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Influence of Gelatin on Bioassayable and Immunoreactive Opsonic Fibronectin[1] (41228)

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Abstract. Plasma fibronectin has a high affinity for denatured collagen (gelatin) and exerts an opsonic influence on phagocytosis of test colloids and clearance of tissue debris by macrophages. This study evaluated the effect of *in vitro* and *in vivo* interaction of gelatin with plasma on measurable bioassayable opsonic activity and immunoreactive fibronectin. Incubation of human, dog, sheep, and rat plasma with gelatin prior to *in vitro* assay decreased ($P < 0.05$) the ability of plasma to augment particle uptake in the liver slice bioassay. Incubation of plasma with gelatin also decreased the concentration of fibronectin that could be detected by electroimmunoassay. Intravenous infusion of gelatin into rats, dogs, and sheep resulted in an acute depression in both bioassayable and immunoreactive opsonic fibronectin for several hours, followed by restoration of both parameters within 24 hr. These observations suggest that deficits of opsonic fibronectin as documented in injured patients by bioassay and electroimmunoassay may not be exclusively related to actual depletion of fibronectin from blood but may be, in part, due to binding of fibronectin to blood-borne material post-trauma, i.e., collagenous tissue debris, whose presence in plasma may limit its detection by both assays.

Reticuloendothelial clearance of blood-borne nonbacterial particulate matter, such as gelatin-coated test colloids, fibrin aggregates, injured cells, and collagenous tissue debris appears to be modulated by opsonic glycoprotein (3, 4, 14, 28–30). This opsonic glycoprotein has been isolated from animal and human plasma and shown to be identical to a high-molecular-weight dimeric glycoprotein called plasma fibronectin or cold-insoluble globulin (4, 30, 33, 37). Opsonic deficiency as documented by liver slice bioassay (31) and electroimmunoassay (3) correlates with impaired hepatic Kupffer cell phagocytic clearance of blood-borne test particulates and is associated with an increased extrahepatic localization especially in the lungs and spleen (16, 23). Purified plasma fibronectin will stimulate the binding and ingestion of gelatin-coated test particles by peritoneal macrophages *in vitro* as recently documented with isotopic and electronmicroscopic techniques (8, 14, 21), as well as stimulate particle phagocytosis *in vivo* (32).

Septic surgical, trauma, and burn patients demonstrate a persistent opsonic fibronectin deficiency which is correlated with organ failure such as pulmonary insufficiency (29, 33, 34). Based on determinations of opsonic activity by liver slice bioassay as well as electroimmunoassay, clinical studies (29, 33, 34) have been initiated to reverse opsonic fibronectin deficiency by infusion of plasma cryoprecipitate whose concentration of plasma fibronectin is typically 8–12 times that of plasma. Reversal of opsonic fibronectin deficiency in septic-in-

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jured patients results in an improvement in pulmonary function, limb blood flow, limb oxygen consumption, and endogenous creatinine clearance (33, 34).

Fibronectin is known to interact with and bind denatured collagen (gelatin) (5, 6, 9, 10). Indeed, a binding site on fibronectin with respect to its interaction with gelatin has been documented (22). Verification of this interaction is indirectly shown by the ability to isolate fibronectin from plasma or serum by affinity chromatography with gelatin–Sepharose columns (9), as well as the rapid opsonization of gelatin-coated test particles by fibronectin prior to macrophage ingestion (14, 33). Since collagenous debris may be elaborated into the circulation of patients after massive soft tissue trauma and/or burn injury, it is possible that the opsonic fibronectin deficiency as detected by bioassay and/or electroimmunoassay in such patients reflects the complexing of opsonic fibronectin to such blood-borne material. This complex may be removed slowly from the circulation as opposed to earlier suspicions of rapid clearance from the blood (33, 34). The presence of such collagenous debris in plasma may alter the detection of opsonic fibronectin by bioassay or immunoassay.

In order to evaluate this concept as well as to further illuminate the mechanisms by which opsonic deficiency can develop, the present study evaluated the influence of gelatin (denatured collagen) on bioassayable and immunoreactive opsonic fibronectin under *in vitro* and *in vivo* conditions. A comparative species analysis was completed in order to document the species specificity of this response.

Methods. *Plasma collection.* Healthy adult sheep (25–50 kg), male Sprague–Dawley rats (250–300 g), and mongrel dogs (10–15 kg) maintained on food and water *ad libitum* served as donors for the collection of heparinized blood. Human blood was collected by venipuncture into heparinized syringes from four healthy volunteers (28–37 years). Samples withdrawn from *in vivo* animal studies also consisted of heparinized blood samples. Plasma samples were obtained after centrifugation of the heparinized whole blood. The pattern observed with plasma or serum is essentially similar except the absolute level of opsonic fibronectin in serum is lower than that observed in plasma (22, 33), due to the covalent binding of fibronectin to fibrin (22).

Immunoreactive opsonic fibronectin. Electroimmunoassay or rocket immunoelectrophoresis was used to quantitate opsonic fibronectin levels (3, 33). Briefly, agarose (Seakem) was dissolved in 0.07 *M* barbital buffer (pH 8.6, 2 m*M* Ca-lactate) to a concentration of 1% by careful heating at 100° while stirring. The 1% agarose was allowed to cool to 63° and warmed monospecific antiserum was added to yield a final antiserum concentration of 0.6% (varies with antibody titer). A 60-ml volume of the agarose–antiserum solution was poured onto a clear, warm, level 5 × 10-in. glass plate yielding a gel thickness of about 1.8 mm. After the agarose had hardened, 3-mm wells were cut into the agarose 1 cm apart and 2 cm from one edge of the plate. Serum or plasma was diluted to 10% with saline and 10-μl aliquots were pipetted into each well. The antigen was electrophoretically moved toward the anode at a voltage of 7.5 V/cm at 4° for 22 hr using a LKB multiphore system.

The electroimmunoassay plates were washed, pressed, dried, and stained as previously described (3). Rocket heights were used as a quantitative index of immunoreactive opsonic fibronectin. A double reciprocal plot of rocket height in millimeters vs micrograms fibronectin was defined using serum of known fibronectin concentrations (3). Immunoreactive opsonic fibronectin was expressed as micrograms per milliliter.

Bioassayable opsonic activity. Bioassayable opsonic activity of plasma or serum was determined by its ability to support uptake of the gelatinized ^{131}I-reticuloendothelium(RE) test lipid emulsion using the liver slice bioassay as previously used in animal (4, 31, 32) and clinical studies (29, 33, 34). With this assay, opsonic activity is decreased after RE blockade (31) and trauma (16) with restoration of opsonic activity correlating with RES phagocytic recovery. Selective RE cell localization of the gelatinized RE test lipid emulsion in such *in vivo* studies has been confirmed by electronmicroscopic analysis (35) and similar studies with the lipid particle have been completed with the liver slice assay (15). The ability of affinity-purified animal and human plasma fibronectin to express opsonic activity as tested in the liver slice bioassay has been extended to observations with isolated peritoneal macrophages where the kinetics of phagocytosis as well as particle internalization as studied by electronmicroscopy has been documented (8, 14). The lipid base for the test emulsion was prepared by blenderizing glycerol, ^{131}I-triolein (Mallinckrodt Nuclear, St. Louis, Mo.) and lecithin in a ratio of 10:10:1, respectively. When used in the assay the anhydrous base was mixed with 0.1% gelatin-supplemented 5% dextrose and water solution (pH 7.4) to yield a 1% emulsion (31). The assay medium consisted of 1.0 ml plasma, 2.0 ml Krebs–Ringer phosphate buffer, and 100 USP units of heparin. Liver slices (200–300 mg) were prepared from normal rat livers utilizing a Stadie–Riggs tissue slicer. The flasks containing the liver slices and medium were supplemented with 2.0 mg of the 1% emulsion and incubated at 37° under 95% O_2 and 5% CO_2 for 30 min in a Dubnoff metabolic shaker. After incubation, the slices were isotopically evaluated for particle uptake as previously described (3, 4, 31, 34). Bioassayable opsonic activity was expressed as a percentage of the emulsion dose removed per 100 mg of wet liver (%ID/100 mg).

In vitro gelatin preincubation. The effect of preincubation of sheep, human, dog, or rat plasma with gelatin (Nutritional Biochemical Corp.) on its opsonic activity, as tested by liver slice bioassay and its fibronectin concentration, as tested by electroimmunoassay, was studied. Fresh rat, dog, human, and sheep plasma was divided into 1.0-ml aliquots. Each aliquot was supplemented with varying concentrations of gelatin prepared to a fixed volume of 300 μl to avoid variations due to dilutional differences. The plasma–gelatin mixture was incubated for 15 min at 37° prior to functional and immunological analysis.

Preincubation of rat liver slice in gelatin-rich solutions. The possibility that gelatin directly affects Kupffer cell function as opposed to binding to fibronectin was tested by a modification of the rat liver slice technique. Liver slices of uniform size (290.1 ± 7.8 mg) were prepared from normal rat livers utilizing a Stadie–Riggs tissue slicer. Each slice was placed in a 25-ml Erlenmeyer flask containing 3.0 ml of Krebs–Ringer phosphate buffer supplemented with 0, 100, 200, or 300 μg of gelatin and incubated under a gas phase of 95% O_2 and 5% CO_2 for 15 min at 37° in a Dubnoff metabolic shaker. Following incubation, the slices were rinsed in cold saline and utilized in the standard bioassay with pooled normal rat plasma and the gelatinized ^{131}I RE test lipid emulsion. The uptake by these slices after a 30-min incubation period was again expressed as the percentage of the emulsion dose removed per 100 mg of wet liver (%ID/100 mg).

In vivo gelatin challenge. Circulating bioassayable and immunoreactive opsonic activity in the rat, dog, and sheep were evaluated at various time intervals after intravenous infusion of soluble gelatin. In view of marked differences in levels of opsonic fibronectin in these three species, as previously determined by immunoassay (25), the gelatin challenge was varied so that the circulating gelatin:fibronectin ratio was approximately equal. The gelatin (Nutritional Biochem. Corp.; ICN Pharm.) was suspended in sterile 5% dextrose and water and adjusted to pH 7.4 prior to injection. The gelatin doses were: 8.7 mg/kg in sheep; 10.6 mg/kg in rats; and 50.0 mg/kg in dogs. These doses were used to provide estimated zero time levels of gelatin equivalent to 200, 300, and 1000 μg/ml of estimated plasma volume in the sheep, rats, and dogs, respectively. Their plasma fibronectin levels by immunoassay typically range between 200 and 250 μg/ml for sheep; 300 and 450 μg/ml for rats; and 900 and 1200 μg/ml for dogs. Blood samples were collected prior to and over a 24-hr postinjection period in order to define the pattern for restoration of opsonic activity. In rats, separate animals were needed at each time interval, since the large volume of blood needed for the bioassay necessitated exsanguination and sacrifice of the rats. In dogs and sheep, each animal acted as its own control.

Statistical analysis of data. Bioassay data were analyzed using the unpaired Student's t test, while the paired Student's t test was applied to the immunoassay data. The confidence level was placed at 90%. Data are expressed as mean ± SE of the mean.

Results. Preincubation of either dog, rat, human, or sheep plasma with gelatin resulted in a significant decrease in bioassayable opsonic activity (Fig. 1). Moreover, in contrast to rat, human, or sheep plasma, which required only a small amount of gelatin to initiate this inhibiting response, it was observed that dog plasma with known high concentrations of fibronectin was only inhibited when the gelatin concentration was increased to a range of 300–1000 µg/ml. Analysis of the plasma prior to and after preincubation with gelatin by electroimmunoassay was also conducted. As indicated in Fig. 2, the detectable concentration of fibronectin was also significantly reduced ($P < 0.05$) when tested by this method. However, the level detectable in dog plasma after gelatin preincubation is still relatively high in agreement with the bioassay data. The findings in Fig. 2 do not distinguish between the influence that gelatin may have on the mobility of fibronectin in an electric field through the agarose used in the electroimmunoassay procedure and the possibility that gelatin may bind important sites on the fibronectin which make the molecule less immunoreactive. These unexpected findings indicate that fibronectin can be present in a plasma sample but its detection by either a functional phagocytic assay or rocket immunoelectrophoresis can be significantly compromised by the presence of denatured collagen (gelatin).

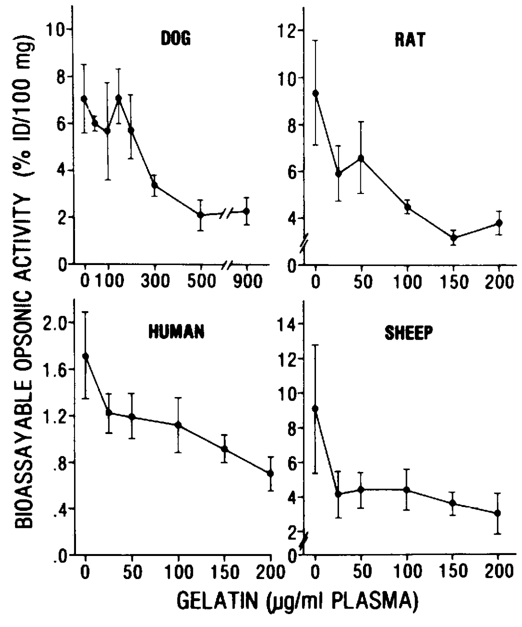

FIG. 1. The effect of preincubation of dog, rat, human, and sheep plasma with varying concentrations of gelatin on the bioassayable opsonic activity of plasma as determined by uptake of the gelatinized ^{131}I RE test lipid emulsion in the liver slice assay. Each point represents the mean ± SE of three to nine assays. Samples were preincubated for 15 min at 37°.

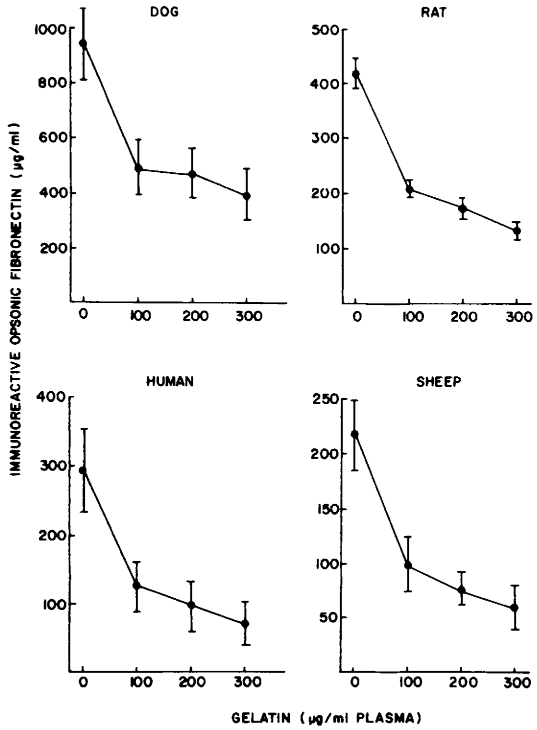

FIG. 2. The effect of preincubation of dog, rat, human, and sheep plasma with varying concentrations of gelatin on immunoassayable opsonic fibronectin as determined by electroimmunoassay. Each point represents the mean ± SE of four assays. Samples were preincubated for 15 min at 37°.

The temporal alterations of immunoreactive and bioassayable opsonic activity *in* *vivo* in the three species following gelatin injection are presented in Figs. 3–5. Opsonic activity in rats was markedly ($P <$ 0.001) decreased at 1 hr with recovery to 70% of control activity 3 hr. Restoration to prechallenge levels was evident within 24 hr. Similar to the response in the rat, there was a parallel decrease in immunoreactive and bioassayable opsonic fibronectin activity following gelatin infusion in sheep and dogs (Figs. 3 and 4). Restoration of biologically active and immunoreactive opsonic fibronectin was minimal, however, in the dog and sheep during the initial 3 hr of the evaluation period. In all three species opsonic fibronectin returned to prechallenge levels within 24 hr after gelatin challenge.

In order to delineate the specificity of this response, the blood level of other common proteins as well as the total serum protein was studied in one of the larger species, i.e., sheep. This species was selected since the decrease of fibronectin was rather pronounced and adequate volumes (10 ml) of blood can be removed serially with undetectable cardiopulmonary influence. As indicated in Table I, there was no change in total protein as determined by modified Biuret after intravenous challenge with the small dose of gelatin. More importantly, albumin, by cellulose acetate electrophoresis (Microzone 110, Beckman Inst.) and fibrinogen as determined by immunoassay were unaltered.

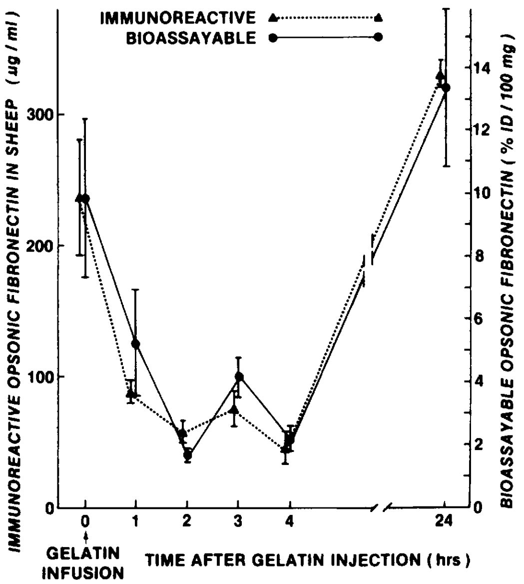

FIG. 4. Circulating bioassayable and immunoreactive plasma opsonic fibronectin in sheep after intravenous infusion of gelatin. The gelatin dose was 8.7 mg/kg body wt. Data are represented as the mean ± SE with three to seven sheep at each point. The decrease in immunoreactive opsonic fibronectin was significant ($P < 0.005$).

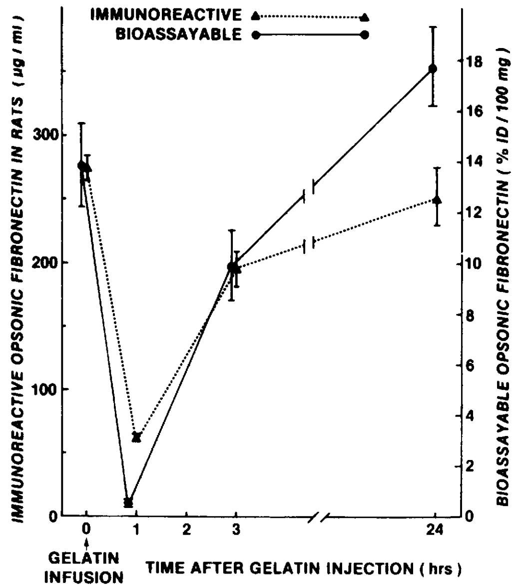

FIG. 3. Circulating bioassayable and immunoreactive plasma opsonic fibronectin in rats after intravenous infusion of gelatin. The gelatin dose was 10.6 mg/kg body wt. Data are represented as the mean ± SE with five to six rats at each point. The acute decrease was highly significant ($P < 0.001$).

The effect of preincubation of liver slices with gelatin on their subsequent phagocytic activity in normal rat plasma, is presented in Table II. The uptake observed at all gelatin levels was not different ($P > 0.05$) from that of controls incubated in a gelatin-free medium or nonincubated slices used fresh.

Discussion. Recent studies by Saba *et al.* (30) and Blumenstock *et al.* (4) have demonstrated that plasma fibronectin or cold-insoluble globulin (27, 37) is identical to opsonic α_2 surface binding (SB) glycoprotein in animals and humans. They have the same molecular weight and amino acid composition (4, 30). The ability for plasma fi-

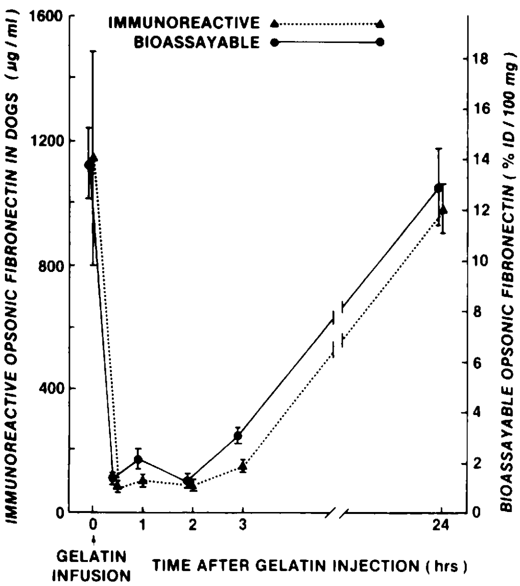

FIG. 5. Circulating bioassayable and immunoreactive plasma opsonic fibronectin in dogs after intravenous infusion of gelatin. The gelatin dose was 50.0 mg/kg body wt. Data are represented as the mean ± SE with five to eight dogs at each point. The depression in bioassayable and immunoreactive opsonic fibronectin was significant ($P < 0.02$ and $P < 0.001$, respectively).

bronectin to act as an opsonin for gelatin-coated particles has been confirmed by Molnar *et al.* (21), Doran *et al.* (8), and Gudewicz *et al.* (14) using peritoneal macrophages and recently reviewed in detail by Mosher (22). Such studies include isotopically labeled particle uptake and electromicroscopic data confirming binding and internalization. Moreover, the findings with the liver slice assay and the isolated peritoneal macrophages are similar in terms of response to plasma fibronectin (2). While most test colloids used have been gelatin-coated, the documentation of binding of fibronectin to *Staphylococcus aureus* (19) as well as the localization of this binding site to a 27-kdalton fragment of fibronectin (23) coupled with the increased bacterial phagocytosis by neutrophils due to fibronectin (20, 22) emphasizes the potential importance of fibronectin in antibacterial defense (33). This protein interacts with foreign and effete particulates to facilitate their removal from the blood and tissue spaces by RE cells, a process viewed as critical to systemic defense against organ microembolism during post-trauma sepsis or disseminated intravascular coagulation (24, 33).

Clinical studies in septic burn and trauma patients have documented a profound deficiency of this protein in the early postinjury period as well as during subsequent sepsis in association with multiple organ failure (29, 30, 33, 34). Reversal of opsonic deficiency can be accomplished in patients by the infusion of fibronectin-rich plasma cryoprecipitate (29). Reversal with the purified protein and/or cryoprecipitate in animals after surgery will also improve RES function (32). In such studies, the degree of deficiency as well as the response of the plasma fibronectin to cryoprecipitate infusion has been assessed by liver slice bioassay as well as electroimmunoassay (29, 34).

Plasma fibronectin exhibits affinity for fibrin and fibrinogen complexes (27), gelatin-coated colloids (3) as well as native and denatured collagen (9, 10). Fibronectin binds with actin (17) and may thus facilitate

TABLE I. CIRCULATING PLASMA TOTAL PROTEIN, ALBUMIN, FIBRINOGEN, AND OPSONIC FIBRONECTIN IN SHEEP PRIOR TO AND FOLLOWING INTRAVENOUS INFUSION OF GELATIN

Time after gelatin (hr)	Total protein[a] (g%)	Albumin[a] (g%)	Fibrinogen[a] (mg/ml)	Fibronectin[a] (µg/ml)
0	6.99 ± 0.45	2.46 ± 0.32	6.51 ± 0.65	237.0 ± 44.0
1	6.83 ± 0.50	2.39 ± 0.38	6.68 ± 1.18	88.5 ± 8.5[b]
2	6.64 ± 0.47	2.42 ± 0.29	6.33 ± 0.87	57.7 ± 8.3[b]
3	6.48 ± 0.45	2.56 ± 0.21	6.33 ± 1.13	75.1 ± 13.7[b]
4	6.47 ± 0.50	2.46 ± 0.36	6.68 ± 1.18	45.5 ± 12.7[b]

[a] A separate group of three sheep was evaluated. Gelatin was infused at a dose of 8.7 mg/kg. Each point represents the mean ± SE. Total protein was measured by the modified Biuret method; albumin by cellulose acetate electrophoresis; fibrinogen by radial immunodiffusion; and opsonic fibronectin by electroimmunoassay.

[b] Significantly decreased as compared to control level.

TABLE II. *In Vitro* Kupffer Cell Phagocytic Activity following Incubation of Rat Liver Slices for 15 Min at 37° in Varying Concentrations of Gelatin as Measured by Liver Slice Bioassay

Group	Percentage ID/100 mg	Significance (P)
Control[a]	13.82 ± 2.40	—
No gelatin (diluent)[b]	12.30 ± 1.55	0.61
100 μg gelatin[b]	13.90 ± 0.87	0.97
200 μg gelatin[b]	15.68 ± 2.15	0.58
300 μg gelatin[b]	17.42 ± 2.20	0.29

[a] The six control liver slices were not preincubated prior to their use in the assay.

[b] Each point represents mean ± SE of six assays with a mean liver slice weight of 290.1 ± 7.8. Each sample was supplemented with 300 μl of either vehicle or varying concentrations of gelatin in vehicle.

clearance of actin from the blood after tissue injury, especially skeletal muscle trauma. Fibronectin appears to have a higher affinity for gelatin (denatured collagen) than for native collagen (6, 9), which suggests that denaturation may expose binding sites which are masked in the native state. Transient RE blockade can be accomplished by injection of particulate matter such as gelatinized colloidal carbon (1), gelatinized RE test lipid emulsion (3, 31), or denatured microaggregated human serum albumin (36). RE blockade is apparently related to a parallel opsonic deficiency (3, 31) and not exclusively saturation of the Kupffer cells (3) as previously reviewed (28).

In the present study, the incubation of gelatin with plasma from sheep, rat, dog, and human results in a significant depression in the ability of the plasma to augment particle phagocytosis. This response was somewhat dose dependent although the dose dependency was not very striking by bioassay. This finding extends the observation of Filkins and Di Luzio (11) using gelatin-coated radiogold. Parallel determinations by electroimmunoassay also reveal this pronounced inhibitory influence of gelatin on immunodetectable fibronectin. Consistent with these *in vitro* findings was the observation that circulating bioassayable and immunoreactive opsonic fibronectin was also depressed *in vivo* in rats, sheep, and dogs after infusion of gelatin. Furthermore, species differences existed, such that dogs with high endogenous plasma fibronectin concentrations of 1050 ± 70 μg/ml required a greater amount of gelatin to produce opsonic depression than did sheep with much lower fibronectin levels of 184 ± 13 μg/ml. These observations collectively suggest that gelatin may bind to plasma fibronectin and that this interaction is responsible for the altered bioassayable opsonic activity and immunodetectable activity of the plasma as determined by electroimmunoassay. Whether gelatin binding to fibronectin alters its mobility in an electric field as used in Laurell rocket immunoelectrophoresis or blocks immunoreactive sites on the fibronectin is unknown.

Intravenous infusion of large doses of gelatin results in depression in particle clearance from the circulation by the RES (12, 26). Such gelatin challenge will also inhibit RES function in sheep and increase the degree of lung vascular injury seen with bacterial sepsis (25). The present data suggest that this depressed RES function may be due to a gelatin-induced depression in opsonic activity due to binding with fibronectin as opposed to early speculation of Koenig *et al.* (18) that gelatin may have some direct inhibitor effect on hepatic Kupffer cells. The present study indicates that a direct inhibitory effect is not very important in that preincubation of rat liver slices with increasing concentrations of gelatin in buffered Krebs–Ringer phosphate results in no decrease in liver slice phagocytic capacity. These findings combined with the *in vivo* and *in vitro* acute depletion of fibronectin by gelatin strongly suggest that gelatin's effect is directly on the opsonic fibronectin. The requirement for such a humoral opsonic factor for normal clearance of gelatinized colloidal carbon by Kupffer cells of the perfused rat liver has been documented (13). The present observations on the *in vitro* influence of gelatin on opsonic activity of human plasma also suggests that the suppression of RES phagocytic function in humans after intravenous gelatin infusion (36) may be mediated, in part, by its previously unsuspected binding to plasma fibronectin. Indeed, the use of gelatin as a plasma expander in shock patients warrants careful reevaluation, especially in view of the relationship between RES depression, opsonic deficiency, and susceptibility to shock and trauma (33).

While significant opsonic recovery was seen in the rat by 3 hr, a slower recovery was seen in the dogs and sheep. The observed recovery of opsonic activity in the dog and sheep may be related to residual gelatin in the circulation. Gelatin is cleared very slowly from the blood (18) and also distributes throughout the extracellular space. Thus, relatively large amounts may continue to circulate and complex with newly synthesized and/or released opsonic fibronectin. While this prolonged depressant effect suggests that gelatin infusion may be a useful approach to inhibit opsonic activity and RES function, one needs to be cautious in this approach. If the opsonin–gelatin complex remains in the blood for a prolonged interval, then the fibronectin could conceivably be available to opsonize other blood-borne particles depending on their affinity for fibronectin resulting in less RE depression than expected. In support of this possibility is the finding that the increased canine intestinal lymph flow during sepsis after particle-induced fibronectin depletion is not observed after infusion of soluble gelatin (7).

The immunoreactive opsonic fibronectin levels in the dog, rat, human, and sheep vary in decreasing concentrations, respectively, in these species (25). Accordingly, one might expect bioassayable opsonic activity to be greatest in the dog and progressively decrease in the rat, human, and sheep. However, since the bioassay employs a rat liver slice, the system's sensitivity may have been greatest for homologous rat plasma and then vary with heterologous plasma according to its concentration. Bioassayable opsonic activity was high for rat plasma and progressively decreased for dog and human plasma. However, bioassayable opsonic activity in sheep was higher than anticipated but the basis is unclear.

Opsonic fibronectin deficiency seen in septic injured patients or disseminated intravascular coagulation (24, 29, 33, 34) may be due to the consumptive utilization of fibronectin during RE clearance of blood-borne particulates as previously postulated. However, the present data suggest that deficiency may also be due to the binding of fibronectin to sites of vascular injury and collagen exposure or the presence of collagenous debris in the blood after soft tissue injury, which may alter detection of fibronectin by standard bioassay or electroimmunoassay.

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