Previous attempts to deliver FVIII via the subcutaneous route have been unsuccessful because FVIII binds to phospholipids, which results in proteolytic cleavage and poor absorption into the circulation.[3] However, Vollack-Hesse et al overcame this pitfall by coadministering FVIII alongside a small recombinant fragment of its binding partner, von Willebrand factor (VWF) in hemophilia A mice. The authors expressed a dimer of the VWF-D'D3 domains with a double repeat of the C-terminal 1238-1268 region that contains a series of *O*-linked glycosylation sites. This fragment, which they name VWF-12, contains the FVIII binding site and has been previously shown to be the minimal fragment required to protect FVIII in the circulation.[4] The authors show that although VWF-12 does not interfere with the function of full-length VWF, it prevents FVIII from binding to phospholipids and components of the subendothelial matrix and protects against proteolytic cleavage. The net result is that when VWF-12 is subcutaneously injected alongside FVIII into hemophilia A mice, absorption of FVIII into the circulation is enhanced. Not surprisingly, the subcutaneous route resulted in a slower time to peak FVIII concentration; however, the half-life of FVIII was increased 2.5-fold over FVIII injected into mice via the IV route (7.2 vs 2.8 hours) and with greater bioavailability. Significantly, it offered up to 24 hours of protection from bleeding. It should be noted that in this study, the concentration of FVIII injected via the subcutaneous route was 5 times higher than that via the intravenous route, but even so, the therapeutic goal was successfully achieved.

The concept of codelivering VWF alongside FVIII has been widely regarded as a means to enhance the half-life of FVIII. Modifications to FIX have resulted in molecules with extended half-lives, but this has proved to be more difficult to achieve with FVIII because its half-life is extrinsically linked to that of VWF, the so called "VWF ceiling."[5] BIVV001 is a novel fusion protein of FVIII and VWF that overcomes the VWF ceiling and has been shown to have a significantly extended half-life.[6] Although VWF-12 does not directly prolong the half-life of FVIII, because endogenous full-length VWF will outcompete it once it is in the circulation, these data from Vollack-Hesse et al clearly show the advantage of exploiting VWF fragments to protect FVIII. In the future, it may be interesting to see how BIVV001 performs after subcutaneous injection.

Subcutaneous delivery of a hemophilia A therapeutic agent has already been achieved by the bispecific antibody emicizumab, which mimics activated FVIII.[7] There are no long-term data on the safety of emicizumab, but there is still a strong rationale for developing regular FVIII products. There are also concerns over the immunogenicity of therapeutics delivered via the subcutaneous route,[8] but Vollack-Hesse et al show in their study that the FVIII/VWF-12 combination was no more immunogenic than FVIII delivered via IV and, in fact, was marginally less immunogenic, which indicates another potential advantage of this delivery route. Many questions still need to be addressed, but these data have great promise and clearly warrant further investigation. Detailed dissection of how this particular VWF fragment protects FVIII will be of scientific interest, and studies to enhance the affinity of VWF-12 for FVIII would also be useful. Ultimately, if successful in humans, this may offer a more patient-friendly alternative to repeated intravenous injections without compromising safety and efficacy.

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REFERENCES

1. Vollack-Hesse N, Oleshko O, Werwitzke S, Solecka-Witulska B, Kannicht C, Tiede A. Recombinant VWF fragments improve bioavailability of subcutaneous factor VIII in hemophilia A mice. *Blood*. 2021;137(8):1072-7081.
2. Perrin GQ, Herzog RW, Markusic DM. Update on clinical gene therapy for hemophilia. *Blood*. 2019;133(5):407-414.
3. Fatouros A, Lidén Y, Sjöström B. Recombinant factor VIII SQ–stability of VIII: C in homogenates from porcine, monkey and human subcutaneous tissue. *J Pharm Pharmacol*. 2000; 52(7):797-805.
4. Yee A, Gildersleeve RD, Gu S, et al. A von Willebrand factor fragment containing the D'D3 domains is sufficient to stabilize coagulation factor VIII in mice. *Blood*. 2014;124(3):445-452.
5. Arruda VR, Doshi BS, Samelson-Jones BJ. Novel approaches to hemophilia therapy: successes and challenges. *Blood*. 2017;130(21): 2251-2256.
6. Seth Chhabra E, Liu T, Kulman J, et al. BIVV001, a new class of factor VIII replacement for hemophilia A that is independent of von Willebrand factor in primates and mice. *Blood*. 2020;135(17):1484-1496.
7. Kitazawa T, Igawa T, Sampei Z, et al. A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model. *Nat Med*. 2012;18(10): 1570-1574.
8. Bittner B, Richter W, Schmidt J. Subcutaneous administration of biotherapeutics: An overview of current challenges and opportunities. *BioDrugs*. 2018;32(5):425-440.

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THROMBOSIS AND HEMOSTASIS

Comment on Samuelson Bannow et al, page 1082

Can HIT testing lose its radioactivity?

Grace M. Lee | Duke University

In this issue of *Blood*, Samuelson Bannow et al describe the results of a multicenter, prospective, blinded study that compared the performance of the serotonin release assay (SRA) against the platelet factor 4 (PF4)-dependent P-selectin expression assay (PEA) for the diagnosis of heparin-induced thrombocytopenia (HIT).[1]

HIT is a potentially life-threatening, prothrombotic, immune complication of heparin caused by immunoglobulin G antibodies that recognize complexes of PF4 and heparin. Thrombocytopenia and thrombosis in HIT are caused by a subset of anti-PF4/heparin antibodies that elicit cellular activation by binding and cross-linking platelet FcγRIIa.[2] Recent studies suggest that antibody binding to FcγRIIa on monocytes[3] and on neutrophils[4] contributes significantly to thrombosis in HIT.

Central to the diagnosis of HIT is laboratory identification of heparin-dependent, platelet-activating antibodies. In most circumstances, HIT testing begins with an immunoassay to detect the presence of

anti-PF4/heparin antibodies. A variety of immunoassays for HIT on various testing platforms are currently available, including solid-phase, particle gel, and latex immunoturbidometric assays. Although these assays all have excellent sensitivity (>98% to 99%), they all suffer from poor positive predictive value (38.7%[5]-55.6%)[6] and high rates of false positives. Therefore, in circumstances in which clinical evaluation and immunoassay results are not adequate to definitively confirm diagnosis, functional testing is often necessary.

Functional testing for HIT is based on the key principal that pathologic, clinically relevant anti-PF4/heparin antibodies are capable of activating platelets. At this time, the gold standard functional test to confirm HIT is the SRA, although other assays with different platelet activation end points are available. In the SRA, platelets from a normal donor are incubated with ^{14}C. After washing, ^{14}C-loaded platelets are incubated with heat-inactivated patient plasma or serum in the presence of buffer, heparin at a therapeutic dose (0.1-0.3 U/mL), or heparin at an excess dose (100 U/mL). Importantly, exogenous PF4 is not required for the SRA, presumably because washed platelets release an adequate amount of PF4 for immune complex formation. After incubation and centrifugation, ^{14}C-serotonin released into the supernatant is quantified by a β-counter.[7] Despite improved sensitivity and specificity (>88% for both) compared with the immunoassays,[8] the SRA is technically challenging, requires reactive platelet donors (only one third of normal subjects are suitable donors for HIT testing),[7] and uses radioactivity. Because of these challenges, the SRA is not readily available at most institutions and is often limited to reference and commercial laboratories. As a result, clinicians are often left to make management decisions that are based on clinical suspicion and immunoassay results alone as they await SRA results, which may take days.

Because of the SRA's limitations, in recent years, a functional assay that does not require radioactivity and that may be more easily adapted by laboratories, the PEA, has been developed. The PEA is a platelet activation assay that uses washed platelets pooled from 3 donors. After incubation with PF4 and patient serum, platelet P-selectin expression is measured by flow cytometry and is expressed as a percentage of maximal P-selectin expression (after treatment with thrombin receptor-activating peptide). In contrast to the SRA, the PEA requires exogenous PF4 to be added, and anti-PF4/heparin antibodies are presumed to recognize complexes of PF4 bound to platelet surface glycosaminoglycans. Previous retrospective studies have shown that the PEA has diagnostic accuracy that is similar to, if not higher than, that of the SRA.[9,10] However, those studies used archived, annotated patient samples for which serologic testing results were already known, and this may have resulted in selection bias.

In their study, Samuelson Bannow et al performed blinded SRA and PEA testing on 409 consecutive adult inpatients with suspected HIT from 2 study sites. Each patient sample was tested using the same donor platelets for both assays. Following a prespecified diagnostic scheme, clinical information and enzyme-linked immunosorbent assay results were used to determine whether the patient was positive, indeterminate, or negative for HIT, allowing for analysis of sensitivity and performance characteristics for both assays. The authors found that the sensitivity and specificity of the PEA was comparable to that of the SRA (sensitivity 76% vs 82% and specificity 96% vs 97% for the PEA vs SRA, respectively). Negative concordance between the PEA and SRA was 0.974 and positive concordance was 0.692. Examination of the discrepant cases showed that 5 of 10 patients among the PEA-positive/SRA-negative patients likely had HIT, and 3 of 8 patients in the SRA-positive/PEA-negative group likely had HIT.

On the basis of their data, Samuelson Bannow et al support the use of the PEA as a functional test for HIT. In contrast to the SRA, the PEA does not require radioactivity, and therefore, may be more readily adopted by laboratories, which will provide clinicians with more rapid results. The importance of minimizing turnaround time for functional testing is important. As highlighted in the Samuelson Bannow et al study, 49% of patients who were treated with an alternative anticoagulant were later deemed to be negative for HIT when functional test results came back negative. One of these patients suffered a clinically relevant non-major bleed. More rapid turnaround of functional testing would potentially minimize the risk of patients being unnecessarily treated with alternative anticoagulants, which are associated with a higher risk for bleeding.

Although Samuelson Bannow et al have established that the PEA is comparable to the SRA in terms of assay performance, several concerns must be addressed before it can be widely implemented. This assay does not require radioactivity, but it remains technically challenging because it requires washed platelets and expertise with platelet flow cytometry. These requirements may not expand the availability of the assay beyond the testing centers that currently offer the SRA, and therefore the issue of turnaround time may not improve. In the Samuelson Bannow et al study, most PEA tests were performed at a single site that had considerable expertise, so it remains to be seen whether assay performance characteristics will be similar in the hands of other operators. Finally, the findings of discordant results in the PEA and SRA are concerning and require further study to fully define the role of the PEA in confirming (or excluding) the diagnosis of HIT. Nevertheless, development and validation of the PEA addresses a long-standing diagnostic challenge in this field.

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REFERENCES

1. Samuelson Bannow B, Warad DM, Jones CG, et al. A prospective, blinded study of a PF4-dependent assay for HIT diagnosis. *Blood*. 2021;137(8):1082-1089.
2. Reilly MP, Taylor SM, Hartman NK, et al. Heparin-induced thrombocytopenia/thrombosis in a transgenic mouse model requires human platelet factor 4 and platelet activation through FcgammaRIIA. *Blood*. 2001;98(8):2442-2447.
3. Tutwiler V, Madeeva D, Ahn HS, et al. Platelet transactivation by monocytes promotes thrombosis in heparin-induced thrombocytopenia. *Blood*. 2016;127(4):464-472.
4. Perdomo J, Leung HHL, Ahmadi Z, et al. Neutrophil activation and NETosis are the major drivers of thrombosis in heparin-induced thrombocytopenia. *Nat Commun*. 2019;10(1):1322.
5. Cuker A, Ortel TL. ASH evidence-based guidelines: is the IgG-specific anti-PF4/heparin ELISA superior to the polyspecific ELISA in the laboratory diagnosis of HIT? *Hematology Am Soc Hematol Educ Program*. 2009;2009:250-252.
6. Warkentin TE, Sheppard JI, Linkins LA, Arnold DM, Nazy I. Performance characteristics of an automated latex immunoturbidimetric assay [HemosIL® HIT-Ab$_{(PF4-H)}$] for the diagnosis of immune heparin-induced thrombocytopenia. *Thromb Res*. 2017;153:108-117.

7. Warkentin TE, Arnold DM, Nazi I, Kelton JG. The platelet serotonin-release assay. *Am J Hematol*. 2015;90(6):564-572.

8. Arepally GM, Ortel TL. Clinical practice. Heparin-induced thrombocytopenia. *N Engl J Med*. 2006;355(8):809-817.

9. Padmanabhan A, Jones CG, Bougie DW, et al. A modified PF4-dependent, CD62p expression assay selectively detects serotonin-releasing antibodies in patients suspected of HIT. *Thromb Haemost*. 2015;114(6):1322-1323.

10. Padmanabhan A, Jones CG, Curtis BR, et al. A novel PF4-dependent platelet activation assay identifies patients likely to have heparin-induced thrombocytopenia/thrombosis. *Chest*. 2016;150(3):506-515.

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TRANSPLANTATION

Comment on Thangavelu et al, page 1090

New hope offered to reduce GVHD

Yi Zhang | Temple University

In this issue of *Blood*, Thangavelu et al have developed a novel pharmacological approach to inhibit graft-versus-host disease (GVHD) by activating retinoid X receptor (RXR) in donor T cells.[1]

The success of allogeneic hematopoietic cell transplantation (allo-HCT) has been hampered by GVHD, which is initiated by host antigen-presenting cells (APCs). GVHD is characterized by the induction of donor effector T cells, which recognize host antigens, and the suppression of functional regulatory T cells (Tregs).[2] Donor effector T-cell expansion is a prerequisite for GVHD induction. Alloreactive effector T cells produce high levels of proinflammatory cytokines (eg, interferon-γ [IFN-γ] and tumor necrosis factor-α) and cytolytic molecules (eg, Fas ligand, perforin, and granzyme B).

Alloreactive effector T cells mediate tissue injury during GVHD and selective inhibition of alloreactive effector T cells by targeting the epigenetic regulator Ezh2 can arrest ongoing GVHD.[3]

$CD4^+$ Tregs are also an important cell population that is needed to control GVHD.[4] FoxP3 expression levels are important for Treg development and function.[5] Natural Tregs (nTregs) develop within the thymus. However, hematopoietic cell transplantation (HCT) grafts may contain insufficient numbers of nTregs to suppress alloreactive T-cell responses, and the use of nTregs in HCT would require ex vivo expansion. In contrast, induced Tregs (iTregs) arise from activated $CD4^+$ T cells in the periphery, but they are often unable to maintain their suppressive activity because of unstable FoxP3 expression. Furthermore, GVHD inflammation and immunosuppressive calcineurin inhibitors (CNIs; used for GVHD prevention) are known to impair Treg expansion and function.[6,7] As such, novel and clinically relevant approaches that could reduce alloreactive effector T-cell expansion and induce stable iTreg generation would be ideal for GVHD prevention.

RXRs are master regulators that control cell growth, differentiation, and survival.[8] Studies have suggested that RXRα signaling in T cells suppresses differentiation of IFN-γ–producing T helper 1 (Th1) cells in mice.[9] Du et al demonstrate that mice with a mutation of Rxra (I273N) have a dramatic decrease in ligand-inducible transactions. Homozygous RXRα I273N mutant mice had severe alopecia, exacerbated Th1 responses, and decreased Tregs' suppressive functions.[9] Interestingly, when naive $CD4^+$ T cells were stimulated under Th1–skewing or mixed Th1/Th2 conditions, RXRα I273N cells produced significantly higher amounts of IFN-γ than their wild-type counterparts, indicating a direct impact of RXRα signaling on inhibiting Th1 cell differentiation.[9] Thus, under physiological conditions, RXRα signaling represses $CD4^+$ T-cell differentiation into Th1 cells while promoting Treg function.

Thangavelu et al demonstrate that administration of the RXR homodimer–selective agonist IRX4204 decreases the generation of Th1 cells and promotes Treg generation, leading to inhibition of GVHD while preserving antileukemia activity. IRX4204 treatment reduced donor T-cell proliferation and Th1 differentiation, decreased intestine injury, and reduced expression of genes critical for regulating proinflammatory pathways (eg, Sema7a, Stat1, Irf1). Notably, IRX4204 activation of RXR signaling leads to enhanced Treg generation and maintenance. The investigators found that IRX4204 treatment in vivo under GVH conditions increased the conversion of donor $FoxP3^-$ T cells into peripheral $FoxP3^+$ Tregs in mice and stabilized them by sustaining FoxP3 expression. The direct effect on Tregs was also confirmed using in vitro cultures of murine and human T cells. IRX4204 failed to prevent acute GVHD in recipients given $CD25^-$ T cells derived from scurfy donor mice that have a deletion of FoxP3. Two important conclusions can be drawn from these findings. IRX4204-mediated repression of GVHD requires the presence of functional Tregs, despite its suppressive effects on Th1 responses against host tissues. Additionally, in contrast to the CNI FK506 (aka tacrolimus)–mediated suppression of Tregs, IRX4204 treatment provides a beneficial effect on Treg generation and maintenance.

The investigators' success in this preclinical study of IRX4204 opens new possibilities for exploring T-cell alloimmunity. For example, what is the mechanism by which IRX4204-activated RXR signaling reduces Th1 differentiation? The investigators correlate the inhibition of Th1 differentiation with decreases in CD98, Glut1, and carnitine palmitoyl-transferase 1 (CPT-1), which are known to be important for regulating T-cell metabolism. However, these molecules are found to be readily upregulated in alloantigen-activated T cells.[10] Will the inhibition of alloreactive T-cell expression of CD98, Glut1, and CPT-1 result in impaired T-cell proliferation? In addition, genetic inactivation of RXRα increases interleukin-12 production by dendritic cells,[9] which may augment effector T-cell responses. Will IRX4204 reduce APC activation and, thereby, modulate Th1 cell responses in vivo?

In summary, the investigators provide clear evidence that activating RXRs with IRX4204 inhibits GVHD via complex effects on alloreactive effector T-cell