Human Fibronectin Metabolism

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Abstract

The metabolic behavior of fibronectin (Fn), a highly adhesive glycoprotein (440,000 mol wt), was studied in eight healthy control subjects and in 11 patients, six of whom were critically ill. Fn was purified from fresh human plasma, radiolabeled, and shown to retain function both in vitro and in vivo. Results showed that, in normal controls, Fn is a rapidly catabolized protein with a fractional catabolic rate (FCR) of 4.81%/h (range, 4.00–6.27), a half-life ($t_{1/2}$) of 25 h (20–30), extravascular/intravascular diffusion ratio (EV/IV) of 2.04 (1.52–3.30), and a synthesis rate (SR) of 0.71 mg/kg body weight per h (0.61–0.87). There was evidence for extravascular catabolism in each subject. Plasma levels correlated with SR but not with $t_{1/2}$ or FCR. Patients had a lower EV/IV ratio, and in two critically ill patients with low plasma Fn concentration the SR was markedly depressed.

These findings suggest that reduced synthesis of Fn, rather than increased FCR or increased extravascular distribution, is responsible for Fn deficiency in critically ill patients.

Introduction

Fibronectin (Fn)[1] is a high molecular weight glycoprotein (440,000 mol wt) that is present on cell surfaces in a multimeric insoluble form and is a major soluble constituent of plasma. Although its physicochemical property as a cold insoluble globulin was observed more than 30 yr ago (1), it is only recently that a potential functional significance has been appreciated. Cellular Fn is a component of the surface of many cells, where it has an important role in cell movement, substrate adhesion, and the maintenance of normal cell morphology and behavior. Plasma Fn interacts with activated Factor XIII, causing it to be convalently cross-linked to fibrin, fibrinogen (to form a cryoprecipitate), or to other Fn molecules. It binds to heparin, the C1q component of complement, amyloid P component, and collagen, and is required for the interaction between fibroblasts and fibrin. The unifying concept for these activities of Fn is that it acts as an adhesive protein (2). These opsonic properties may play an important role in removing plasma debris by facilitating uptake by the reticuloendothelial system (3).

Low levels of Fn have been observed in patients with sepsis (associated with burns, trauma, and acute leukemia) and in critically ill patients with intravascular coagulation (4–6). Saba and co-workers (3) found that these reduced levels correlated with the severity of clinical illness. However, studies in experimental animals have shown no reduction in bioassayable Fn after intravenous administration of endotoxin (7) and the induction of *Escherichia coli* bacteraemia (8); both procedures caused a fall in the phagocytic index. Despite this conflicting evidence, Fn-rich products (such as cryoprecipitates) have been used in the treatment of patients with septic trauma. Some groups have reported an improvement in several clinical and laboratory parameters after such therapy (3). However, an accurate assessment of this treatment is difficult in patients with multi-organ failure, and the optimum regimen for Fn-supplementation is unknown. Nevertheless, the growing recognition of the biological importance of Fn and its by-products makes the clarification of its in vivo behavior of fundamental and clinical importance. In this report, we have examined the metabolism of a highly purified functionally active preparation of radiolabeled plasma Fn by performing turnover studies in experimental animals, normal human controls, and critically ill patients.

Methods

Patients

Metabolic studies were performed in 11 patients. Six were critically ill in an intensive care unit and five had stable chronic disease. Two of the latter had active rheumatoid arthritis; one had mesangial proliferative glomerulonephritis; one had diabetic nephropathy; and one had progressive systemic sclerosis. These five patients were selected for study as a control group for the critically ill patients and because of the possible involvement of Fn in the pathogenesis of their disease. Table I lists the diagnosis and outcome of each patient and Table II shows the laboratory data on entry to the study. The critically ill patients remained stable during the study; that is, none were suffering abnormal loss of blood or other body fluids. Eight control subjects were selected from normal hospital, medical, and laboratory staff and, for each control subject used, one or more patients were studied simultaneously. The controls were not matched for age and sex. Six were males and two were females and age range was 20–40 yr.

The turnover protocol was approved by the hospital's ethics committee and, in all cases, informed consent was obtained from either the subject or the nearest relative before starting the study. Thyroid uptake of unbound iodide was blocked by the administration of oral potassium iodide or intravenous sodium iodide daily for at least 24 h before the injection of radioactive material and throughout the sampling period.

Plasma fibronectin was measured by radial immunodiffusion against monospecific antiserum (Cappel Laboratories, Cochranville, PA) using dilutions of purified Fn and Standard Plasma (Calbiochem-Behring Corp., La Jolla, CA) as controls.

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1. *Abbreviations used in this paper:* EV/IV, extravascular to intravascular diffusion ratio; FCR, fractional catabolic rate; Fn, fibronectin; IV, intravascular; PAGE, polyacrylamide gel electrophoresis; SR, synthesis rate.

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Table I. Patients' Clinical Data

Subject*	Sex/age	Diagnosis	Complications	Outcome
	yr			
1	M/43	Gas gangrene	ARF‡, rhabdomyolysis, septicaemia	Death
2	M/83	Caecal volvulus	Peritonitis, pneumonia	Hemicolectomy Alive
3	M/57	Perforated duodenal ulcer	ARF Fungal septicaemia	Lymphoma Alive
4	M/61	Fractured femur	Pneumonia, ARF, septicemia	Alive
5	M/38	Haemorrhagic pancreatitis	Pancreatic abscess	Alive
6	M/40	Meningioma	Pneumonia, septicaemia	Death
7	M/55	Rheumatoid arthritis	Acute relapse	Controlled on drug therapy
8	M/66	Rheumatoid arthritis	Acute relapse	Controlled on drug therapy
9	F/27	Progressive systemic sclerosis	Skin, gut, renal involvement	Alive
10	F/29	Diabetes—type I	Diabetic nephropathy	Unchanged
11	F/20	Mesangial IgA nephropathy	Haematuria	Unchanged

* Subjects 1–6 were critically ill in an intensive care unit. Subjects 7–11 were stable ward patients with no evidence of infection. ‡ ARF, acute renal failure.

Preparation of labeled Fn

Fn was prepared from plasma donated by a healthy volunteer, who was negative for hepatitis-B surface antigen. 100 ml of EDTA plasma was passed down a 6 × 2.5 cm column of gelatin-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) at 4°C, with a flow rate of 40 ml/h. This column was washed with Tris buffer (0.1 M Tris HCl, pH 7.2, containing 0.15 M NaCl) until base-line OD_{280} was reached, and Fn was then eluted with the same buffer containing 4.5 M urea. After dialysis (against Tris buffer), Fn was labeled with ^{125}I (Amersham Corp., Arlington Heights, IL) by the lactoperoxidase method (9) and was again passed down a gelatin-Sepharose column to separate free iodide. Fn was eluted as before, dialyzed against Tris buffer containing 1 M urea and 250 mg chloramphenicol/liter, and stored in aliquots in liquid nitrogen. Specific activity was 0.1 μCi/μg. All buffers were prepared with pyrogen-free water and were millipore-filtered before use.

Radiolabeled Fn was sterilized by millipore filtration (0.22 μm). No growth was observed after culture under aerobic and anaerobic conditions and tests for pyrogenic activity in rabbits were negative.

Purity and functional activity of Fn

Purity was tested by double immunodiffusion in agarose using anti-whole human serum (Dako Corp., Santa Barbara, CA) supplemented with monospecific anti-Fn antiserum (Cappel Laboratories) and by polyacrylamide gel electrophoresis (PAGE) in a 4–30% gradient and in sodium dodecyl sulfate (SDS) under reduced and nonreduced conditions (10). The following methods were used to assess functional activity:

*Table II. Patients' Laboratory Data**

Subject	Serum creatinine	Serum albumin	Hb	Platelets × 109/liter	Prothrombin ratio	S.Fn
	mmol/liter	*g/liter*	*g/liter*			*g/liter*
1	0.26	27	101	66	1.3	0.10
2	0.10	40	133	200	1.4	0.37
3	1.40	33	96	225	1.1	0.30
4	0.55	37	85	275	1.3	0.26
5	0.11	35	154	270	1.2	0.37
6	0.08	33	159	200	1.2	0.36
7	0.10	37	135	310	1.1	0.35
8	0.08	38	130	315	1.0	0.35
9	0.09	36	145	200	1.1	0.37
10	0.12	36	144	300	1.1	0.40
11	0.10	36	123	250	0.9	0.35
Normal range	0.06–0.12	35–45	115–165	160–350	0.8–1.2	0.28–0.42

* Results obtained from samples taken on admission to the study. ‡ For serum creatinine, 1 mmol/liter = 0.88 mg/100 ml. S.Fn, serum fibronectin.

Gelatin adhesion. The effect of radiolabeling on Fn-function was assessed by testing its ability to mediate cell-substrate adhesion. Purified Fn (labeled and unlabeled) in phosphate-buffered saline (PBS) was added in various dilutions to 2×10^5/ml Raji cells in gelatin-coated plastic microtiter wells (Nunc Plastics, Roskilde, Denmark) (the gelatin coating was performed by overnight incubation of 10 μg/ml of gelatin in complement fixation diluent [Oxoid Ltd., London] at 40°C). The Raji cell concentration gave a suitable monolayer of cells on the bottom of the well. The cell-Fn mixture was incubated for 2 h at 37°C in 5% CO_2 in air. After gentle washing with PBS, adherent cells were then counted using an inverse microscope.

In vivo studies in experimental animals. These experiments were performed in 2.5–3.0 kg New Zealand white rabbits pretreated with potassium iodide drinking water to block thyroidal uptake. First, in vivo metabolic behavior was examined after the injection of ~10 μCi ^{125}I-Fn into a peripheral ear vein. Serial samples were taken from the contralateral ear over 48 h and plasma was separated and processed as previously described (11). Protein-bound radioactivity was measured in a gamma counter. The plasma disappearance curve was then constructed and half-life ($t_{1/2}$) was calculated from the slope of the final exponential. Second, radiolabeled Fn was screened by injecting ~60 μCi of the preparation into a 2.5-kg rabbit, which was then bled 16 h later into 0.02 M EDTA. 6 ml of this ^{125}I-Fn containing plasma (~1.5 μCi of ^{125}I) was then injected into a second rabbit and the disappearance curve was compared with that observed in the first animal (with unscreened ^{125}I-Fn).

Finally, the generation of Fn fragments in vivo was investigated by injection of 100 μCi of ^{125}I into two rabbits. EDTA plasma samples were taken from these animals at 15 min, 1 h, and 5 h. These samples and a sample of the injected ^{125}I-Fn were then subjected to SDS-PAGE analysis in a 9% gel under reduced conditions. The gel was stained with Coomassie Blue, dried, and cut into 2-mm sections, which were counted for radioactivity. The radioactive peaks were compared with the position of SDS-6H molecular weight standards (Sigma Chemical Co., St. Louis, MO). These conditions were chosen to detect the presence of breakdown products, knowing that the standards remain linear only over a fivefold difference in molecular weight and give a sigmoid curve in the higher and lower range (12).

Human metabolic studies

Patients and controls each received ~6 μCi of ^{125}I-Fn. Studies were continued for 3–5 d (or until <7% protein-bound radioactivity remained in the plasma). Plasma and urine samples were processed as previously described (11). Each subject had at least one urine sample tested for protein-bound radioactivity: 2 ml of urine was precipitated with trichloracetic acid, using normal plasma as a carrier, and the precipitated radioactivity was then counted. After plasma separation, the erythrocyte-bound radioactivity was also measured, after washing the cells three times in complement fixation diluent. Serum concentrations of Fn were measured during the study and only patients in a steady state (i.e., <15% variation) were included. The plasma curve was calculated assuming the first point to be 97% of the injected dose of protein-bound radioactivity, To.

Urine and plasma data were analysed by: (*a*) calculation of urine/plasma ratios (i.e., the metabolic clearance method of Berson & Yalow [13]), and (*b*) the integrated rate equations method of Nosslin (14). In those cases without adequate urine collections, metabolic parameters were calculated from the plasma disappearance curve as described by Matthews (15). These methods enabled us to calculate half-life and fractional catabolic rate (FCR). Fn synthesis was then calculated from the FCR, plasma volume, and serum concentration. Plasma volume was calculated from the protein-bound radioactivity in both the first plasma sample and the injected dose. Nosslin's (14) and Matthews' (15) methods also enabled us to calculate compartmental distribution (extravascular/intravascular [EV/IV] ratio) of the labeled protein.

In two of the control subjects (14 and 15 in Table III), whole body radioactivity was also counted using a whole body counter and compared with the plasma disappearance curve.

Statistical methods

The Student's *t* test for paired or unpaired data was used to test the significance of differences obtained in metabolic studies. Correlations were calculated by linear regression and determination of the *r* coefficient.

Results

Analysis of purified Fn by double diffusion in agarose (against anti-whole human serum supplemented with monospecific anti-Fn) showed a single precipitin line. SDS-PAGE studies showed a single band at ~440,000 mol wt in unreduced conditions and at 220,000 mol wt in reduced conditions, indicating that the purified Fn was a dimer of two subunits of 220,000 mol wt (2). After iodination, >95% of radioactivity was precipitated by incubating with anti-Fn. Functional activity of Fn was retained after labeling: Fig. 1 shows no significant difference in cell adhesive properties between labeled and radiolabeled Fn. The turnover characteristics of screened and unscreened preparations of ^{125}I-Fn (in rabbits) are shown in Fig. 2. Plasma disappearance curves for the two preparations were comparable, indicating the absence of significant amounts of denatured (or aggregated) protein in the original preparation. Both curves also showed a high coefficient of linearity (r = 0.99).

Table III. Fn Metabolism in Control Subjects

			FCR			EV/IV ratio			
Subject	S.Fn*	Half-life	Matthews' analysis	Metabolic clearance	Nosslin's analysis	Matthews' analysis	Nosslin's analysis	Synthesis rate	EV catabolic ratio (Nosslin)
	g/liter	*h*		*% per h*				*mg/kg per h*	
12	0.28	24	6.36	6.75	6.27	0.70	1.58	0.62	0.37
13	0.43	29	4.96	4.78	4.26	0.78	1.33	0.71	0.43
14	0.40	28	4.21	4.81	4.00	0.51	1.52	0.61	0.87
15	0.38	30	4.67	4.79	4.40	0.84	1.73	0.75	0.84
16	0.33	26	5.06	4.96	4.28	0.53	2.05	0.50	0.57
17	0.38	20	6.87	4.07	4.79	0.80	2.60	0.75	0.60
18	0.36	20	6.98	4.97	6.25	0.73	3.30	0.87	0.57
19	0.37	22	5.30	4.10	4.20	0.46	2.20	0.86	0.80
Mean	0.37	25	5.55	4.90	4.81	0.67	2.04	0.71	0.63

* S.Fn, serum fibronectin.

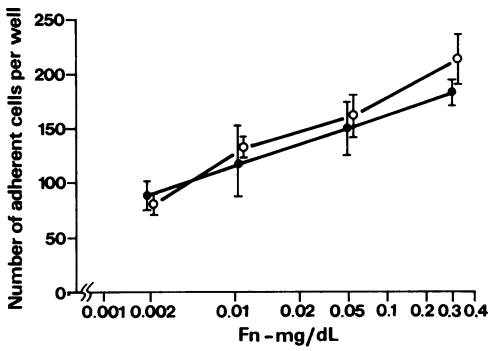

Figure 1. Fn-induced cell-substrate adhesion. ^{125}I-Fn (●) retained the ability to induce Raji cell adhesion to gelatin-coated plastic wells. (○, unlabeled Fn.)

Analysis of serial plasma samples by SDS-PAGE in two rabbits showed production of similar metabolic fragments during the 5-h period of study (Fig. 3). The major component was identical to that obtained with the unlabeled preparation and shows maintenance of molecular integrity during the metabolic studies.

Human metabolism. Metabolic parameters for control subjects are shown in Table III. There was good agreement between the metabolic clearance method and Nosslin's method (14) in the calculation of FCR (t = 0.02). Significant differences were obtained between Matthews' (15) and Nosslin's (14) methods for FCR (t = 3.30; $P < 0.02$) and EV/IV ratio (t = 5.86; $P < 0.001$). Nosslin's extracascular catabolic ratio (K2/K2 + K4) was less than one, which shows a significant degree of extravascular catabolism. The ratio of free to protein-bound radioactivity in the plasma remained relatively constant during the study: the coefficient of variation of this ratio ranged between 8 and 15% in control subjects and in those patients with normal renal function.

Patients' metabolic data is shown in Table IV. Adequate urine was obtained in only two patients; the remainder had impaired renal function or incomplete urine collections. There was no significant difference between the half-life, synthesis rate (SR), or FCR (as calculated by Matthews' method [15])

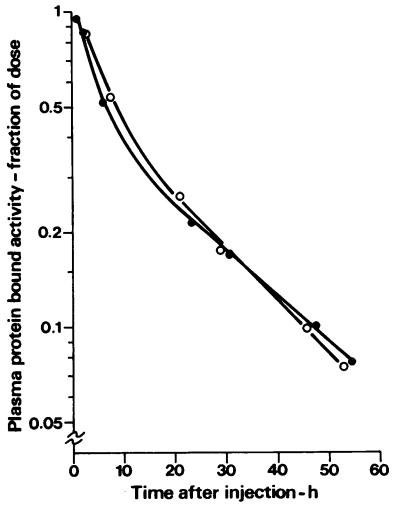

Figure 2. ^{125}I-Fn disappearance curve in experimental animals. Screened (○) and unscreened (●) preparations of ^{125}I-Fn showing similar plasma disappearance curves.

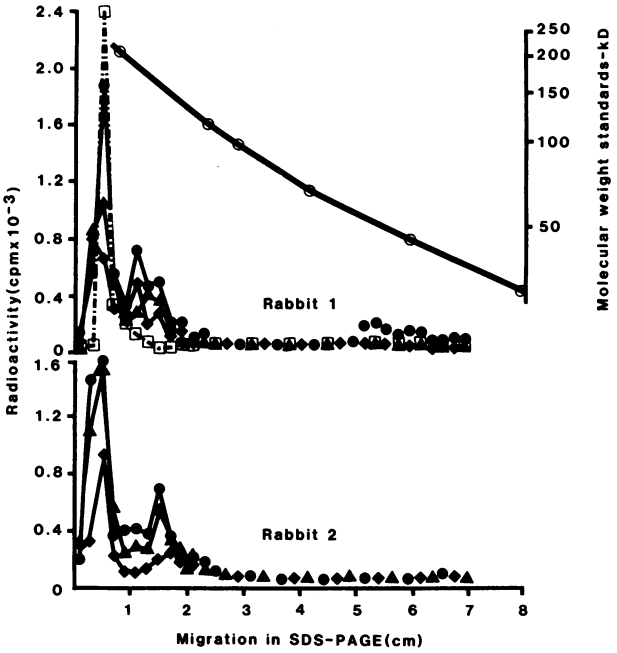

Figure 3. PAGE analysis of ^{125}I-Fn in plasma. Similar metabolic fragments were produced over 5 h after intravenous injection of labeled Fn in experimental animals. □, ^{125}I-Fn before injection; ○, molecular weight standards. Plasma samples taken at 10 min (●), 60 min (▲), and 5 h (♦).

in controls and patients (t = 0.73, 1.10, and 1.50, respectively). However, patients had a significantly lower EV/IV ratio (t = 2.46; $P < 0.05$). Patients 1 and 4 had low serum levels of Fn and these also had the lowest synthesis rates in the whole study. Their FCRs and EV/IV ratios were not increased. Serum Fn levels correlated significantly with SR (r = 0.59; $P < 0.01$) but not with half-life, FCR, or EV/IV ratio.

Whole body counts in the two control subjects showed that the slopes of the whole body curves were comparable to those of the plasma curves: slopes were −0.025 and −0.0245, respectively, for subject 14; and −0.020 and −0.023, respectively, for subject 15 (Fig. 4).

Discussion

This study shows that Fn is a rapidly catabolized plasma protein with an FCR of 4.81% per h (range, 4.0 to 6.27). Although protein denaturation is difficult to prove absolutely, preliminary experiments confirmed the suitability of our radiolabeled Fn preparation for in vivo metabolic studies. Before injection, purity was shown by several tests: a single precipitin line against antiwhole human serum on double immunodiffusion agarose gel; a single reducible band on radioautography of SDS-PAGE (a pattern confirmed previously); and >90% precipitation with monospecific anti-Fn antibodies. A classical adhesive function of the plasma Fn molecule was preserved after purification and iodination. Furthermore, in vivo, metabolic behavior confirmed the viability of the radiolabeled material. After injection, there was a short equilibration period (i.e., <24 h) before a final slope of disappearance was reached; this slope showed a highly significant coefficient (r) of linearity. In control subjects this curve paralleled the whole body radioactivity curve. The release of free iodine from the Fn molecule was uniform (as judged by free/protein-bound radioactivity ratios and calculation of metabolic clearance), suggesting a

Table IV. Fn Metabolism in Patients

Subject	Half-life	FCR			EV/IV ratio		Synthesis rate	EV catabolic ratio (Nosslin)
		Matthews' analysis	Metabolic clearance	Nosslin's analysis	Matthews' analysis	Nosslin's analysis		
	h		*% per h*				*mg/kg per h*	
1	21	5.28	*	*	0.52	*	0.36	*
2	21	5.18	*	*	0.43	*	1.11	*
3	22	4.74	*	*	0.32	*	0.68	*
4	29	3.0	*	*	0.22	*	0.31	*
5	21	5.89	*	*	0.44	*	0.85	*
6	21	4.30	*	*	0.22	*	0.75	*
7	25	5.00	*	*	0.47	*	1.07	*
8	25	5.50	4.52	4.71	0.78	2.2	0.94	0.64
9	24	5.14	*	*	0.65	*	1.11	*
10	28	4.26	*	*	0.57	*	0.86	*
11	24	5.44	4.63	5.26	0.64	1.5	0.65	0.59

* No results obtained because of impaired renal function or incomplete urine collections.

minimal degree of protein denaturation. In experimental animals, the screening of radiolabeled Fn for 16 h produced no change in the pattern of plasma disappearance. Such a procedure eliminates denatured material from subsequently injected plasma aliquots. We have previously used such a procedure to eliminate denatured material from a preparation of ^{125}I-C3 (11). In those experiments, the initial rate of decline of radioactivity (after injection) was grossly reduced after screening, whereas in this study the slope of disappearance remained unchanged.

In normal volunteers, Fn had a rapid turnover rate. Values for half-life and FCR suggest a metabolic rate greater than other immunoreactive proteins whose turnover characteristics have been investigated. Table V shows a comparison of metabolic parameters for some of these proteins (16–24). FCR, as calculated by three different methods, showed >100% of the plasma pool of Fn to be catabolized within 24 h. Considerable variation was observed in EV/IV distribution as measured by Matthews' exponential analysis (15) and Nosslin's (14) integrated rate equations method. The former suggested ~60% of the radiolabeled protein to reside within the plasma compartment, while Nosslin's analysis showed EV/IV ratios to be >1.5, suggesting the majority of the injected material to reside in an extravascular site. This difference may be explained by an inherent weakness in applying Matthews' method (15) to rapidly catabolized proteins where there is retention of non-protein-bound tracer in intravascular and extravascular sites (25). Correcting this problem would require an estimate of the iodide space by the use of another iodide isotope—a study that we did not perform. In the case of Fn, it should be recognized that additional sites within the plasma compartment (such as circulating and fixed cell surfaces) could explain this high ratio. An argument against this possibility was the absence of significant radioactivity in aliquots of washed cells collected at the time of routine sampling. Also, the decline in the whole body radioactivity coincided closely with the rate of plasma disappearance and there was complete retrieval of free ^{125}I from urine samples. The synthesis of Fn was shown to be between 0.50 and 0.87 mg/kg per h in normal subjects. Such a rate is comparable to that of fibrinogen and is slightly in

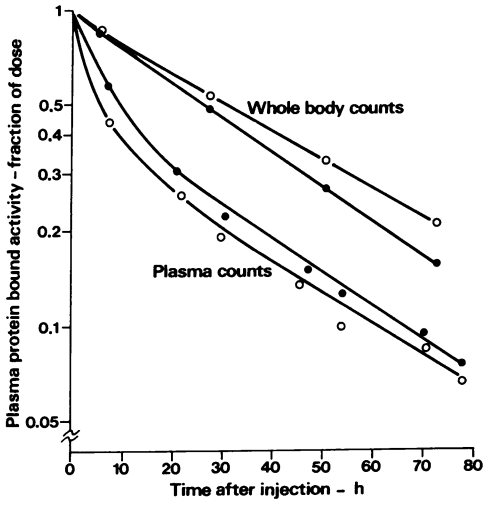

Figure 4. ^{125}I-Fn plasma and whole body disappearance curves in controls. Subject 14 (●) and subject 15 (○) had comparable disappearance curves for plasma and whole body radioactivity.

*Table V. Comparison of Metabolic Parameters for Some Plasma Proteins**

	Half-life	FCR	Percent in IV pool	Synthesis
	d	*% per d*		*mg/kg per d*
Albumin	16.5	8	41	160
IgG 1, 2, 4	21	7.5	55	30
IgG 3	7	16.8	64	—
IgM	5.1	10	75	3.2–16.9
IgA	6.4	22	40	2.7–55
Fibrinogen	4	22	86	35
C1q	1.25	67	66	4.3
C4	2.5	50	62	11
C3	3	40	58	19
C5	2.6	40	66	2.1
B	3	47	47	4.32
H	3.2	32	65	8.9
Fn (our data)	1	115	33	17

* Data collated from references 16–24.

excess of C3. Although this parameter is calculated indirectly, it should be stressed that the primary values used for this calculation were well substantiated: there was good agreement among the methods for calculating FCR, and values for plasma volume (in normal subjects) ranged between 40 and 50 ml/kg. Previous studies of Fn metabolism in experimental animals have shown variations in half-life and distribution. Sherman and Lee (26) studied rabbit Fn in rabbits and found a t½ of 71 h and a predominantly intravascular protein with 80% residing in the IV compartment (or EV/IV ratio of 0.25). However, our findings were similar to those reported by Deno et al. (27). They studied in vivo labeled rat Fn and found a t½ of 21 h with 62% of the labeled protein in the IV compartment (or EV/IV ratio of 0.63) (see Results: in rabbits, t½ = 20 h; in humans, t½ = 25 h; and EV/IV ratio is 0.67, using plasma curve analysis). Such observed differences may be the result of studying different species as well as differences caused by the use of autologous and heterologous proteins.

Mathematical models used in our calculations assume a steady state and this criteria was applied to the selection of patients. The critically ill patients remained stable during the study and the other patients acted as controls for this critically ill group and also examined the effect of an acute phase response on Fn turnover. Whilst we have not attempted to define a role for Fn deficiency in the pathophysiology of critically ill patients (in fact, our data neither confirm nor deny such a role), metabolic studies of Fn have therapeutic implications. They provide a rational basis for the choice of dose of Fn-concentrates for IV infusion in immunologically susceptible patients. Knowledge of plasma pool size, t½, and serum concentration permits a reasonably accurate assessment of the dose needed to elevate significantly the level of Fn in the circulation. Our finding that decreased synthesis, rather than increased FCR, was responsible for the reduced level in critically ill patients implies that the dose of the infusion (rather than its frequency) would need to be increased in such patients. Experiments to determine the plasma levels needed to produce significant improvement in Fn-related functions would further enhance its potential value as a therapeutic agent. The relative ease of preparation of biologically active Fn warrants consideration in preparing such infusates, as this would obviate the risk of denaturation inherent in producing IV concentrates (primarily for other purposes) and also the potential loss of Fn function as a result of binding to other plasma constituents in the infusate.

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