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Key words: Diamond-Blackfan anemia, RPS19, deletion, MLPA.

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Immune-mediated pure red cell aplasia in renal transplant recipients

Pure red cell aplasia (PRCA) is defined by an isolated severe anemia, contrasting with a normal white blood cell and platelet counts, severe reticulocytopenia and a selective absence of erythroid progenitors in the bone marrow smears.[1] Although an immune origin is involved in the majority of cases,[1] the diagnosis of immune-mediated cytopenia is rarely considered in organ transplant recipients, given the immunocompromised status. Etiologies of post-transplant PRCA are rather dominated by chronic parvovirus B19 infections[2] and drug-induced bone marrow toxicity.[3] However, in most cases reported in the literature with drug-induced PRCA, cyclosporine A (CyA) was introduced to replace the incriminated drug. We, therefore, hypothesized that some post-transplant PRCA may be immune-mediated, especially in renal transplant recipients treated with a calcineurin inhibitor (CNI)-free regimen. This hypothesis is supported by an unexpectedly high frequency of LGL-like clonal disorders in organ transplant recipients,[4] and by a recent report of autoimmune cytopenia occurring in up to 5.6% of pancreas transplant recipients receiving a calcineurin inhibitor-free regimen.[5] We report here 3 renal transplant recipients admitted for PRCA, in whom thorough investigations provided compelling evidence of immune mechanisms. End stage renal failure was related to chronic primary glomerulonephritis in all of them, and they all received a first renal transplant in the early '80s. Between 1980 and 1990, 489 out of the 611 patients receiving a renal transplant in our center were given a calcineurin inhibitor (CNI)-free immunosuppressive regimen, including the 3 cases who later experienced immune-mediated PRCA.

The 3 patients were admitted for severe aregenerative anemia, while treated with prednisone and azathioprine for several years. Bone marrow smears confirmed the diagnosis of PRCA. None of them was given recombinant erythropoietin and the high erythropoietin levels found in our patients excluded the hypothesis of anti-erythropoietin antibodies (Table 1). Parvovirus B19 infection is a well-identified cause of PRCA in immunocompromized patients.[2] However, both serology and specific PCR ruled out this hypothesis in our 3 cases. A specific azathioprine-

Table 1. Laboratory examination of 3 patients with immune-mediated PRCA. Erythroid progenitors cultures were performed with 5×10^5 cells/mL. Values are means of duplicate culture of day 7 CFU-E-derived colonies per 5×10^5 plated cells.

	Normal Values	*Case 1*	*Case 2*	*Case 3*
		1st flare / 2nd flare		
Mean corpuscular volume (fL)	80-100	105	105	100
Erythropoietin (mU/mL)	4-14	1666 / 1920	ND	1220
Parvovirus B19 IgM		– / ND	–	–
IgG		+ / ND	+	+
PCR		– / –	–	–
T-cell clone		+ / +	+	+
LGL population		ND +	ND	ND
Coomb's reaction		– / –	–	–
ANA		–	–	–
CFU-E-derived colonies	100-1,000			
patient's serum		155 / ND	135	ND
control's serum		105 / ND	65	ND

ANA: anti-nuclear antibody; LGL: large granular lymphocyte; ND: not done; PCR: polychain reaction; (+), positive; (-), negative.

induced toxicity on erythroid progenitors was considered.[3] However, in our patients, red blood pack requirement did not decrease several weeks after the interruption of azathioprine. In addition, remission of the first episode of erythroid aplasia in case 1 was sustained over a period of ten years while azathioprine had been reintroduced (Figure 1). In the same case, remission of the second flare was obtained without discontinuing azathioprine treatment (Figure 1). Altogether these observations strongly argued against a direct toxic effect

Several results argued for an immune mechanism. First and foremost, a burst of reticulocytes occurred a few days after CyA introduction (Figure 1), in agreement with the remission achieved after introduction of other immunosuppressive drugs in other cases reported in the literature.[3] In addition, PRCA has never been described in patients receiving azathioprine associated with CyA, although this combination has been widely used since the late '80s in organ transplantations. Second, the normal *in vitro* growth of erythroid progenitors (Table 1),[6] as observed in our patients, was previously shown to be highly predictive for a response to immunosuppressive drugs.[6,7] The absence of circulating inhibitor (antibodies) of erythropoiesis in our cases, as assessed by autologous bone marrow culture with patient's sera, was not incompatible with immune mediation. Indeed cell-mediated response is likely involved in autoimmune PRCA not related to anti-erythropoietin antibodies.[1,8] Although *in vitro* inhibition of erythroid progenitors growth by autologous T cells would have brought conclusive arguments in favor of immune cell-mediated PRCA, this assay could not be performed in our patients. However, to date such assay has not been standardized because of technical limitations that make the method poorly reliable.

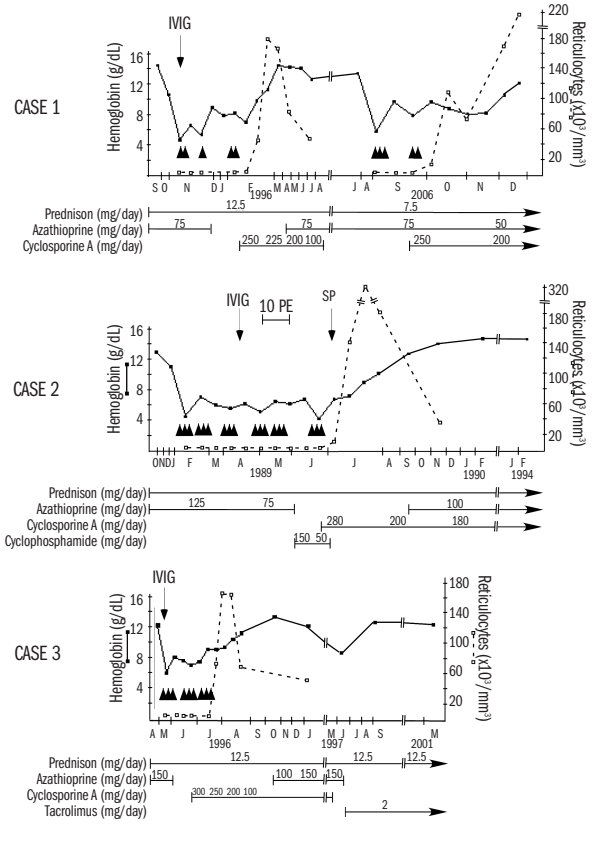

Figure 1. Clinical course of 3 renal transplant recipients with immune-mediated PRCA. Hemoglobin levels (■) and reticulocyte counts (□) over time (months/years). Intravenous immunosuppression treatments and packed red blood cell transfusions are indicated by arrows (➤) and triangles (▲) respectively. IVIG, intravenous immunoglobulin; PE, plasma exchanges; SP, steroid pulses.

The 3 patients underwent chest X-ray and study of TCR γ chain rearrangement[9] to look for associated diseases, such as thymic hyperplasia or T-cell lymphoproliferations. Chest X-ray ruled out thymoma and TCR γ chain rearrangement identified a small number of clones in all cases. The absence of tumor mass, normal medullar cytology and long-term follow-up excluded aggressive T-cell malignancies. These clones may correspond to indolent $CD3^+CD8^+CD57^+$ LGL populations, which are frequently found in association with PRCA.[10] A lymphocyte immunophenotype study was performed in case 1, while the PRCA remission had been obtained with CyA for one year, and disclosed a LGL γδ population accounting for 11% of the total lymphocytes. Whether the clones identified in our patients were involved in the self-reactive response against erythroid progenitors is uncertain. Of note, the persistence of clones despite remission of the PRCA does not necessarily rule out a causal link. CyA might have blocked the pathogenic effect of the self-reactive clones, without interfering with their maintenance. Indeed CyA has been shown to be more efficient in controlling T-LGL proliferation and related cytopenia than traditional anti-leukemic therapy.[11]

The role of CNI in controlling self-reactive clones in transplanted patients is further supported by a recent report that 20 out of 357 (5.6%) pancreas transplant recipients experienced immune-mediated cytopenia while receiving a calcineurin inhibitor-free regimen.[5] The high frequency of autoimmune cytopenia in this series is striking and may be related to lymphopenic induction by alemtuzumab, which has already been associated with increased autoimmune thyroid disease in renal transplant recipients.[12] Mechanisms of lymphopenia-induced autoimmunity are well described and it is likely that calcineurin inhibitors may be critical to control the expansion of pathogenic self-reactive clones in this setting. In conclusion, clinicians should be aware that immune-mediated PRCA may be an unexpected cause of anemia in transplanted immunosuppressed patients and that cyclosporine may be efficient in ameliorating the condition. These cases also raise questions about the significance of clonal T-LGL-expansions in organ recipients. Whether they correspond to T-cell clonopathies of unknown significance (TCUS) or to lymphoproliferative disorders, and whether they emerge from auto, allo or viral antigens are important issues that require further studies.

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Nonsense mutations of the α-spectrin gene in hereditary pyropoikilocytosis

Hereditary pyropoikilocytosis (HPP) is an inherited hemolytic anemia characterized by peripheral blood smear findings reminiscent of those seen in patients suffering severe thermal burns.[1,2] Erythrocytes from most HPP patients exhibit qualitative and quantitative abnormalities of the erythrocyte membrane protein spectrin, the principal structural component of the erythrocyte membrane skeleton. Qualitative spectrin defects are typically associated with missense mutations that lead to abnormal spectrin self-association, a process critical for membrane structure and function.[3]

The pathogenesis of qualitative spectrin defects, *i.e.* spectrin deficiency, in HPP erythrocytes is poorly understood. Whereas some HPP patients are compound heterozygotes or homozygotes for missense mutations in spectrin, others are heterozygotes for a missense mutation and possess a second, *thalassemia-like* α-spectrin allele *in trans* to the missense mutation.[4-6] This production-defective allele is associated with decreased or absent accumulation of α-spectrin on the erythrocyte membrane. With the exception of the original case described by Zarkowsky *et al.*, the molecular basis of the production-defective α-spectrin allele in HPP is unknown.

We studied HPP probands from 2 HE/HPP kindreds. Both probands had typical hereditary pyropoikilocytosis. Laboratory findings included compensated hemolytic anemia, marked microcytosis (MCV<75 fL), and typical blood smears with erythrocyte morphology including elliptocytes, poikilocytes, microspherocytes, and fragmented cells. Informed consent was obtained in accordance with the Declaration of Helsinki.

One-dimensional SDS-PAGE analyses of erythrocyte membranes from both probands were qualitatively normal (*data not shown*). Quantitative analyses of spectrin content, measured by spectrin/band 3 ratios, demonstrated spectrin deficiency in both probands (Table 1). These are values typically seen in patients with HPP.

Limited tryptic digestion of normal spectrin followed by two-dimensional gel electrophoresis yields a pattern of five major proteolytically resistant domains of α-spectrin and 4 proteolytically resistant domains of β-spectrin.[7] The 80kDa αI domain encodes the NH_2-terminus of α-spectrin which interacts with sequences from the 17th repeat of β-spectrin to form the αβ binding site for spectrin self-association. Most HPP-associated spectrin mutations affect the 80kDa αI domain and yield peptide maps containing one or more fragments of the domain.

Both HPP probands exhibited abnormal tryptic spectrin maps, with the αI/50a kDa variant peptide. No normal αI 80kDa peptide was seen on maps from either of the HPP probands, implying homozygosity for the underlying

Table 1. Biochemical and genetic studies in hereditary pyropoikilocytosis patients.

	Patient 1	*Patient 2*
Spectrin/band 3 ratio	0.76+0.04	0.71+0.05
Spectrin tryptic phenotype	αI/50a kDa	αI/50a kDa
Missense mutation		
Exon number	Five	Six
DNA	CTG-CCG	CTG-CCG
Codon	Leu207Pro	Leu260Pro
Nonsense mutation		
Exon number	Forty-three	Thirty-five
DNA	GAA-TGA	CGA-TGA
Codon	Glu2018Stop	Arg1659Stop