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CAN WE IMPROVE CLINICAL SPECIFITY FOR HIT IMMUNOASSAYS?

J. J. AMIRAL[*1], A. M. VISSAC[1]
[1]RESEARCH, HYPHEN BioMed, Neuville sur Oise, France

Immunoassays used for confirming or diagnosing HIT detect many asymptomatic heparin dependent antibodies (usually targeted to PF4, and sometimes to IL-8 or other chemokines complexed to heparin). Asymtomatic antibodies are usually IgM or IgA isotypes, but some IgG isotypes are also asymptomatic, and conversely some (few) IgA (IgM?) isotypes could be pathogenic. Measuring only IgG isotypes improves clinical specificity. However, characterization of this iatrogenic complication requires to combine the clinical score with functional and/or immunological tests for heparin dependent antibodies. Our goal was to evaluate which analytical factors could be associated with heparin dependent antibody pathogenicity. Affinity purification of antibodies from some patients with definitely characterized HIT, and their testing in functional assays, suggested that those antibodies presenting with the highest affinity are the most pathogenic, as they exhibit the highest capacity to positive SRA. Recently, we introduced a dynamic assay, where the immune complex between PF4 and HIT antibodies forms on immobilized heparin, thus mimicking the in vivo conditions. We then designed immunoassay variants aiming to detect only HIT antibodies with the highest affinity. This was achieved by using a more chaotropic assay diluent. This approach allowed to distinguish between patients and selecting those having the antibodies with high avidity and affinity, and potentially the most harmful.In these conditions,some heparin dependent antibodies were almost negativate, whilst other remained highly positive. These preliminary results suggest that pathogenic effect of antibodies could be gratduated by classifying them through their affinity for the heparin dependent antigen. This could allow developing diagnostic approaches more specific of the immediate clinical risk for patients. Anyhow, as HIT is a very rapid evolving context, great caution is necessary before excluding this risk in antibody positive patients.

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AM Vissac, J. Amiral

HYPHEN BioMed Research, 95000 Neuville sur Oise (France)

OBJECTIVE OF THE STUDY

Immunoassays for Heparin induced Thrombocytopenia (HIT) offer a good sensitivity but poor clinical specificity as many antibodies are asymptomatic, including IgG isotypes without platelet activation activity[1].

Improvement of HIT immunoassays is required for:

- Obtaining high Sensitivity/Specificity for « pathogenic » Heparin dependent Antibodies associated with clinical HIT.
- Avoiding interference of «false» antibodies («sticky·samples»).
- Differentiating symptomatic from asymptomatic heparin dependent antibodies.

The goal of this study was to evaluate which analytical factors could be associated with heparin dependent antibody pathogenicity, then to use these findings for developing more clinically relevant immunoassays for HIT.

BACKGROUND

Affinity purified heparin dependent antibodies from 3 patients with HIT showed that only the high avidity IgG fraction produced high positive SRA tests, whilst the lower affinity IgG fraction, although it bound identically to H-PF4, was unable to activate platelets (SRA negative)[2].

From this observation, we are developing an immunoassay approach by privileging binding of antibodies with the highest affinity to the heparin dependent antigen, mainly PF4[3].

Asymptomatic antibodies presenting the lowest affinity for heparin dependent antigen are expected to yield negative or weak positive responses.

MATERIALS & METHODS

Dynamic assay (Zymutest HIA IgG,A,M or IgG) in which immune complexes between PF4 (provided by platelet lysates) and HIT antibodies (when present in the assayed specimen) are formed with immobilized heparin fixed onto the plate through Protamine Sulfate (PS) (heparin being in large excess), or with biotinylated heparin and immobilized Streptavidin[4](SA). An assay variant was designed by increasing chaotropicity of sample diluent, in order to favour the binding of antibodies with the highest affinity.

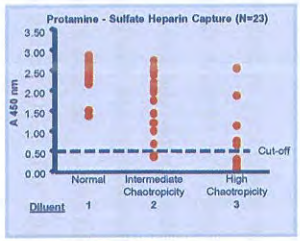
Only IgG isotypes were measured.

Samples tested:

- 23 samples from patients with HIT suspicion were tested.
- All the patients were PAT and SRA positive, and platelet counts were decreased by > 50%.

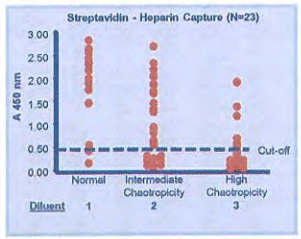
RESULTS

Incidence of Sample Diluent chaotropicity on antibody binding to:



→ Heparin Protamine Sulfate

When excess of Heparin with Protamine sulfate is used for coating, all the samples have an OD>1.00 with diluent no.1 and only 5 remain positive with diluent no.3. These patients are those presenting the most severe clinical symptoms with the highest HIT score. With diluent no.2, 19 out of 23 keep an OD >1.00.The OD doesn't decrease for 2 samples (these 2 samples were later demonstrated to have antibodies to Protamine Sulfate).

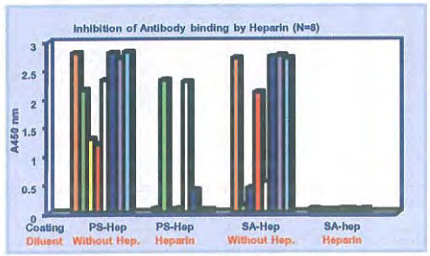


→ Biotinylated-Heparin-Streptavidin

With the Biotinylated - Heparin - Streptavidin system, only 20 samples have an OD>1.00 with the diluent no.1, but 2 are borderline (A450 of about 0.50) and 1 is negative. OD decreases for almost all specimen with diluent no.3, but 4 remain highly positive (OD>1.00).

Interestingly, with diluent no.2 of intermediate chaotropicity, only patients with a definite HIT remain highly positive (A450>1.00), which represents 15 out of the 23 patients.

Confirmation of heparin specificity with heparin inhibition study



The inhibition of antibody binding by heparin (2 IU/ml in the reactive well) was tested on 8 samples.

→When the Streptavidin system is used for coating, antibody binding was completely inhibited for all the 8 samples, but inhibition occurred only for 6 samples with the Protamine Sulfate coating.

→The two non inhibited samples were further demonstrated to have antibodies to Protamine Sulfate.

CONCLUSIONS

- HIT Antibodies «are» pathogenic when they bind to heparin dependent antigen (PF4 mainly) present in blood and onto blood cell surfaces. The most harmful are those exhibiting the highest avidity.
- Using biotinylated heparin immobilized onto the microELISA plate through Streptavidin, and in presence of platelet lysate, the most potentially pathogenic IgGs can be identified by using buffers with increasing chaotropicity. This approach improves the clinical specificity of measured antibodies.
- New laboratory assays could be designed from this observation, but extensive clinical studies are required for documenting the correlation of analytical measurements with severity of HIT clinical context.
- Should we «disregard» asymptomatic antibodies?

DISCUSSIONS

This approach allows distinguishing between patients and selecting those having antibodies with high avidity and affinity, and potentially the most harmful.

- In these conditions, some heparin dependent antibodies became almost negative, whilst other remained highly positive. Presence of antibodies to Protamine Sulfate is evidenced in 2 of the patients.
- These preliminary results suggest that the pathogenic effect of antibodies could be graduated by classifying them through their affinity for the heparin dependent antigen, and by testing preferentially their binding to biotinylated Heparin with Streptavidin.
- This could allow developing diagnostic approaches more specific for the immediate clinical risk of HIT in patients, or for a diagnosis correlating better with the clinical status.

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