Induction of Acalculous Cholecystitis and Pneumonitis in Dogs Following Inhalation of Constituents of Cigarette Smoke Condensate

LARRY DILLON, MD, FRANK GLENN, MD, and CARL G. BECKER, MD

From the Departments of Pathology and Surgery, New York Hospital–Cornell University Medical Center, New York, New York

In previous studies in this laboratory it was demonstrated that 1) constituents of the water-soluble phase of cigarette smoke condensate can activate Hageman-factor–dependent pathways of coagulation, fibrinolysis, and kinin generation[1]; and 2) that *in vivo* activation of Hageman-factor–dependent pathways by intravenous injection of plant polyphenols in dogs and African Green monkeys can induce acute acalculous cholecystitis and alveolitis.[2] The purpose of this communication is to report that inhalation of the water-soluble, nondialyzable constituents of cigarette smoke condensate, or "tar," can activate Hageman-factor–dependent pathways in the dog and induce acute acalculous cholecystitis, pneumonitis, and the formation of thrombi in branches of pulmonary vessels. (Am J Pathol 1982, 109: 253–258)

Materials and Methods

Preparation of Tar-Derived Material (TDM)

Cigarette smoke condensate prepared from 1R2 cigarettes was supplied through the courtesy of Dr. Thomas Osdene, Philip Morris Tobacco Co., Richmond, Virginia. Thirty-three grams of condensate was extracted for 18 hours with stirring in 400 ml of barbital-acetate-NaCl buffer, pH 8.9, at 56–60 C. pH was maintained by dropwise addition at intervals of 1N NaOH. The solution was clarified by centrifugation and the supernate delipidized by sequential extraction with petroleum ether and diethyl ether. The aqueous phase was then dialyzed exhaustively against distilled water at 4 C and then lyophylized. The yield of highly water-soluble powder is approximately 30 mg/g of tar. The lowest molecular weight species in this material is 2500 daltons, as determined by exclusion chromatography on a 2.5× 100-cm column of Bio-Gel P-10 (Bio Rad Laboratories, Richmond, Calif) equilibrated with phosphate-buffered physiologic saline, pH 7.4 (PBS). Prior to use in experiments described below, TDM was weighed, dissolved in sterile pyrogen-free saline, and passed through a sterile Millipore filter of 0.25-μ pore size.

Effect of Exposure of Dog Plasma to TDM on Partial Thromboplastin Time and Fibrinolytic Activity

Partial Thromboplastin Time

Whole blood was obtained from normal mongrel dogs or from dogs used as experimental subjects by sterile venipuncture and anticoagulated with Na citrate. Platelet-poor plasma was obtained by centrifugation. All procedures were performed with plastic tubes and pipettes.

A fibrometer (BBL, Baltimore, Md, plastic cups, plastic pipette tips, and the following reagents were

Supported in part by NHLBI Research Grants HL 01803 and HL 18828, the Theodore Dubin Fund of Cornell University Medical College, and grants from the Cross Foundation and the Harvey and Beverly Karp Foundation.

Dr. Dillon was fellow of the Lewis Cass Ledyard Fund of New York Hospital.

Dr. Glenn died January 13, 1982.

Accepted for publication June 25, 1982.

Address reprint requests to Dr. Carl G. Becker, Department of Pathology, New York Hospital-Cornell Medical Center, 1300 York Avenue, New York, New York 10021.

0002-9440/82/1210-0253$01.10

used: celite (Filter Aid, Fisher Scientific Co., Pittsburgh, Pa) in PBS; rabbit brain cephalin (Sigma Chemical Co., St. Louis, Mo) in glyoxaline buffer; and 0.05 M $CaCl_2$ in glyoxaline buffer. In studies of the effect of TDM added to normal dog plasma *in vitro*, 0.1 ml of plasma was pipetted in a plastic cup and warmed at 37 C for 60 seconds. Celite suspension, 0.1 ml, TDM in PBS, or PBS as control and 0.05 ml of cephalin suspension was added; and the mixture was incubated for 60 seconds. $CaCl_2$, 0.05 ml, was then added, and the timer and probes were started. The timer stops when a clot forms. In studies of plasma samples taken from dogs during the course of the experiments described below, 0.1 ml of plasma was warmed for 60 seconds at 37 C. Celite suspension, 0.1 ml, or PBS and 0.05 ml of cephalin suspension was then added and incubated for another 60 seconds before addition of 0.05 ml of $CaCl_2$ solution and activation of the probes and timer.

The celite-activated partial thromboplastin time (PTT) of normal dogs ranged between 35 and 62 seconds. The unactivated partial thromboplastin time ranged between 65 and 185 seconds. All PTTs represent the mean of two determinations, and the coefficient of variation between duplicate samples was approximately 6%. Because of variation in PTT between different dogs, in experiments described below a baseline plasma sample was obtained from each dog, and subsequent samples were compared with it in measurement of unactivated PTT. The data are, therefore, expressed as percentage change from baseline.

Fibrinolytic Activity

In studies of the effect of addition of TDM on the fibrinolytic activity of normal dog plasma, 0.1 ml of TDM diluted in PBS or PBS as control was added to 0.1 ml of plasma. To this was added 2.0 ml of Na acetate buffer, pH 4.5; and the mixture was incubated at 37 C for 60 minutes. Plasma, 0.5 ml, was also mixed with 0.5 ml of celite suspension and 10 ml of acetate buffer and incubated for 60 minutes at 37 C. The euglobulin precipitate was pelleted by centrifugation and redissolved in barbital buffer, pH 8.4, in volume equal to the initial aliquot of plasma. The euglobulin precipitate from plasma samples incubated with celite was serially diluted in twofold steps in barbital buffer, and 0.02 ml of each of these dilutions was added to 4-mm wells cut in fibrin plates, prepared from fibrinogen free of plasminogen, as described by Granelli-Piperno and Reich.[3] Aliquots (0.02 ml) of the redissolved euglobulin precipitate from plasma incubated with dilutions of TDM or of PBS were also placed in such wells. The plates were sealed and incubated for 16 hours at 37 C. The diameter of the clear zones indicating fibrinolysis was measured. A standard curve was prepared in which the diameters of zones of fibrinolysis produced by serial twofold dilutions of euglobulin precipitate from plasma incubated with celite was plotted against the reciprocal of the dilution on semilog paper. The zone of fibrinolysis produced by 1:1 dilution of celite-treated plasma was taken as 100% of maximal activity, the 1:2 dilution as 50%, and so forth. Zones of fibrinolysis produced by plasma treated with dilutions of TDM or PBS were compared with this curve.

These curves were linear and highly reproducible. The coefficient of variation between duplicate samples was 4.5%.

In the study of plasma samples taken from dogs during the course of experiments described below, we prepared the euglobulin precipitate from baseline and subsequent samples from each dog by diluting 0.1 ml of plasma in 2 ml of acetate buffer and incubating it at 4 C for 60 minutes. The euglobulin precipitate was dissolved in 0.1 ml of barbital buffer, and 0.02 ml of this was placed in wells in fibrin plates as described above. The zones of fibrinolytic activity were compared with a standard curve prepared from the baseline sample treated with celite as described above.

Experimental Plan

Ten mongrel male dogs weighing between 8 and 15 kg were used in these experiments. They were divided into four groups: dogs that were subjected to laparotomy and inhalation of TDM in PBS (3 dogs) or PBS (2 dogs) and those not subjected to laparotomy that inhaled TDM in PBS (3 dogs) or PBS (2 dogs).

All animals were anesthetized by intravenous injection of pentobarbital. Laparatomy was performed under aseptic conditions on dogs in groups described above. The gallbladder, stomach, duodenum, small bowel, colon, liver, and spleen were clearly visible in the operative field. We placed a catheter in the femoral artery of all dogs to obtain blood samples, which were taken at the start of each experiment and at 15, 30, 45, and 60 minutes thereafter. An endotracheal tube connected to a two-way valve was inserted in all dogs, which was in turn connected to a DeVilbiss nebulizer (Somerset, Pa). Either PBS or TDM dissolved in PBS was placed in the nebulization chamber, and the machine was operated at the maximal setting for 60 minutes. The volume of material in the chamber was measured before and after each

experiment, and the quantity of TDM inhaled was calculated. The quantity of TDM inhaled was approximately 0.56 mg/kg. The yield of TDM was 30 mg/g of cigarette smoke condensate, or "tar." Therefore, each dog exposed to TDM inhaled a quantity equivalent to having smoked continuously one cigarette of 20 mg "tar" content/kg body weight/hour.

At the end of this time the dogs were sacrificed by injection of a lethal dose of sodium pentobarbital. They were subjected to complete postmortem examination; and specimens of gallbladder, liver, stomach, duodenum, small bowel, colon, mesentery, kidney, skeletal muscle, diaphragm, skin, heart, and lung were fixed in Zenker's solution made 5% in acetic acid, washed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light-microscopic examination. Photomicrographs were taken on a Leitz photomicroscope (E. Leitz, Inc., Rockleigh, NJ) with Kodachrome II film (Eastman Kodak Co., Rochester, NY).

Results

It can be seen in Table 1 that addition of TDM to normal dog plasma in appropriate concentration can shorten PTT and generate fibrinolytic activity, in concentrations ranging between 67 μg/ml and 0.9 μg/ml and 333 μg/ml and 0.9 μg/ml, respectively. High concentrations of TDM prolonged PTT and inhibited activation of fibrinolysis.

Morphologic Observations

Within approximately 10 minutes after the start of inhalation of TDM in PBS vapor the body and fundus of the gallbladder became pale because of segmental spasm of small arteries. In general, there was no change in the ampulla or its vessels. After approximately 15–20 minutes lymphatic vessels of the body and fundus appeared greatly dilated. The serosal surface developed a reticulated or striated appearance, presumably as a result of the spreading apart of connective tissue bundles by edema fluid. Between 30 and 60 minutes after the start of the experiment focal hemorrhage became apparent on the serosal surface of the body and fundus of the gall bladders of those dogs that inhaled TDM in PBS vapor (Figure 1). These changes were not observed in dogs that were similarly laparatomized but inhaled PBS vapor as control. No changes were observed grossly in the other abdominal viscera of dogs that inhaled either TDM or PBS.

At necropsy of dogs that had inhaled TDM, laparatomized or not, the wall of the body and fundus of the gallbladder was edematous and contained focal hemorrhages in the serosa. Focal subpleural hemorrhages were also present in dogs that had inhaled TDM, whether or not they had been laparatomized. In addition, in the few minutes between sacrifice and removal of organs, blood had already clotted into a cast of the vascular tree of those dogs that had inhaled TDM. None of these changes were observed in dogs that had inhaled only PBS vapor, whether or not they had been laparatomized.

Table 1—Effect of TDM on Partial Thromboplastin Time and Fibrinolytic Activity of Dog Plasma *in Vitro*

Activator substance	Concentration in mixture	% Change in PTT	% Maximal fibrinolytic activity
Celite	1.66 mg/ml	↓ 100	100
PBS		0	25
TDM	1.66 mg/ml	↑ 256	12.5
	333 μg/ml	↑ 125	50
	67 μg/ml	↓ 15.6	50
	13.3 μg/ml	↓ 30	30
	2.7 μg/ml	↓ 13	30
	908 ng/ml	↓ 5	29

On microscopic examination of the gallbladders of dogs that had inhaled TDM, marked edema of the submucosa, muscularis, and serosa was observed (Figures 2 and 3). Small veins were engorged with polymorphonuclear neutrophils that were adherent to endothelium, between endothelial cells, and extravasated (Figure 4). Focal hemorrhage was present around some vessels. In some small veins endothelial denudation had occurred, and polymorphonuclear neutrophils replaced endothelial cells (Figure 5). Although these changes were most marked in the serosa and muscularis, emigration of polymorphonuclear neutrophils was also present in the submucosa (Figure 4). Lymphatic vessels were also greatly dilated, especially in the serosa. Sequestration of polymorphonuclear neutrophils was present in the pulmonary vessels (Figure 6), especially alveolar capillaries. Aggregation of polymorphonuclear neutrophils and platelets occluded some vessels (Figure 7), and occasional thrombi were found in small pulmonary veins (Figure 8). This was associated with focal injury, as evidenced by extravasation of edema fluid and, focally, erythrocytes (Figure 9).

Inflammatory changes like those described above were not present in other organs examined microscopically, including heart, liver, spleen, kidney, stomach, small and large bowel, skeletal muscle, and skin.

No changes were observed in the gallbladder, lungs,

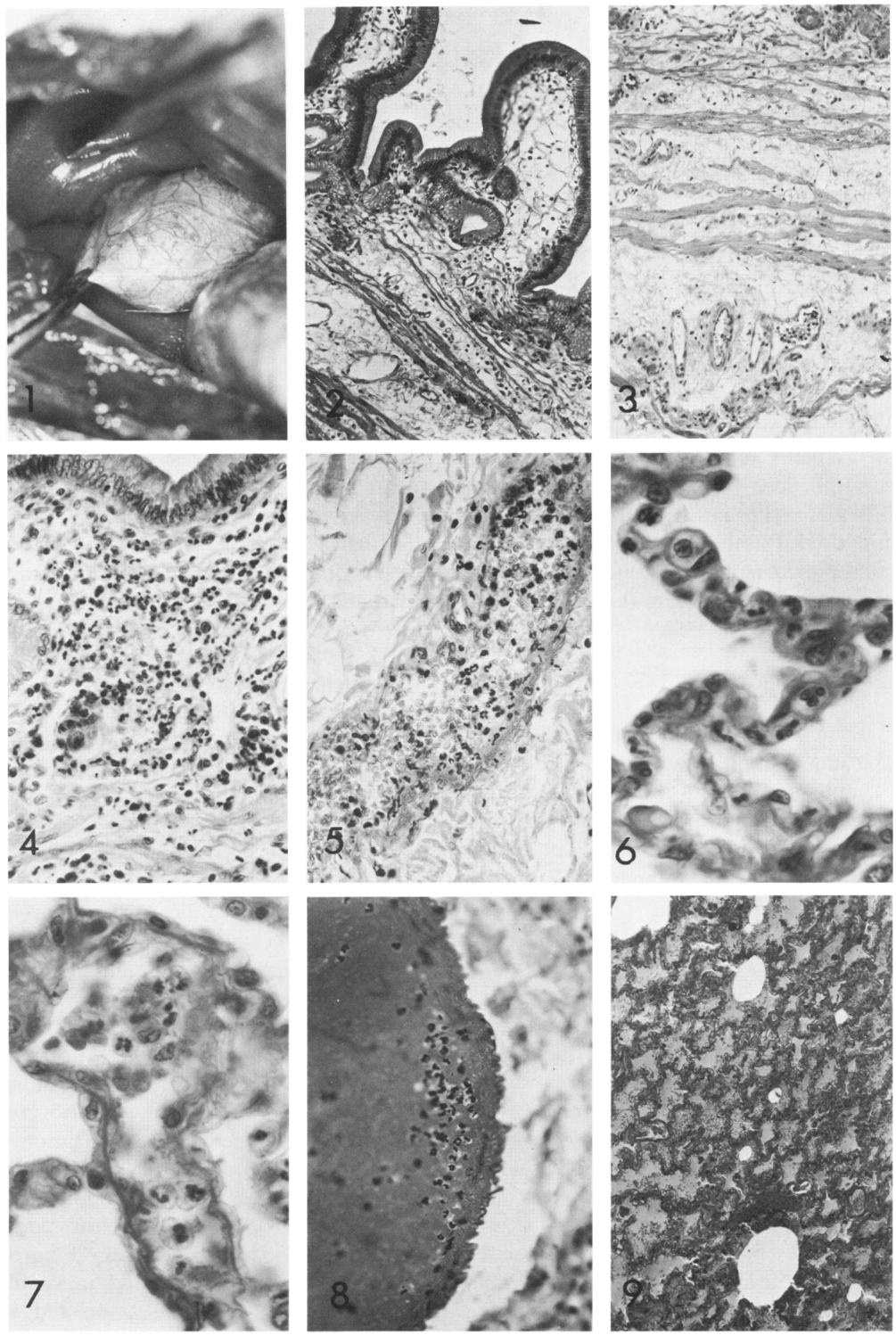

Figure 1 – Gallbladder in Dog 71 after 60 minutes of inhalation of TDM. The serosal surface is pale and petechiae are present. **Figure 2** – Gallbladder of dog 69 after 60 minutes of inhalation of TDM. The submucosa and muscularis are edematous. (H & E, × 156) **Figure 3** – Gallbladder of Dog 69 after 60 minutes of inhalation of TDM. Smooth muscle bundles are separated by edema. Polymorphonuclear neutrophils are aggregated and marginated in a small serosal vein. (H & E, × 156) **Figure 4** – Gallbladder of Dog 71 after 60 minutes of inhalation of TDM. Polymorphonuclear neutrophils are extravasated in the submucosa. (H & E, × 312) **Figure 5** – Small vein in gallbladder of Dog 69 after 60 minutes of inhalation of TDM. Polymorphonuclear neutrophils are adherent to the vessel wall, in some places replacing denuded endothelium. Others have emigrated. (H & E, × 500) **Figure 6** – Lung from Dog 69, which had inhaled TDM for 60 minutes. Polymorphonuclear neutrophils are sequestered in alveolar capillaries. (H & E, × 1250) **Figure 7** – Lung from Dog 71, which had inhaled TDM for 60 minutes. A pulmonary vessel is partially occluded by aggregated polymorphonuclear neutrophils and platelets. Polymorphonuclear neutrophils are adherent to the endothelium of this vessel. (H & E, × 1250) **Figure 8** – A pulmonary vein from Dog 70, which had inhaled TDM for 60 minutes, is distended with a thrombus. Polymorphonuclear neutrophils are at the interface between the thrombus and the vascular wall. (H & E, × 312) **Figure 9** – Alveoli contain edema fluid and some extravasated erythrocytes in the lung of Dog 66, which had inhaled TDM for 60 minutes. (H & E, × 156)

or other viscera of dogs that had inhaled PBS vapor under the same experimental conditions.

Studies of Plasma

The effects of inhaling TDM in PBS vapor on PTT and fibrinolytic activity of plasma are shown in Table 2. The unactivated PTT of plasma from dogs which had inhaled TDM in PBS vapor for 60 minutes was shortened (↓ 40.33% ± 22.46%) and fibrinolytic activity was enhanced (35.83% ± 32.46% of maximal activity). In contrast, only 1 of 4 dogs that had inhaled PBS vapor exhibited any decrease in unactivated PTT or enhanced fibrinolytic activity, and even that change was less than had occurred in plasma of dogs that had inhaled TDM in PBS vapor. These changes are statistically significant. For PTT data, $t = 3.092$, $P < 0.001$. For fibrinolytic data, $t = 12.066$, $P < 0.001$. Shortening of PTT and enhanced fibrinolytic activity were apparent in some plasma samples as early as 30 minutes after the start of inhalation but were demonstrable in all samples by 60 minutes. These changes in PTT and fibrinolytic activity were not influenced by whether or not the dogs had been subjected to laparotomy ($t = 0.1614$, $P > 0.20$ and $t = 0.93$, $0.20 > P > 0.10$, respectively).

These data indicate that inhalation of TDM resulted in activation of pathways of coagulation and fibrinolysis dependent on Factor XII.

Table 2

Dog No.	Inhalant	Laparotomy	% Change PTT	% Maximum fibrinolytic activity
70	TDM	+	↓ 76	100
71	TDM	+	↓ 30	17
72	TDM	+	↓ 20	28
69	TDM	–	↓ 58	30
66	TDM	–	↓ 20	30
64	TDM	–	↓ 38	10
73	PBS	–	↓ 15	10
65	PBS	–	0	0
63	PBS	+	0	0
61	PBS	+	0	0

Discussion

The occurrence of acute acalculous cholecystitis among hospitalized patients is associated clinically with bacterial sepsis, major trauma, including burns or major surgery, and cancer. These conditions might initiate activation of pathways dependent on Factor XII, or Hageman factor, through exposure of plasma to bacterial endotoxin or to collagen, release of proteolytic enzymes from injured tissue, or the effects of plasminogen activator on tumor cells. Acute, acalculous cholecystitis among hospitalized patients has been increasing since 1950.[4]

In previously reported studies[2] it was demonstrated that intravenous injection of ellagic acid or rutin, plant-derived polyphenols known to activate Factor-XII-dependent pathways,[1,5] resulted in the development of acute acalculous cholecystitis and pneumonitis in dogs and African Green monkeys. These changes were associated with shortening of unactivated partial thrombplastin time and of the euglobulin clot lysis time of plasma, indicating that Factor-XII-dependent pathways had been activated.

In the experiments reported herein it was observed that the addition of the nondialyzable, delipidized, water-soluble fraction of cigarette smoke condensate could shorten the PTT and generate fibrinolytic activity in normal dog plasma *in vitro*. This result was expected, because it had been previously demonstrated that polyphenol containing constituents of this fraction could activate Factor-XII-dependent pathways of coagulation fibrinolysis and kinin generation in normal human plasma *in vitro*.[1] It was further observed that inhalation of TDM in PBS vapor by dogs resulted in shortening of unactivated partial thromboplastin time and generation of fibrinolytic activity of plasma. These changes became apparent as early as 30 minutes after the start of inhalation and had occurred in all dogs after 60 minutes of inhalation. Dogs that inhaled TDM also developed inflammatory changes in the wall of the body and fundus of the gallbladder, pneumonitis, and occasional thrombi in pulmonary vessels. As in experiments in which ellagic acid or rutin was injected intravenously, the blood vessels of organs other than the gallbladder and lung displayed no changes by light microscopy. No change occurred in the gallbladders and lungs of 4 dogs that had inhaled PBS vapor as control; and only 1 of these dogs developed modest shortening of PTT and enhanced fibrinolytic activity of plasma.

These data indicate that blood vessels of the gallbladder and lung are especially susceptible to injury consequent to *in vivo* activation of Factor-XII-dependent pathways. The nature of this increased susceptibility is obscure. It can be speculated that it might be related to increased sensitivity to products of activation of the complement system, especially those with anaphylatoxic activity, since it has been recently reported that Factor XII fragments can activate the first component of human complement[6] and that kallikrein can cleave rabbit C5a from C5.[7] This could not be studied in these experiments, because purified complement components from dog plasma were not available. It might also be related to in-

creased sensitivity of these vascular beds to bradykinin. It is known that certain blood vessels, ductus arteriosus, and umbilical artery, contract rather than dilate in response to bradykinin.[8,9] The first response visible in the gallbladders of dogs that inhaled TDM was vasospasm. The question arises as to whether vasospasm leading to focal endothelial damage is the initial step in the development of selective vascular injury. Endothelial injury consequent to vasospasm has been described by Joris and Majno.[10]

Although white blood cell count and platelet count were not measured in these experiments, in other experiments in rats[11] and rabbits,[12] inhalation of TDM was followed by a precipitous fall in white blood cell count and platelet count. These returned to normal within approximately 2–3 hours after cessation of inhalation of TDM, also suggesting that complement may have been activated. It has also been reported that kallikrein can cause aggregation of polymorphonuclear neutrophils,[13] which may in part be responsible for the fall in white blood cell count described by Alonso et al[11] and Firpo et al.[12]

The observations described herein also suggest that cigarette smoking might be linked to gallbladder disease. To our knowledge there are no published epidemiologic studies that suggest such a relationship; nor are there any that suggest the contrary. Admittedly, in these experiments the degree of exposure to tar constituents was high, but changes in the gallbladder appeared rapidly. It can be hypothesized that years of smoking might produce focal injury of the gallbladder wall, allowing stone formation in persons with certain patterns of lipid and bile acid metabolism. Mucosal injury was not observed under these experimental conditions. However, the fact that polymorphonuclear neutrophils were extravasated in the vicinity of the basal lamina of the mucosa indicates that such injury might become apparent later. The hypothesis that cigarette smoking may be linked etiologically with gallbladder disease in man should be tested, because women are at greater risk of developing gallbladder disease, and the largest increase in cigarette sales has been among young women.

Finally, data presented herein indicate that inhalation of constituents of tar capable of activating Factor-XII–dependent pathways may be important in the pathogenesis of chronic pulmonary disease associated with cigarette smoking. Also, other commonly ingested substances such as coffee and chocolate contain constituents antigenically similar to those in tobacco tar and also capable of activating Factor-XII–dependent pathways, and it is conceivable that absorption of these materials might also contribute to the development of gallbladder disease.[14]

References

1. Becker CG, Dubin T: Activation of factor XII by tobacco glycoprotein. J Exp Med 1977, 146:457–467
2. Becker CG, Dubin T, Glenn F: Induction of cholecystitis by activation of factor XII. J Exp Med 1980, 151: 81–90
3. Granelli-Piperno A, Reich E: A study of protease and protease-inhibitor complexes in biological fluids. J Exp Med 1978, 148:223–234
4. Glenn F, Becker CG: Acute acalculous cholecystitis: An increasing entity. Ann Surg 1982, 195:131–136
5. Ratnoff OD, Crum JD: Activation of Hageman factor by solutions of ellagic acid. J Lab Clin Med 1964, 63: 359–377
6. Ghebrehiwet B, Silverberg M, Kaplan AP: Activation of the classical pathway of complement by Hageman factor fragment. J Exp Med 1981, 153:665–676
7. Wiggins RC, Giclas P, Henson PM: Chemotactic activity generated from the fifth component of complement by plasma kallikrein of the rabbit. J Exp Med 1981, 153:1391–1404
8. Kovalcik V: The response of the isolated ductur arteriosus to oxygen and anoxia. J Physiol (Lond) 1963, 169: 185–197
9. Davignon J, Lorenz RR, Shepherd JT: Response of human umbilical artery to changes in transmural pressure. Am J Physiol 1965, 209:51–59
10. Joris I, Majno G: Endothelial changes induced by arterial spasm. Am J Pathol 1981, 102:346–358
11. Alonso DR, Becker CG: Cigarette smoke condensate causes focal myocardial necrosis and pulmonary injury in rabbits. Fed Proc 1981, 40:758
12. Firpo A, Becker CG, Alonso DR: Induction of uteroplacental injury in rats by injection or inhalation of soluble constituents of cigarette tar. Circulation 1981, 64 (Suppl IV):54
13. Shapira M, Despland E, Scott CG, Boxer LA, Colman RW: Purified human plasma kallikrein aggregates human blood neutrophiles. J Clin Invest 1982, 69:1199–1202
14. Becker CG, Van Hamont N, Wagner M: Tobacco, cocoa, coffee and ragweed: Cross-reacting allergens that activate factor XII dependent pathways. Blood 1981, 58:861–867

Acknowledgments

The authors are indebted to Miss Mary Wagner and to Mr. Richard Terek for technical assistance; to Ms. Rebecca Bethea for preparation of excellent histologic specimens; and to Mrs. Barbara Whyte for manuscript preparation.