PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **180**, 149–154 (1985)

The Relationship between Plasma Fibronectin Levels and Autoimmune Disease Activity in MRL/l Mice (42157)

KEVIN CONNOLLY,[1] VERA J. STECHER, JEFFREY K. SAELENS, AND JOHN E. KAPLAN*

*Sterling-Winthrop Research Institute, Rensselaer, New York 12144, and *Albany Medical College, Albany, New York 12208*

Abstract. Plasma fibronectin levels increased significantly over time in MRL/l mice with progressive autoimmune disease. At 100 and 120 days of age both male and female MRL/l mice exhibited significantly higher fibronectin (Fn) levels than the more resistant MRL/n controls. Male mice at early time points had Fn levels no greater than controls due perhaps to the later onset of disease in MRL/l males. In contrast, female MRL/l mice, when compared with MRL/n controls, had higher Fn levels from 40 days of age. The proteinuria in these animals was also above MRL/n controls from the first time point taken (Day 40). In a temporal study with female MRL/l mice, Fn levels peaked at age 120 days and reflected the pattern of the survival curve, indicating that plasma Fn levels have an association with disease activity.
© 1985 Society for Experimental Biology and Medicine.

Fibronectin (Fn) is a 440,000 mol wt protein found in abnormally high concentrations in the synovial fluid (1–3) and pannus tissue (4, 5) of arthritics. Because of its opsonic (6), chemotactic (7, 8), and adhesive (9) properties, it has been suggested that Fn may contribute to the pathophysiology of rheumatic diseases (10, 11). In the adjuvant arthritic rat model, we have shown that arthritic plasma Fn levels are double those of normal controls, and that Fn levels are maintained at high levels for more than 120 days (submitted for publication). In addition to the adjuvant arthritic rat model, another model of autoimmune disease is the MRL/l mouse (12). Both males and females develop severe immune complex glomerulonephritis, succumbing to renal failure within 180 days. However, the disease in male MRL/l mice appears slightly later than in females (12). Like the New Zealand Black mice and the BXSB mice (13), the MRL/l strain is used as a model of systemic lupus erythematosus (SLE) (12).

The MRL/n strain, while genetically similar to the MRL/l mouse, does not possess the lymphoproliferative (lpr) gene and consequently develops autoimmune disease at a much later date. Results in this paper show that MRL/l mice, which have an earlier onset of disease, also have significantly higher Fn levels than their MRL/n counterparts. Thus plasma Fn levels appear to have an association with autoimmune disease activity in MRL/l mice.

Materials and Methods. *Animals.* The MRL/l and MRL/n mice were obtained originally from Jackson Laboratories, Bar Harbor, Maine, and have been maintained by brother–sister matings since 1979 at Sterling-Winthrop Research Institute. The recessive lymphoproliferative gene present in the MRL/l mouse (lpr/lpr) appears to account for the rapid onset of autoimmune disease (12). The MRL/n mouse (+/+) was used as a control since it bears 90% of the MRL/l genome but lacks the lpr gene.

Plasma preparation. A 0.3-ml sample of blood was obtained by retro-orbital plexus bleeding using a capillary pipet. Blood was immediately mixed in microvials with 0.03 ml sodium citrate (0.0185 g/ml), and centrifuged 5 min in a tabletop centrifuge (Fisher Scientific, Fair Lawn, N.J.). Whole plasma was removed, stored at −70°C, and assayed for Fn within 2 weeks.

Fibronectin purification. The concentration of Fn in the rat plasma standard was obtained using affinity chromatography (14, 15). Rat plasma was collected by heart puncture using sodium citrate as an anticoagulant. Plasma

[1] To whom correspondence should be addressed: Immunopharmacology, Sterling-Winthrop Research Institute, Rensselaer, N.Y. 12144.

0037-9727/85 $1.50
Copyright © 1985 by the Society for Experimental Biology and Medicine.
All rights reserved.

was incubated for 60 min at 4°C with barium chloride (15 mg/ml) and 3 m*M* benzamidine hydrochloride to precipitate out the vitamin K complex. Plasma was centrifuged at 12,000*g* for 30 min and the supernatant incubated with ammonium sulfate (9.5 mg/ml) to cause precipitation of barium sulfate. The sample was centrifuged at 12,000*g* for 30 min and the precipitate discarded. Approximately 200 ml of this treated plasma was applied to a gelatin Sepharose 4B (Pharmacia) column (30-ml bed volume) at a flow rate of 25 ml/hr. The column was extensively washed with a phosphate-buffered saline solution (1 *M* NaCl, pH 7.4) plus benzamidine hydrochloride (3 m*M*). The Fn was then eluted with 4 *M* urea–Tris buffer (pH 7.4. Trisma Base 0.075 *M*) and dialyzed against PBS (pH 7.4). The dialysate was passed again over the gelatin-sepharose column to further purify the Fn. When the purified Fn was assayed using SDS slab gel electrophoresis, it produced a 220,000 mol wt "double" band characteristic of the purified Fn dimer. The purified Fn was dialyzed in 0.01 *M* Caps (3-cyclohexylamino propane sulfonic acid, Sigma, St. Louis, Mo.), quantitated spectrophotometrically (280 nm), and stored (−70°C) at no higher a concentration than 3 mg/ml.

Preparation of Fn antibody affinity column. A 10-g quantity of CNBr activated Sepharose 4B (Pharmacia, Piscataway, N.J.) was added to a large Buchner funnel and washed with 2 liters of 0.001 *M* HCl to remove dextran and lactose residues. A 100 mg quantity of purified Fn (3 mg/ml) was dialyzed against a sodium bicarbonate buffer (0.1 *M* $NaHCO_3$, 0.5 *M* NaCl) and added to a tube containing the washed Sepharose.

The mixture of Sepharose and Fn was agitated gently, end over end, overnight at 4°C. The solution was then decanted and washed with 400 ml of sodium bicarbonate buffer. The Sepharose was placed in a large tube and allowed to react 2 hr at room temperature with a 1 *M* solution of ethanolamine (Sigma, St. Louis, Mo.) in sodium bicarbonate buffer. After 2 hr of gentle agitation, the Fn-conjugated Sepharose was allowed to settle and the ethanolamine supernatant removed. The ethanolamine was replaced by a 0.1 *M* solution of sodium acetate (pH 4.0) in 1.0 *M* NaCl. After a 1-hr agitation the sodium acetate solution was removed and replaced by 0.1 *M* borate buffer (pH 8.0) in 1.0 *M* NaCl. Following a 1-hr room temperature agitation, the borate buffer was removed and the acetate, borate wash cycle repeated two additional times. The Fn-conjugated Sepharose was finally resuspended in degassed PBS (pH 7.4) and poured into a column where it was allowed to equilibrate with PBS.

Preparation of antibody. The available stock of anti-rat Fn antibody showed high cross-reactivity with mouse Fn and was therefore used in all electroimmunoassays. To derive Fn antibody, affinity column (14) purified rat Fn (2 mg/ml) was mixed 1:1 with complete or incomplete Freund's adjuvant. On Days 1 and 8, 0.5 ml of the complete adjuvant (plus Fn) was injected subcutaneously into each of two sites along the flank of a goat. The procedure was repeated on Day 15 using Fn plus incomplete adjuvant. On Day 22, the goat received one subcutaneous and one im injection of 0.5 ml Fn plus incomplete adjuvant. Ten days later, 250–500 ml of whole blood was taken from the jugular vein. Blood was allowed to clot for 1 hr at room temperature, then rimmed and incubated overnight at 4°C and centrifuged to separate serum from the clot. Goat anti-rat Fn antibody was obtained from this serum using affinity chromatography (15).

Fn antibody was precipitated from the goat antiserum with a saturated ammonium sulfate solution. For every 100 ml of serum, 57 ml of ammonium sulfate solution was added. The serum was stirred overnight at 4°C to precipitate antibody. The solution was centrifuged at 11,000*g* for 30 min, resuspended in one-fifth the original serum volume, using 0.01 *M* PBS (pH 7.2). The antibody suspension was then dialyzed three times against 4 liters of 0.01 *M* PBS and applied over the Fn column. The column was washed with PBS (pH 7.2) and the Fn antibody eluted with 0.2 *M* sodium acetate. The eluate was immediately dialyzed against distilled water and lyophilized. The antibody specificity against Fn was monitored by Ouchterlony immunodiffusion technique and showed no cross-reactivity with rat fibrin, serum albumin, or collagen.

Quantitation of fibronectin. Fn levels in plasma test samples were quantitated using the technique of rocket electrophoresis (16, 17). Agarose (630 mg) was dissolved in 63 ml of boiling Tris–Tricine buffer (9.8 g/liter Trizma base; 4.3 g/liter Tricine; 0.106 g/liter calcium lactate; 0.2 g/liter sodium azide) and cooled to 63°C. Between 0.1 and 0.2 ml of Fn antibody (8 mg/ml) was mixed in the liquid gel, which was then poured on a Gel Bond film (FMC Corp., Rockland, Maine). Wells (2 mm) were punched in the solidified gel: 10 μl samples of plasma diluted 1:10 in Tris–Tricine buffer (pH 8.8) were then applied. At each corner of the plate were run a series of internal mouse standards originally calibrated against known Fn standards kindly supplied by Dr. Thomas Saba, Albany Medical College. The gel was run 21 hr on a cooling plate (LKB, Gaithersburg, Maryland) then dried and stained with Coomassie brilliant blue R-250 (Bio-Rad, Richmond, Calif.). The heights of the sample "rocket" peaks were compared to the height of the known internal standards to determine Fn concentration of the sample.

Determination of urine total protein content. The protein binding property of Coomassie brillant blue G-250 (Bio-Rad) was used to spectrophotometrically quantitate total protein in the urines of MRL/l and MRL/n mice (18, 19). Coomassie G-250 was freshly prepared on the day of the assay by adding together 100 mg of Coomassie, 50 ml 95% ethanol, 100 ml 85% phosphoric acid, and 150 ml of H_2O. The test is accurate in the range of 10–100 μg of protein/5 ml of dye. Urine samples were diluted appropriately. Samples plus dye were incubated 2 min at room temperature and read within 1 hr on a Zeiss PM6 spectrophotometer. Protein content of the samples was derived by comparison to the dilution curve from a series of rat serum albumin standards.

Statistics. Students *t* test was used to derive significance between MRL/l and MRL/n mice ($P < 0.05$). Numbers represent means ± SEM.

Results. *Comparison between plasma fibronectin levels in female MRL/l mice and female MRL/n controls.* Female MRL/l mice suffer from chronic systemic autoimmune disease which becomes increasingly severe as the animals age (12). Figure 1 illustrates that plasma Fn levels also rose as the animals aged, from a low of 416 μg/ml for 40-day-old animals, to a high of 819 μg/ml for 120-day-old mice. At all time points, Fn levels from MRL/l females were higher than those from MRL/n controls, though at Days 140 and 160, Fn levels in female MRL/l mice had fallen to 717 μg/ml. Conversely, by Day 160, Fn levels in female MRL/n mice had increased from a low of 378 to 625 μg/ml.

Comparison between plasma fibronectin levels in male MRL/l mice and male MRL/n controls. Male MRL/l mice, like their female counterparts, also develop autoimmune disease, albeit, with a somewhat delayed onset (12). Figure 2 demonstrates that male MRL/l mice, like females (Fig. 1), exhibited elevated levels of Fn over time. From Day 100, Fn levels in male MRL/l mice were significantly higher than those in the MRL/n controls. At

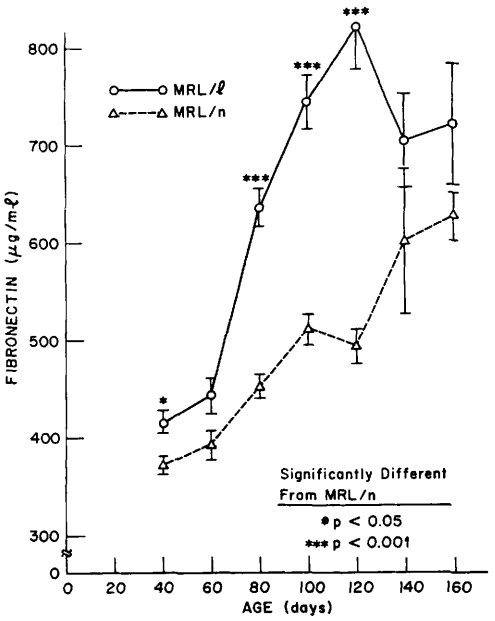

FIG. 1. Comparison between plasma fibronectin levels in female MRL/l mice and MRL/n controls. Each timepoint was taken from a separate group of 20 mice. Fn levels represent the means ± SEM of 20 individual animals bled from the retro-orbital plexus. Fibronectin level was quantitated in μg/ml using electroimmunoassay (18, 19) as described under Materials and Methods. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Statistical analysis was performed on a comparison of MRL/l mice and MRL/n controls.

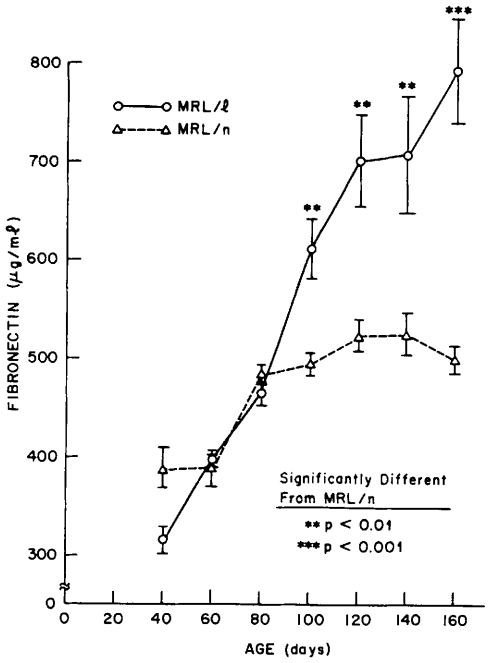

FIG. 2. Comparison between plasma fibronectin levels in male MRL/l mice and MRL/n controls. For protocol see Fig. 1 and Materials and Methods.

the earler 60- and 80-day time points, male MRL/l and MRL/n Fn levels were not significantly different.

The relationship between fibronectin levels and the mortality rate in aging female MRL/l mice. Figure 3 shows the mortality curve for 45 female MRL/l mice. Fn levels in this group of animals was followed by taking sequential retro-orbital plexus bleedings at the time points indicated. Fn levels rose as the animals aged and began to die. Fn, at its highest level (Day 120), corresponded to the survival curve at its steepest point (i.e., the time when the highest number of animals was dying).

Proteinuria in aging female MRL/l and MRL/n mice. Early kidney dysfunction is a hallmark of autoimmune disease in MRL/l mice. In Fig. 4, this parameter was assessed by examining levels of protein in the urine of female MRL/l and MRL/n mice over time. At all time points, protein levels, like Fn levels (Fig. 1), were elevated in female MRL/l mice when compared to MRL/n controls. As was seen in the case of Fn levels (Fig. 3), protein levels dropped off at the later time points.

Discussion. Evidence is accumulating that high Fn concentrations in plasma, joint tissue, or fluid are associated with various rheumatic diseases (10). In arthritics, a high fibronectin concentration is found in synovial fluid (1–3), rheumatoid pannus (4, 5), and synovial joint tissue (4, 5). We now report that the MRL/l mouse model is another case where a high Fn level was associated with severe rheumatic disease. Results showed that Fn levels in both male and female MRL/l mice increased significantly over time (Figs. 1 and 2), Fn levels in both male and female MRL/l mice were significantly higher than those for MRL/n controls (Figs. 1 and 2), the curve of Fn level over time matched the MRL/l survival curve (Fig. 3) and fibronectin levels as well as proteinuria were abnormally high in female MRL/l mice from Day 40, the earliest time point taken (Fig. 4). The fact that proteinuria does not increase over time but rather is maintained at a high level from Day 40 may indicate that proteinuria is

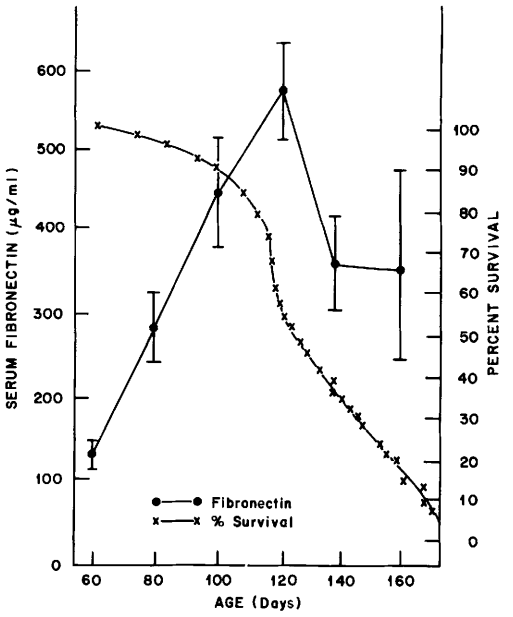

FIG. 3. Fibronectin level vs mortality rate in aging female MRL/l mice. A group of 50 female MRL/l mice were followed from birth to death and retro-orbital bleedings taken every 20 days (beginning 40 days of age) for use in relating Fn level to the rate of death.

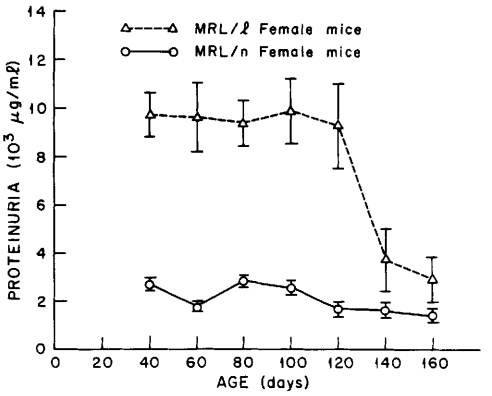

FIG. 4. Proteinuria in aging MRL/l mice. Urine samples were taken over the course of an 8-hr day. Time points represent the mean ± SEM of each group of 20 individual female animals bled at the time points indicated in Fig. 1. Proteinuria was measured spectrophotometrically using Coomassie blue G-250 protein stain (20, 21).

associated with disease onset but does not necessarily reflect disease severity. Plasma fibronectin, on the other hand, was elevated from Day 40 but, in addition, increased over time as the animals sickened and died. The decrease in female MRL/l mortality rate, Fn level, and proteinuria at 140–160 days may represent animals with a greater resistance to the disease.

Conversely, the increase in female MRL/n Fn level at 140–160 days may reflect the late onset of autoimmune disease in MRL/n mice. In contrast to female MRL/l mice, male MRL/l mice at the early time points had relatively low Fn levels that did not significantly differ from the male MRL/n controls (Fig. 2). The difference in the pattern of Fn levels in male and female MRL/l mice might reflect the somewhat later onset of the disease in male mice (12). Late onset of the disease in males may also explain why Fn levels in MRL/l male mice had not peaked by Day 160 like their female MRL/l counterparts (Fig. 1). The results indicate that plasma Fn levels increase as autoimmune mice age and become increasingly debilitated by disease. Thus Fn levels appear to be associated with disease activity in mice with a lupus-like syndrome.

This is compatible with reports that high Fn levels have also been observed in the plasma of lupus patients (20). It would be interesting to follow Fn levels over time and try and correlate those results with other parameters of autoimmune disease like circulating levels of IgG, IgM, anti-DNA, and anti-poly(A) antibodies (21) as well as histologic examination of joints and kidneys.

The authors acknowledge the indefatigable efforts of Don Pruden in providing the technical expertise for this project.

1. Carsons S, Mosesson MW, Diamond HS. Detection and quantitation of fibronectin in synovial fluid from patients with rheumatic disease. Arthritis Rheum **24**:1261–1267, 1981.
2. Lu-Steffes M, Iammartino AJ, Schmid FR, Castor CW, Davis L, Entwistle R, Anderson B. Fibronectin in rheumatoid and non-rheumatoid arthritis synovial fluids and in synovial fluid cryoproteins. Ann Clin Lab Sci **12**:178–185, 1982.
3. Scott DL, Farr M, Crockson AP, Walton KW. Synovial fluid and plasma fibronectin levels in rheumatoid arthritis. Clin Sci **62**:71–76, 1981.
4. Vartio J, Vaheri A, Von Essen R, Isomaki H, Stenman S. Fibronectin in synovial fluid and tissue in rheumatoid arthritis. Eur J Clin Invest **11**:207–212, 1981.
5. Scott DL, Wainwright AC, Walton KW, Williamson N. Significance of fibronectin in rheumatoid arthritis and osteoarthrosis. Ann Rheum Dis **40**:142–153, 1981.
6. Hynes RO, Yamada KM. Fibronectins: Multifunctional modular glycoproteins. J Cell Biol **95**:369–377, 1982.
7. Norris DA, Clark RAF, Swigart IM, Huff JC, Weston WL, Howell SE. Fibronectin fragments are chemotactic for human peripheral blood monocytes. J Immunol **129**:1612–1618, 1982.
8. Mosesson MW, Amrani DL. The structure and biologic activities of plasma fibronectin. Blood **56**: 145–158, 1980.
9. Akiyama SK, Yamada KM, Hayashi M. The structure of fibronectin and its role in cellular adhesion. J Supramol Struct Cell Biochem **16**:345–358, 1981.
10. Scott DL, Delamere JP, Walton KW. The distribution of fibronectin in the pannus in rheumatoid arthritis. Brit J Exp Pathol **62**:362–368, 1981.
11. Weissmann G. Activation of neutrophils and the lesions of rheumatoid arthritis. J Lab Clin Med **100**: 322–333, 1982.
12. Andrews BS, Eisenburg RA, Theofilopoulos AN, Izui S, Wilson CB, McConahey PJ, Murphy ED, Roths JB, Dixon FJ. Spontaneous murine lupus-like syndromes. Clinical and immunopathological manifestions in several strains. J Exp Med **148**:1198–1215, 1978.
13. Howie JB, Simpson RO. Autoimmune disease in

NZB mice and their hybrids. In: Dubois EL (ed.), Lupus Erythematosus. Univ Southern California Press, p124, 1976.
14. Weiss RE, Reddi AH. Isolation and characterization of rat plasma fibronectin. J Biochem **197**:529–534, 1981.
15. Todd-Kulikowski HD, Parsons RG. A stable sensitive assay for human fibronectin. J Immunol Methods **44**:333–341, 1981.
16. Laurell CB. Electroimmunoassay. Scand J Clin Lab Invest **29**(Suppl 124):21–37, 1972.
17. Blumenstock F, Weber P, Saba TM, Laffin R. Electroimmunoassay of alpha-2 opsonic protein during reticuloenthelial blockade. Amer J Physiol **232**: R80–R87, 1977.
18. Bradford MM. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann Biochem **72**:248–254, 1976.
19. Nesbitt J, Sauer D, Culpepper R. Use of the Bradford dye-binding method for cerebrospinal fluid and urine total protein determinations. J Amer Med Tech **40**: 278–279, 1978.
20. Parente D, Carsons SE, Lavietes BB, Diamond HS, Steinman CR. Fibronectin, a DNA binding protein, is elevated in systemic lupus erythematosus. Arthritis Rheum **25**:S41, 1982.
21. Ackerman NR, Trimble B, Kowalski WJ, Sadrkian J, Miram L, Terrell TG. The effects of D-penicillamine in the MRL/l mouse. In: Otterness I, Capetola R, Wong S, (eds.) Advances in Inflammation Research. New York, Raven Press, Vol 7:pp277–286, 1984.

Received February 12, 1985. P.S.E.B.M. 1985, Vol. 180.
Accepted May 13, 1985.