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RESEARCH ARTICLE

Standardization of HIT Diagnostic Assays, with K070, a chimeric human-mouse antibody, mimicking heparin dependent pathogenic antibodies

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ABSTRACT

This study reports the K070 monoclonal antibody, a human-mouse recombinant chimeric monoclonal antibody, IgG1 isotype, targeted to Heparin and PF4 complexes, which mimics Heparin Induced Thrombocytopenia. This antibody has a strong reactivity to Heparin-PF4 complexes, and a much weaker one to PF4 alone. When plasmas spiked with this antibody at concentrations ≥ 0.5 µg/ml are tested at the standard immunoassay dilution, a dose dependent reactivity is obtained. This opens the perspective to use this material to calibrate immunoassays for a quantitative measurement of heparin dependent antibodies, when the disease is suspected. Furthermore, when plasmas are spiked at a higher concentration, from 20 to 200 µg/ml, they test positive in functional assays, like Platelet Aggregation/Activation Tests or Flow Cytometry methods, in presence of a low (0.1-1.0 IU/ml) but not a high (10-100 IU/ml) heparin concentration. In functional assays K070 reactivity level depends on the platelet rich plasmas used. In Platelet Aggregometry Tests some platelet rich plasmas are already positive when tested with plasmas supplemented with 20 µg/ml, whilst others require 100 to 200 µg/ml. At this latter concentration, all tested plasmas generate a strong positive responses with functional assays. K070 is then a useful antibody for calibration of immunoassays and standardization of functional methods used to diagnose Heparin Induced Thrombocytopenia.

Keywords: K070 HIT mimicking antibody; Functional assays; Immunoassays; Calibration; Heparin Induced Thrombocytopenia; HPF4 complexes.

Introduction

Heparin Induced Thrombocytopenia (HIT) is a severe, and life-threatening complication of heparin therapy[1] , which requires an immediate heparin withdrawal, a rapid diagnostic and its confirmation[2] along with a clinical risk appraisal, established as a clinical score for HIT probability[3] when this clinical complication is suspected[4]. Its occurrence is higher in Unfractionated Heparin (UFH) treated patients than in those receiving a Low Molecular Weight Heparin therapy [5]. Affected symptomatic patients can develop thrombocytopenia, with or without thrombosis, 5 to 15 days following the initiation of heparin therapy, caused by the presence of allo-antibodies developed to Heparin and Platelet Factor 4 (HPF4) complexes[6,7], however those antibodies can remain asymptomatic in many patients[8,9]. The anti-HPF4 Elisa has therefore become a major laboratory method for excluding HIT, when this clinical complication is suspected in heparin treated patients, and to permit patients benefiting from that anticoagulant[10]. In presence of anti-HPF4 antibodies, the diagnosis of HIT must be confirmed later using functional assays to differentiate pathogenic from asymptomatic antibodies in positive patients[11]. Using positive plasmas from patients with diagnosed HIT as assay controls is a current practice of high usefulness in clinical laboratories to validate and standardize diagnostic methods for HIT. In addition, HIT plasmas confirmed positive are useful for selecting the good responders platelet donors, for performing platelet activation functional assays, as all platelet donors are not equivalent[12] This is usually achieved by aliquoting and freezing plasmas from HIT positive patients, duly characterized, and using them in each testing series. However, this source is restrictive, patient-dependent, and cannot be widely extended to laboratories [13]. Therefore, PF4 dependent Enzyme Linked Immuno Sorbent Assays (ELISAs), usually include positive controls, artificially obtained, and which can be used to control the assay performances. In 2000, Arepally and her group reported a mouse monoclonal antibody (KKO) that mimics HIT[14]: it binds to HPF4 complexes, but poorly to PF4 alone; it can activate platelets in presence of low but not high heparin concentrations, and this activation is abrogated by antibodies to Fcɣ-RIIA platelet IgG receptor[14]. Although highly useful for research studies on HIT, that mouse antibody presented little usefulness for routine laboratory assays, because it differs from human antibodies developed in HIT, due to the absence of the human IgG Fc fragment. In attempts to introduce a stable control material for HIT diagnosis, a chimeric mouse-human monoclonal antibody (5B9), expressing the human Fc fragment was produced in transgenic mice, as it was first described in 2017, and reported later in a multicentric study to behave like human pathogenic anti-HPF4 antibody, mimicking HIT, in various laboratory assays[15,16]. Like KKO, this antibody reacts with HPF4 complexes, to a similar epitope, as it was demonstrated by the dose-dependent inhibition of 5B9 binding to HPF4 complexes by KKO[15]. When evaluated in the multicentric study, this 5B9 antibody was reactive in functional and immunological methods, and it was proposed as a control material for HIT functional tests and immunoassays, when used in the purified form[16]. There is then an essential and definite need for a humanized heparin dependent antibody, which mimics HIT when spiked in plasma, to allow standardization of performances and optimization of laboratory

diagnostic assays, whether functional or immunological[17]. In this article, we report a new chimeric mouse-human monoclonal antibody (K070), which behaves like human heparin dependent antibodies developed in HIT, and which can be used as control material for anti-HPF4 immunoassays, or for functional assays like platelet activation tests (PAT), whether this antibody is used in the purified form or spiked in plasma. It offers a special usefulness to select blood donors for platelets offering the right reactivity to heparin dependent antibodies, and which are appropriate for functional HIT diagnostic methods[18]. In addition, further studies showed that the anti-HPF4 antibody concentration tested with immunoassays, and their affinity, are critical risk factors for development of thrombocytopenia and thrombosis[19,20]. Therefore, another application of that antibody when spiked in plasma, at a constant concentration, is to use it as a calibrator for anti-HPF4 immunoassays: it can then allow performing quantitative measurements of anti-HPF4 antibody concentration, offering a better relationship with their pathogenicity. This report then presents the performances of the K070 monoclonal antibody, and how it can help to standardize HIT laboratory assays, to improve the interpretation of their results, and to optimize their internal quality control, therefore offering a new tool for a better consistency for laboratory testing of HIT, and overcoming the current issues[21].

Materials and methods:

Materials and reagents:

PF4 was purified from human fresh platelet concentrates using a combination of heparin affinity and size-exclusion chromatography[22,23]. PF4 was obtained in the tetrameric presentation as previously reported[22,23]. Enoxaparin (Lovenox®) and unfractionated heparin (UFH, Heparin Sodium, Choay) were from Sanofi (Vitry-sur-Seine, France). Complexes between PF4 and enoxaparin were prepared as previously described, using 175 µg enoxaparin per mg of PF4 to generate the stoichiometric HPF4 complexes, presenting the strongest binding to heparin dependent antibodies developed in HIT[22,23]. The recombinant mouse-human chimeric monoclonal antibody (MoAb), with the code K070, was provided by Sysmex Corporation (Kobe, Japan): this antibody was obtained by combining the variable domain of a mouse monoclonal antibody to HPF4 complexes, with the constant domain of human IgG1, heavy and light chains (manufacturer information). This antibody is characterized by its strong reactivity to HPF4 complexes along with a poor binding to PF4 alone. A polyclonal chimeric rabbit-human IgG antibody was obtained by reticulating with glutaraldehyde affinity purified rabbit antibodies to human PF4 with a same concentration of human IgGs (RH chimera) as previously reported[24]. The 5B9 chimeric MoAb was obtained from Diagnostica Stago (Asnières, France), and used for a comparison study with K070 with the Flow Cytometry technique. Testing of IgG isotype antibodies to PF4 and HPF4 by ELISA was performed as already described, using PF4 or HPF4 complexes bound to 96-well micro-plates (NUNC, maxisorp, type 1 (Dutscher, Bernolsheim, France). For this technique, the tag antibody is a goat affinity purified immunoglobulin, specific for human IgG Fcγ domain, and coupled with peroxidase. Assay diluent used for testing antibodies is a 0.05 M phosphate, 0.15 M sodium chloride buffer at

pH 7.40, supplemented with 10% goat serum and 0.05 % tween 20, to avoid any non-specific interaction. Zymutest HIA (Heparin Induced Antibodies), IgG, and Zymutest PF4 were from HYPHEN BioMed (Neuville sur Oise, France). Normal human plasma pool, obtained from Precision Biologic (Cryocheck, Halifax, Canada), was spiked with K070 at concentrations from 2 to 200 µg/ml final concentration. Individual normal citrated human plasmas, or normal citrate anticoagulated blood were obtained from Etablissement Français du Sang (EFS, Pontoise, France) from healthy donors, duly informed and who gave their consent. Blood was kept at room temperature and Platelet Rich Plasma (PRP) was prepared within 4 hours following donation, through a centrifugation at 200 g, for 5 minutes at room temperature, and used within the following 2 hours. Platelet counts on PRP were performed using a Sysmex hematology analyzer KX-21 N (Sysmex, Villepinte, France). Platelet aggregometry tests were performed using siliconized glass tubes with stir-bars, and the SD-Innovation TA-8V 8-channel thrombo-aggregometer TA-8V (Frouard, France). Citrated plasmas from patients with suspected HIT, and tested positive for anti-HPF4 antibodies, were provided by the University Hospital of Rennes (Rennes, France).

Methods:

Anti-PF4 or Anti-HPF4 human IgG ELISAs were performed as already reported[22,23]. Zymutest HIA, IgG was conducted according to the assay package insert for testing the various patients' plasmas, and antibodies or chimeras in dilution buffer or spiked in plasma. A concentration range of K070 from 0 to > 100 ng/ml, was tested in dilution buffer or in plasma for IgG isotype antibodies using the Anti-PF4 or Anti-HPF4 ELISAs, or the Zymutest HIA, IgG kit. The rabbit-human IgG chimeric antibody was tested at a concentration range from 0.1 to 5.0 µg/ml. The K070 antibody spiked in the cryocheck normal pool plasma was tested with various normal platelet rich plasmas (PRPs) using the PAT or FCM assays. PATs were performed in the absence or the presence of UFH at a low (0.3 IU/ml) or a high (100 IU/ml) concentration using freshly prepared normal PRPs, and methods previously reported in the litterature[25]. In practice, in a siliconized glass micro-tube containing a micro-stir bar, 200 µl of PRP were introduced, then 44 µl of the UFH solution at 0.3 or 100 IU/ml, and 200 µl of the tested plasma or plasma spiked with K070, and platelet aggregation was followed for 35 minutes under stirring. Various concentrations of K070 were tested, from 10 to 200 µg/ml in citrated plasma pool. For poor PRPs' responders, additional studies were performed by supplementing them with purified PF4, at concentrations from 1.0 to 20.0 µg/ml, and performing again PAT or FCM. Flow cytometry measurements were performed using the EMOSIS HIT Confirm assay (Ilkirch-Graffenstaden, France) as previously described[26,27]. Briefly, tested plasmas or samples containing heparin dependent IgG antibodies are incubated with various PRPs at a low (0.3 IU/ml), or a high (100 IU/ml) heparin concentration, with an anti-CD-(Cluster Differentiation)-41-PhycoErhytrin (PE) fluorescent conjugate to identify platelets, and an anti-P selectin monoclonal antibody, tagged with the FITC fluorescent label to detect platelets. When activated, platelets expose P-selectin in a dose-dependent manner, and they bind the fluorescent antibody. The fluorescence intensity of activated platelets is

then measured by flow cytometry. Results are expressed as % HEPLA (Heparin Platelet Activation Index), which indicates the amount of activated platelets. There is a high variability for platelet activation/aggregation induced by heparin dependent antibodies in presence of a low heparin concentration, and results are highly dependent on platelet donor as reported in many studies[25,28]. For FCM, variability is slightly lower, and for that study PRPs reactivity (good responsiveness) was previously tested with a known HIT-positive patients' plasma: 9 PRPs were used, from which 7 were good responders, and 2 poor. For PAT there was no selection of platelet donors. The high variability of PRPs used is noted with all platelet activation assays[27,28]. PRPs were tested for PF4 in the corresponding Platelet Poor Plasmas (PPP), and for platelet count. Heparin Induced Platelet Activation (HIPA) was performed at external laboratories using methodologies already reported[29–31]. To test the suitability of using chimeras to calibrate Elisas for quantitatively measuring heparin dependent antibodies in plasmas from patients with HIT, a calibration curve was prepared with K070 or the RH-chimera, and antibody concentrations were tested in 10 different HIT patient plasmas, and expressed against each calibrator. Then, plasmas were all pre-diluted to a same measured concentration, and serial dilutions were prepared and tested. The dose-response curves were compared for patient-to-patient, and with chimeras.

Results:

Antibody reactivity:

The binding of K070 to PF4, HPF4 or onto the Zymutest HIA, IgG kit coated plate was tested as shown on figure 1. This reactivity is the same whether the antibody is tested either in the purified form or spiked in plasma, then deluted with the assay dilution buffer containing 10% goat serum. The antibody reactivity is much higher to HPF4 than to PF4 and our data comply with the K070 characteristics claimed by the manufacturer. Tested with ELISA, K070 behaves in a very similar manner to other reported HIT-like antibodies, like the 5B9 MoAb or the KKO mouse MoAb, with an almost total specificity to HPF4 complexes, and a negligeable binding to PF4 alone.

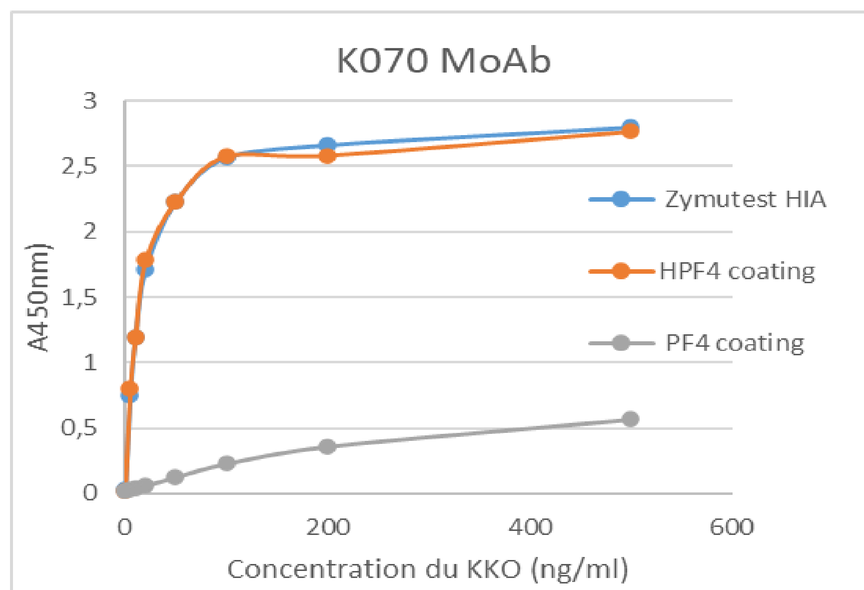

Figure 1: Dose-response reactivity of the K070 MoAb to Platelet Factor 4 (PF4), to Heparin (enoxaparin)-PF4 (HPF4) complexes using the ELISA method (left panel), or in the Zymutest HIA, IgG kit. A significant absorbance at 450 nm (A450) of 0.80 is already obtained for a K070 concentration of only 5 ng/ml, whilst there is no significant reactivity to PF4.

K070 has a very high reactivity in the Elisa, as it generates a strong response, with an absorbance at 450 nm (A450) > 1.00 for the concentration of 1.00 µg/ml, tested diluted 1:100 with the assay diluent, which corresponds then to 10 ng/ml in the assayed dilution. This anti-HPF4 binding was inhibited by UFH in the tested dilution, and a 0.2 IU/ml concentration inhibited > 75 % of binding. Conversely, the rabbit-human reticulated IgG chimera, which carries the anti-PF4 antibody site along with the whole rabbit IgG molecule, and the human IgG global epitopes, shows a slightly higher reactivity to PF4 than to HPF4, as shown on figure 2.

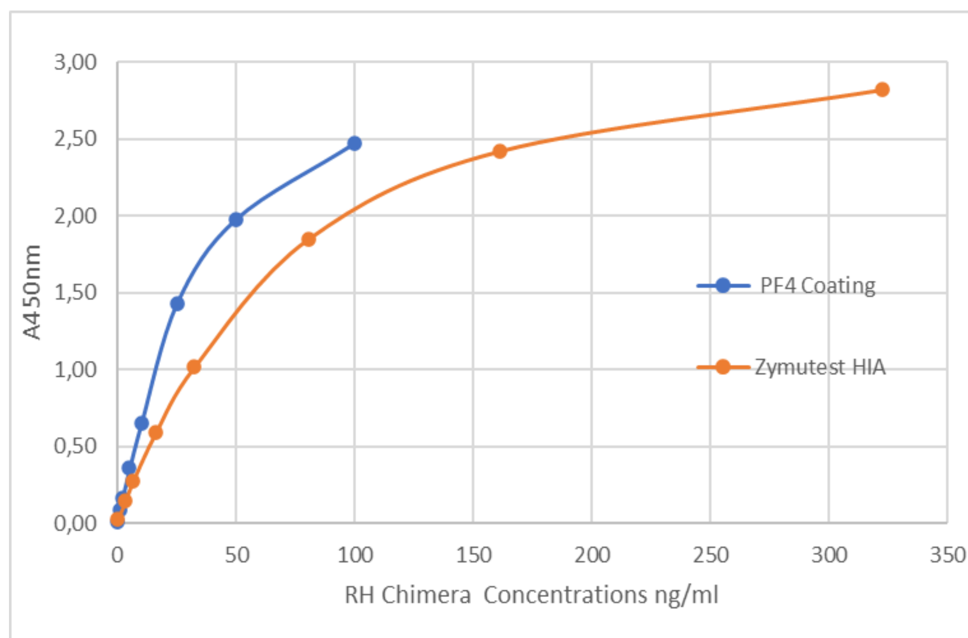

Figure 2: Dose-response curves for the binding of the RH rabbit-human antibody chimera to Platelet Factor 4 (PF4) or the HPF4 complexes, tested for IgG isotype.

However, the RH-chimeric antibody must be used at a much higher concentration than that of K070, and the A450 > 1.00 is obtained for a concentration of about 20 ng/ml for binding to PF4, and slightly above 50 ng/ml for the binding to HPF4. This lower reactivity, as compared to that of K070, results from the reticulation process, which hinders some antibody structures, or introduces a steric hindrance for many antibody sites. In both cases a good dose response curve is obtained when increasing antibody concentrations are used.

Platelet aggregation studies:

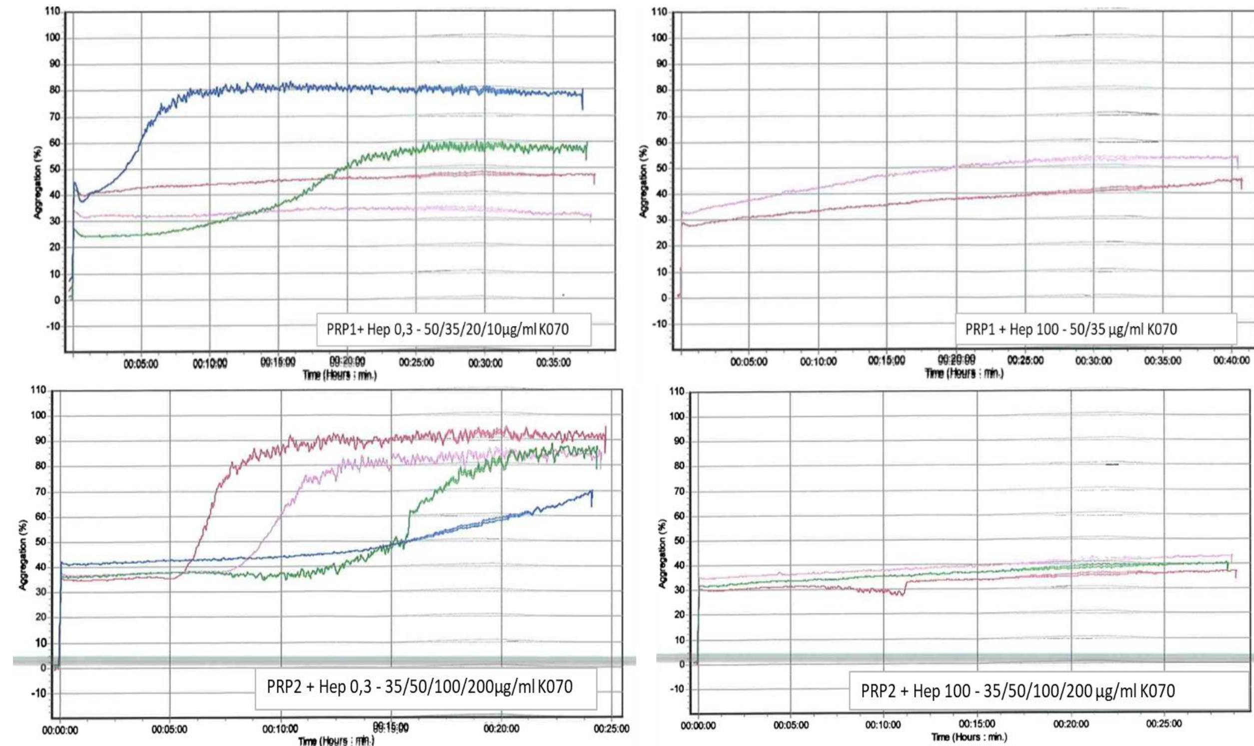

Figure 3: Platelet Aggregation Tests with PRP1 performed at 0.3 and 100 IU/ml of unfractionated heparin (UFH) using K070 concentrations from 10 to 50 µg/ml are positive from 35 µg/ml and abrogated by heparin at 100 IU/ml. For PRP2, there is a dose dependent increase of platelet aggregation intensity with K070 concentrations, inhibited by high UFH.

As reported for other platelet activation or aggregation tests, there is a high variability from donor to donor for not completely identified reasons, which could involve FcɣRIIA density and phenotype or other platelet surface glycoproteins. To illustrate this, figure 3 shows for 2 PRPs the aggregation profiles of 2 PRPs tested with various K070 concentrations. Figure 4 shows for 7 PRPs from blood donors, randomly recruited without any selection criteria, the platelet aggregation tests obtained with 50 µg/ml K070 in cryocheck plasma pool and 0.3 IU/ml UFH. All aggregations are inhibited with 100 IU/ml UFH (results not shown). Platelet aggregability is platelet donor dependent. Two PRPs show a high responsiveness, 3 others a good one, and 2 are unresponsive with 50 µg/ml K070.

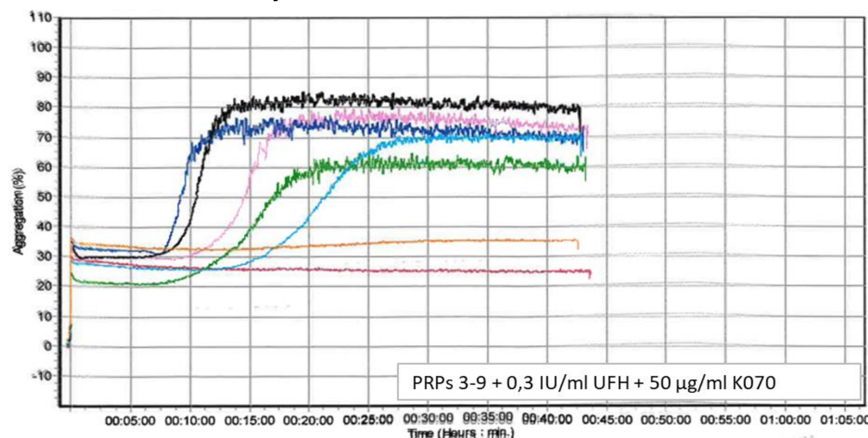

Figure 4: PAT on 7 PRPs from normal blood donors, analyzed with 0.3 IU/ml UFH and using 50 µg/ml K070: a high variability is observed from platelet donor-to-donor when PRPs are tested in the same conditions.

Trials were performed by adding increasing concentrations of PF4, from 1.0 to 10.0 µg/ml final concentration, in the poor responder PRPs. This did not succeed in inducing any platelet aggregation. PF4 was tested in the 7 PRPs to check if there is any relationship between ex-vivo release of PF4 and PRPs reactivity. PF4 ranged from < 100 to > 500 ng/ml in the various PPPs, but there was no relationship noted between PF4 concentration and PAT reactivity. In addition, testing for platelet counts did not show any association with PAT.

As shown on figure 5, in presence of 0.3 IU/ml UFH, addition of PF4 for concentrations from 1 to 20 µg/ml failed in inducing any platelet aggregation. However, increasing the K070 concentration to 100, or 200 µg/ml succeeded in promoting the platelet aggregation with the 2 poor responders' PRPs. All tested PRPs then produced positive PAT tests when the K070 concentration in tested plasma was of 100 µg/ml or higher.

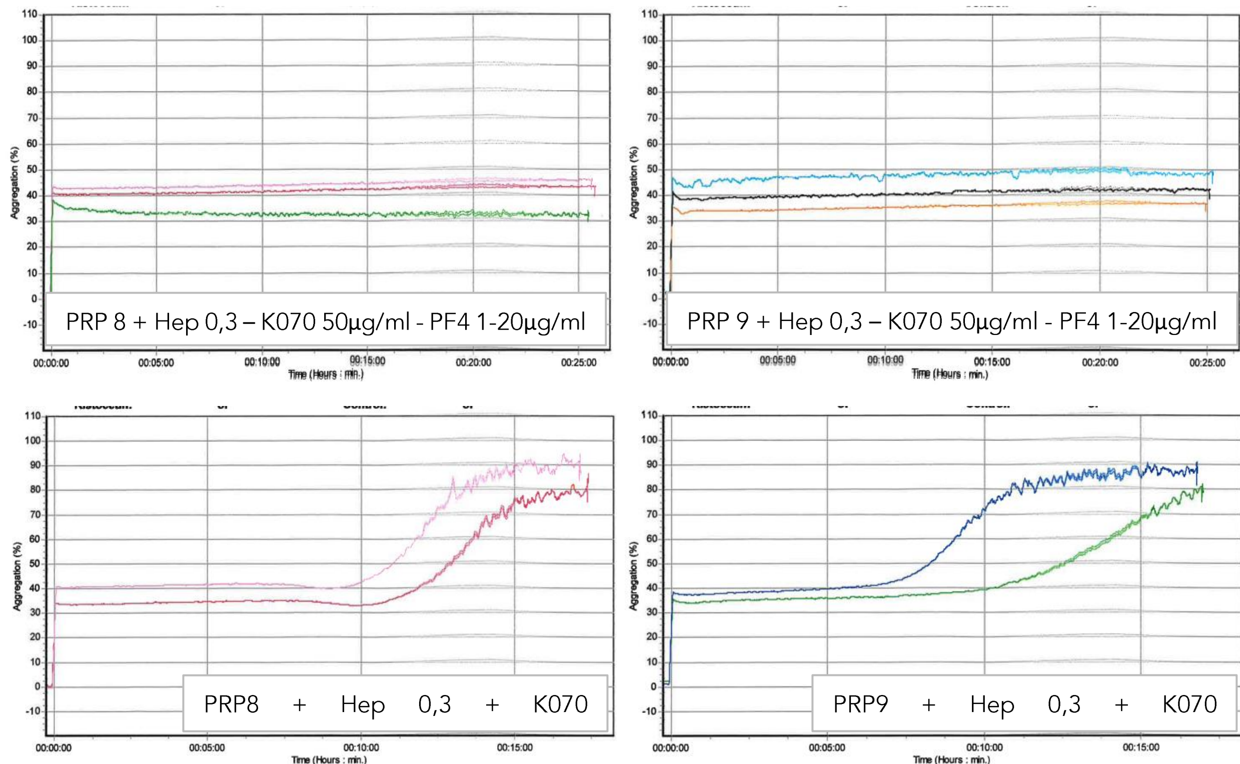

Figure 5: In presence of 0.3 IU/ml of UFH, PAT results with the 2 PRPs, out of the 9 tested , which were negative for PAT with 50 µg/ml K070. Addition of PF4 (1 to 20 µg/ml) in these 2 poor responders' PRPs did not succeed to activate PAT; by contrast, increasing the K070 concentration to 100 or 200 µg/ml induced a positive PAT response.

Figures 4 and 5 show that for each donor the platelet aggregation intensity is K070 MoAb dose-dependent, few PRPs being activatable with only 20 to 30 µg/ml of K070. When the MoAb concentration increases, more PRPs become positive, and all those tested were positive using the 100 or 200 µg/ml concentration. It is interesting to note the high K070 concentration difference to get positivity in the immunoassay and the PAT: there is a 20 to > 100

fold higher antibody concentration required for rendering the PAT result positive, depending on the PRP used, as compared with the ELISA (already clearly positive for a concentration ≥ 1.0 µg/ml in undiluted plasma). For the poor PRP responders, trials of adding PF4 to a final concentration from 1.00 to 20.0 µg/ml did not succeed to improve the platelet response in presence of K070.

Flow cytometry testing:

K070 was tested comparatively to the 5B9 MoAb, both either in the purified form or spiked in plasma. When tested with the FCM method, the K070 MoAb showed a dose-dependent activation of platelets, in presence of a low, but not a high heparin concentration. Nine PRPs were tested. The 5B9 MoAb was tested with the same conditions than K070, both at concentrations from 10 to 30 µg/ml. Figure 6 shows the mean % HEPLA for the various PRPs, with both K070 or 5B9 MoAbs, at the concentrations of 30 µg/ml, when tested with 0.3 IU/ml UFH. In presence of 100 IU/ml of heparin, all reactivities were abrogated.

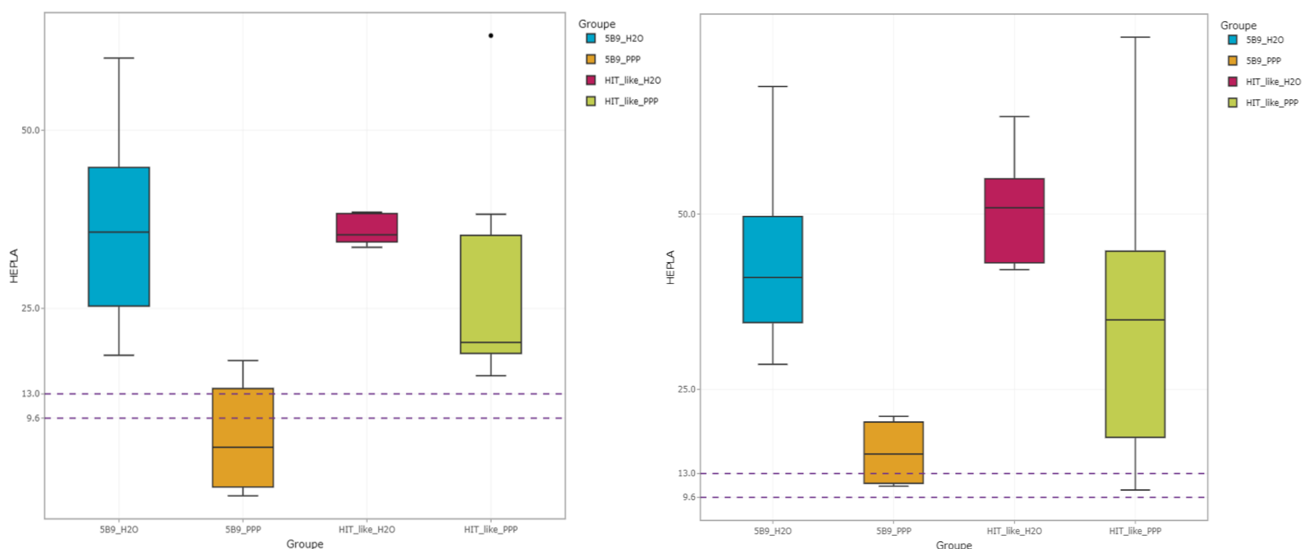

Figure 6: Flow cytometry tests obtained with 9 different PRPs (mean values and standard deviation) using the 2 HIT-like MoAbs, 5B9 and K070, at 20 and 30 µg/ml; results are expressed as % HEPLA (> 13 % corresponds to positive results; < 9.5 % to negative results; and there is a grey zone between 9.5 % and 13 %).

Five out of the 9 PRPs were good responders, 2 moderate, and the last 2, poor responders. Both MoAbs 5B9 and K070 showed a good activation of platelets in presence of the low heparin concentration (0.3 IU/ml), and their activity was higher when tested in the purified form than when spiked in plasma. Both antibodies already activated PRPs at the concentration of 20 or 30 µg/ml, when tested in buffer, but 5 B9 was negative at the 20 µg/ml concentration when spiked in plasma, whilst K070 was clearly positive. At the concentration of 30 µg/ml, 5B9 in plasma became weakly positive, and K070 showed a higher positive response, as shown on figure 6. When K070 was tested with the HIPA technique (external testing, data not shown) results were positive with at least 2 out of the 4 washed platelet preparations from the concentration of 20 µg/ml or higher (results not shown), and almost all platelet preparations tend to be aggregated with higher K070 concentrations.

Calibration of immunoassays:

For this approach, HIT patients' plasmas were tested using the RH-chimera or the K070 calibration, and the anti-HPF4 IgG concentration was assigned to those plasmas with each calibrator. Each plasma was then diluted to the same concentration, corresponding to the high range of the ELISA reactivity, and serial dilutions were performed with the assay dilution buffer.

Tested with the PF4, HPF4 capture ELISAs, or the Zymutest HIA, IgG, kit the RH-chimera, rabbit-human reticulated IgGs, the K070 MoAb, or the serial dilutions of plasmas from patients with heparin dependent antibodies produced dose-responses curves superimposable, showing the same reactivity and slope to the capture HPF4 antigen for a better understanding: "Patients' plasmas were diluted to give an absorbance in the upper zone of the immunoassay reactivity range, and then serial 2-step dilutions were performed." For these plasmas from HIT-suspected patients, the same plasma antibody concentration is obtained whether the dilution tested is, as shown on figure 7. The same dose-response curves were obtained with the patients' plasmas, when the calibration was performed with the RH chimera, and similar results were generated with the K070 MoAb.

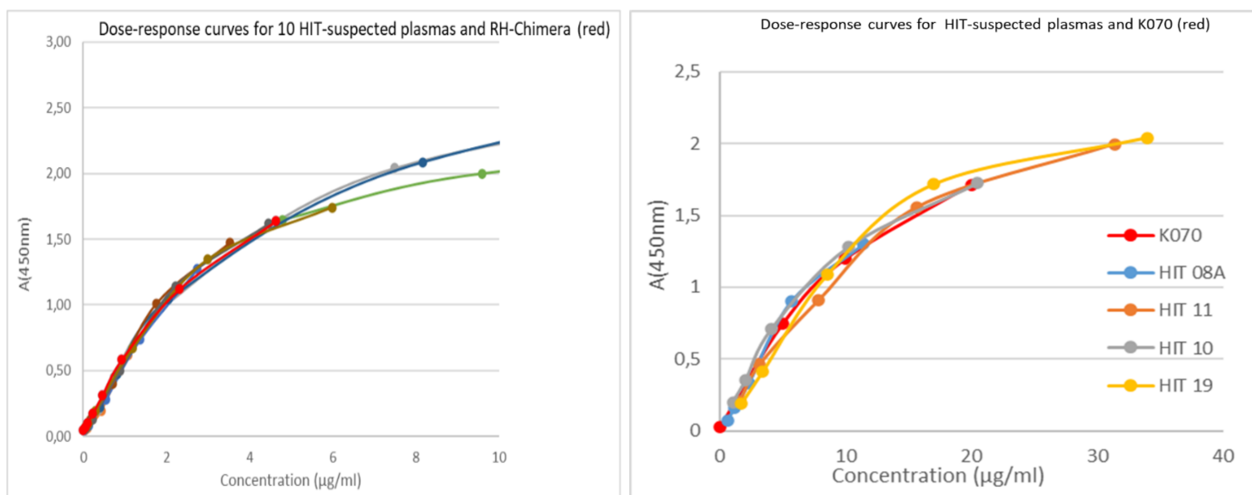

Figure 7: Dose-response curves with the RH-chimera and 10 different HIT -suspected patients' plasma (left panel), and with the K070 MoAb and other HIT-suspected patients' plasmas. The RH-Chimera concentrations are only a rough estimate due to the technology used, whilst the K070 MoAb is fully homogeneous and allows assigning an anti-HPF4 concentration to tested plasmas.

The use of K070 MoAb provides more reliable and accurate results than the RH-chimera, which is a large multimolecular IgG reticulated complex, with lot-to-lot variability, and a poor reactivity due to hindered antibody sites. Therefore, when this K070 MoAb is used as calibrator to quantitate anti-HPF4 antibodies in HIT-suspected plasmas, measured anti-HPF4 IgG antibody concentrations are in the range of < 200 ng/ml to > 4,000 ng/ml in plasma, when corrected for the dilution tested. This quantitative measurement of anti-HPF4 IgGs could allow establishing a more accurate relationship between antibody concentration and its pathogenicity.

Discussion:

In this article we report the laboratory performances of a new mouse-human chimeric recombinant monoclonal antibody, coded

K070, containing the mouse variable domain of a MoAb to HPF4 complexes and the human IgG1 constant domain, and which exhibits very useful laboratory performances when tested, spiked in plasma, for HIT assays, whether functional, and based on platelet activation /aggregation, or immunoassays. When spiked in citrated plasma, this K070 behaves like a plasma from a symptomatic patient with HIT. This antibody is available always with the same constant characteristics over time, and can be obtained at high amounts, which makes it usable for all HIT laboratory testing methods. It then offers a reference material, which is of high usefulness for standardizing diagnostic approaches. Our laboratory investigations were focused on: i) antibody behavior in HPF4 or PF4 Elisas; ii) the Zymutest HIA, IgG kit ; iii) using PAT or FCM with various PRPs obtained from unselected blood donors. FCM testing was performed through a collaboration study with Emosis, using the Emosis HIT confirm test[26,27], and including various PRPs from unselected blood donors. In addition, external studies were performed with the HIPA method, and only the preliminary results obtained are discussed in this report, the goal being to verify the behavior of this antibody in that functional HIT assay, currently used in many laboratories. As HIPA works with washed platelets, K070 was already mildly positive at 6.5 µg/ml on the 4 tested platelet preparations (all tested positive with a control HIT serum), and platelet aggregation time shortened with increasing doses, up to 100 µg/ml. Platelet aggregation was noted even in the absence of heparin with the higher K070 concentrations, but all tests became negative in presence of the high UFH concentration, which confirms the K070 specific effect.

The tested Elisa methods showed a very strong reactivity of K070 with microplates coated with HPF4 complexes, or with the Zymutest HIA, IgG kit, producing a A450 > 1.00 for 10 ng/ml in the tested dilution, and the reactivity was negligeable with plates coated with PF4 alone. This K070 then behaves like typical heparin dependent antibodies, specific for HPF4 complexes, detected in patients with HIT, and also present in some asymptomatic patients. However, heparin induced antibodies are very heterogenous in asymptomatic or symptomatic patients, many presenting anti-PF4 antibodies in addition to those to HPF4[22]. K070 has also the same immunoreactivity profile to PF4 or HPF4 complexes profile than the other reported antibodies, KKO[14] or 5B9[15,16]. KKO is a murine monoclonal antibody, which has been extremely helpful for HIT laboratory studies, but which cannot be directly assimilated to a human antibody. 5B9 was developed with the human Fc fragment and the variable domain of a mouse MoAb, and it has been proposed as a control for HIT functional assays or immunoassays. In a multicentric assay, it was evaluated in the purified form with various HIT functional assays, including C14-Serotonin Release Assay (SRA), PAT (also named Light Transmission Aggregometry), Heparin Induced Multiple Electrode Assay (HIMEA) and FCM. In our study, we used only PAT and FCM, and the satisfactory behavior of K070 in HIPA was tested externally. With PAT, 5 out of the seven PRPs aggregated strongly with 50 µg/ml of K070 in plasma, whilst 2 PRPs failed to aggregate. Increasing the K070 concentration to 100 µg/ml succeeded in aggregating these 2 weak responders PRPs. It must be noted that K070 concentrations are indicated for the tested plasma, whilst in the reactive PRP

mixture they are 2.44-fold lower, i.e. 20.5 µg/ml for the tested 50 µg/ml plasma concentration. K070 was compared with 5B9 only in the FCM method. It was tested at various concentrations in the assay reactive tube (10 to 30 µg/ml), with 8 different unselected PRPs, and either in the purified form or spiked in the plasma pool. Tested at the same concentrations (10 to 30 µg/ml in the final PRP mixture), 5B9 and K070 induced equivalent platelet activation responses, especially at 20 and 30 µg/ml, when they are used in the purified form. However, when spiked in plasma reactivity was much lower for 5B9, whilst it was only moderately affected for K070, all PRPs being positive at 20 and 30 µg/ml. On contrast, with 5B9 in plasma, all PRPs were below the positive threshold with 20 µg/ml, and they became slightly positive with 30 µg/ml. This different behavior in the plasma matrix is probably due to the IgG Fc of both MoAbs, which human IgG1 epitopes are provided by the transgenic mice and concern the heavy chain for 5B9, and are recombinant and concern the IgG constant domain for K070. This can impact the binding of Fcɣ-RIIA platelet receptors within a high IgG concentration environment[32].

Lastly, when diluted with the assay buffer for giving the same reactivity (in the upper range of the Elisas with an A450 of about 2.00), and tested with the anti-HPF4 Elisa, or the Zymutest HIA, IgG kit, plasmas from HIT patients, the RH chimera or the K070 recombinant chimeric MoAb generated fully superimposable dose-response curves. This opens the possibility to use chimeras for calibrating immunoassays. However, it is inconvenient to assign a ponderal value to the RH chimera, as it is a multimolecular complex between rabbit affinity purified antibodies and human purified IgGs, many anti-PF4 sites being hindered. In addition, its reticulated structure is too different from the human IgGs in the tested plasmas from patients with HIT. Conversely, K070 has a very close structure to those heparin dependent antibodies in tested patients, with the full constant IgG1 domain, and only the variable domain of a mouse MoAb to HPF4. Its ponderal concentration can be very accurately measured. Therefore, this antibody is a good candidate for proposing a plasma calibrator for quantitating anti-HPF4 antibodies present in HIT asymptomatic or symptomatic patients. This can contribute to a better immunoassay standardization than the simple use of A450 for graduating the heparin dependent antibody concentration, inasmuch that latter is associated with pathogenicity. Various studies reported that all symptomatic patients had an enough high anti-HPF4 IgG concentration, with an A450 > 1.00 in Elisa[19,33]. However, patients with HIT can develop anti-HPF4 with different affinities, and different effect to activate platelets, as shown with affinity purified antibodies: only those with the highest affinity were able to activate platelets in presence of heparin[34]. This means that only a subset of anti-HPF4 antibodies are pathogenic, and additional studies must investigate the diagnostic value of the quantitative measurement of anti-HPF4 antibodies and their association with disease severity.

The objective of this preliminary study was to show that K070 spiked in citrated plasma can be used as a control plasma for HIT diagnostic tests, whether immunoassays or functional, as it mimics a plasma from a patient with HIT. All laboratory diagnostic methods were not tested, but the antibody behavior was consistent within all assays, which fully supports its value in that

application. Complementary investigations are required to document its reactivity in other assays like the SRA, the various chemiluminescent immunoassays (internal data, not presented, showed a good reactivity with a chemiluminescent method), or the lateral immune-filtration devices, like the STIC® expert[35]. This work did not investigate any effect of antibodies on blood cells, or experimental animals, as this was not the goal, and it has been restricted to antibody performances in laboratory assays. Complementary investigations can be conducted, when the antibody becomes publicly available. A special focus on the role of its specific IgG1 structure in platelet activation or, producing HIT-like symptoms would present a high interest.

Conclusion:

The K070 mouse-human chimeric recombinant monoclonal antibody, highly reactive to HPF4 complexes, and poorly with PF4 can be used as a control material for HIT laboratory diagnostic methods, whether functional or immunological, opening the possibility to use a material mimicking plasmas from patients with HIT. It is also a good candidate for calibrating the various assays, and then rendering quantitative concentrations for heparin dependent antibodies, which can facilitate the studies between antibody concentration and its pathogenicity. Additional studies are needed to confirm that application.

Conflict of Interest Statement:

None

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Contributions:

JAM designed the study and wrote the article; NBO and ELE prepared, realized and analyzed the laboratory studies and reviewed the article; KKO contributed to the assay design and study supervision, and analyze the results, and reviewed the report.

Disclosures:

JAM is a scientific and technical consultant for HYPHEN BioMed (Neuville sur Oise, France) and Sysmex Corporation (Kobe, Japan); NBO, ELE and KKO are employed by HYPHEN BIOMED.

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Abbreviations:

CD: Cluster Differentiation
ELISA: Enzyme Linked Immuno-Sorbent Assay
FCM: Flow CytoMetry
HEPLA: Heparin Platelet Activation
HIA: Heparin Induced Antibodies
HIPA: Heparin Induced Platelet Activation
HIT: Heparin Induced Thrombocytopenia
HITT: Heparin Induced Thrombocytopenia and Thrombosis
HPF4: Heparin Platelet Factor 4 complexes
IgG: Isotype G immunoglobulin
MoAb: Monoclonal Antibody
PAT: Platelet Aggregometry Test.
PE: PhycoErhytrin
PF4: Platelet Factor 4
PPP: Platelet Poor Plasma
PRP: Platelet Rich Plasma
SRA: C14 Serotonin Release
UFH: Unfractionated Heparin

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