WITH THE INCREASING integration of molecular genetics into the study of human diseases we have come to almost expect that, whenever a new gene is cloned, the next paper will report the consequences of ‘knocking out’ the gene. In a way, we are thus witnessing the logical updating of a century-old approach to the study of physiology. To define the function of an organ, a simple way was to remove it surgically; today, targeted homologous recombination has proven a powerful micro-surgical technique to remove the function of one gene at a time.1 However, lest we lose sight of our purpose, it is useful to draw a distinction. When a gene is isolated by positional cloning, we often have no idea of its normal function: therefore, knocking it out can suddenly provide an entirely new insight. When a gene is isolated instead on the basis of its known biochemical function, and the abnormality of that function is already known to cause a certain disease, no immediate surprise is to be expected. However, the knock-out approach can be still used to identify subtle pathophysiological features of the disease concerned, and especially to obtain an animal model lending itself to experimental manipulations and ultimately to investigating new forms of therapy.2

The PIG-A gene was cloned by the group of Miyata et al3 in 1993 by an elegant approach: the functional complementation of a cell line with a known defect in the biosynthesis of glycosyl phosphatidyl inositol (GPI) anchors. At the time it was also already known that the biochemical lesion in paroxysmal nocturnal hemoglobinuria (PNH) cells was in this metabolic pathway,4 and it became immediately clear that the ‘PNH gene’ had been really identified.5,6 We did not need to wait for any knock-out experiment to understand that a frameshift mutation of PIG-A would completely abrogate GPI biosynthesis,7 which would prevent CD59 from appearing on the surface of red blood cells (RBCs), which would cause these RBCs to be highly vulnerable to the final stage of the complement activation cascade. Complement activation can be triggered by an antigen-antibody reaction or, through the alternative pathway, by the flimsiest of excuses: if CD59 is lacking this ends up in a large number of RBCs being destroyed. However, there are of course two important differences between PNH and other hemolytic anemias due to intracorpuscular abnormalities of the RBCs: (1) Because PNH is caused by a somatic mutation in a hematopoietic stem cell,8 rather than by an inherited mutation, the other cells in the body are not affected. (2) Even within the hematopoietic system, because only one or very few9 stem cells can be expected to have PIG-A mutations, there is always a residual population of cells with normal PIG-A and, therefore, normal GPI-linked molecules. Indeed, as originally suggested a decade ago,10 and as more recently highlighted in several recent reviews,11-15 a central issue to understanding PNH is to pinpoint the factors that determine the balance between the normal and the PNH hematopoietic populations. Thus, knock-out animals were needed not so much to prove that PIG-A mutations cause PNH, or to define more precisely the function of PIG-A, but very much to provide an animal model in which other components of the disease process could be investigated.

Three groups promptly targeted pig-a in male mouse embryonic stem (ES) cells and, thanks to the fact that this gene is X-linked, they promptly obtained ES cells that were genetically and phenotypically GPI-negative and that proved viable.16-18 However, when these cells were used to produce pig-a null mice, the problem arising was similar to that confronting an over-enthusiastic physiologist who, a century ago, having successfully defined the function of the pancreas and of the pituitary gland by surgically removing them, decided to do the same with the liver or the heart. We all found that high-contribution chimeric embryos died in utero and, therefore, germ-line transmission could not be obtained. Low-contribution pig-a null mice were born, but their low numbers of GPI-negative blood cells failed to provide a valid model of the human disease PNH.

In this issue of Blood two papers report success in producing such a model, based on the technique of conditional knock-out, using the system known in jargon as cre-lox. Murakami et al19 have crossed male mice, in which two lox sequences had been engineered to flank exon 6 of pig-a, with female mice transgenic for cre driven by the universally active cytomegalovirus (CMV) promoter. The resulting females, heterozygous for a pig-a gene vulnerable to inactivation, died late in fetal life with brain abnormalities: but before they died, hematopoietic cells from their liver were used to reconstitute hematopoiesis in...
syngeneic irradiated recipient mice. Because, following X chromosome inactivation, these liver cells were a mosaic of cells with a normal and a knocked out pig-a, the transplanted animals had both normal and PNH cells in their blood. Instead, Tremml et al20 crossed mice (both males and females) in which two lox sequences had been engineered to flank exon 2 of pig-a, with mice (females and males) transgenic for cre driven by the EIIa promoter, which is expected to drive gene expression only in the very early, preimplantation stage of embryonic life. In this case the offspring were born live, and the proportion of PNH cells in their blood presumably reflected the proportion of stem cells arising from cells in which, in the early embryo, cre expression had knocked out pig-a. Thus, both of these two different approaches have yielded the desired animal model;

<table>
<thead>
<tr>
<th>Table 1. Two Mouse Models of PNH</th>
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<tr>
<td>Murakami et al19</td>
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<tr>
<td>No. of mice studied</td>
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<tr>
<td>Sex</td>
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<tr>
<td>PNH phenotype, %</td>
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<tr>
<td>(Range) mean PNH RBC, %‡</td>
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<td>(Range) mean PNH PMN, %‡</td>
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<td>Mean PNH T cells, %‡</td>
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<td>(Range) mean PNH B cells, %‡</td>
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*From female donors.
†Defined as having at least 5% PNH cells.
‡At 32 weeks.

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Fig 1. PNH in mice and humans. The two upper panels represent in cartoon form the mouse models constructed in refs 19 and 20, respectively. Although all cells have the same genotype, their phenotype differs depending on which X chromosome has been inactivated: white circles indicate cells in which the X chromosome with the normal pig-a gene is active; gray circles indicate cells in which the X chromosome with the inactivated pig-a gene (pig-a°) is active. As a result, in the peripheral blood there is a mixture of normal (white) and PNH (gray) blood cells. The lower proportion of PNH cells in the model by Tremml et al is caused by the fact that cre, driven by EIIa, does not knock out pig-a in every cell (Tremml et al have produced also male mice with PNH [see text].) In both models the proportion of PNH cells in the peripheral blood depends on the proportion of cells in which cre-mediated recombination has taken place: there is no evidence of selection thereafter. The bottom panel represents in cartoon form what happens in the human disease, PNH. Here, unlike in the mice, the entire PNH population arises usually from a single stem cell in which a somatic mutation has taken place: therefore, selection must take place at the level of stem cells in order for a large proportion of the blood cells to have the PNH phenotype. Here only one allele of the PIG-A gene is shown because the entire process takes place after X-inactivation, when somatic cells are functionally haploid for most X-linked genes in both males and females.
and the first and gratifying observation from reading the two papers is that the results are in good agreement (see Table 1).

In fact, the two approaches differ in a non-trivial way (see Fig 1). Because the EIIa promoter is not tissue-specific, the mice produced by Tremml et al must be mosaics for pig-a inactivation in all tissues: unlike the case of patients with PNH, in whom only the hematopoietic tissue is involved. (However, in a separate report\(^2\) the same group shows that in most tissues in the majority of cells the X chromosome with the normal pig-a gene is the active one, indicating negative selection against the GPI-negative cells early in organogenesis.) In this respect the mice produced by Murakami et al are more like PNH patients, because their nonhematopoietic tissues have not been manipulated. On the other hand, the transplantation procedure required the use of irradiation, and the authors themselves point out that in theory this might affect the fate of the transplanted cells: for instance, through an effect on stromal cells. No irradiation was used in Tremml’s experiments. The fact that the results obtained are similar virtually eliminates a significant role of these potentially confounding variables, and the data in the two papers further corroborate one another. Therefore I will choose to consider the two papers together in looking at them as a model of human PNH.

Three main points have emerged. First, the mice have two discrete populations of RBCs, granulocytes, monocytes, and lymphocytes: in first approximation, the flow cytometry patterns are remarkably similar to those seen in patients with PNH. Second, the mice are not anemic. This may be at first sight disappointing; however, in vitro the RBCs display a perfect mimicry of the classical PNH phenotype, with a marked shift in susceptibility to complement. Indeed, although the proportions of RBCs are lower in the mice studied by Tremml et al (Table 1), they have been able to produce a positive micro-Ham test, and they show convincingly, by analyzing reticulocytes, that the life span of the PNH RBC population is significantly shortened in vivo. Thus, although, by looking at their cages, it does not seem the mice really have hemoglobinuria, the RBC phenotype of the human disease is fully reproduced (and, who knows, perhaps hemoglobinuria would turn up if the mice were allowed out of the sheltered environment of a high-class research animal house). Third, and perhaps least expected, both reports show a remarkably high proportion of PNH B cells and T cells. In fact, Murakami et al find that almost all the T cells are PNH, and their detailed analysis suggests that the takeover occurs after the cells have entered the thymus, implying a paradoxical advantage of T cells lacking GPI-linked proteins at this particular stage in development.

Thus, there is no doubt that we have a good model of the hematological picture of PNH at a particular point in time. But what is the clinical course of the mice patients (or patient mice)? On this point, we get two important messages: (1) Once the mice are adult, the proportion of PNH cells is remarkably stable. (2) At least until now, the mice have not developed leukemia. For a whole generation we have been inspired by William Dameshek’s classic 1967 editorial\(^2\) in this journal, in which, while giving full credit to Dacie and his colleagues\(^2\) for first outlining the close links between PNH and acquired aplastic anemia (AAA), he explored himself what may be in common between PNH, AAA, and “hypoplastic leukemia.” Two years later, as editor of Blood, Dameshek succeeded in bringing together in one special issue of the journal (published ‘to whittle down our rather impressive backlog’ of manuscripts) 3 individual case reports of PNH that ‘evolved’ to acute myeloid leukemia (AML). In a second editorial\(^2\) in that issue he developed clearly the notion that, although PNH was traditionally classified among hemolytic anemias, it was ‘a disorder of the entire bone marrow,’ and he suggested it was ‘a candidate’ myeloproliferative disorder (MPD). This notion seemed to become corroborated when it became clear that PNH was a clonal disease\(^2\); indeed, because leukemias are clonal \textit{par excellence}, the word ‘clonal’ is often used as though it were synonymous to neoplastic, or malignant.

Dameshek made no secret of his distaste for “pigeonholes,” and he was more sympathetic to ‘lumpers’ than to ‘splitters.’ Therefore, bringing together AAA, PNH, MPD, hypoplastic leukemia (now myelodysplastic syndrome [MDS]?), and Di Guglielmo’s disease (now AML-M6) appealed to him. As a credit to his vision, it is now abundantly confirmed that PNH shares with MDS, MPD, and AML the feature of being clonal; and with AAA, the feature of bone marrow failure. However, one generation later, we have several reasons to say that the link of PNH to AAA is closer than its link to MPD: (1) Evolution to AML is common in MPD and in MDS; but, contrary to Dameshek’s expectation, it is rare in human PNH,\(^2\) and it has not been a feature of murine PNH. (2) ‘Clonal expansion’ is progressive and usually inexorable in MDS or in MPD: by contrast, PNH clones expand up to a point, and then tend to be stable. The mouse data are remarkably similar in this respect. (3) In a number of well-documented patients PNH has evolved to AAA: this rarely happens in MDS and practically never in MPD. All these data are in keeping with the notion that MDS and MPD clones (let alone AML clones) have an unconditional or absolute growth advantage, whereas PNH clones have a relative or conditional growth advantage, which is made prominent by the marrow environment of AAA.

On this third point we do not yet have help from the mouse models, because the mice do not have AAA. In fact, in the model mice the PNH cell population arises from a substantial number of mutated stem cells, and the size of the PNH population depends on the number of cells that were transplanted, or from the number of cells in which \textit{cre} activation has caused inactivation of \textit{pig-a} (see Fig 1). By contrast, in human PNH the PNH cell population arises from the expansion of a single mutant stem cell. It has been suggested that this expansion results from the fact that PNH stem cells are able to escape from damage inflicted to normal stem cells by a specific pathogenic mechanism\(^2\); the best candidate being an autoimmune mechanism.\(^10,11\) Thus, PNH cells have a conditional growth advantage if, and only if, such a process is operating. This pathogenetic model has recently received support from the finding that PNH microclones are present in normal people: but in normal people they fail to expand.\(^2\) The logical implication of this model with respect to management is that the best way to treat PNH is to treat the underlying AAA. In fact, treatment with antilymphocyte globulin has been beneficial in individual cases\(^2\,\,\,30\); and there is evidence that PNH does not recur after an
allogeneic bone marrow transplant (BMT) even without a fully myeloablative conditioning. This suggests that BMT helps the patient by providing transient intensive immune suppression together with new stem cells: eradication of the PNH clone(s) is not necessary. By contrast, recurrence has occurred after a syngeneic BMT performed without any conditioning, because this treatment, although it provided new stem cells, did not provide immune suppression.

As mentioned earlier, we already knew that the PNH abnormality resulted from a PIG-A mutation. However, having reproduced the phenotype by targeting the pig-a gene has provided formal proof that such a mutation is necessary and sufficient to produce that phenotype: a clear analogy to Koch’s postulate for the etiology of an infectious disease. We now need to reproduce in the mice those conditions which, in humans, are able to select for the cells with the PNH abnormality. It would not be right to second-guess the two groups that have developed these models, but I would be surprised if they were not already hard at work in finding ways to induce AAM in their mice, probably by some immunological approach.

ACKNOWLEDGMENT

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