SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

NEW ASPECTS OF HEPARIN-INDUCED THROMBOCYTOPENIA

by **Zsolt Oláh**

Supervisor: Zoltán Boda

UNIVERSITY OF DEBRECEN
KÁLMÁN LAKI DOCTORAL SCHOOL OF THROMBOSIS, HEMOSTASIS, AND VASCULAR DISEASES

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New aspects of heparin-induced thrombocytopenia

By Zsolt Oláh, M.D.

Supervisor: Zoltan Boda, D.Sc.

Kálmán Laki Doctoral School of Thrombosis, Hemostasis, and Vascular Diseases
University of Debrecen

Head of the **Examination Committee**:	György Balla, M.H.A.Sc.
Members of the Examination Committee:	Csongor Kiss, D.Sc.
	Éva Ajzner, Ph.D.

The Examination takes place at the Library of Department of Pediatrics, Medical and Health Science Center, University of Debrecen
at 11.00 a.m., 27 November, 2012

Head of the **Defense Committee**:	György Balla, M.H.A.Sc.
Reviewers:	Klára Vezendi, Ph.D.
	Éva Katona, Ph.D.
Members of the Defense Committee:	Csongor Kiss, D.Sc.
	Éva Ajzner, Ph.D.

The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen
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INTRODUCTION

Heparin was discovered approximately a century ago and has been used for the therapy and prophylaxis of venous thromboembolism since the late 1930s. Heparin-induced thrombocytopenia (HIT) is a rare, but life-threatening complication of heparin therapy. It is an immune-mediated disorder caused by platelet activating IgG type autoantibodies to platelet factor 4 (PF4)/heparin complexes. PF4 bound to heparin expresses immunogenic neoepitopes that can provoke the production of IgG type antibodies. Then, PF4/heparin/IgG complexes can induce strong platelet activation via their FcγIIa receptors resulting in further release of PF4, procoagulant proteins and microparticles. Development of thrombocytopenia can be explained primarily by consumption. HIT antibodies can also activate endothelial cells and monocytes leading to further procoagulant changes. Based on these mechanisms, despite the thrombocytopenia and administration of heparin, HIT is associated with a high risk of arterial and venous thromboembolism.

However, clinically overt HIT develops only in the small proportion of the cases associated with the presence of antibodies to PF4/heparin complexes. Therefore, detection of autoantibodies usually is required for the diagnosis, but itself alone cannot prove HIT.

HIT usually features a real or relative (platelet count fall > 50% of the baseline) thrombocytopenia associated with new arterial or venous thrombosis (HITT) in 50% of the cases during heparin therapy. Different types of HIT are known according to the temporal development of thrombocytopenia. Typical onset HIT occurs between days 5 and 10 of heparin administration. Rapid fall in the platelet count appears within 24 hours after the initiation of heparin in cases of rapid onset HIT. Only few and sporadic cases of delayed onset HIT and spontaneous HIT were reported.

Other causes of thrombocytopenia should be considered in cases of suspected HIT (EDTA-induced thrombocytopenia, antiphospholipid syndrome, sepsis etc). Suspicion of HIT usually arises from the clinical course. The 4T's and HEP score modells can help to estimate the likelihood of HIT based on the clinical picture. Two major categories of laboratory assays are available for the detection of antibodies against PF4/heparin complexes. Functional tests detect evidences of platelet activation in the presence of heparin demonstrating HIT antibodies indirectly. Immunological assays, primarily the ELISA tests specifically and directly identify antibodies to PF4/heparin. These tests are widely available and very sensitive with a moderate specificity, whereas functional tests have got high specificity but limited availability. Antibodies to PF4/heparin complexes can be detected for 3-4 months after the episode of HIT.

Early diagnosis and prevention of further complications should be the main purposes of HIT/T management. When HIT is suspected clinically, all types of heparin should be withheld and one of the direct thrombin inhibitors (DTI) (recombinant hirudin, bivalirudin, argatroban) or factor Xa (FXa) inhibitors (danaparoid, fondaparinux) initiated. The anticoagulant effect of DTIs is monitored by using the activated partial thromboplastin time (aPTT). If monitoring of indirect FXa inhibitors is required, the anti-Xa level is the recommended test. Special attention should be exhibited regarding the initiation of vitamin K antagonist (VKA) due to the high risk of coumarin-induced skin necrosis and venous limb gangrene in HITT.

It may be very difficult to make a distinction between HITT and antiphospholipid syndrome (APS) in the clinical practice. HITT and APS have substantial overlaps and similarities regarding both the pathomechanism and clinical signs. Laboratory tests may also cause confusions, since antibodies to PF4 without any potential to induce HIT are often detected by ELISA in APS. Theoretically, the incidence of antiphospholipid antibodies (APAs) may also be higher in HIT. However, large study has not been published in this topic.

The main clinical manifestations of APS are thromboses and/or obstetric complications associated with the presence of persistent IgG and/or IgM type APAs, such as anti-cardiolipin (aCL), anti-β2GPI antibodies and/or lupus anticoagulant (LA). Although aCL and anti-β2GPI are detected by ELISA, detection of LA is much more complicated.

For LA screening, two different phospholipid-dependent laboratory tests that represent different coagulation pathways should be performed. Diluted Russell viper venom time (dRVVT) and an aPTT-based assay with low phospholipid content are the recommended tests.

Diluted prothrombin time (dPT) using recombinant thromboplastin reagent also provides a widely used, reliable assay with high sensitivity for LA.

Laboratory tests for LA can be influenced by different anticoagulants due to the prolonged basal clotting times. Limited amount of information is available about the effect of the widely used low molecular weight heparins (LMWH) and anticoagulants used in HIT such as DTIs or FXa inhibitors on LA tests. However, in certain medical conditions detection of LA may be required immediately regardless of the acute thrombotic event or the treatment. Accordingly, it is important to have exact information about the effects of different anticoagulant drugs on the interpretation of LA tests. In vitro experiments demonstrated that concentrations of heparins or DTIs in plasma within the therapeutic range could potentially modify the results and interpretation of dRVVT based on the examination of common coagulation pathway and LA sensitive aPTT tests based on the examination of intrinsic coagulation pathway. Lepirudin and the FXa inhibitors used in HIT have not significant effect on prothrombin time. Therefore, we intended to examine whether these drugs have effect on LA tests based on the examination of extrinsic coagulation pathway. These experiments were also important because the dPT-based LA test is part of the algorythm of LA detection in the University of Debrecen.

OBJECTIVES

1. To investigate the presence of antiphospholipid antibodies in HIT/T. We retrospectively analyzed data of our patients hospitalized for HIT/T between 2004 and 2011 in the 2nd Department of Medicine of the University of Debrecen.
2. To analyze, report in details and highlight HIT cases having unique peculiarities and rare manifestations or previously unreported associations of these rare symptoms with scientific significance.
3. To determine the effect of parenteral anticoagulant drugs used in HIT (lepirudin, danaparoid, argatroban and fondaparinux) and enoxaparin on dPT ratio and interpretation of LA tests based on dPT.

MATERIALS AND METHODS

Retrospective study of our patients with HIT/T

Patients: We retrospectively studied 12 patients with the diagnosis of HIT/T who were hospitalized in the 2nd Department of Medicine of the University of Debrecen between 2004 and 2011. Heparin-induced thrombocytopenia was considered present if all of the following criteria were met: 1. a 50% or greater decrease in the platelet count under heparin therapy; and 2. the likelihood of HIT was high based on the clinical course according to 4Ts and/or HEP score system (4Ts and or HEP score ≥ 5); and 3. a positive serologic test for HIT.

Methods: The available laboratory tests for HIT antibodies were not constant and they were changing and improved in time.

Particle gel immunoassay (PaGIA) (DiaMed AG) and/or ELISA (Zymutest HIA IgG, Hyphen BioMed) were performed as immunological tests for HIT antibodies in case of each patient.

One of the following functional tests for HIT was performed if immunological test(s) had provided a positive result.

Heparin-induced platelet aggregation (HPAG): Platelet aggregation was measured using a Chrono-Log 660 aggregometer (Havertown PA). Patient's platelet-poor plasma (PPP) and heparin was added to healthy voluntary blood donor's platelet rich plasma, then platelet aggregation was measured. Development of platelet aggregation in the presence of low concentration heparin (0.3 IU/mL) suggests the diagnosis of HIT/T.

Flow cytometric assay for HIT: The increased ability of activated platelets to bind annexin-V was measured in the presence of heparin by flow cytometry (FACScan, Beckton Dickinson).

Detection of LA: Measurements of LA-sensitive aPTT (PTT-LA, Diagnostica Stago) and dPT ratio (Siemens) were performed as screening tests for LA according to the manufacturer's instructions. dPT ratio was determined using 1:50 and 1:500 dilutions of thromboplastin (Innovin). LA-sensitive aPTT with

1:1 proportion of patient PPP:normal pooled plasma was carried out as a mixing study, while hexagonal phospholipid test (Staclot LA, Diagnostica Stago) was used as a confirmatory step.

Detection of aCL and anti-β2GPI antibodies: Both aCL (Quanta Lite™ ACA IgG III, IgM III) and anti-β2GPI (Quanta Lite™ β2 GPI IgG, IgM, INOVA Diagnostics Inc.) antibodies were detected by ELISA.

Effects of alternative anticoagulants used in HIT and enoxaparin on the lupus anticoagulant screening tests based on dPT

LA-negative group. After written informed consent, a blood sample was obtained from 25 healthy voluntary blood donors with no recent use of non-steroidal anti-inflammatory drugs or any type of anticoagulants.

LA-positive group. Adults previously identified as positive for LA in the absence of oral or parenteral anticoagulant therapy served as the blood donors of LA-positive samples in the study. LA was diagnosed according to the revised and standardized criteria proposed by the International Society of Thrombosis and Haemostasis.

Platelet-poor plasma of both LA-negative and LA-positive individuals was used to determine the in vitro effects of different anticoagulants on dPT and dPT ratio. Normal pooled plasma was used to investigate the therapeutic concentrations of DTIs, as well as the effect of thromboplastin reagent dilution on dPT ratio.

Anticoagulants. Lepirudin (50 mg Refludan) and argatroban (250 mg/2.5 mL Argatra) were prepared according to the manufacturer's instructions. Single-dose, prefilled syringes of fondaparinux (2.5 mg/0.5 mL Arixtra), enoxaparin (10 000 anti-Xa units/mL Clexane) and danaparoid solution (750 anti-Xa units/0.6 mL Orgaran) did not require additional preparation.

Increasing concentrations including the therapeutic range of these drugs were added to plasma samples. Samples containing factor Xa inhibitors were

investigated in the absence as well as in the presence of heparinase (Hepzym, Siemens).

Coagulation tests. Prothrombin time (PT) and aPTT were determined on an automated coagulometer (BCS-XP, Siemens) using Innovin and Pathromtin SL (Siemens), respectively.

Factor Xa inhibitor concentrations of the supplemented plasma samples were determined using anti-Xa assay (Berichrom Heparin, Siemens). A Siemens LMWH calibrator was used to determine the concentration of enoxaparin and danaparoid. A fondaparinux calibrator (Diagnostica Stago) was applied for measuring the concentration of fondaparinux.

Diluted prothrombin time was determined on BCS-XP coagulometer using different dilutions of Innovin reagent (1:50, 1:100, 1:200, 1:500; diluted with physiologic saline solution). Diluted prothrombin time ratio, calculated as the dPT of plasma under investigation (test plasma) divided by the mean value of pooled normal plasma, was used for LA-screening. A dPT ratio of 1.2 is used in our laboratory as the cutoff value, based on our previous experiments. A dPT ratio higher than 1.2 suggests the presence of LA.

Statistics. To assess the correlation between dPT ratio and drug concentrations or coagulation time ratios the Pearson correlation coefficient (r) was calculated using linear regression analysis. Correlation was considered to be statistically significant at $p < 0.05$. Statistical analysis was done with GraphPad Prism 4 software (GraphPad Software).

The study was approved by the local ethics committee of the university.

RESULTS

12 patients with HIT were hospitalized at our department between 2004 and 2011. Laboratory assessment for APAs were performed in 10 of 12 cases and eight of them had got at least one type of APA (aCL, anti-β2GPI and/or LA). Only LA tests with positive results were performed in four patients; three of them were treated with lepirudin. Although lepirudin can potentially modify the interpretation of dRVVT and LA-sensitive aPTT tests, its effect on dPT based LA tests was not known. Therefore, before further evaluation of our patients' data, in vitro experiments were planned to determine the impact of different anticoagulants used in HIT on LA testing based on dPT.

Effects of alternative anticoagulants used in HIT and enoxaparin on the lupus anticoagulant screening tests based on dPT

Therapeutic concentrations of DTIs and their effects on coagulation tests

Argatroban and lepirudin were added to pooled normal plasma and dose-response curves were generated by measuring the aPTT and PT. Dose-response curves of aPTT fitted by nonlinear regression were similar for both lepirudin and argatroban. For lepirudin 1.5 to 2.0 times the baseline aPTT has been considered therapeutic for anticoagulation, which corresponds to 0.26 to 0.67 μg/mL lepirudin concentrations in our study. PT showed no significant prolongation with increasing concentration of lepirudin. The recommended target aPTT is 1.5- to 3.0-fold of the baseline value during argatroban treatment, which corresponds to 0.3 to 2.0 μg/mL concentrations according to our data. In the case of argatroban there was a pronounced PT prolongation.

Effect of thromboplastin reagent dilution on dPT and dPT ratio

Because laboratories may use various reagent concentrations, we determined their impact on dPT values at 4 different thromboplastin dilutions: 1:50, 1:100, 1:200, and 1:500. Using pooled normal plasma, these experiments were performed in the presence of predefined final concentrations of each drug. Our

data indicated a linear correlation between the dPT ratio and the degree of Innovin reagent dilutions in the case of each drug except for lepirudin. Increasing dilutions of thromboplastin resulted in increasingly longer dPT and a higher dPT ratio. In view of these findings, the 2 markedly different (1:50 and 1:500) dilutions of Innovin were applied to cover the whole range of the commonly used dilutions in our subsequent experiments.

Effect of DTIs on dPT ratio in LA-negative samples

In the presence of DTIs, the dPT ratio was increased notably in a dose-dependent manner. In the case of the individual LA-negative plasma samples containing clinically relevant concentrations of lepirudin, dPT ratio showed a linear increase in relation to increasing aPTT ratio. aPTT ratios greater than 1.19 (above 0.03 μg/mL lepirudin) and 1.38 (above 0.15 μg/mL lepirudin) resulted in a dPT ratio greater than 1.2 in 1:500 and 1:50 Innovin dilutions, respectively. These results show that dPT ratio exceeds the cutoff value even in the presence of subtherapeutic lepirudin concentrations.

The cutoff value of the dPT ratio had already been reached at the lowest (subtherapeutic) argatroban concentration (0.2 μg/mL) in our study, corresponding to a 1.4 aPTT ratio with both 1:50 and 1:500 Innovin reagent dilutions, leading to false-positive interpretation of LA.

Effect of factor Xa inhibitors on dPT ratio in LA-negative samples

The dPT ratio showed a linear increase in relation to increasing anti-Xa values in samples spiked with the different factor Xa inhibitors. However, there were differences in the slope of fitted regression lines. The calculated anti-Xa activities required to achieve 1.2 dPT ratio with 1:500 Innovin reagent dilution were as follows: 0.32 IU/mL anti-Xa, 0.74 μg/mL and 0.13 IU/mL anti-Xa in the presence of danaparoid, fondaparinux and enoxaparin, respectively. In the same context, the calculated anti-Xa activities with 1:50 Innovin reagent were 0.87 IU/mL anti-Xa, 1.59 μg/mL and 0.44 IU/mL anti-Xa in the presence of danaparoid, fondaparinux and enoxaparin, respectively.

dPT ratios returned to the basal value after heparinase treatment only in samples containing enoxaparin, whereas in the presence of fondaparinux and danaparoid, dPT ratio decreased, but remained elevated, maintaining the false-positive results in spite of the presence of heparinase.

Effect of anticoagulants on dPT and dPT ratio in LA-positive samples

In cases of LA-positive samples, the dPT ratio showed further increase after using any of the studied anticoagulants. However, similarly to LA-negative samples, dPT ratio also decreased to the baseline value after addition of heparinase without causing false-negative result in the presence of enoxaparin.

Retrospective study of our patients with HIT/T

Patients being tested for only LA under DTI, danaparoid or fondaparinux therapy (3/12) or not tested for APAs at all (2/12) were excluded from the further investigation according to our in vitro experiments.

Subjects of the study had severe HIT associated with arterial (1/7) or venous (5/7) thrombosis except for one patient who presented with only thrombocytopenia as a clinical sign of HIT. Venous limb gangrene was developed in three cases. At least one type of APAs (anti-β2GPI and/or aCL in medium or high titres, LA) was revealed in four of the seven patients. APAs were not present in two subjects, while IgM type aCL was detected near the upper limit of the low titre in one patient.

Two patients' case merits considerable attention, since APAs could not be detected three months after the initial evaluation and APS could be ruled out retrospectively. Therefore these cases were found to be worth evaluating and reporting in details.

Case report (P1)

A 76-year-old man had surgery for traumatic left hip fracture; his preoperative platelet count was 160×10^9/L. Six hours after surgery, subcutaneous (sc) enoxaparin was started (60 mg/day) with the aim of VTE prophylaxis. Four days

later acenocoumarol was restarted and added to enoxaparin because of previous recurrent deep vein thromboses (DVT). Eight days postoperatively, he had swelling and pain of the left leg, which rapidly progressed to limb gangrene. Compression ultrasonography showed acute proximal DVT whith intact arterial circulation. Coumarin-related complication (e.g. coumarin-induced skin necrosis) was suspected, so acenocoumarol was discontinued and enoxaparin dose increased to achieve therapeutic anticoagulation (60 mg bid). On postoperative day 12, the platelet count fell to 51×10^9/L. Although the clinical course was consistent with heparin-induced thrombocytopenia with thrombosis, both the PaGIA and ELISA for antibodies to PF4/heparin complexes were negative. In addition, LA and high titre of IgM and low titre of IgG type aCL were detected. On the basis of the laboratory results, enoxaparin therapy was continued. However, on day 14 postoperatively, a necrotic skin lesion developed at the injection sites of enoxaparin, whereas the PaGIA remained negative as an evidence of the absence of conventional HIT antibodies. Despite the result of PaGIA, low concentrations of enoxaparin induced in vitro platelet aggregation (HPAG) supporting the diagnosis of HITT with a coumarin-induced limb gangrene. Recombinant hirudin was started and enoxaparin stopped, resulting in improvement in clinical symptoms and platelet count.

The patient received debridement and reconstructive surgeries with skin mesh graft at the sites of skin necrosis and limb gangrene. However, amputation of two toes of the left leg was necessary. At final follow-up, wounds were healed completely. Three months after the initial evaluation, laboratory tests for APAs became negative and careful transition to acenocoumarol therapy was done with overlapping hirudin.

Case report (P2)

A 64-year-old woman was hospitalized because of methicillin resistant *Staphylococcus epidermidis* (MRSE) sepsis, which was resolved by the pathogen- and susceptibility-directed therapy. During the hospitalization, the

patient did not receive any type of heparin. Platelet count varied between 228×10^9/L and 314×10^9/L. When the patient was discharged, the platelet count was 259×10^9/L. Five days later, her family physician prescribed prophylactic dose of enoxaparin (40 mg od, sc.). This was the patient's first exposure to heparin. Thirty-six hours later, after two sc. injections of enoxaparin, she was admitted to our department with a platelet count of 86×10^9/L.

She presented skin necrosis at the sites of enoxaparin injections on both upper arms. Within 24 hours, limb gangrenes developed involving the acral parts of left limbs. Compression ultrasonography revealed acute DVT in both left extremities, whereas arterial circulation was perfect. In addition, presence of medium or high titer of IgG and IgM type aCL antibodies was revealed raising the suspicion of APS beside HITT. Anti-β2GPI and LA could not be detected. PaGIA and ELISA for antibodies to PF4/heparin complexes were found to be strongly positive proving the high amount of heparin-dependent IgG type HIT antibodies in the plasma.

The diagnosis of HIT was confirmed by flow cytometric functional HIT assays. Despite the absence of previous heparin exposure, the clinical course and laboratory tests were consistent with rapid-onset HIT and thrombosis associated with venous limb gangrenes and heparin-induced skin necrosis. Accordingly, enoxaparin was discontinued and recombinant hirudin therapy was initiated. Forty-eight hours after enoxaparin cessation, platelet count returned to normal range (196×10^9/L). However, amputation of the left arm and foot was inevitable due to limb gangrenes. Finally, careful transition to acenocoumarol therapy was performed with overlapping hirudin. Three months after the first measurement, laboratory tests for APAs became negative and ruled out the diagnosis of APS.

DISCUSSION

A high incidence of APAs, particularly aCL and LA was observed in our patients with HIT/T. Presence of APAs with the clinical course of HIT could cause a serious differential diagnostic problem of HITT and APS. Furthermore, these patients usually receive parenteral anticoagulant that can influence the interpretation of LA tests complicating the situation. Therefore, before further evaluation of our patients' data, in vitro experiments were planned to determine the impact of enoxaparin and different anticoagulants used in HIT on LA testing based on dPT.

Effects of alternative anticoagulants used in HIT and enoxaparin on the lupus anticoagulant screening tests based on dPT

dPT using recombinant thromboplastin is a widely performed, well-documented, and sensitive screening test for LA. Different dilutions of the recombinant thromboplastin Innovin are used for determination of dPT. We demonstrated that increasing dilutions of thromboplastin resulted in increasingly higher dPT ratio in the presence of the investigated anticoagulants. In our study two markedly different dilutions (1:50 and 1:500) of the recombinant thromboplastin Innovin were applied to cover the commonly used range.

DTIs such as argatroban or lepirudin dramatically increased dPT and dPT ratio causing false-positive results already at subtherapeutic concentrations. In view of this effect, laboratory tests based on dPT are unreliable for detecting LA in the presence of these drugs.

Increasing concentrations of factor Xa inhibitors linearly increased the dPT ratios and potentially were able to cause false-positive results (dPT ratio>1.2). However, different drugs have different impacts on the dPT ratio. Enoxaparin caused the greatest prolongation of dPT, followed by danaparoid and fondaparinux.

Heparinase was able to quench each of the factor Xa inhibitors measured by anti-Xa activity. In spite of the reduced anti-Xa activity, dPT values returned to the baseline level and the dPT ratio could be determined precisely only in the case of enoxaparin. Thus, when the effect of LMWH is neutralized by heparinase, an LA test based on dPT seems to be useful as a screening assay in the diagnosis of LA during enoxaparin therapy. On the contrary, heparinase cannot completely inhibit the effect of danaparoid and fondaparinux on dPT ratio.

Patients being tested for only LA under DTI or FXa inhibitors therapy were excluded from the further investigations according to the results of our in vitro studies. LA positivity, however, could be resulted from the laboratory interference caused by anticoagulants in their cases. Nevertheless, subjects under enoxaparin therapy were considered as an exception and continued the study, if LMWH was neutralized in vitro with heparinase.

Hereafter, new and rare aspects of HIT are emphasized on the basis of the seven eligible subjects' data. Knowledge of these new facts can be important in the understanding of pathomechanism, diagnostics and management of HIT.

Retrospective study of our patients with HIT/T

<u>HIT associated with positivity of laboratory tests for antiphospholipid antibodies</u>

Seven patients with HIT were examined. Four of them had LA or aCL in high titre, while APAs in low titre were also present in the most cases. This phenomenon may have three possible explanations: 1. frequent association of HIT/T and APS; 2. cross reactivity of HIT antibodies with APAs; 3. high prevalence and transient presence of real APAs in HIT/T without APS.

The two case reports presented in details do not support the conception of the real association of HIT/T and APS. Development of severe HIT with massive arterial or venous thrombosis, association of the rare heparin-induced skin necrosis and venous limb gangrene and the transient presence of APAs limited to the acute phase of HIT should raise the possible casual role of APAs in the

pathomechanism of HITT. Prognostic value of APAs should also be considered. The detailed cases are the first reports on the occurrence of transient APAs positivity in patients with HIT/T. The knowledge of this phenomenon can be important in the differential diagnosis of HIT/T and APS. According to our results, further investigations should be performed to clarify the possible role of APAs in HIT/T.

Rare forms and clinical manifestations of HIT/T in our patients

Heparin-induced thrombocytopenia without antibodies to PF4/heparin complex

PF4/heparin complex is the most common and conventional target of HIT antibodies. However, in less than 5% of cases, despite positive platelet activation test, these classical HIT antibodies are not detectable by immunoassays. Antibodies to PF4-related chemokines, such as neutrophil-activating peptide-2 or interleukin-8 are found in these cases and considered as casual factors in the development of HIT/T. A PF4-related chemokine could also play the key role in patient P1 explaining the positive result of HPAG and repeatedly negative results of immunoassays. Accordingly, one of the functional HIT tests should also be performed in cases of suspected HIT/T even when the immunoassays are negative.

Association of HITT and venous limb gangrene

Venous limb gangrene usually involves the acral part of the limb affected by DVT, whereas arterial circulation is intact. Its pathomechanism corresponds with coumarin-induced skin necrosis, however, the relatively high prevalence in HITT (12.1%) can be explained by additional thrombin generation and procoagulant conditions associated with heparin-induced thrombocytopenia. The incidence of venous limb gangrene can be diminished with the adequate management of HITT. Of our HIT cases, three subjects developed venous limb gangrene. The P2 patient requires special attention, since the severe venous limb gangrenes, which affected two limbs and led to amputations developed despite the absence of VKA therapy. Although, this phenomenon is known in theory,

we have not found a well documented case report in the literature. Venous limb gangrene in the absence of VKA therapy occurs very rarely in HIT, so additional procoagulant factors should be sought in the background. In our case this factor could be due to the presence of APAs beside HITT.

Rapid-onset HITT without previous heparin exposure

Traditionally, rapid-onset HIT is suggested to be caused by the previously produced and circulating PF4/heparin-dependent IgG antibodies resulting from a recent immunizing exposure to heparin. However, Greinacher et al. showed that PF4 can also bind to surface polyanions of various bacteria and the human anti-PF4/heparin antibodies crossreact with these PF4-coated organisms. In their study, mice could develop anti-PF4/heparin antibodies during chronic artificial peritonitis and subsequent polymicrobial sepsis without heparin application. Based on these experiments, it is presumed that preimmunization against PF4/heparin can occur in the presence of other polyanions with ability to bind to PF4. This theory can explain the pathomechanism of rapid-onset HIT developing after septicemia in P2 subject by the following sequence of events: 1. PF4 released from activated platelets during the sepsis could bind to the surface polyanions of MRSE bacteria and expose neoepitopes; 2. primary immune response against these neoepitopes of PF4/MRSE bacteria complexes resulted in mainly IgM, then IgG antibodies which could crossreact with PF4/heparin complexes; 3. enoxaparin exposure, formation of PF4/enoxaparin complexes; 4. circulating IgG antibodies to PF4/MRSE produced during the sepsis could bind to PF4/enoxaparin complexes; 5. rapid-onset HITT developed without previous heparin exposure.

Our case (P2) represents the first, well-documented example of rapid onset HITT developing after septicemia without previous heparin exposure and advocates the new theory of Greinacher's team. Furthermore, this case calls the attention that rapid-onset HIT cannot be excluded based on the absence of previous exposure to heparin.

SUMMARY – NEW CONCLUSIONS

Effects of alternative anticoagulants used in heparin-induced thrombocytopenia (HIT) and enoxaparin on the lupus anticoagulant (LA) screening tests based on diluted prothrombin time

- Direct thrombin inhibitors (DTIs) such as argatroban or lepirudin dramatically increase diluted prothrombin time (dPT) and dPT ratio causing false positive results already at subtherapeutic concentrations. Laboratory tests based on dPT are unreliable for detecting LA in the presence of these drugs.
- The predominantly factor Xa (FXa) inhibitors such as enoxaparin, danaparoid or fondaparinux increase dPT and dPT ratio by different but significant extent, potentially leading to false positive results. Laboratory tests based on dPT are also unreliable for detecting LA in the presence of these drugs.
- Although heparinase was able to quench each of factor Xa inhibitors even in the therapeutic range measured by anti-Xa activity, dPT values and dPT ratios returned to the baseline level only in the case of enoxaparin.
- When the effect of LMWH is neutralized by heparinase, LA test based on dPT remain a reliable and precise screening assay in the diagnosis of LA during enoxaparin therapy. On the contrary, heparinase cannot completely inhibit the effect of danaparoid and fondaparinux on dPT ratio maintaining the false positive results.

Retrospective study of our patients with HIT/T

- Transient positivity of antiphospholipid antibodies (APAs) can be associated with HIT even in the absence of antiphospholipid syndrome (APS) leading to severe differential diagnostic problems in the clinical

practice. This fact should be considered during the diagnosis and differential diagnosis of HIT.

- According to our study, we should raise the possible casual role of APAs in the pathomechanism of thrombosis in HITT and other rare complications such as heparin-induced skin necrosis or venous limb gangrene. Prognostic value of APAs should also be considered in cases of HIT/T.
- Occasionally the diagnosis of HIT/T cannot be excluded even in the absence of the classical HIT antibodies to PF4/heparin(polyanion) complexes. Therefore, one of the functional HIT tests should be performed in each case with suspected HIT/T even if the immunoassays with high sensitivity are negative.
- Although, the development of venous limb gangrene is usually a coumarin-related complication in HITT, very rarely it can occur in the absence of coumarin therapy.
- Previous heparin exposure is not required for the diagnosis of rapid-onset HITT partly due to the possible preimmunization by PF4 bound to bacterial polyanions.

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Candidate: Zsolt Oláh

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List of publications related to the dissertation

1. **Oláh, Z.**, Kerényi, A., Kappelmayer, J., Schlammadinger, Á., Rázsó, K., Boda, Z.: Rapid-onset heparin-induced thrombocytopenia without previous heparin exposure.
   Platelets. 23 (6), 495-498, 2012.
   DOI: http://dx.doi.org/10.3109/09537104.2011.650245
   IF:1.847 (2011)

2. **Oláh, Z.**, Szarvas, M., Bereczky, Z., Kerényi, A., Kappelmayer, J., Boda, Z.: Direct thrombin inhibitors and factor Xa inhibitors can influence the diluted prothrombin time used as the initial screen for lupus anticoagulant.
   Arch. Pathol. Lab. Med. "közlésre elfogadva", 2012.
   IF:2.577 (2011)

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