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The Effect of Glucocorticoids on Plasma Fibronectin Levels in Normal and Arthritic Rats (42343)

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Abstract. In vivo studies with normal and adjuvant-induced arthritic rats were undertaken in order to measure the effects of glucocorticoids on paw inflammation and plasma fibronectin (Fn) levels. Dexamethasone, methylprednisolone, and corticosterone all enhanced plasma Fn levels in normal animals. All drugs also significantly decreased inflammation in arthritic rats as measured by paw swelling. Of the three glucocorticoids, only corticosterone did not significantly enhance Fn levels in arthritic rats, possibly due to its lesser potency and narrow therapeutic window.

Adjuvant-induced arthritis in rats has many of the characteristics of rheumatoid arthritis (1). It is a chronic, progressive, deforming arthritis of the peripheral joints, with a primary mononuclear cell response consisting of bone and joint space invasion by pannus (2). In the rat adjuvant arthritis model, we have shown that chronic, systemic inflammation is preceded by an increase in the level of plasma fibronectin (Fn) (3). Within 24 hr of adjuvant injection, rat plasma Fn levels rise from normal levels of approximately 350 to almost 700 μg/ml. The twofold increase in plasma Fn in adjuvant-induced arthritic rats is sustained throughout the course of the disease, indicating that plasma Fn levels have an association with disease activity. When arthritic animals were treated with nonsteroidal anti-inflammatory drugs (NSAIDs), inflammation of the paws was significantly decreased without an accompanying decrease in plasma Fn (4). This may be related to the clinical profile of NSAIDs as drugs providing symptomatic relief without affecting the underlying progression of the disease (5).

The glucocorticoids are another class of drugs used as anti-inflammatory agents in the treatment of arthritis (6). *In vitro* studies have demonstrated that dexamethasone enhances Fn production in cultures of chick hepatocytes (7), rat hepatocytes (8), rat hepatomas (9), and SV40-transformed human fibroblasts (10). In the following *in vivo* study, normal and adjuvant-induced arthritic rats were given daily doses of dexamethasone, methylprednisolone, or corticosterone in order to determine how paw inflammation and Fn levels are affected by these drugs. In all normal groups, glucocorticoids significantly enhanced plasma Fn levels, paralleling the way dexamethasone increased Fn production *in vitro* (7–10). In all arthritic groups, glucocorticoids significantly decreased paw inflammation in a manner similar to the anti-inflammatory effect of NSAIDs (5) and steroids (6) in the clinic. With the exception of corticosterone, glucocorticoids significantly enhanced plasma Fn levels in arthritic rats. The efficacy of steroid treatment is discussed in view of the possible involvement of Fn in the pathophysiology of rheumatoid arthritis (11).

Materials and methods. *Animals.* Male, outbred, Sprague–Dawley rats (approximately 300 g) were obtained from Charles River Laboratories.

Induction and measurement of adjuvant arthritis. Freund's complete adjuvant was prepared by adding 100 mg of *Mycobacterium tuberculosis* (Difco Laboratories, Detroit, Mich.) to 15.6 ml of heavy paraffin oil (Fisher Scientific, Fair Lawn, N.J.). The *Mycobacterium* was then ground in a homogenizer (Eberbach Corp., Ann Arbor, Mich.) followed by addition of 1 ml of saline. The mixture was thoroughly emulsified by pulsing for 30 sec with a Polytron (Brinkman Instruments, Westbury, N.Y.). Each rat was injected in the right hind footpad with 300 μg of *Mycobacterium* in a 0.05-ml volume. The systemic nature of the disease was assessed by measuring uninjected (left) hind paw swelling 16 to 21 days after adjuvant injection. The degree of

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swelling was measured by mercury displacement (Buxco Electronics, N.J.) by dipping paws in a reservoir of mercury and recording on a polygraph the amount (ml) of mercury displaced.

Drug dosing. Drug dosing was begun on Day 3, two days after adjuvant injection. Daily dosing continued for 15–17 days at which time the experiment was terminated. Dexamethasone and methylprednisolone were administered orally in 1% gum tragacanth. Corticosterone was delivered subcutaneously in an ethanol:cottonseed oil (10:90) vehicle. All drugs were administered as base.

Plasma preparation. Just prior to termination of each experiment, rat paws were measured and a 0.1-ml sample of blood was obtained by cardiac puncture using a 1-cc syringe and a 27-gauge needle. Blood was immediately mixed in microvials with 0.01 ml sodium citrate (18.5 mg/ml), and centrifuged 5 min in a tabletop centrifuge (Fisher Scientific). Whole plasma was removed, stored at −70°C, and assayed for Fn within 2 weeks.

Fibronectin purification. An affinity chromatography procedure (12) was followed to obtain purified Fn used in the preparation of an Fn Sepharose column, in addition to its use as an antigen in the preparation of Fn antibody. Rat plasma was collected by heart puncture using sodium citrate as an anticoagulant. After treatment with barium chloride and ammonium sulfate, approximately 200 ml of rat plasma was applied to a gelatin Sepharose 4B (Pharmacia, Piscataway, N.J.) column. The Fn was eluted with 4 *M* urea–Tris buffer (pH 7.4, Trizma base 0.075 *M*) and dialyzed against PBS (pH 7.4). The purified Fn was dialyzed at pH 10.5 in 0.01 *M* CAPS (3-cyclohexylamino-1-propanesulfonic acid; Sigma, St. Louis, Mo.), quantitated spectrophotometrically (280 nm), and stored (−70°C) at a concentration not higher than 3 mg/ml.

Preparation of Fn Sepharose column. A 10-g quantity of CNBr-activated Sepharose 4B (Pharmacia) was added to a 100-mg quantity of purified Fn (3 mg/ml). Standard procedures for protein conjugation were followed as previously described (12). The Fn-conjugated Sepharose was resuspended in degassed PBS (pH 7.4) and poured into a column where it was allowed to equilibrate with PBS.

Preparation of antibody. To derive Fn antibody, affinity column (13)-purified rat Fn (2 mg/ml) was mixed 1:1 with complete or incomplete Freund's adjuvant and injected into a goat. Goat anti-rat Fn antibody was obtained from this serum using affinity chromatography (12). 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 immunoelectrophoresis (12, 13). Agarose (630 mg) was dissolved in 63 ml of boiling Tris–Tricine buffer and cooled to 63°C. The Fn antibody was mixed in the liquid gel, which was then poured on a Gel Bond film (FMC Corp., Rockland, Maine). Wells were punched in the solidified gel and 10-μl samples of plasma diluted 1:10 in Tris–Tricine buffer (pH 8.8) were then applied. A series of internal standards was run at each corner of the plate. This consisted of a sample from a rat plasma pool (330 μg/ml) diluted with Tris–Tricine buffer to 40, 20, 10, and 5% concentrations. The rat plasma pool was originally calibrated against known Fn standards kindly supplied by Dr. John Kaplan and Dr. Thomas Saba, Albany Medical College. The gel was run 21 hr on a cooling plate (LKB, Gaithersburg, Md.) and 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 internal standards to determine Fn concentrations of the samples.

Statistics. Student's *t* test was used to derive significance between groups ($n = 10$) at $P < 0.05$, $P < 0.01$, $P < 0.001$.

Results. *Effect of dexamethasone on fibronectin and paw volume in normal and arthritic rats.* As shown in Fig. 1, a 15-day dosing regimen led to significantly increased levels of plasma Fn in normal rats. The degree of Fn increase was dose dependent, although dexamethasone, even at the lowest dose tested (0.02 mg/kg), significantly increased Fn levels ($P < 0.001$) in normal rats. Adrenalectomy of normal rats significantly lowered plasma Fn levels from 487 μg/ml in sham adrenalectomized rats to 363 μg/ml in adrenalectomized rats (Table 1). Dexamethasone treatment of adrenalectomized rats raised Fn levels significantly above sham adrenalectomized con-

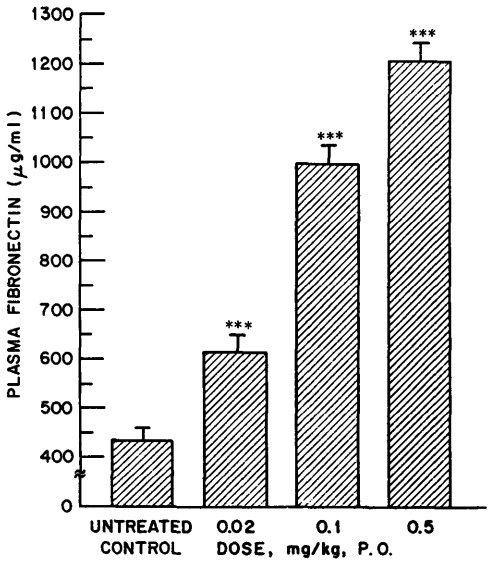

FIG. 1. Dexamethasone enhancement of plasma fibronectin in normal rats. Dexamethasone at a dose of 0.02, 0.1, and 0.5 mg/kg was administered orally to groups of 10 outbred Sprague–Dawley rats once daily for 15 days. Drug was mixed with a 1% gum tragacanth vehicle. On the 15th day of dosing, animals were bled from the heart and the plasma assessed for Fn using rocket immunoelectrophoresis. ***$P < 0.001$ compared to untreated normal animals.

trols. When given to arthritic rats, all doses of dexamethasone had significant anti-inflammatory activity as measured by inhibition of paw swelling (Fig. 2). It also significantly increased the already high plasma Fn levels in arthritic rats. Dexamethasone-treated rats showed a dose-dependent loss in adrenal and total body weight (data not shown).

Effect of methylprednisolone on fibronectin and paw volume in normal and arthritic rats. Oral administration of methylprednisolone for 17 days resulted in an identical pattern of anti-inflammatory activity and enhanced plasma Fn levels as had been seen with dexamethasone. Normal animals treated with methylprednisolone exhibited a dose-dependent increase in plasma Fn (Fig. 3). When dosing arthritic animals with methylprednisolone, as inflammation (i.e., paw swelling) was inhibited, plasma Fn levels rose significantly (Fig. 4). As had been observed for dexamethasone-treated animals, methylprednisolone caused a dose-dependent loss in adrenal and total body weight (data not shown).

Effect of corticosterone on adrenal weight, total body weight, and paw volume. It was necessary to strike a balance between corticosterone's anti-inflammatory properties and the

TABLE I. PLASMA FIBRONECTIN LEVELS IN NORMAL ADRENALECTOMIZED AND DEXAMETHASONE-TREATED ADRENALECTOMIZED RATS[a]

Surgical sham control	Adrenalectomized	Adrenalectomized + dexamethasone (0.1 mg/kg)
487 ± 27	363 ± 11[b]	778 ± 33[b]

[a] Male rats (265–285 g) were divided into the following three groups of 15 animals each: (1) sham adrenalectomized; (2) adrenalectomized; (3) adrenalectomized plus dexamethasone (0.1 mg/kg). Animals were medicated (po) for 15 days with dexamethasone or vehicle (1% gum tragacanth) starting the day following surgery. At the time of autopsy, blood was obtained via cardiac puncture. Plasma Fn (μg/ml) levels were determined using electroimmunoassay and the results expressed as means ± SEM.

[b] $P \leq 0.01$ compared to surgical sham control.

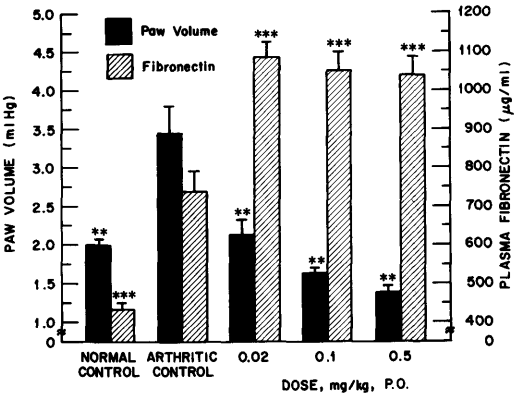

FIG. 2. Effect of dexamethasone on fibronectin and paw volume in arthritic rats. On Day 1, rats were injected in the right hind paw with 0.05 ml of complete Freund's adjuvant composed of 100 mg of *Mycobacterium tuberculosis* ground and emulsified in 15.6 ml of heavy paraffin oil and 1 ml of saline. From Day 3 through Day 18, animals were dosed orally once daily with dexamethasone suspended in 1% gum tragacanth. The volume of drug plus vehicle was calculated so that animals received 1 ml of drug plus vehicle/100 g body wt. Rats in each group of 10 were bled from the heart on Day 18 and plasma Fn levels obtained using rocket immunoelectrophoresis. Just prior to bleeding, animals were assayed for systemic inflammation by measuring the volume of the uninjected left paw using mercury displacement. **$P < 0.01$, ***$P < 0.001$ compared to untreated arthritic animals.

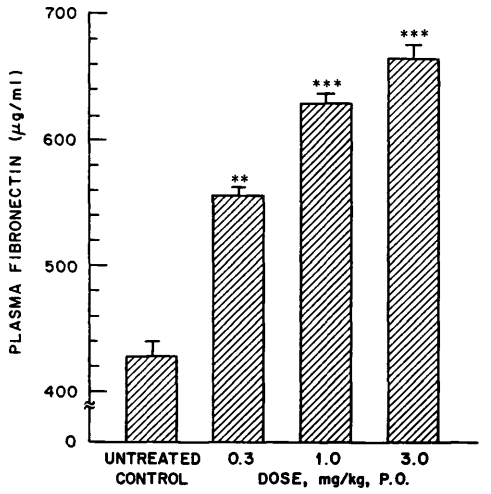

FIG. 3. Methylprednisolone enhancement of plasma fibronectin in normal rats. Methylprednisolone at doses of 0.3, 1.0, and 3.0 mg/kg was given to normal rats (n = 10) as described in Fig. 1 except that oral dosing was maintained for 17 days. Plasma fibronectin determination was made as described in Fig. 1 and under Material and Methods. $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated normal animals.

characteristic "catabolic" effect (14, 15) which results in severe weight loss. Corticosterone administered subcutaneously for 15 days induced a significant body weight loss in normal and arthritic rats (Table 2). All three doses (8, 20, and 50 mg/kg) exhibited biological activity as measured by significant decreases in adrenal weight. However, only the middle dose possessed antiinflammatory activity (significant inhibition of paw swelling) without concomitant severe weight loss. Therefore, a dose of 20 mg/kg was chosen in order to assess the effect of corticosterone on plasma Fn levels over time.

Effect of corticosterone on fibronectin in normal and arthritic rats. Subcutaneous injections of corticosterone were given daily for 16 days to normal rats. Plasma Fn levels were significantly increased by Day 3, the first time point taken (Fig. 5). Ocular orbital plexus bleedings done on Days 6, 10, and 16 showed that increased plasma Fn levels were sustained. Although corticosterone at 20 mg/kg was able to enhance Fn levels in normal rats, this dose, which exhibited anti-inflammatory activity (Table 2), failed to enhance plasma Fn levels in arthritic rats even after 16 days of administration (Fig. 6). However, Fn levels in arthritic rats were already high compared to normal rat Fn levels (825 vs 410 μg/ml). It appears that this dose of corticosterone is sufficient to significantly raise normal Fn levels but is not able to boost the already high levels of Fn in arthritic rats.

Discussion. Due to their potent anti-inflammatory effects, glucocorticoids have long been used in the treatment of rheumatoid arthritis (16). Although treatment with glucocorticoids ameliorates pain, it does not attack the underlying progression of rheumatoid disease as measured by radiography (17). NSAIDs also exhibit this pattern of activity, reducing inflammation and pain, without halting or reversing the progression of joint disease (5).

In the rat model of adjuvant-induced arthritis, we found that plasma Fn levels are significantly higher than normal and are maintained at this high level for the duration of the disease (3). Postulating that Fn could be used as a disease marker, we have shown that NSAIDs which, in humans, provide symptomatic relief without affecting the underlying progression of the disease, do not alter high plasma Fn levels in arthritic rats (4).

It was, therefore, of interest to determine how another class of antirheumatic com-

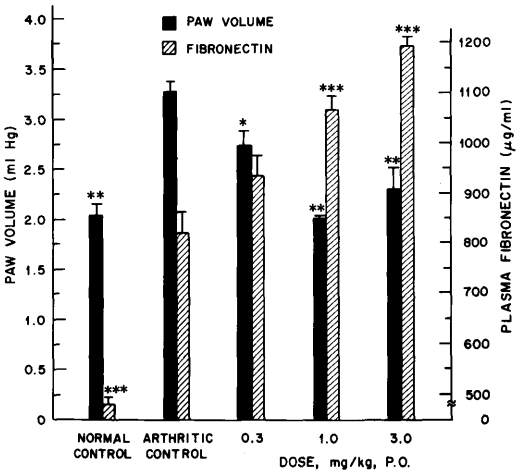

FIG. 4. Effect of methylprednisolone on fibronectin and paw volume in arthritic rats. Groups of 10 rats injected on Day 1 with Freund's complete adjuvant received the indicated oral dose of methylprednisolone as described in Fig. 3. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to untreated arthritic animals.

TABLE II. EFFECT OF CORTICOSTERONE ON ADRENAL WEIGHT, TOTAL BODY WEIGHT, AND PAW VOLUME OF NORMAL AND ARTHRITIC RATS

Group	Corticosterone dose (mg/kg)[a]	Adrenal weight (mg)	Body weight change (g)	Left paw volume (ml Hg)
Normal	Vehicle control	49.5 ± 2.6	+66	1.96 ± 0.04
	8	22.4 ± 0.8[b]	+52	1.84 ± 0.05
	20	17.5 ± 0.6[b]	−6[b]	1.62 ± 0.04[c]
	50	12.2 ± 0.6[b]	−61[b]	1.46 ± 0.04[d]
Arthritic	Vehicle control	62.4 ± 6.2	+2	2.44 ± 0.2
	8	27.9 ± 2.5[b]	+3	2.19 ± 0.2
	20	15.7 ± 1.4[b]	−13	1.68 ± 0.05[d]
	50	16.6 ± 2.0[b]	−64[b]	1.39 ± 0.02[d]

[a] Animals received daily subcutaneous injections of corticosterone suspended in 10% ethanol and 90% cottonseed oil. After 15 days animals were weighed, bled from the heart for determination of plasma Fn, and autopsied for adrenal weight loss.
[b] $P < 0.001$.
[c] $P < 0.05$.
[d] $P < 0.01$.

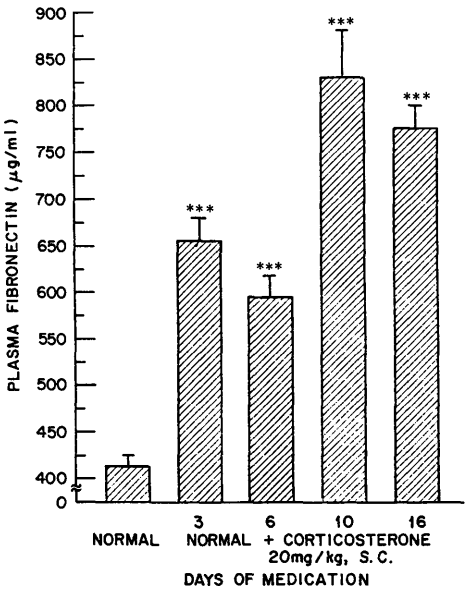

FIG. 5. Corticosterone enhancement of fibronectin levels in normal rats. Corticosterone (20 mg/kg) was delivered subcutaneously in a 10% ethanol, 90% cottonseed oil vehicle. Drug was administered once daily for 16 days and each of the 10 animals was bled by retroorbital plexus bleeding on Days 3, 6, 10, and 16. Two days prior to dosing, a blood sample was taken from each animal and used as the normal control to establish basal levels of plasma Fn. It had been shown previously that the trauma of repeated bleedings was not of itself sufficient to increase plasma Fn levels (results not shown). ***$P < 0.001$ compared to untreated normal animals.

pounds, the glucocorticoids, affected rat plasma Fn levels. All glucocorticoids tested significantly increased plasma Fn levels in normal animals. All three drugs exhibited characteristic anti-inflammatory activity, as indicated by decreased paw swelling in the adjuvant arthritic rat. In addition, the two most potent glucocorticoids, dexamethasone and methylprednisolone, also significantly enhanced plasma Fn levels in arthritic rats. The failure of corticosterone to enhance Fn levels in arthritic rats may be due to its comparative lack of potency and inability to boost already heightened levels of plasma Fn in arthritic rats.

Endogenous levels of glucocorticoids appear to increase the level of plasma Fn, since adrenalectomy of normal rats resulted in a significant decrease in the concentration of plasma Fn which was increased by administration of dexamethasone. It is possible that endogenous glucocorticoid levels are elevated in rats with adjuvant-induced arthritis which could, in turn, account for the elevated levels of Fn in these animals. If so, the levels of exogenous corticosterone injected may not have been sufficient to raise the already elevated Fn levels in these animals. However, the levels of dexamethasone and methylprednisolone administered may have been high enough to overcome the potentially elevated levels of endogenous glucocorticoids.

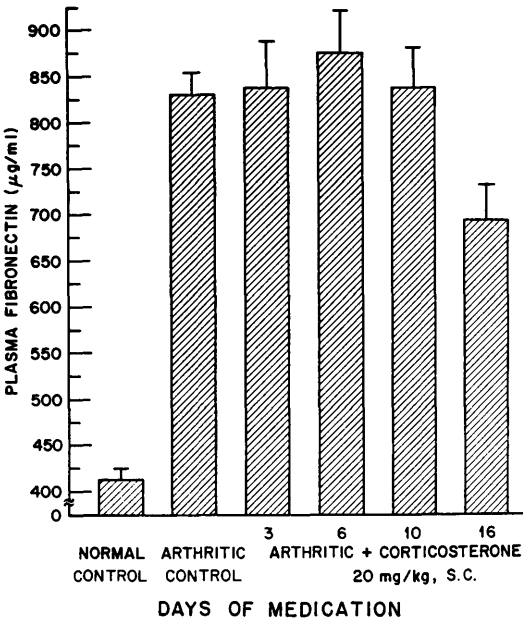

FIG. 6. Effect of corticosterone on fibronectin in arthritic rats. Groups of 10 rats were injected with 300 μg of *Mycobacterium tuberculosis* in an oil and water emulsion. Two days after injection, a blood sample was taken from each animal and used as the adjuvant control to establish basal levels of plasma Fn. Drug treatment was then begun and continued for the next 16 days. Corticosterone (20 mg/kg) was administered once daily and each of the 10 animals was bled by retroorbital plexus bleeding on Days 3, 6, 10, and 16.

The significant increase in rat plasma Fn levels following steroid treatment may be due to an effect upon liver metabolism. Normal plasma Fn levels, averaging approximately 300 μg/ml (18), are maintained by liver synthesis (19). Plasma fibronectin levels in the arthritic rat are twice normal levels (3), possibly due in part to Fn release from the diseased joints. This is supported by clinical reports of high Fn levels in the synovial fluid (20, 21) and the joint tissue (22, 23) of arthritics. There has been much speculation that Fn may be directly involved in the pathophysiology of arthritic disease (11) because it possesses a broad repertoire of biological activities including enhancement of leukocyte phagocytosis, chemotaxis, adhesion, and binding to fibrinogen and collagen. In view of the possible involvement of Fn in the actual progression of disease, its presence in high concentrations may exacerbate rather than ameliorate disease. If glucocorticoid treatment enhances Fn levels, such treatment may not be beneficial to the patient in the long term even though the patient receives immediate relief from pain.

Most researchers do not challenge the efficacy of glucocorticoids in the treatment of arthritis. However, there are reported instances where joint deterioration following steroid treatment (24–26) cannot be explained by osteoporosis or joint overuse as a result of pain reduction (27). In these instances, one might speculate that joint deterioration is partly due to high levels of Fn which bind leukocytes to cartilage. It has been hypothesized that the Fn-mediated bond between leucocyte and cartilage allows formation of a sheltered microenvironment conducive to tissue degradation by proteolytic enzymes which normally would be rendered inactive by endogenous inhibitors (e.g., α_2-macroglobulin) (11). Alternatively, a certain basal level of Fn may be desirable since Fn may be involved in matrix repair or modulation of cells active in the repair process. While it is intriguing to speculate upon the actual involvement of Fn in the pathophysiology of rheumatoid arthritis, at this time we can only state that Fn appears to be a reliable marker associated with some autoimmune diseases (3, 4, 12).

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