Leukemia (2001) 15, 1148–1152

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EDITORIAL

The cellular pathogenesis of paroxysmal nocturnal haemoglobinuria

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Paroxysmal nocturnal haemoglobinuria (PNH) is a unique disorder characterised by the triad of intravascular haemolysis, thrombosis and bone marrow failure. In the early seventies it was shown that PNH is a clonal disease; and in the nineties the molecular basis of the PNH abnormality was elucidated. However, what makes a PNH clone expand is still not known. Here, we suggest that this is due to somatic cell selection, resulting from the presence in the patient of autoreactive T cells that target glycosylphosphatidylinositol (GPI) in the context of an MHC-like molecule on the surface of haemopoietic stem cells. PNH cells would escape damage precisely because they have lost most or all of their ability to produce GPI. *Leukemia* (2001) **15,** 1148–1152.
Keywords: paroxysmal nocturnal haemoglobinuria (PNH); stem cells; clonal selection; autoreactive T cells; glycosylphosphatidylinositol (GPI); CD1d

Introduction

If we look back at our efforts to understand the nature of a disease condition, there are few examples with such a variegated history as paroxysmal nocturnal haemoglobinuria (PNH). In fact, whenever a new advance in PNH has answered a specific question, it has posed at the same time a new question. For instance, the early finding of haemoglobinuria clearly indicated intravascular haemolysis,[1] but the cause was puzzling because a high-titre autoantibody was not found. Next it was shown that haemolysis was due to abnormal susceptibility to complement,[2,3] but one wondered why this abnormality was limited to only a subset of all red cells. This led to a successful test of the notion that PNH was a clonal disorder due to a somatic mutation:[4,5] but very soon this seemed to clash with the finding that not just one, but so many different surface proteins were deficient in PNH cells. This paradox was solved by the finding that all these proteins had a common non-protein moiety, a glycolipid which has since become known as the glucosylphosphatidylinositol (GPI) anchor, because it tethers each one of these proteins to the cell's surface by virtue of the fact that its fatty acid residues are embedded in the membrane's lipid bilayer[6] (see Figure 1). Thus, deficiency of one of the enzymes required for the biosynthesis of GPI became a good candidate for explaining the PNH phenotype, but it was puzzling how a single mutation (ie on one allele only), could cause complete deficiency of an enzyme. This difficulty was solved when the biosynthetic gene *PIG-A* was cloned and mapped to the X chromosome, thus neatly explaining how one hit could cause complete loss of function of the gene product.[7] Today, we can define PNH as an acquired clonal disorder of the haemopoietic stem cell (HSC). Somatic mutations in the X-linked *PIG-A* gene of one or more HSCs result in the deficiency of GPI-anchored proteins from the surface of blood cells. The mutated (PNH) HSC(s) are able to expand to such an extent that in a patient with PNH the PNH blood cells may comprise up to >90% of the total haemopoiesis. However, we still do not know what determines (1) the size of the PNH clone, (2) the severity of the patient's pancytopenia and (3) the increased propensity to venous thrombosis: ie the three most important features of this condition in terms of clinical course and clini-

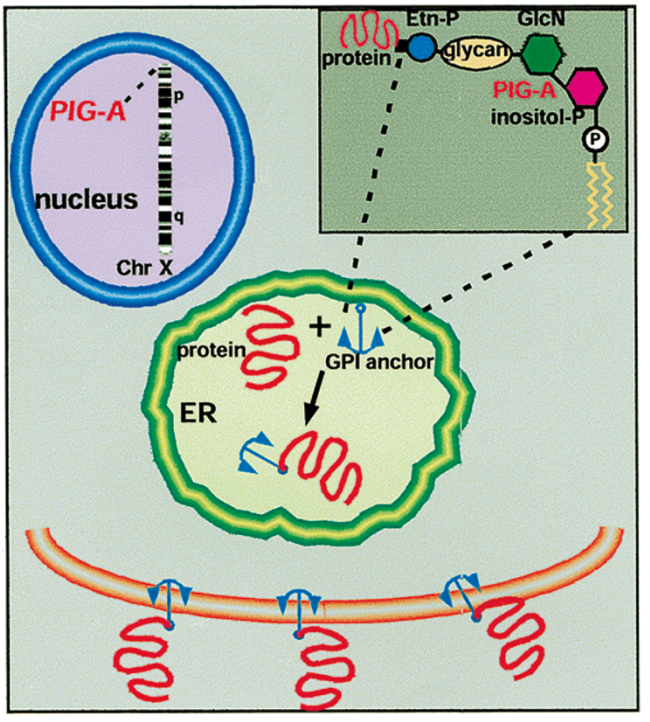

Figure 1 *PIG-A*, GPI anchors, GPI-anchored proteins and PNH. PIG-A is a protein encoded by the X-linked gene *PIG-A*. PIG-A is a member of a multi-subunit enzymatic complex which catalyzes, in the endoplasmic reticulum (ER) the first step in the biosynthesis of GPI: the addition of acetylglucosamine (GlcN) to phosphatidylinositol (inositol-P; inset). The synthesis of the GPI anchor is completed by the serial addition of a glycan moiety consisting of three mannose molecules and a molecule of phosphoethanolamine (Etn-P), to which, through a transpeptidation reaction, proteins with the appropriate carboxy-terminal amino acid motif are attached covalently. The GPI–protein complex subsequently travels to the cell surface, where the protein becomes 'anchored' to the lipid bilayer through GPI. In PNH, *PIG-A* has suffered a somatic mutation in one or few HSCs. As a result, very little GPI is synthesised, or none at all, with consequent severe deficiency of GPI-anchored proteins on the surface of the mutated HSCs and their progeny.

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Received 22 March 2001; accepted 22 April 2001

cal outcome.[8] Here, we advance a specific hypothesis that may help to answer these questions.

The vast expansion of a PNH clone that is seen in a patient with PNH cannot take place without a growth advantage. We have previously suggested that this growth advantage, rather than being absolute (as it is in leukaemia), might be relative or conditional, ie dependent on a particular bone marrow environment.[9] Specifically, an injury to the normal (GPI^+) HSC might spare PNH (GPI^-) HSC. While one might certainly conceive of physical, chemical or biological agents being able to discriminate between GPI^+ and GPI^- HSC, an obvious possibility is that the damaging agent is part of an auto-immune process (see Figure 2) especially, since this is a widely accepted pathogenetic mechanism in the closely related condition, idiopathic aplastic anaemia (IAA).[10] Here, we explore this model, leading on to the notion that the target of this auto-immune process could be the GPI molecule itself on HSCs.

PIG-A mutations are necessary but not sufficient to cause PNH disease

First, we summarize the current evidence that, once a *PIG-A* mutation has taken place, there must be a mechanism for positive selection of the mutant cells, or negative selection against the non-mutated cells (dual pathogenesis of PNH). There are essentially four lines of evidence. (1) *PIG-A* mutations are found regularly in normal people.[11] (2) Non-mutated hematopoietic cells are decreased in absolute terms in patients with PNH (as in IAA).[12] (3) Patients with PNH not infrequently have more than one mutant clone; and sometimes a different PNH clone can be shown to be dominant in the same patient at different points in time.[13] (4) In mice with targeted inactivation of the *pig-a* gene the PNH cell population does not increase in time, and often tends to disappear.[14–16] This last finding does not support positive selection for mutant cells; by implication, it favours instead the notion that negative selection against non-mutant cells dominates the picture in human PNH patients.

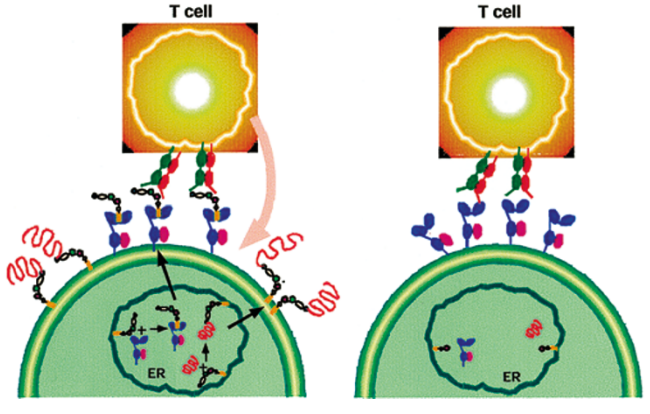

Figure 2 GPI-specific, CD1d-restricted T cells in the pathogenesis of PNH. In this hypothetical model, GPI-specific, CD1d-restricted T cells target and deplete GPI^+ (ie non-mutated) HSCs (left). However, GPI^- HSCS, because they fail to synthesise GPI, escape the T cell attack (right), expand and contribute to haemopoiesis to various degrees.

What is the target and what is the mechanism of negative selection?

We will make the specific assumption that an autoreactive population of cytotoxic T cells inhibits the growth or causes the demise of non-mutated HSC: ie we postulate that these cells have a differential effect on GPI^+ and GPI^- HSCs (escape model).[9] An increased frequency of clonal T cell expansions in patients with PNH is in line with this concept.[17] In addition, we have recently observed two patients with an association of T-LGL and PNH.[18] According to this model, we consider four possible alternative targets and cognate mechanisms of selection.

The deficiency of GPI-anchored molecules from the surface of PNH cells helps in itself to protect them from T cell-mediated attack

Indeed, it is conceivable that a GPI-linked protein is essential for effective recognition or killing by a cytotoxic T cell. However, we and others have shown in internally controlled experiments that there is no significant difference in the ability of T cells or NK cells to kill PNH haemopoietic cells *vs* normal, isogenic haemopoietic cells.[19]

The target of autoaggressive T cells is an epitope belonging to the PIG-A protein itself

Because PIG-A is an intracellular protein it can become itself antigenic only if critical peptide(s) arising through endogenous processing pathways are then displayed to pathogenic cytotoxic T cells in the groove of an HLA class I molecule.[20] Thus, non-PNH HSCs would process and present the putative PIG-A-derived peptide and become targets of T cells; in contrast, PNH HSCs would escape the T cell attack because, by failing to produce the PIG-A protein as a result of a *PIG-A* mutation they would not present the critical peptide. A strong argument against this possibility is the fact that PIG-A is ubiquitously expressed:[7] therefore, the immune process would be expected to damage cells in a variety of tissues. Instead, PNH is strictly a disease of haemopoiesis. In addition, there are many missense mutations in *PIG-A* in PNH,[21] and at least some of them would still produce the putative critical peptide(s). Therefore, it is unlikely that PIG-A is the target we are looking for.

The target of autoaggressive T cells is an epitope derived from a GPI-anchored protein

Although proteins destined to be GPI-linked fail to land on the surface of PNH cells because they do not find the anchor, they are as efficiently transcribed and translated in PNH cells as in non-PNH cells. This would result, in PNH cells, in the accumulation of unprocessed proteins which, in association with chaperones residing in the endoplasmic reticulum, are transferred to the cytoplasm where they are degraded.[22] Thus, although it has been suggested that PNH cells may present a critical epitope less efficiently than GPI^+ cells,[23,24] there is no experimental evidence to support this notion. Therefore, peptides from GPI-linked proteins do not seem at the moment good candidate targets either.

GPI itself is the target of autoaggressive T cells

It is now well established that, just as HLA molecules present peptide antigens, the β2-microglobulin-associated CD1 molecules (CD1a, CD1b, CD1c, CD1d), expressed on professional antigen presenting cells (APC; for example, dendritic cells), present lipids or glycolipids to T cells.[25] CD1a, b and c-restricted, lipid-specific immune responses have been implicated in the control of intracellular pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae*.[25] Similarly, CD1d-restricted, glycolipid-specific T cell responses may be involved in the pathogenesis of autoimmune disease,[26] as well as in the immunity against intracellular pathogens[27] and possibly against certain types of cancer.[28] A finding potentially very pertinent to PNH is the that murine CD1d contains GPI in its presentation groove,[29] and parasite GPI may be the target of protective T cell immunity in murine malaria.[30]

On the basis of these data, we favour the hypothesis that CD1d-restricted, GPI-specific T cells are central to the pathogenesis of PNH. Specifically, we envisage that some of the expanded T cell clones – which we have demonstrated in PNH patients – recognize GPI presented in the context of CD1d by HSCs, and thus attack these cells. By contrast, the PNH HSC, since they synthesise very little GPI, or none at all, will be able to escape the T cell attack. This model implies that pluripotential HSCs must express CD1d at some stage. This has not yet been tested.

This hypothesis might also help to explain the pathogenesis of venous thrombosis, one of the most dreaded complications of PNH, since it affects up to 40% of patients and it is a leading cause of mortality.[8] For reasons which have been obscure hitherto, in PNH venous thrombosis has a special predilection for intra-abdominal and cerebral veins.[8] Interestingly, CD1d, among other tissues, is also expressed in the muscle layer of abdominal blood vessels.[31]

Testing hypothesis 4

The crucial proof for this model must consist in demonstrating the presence in PNH patients of autoreactive T cells that recognize the GPI molecule. The identification of antigen-specific T cells in clinical samples has relied traditionally on stimulating their growth by having the appropriate antigenic peptide presented by appropriate APC. The functional readout consists in cytotoxicity, proliferation assays or cytokine secretion assays. A recent powerful advance has been the introduction of HLA class I-peptide tetrameric complexes[32,33] (Figure 3a). By using tetramer technology it is possible to identify, quantitate and sort specific $CD8^+$ T cells with a sensitivity as high as 0.01%.[34,35]

We propose therefore that using CD1d/β2m/GPI tetramers would be the best way to experimentally test our hypothesis. In order to provide proof of principle that this approach is feasible, we have already constructed CD1d tetramers, with the glycolipid α-galactosylceramide (α-GalCer) in the presentation groove. The sensitivity of this technique has been validated[36] (see Figure 3b). The next step would be to construct CD1d tetramers with GPI instead of α-GalCer, and use them to stain lymphocytes from PNH patients and control individuals. CD1d/GPI $tetramer^+$ T cells can be then flow-sorted, and their specificity confirmed in proliferation, cytotoxicity or cytokine secretion assays in the presence of CD1d-expressing APC pulsed with GPI. Finally, the role of these cells in the pathogenesis of PNH could be proved directly by showing, in functional assays, that they are able to inhibit normal haemopoiesis but not PNH haemopoiesis.

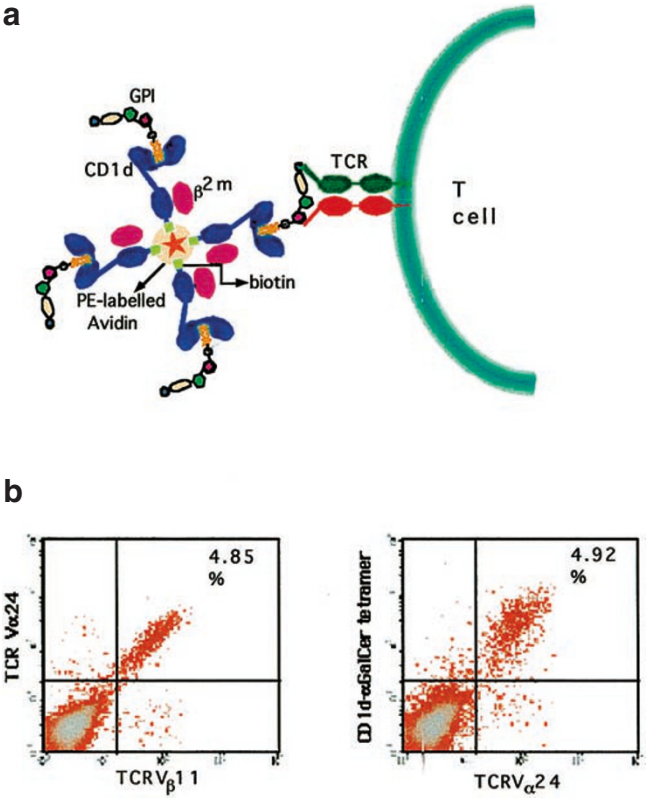

Figure 3 Testing of the hypothesis: identification of GPI-specific, CD1d-restricted T cells using tetramer technology. (a) The basic structural unit of a tetramer is a tri-partite complex (referred to as monomer) of an HLA class I heavy chain (replaced by CD1d in this case), β2-microglobulin and a peptide (replaced by a glycolipid in this case), which must be correctly refolded *in vitro*. A biotinylation signal oligopeptide was engineered at the carboxy-terminus of CD1d in order to facilitate the enzymatic biotinylation of the monomeric complexes, which in turn enables us to form and purify tetramers by the addition of the tetravalent, phycoerythrin (PE)-conjugated (Extra)Avidin. The tetrameric complex binds to its cognate T cell receptor and thus tags the T cells of interest, which can be identified, counted and characterised *in vitro* as well as *ex vivo* in terms of their immunophenotype, with a sensitivity as low as 0.01%. (b) We have constructed a CD1d-α-GalCer tetramer which binds to T cells specific for the glycolipid α-GalCer. Characteristically, such T cells bear a T cell receptor made of the un-mutated α-chain Vα24 and the semi-invariant βchain Vβ11. The frequency of TCR $V\alpha24^+/V\beta11^+$ T cells was increased *in vitro* by culturing blood lymphocytes from a normal donor in the presence of antigen presenting cells, α-GalCer and interleukin-2. Staining with PE-labelled anti-TCR Vα24 and FITC-labelled anti-TCR Vβ11 and analysis by flow cytometry revealed that 4.85% of the cells were TCR $V\alpha24^+/V\beta11^+$ (left); staining with the CD1d-αGalCer tetramer showed that a similar proportion of cells was CD1d-αGalCer $tetramer^+$/TCR $V\alpha24^+$ (right). For the identification of GPI-specific, CD1d-restricted T cells in PNH patients, we will use CD1d tetramers containing GPI instead of α-GalCer.

Conclusion

At the time the escape model for the pathogenesis of PNH was first proposed, more than a decade ago,[9] it was merely a concept based on the need to explain why the PNH clone was able to expand, and yet it did not have the features of

leukaemia. The model has proved to have heuristic value, because we now know the molecular basis for the PNH abnormality, we know that PNH clones are present but do not expand in normal people,[11] and we know that PNH cells tend to disappear rather than to expand in mouse model systems.[14–16] Moreover, we have evidence that the conditional selective agent we had postulated may consist in T cell clones, because we have demonstrated such clones in a substantial proportion of PNH patients.[17] CD1d tetramer technology may help to prove that these clones have the properties that we have hypothetically ascribed to them. Most important, if the hypothesis is correct, this may open up new possibilities for the treatment of PNH, and possibly of other conditions in which similar pathogenetic mechanisms are in operation.

Acknowledgements

We warmly thank all the present and past members of the Human Molecular Genetics Laboratory in the Department of Human Genetics at MSKCC, New York; the ideas discussed in this paper have developed through the hard work of everyone in the PNH group. In particular, we thank David Araten, Monica Bessler, Khedoudja Nafa, Rosario Notaro and Vittorio Rosti. We also thank immensely Vincenzo Cerundolo and Stephan Gadola of Oxford, UK, for their role in the CD1 tetramer work. Finally, we thank Irene AG Roberts, London, for her enthusiastic support of our endeavours; and Bruno Rotoli of Napoli, Italy with whom for two decades we have shared efforts to shed light on the cellular pathogenesis of PNH. The authors' research work has been supported by a grant from the National Institutes of Health (No. RO1 HL56778).

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