Comparative Effects of Endotoxin and Gelatin on Reticuloendothelial Activity.* (32237)

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Gelatin, an acid or alkaline hydrolysis product of collagen, is widely employed as a stabilizing agent for inert colloids used to evaluate the phagocytic function of the reticuloendothelial system (RES). Indeed, when gelatin-stabilized colloids such as carbon or gold are phagocytized, the RES may be envisaged as "recognizing" and engulfing gelatinized entities rather than specific colloids. Although numerous studies have indicated an effect of the gelatin content on colloid intravascular removal rates(1-4), only recently have *in vitro* models such as the isolated perfused rat liver (5,6) and liver slices(7,8) allowed analysis of the mechanism of gelatin's influence on phagocytosis. On the basis of *in vitro* studies it appears that gelatin's mode of action involves union with a plasma phagocytosis-promoting-factor or "opsonin"(7).

The plasma constituent which reacts with gelatin and in some fashion thereby enhances phagocytosis by the RES has not been characterized. Perhaps it is similar to the "nonspecific opsonins" often espoused in leucocyte phagocytosis(9). In contrast, Murray(10) has suggested that the opsonin is specific agglutinating antibody to gelatin. Since a recent study indicated that germ-free rats possess the gelatin opsonin, a microbially induced agglutinin is improbable(11). Thus if the gelatin opsonin is immunological in character, it would appear to be a 'natural' antibody. In addition, since heparin is involved in the opsonization of gelatinized colloids, we have suggested that a heparin-gelatin-opsonin reaction scheme may be implicated(8). Recent *in vivo* studies have substantiated the influence of heparin and gelatin interactions on intravascular phagocytosis(12).

Because gelatin and gelatin-stabilized colloids are often pyrogenic(13,14) it is plausible that a pyrogen in gelatin is the active ingredient in the opsonin reaction(14). Due to the implications of this hypothesis to the opsonin concept, as well as the mode of physiologic disposition of endotoxins, the present study was undertaken to evaluate the importance of gelatin pyrogenicity in its interaction with the RES. While potent effects of endotoxin on the RES were demonstrated, the pyrogen content of gelatin *per se* was not substantiated as the primary determinant of gelatin's influence on the RES.

Methods. Intravascular clearance half-times for colloidal carbon (C11/1431a, Pelikan-Werke, Hanover, Germany) were measured in male Holtzman rats of 250-300 g body weight. The basic procedure employed was modified from Biozzi *et al*(15) and described in detail previously(12). In essence, 16 mg/100 g of colloidal carbon was injected intravenously and timed blood samples were obtained, lyzed and the carbon concentrations determined spectrophotometrically. Intravascular half-times were calculated from semi-logarithmic plots of carbon concentration versus time in minutes.

Pyrogen assays were performed in male New Zealand white rabbits of 2-2.5 kg body weight by measuring rectal temperatures with a thermistor probe. Pyrogen test injections of endotoxin or various gelatins were made into the marginal ear vein and the fever was quantitated as the integrated area under a plot of time in hours *vs* rectal temperature in °F. As suggested by Keene *et al*(16) a fever index of 100 was assigned to a 1°F rise maintained for 1 hr. The isoelectric pH ranges of various gelatins were estimated using turbidimetric measurements(17) of 3% to 10% gelatin solutions in saline at 375 mμ in a Beckman Model B Spectrophotometer. Gelatin preparations were purchased from J. T. Baker Chemical Co., Phillipsburg, N. J.; Coleman, Matheson and Bell, Rutherford, N. J.; Difco Laboratories, Detroit, Mich.; Gunther-Wagner, Hanover, Germany; Knox

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TABLE I. Effects of Acid- and Alkaline-Precursor Commercial Gelatins on Carbon Phagocytosis† in the Rat and Rectal Temperature in Rabbits.

Gelatin	Rat colloidal carbon half-time (min)	Rabbit 5-hr fever index‡	Gelatin isoelectric pH range
Sigma	148* ± 20 (10)	457* ± 82 (5)	8–9
Difco	72* ± 12 (7)	627* ± 51 (5)	8–9
Nut. Bio. Corp.	70* ± 10 (13)	600* ± 59 (11)	8–9
Knox	48 ± 5 (7)	866* ± 110 (5)	4–5
Cole.-Math.-Bell	40 ± 4 (5)	348* ± 29 (5)	4–5
J. T. Baker	36 ± 6 (9)	191 ± 77 (5)	4–5
Gunther-Wagner	29 ± 4 (6)	529* ± 83 (5)	4–5
None (saline control)	39 ± 4 (7)	95 ± 39 (5)	

* $p < .05$ as compared to saline control group.
Values are means ± S.E. Number of animals per group in parentheses.
† Colloidal carbon was administered 30 min after intravenous injection of 100 mg of the various gelatins.
‡ 100 mg of gelatin in saline was administered intravenously and fever index calculated such that 100 = 1°F/1 hr.

Gelatin Co., Inc., Camden, N. J.; Nutritional Biochemicals Corp., Cleveland, Ohio; and Sigma Chemical Co., St. Louis, Mo. Endotoxin was obtained from Difco Laboratories as the Boivin lipopolysaccharide of *Salmonella enteriditis*, Lot Nos. 181150 and 171033.

Half-time values and fever indices were evaluated for statistical significance at the 95% level of confidence using Student's "t" test.

Results. Effect of pre-treatment with various gelatins on colloidal carbon phagocytosis. Many laboratories which employ the procedure of Biozzi *et al*(15) to evaluate RE function with colloidal carbon dilute the commercial Pelikan to a suitable injection concentration with gelatin solutions. To evaluate the variability of individual commercial gelatin preparations, 7 gelatins from various suppliers were tested for their effects on colloidal carbon phagocytosis. As indicated in Table I, only 3 of 7 gelatin preparations administered intravenously depressed a subsequent phagocytic test dose of colloidal carbon. Since we contend the mechanism of gelatin's action involves combination with a plasma opsonin, these data may reflect that only certain gelatins can react with opsonin, deplete the circulating supply, and thus reduce the rate of removal of a subsequent carbon test injection.

Pyrogenicity of commercial gelatins and Pelikan ink. One attribute of the various gelatins which might impart opsonin specificity is their relative pyrogen content. To evaluate this point, the 7 gelatins were tested for pyrogenicity in rabbits and a correlation computed between pyrogenicity and depression of carbon removal (Table I). No significant correlation ($r = +0.143$) existed between gelatin pyrogenicity and carbon clearance inhibition—*e.g.*, Knox gelatin was a potent pyrogen but no significant effect on carbon clearance. However, all 3 gelatins which depressed carbon phagocytosis were acid precursor types with alkaline isoelectric ranges. Perhaps, therefore the chemical nature and charge of the gelatin are the determining influence in moderating phagocytic activity.

To assess the nature of the pyrogen in gelatin, cross-tolerance experiments were performed between a pyrogenic gelatin (Nutritional Biochemicals Corp.) and endotoxin. As indicated in Table II, tolerance was induced both to endotoxin and gelatin pyrogenicity. Furthermore, definite cross-tolerance was manifest between rabbits tolerant to endotoxin as well as gelatin pyrogenicity. In addition, commercial Pelikan ink was pyrogenic in rabbits and 80 mg/kg carbon dose was about equivalent in pyrogenicity to 0.6 μg of *S. enteriditis* lipopolysaccharide as compared on a dose-response curve (Fig. 1).

Comparative effects of endotoxin and gelatin on intravascular carbon phagocytosis. To evaluate further the role of gelatin pyrogenicity in its interaction with the RES, studies were performed which directly com-

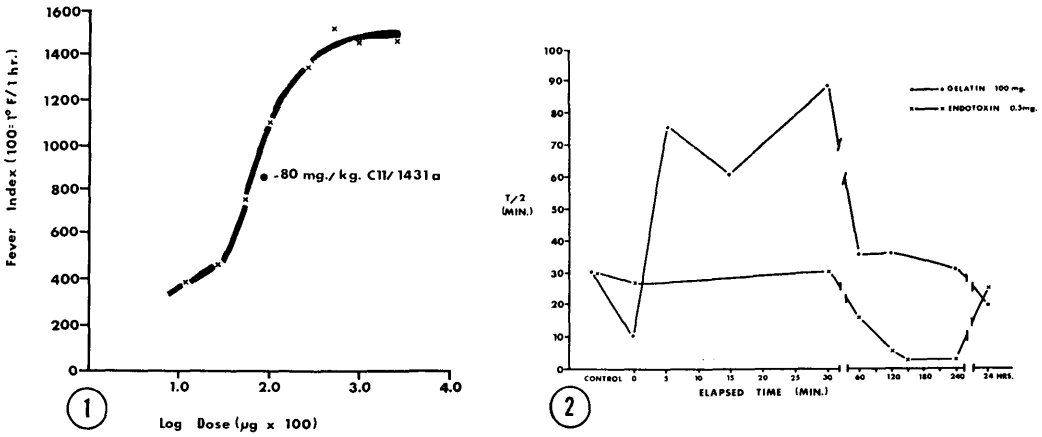

FIG. 1. Pyrogenicity of colloidal carbon ink (C11/1431a) as compared to dose-response curve for *Salmonella enteriditis* endotoxin pyrogenicity. 5 hour fever in dex calculated from average of 5 rabbits per point.

FIG. 2. Time course of effects of a single intravenous injection of gelatin (Nutritional Biochemicals Corp.) or endotoxin (*S. enteriditis*) on vascular clearance of colloidal carbon (16 mg/100 g C11/1431a). Gelatin or endotoxin was administered in colloidal carbon to obtain the zero time values. 6 to 12 rats per point.

pared the response of the RES to gelatin and endotoxin in both acute and chronic injection regimens. As indicated in Fig. 2, intravenous pre-treatment with 100 mg of gelatin (Nutritional Biochemicals Corp.) produced a transient depression in carbon clearing ability which was in effect for about 60 minutes and then receded. In addition, as reported previously(12), gelatin incorporation into the carbon test dose significantly reduced the half-time as indicated by the zero time value in Fig. 2. In contrast to gelatin, endotoxin (0.5 mg, *S. enteriditis*) produced neither an augmentation of clearance when incorporated into the test dose, nor an early depression of phagocytosis analogous to gelatin; however, the removal of colloidal carbon was significantly enhanced 2 and 4 hours after endotoxin administration. Normal phagocytic activity was however manifested at 24 hours after endotoxin administration.

TABLE II. Cross-Tolerance of Fever Responses to Intravenous Administration of Gelatin and *S. enteriditis* Lipopolysaccharide (LPS).

Group		Pyrogenic test agent	5-Hour fever index (mean ± S.E.)
Saline controls*	(17)	LPS	1340 ± 69
" "	(16)	Gelatin	635 ± 61
Chronic LPS	(7)	LPS	463 ± 75
" "	(6)	Gelatin	121 ± 43
Chronic gelatin	(7)	LPS	553 ± 45
" "	(6)	Gelatin	203 ± 47

Chronic injection regimens:

Days 1-6: Gelatin—100, 100, 200, 200, 400, 400 (mg/day)
LPS—2.5, 2.5, 5.0, 5.0, 10, 10 (μg/day)

Days 7: Pyrogenic test agent dose—Gelatin 100 mg; LPS 2.5 μg

No. of rabbits per group in parentheses.

* Saline controls received 1 ml of pyrogen-free saline daily for 6 days.

When multiple injections of gelatin (Nutritional Biochemicals Corp.) were administered, no striking effects were observed on either carbon phagocytosis or splenic weights (Table III). In contrast the chronic intravenous injection of endotoxin markedly augmented carbon clearances and produced a striking splenomegaly which is a sensitive indication of RES proliferation. No significant changes in body weights were observed in the endotoxin group. Thus gelatin and endotoxin were not comparable in their effects on the RES in either an acute or chronic model.

Discussion. The reticuloendothelial system (RES) has long been implicated in the physiological and pathological effects of endotoxin (18,19). In addition, Freedman(20) and Greisman *et al*(21) have recently implicated an opsonin with high endotoxin specificity in

TABLE III. Alterations in Phagocytic Activity and Spleen Weights After Chronic Endotoxin and Gelatin Injections.

Group	Colloidal carbon half-time (min)	Spleen (% body wt)
Control (12)	27 ± 6	.30 ± .08
Gelatin (8)	22 ± 4	.38 ± .09
Endotoxin (6)	4 ± 1*	.95 ± .18*

Chronic injection regimens:

Days 1-6: Gelatin—100, 200, 200, 200, 400, 400 (mg/day)
LPS—0.5, 0.5, 1.0, 1.0, 2.0, 2.0 (mg/day)

Days 7: (Clearance of 16 mg/100 g colloidal carbon and spleens weighed)

No. of rats per group in parentheses. Values are means ± S.E.

* p value <.001 as compared to either of the above groups.

the mechanism of endotoxin fever tolerance. Thus, since gelatin and commercial Pelikan ink are pyrogenic, the suggestion that opsonins may be interacting with endotoxin in the carbon-gel preparation seemed reasonable and worthy of evaluation. The data presented in these studies however do not substantiate a significant role for gelatin pyrogenicity in the effects of gelatin on RES phagocytosis. Rather it appears more likely that the chemical nature of the gelatin as reflected in the isoelectric pH value may determine its interaction with plasma opsonin. This suggestion may explain the diverse effects of different gelatins reported in the literature(22-24). Furthermore since acid-precursor gelatins are sensitive to the presence of small ions, the influence of various ions in the suspending media(25) on carbon clearance may reflect effects on gelatin charge and hence opsonin binding. Since heparin is involved in the opsonization process a complex between heparin, acid-precursor gelatin, and plasma opsonin appears feasible.

The marked augmentation of carbon clearance at 2 hours after endotoxin confirms the recent observation of Arredondo and Kampschmidt(26). This hyperphagocytic state is strikingly contrary to the hypofunction which accompanies endotoxin administration in the mouse and rabbit(18) and merits further study.

The chemical nature of the gelatin preparation and fact that various gelatins as well as colloidal carbon may contain endotoxin should be considered in evaluating studies employing these agents for colloid stabilizers and RE functional assessment.

Summary. Colloidal carbon ink (C11/1431a) and various commercial gelatin preparations were pyrogenic in rabbits. Cross-tolerance studies indicated the existence of endotoxin in pyrogenic gelatins. Three of seven gelatin preparations depressed carbon phagocytosis in rats when administered 30 minutes prior to the clearance test; however, no significant correlation existed between gelatin pyrogenicity and depression of phagocytosis. All 3 RE interacting gelatins had alkaline isoelectric ranges. Gelatin pretreatment depressed a subsequent carbon clearance while endotoxin induced a marked but delayed increase in carbon phagocytosis. Chronic injections of endotoxin markedly stimulated the RES as reflected in enhanced phagocytosis and splenomegaly; in contrast, chronic gelatin administration produced no such effects. It appears that the influence of gelatin on the RES is not mediated solely by a pyrogenic endotoxin contaminant.

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Species Differences in Methemoglobin Levels Produced by Administration of Monomethylhydrazine.* (32238)

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Previous studies of the effects of monomethylhydrazine (MMH) demonstrated that significant levels of methemoglobin were found in the blood of anesthetized dogs receiving I.V. injections of MMH(1,2). Additional experiments demonstrated that methemoglobin were also produced *in vitro* during incubation of MMH with either canine or human blood(2).

Further studies have been conducted with rats, rabbits, and guinea pigs. The results reported here show that in blood of these 3 species methemoglobin levels found *in vivo* after injection of MMH are markedly lower than previously found in dogs. This species difference was demonstrable also during *in vitro* incubations, when peak methemoglobin levels in rat, rabbit, or guinea pig blood incubated with MMH were much lower than in human or canine blood similarly incubated with MMH.

Procedures. In vivo *studies.* To determine the effects of MMH on methemoglobin levels *in vivo,* methemoglobin content was measured in blood samples taken just before and at intervals after injection of either MMH solution or physiological saline solution.

All studies utilized male animals. Rats were of Sprague-Dawley descent, guinea pigs were albinos of the Hartley strain, and rabbits were New Zealand whites. Monomethylhydrazine (MMH) was obtained from the Eastman Kodak Co. Appropriate dilutions were prepared fresh daily from liquid MMH using physiological saline solution as a diluent. Control animals received injections of corresponding volumes of physiological saline solution. Guinea pigs were injected *via* cardiac puncture during CO_2 anesthesia; rats were injected intravenously *via* the tail vein, and rabbits *via* the marginal vein of the ear. Blood samples were obtained from rats by bleeding from the tail, from guinea pigs by cardiac puncture during CO_2 anesthesia, and from rabbits by bleeding from the marginal vein of an ear.

Blood samples were taken at zero time just before injection of either MMH or saline solution and at selected times thereafter.

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