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Alteration of RES Phagocytic Function by Pyrogen Contaminated Colloidal Carbon.* (29992)

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Various colloidal preparations have been used to study the phagocytic function of the reticuloendothelial system (RES) often with variable and conflicting results. One prerequisite for such studies is that the colloid be non-toxic for RE-cells and the animal(1). Halpern *et al*(2) found that the toxic component of India ink was an organic shellac. They subsequently obtained a special preparation of India ink in which "fish glue" was substituted for shellac. This special ink ("Special Biological Ink" C11 1431a) is available commercially and is widely used in studies of RES phagocytic function. From the literature concerning pyrogenic therapy, it is apparent that proteinaceous substances free of pyrogen are exceptional. Seibert's work(3) is outstanding in showing that pyrogens are almost exclusively products of Gram-negative bacteria.

In preliminary studies we observed considerable pyrogenic response in rabbits after intravenous injection of the special biological ink. This report gives the results of further studies relating to this observation.

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Materials and methods. Colloidal carbon. "Special Biological Ink": C11 1431a (Gunther Wagner Co., Hannover, Germany) was obtained from the John Henschel Co., New York. It was prepared in 1% gelatin (Difco, certified) to a carbon concentration of 16 mg/ml after the large aggregates had been removed by centrifugation at 2,930 *g* for 15 minutes. The dry weight of carbon remaining was 110 mg/ml. The carbon suspension was then autoclaved at 15 psi for 15 minutes and stored in sealed 10 ml glass vials at 4°C. It was warmed to 37°C and thoroughly mixed before use.

Endotoxin. The purified *Escherichia coli* 08 $C008_{2158}S_5$ endotoxin was obtained from Dr. Otto Westphal, Max Planck Institute, Freiburg, West Germany. The dose used, 0.2 μg/kg, was equivalent to 25 MPD-3/kg (Minimal Pyrogenic Dose at 3 hours)(4).

Test animals. American-Dutch rabbits were obtained from a single supplier. They were housed in air-conditioned quarters and the clearance tests were performed in a similar environment. Adult animals were selected to weigh 1.0 to 1.5 kg. Young animals weighed on the average 0.3 kg.

Cortisone treatment of rabbits. A group

of normal adult rabbits, 1.5 kg avg wt, were injected intramuscularly with 16 mg/kg of cortisone (cortisone acetate, Merck) daily for 3 days. The third injection was given 1 hour before clearance of pyrogen tests.

Alkali treatment of the special ink. An equal volume of 10 N sodium hydroxide was added to a measured volume of the supernatant after 15 minutes' centrifugation of the ink at 2930 *g*. These reactants were heated slowly to boiling and refluxed for 15 minutes, after which an additional volume of alkali was added and refluxed for 15 minutes. The mixture was cooled and centrifuged at 1100 *g* for 30 minutes. The precipitate was resuspended in 100 ml of sterile pyrogen-free saline and centrifuged at 700 *g* for 20 minutes. The precipitate was resuspended in 50 ml of saline and heated to boiling. The final centrifugation was done while the suspension remained hot, at 700 *g* for 20 minutes. The final precipitate was resuspended in a small volume of pyrogen-free saline, adjusted to pH 7.0, and brought to original volume with additional saline. For pyrogen tests, the hydrolyzed carbon suspension was further diluted to 16 mg/ml with sterile homologous rabbit serum or with 1% pyrogen-free Knox gelatin solution (Knox Gelatin Protein Products, Inc., Camden, N. J.) just before use; saline diluted suspensions of hydrolyzed carbon without added colloids were not sufficiently stabilized. Microscopic examination did not reveal surface changes of the carbon particles after treatment.

Clearance test. The method was similar to that of Biozzi, Benacerraf and Halpern(5). Since our animals are fed in the morning, the rabbits were weighed on the evening before the test, to facilitate dose quantitation. They were conditioned in a retaining rack designed for pyrogen studies 3 to 5 hr one day or more prior to testing. On the morning of the test, the animals were positioned in the rack and rectal electrodes for fever measurement were connected. Animals with temperatures lower than 100°F or higher than 102°F after 1 hour in this position were not used. It was routine procedure to measure temperatures at 30-minute intervals following an initial 1-hour rest period before clearance tests and to continue for 5 hours. Control samples of blood were taken from a marginal ear vein of each animal using standard diameter capillary tubes (Scientific Products, Evanston, Ill.) previously rinsed with heparin. These tubes containing 0.01 ml of blood were immediately dropped into test tubes containing 2.0 ml of sodium carbonate and mixed to lyse the erythrocytes. The dose of colloidal carbon was then injected into a marginal vein of the opposite ear. The 0-time test samples of blood were taken immediately, and at 10-minute intervals for 30 minutes in the same manner described for control samples. Clearance of colloidal carbon from the blood is exponential with respect to time. The rate constant "K," referred to as the phagocytic index, was calculated by the following equation:

$$K = \log C_o - \log C/T - T_o$$

where C_o and C are blood carbon concentrations at time T_o and T. Blood carbon concentrations were estimated from optical density readings by comparison with a standard curve. Readings were made on the Beckman DB spectrophotometer at wavelength 710 mμ. K-values are presented as the mean for 3 readings at 10, 20, and 30 minutes. It has been our experience that mean K-values for groups of 5 or more healthy control animals may vary by a factor of ± 0.005.

All glassware was handled under conditions to avoid pyrogen contamination and water used for solutions was deionized distilled and pyrogen free.

Results. Pyrogenic activity of special biological ink: C11 1431a. Fig. 1 shows a plot of rabbit fever responses to the preparation in 2 different diluents. Curve (b) showing the highest peak, 3.4°F at 4 hours, was the response to 8 mg carbon/100 g body weight, diluted in 1% gelatin (Difco) in saline. Curve (a) showing the second highest peak, 2.5°F at 1½ hours, was the response to the same dose of carbon diluted in pyrogen-free, physiologic saline. The greater response induced by the gelatin suspended preparation is a combined effect, since we observed that Difco gelatin is also pyrogenic. These results and the shapes of the fever curves in-

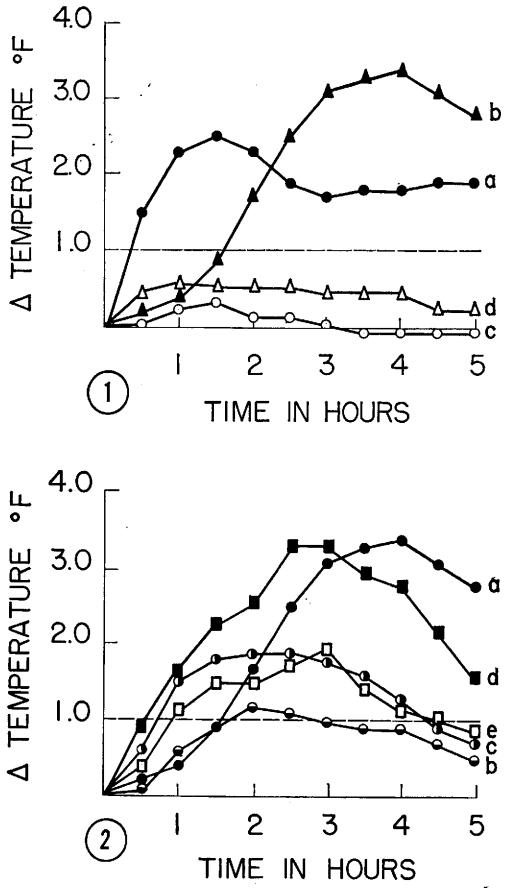

FIG. 1. Pyrogenic activity of special biological ink C11 1431a and its inactivation by treatment with alkali. 8 mg/100 g carbon (C11 1431a) was administered intravenously after preparation as follows: curve a, diluted in pyrogen-free saline; b, diluted in 1% gelatin (Difco); c, alkali treated and resuspended in homologous serum; d, alkali treated and resuspended in 1% gelatin (Knox). Each curve represents the mean fever response of at least 5 adult rabbits (1.0-1.5 kg).

FIG. 2. Minimal pyrogenic activity of special biological ink C11 1431a in young rabbits (0.3 kg avg) and in cortisone-treated adult rabbits. 8 mg/100 g carbon (C11 1431a) diluted in 1% gelatin (Difco) was administered intravenously to rabbits as follows: a, non-treated adults; b, non-treated young; c, cortisone-treated adults. Curves (d) and (e) represent responses of non-treated and cortisone-treated adults respectively to a small dose (0.2 μg/kg) of purified endotoxin. Each curve represents the mean fever response of at least 5 rabbits.

dicated pyrogen contamination of the special ink.

Enhancement of carbon clearance by repeated injection. On the basis of the ability of endotoxin to stimulate the rate of carbon clearance(6), the ability of colloidal carbon alone, after repeated injections, to enhance homologous clearance was tested. Table I shows results obtained with a group of rabbits given 3 intravenous injections of carbon. The rate of clearance of each of 3 spaced doses, initial, 3-hour, and 48-hour, was measured. The rate of clearance of the 48-hour dose (0.015) was twice that of the initial and 3-hour doses (0.007 and 0.006). The fact that the rate at 3 hours for the second dose of carbon, making a total of 32 mg/100 g, was not slower than the initial dose points out the difficulty of complete physical blockade of the RES in rabbits, and the stimulation at 48 hours was not unlike that observed in endotoxin treated rabbits. Autopsy performed on these animals revealed carbon congestion of all internal organs, with high concentrations in the bone marrow.

Enhanced clearance rate and minimal pyrogenicity of colloidal carbon in young rabbits. Studies in this laboratory(7) revealed that young rabbits are more resistant to lethal and pyrogenic effects of endotoxin than are adults. It was suggested that adult rabbits are more hypersensitive to endotoxin than the young. With these results in mind, carbon clearance experiments were done in young rabbits. Fig. 2 reveals that only a minimal fever response was induced in the young rabbits (b) as compared with adult controls (a). The fever response induced in young rabbits of 1.2°F at 2 hours is just above the range of normal variation and the 3-hour hypersensitive phase (7) of the curve is absent. Table II shows that the carbon clearance rate is twice as fast as that of adults. These results show that the colloid is less toxic to the young rabbit, and is a factor which may be associated with their more rapid clearance rate.

TABLE I. Enhanced Rate of Clearance as a Result of 3 Spaced Injections of Colloidal Carbon* in Adult Rabbits.†

Interval between carbon injections	Phagocytic index (K)
Initial	.007
3 hr	.006
48 "	.015

* 16 mg/100 g body weight.

† The group consisted of 7 animals; 1 died prior to the 48-hr test.

TABLE II. Enhanced Rate of Clearance of Colloidal Carbon* by Young Rabbits.†

Age	Phagocytic index (K)	Body wt
Adult	.028	1.3 kg avg
Young	.046	.3 " "

* 8 mg/100 g body weight.
† 8 and 9 animals in each group respectively.

Enhanced clearance rate and reduced pyrogenicity of colloidal carbon in cortisone treated adult rabbits. From results of the previous experiment, it appeared that adult rabbits would clear the colloid at a faster rate if it could be made less toxic. Chemical inactivation of the colloid pyrogen was discarded after a few attempts, since the colloidal state of the particles was destroyed making them unsuitable for clearance tests. As an alternative method, modification of host response to pyrogen by cortisone treatment was done. A comparison of the fever responses of cortisone treated adult rabbits to a small dose (25 MPD-3/kg) of purified endotoxin and to the clearance test dose (8 mg/100 g) of carbon is presented in Fig. 2. The cortisone treated groups (e and c) responded with considerably less fever than control animals (a and d) in both cases. As predicted, the clearance rate of colloidal carbon in cortisone treated rabbits (Table III) was about twice that of non-treated control animals.

Inactivation of pyrogen in colloidal carbon by alkali treatment. The exceptional heat stability of pyrogens or endotoxins is well known. Their pyrogenic activity, however, can be inactivated by heating in acid or alkali. As shown in Fig. 1, colloidal carbon treated with sodium hydroxide and resuspended in either normal homologous rabbit serum (c) or pyrogen-free gelatin (d) did not induce fever.

TABLE III. Enhanced Rate of Clearance of Colloidal Carbon* by Cortisone Treated Adult Rabbits.†

Treatment	Phagocytic index (K)
Controls	.028
Cortisone treated	.049

* 8 mg/100 g body weight.
† 8 and 10 animals in each group respectively.

Detection of bacteria in colloidal carbon. Routine bacteriological isolation techniques were employed. A newly purchased batch of special biological ink: C11 1431a, which was sealed on arrival, contained 1,000 viable bacteria per ml by the pour-plate method. Direct microscopic examination of the sediment after centrifugation at 2,930 *g* revealed both Gram-positive and Gram-negative species.

Discussion. The special ink used in this study, and used widely in RES studies, was originally prepared to eliminate a toxic shellac component(5) found in conventional India ink of the same brand. Our observations indicate that the special preparation (Special Biological Ink: C11 1431a) contains a toxic component of biological origin.

While this work was in progress, pyrogen contamination of several brands of India ink was reported(8); however, the special ink with which our study has been concerned was not specified. Our pyrogen test of the special colloidal carbon resulted in biphasic fever curves characteristic of endotoxin(4,7). We found that a series of injections of the preparation alone resulted in an increased rate of clearance of the same colloid (Table I). A colloid loading effect, in which the rate of clearance was found to return to normal after an initial condition of RES blockade with saccharated iron oxide has been reported(9); but with preparation C11 1431a, we observed an enhanced rate of clearance at 48 hours, a time at which purified endotoxin greatly stimulates RES phagocytosis. The reduced toxic effects of the colloid in young rabbits (Fig. 2) is in agreement with another report from this laboratory(7) which showed that young rabbits are more resistant to endotoxin due to a suggested lack of development of hypersensitivity. Conceivably related to this is our finding that the young rabbit is able to phagocytize the colloid at a more rapid rate than the adult (Table II). It has been reported that young animals have "more active RE-cells"(1). We agree, but with further clarification, *i.e.*, RE-cells of the young rabbit are less adversely affected by the toxic component of the colloid than the more hypersensitive adult. This is supported by our finding that modification of the response of adult rabbits to colloidal carbon-

toxicity by cortisone treatment (Fig. 2) resulted in enhanced clearance (Table III) which was similar in degree to that of young rabbits. Protection of spleen cells from the cytotoxicity of endotoxin has been demonstrated *in vitro*(10).

It is likely that the "non-specific opsonins" thought to operate against any and all "inert" colloidal particles(11) are directed toward contaminating pyrogens or endotoxin present on these colloids.

The ideal proof that the pyrogen is one of the known endotoxins would be a demonstration of cross tolerance(7). Our experiments designed to test this hypothesis were inconclusive. In one such test, animals made tolerant to *E. coli* (C008) endotoxin were also tolerant to the carbon pyrogen, but attempts to repeat this effect failed. A degree of specificity of endotoxin pyrogenic tolerance was recently reported from this laboratory(7). Endotoxin from one Gram-negative species was found to confer only partial cross tolerance to the endotoxin of another Gram-negative species. It is conceivable that endotoxin produced in mixed culture, as was likely the case due to accidental contamination in manufacture of colloidal carbon, would give only occasional and partial cross tolerance.

Inactivation of the pyrogen in colloidal carbon by alkaline treatment (Fig. 1) which did not destroy the carbon particles makes it doubtful that particles *per se* are pyrogenic. The most likely explanation is that there are few, if any, pyrogen-free or "inert" colloids. Additional excellent evidence for this concept is the recent work of Kobayashi and Friedman(12), which showed that the pyrogenic effect induced in rabbits by polystyrene latex particles could also be eliminated by alkaline and acid treatment without destruction of the particles. Adsorption of acid-treated particles with endotoxin restored pyrogenicity, which was not removed by conventional washings with pyrogen-free saline.

Summary. Evidence is presented that a special preparation of colloidal carbon (C11 1431a), which is used widely in studies of phagocytic function of the reticuloendothelial system, contains pyrogen. The carbon preparation induced febrile responses in adult rabbits with characteristics of endotoxin; this activity was eliminated by treatment with alkali. Repeated injections of the preparation stimulated its rate of clearance. Young rabbits showed minimal fever responses to the preparation and exhibited enhanced clearance rates in comparison with adults. Cortisone treated adult rabbits showed clearance rates and fever responses similar to that of young rabbits. It is suggested on the basis of these altered responses that pyrogen contained in the carbon preparation can influence tests in which it is used.

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