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MASSIVE APOPTOSIS OF BONE MARROW CELLS IN APLASTIC ANAEMIA

The pathogenesis of aplastic anaemia (AA) is still incompletely understood. Bone marrow (BM) hypoplasia is a hallmark of AA and one of the considered mechanisms of hypoplasia is an increased apoptosis of haematopoietic progenitors. However, definitive evidence supporting this is largely lacking (Ismail *et al*, 2001).

We have studied *in situ* apoptosis of BM mononuclear cells (BMMNCs) in the BM biopsies of 10 AA patients. All the patients had acquired AA and none of the patients had received prior immunosuppressive treatment. Two patients had very severe AA (VSAA), five had severe AA (SAA) and three had non-severe AA (NSAA) (Camitta *et al*, 1979; Bacigalupo *et al*, 1988). Ten age-/sex-matched lymphoma patients with no infiltration of BM by lymphomatous cells, as confirmed by microscopy and immunophenotyping, served as controls. Apoptosis was detected by TdT-mediated dUTP nick end labelling (TUNEL) assay using the '*in situ* cell death detection kit-Fluorescein' (Boehringer Mannheim, Germany). The fluorescent signal was converted into light microscopy by treating the sections with antifluorescein-alkaline phosphatase (Boehringer) and its substrate, fast red (Sigma, St Louis, MO, USA). Mayer's Haematoxylin was used as counter-stain. The number of apoptotic BMMNCs were 54·14 ± 13·22% in the biopsies of patients (Fig 1A) and 3·78 ± 1·85% in those of controls (Fig 1B) ($P < 0·001$). The apoptotic index observed in patients with VSAA and SAA was significantly higher than in those with NSAA (61·0 ± 10% versus 39·0 ± 4%, $P < 0·05$), suggesting a correlation between severity of the disease and degree of apoptosis.

In a previous study, Callera & Falcao (1997), using the same method, reported significantly increased apoptosis in the biopsies of AA patients compared with controls. However, the apoptotic index observed by this group (8·19 ± 1·45%) was much lower than ours (54·14 ± 13·22%). This may be due to differences in the clinical spectrum of patients and the protocol of the method used. All the patients included in our study were untreated and most of them (7/10) had SAA/VSAA. However, most of the patients (6/11) of Callera & Falcao (1997) had been previously treated and had moderate disease (7/11). Use of suboptimal concentration of Proteinase K (Sigma) or treatment time of tissue sections with the enzyme can give false results. We determined the optimal concentration as well as treatment time of Proteinase K (20 μg/ml for 30 min) by studying apoptosis in control biopsies treated with graded concentrations of DNase I (Sigma). However, the concentration and time period of incubation with Proteinase K was not clear in the report of Callera & Falcao (1997).

The massive BMMNC apoptosis that we observed was in accordance with a previous report showing a significant increase in haematopoietic cells bearing Fas antigen (56 ± 9%) (Maciejewski *et al*, 1995). Furthermore, our data corroborates with two recent reports showing significant apoptosis/death of $CD34^+$ cells in BM of AA patients (Killick *et al*, 2000; Ismail *et al*, 2001).

In conclusion, we have observed massive apoptosis of BMMNCs in our patients, suggesting that apoptotic death of marrow cells may be a major cause of BM hypoplasia in AA. Further studies on phenotypic characterization of apoptotic

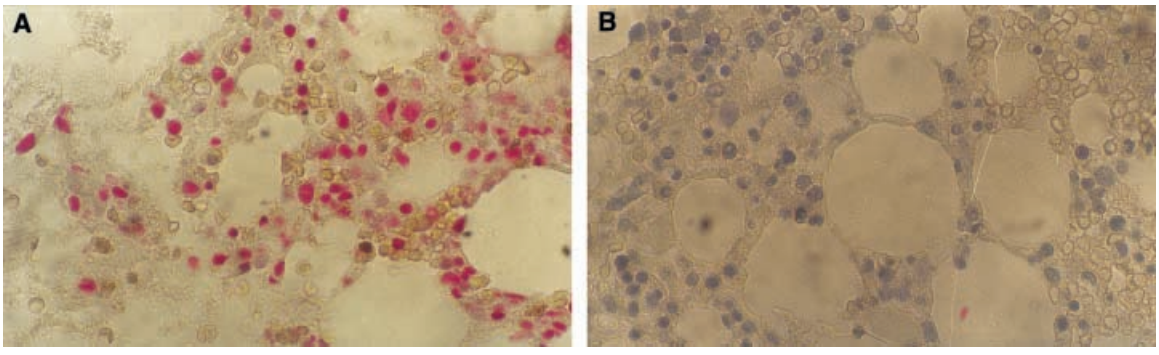

Fig 1. (A) Representative photomicrograph showing TUNEL staining of BM biopsy of an AA patient (×100). The pink nuclei represent the apoptotic cells and purple nuclei represent non-apoptotic cells. (B) Representative photomicrograph showing TUNEL staining of BM biopsy of a control (×100). The purple nuclei represent non-apoptotic cells.

marrow cells and precise mechanism(s) of their apoptosis will provide important new insights in understanding the pathophysiology of the disease.

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Keywords: aplastic anaemia, apoptosis, bone marrow biopsy, bone marrow mononuclear cells, TUNEL assay.

POST-TRANSFUSION PURPURA WITHOUT DETECTABLE ANTIBODIES: THEIR ADSORPTION FROM THE PLASMA BY MULTIPLE INCOMPATIBLE PLATELET TRANSFUSIONS

About 200 cases of post-transfusion purpura (PTP) have been reported (Mueller-Eckhardt, 1986; Kunicki & Beardsley, 1989; Taaning & Svejgaard, 1994). This syndrome occurs most frequently in women, presumably due to immunization during previous pregnancies (Mueller-Eckhardt, 1986). The target antigen in most cases is human platelet antigen 1a (HPA-1a). PTP may occur more frequently than has been recognized, as its clinical diagnosis is based on a sudden drop of platelets after transfusion, which does not occur in pre-existing thrombocytopenia. We describe a female patient who had preformed HPA-1a antibodies which were not detectable at the time of severe thrombocytopenia, presumably due to their adsorption by continuously administered platelet concentrates.

A 49-year-old mother of two children with liver cirrhosis was scheduled for liver transplantation. HPA-1a antibodies had been detected 2 years earlier. In the preceding year she had received 14 units of filtered packed red blood cells (PRBC), which were unmatched for the HPA system, during surgery for a duodenal ulcer. No platelet concentrates (PC) were transfused. On the day of the liver transplantation, her haemoglobin was 11·2 g/dl and the platelet count was 40×10^9/l. The report on the platelet antibodies had not been forwarded from the laboratory. The patient received 8 units of filtered PRBC, 6 units of frozen plasma and one leucocyte-filtered PC intraoperatively. The platelet count dropped to $< 10 \times 10^9$/l (Fig 1). There was no increase of platelet counts despite daily transfusions with unselected single donor apheresis PC, retrospectively typed as HPA-1a. An antibody screening by the monoclonal antibody-specific immobilization of platelet antigens (MAIPA) assay (Kurz *et al*, 2001) on sera taken on two different days revealed human leucocyte antigen (HLA) antibodies and a positive cross-match with the first PC due to these antibodies, but no other platelet antibodies. However, the patient was genotyped HPA-1bb, 2aa, 3aa, 5aa and a diagnosis of PTP was presumed. All transfusions were stopped. She received intravenous immunoglobulin

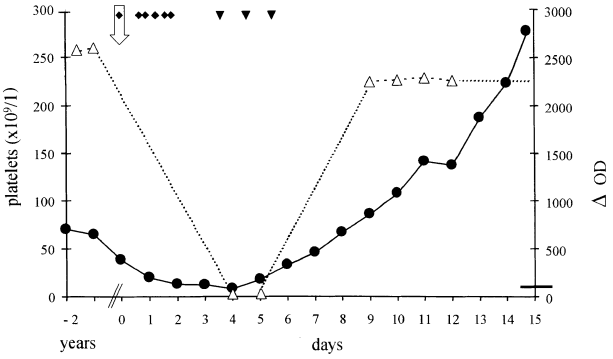

Fig 1. The course of HPA-1a antibodies (△) and peripheral platelet counts (●) are shown. An optical density at 492 nm, after subtraction of blanks (△ OD), of more than 100 (—) by the MAIPA assay was defined as a positive result. A total of six platelet concentrates (◆) were transfused. One was given during surgery and five others within the first 3 d. … indicates the day of surgery, and ▼ indicates the days of i.v.Ig treatment (1 g/kg body weight).

(i.v.Ig) for three consecutive days and platelet counts rose rapidly (Fig 1). Eight further PRBC from HPA-1bb donors were well tolerated. A repeat evaluation of all sera within the same MAIPA assay confirmed the presence of HPA-1a antibodies, but only in sera obtained before surgery and after recovery (Fig 1). The patient was discharged from hospital 3 weeks later with a platelet count of 262×10^9/l.

Complex clinical situations with thrombocytopenia, such as in patients undergoing chemotherapy, or in patients with septicaemia or liver cirrhosis (Stiegler *et al*, 1998), may mask PTP, which typically is indicated by a sudden drop of platelet counts. Failure of platelet transfusions to increase the platelet count may be interpreted as refractoriness to platelet concentrates, but not as PTP. In this case we assumed that intensive transfusions of incompatible platelets adsorbed the antibodies from the plasma (Nagasawa *et al*, 1978), resulting in a negative test result. A diagnosis of PTP was considered, however, because the patient was genotyped HPA-1bb and therefore regarded at high risk for PTP.

It is assumed that PTP is due to boosting of alloantibodies, resulting in a premature clearance of the transfused and autologous platelets. In this case we cannot document boosting of the antibodies. In contrast, we showed the presence of the antibodies prior to transfusion. Whether or not the boosting of antibodies is a prerequisite for PTP is not known, as the absence of antibodies prior to transfusions has only been documented in a few case of PTP (Mueller-Eckhardt, 1986). However, the drop of platelets after transfusion of the first PC below pretransfusion levels and the rapid response to i.v.Ig support the assumption of PTP. Thus, this case illustrates that PTP may not be recognized if the diagnosis is based on the detection of platelet antibodies, which can be adsorbed from the plasma by transfused platelets.

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Keywords: post-transfusion purpura, HPA-1a antibodies, platelet transfusion, MAIPA, thrombocytopenia.

HEPATOSPLENIC γδ T-CELL LYMPHOMA: COMPLETE RESPONSE INDUCED BY TREATMENT WITH PENTOSTATIN

Hepatosplenic γδ T-cell lymphoma (HSγδTCL) is a rare but distinct entity (Farcet *et al*, 1990), which mainly affects young males and is characterized by hepatosplenomegaly but no or little lymphoadenopathy, frequent B symptoms, thrombocytopenia and poor response to conventional chemotherapy (Wong *et al*, 1995). A wide and heterogeneous array of therapies has been applied: monotherapy with steroids or alkylating agent, fludarabine, splenectomy, polychemotherapy and bone marrow transplantation. Despite different therapeutic approaches, complete remission (CR) is rarely obtained and the few responding patients relapse early with virtually all patients dying within 2 years (Weidmann, 2000). Pentostatine is a potent inhibitor of adenosine deaminase with a marked cytotoxic activity on T lymphocytes and has been shown to be effective in treatment of some histotypes of T-cell lymphomas (Kurzrock, 2000). Recent evidence displayed sensitivity of γδ cells to pentostatine *in vitro* (Aldinucci *et al*, 2000) and prompted us to treat a patient affected with HSγδTSL using this drug.

In April 1999, a 42-year-old Caucasian man was admitted to our ward because of night sweats, weight loss, dull pain at left ipocondrium, mild anaemia and thrombocytopenia discovered 2 weeks previously. Physical examination highlighted massive splenomegaly (20 cm below left costal margin) and hepatomegaly (7 cm below costal margin at midclavicular line). Haematological and biochemical tests revealed mild normochromic anaemia (Hb 10 g/dl) and thrombocytopenia (60×10^9/l), severe neutropenia and lymphocytopenia (white blood cells, $1{\cdot}1 \times 10^9$/l; neutrophils, $0{\cdot}4 \times 10^9$/l; lymphocytes, $0{\cdot}6 \times 10^9$/l). Lactate dehydrogenase and beta2 microglobulin were within normal range. Virological tests documented an acquired immunity for hepatitis B virus and Epstein–Barr

virus, and were negative for hepatitis C virus and human immunodeficiency virus infection. Bone marrow aspiration showed a 40% infiltrate consisting of medium-sized lymphocytes with slightly indented nuclei, small nucleolus and a small rim of lightly basophilic cytoplasm; the immunophenotype was as follows: $CD3^+$, $CD2^+$, $CD8^+$, $CD4^-$, $CD5^-$, $CD10^-$, $CD56/16^+$, TCR αβ, TCR $\gamma\delta1^+$.

A bone marrow trephine biopsy demonstrated a characteristic exclusively intrasinusoidal infiltrate consisting of medium-sized T lymphocytes (immunohistochemistry ABC Strept LCA^+, $CD20^-$, $CD45RO^+$, $CD45RA^-$).

The diagnosis of HSγδTSL was made and the patient was treated with pentostatin (4 mg/m^2 intravenously every 2 weeks for 6 months). The disappearance of B symptoms and shrinkage of the spleen rapidly followed the first administration of pentostatin. At restaging, performed after 10 courses of pentostatin, the patient was found to be in complete remission (neither lymph nodes nor liver or spleen enlargement were detected using a computerized tomography scan and the bone marrow infiltrate was completely clear). There was no significant treatment-related toxicity. The patient received another two monthly courses of pentostatin and subsequently underwent a successful mobilization of peripheral blood stem cells (PBSC) using cyclophosphamide 7 g/m^2 plus granulocyte colony-stimulating factor (10 μg/kg/d) with a yield of $6{\cdot}5 \times 10^6$/kg $CD34^+$. In January 2000, high-dose therapy was administered with idarubicin (21 mg/m^2, d −9 to d 8 and melphalan 140 mg/m^2, d −3) followed by reinfusion of the PBSC. Twelve months after transplant the patient is well and still in CR.

In conclusion, the case reported here, which showed an impressive responsiveness to pentostatin, suggests that this purine nucleoside analogue can obtain a CR with negligible toxicity. Moreover, pretreatment with pentostatin did not impair the collection of PBSC with the schedule used in this patient. To the best of our knowledge this is the first case treated successfully with pentostatin. We think that it could be reasonable to consider therapy with pentostatin as a possible option for induction therapy of patients affected by HSγδTCL.

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Keywords: hepatosplenic, γδ T-cell lymphoma, pentostatin.

EFFICACY OF THALIDOMIDE IN THE TREATMENT OF VAD-REFRACTORY PLASMA CELL LEUKAEMIA APPEARING AFTER AUTOLOGOUS STEM CELL TRANSPLANTATION FOR MULTIPLE MYELOMA

Positive results have been reported with thalidomide in patients with refractory multiple myeloma (MM) (Singhal *et al*, 1999). However, the efficacy of this drug in plasma cell leukaemia (PCL), the most aggressive form of MM, has not been yet widely evaluated. We report here the significant activity of thalidomide in a patient with VAD (vincristine, doxorubicin, dexamethasone)-refractory PCL, appearing several months after autologous stem cell transplantation for MM.

This 67-year-old woman presented in January 2000 with a diagnosis of Durie–Salmon stage III MM. Laboratory investigations showed anaemia [haemoglobin (Hb) 8·7 g/dl], monoclonal paraprotein (IgG kappa, 40 g/l), and normal C-reactive protein, beta2-microglobulin, calcium and creatinine levels. No Bence–Jones proteinuria was noted. A bone marrow examination revealed 28% plasma cells. Radiographs found several femoral and vertebral osteolytic lesions. She received three courses of VAD combined with pamidronate infusions. Following this treatment, the paraprotein concentration fell to 16 g/l, the Hb level raised to 10·5 g/dl and only 7% plasma cells were seen on marrow smears. After an additional VAD course, the patient was treated with 4 g/m^2 cyclophosphamide in order to collect peripheral blood stem cells. The target dose of progenitors was obtained using only two leukaphereses. After a fifth course of VAD, an autologous transplant was performed in August 2000 with high-dose melphalan (200 mg/m^2) as the preparative regimen. The post-transplant medications included clodronate, folic acid and penicillin. The patient was then considered to be in

complete response (normal blood counts, near absence of paraprotein on serum electrophoresis, 1% marrow plasmacytosis).

Her follow-up was unremarkable until she complained of progressively worsening pain in her left arm and was admitted to our department on July 3 2001. A large osteolytic lesion of the humerus was evidenced on X-ray. On the skeletal survey, no progression was evidenced concerning the other osteolytic lesions. The haemogram was normal, but an increase in monoclonal paraprotein (14 g/l) and bone marrow plasma cells (28%) confirmed the MM relapse. VAD protocol was introduced again (first course: July 3 2001) and the patient received local radiotherapy (45 Gy). However, 4 weeks after VAD initiation, the lactate dehydrogenase level was 1246 IU/l (upper normal limit: 618 IU/l) and the haemogram showed a hyperleucocytosis $15{\cdot}4 \times 10^9$/l with 20% circulating plasma cells ($3{\cdot}08 \times 10^9$/l). Bone marrow aspirate revealed a massive infiltration by 90% plasma cells. A diagnosis of secondary PCL was made. Considering the VAD-refractoriness, thalidomide (50 mg capsules, Laphal laboratory, Allauch, France) was initiated under compassionate use at 200 mg/d in two divided doses on August 7. On August 17, our patient developed fever > 39°C in a pancytopenic setting: $0{\cdot}9 \times 10^9$/l leucocytes (16% neutrophils, only 1% peripheral myeloma cells), 10·9 g/dl Hb and 6×10^9/l platelets. A coagulase-negative staphylococcus septicaemia was evidenced. This episode resolved under intravenous antibiotics, transfusions and five injections of granulocyte colony-stimulating factor. During this period, the dose of thalidomide was escalated to 300 mg/d and dexamethasone 40 mg/d was added for 4 d. This latter agent induced a significant and prolonged hyperglycaemia and its further use was precluded. Four weeks later, the haemogram was markedly better, with $4{\cdot}3 \times 10^9$/l leucocytes (78% neutrophils, absence of plasma cells), Hb 10 g/dl, 279×10^9/l platelets. From October 2, the administered dose of thalidomide was increased to 400 mg/d. At the time of writing, more than 3 months after thalidomide initiation, the patient has remained symptom-free, in good haematological response [$4{\cdot}7 \times 10^9$/l leucocytes (73% neutrophils, no circulating plasma cells), 10·7 g/dl Hb, 172×10^9/l platelets, 8% marrow plasma cells, serum paraprotein level at 11 g/l]. No significant side-effects were noted.

PCL represents an extremely aggressive entity which can appear either *de novo* (primary form) or terminating the course of MM (secondary form). This disease is characterized by $> 2 \times 10^9$/l circulating plasma cells. Melphalan-based treatments are poorly effective (about 2 months median survival using the Alexanian scheme) (Dimopoulos *et al*, 1994). Thalidomide has been validated recently for treating refractory MM (Singhal *et al*, 1999). Apart from its anti-angiogenic, immunomodulatory and myeloma cell pro-apoptotic properties, this agent modulates adhesive interactions between tumour cells and the bone marrow microenvironnement (Hideshima *et al*, 2000). Interestingly, some adhesion molecules are lost on leukaemic plasma cells, explaining their escape from the marrow. A direct dose-dependent effect of thalidomide on MM cells has been suggested (Hideshima *et al*, 2000). Furthermore, an *in vitro* synergy between this component and dexamethasone has been demonstrated (Hideshima *et al*, 2000).

Here, we had to manage an aggressive MM progression associated with PCL features < 12 months after autografting. Of note, this evolution occurred following therapy with the VAD regimen, which is thought to be the most potent treatment of PCL (Dimopoulos *et al*, 1994). However, the efficacy of thalidomide in VAD-resistant MM cases was clearly demonstrated in the pivotal study (Singhal *et al*, 1999). We administered thalidomide in two daily doses in order to try to improve time to achieve remission, response rate and tolerance (Juliusson *et al*, 2000; Bladé *et al*, 2001). The response, obtained with only 200 mg/d after a brief period of aplasia, was rapid, durable and subsequently not associated with significant side-effects.

Bladé *et al* (2001) have recently pointed out that thalidomide had no effect in soft-tissue plasmacytomas. This suggested that the effectiveness of the drug depends on the tumour location (marrow *vs* extramedullary sites) (Bladé *et al*, 2001). Thus, thalidomide appears to be an attractive agent in PCL, the most severe form of plasma cell disorders, but its best modalities of use (dose, association with other agents) remain to be determined in larger series.

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Keywords: thalidomide, plasma cell leukaemia, multiple myeloma.

RED CELL LIFESPAN ESTIMATION BY ^{51}Cr LABELLING

A recent review (Dacie, 2001) prompts me to try and put the estimation of red cell lifespan by ^{51}Cr labelling into perspective. In truth, this investigation is now so infrequently attempted that its technical limitations have been forgotten and there is a danger that it might be accepted at face value. While it was undoubtedly an advance on Ashby's original attempt, it was never a reliable way of measuring red cell lifespan.

At a simplistic level the method appears straightforward. The subjects own red cells are incubated *in vitro* with a solution of ^{51}Cr chromium chloride. At intervals after reinjection the amount of ^{51}Cr remaining in the circulation is measured and from this an estimation of the red cell lifespan is calculated.

In truth what is calculated is the half-life of ^{51}Cr in the circulation. The reason why this does not equate with red cell lifespan is that ^{51}Cr is lost from the red cells in the circulation at a rate which is equal to or greater than the rate at which it is lost as red cells are cleared. Worse still, this rate of elution is unpredictable. Some studies have attempted to correct this by using an elution correction factor of 1% per day – about the same rate as red cells might be expected to be cleared. This figure was based on a study of 20 patients in one laboratory a long time ago (Mollinson, 1961). Even then it showed that the elution rate varied over a twofold range, that is, in some patients the loss of chromium by this route could be double the rate of red cell clearance.

When ^{59}Fe kinetic studies were available to assess true red cell lifespan they showed the inherent unreliability of the method as a means of assessing red cell lifespan, although their impracticability did not offer a clinical alternative (Napier *et al*, 1979). At the moment, the reticulocyte percentage is the only practical (but indirect) assessment of red cell lifespan available. This is not because it reflects erythropoietic compensation for haemolysis, but simply because it indicates a change in the proportion of young to old red cells. Such limited data suggests that when the reticulocyte percentage exceeds 2·5% the red cell lifespan is likely to be < 30 d.

Notwithstanding this, it is time to remove the ^{51}Cr assessment of red cell lifespan from haematological practice and to recognize that any results or interpretation that have been based on such a fundamentally flawed technique will have been equally flawed.

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Keywords: red cell lifespan, estimation, errors.

TREATMENT OF PRIMARY CHRONIC COLD AGGLUTININ DISEASE WITH RITUXIMAB: MAINTENANCE THERAPY MAY IMPROVE THE RESULTS

Berensten *et al* (2001) recently reported on the efficacy of rituximab in primary chronic cold agglutinin disease (CAD). In 4/6 patients CAD was associated with lymphoplasmocytic lymphoma and these patients had favourable responses. No response was observed in the two remaining patients with no signs of lymphoma. Three responding patients have been previously reported (Lee & Klueck, 1998; Bauduer, 2001; Layios *et al*, 2001) and all three had also low-grade lymphoma-associated CAD, suggesting that the results may be better for this condition. Therefore, we agree with the statement of Berensten *et al* (2001) that 'the failure of two patients to respond may possibly relate to absent or atypical signs of lymphoproliferative disease'.

We report the case of a patient with refractory CAD not associated with any sign of lymphoproliferative disease who poorly responded to induction therapy with rituximab but who went into complete remission after 6 months of maintenance therapy.

A 77-year-old Caucasian woman presented in October 1991 for weakness and dyspnoea. There were no enlarged lymph nodes or hepatosplenomegaly. Haemoglobin was 6·9 g/dl and the reticulocyte count was 222×10^9/l. The lactate dehydrogenase (LDH) level was 929 UI/l (normal < 220 UI/l) and haptoglobin was below the detection limit. The direct antiglobulin test was positive for polyvalent serum and complement. Cold agglutinins of the IgM type showing anti-I specificity were positive with a titre of 1/2072. No abnormal cell population was detected using lymphocyte immunophenotyping and the bone marrow smears were unremarkable. Serum protein electrophoresis and immunofixation were normal. She had received prednisolone (1 mg/kg/d) from November 1991 until February 1994 associated with cyclophosphamide (150 mg/d) from January 1992 until February 1994, with complete resolution of signs of haemolysis. She remained in stable complete remission until January 2001, when acute relapse occurred.

Haemoglobin was 6·7 g/dl, LDH was 989 UI/l and haptoglobin was below the detection limit. The direct antiglobulin test was positive for polyvalent serum and complement. Cold agglutinins were positive with a titre of > 1/2400. Treatment with corticosteroids and cyclophosphamide was resumed but did not improve the haemolysis and the patient required multiple transfusions. She then received four weekly doses of rituximab at 375 mg/m^2 each (Mabthera, Laboratoires Roche, France) from February 2001, with good tolerance. Cyclophosphamide was stopped and corticosteroids tapered. Red cell transfusions were stopped after the first rituximab infusion and the haemoglobin level raised to 10·5 g/dl at the end of March. Unfortunately, haemolytic signs reappeared (haemoglobin 8·8 g/dl, LDH 940 UI/l, haptoglobin < 0·08 g/l) in April 2001, 6 weeks after the last rituximab infusion. It was then decided to resume the treatment with rituximab using a different schedule: one injection every 2 weeks for 2 months, then progressively tapered to every 3, 4 and, finally, 6 weeks. A complete remission was achieved in 6 months. Haemoglobin, reticulocytes, LDH and haptoglobin were all within the normal range. At the time of writing the patient is still doing well in complete remission with one maintenance injection every 2 months.

CAD is a difficult disease to treat. The preliminary results observed with rituximab are very promising, but it seems that the response is less favourable in patients without associated signs of lymphoproliferative disease. Our case report highlights, for the first time, that complete remission can be achieved, even in this particular setting, using induction therapy with rituximab followed by a maintenance phase. The use of maintenance therapy seems promising to further improve the results of rituximab in CAD, although further studies are warranted to permit any general statement about the value of maintenance therapy.

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