AACC Annual Meeting | July 30, 2014, Chicago, IL, Poster B-355

A step towards D-dimer assays' standardization:
Antibodies with Equal Specificity to D-dimer and High Molecular Weight Fibrin Degradation Products

A. Kogan[1], K. Mukharyamova[1], A. Kara[2], A. Bereznikova[1], A. Katrukha[1]

[1]HyTest LTD, Turku, Finland, [2]School of Biology, Moscow State University, Moscow, Russian Federation

Introduction

FIBRIN DEGRADATION IN BLOOD results in a wide spectrum of fibrin degradation products (FDP) of different molecular weights in which D-dimer is the smallest one (180 kDa). The D-dimer test implies the detection of D-dimer and all kinds of FDP. The kits currently used for D-dimer determination show different results in plasma samples of the same patients because of different specificities of MAbs used in the kits to D-dimer and to FDP. That is why currently neither D-dimer nor FDP can be used as universal standards or reference materials in DD-assays. So far all standardization and harmonization attempts have not resulted in satisfying results and this is a continuous cause of problems in daily clinical practice. We believe that for an accurate determination of all FDP and D-dimer in plasma and for the standardization of D-dimer assays, MAbs that are utilized in commercial assays should recognize FDP and D-dimer with equal specificity.
The aim of this work was to design sandwich immunoassay with equal specificity to D-dimer and to FDP.

Materials and Methods

MONOCLONAL ANTIBODIES: Hybridoma clones have been derived from hybridization of Sp2/0 myeloma cells with spleen cells of Balb/c mice immunized with D-dimer by a standard hybridoma technique. mAbs were selected in direct ELISA being tested with D-dimer (selected antibodies with positive response) and with fibrinogen (selected antibodies with negative response). Selected mAbs were tested in pairs (one-step sandwich fluoroimmunoassay) with fibrinogen, FDP, and D-dimer.

FDP PREPARATION: Main stages were:
- Fibrinogen purification from human plasma (ammonium sulphate precipitation followed by ethanol precipitation)
- Fibrinogen clotting by thrombin
- Partial fibrin clot lysis by plasmin

D-DIMER PREPARATION WITH THE SAME MOLAR AMOUNT OF CROSS-LINKED MATERIALS AS IN FDP PREPARATION. Liquid fraction of FDP was withdrawn from FDP preparation and divided into two portions. In the first portion the reaction was stopped by PMSF (to preserve FDP fraction); in the second portion the reaction continued for another 24 hours (to obtain the sample containing D-dimer fraction mainly).

HPLC GEL FILTRATION was performed on the Superdex200 16/60 column using AKTA-FPLC system (Amersham Pharmacia Biotech) in 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl at the eluent rate of 2 ml/min. Fraction volume – 1 ml.

ONE-STEP IMMUNOFLUORESCENT ASSAY was performed as follows:
Capture MAbs: 1 μg/100 μl/well in PBS, 30 minute incubation, triple washing
Detection MAbs: 400 ng/75 μl/well of Eu-labeled MAbs in Delfia assay buffer (Wallac, Finland)
Sample: 25 μl immediately after the adding the detection MAbs, 1 hour incubation
Signal detection: in 1420 Multilabel Counter Victor (Wallac, Finland) using Lanfia solution

EDTA PLASMA SAMPLES from patients and healthy volunteers were prepared by a routine procedure.

Results and Discussion

MONOCLONAL ANTIBODIES. 22 D-dimer specific mAbs without crossreaction with fibrinogen were obtained and selected for testing in one-step sandwich immunofluorescent assay.

PREPARATION OF DD AND FDP WITH EQUAL MOLAR CONTENT OF CROSS-LINKED MATERIAL. D-dimer and FDP solutions were prepared as described in Methods and analyzed using PAGE in 3-10% gradient gel by Laemmly's method (Fig. 1). FDP preparation contained mainly high molecular weight fibrin degradation products and much less D-dimer. On the contrary, D-dimer preparation contained mainly D-dimer and little if any FDP.

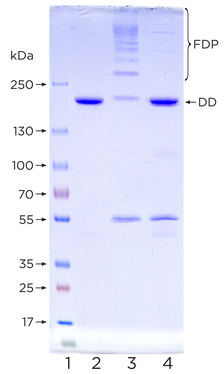
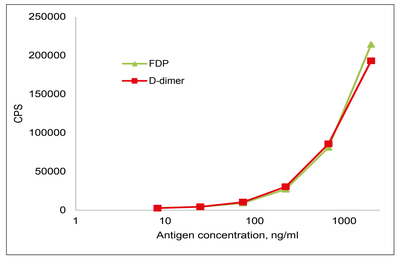


FIGURE 1. 1: MW standards, 2: D-dimer (standard), 3: FDP preparation, 4: D-dimer produced from FDP fraction by lysis.



MABS SELECTION. All possible two-site combinations of 22 selected MAbs were tested with DD and FDP preparations. Most of the assays had high specificity to FDP and low to DD. Much less assays were more specific to DD than to FDP. Only one mAbs combination DD189-DD255 was equally specific to DD and FDP giving an equal response with FDP and D-dimer in the range of the antigen concentrations from 20 to 1000 ng/ml. (Fig. 2).

FIGURE 2: Antibody pair DD189-DD255 detects FDP and D-dimer with equal specificity.

DETECTION OF FDP AND DD IN HPLC GEL FILTRATION PROFILES. Proteins from FDP and DD preparations were separated by gel filtration method. Protein fractions were analysed by DD189-DD255 assay. On the FDP profile the activity peak completely coincided with the FDP protein peak and with an additional small low molecular weight peak (Fig. 3a). Although a wide spectrum of FDP is seen on PAGE, the Superdex200 column does not permit to separate particular FDP forms, and they elute as a single protein and activity peak. On the DD profile the activity peak also coincided with the major protein peak corresponding to DD. No FDP was detected in the DD preparation profile (Fig. 3b). We believe that the small activity/protein peak in the FDP profile probably belongs to DD bound to E-fragment since its molecular weight is a bit higher than DD molecular weight in the DD profile.

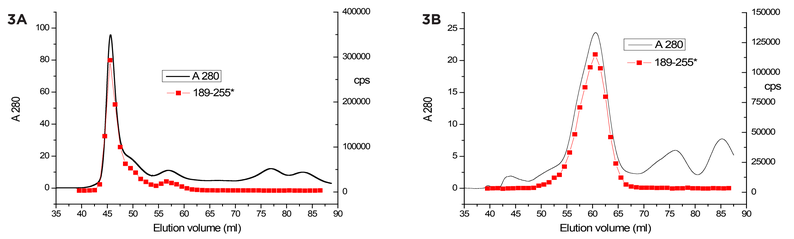


FIGURE 3. Immunoreactivity of the proteins from two preparations of fibrin degradation products separated by gel filtration method (measured by the DD189-DD255 assay).
3a: Proteins from FDP preparation. 3b: Proteins from DD preparation.

D-DIMER MEASUREMENTS IN PLASMAS OF PATIENTS WITH DIFFERENT DISORDERS. The plasmas of patients with thrombosis, thromboembolism, disseminated intravascular coagulation (DIC), and patients who had undergone surgical operations were tested by DD189-DD255* assay. The measurements were performed as described in "Methods" by a one-step assay using patients plasmas diluted 1:10 and purified D-dimer (HyTest, Finland) as a calibrator. Results are summarized in the Table 1.

Disorder	n	Mean DD (μg/ml)	Range
Sepsis	15	2.5	0.52 – 4.9
Thrombosis	8	3.1	0.98 – 6.9
Surgical operation (Before/After)	9	0.28/2.6	0.25-0.32/1.2-3.6
Pulmonary thromboembolism	7	2.7	1.3 – 6.7

TABLE 1. D-dimer detection in plasma samples of patients with different disorders by the DD189-DD255 assay.

In order to evaluate the ratio between DD and FDP in plasmas of different patients' groups, the proteins of plasma samples were separated by gel filtration method, and resulting profiles were analyzed by the DD189-DD255 assay that equally recognizes FDP and DD. It was found that in plasmas of patients with thrombosis the main component of fibrin-derived material was FDP and to a less degree DD. On the contrary in blood of patients one day after surgical operations DD was comparable or even exceeded the FDP levels. The typical profiles are shown in Figs. 4a and 4b.

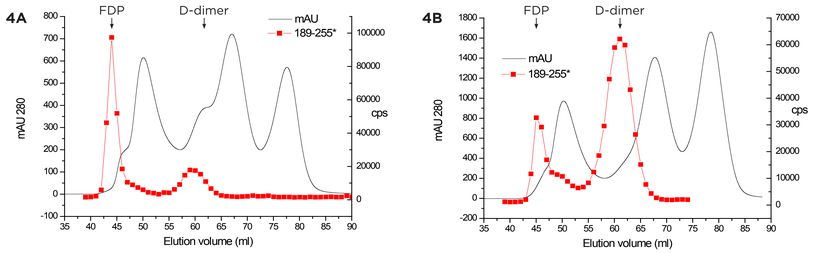


FIGURE 4. HPLC gel filtration of plasma samples from patients with thrombosis (A) and after a surgical operation (B). 200-500 μl of plasma was applied to the Superdex 200 column 16/60 at a flow rate of 1 ml/min. 1 ml fractions were analyzed by the DD189-DD255 pair in a one-step assay as described in Figure 2.

Conclusions

1. The immunoassay utilizing mAb DD189 as capture and mAb DD255 as detection has equal specificity to DD and to FDP.
2. Because of the different ratio of DD and FDP in the blood of patients with different diseases precise measurement of fibrin-derived materials in patients' blood is possible only by the assays that have equal specificity to DD and to FDP.
3. Standardization of DD assays is possible only in case when all assays subjected to standardization have equal specificity to DD and to FDP. In this case both forms of fibrin-derived materials (DD or FDP) could be used as a standard.

HyTest Ltd Intelligate 1, 6th floor, Joukahaisenkatu 6, FI-20520 Turku, FINLAND | Tel. +358 2 512 0900, Fax +358 2 512 0909, e-mail: hytest@hytest.fi, **www.hytest.fi**