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section 22 Haematological disorders 5348 There are anecdotal reports on the use of monoclonal antibody therapy with alemtuzumab (anti-CD52) and rituximab (anti- CD20). Alemtuzumab has been used in combination with CSA in a small number of patients. However, relapse may occur and con- tinued ‘maintenance’ with CSA may be required. Patients need to be carefully monitored for infections. Anecdotal responses have been reported with rituximab notably in patients with underlying B-cell lymphoproliferative disorders. Likely future developments With the advent of next-generation, high-throughput DNA sequen- cing, and the success of the 100K Genome project, increasing num- bers of genes involved in inherited BMF disorders will be identified. This will form the basis of a routine clinical screening test for all newly presenting patients, and is likely to have a major impact on clinical management decisions. It is predicted that more patients with apparent acquired BMF will have a mutation in one or more genes that are involved in the pathogenesis of BMF. Further understanding of the immunological changes that occur in acquired AA is likely to result in the availability of predictive tools for response to immunosuppressive therapy and for later relapse. Alternative approaches to HSCT for those patients lacking a suit- ably matched sibling or volunteer unrelated donor may include further evaluation of haploidentical HSCT with the use of post- transplantation high-dose cyclophosphamide. It is almost always possible to find a potential haploidentical donor. Novel cellular ther- apies using, for example, ex vivo expanded autologous Tregs are in development following the in vitro expansion of AA Tregs that are functional, stable and polyclonal. As the success of HSCT continues to improve, it is likely that this treatment is already being extended to older patients. FURTHER READING Alter BP (2005). Bone marrow failure: a child is not just a small adult (but an adult can have a childhood disease). Hematology, 2005, 96–103. Bacigalupo A, et al. (2015). For the Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation (WPSAA-EBMT). Current outcome of HLA identical sibling vs. un- related donor transplants in severe aplastic anemia: an EBMT ana- lysis.

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 marrow failure is always present; and sometimes the disease may be preceded by or may evolve to bone marrow aplasia indistinguishable from acquired aplastic anaemia. Definitive diagnosis is based on demonstrating the presence of a discrete population of ‘PNH red blood cells’ by flow cytometry using anti-CD59. In most cases, espe- cially when the patient is transfusion dependent and/or has severe signs and symptoms, there is an indication for long-term treatment with the complement inhibitor eculizumab. Definition Paroxysmal nocturnal haemoglobinuria (PNH) is an acquired chronic disorder characterized by

persistent intravascular haem- olysis, subject to recurrent exacerbations, often associated with cytopenias, and with a distinct tendency to venous thrombosis. The triad of haemolytic anaemia, pancytopenia, and thrombosis makes PNH a truly unique clinical condition: however, even in the absence of one or more of these manifestations a conclusive diagnosis can be made by appropriate laboratory investigations. Epidemiology PNH is encountered in all populations throughout the world, and it can affect people of all socioeconomic groups. The prevalence of PNH is not accurately known: however, it is rarer than the re- lated disorder, acquired aplastic anaemia (AAA). An estimate of the prevalence of PNH is between 1 in 100 000 and 1 in 1 million. Like AAA, PNH may be somewhat less rare in South-East and East Asia. Most patients present as young adults, but we have seen PNH in a 2-year-old child and in people in their seventies. PNH has never been reported as a congenital disease, and there is only one isolated case with inherited susceptibility. The sex ratio is not far from even. Clinical features The patient may seek medical attention because, one morning, he or she has ‘passed blood instead of urine’. This distressing or frightening event—the direct evidence of haemoglobinuria—may be regarded as the classic presentation; however, more frequently the patient presents as a problem in the differential diagnosis of anaemia, whether symptomatic or discovered incidentally. The anaemia may be associated with jaundice, with neutropenia, with thrombocytopenia, or any combination of these. Recurrent attacks of abdominal pain are not uncommon; dysphagia and erectile dys- function less common. In some patients, venous thrombosis may be the first clinical manifestation. Although any vein may be af- fected, the most common localization is intra-abdominal, and when thrombosis affects the hepatic veins, it may produce acute hepatomegaly and ascites—that is, Budd–Chiari syndrome. The natural history of PNH can extend over decades. In the past, with only supportive treatment, the median survival was estimated to be about 10 to 20 years (Fig. 22.5.3.1), with the most common causes of death being thrombosis, infection associated with severe neutropenia, and haemorrhage associated with severe thrombo- cytopenia. With contemporary treatment including eculizumab (see ‘Eculizumab’), the lifespan may be nearing normal. A patient with PNH may have a history of previous AAA. In fact, with improved laboratory diagnosis the transition from AAA to PNH is being documented increasingly, to the extent that PNH evolving on a background of bone marrow failure may be the rule rather than the exception. Conversely, a picture of overt AAA may develop after years of PNH. Rarely (estimated at 1–2% of all cases), PNH may terminate in acute myeloid leukaemia. By contrast, full spontaneous recovery from PNH has also been well documented. Laboratory investigations and diagnosis The most consistent finding in the blood count is anaemia, which may range from mild to moderate to very severe. The anaemia is usually normo-macrocytic; a high mean cell volume is usually largely ac- counted for by reticulocytosis, which may be quite marked—up to 20%. The anaemia may become microcytic if the patient is allowed to become iron deficient as a result of chronic urinary blood loss through haemoglobinuria. The red cell morphology is otherwise usu- ally normal. There may be neutropenia and/or thrombocytopenia. Unconjugated bilirubin is mildly or moderately elevated, lactate dehydrogenase is typically markedly elevated, and haptoglobin is usually undetectable. Haemoglobinuria may be overt in a random urine sample; if it is not, it may be helpful to obtain serial urine samples, since haemoglobinuria can vary dramatically from day to day, and even from hour to hour (it is more common, but not always, in the early morning: hence the adjec- tive ‘nocturnal’). Obviously, haemoglobinuria must be distinguished from haematuria and other causes of dark urine (Table 22.5.3.1). Surprisingly, even today a patient may undergo extensive urological investigations before it is realized that he/she has PNH. There may be free haemoglobin in the serum, and sometimes this is so high as to interfere with clinical chemistry. These findings clearly indicate a

haemolytic anaemia with intravascular haemolysis. The bone marrow is usually cellular, with marked to massive erythroid Survival (%) Median survival allo-BMT (Raiola et al.) 100 75 50 25 Years 1 5 10 15 20 25 Supportive care (Hillmen et al.) allo-BMT (IBMTR) allo-BMT (IBMTR) extrapolated Fig. 22.5.3.1 PNH is a chronic disorder, the time course of which is often measured in decades. From a series of 80 patients who received only minimal supportive treatment, a median survival of about 10 years had been estimated. Allogeneic bone marrow transplantation is still associated with significant mortality and may have reduced the survival of some patients, but more encouraging results have been reported on a small series from a single centre (Raiola et al.). The time course and survival have changed markedly with the introduction of eculizumab (Fig. 22.5.3.7), but the data shown here are still relevant to countries where this drug is not available.

section 22 Haematological disorders 5350 hyperplasia, often with mild to moderate dyserythropoietic fea- tures. However, at some stage of the disease the marrow may become hypocellular or even frankly aplastic. The definitive diagnosis of PNH must be based on demonstrating that a substantial proportion of the patient’s red cells have an increased susceptibility to complement due to the deficiency on their surface of proteins (particularly CD59 and CD55) that normally protect the red cells from activated complement. For decades this has been done reliably by using the acidified serum (Ham–Dacie) test. Nowadays, the gold standard is flow cytometry that will display a bimodal (some- times trimodal) distribution of red cells. Such analysis is also applied to granulocytes (Fig. 22.5.3.2), revealing that the proportion of affected granulocytes is almost always greater than that of affected red cells. Pathophysiology Haemolysis Haemolysis in PNH is due to an intrinsic abnormality of the red cell which makes it exquisitely sensitive to activated complement, whether it is activated through the alternative pathway or through the classic pathway. Activation through the so-called tick-over com- ponent of the alternative pathway explains why there is chronic haemolysis in PNH (Fig. 22.5.3.3, top panel). Activation through the classic pathway, triggered by an antigen–antibody reaction, ex- plains why haemolysis can be dramatically exacerbated (with a con- sequent paroxysm of haemoglobinuria) in the course of a viral or bacterial infection. Hyper-susceptibility to complement is due to the deficiency of several protective membrane proteins, of which CD59 is the most important, mainly because it hinders the insertion into the membrane of C9 polymers. The molecular basis for the deficiency of these proteins has been pinpointed not to a defect in any of the respective genes, but rather to the shortage of a glycolipid molecule, glycosylphosphatidylinositol (GPI), which through a peptide bond anchors these proteins to the surface membrane of cells. The shortage of GPI is due in turn to a mutation in an X-linked gene, called PIGA, required for an early step in GPI biosynthesis. In virtually each patient the PIGA mutation is different. This is not surprising since these mutations are not inherited: rather, each one is a somatic mutation that takes place de novo in a haemopoi- etic stem cell. As a result, the patient’s bone marrow is a mosaic of mutant and nonmutant cells, and the peripheral blood always contains both GPI-negative PNH cells and GPI-positive cells (Fig. 22.5.3.2). Thrombosis This is one of the most immediately life-threatening complications of PNH and yet one of the least understood pathogenetically. It could be due to impaired fibrinolysis because the urokinase plas- minogen activator receptor (uPAR) is a GPI-linked protein, or to activated complement causing hypercoagulability, or to activated complement and haemoglobin in the plasma causing hyperactivity of platelets, or any combination of these. Table 22.5.3.1 Differential diagnosis of dark urine Different sorts of dark urine Causes Additional tests Possible diagnosis Haematuria Many Clears on centrifugation Mostly urinary tract or glomerular pathology Myoglobinuria Rhabdomyolysis Ultrafiltration; spectroscopy March myoglobinuria Haemoglobinuria

Intravascular haemolysis Repeat crossmatch Incompatible blood transfusion Donath–Landsteiner antibody Paroxysmal cold haemoglobinuria G6PD activity G6PD deficiency Blood film for malaria parasites Blood cultures Red cell morphology ‘Blackwater fever’ Clostridium perfringens septicaemia Microangiopathic haemolytic anaemia Ham; flow cytometry for CD59 PNH G6PD, glucose-6-phosphate dehydrogenase; PNH, paroxysmal nocturnal haemoglobinuria. Erythrocytes 33% 400 Counts 300 200 100 0 67% Granulocytes 60 40 20 15 10 5 Normal PNH patient Counts Counts Counts 15 M2 CD59–FITC M1 M2 CD59–FITC M1 0 CD59–FITC 400 300 200 100 0 95% 5% CD59–FITC M2 M1 Fig. 22.5.3.2 Flow cytometry analysis of blood cells in a patient with PNH. On the left, red cells and granulocytes from a normal person display a unimodal distribution of surface expression of the GPI-linked protein CD59, which protects red cells against complement-mediated lysis. On the right, a similar analysis reveals a clearly bimodal distribution in a patient with PNH, and from this analysis the size of the PNH cell population can be quantified. FITC, fluorescein-isothiocyanate. Courtesy of Dr David Araten.

22.5.3 Paroxysmal nocturnal haemoglobinuria 5351 Bone marrow failure and the relationship between PNH and AAA PNH has an intimate link to AAA, which manifests in several ways. Firstly, as stated previously, PNH is often preceded by AAA, and sometimes a patient with PNH becomes ‘less haemolytic’, ‘more pancytopenic’, and ultimately evolves to frank AAA. Secondly, in- tensive immunosuppression is a standard-of-care treatment in AAA, and a beneficial response to the same treatment can be seen also in patients with PNH. Thirdly, in terms of pathogenesis, AAA is regarded as an organ-specific autoimmune disease mediated by ‘activated’ cytotoxic (CD8+) T lymphocytes which are able to inhibit haemopoietic stem cells. GPI-specific CD1d-restricted T cells have been demonstrated in both PNH and AAA. It therefore seems that an element of bone marrow failure is the rule rather than the exception in PNH: an extreme view is that PNH is a form of AAA in which bone marrow failure is masked by the enormous expansion of the PNH clone that populates the patient’s bone marrow. In other words, two different mechanisms cooperate in producing PNH (Fig. 22.5.3.4): autoimmune damage to stem cells, and a somatic mutation in the PIGA gene. This notion is sup- ported by two further lines of evidence: (1) by targeted inactivation of the Piga gene in mouse embryonic stem cells one can produce mice with a PNH cell population, but this population does not grow further as it does in patients with PNH; and (2) by using refined flow cytometry technology, PNH cells harbouring PIGA mutations can be demonstrated in normal people at a frequency in the order of 10 per 1 million. Both these findings indicate that some other factor is required, in addition to a somatic mutation in the PIGA gene, in order to cause expansion of a PIGA mutant clone and thus PNH. Most likely, the same cytotoxic damage to stem cells that would otherwise cause AAA spares the PNH stem cells, thus allowing the PNH clone to grow to the size when it gives clinical PNH. The pre- cise mechanism whereby the PNH stem cells escape damage is not yet known, but one possibility is that the GPI-specific T cells men- tioned previously damage GPI-positive stem cells, whereas they are harmless for the GPI-negative stem cell from which the PNH clone originates. Complications The most important complication is thrombosis, which is nearly always venous, and can be life-threatening especially if it affects either the abdominal veins (Fig. 22.5.3.5) or the intracranial veins. The Budd–Chiari syndrome, because of its characteristic clinical picture, is usually easy to recognize: however, in PNH it is some- times associated with portal vein thrombosis, and this may limit the extent of liver enlargement. Thrombosis of the splenic vein should be suspected whenever a patient with PNH has, or develops, splenomegaly. Thrombosis of one of the mesenteric veins is much more difficult to diagnose clinically. Appropriate investigations include Doppler ultrasonography, contrast-enhanced CT, and magnetic resonance

imaging venography (for this purpose, pos- sibly the most sensitive imaging technique). Recognizing venous thrombosis is of great practical importance, because thrombolytic Alternative pathway C5 C3 C3 Eculizumab C5 C3 Alternative pathway C3 Coombs' ++ Fig. 22.5.3.3 Red cells and complement in PNH. Top: in PNH in the steady state, erythrocytes, by virtue of being deficient in the complement regulators CD55 and CD59, suffer as a result of spontaneous (tick-over) complement activation, with consequent intravascular haemolysis through formation of the membrane attack complex (MAC); exacerbated haemolysis ('paroxysm') will result from activation of extra complement through the classical pathway. Bottom: on eculizumab, PNH erythrocytes are protected from haemolysis through C5 blockade, but continuing upstream complement activation may lead to C3 opsonization (positive Coombs' test) and consequent extravascular haemolysis. Modified from Luzzatto L, Risitano AM, Notaro R (2010). Paroxysmal nocturnal hemoglobinuria and eculizumab. Haematologica, 95, 523–6. Target: GPI PIGA mutation in a HSC Subclinical GPI-negative clone Expansion of GPI- negative clone APLASTIC ANAEMIA Target: other molecules T cell-mediated autoimmune attack against HSC PNH Fig. 22.5.3.4 The role of somatic mutation and bone marrow failure in causing PNH. This diagram illustrates that two separate factors are required to bring about PNH as a clinical disease. On the one hand, a PIGA mutation on its own will produce a PNH clone, but there will be no basis for it to expand; on the other hand, damage to haemopoietic stem cells (HSC) can cause aplastic anaemia without PNH. When both factors cooperate, and if the damage to HSC is GPI mediated, then there will be selective expansion of the PNH clone. A patient with PNH often has a history of aplastic anaemia, and whether aplastic anaemia or PNH is diagnosed first will depend on the timing of the PIGA mutation as well as on the kinetics of BMF and of the expansion of the PIGA mutant clone.

section 22 Haematological disorders 5352 therapy with tissue plasminogen activator has been carried out successfully even after 6 weeks from the onset of signs and symp- toms (Fig. 22.5.3.5). Management Bone marrow transplantation Unlike other acquired haemolytic anaemias, PNH may be lifelong, and this is important in our approach to management. The only form of treatment that can provide a cure for PNH is allogeneic bone marrow transplantation, which should be offered for consid- eration to any young patient with PNH for whom a human leuco- cyte antigen-identical sibling is available. Results similar to those for AAA can be expected, with long-term disease-free survival ranging from 60 to 100% in the few series that have been published. By con- trast, in a few cases in which bone marrow transplantation has been carried out from unrelated donors the outcome in several PNH pa- tients has not been good (Fig. 22.5.3.1). Eculizumab A major advance in the management of PNH has been the introduc- tion of complement blockade by the use of a humanized monoclonal antibody, eculizumab, which targets the C5 component of com- plement (Fig. 22.5.3.3, bottom panel). This has proven effective in controlling intravascular haemolysis, hence haemoglobinuria disap- pears in most patients, and at least one-half of the patients who were transfusion dependent no longer require transfusions, and in others the need for transfusions is significantly reduced (Fig. 22.5.3.6). In addition, distressing symptoms such as abdominal pain are relieved and hence quality of life improves. However, given its mechanism of action, eculizumab is not a curative treatment: its benefits will last as long as the agent is administered through an intravenous infusion at fortnightly intervals. Since only the distal complement pathway is blocked, in PNH patients on eculizumab C3 fragments will bind to PNH red cells that, being so opsonized, become susceptible to phagocytosis by macrophages (Fig. 22.5.3.3). Thus, patients on eculizumab still have haemolysis, but this is usually milder than what they had before eculizumab and, being extravascular, it is far less symptomatic. It is also important to note that (unlike

intravascular haemolysis) there is no iron loss with extravascular haemolysis, hence for the first time PNH patients are at risk of iron overload if they still require blood transfusion whilst on eculizumab. As the distal complement pathway is blocked in patients on eculizumab, they are at an increased risk for infection by menin- gococcus, hence immunization against this organism is mandatory before starting eculizumab. In most patients this treatment has been remarkably free of serious side effects, but there have been a few instances of severe infection which require immediate antibiotic treatment. A very significant extra advantage of eculizumab treatment is that it also decreases the risk of thrombosis, which is especially (a) (b) Fig. 22.5.3.5 Abdominal vein thrombosis in PNH can resolve with thrombolytic therapy. (a) Extensive thrombus in the inferior vena cava in a patient with known PNH who had developed Budd–Chiari syndrome a few days earlier: it is not infrequent in PNH for thrombosis to involve multiple veins in the abdomen all at once. (b) A thrombus-free vena cava 2 days after an intravenous infusion of tissue plasminogen activator. Courtesy of Raymond Thertulien; see also Haematologica 97: 344, 2012. Hgb≥11 33.3% 8≤Hgb<11 46.3% ≤50% 14.8%

> 50% 5,6% n = 54 T r a n s f u s i o n i n d e p e nd e n c e T r a n s f u s i o n d e p e n d e n c e Fig. 22.5.3.6 Effect of eculizumab treatment on blood transfusion requirements in PNH. About two-thirds of the patients are or have become transfusion independent. The smaller sectors indicate patients who still require blood transfusion, subdivided according to whether the requirement is greater or less than one-half of what it was before eculizumab treatment. Updated 2013 from Risitano AM et al. (2009).

22.5.3 Paroxysmal nocturnal haemoglobinuria 5353 important because patients with PNH are not fully protected from thrombosis even by painstaking anticoagulant treatment. Most patients on eculizumab have not only a better quality of life but probably an increased survival (Fig. 22.5.3.7). Several suc- cessful pregnancies have been supervised in PNH patients on eculizumab. Supportive care Eculizumab is not available in many countries because it is very expensive. It is therefore appropriate to remember that, before its introduction, supportive management supervised by somebody who has experience of PNH can help patients to ‘live with PNH’ for years, sometimes for decades, and sometimes with a good quality of life. The mainstay of support is the transfusion of filtered red cells whenever necessary. Folic acid supplements (≥3 mg/day) are mandatory; the serum iron concentration should be checked periodically and iron supplements added as indicated. There is no evidence that prednisone (which used to be administered at a dose of 15 to 30 mg on alternate days) decreases the rate of haem- olysis, and long-term administration of prednisone, even at a low dosage, is contraindicated in view of its well-known serious po- tential side effects (a short course of prednisone may sometimes appear helpful in dealing with an episode of massive haemo- globinuria associated with intercurrent infection). Any patient who has had a deep vein thrombosis should be given anticoagu- lant prophylaxis. Bone marrow failure Eculizumab will have clearly no effect on the bone marrow failure component of PNH. When the manifestations of bone marrow failure predominate, the approach to treating PNH becomes similar to that indicated for AAA: accordingly, a logical option is intensive immunosuppressive treatment with antilymphocyte globulin (ALG) and ciclosporin. Although no formal trial has ever been conducted, this approach has particularly helped to relieve severe thrombo- cytopenia and/or neutropenia in patients in whom these were the main problem(s): by contrast, there is often little beneficial effect

on the haemolysis itself. Thus, the therapeutic effects of ALG and eculizumab are in a sense complementary. New approaches to in- hibit complement in PNH are currently being investigated.

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