCP, TPS: analyzed bone marrow biopsy specimens, interpreted and described morphologic findings and edited the manuscript; TE: analyzed and interpreted blood bank data, described immunohematologic findings and edited the manuscript; ACG, RWG, DS: substantially contributed to initial conception of research, study design and edited the manuscript; JvB: analyzed and interpreted infectious disease data and edited the manuscript; MTR, RBH: provided clinical data for RCA and AIHA patients, interpreted clinical data and provided intellectual input; NSK: substantially contributed to initial conception of research and study design, revised drafts for important intellectual concepts, edited and finalized the manuscript.

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Conflict of Interest

The authors reported no potential conflicts of interest.

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