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Rate-Nephelometric Determination of Fibronectin in Plasma

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We describe a kinetic immunonephelometric method for the determination of fibronectin in human plasma, used with the Beckman ICS rate nephelometer. The method is rapid and cost-effective. Two commercially available controls stated by the manufacturer to contain 200 and 295 mg/L were found to contain 198 and 290 mg/L, respectively. Mean analytical recovery was 104%. Within-run precision (CV) for normal samples was 3.8%, between-day precision 5.1%. For samples containing subnormal concentrations of fibronectin, these figures were 3.8% and 6.7%, respectively. Results by the method described here agreed and correlated well with those by a commercially available turbidimetric assay. With appropriately diluted samples, the range of measurement is 40 to 1000 mg/L. Normal values for women and men were 286 (SD 84) and 340 (SD 55) mg/L, respectively, in good agreement with values published by others.

Additional Keyphrases: *immunoassay · reference interval · sex-related differences · glycoproteins*

Fibronectin was first described in 1948 by Morrisson et al., who called it "cold-insoluble globulin" (*1*). The next to report on this protein were Mosesson and Umfleet, in 1966 (*2*). Since 1970, several papers have described a glycoprotein that is present in dimeric form in plasma and in multimeric form on cell surfaces (e.g., fibroblasts). Although given different names by different authors (e.g., cell surface protein, opsonic protein), the protein appeared to be one and the same; it was eventually uniformly named fibronectin (*3*).

Its synthesis and physiological role have been extensively reviewed (*4, 5*). Its relative molecular mass has been estimated, and structural and sequencing studies have been done (*6*). Normal values of plasma fibronectin in relation to age and sex have been determined by Eriksen et al. (*7*). Various techniques for measuring fibronectin have been described (*8–11*).

The growing understanding of the importance of the role of fibronectin in the monocytic macrophage system-related host-defense mechanisms (*12*) has prompted increasing interest in its measurement in blood plasma, not only reliably and sensitively, but also rapidly and cost-effectively.

Here we describe such a procedure, involving kinetic rate nephelometry.

Materials and Methods

Instrumentation and reagents. We used a Beckman Immunochemistry System (ICS; Beckman Instruments, Brea, CA), and nephelometric-grade reagents. Phosphate-buffered isotonic saline (PBS; phosphate concentration 20 mmol/L, pH 7.0) was used as diluent; PBS containing 40 mL of polyethylene glycol per liter as polymer enhancer was the reaction buffer, also from Beckman Instruments. For turbidimetry we used an Hitachi 705 Selective Multianalyzing Clinical Chemistry System and a commercial turbidimetric assay for fibronectin (product no. 401218) adapted for use with the Hitachi 705 (both from Boehringer Mannheim, Mannheim, F.R.G.).

Preparation of the sample. Blood was sampled by venipuncture into tubes containing EDTA as anticoagulant. The blood was centrifuged and the separated plasma was promptly frozen and stored at −70 °C (*11*). No preservatives were added. Shortly before analysis, the plasma samples were allowed to thaw at 37 °C (*7*), then diluted 36-fold in PBS (unless otherwise indicated below). When the fibronectin content is subnormal, 18-fold or smaller dilutions may be required.

Neither sterile filtration of plasma through a 0.22-μm (av. pore size) filter (Millex GV; Millipore Corp., Bedford, MA.) nor removal of plasma lipids by treatment with "Lipoclean" (Behringwerke AG, Marburg, F.R.G.), or both, improved the results.

Antiserum. Rabbit antiserum to human fibronectin (product no. A 245, lot no. 042, Dakopatts A.S., Glostrup, Denmark; provided by Amstelstad BV, Zwanenburg, Holland) was checked for specificity by immunoelectrophoresis. The optimal dilution of the antiserum in PBS was five- to 10-fold. Unless stated otherwise, we used antiserum diluted sevenfold in PBS. The diluted antiserum was stable for several days at 4 °C.

Neither dilution of the antiserum in 40 g/L solutions of human or bovine albumin nor sterile filtration influenced the results.

Calibrators and controls. A human fibronectin standard series and a fibronectin control solution (product no. 401218) were obtained from Boehringer Mannheim, Penzberg, F.R.G. A second control (Protein Standard Plasma, product no. OTFI 06-07) was purchased from Behringwerke AG. Calibrators and controls were diluted 36-fold in PBS before use.

Manual mode rate nephelometry. Unless stated otherwise, the Beckman ICS nephelometer was used in the manual rate mode (manual mode card M-44), according to the manufacturer's instructions. We placed 42 μL of diluted antiserum in a disposable reaction cell that already contained 600 μL of buffer and 42 μL of diluted plasma or standard solution, and pressed the "start" button 8 to 10 s later. Mean values of duplicate determinations are reported here.

Results and Discussion

Figure 1 depicts a typical calibration curve.

The data we obtained for a series of dilutions of a normal plasma with antiserum diluted 21-fold suggest that the calibration curve corresponds to the utmost left-hand part of the Heidelberger curve (*13;* see also Figure 2), so no falsely low results will be obtained when plasma with very high fibronectin content is assayed.

We studied the course of the antigen–antibody reaction by measuring the total light scatter at several intervals after

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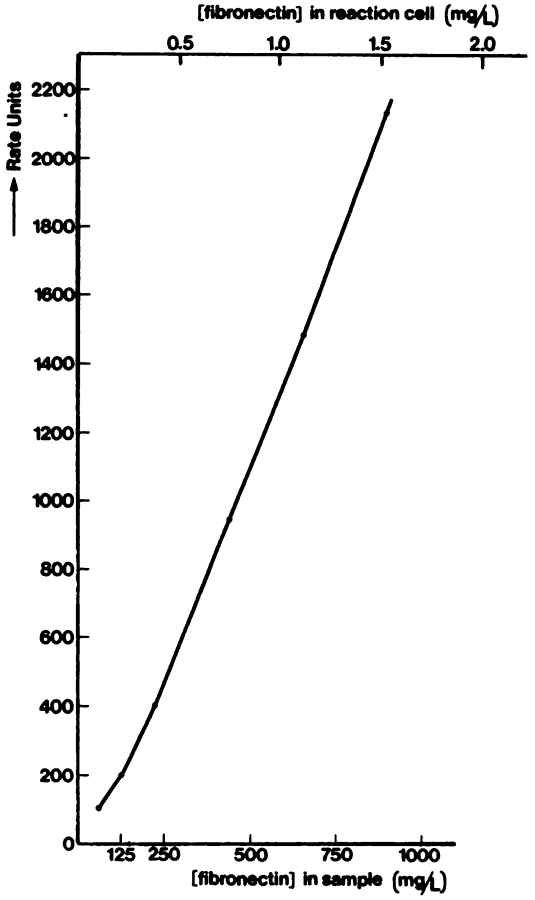

Fig. 1. Standard curve for fibronectin, obtained with anti-human fibronectin antiserum from rabbit, diluted sevenfold with PBS, and fibronectin standards of 125, 250, 500, 750, and 1000 mg/L, diluted 36-fold in PBS

Lower abscissa, the concentrations of the standards; *upper abscissa*, the fibronectin concentration in the reaction cell, calculated for each standard

antiserum was added to a normal plasma, using the Beckman ICS in the "Scatter Mode." The results are shown in Figure 3*A*. When the rates of change of the measured scatter signals with time (ΔS.U./Δt) are calculated—which is, in fact, the procedure carried out by the Beckman ICS in the kinetic "Rate Mode"—the resulting curves are like that in Figure 3*B*. After an initial peak 15 to 30 s after the addition and mixing of antiserum, the peak-rate value of the antigen–antibody reaction is reached between 45 and 75 s after starting the reaction. The "peak-following" procedure of the Beckman ICS starts after 30 s, so the instrument neglects the mixing peak and reads the true peak-rate value of the reaction.

However, with some plasmas (e.g., from sepsis patients or some plasmas with very low fibronectin content) the peak-following procedure may start too early, leading to erroneously high readings or "unstable sample" messages. This problem is easily overcome by waiting for 8 to 10 s after adding the antiserum before pressing the "start" button.

When the fibronectin or antibody concentrations (or both) are too low, the instrument can no longer distinguish the peak-rate signals from background noise, and erroneous readings may result. If quantitative results are to be assuredly reproducible, antiserum dilutions greater than eight- to 10-fold therefore cannot be used. For the same reason, 36-fold diluted plasma samples that give readings below 90 "rate units" should be reassayed in a smaller dilution. Furthermore, mean values of duplicate determinations should be used.

A minimum concentration of 40 mg/L in plasma can be

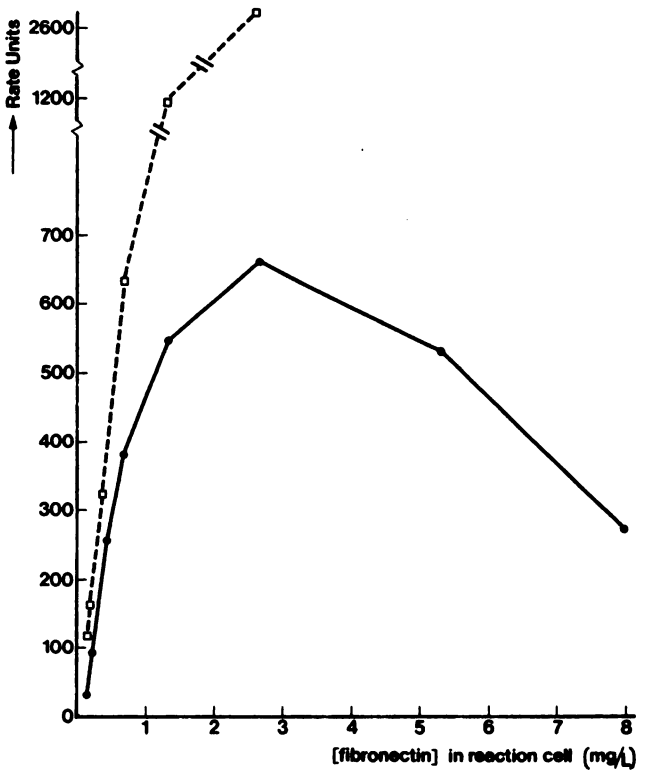

Fig. 2. Relation between the measured rate of change of scatter ("rate units") and the fibronectin concentration in a normal plasma (fibronectin concentration 260 mg/L) diluted two- to 108-fold and assayed with antiserum diluted 21-fold (●) and sevenfold (□)

The *abscissa* represents the actual fibronectin concentration in the reaction cell

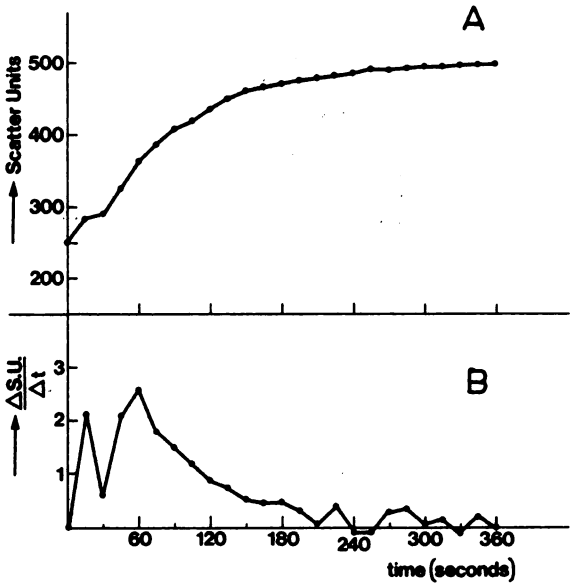

Fig. 3. Course of antigen–antibody complex formation, determined with the Beckman ICS in the "scatter mode," for a normal plasma sample (fibronectin concentration 288 mg/L) reacted with antiserum diluted fivefold

A, measured scatter (Scatter Units, S.U.) vs time elapsed since the addition of antiserum at t = 0; *B*, the rate of change of scatter with time

determined accurately if the sample is assayed in 12-fold dilution. Figure 4 illustrates the effect of using dilutions other than 36-fold, i.e., for a normal plasma diluted 72-, 36-, 18-, and 12-fold in PBS. As shown, the calculated values and those read from the calibration curve were linearly related.

Accuracy. We know of no officially certified reference preparation of fibronectin. Thus, to check the accuracy of our nephelometric method, we used commercial preparations

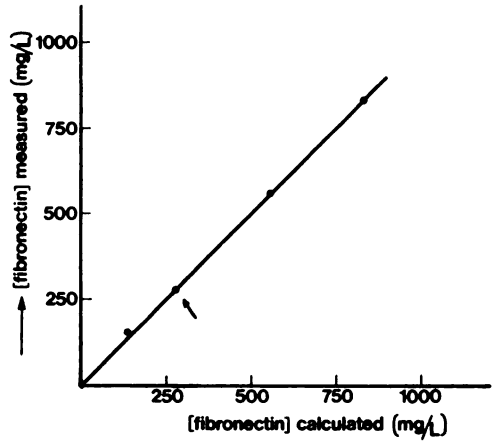

Fig. 4. Effect of diluting a normal plasma 72-, 36-, 18-, and 12-fold in PBS before measuring the fibronectin content
The *arrow* marks the result obtained in 36-fold diluted plasma

from Boehringer Mannheim and Behringwerke, with fibronectin concentrations specified to be 200 (SD 40; measured turbidimetrically) and 295 (measured with radial immunodiffusion) mg/L, respectively. Using the method described here, we found the fibronectin concentrations of these controls to be 198 (SD 8) mg/L (n = 14) and 290 (SD 20) mg/L (n = 11), respectively.

Analytical recovery. When we added different amounts of the highest-concentration standard or a human plasma with high fibronectin content to samples of normal plasma and assayed them, we could account for 104% of the added fibronectin.

Precision. To evaluate the within-run variability of our fibronectin assay, we assayed a normal plasma pool ($\bar{x}$ = 298 mg/L) and a plasma with low fibronectin content ($\bar{x}$ = 111 mg/L) 19 times in one run. The CV was 3.8% for both. To establish the between-day variability, aliquots of another normal plasma pool ($\bar{x}$ = 343 mg/L), which were stored at −70 °C, were assayed on 12 different days during two months. We also assayed aliquots of five plasmas with low concentrations of fibronectin (range 101–122 mg/L) on four consecutive days. The CV averaged 5.1% for the normal plasma pool, 6.7% for the samples with low fibronectin content.

Comparison of rate-nephelometric method with commercial turbidimetric assay. We compared the present method (y) with the commercially available turbidimetric assay (x). Results for 36 samples (range, about 100 to about 700 mg/L) agreed and correlated well: $y = 0.82x + 38$ ($r = 0.912$; $\bar{x}$ = 370, SD 126 mg/L; $\bar{y}$ = 342, SD 113 mg/L). Current reagent costs for these two methods are $0.25 and $1.70 for the nephelometric and turbidimetric methods, respectively.

Reference values. Fibronectin was measured in plasma samples from 19 apparently healthy women, ages 23 to 49 years, and 18 apparently healthy men, ages 23 to 50 years. Both of these reference groups comprised nurses, laboratory personnel, and outpatients with no apparent clinical signs of diseases known to influence fibronectin concentrations. We found mean (and SD) reference values of 286 (84) mg/L for the women (range: 107 to 470 mg/L) and 340 (55) mg/L for the men (range: 245 to 419 mg/L). This sex-related difference is significant (SE diff. = 23.2; $0.02 < p < 0.05$, Student's t-test).

These values and the significant difference between sexes are both in agreement with the results of Eriksen et al. (7).

In conclusion, kinetic immunonephelometry as described here is a rapid, cost-effective method for accurate, precise determinations of fibronectin in human plasma.

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