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Hemorrhagic Diathesis in Laboratory Rodents* (32985)

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A disease characterized by spontaneous hemorrhage and death affected a large portion of newly established breeding colonies of specific-pathogen-free (SPF) mice and rats. The subsequent occurrence of an identical problem in similar mice and rats at other laboratories and the increasing use of the SPF animal for research emphasized the importance of this problem and the need to carefully define experimental animals.

Clinical history. Our Division maintains approximately 2,000 pairs of breeding mice and rats and a total of 30,000 rodents in 40 animal rooms. All are housed as pairs in plastic cages fitted with stainless steel tops; white pine shavings are used as bedding; a commercial diet with a 10% fat content is fed; water and bottles are sterilized by autoclaving.

The hemorrhagic disease occurred only in two rooms that contained cesarean-derived mice and rats.

The affected rat colony (SD/Anl-SPF) was started from 54 male and 54 female germfree Sprague-Dawley weanling rats purchased from a commercial breeder. They were removed from plastic isolators prior to shipment to the Laboratory and at 12 weeks of age they were paired and bred. Each pair produced a litter with very few losses among the progeny, and none among the adults.

At the same time an SPF mouse colony (CF no.1/Anl-SPF) was initiated by foster nursing cesarean-derived CF no.1/Anl neonates on purchased germfree dams. Twenty-six females and 13 males were removed from plastic isolators and bred at 8 weeks of age.

At 16 weeks of age, one of the original SPF male rats bled to death from a minor facial laceration. Within the next 4 weeks, 43 more males and three females died. Most showed no signs of illness before death. A few males showed anemia and hemorrhage of one or more of the following: conjuctiva, external nares, urethra, facial skin, subcutis tunica vaginalis visible through the scrotum. A few also had posterior paralysis or paresis. Bleeding from the vulva was noted in the affected females.

During the 7 months after the first death, 120 adult rats died; 98 males and 22 females. The signs remained constant but their severity diminished with time. Approximately two-thirds of the deaths occurred within the first 90 days.

When the SPF mice were 10 weeks old, four of the males died. Wthin 10 days, the remaining nine males died with signs that included dyspnea, depression, and generalized anemia. A few days after the last male died, a female died showing similar signs preceding death.

During the same 7 months that the problem was studied in the rat colony, 200 mice died; 148 males and 52 females.

Materials and Methods. Pathology. All

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TABLE I. Reagent Factor Content.

Reagent	II (Prothrombin)	V (Proaccelerin)	VII (Proconvertin)	X (Stuart)
Oxalated plasma	+	+	+	+
$BaSO_4$ adsorbed plasma	—	+	—	—
Aged serum	—	—	+	+
Aged plasma	+	—	+	+
$BaSO_4$ adsorbed plasma + aged serum (1:1)	—	+	+	+

decedents and selected clinically affected animals were necropsied. Major organs and tissues and all those exhibiting hemorrhages or other lesions were fixed in formalin, sectioned, stained with hematoxylin–eosin and examined microscopically.

Treatment. About 10 weeks after the first death, treatment of a portion of the mice and rats with vitamin K (menadione sodium bisulfite)[1] was initiated. Two-thirds of the animals in each colony were given weekly subcutaneous injections of 5 μg of menadione; the other one-third remained untreated. After 10 weeks menadione was added to the diet at a rate of 2.83 ppm.

Hematology. Complete blood counts and indirect platelet counts were made on 26 asymptomatic male SPF rats and 10 similar male SPF mice and a comparable number of conventional animals.

Whole blood clotting times were determined on 16 conventional and 27 apparently normal, untreated SPF adult rats. Clotting time was measured by demonstrating formation of a fibrin thread in capillary tubes.

"One-stage" prothrombin time determinations (PT_1) were performed on the 16 conventional rats and 34 untreated male SPF rats and on 10 conventional and 10 untreated male SPF mice. All determinations were made according to the method of Quick *et al.* (1) using thromboplastin[2] extract. All tests were run in duplicate; the average prothrombin time was recorded in seconds. Control plasma was run as a reference on all thromboplastin samples.

Differential tests to determine the coagulation defect were performed on plasma samples from 23 clinically affected SPF rats and 10 affected SPF mice with abnormal, one-stage prothrombin times. Procedures outlined by Mandel (2) were used. To 0.9 ml of abnormal plasma, 0.1 ml of known plasma factor "reagent" was added. One-stage prothrombin times were run on the abnormal plasma plus "reagent" and any correction of the prolonged PT_1 was noted. Table I lists the specific clotting factors present in the reagents used.

Duplicate one-stage prothrombin times were also run on 15 male SPF rats prior to subcutaneous injection with 1 mg of menadione sodium bisulfite and again 24 hours after treatment.

Microbiology. Contents of both the ileum and colon from 10 mice and 12 rats from the SPF colonies were cultured aerobically and anaerobically during the first 4 weeks after the onset of hemorrhagic signs and lesions. Phenyl ethyl alcohol agar[3], eosin methylene blue agar[3], tryptic soy agar[3] with 10% defibrinated sheep blood and thioglycolate broth[3] were used for the microbiologic procedures.

Analysis of bedding. Chromatographic analyses of the bedding used for the cesarean-derived germfree mice were made. The procedure used was similar to that described by Meier *et al.* (3). In contrast to their procedure, however, butanol–acetic acid–water chromatograms were included, and periodic acid spray, followed by ammonical silver nitrate, was used. Aniline phthalate also was used.

Results. Pathology. Hemorrhage was the primary lesion but mild to moderate hepatic

[1] Hykinone, Abbott Laboratories, North Chicago, Illinois.

[2] Simplastin, Warner-Chilcott, Morris Plains, New Jersey.

[3] Difco Laboratories, Detroit, Michigan.

TABLE II. Necropsy Findings in Affected SPF Rodents.

	Rats		Mice	
	98 ♂	22 ♀	148 ♂	52 ♀
Hemorrhage				
Urogenital	35	15	—	37
Central nervous system	43	1	19	—
Subcutaneous	24	1	16	—
Hemothorax	11	—	93	3
Pulmonary and pleural	—	—	5	—
Peritoneal and retroperitoneal	9	—	7	—
Myocardial	1	—	65	11
Pregnant	—	15	—	6
Decomposed	12	2	7	6
Anemia, generalized, with hemorrhage	—	—	—	1

TABLE III. Mortality of SPF Rodents Before and After Treatment with Menadione.

	Before treatment		After treatment			
	Males	Females	Males		Females	
Species			Untreated	Treated	Untreated	Treated
Rat	48	5	44	6	14	3
Mouse	18	10	85	15	6	6

degeneration and hepatomegaly were also noted. Sometimes the hemorrhages were multiple, sometimes only a single lesion was present. Table II gives a summary of the incidence of hemorrhage and other necropsy findings.

The sites of hemorrhage were decidedly different between the male rats and mice and between males and females of the same species. The main site of hemorrhage in the females was the gravid uterus while in the males many areas were affected.

The only consistent microscopic lesion, other than hemorrhage and varying degrees of hepatic degeneration, was marked myocarditis in all 26 male mice examined (Figs. 1 and 2). This lesion was present in only one of the 12 female mice examined and in none of the six female rats examined. Heart lesions were demonstrated in 4 of 23 male rats examined but, except for one, these lesions were limited in extent and mild in severity.

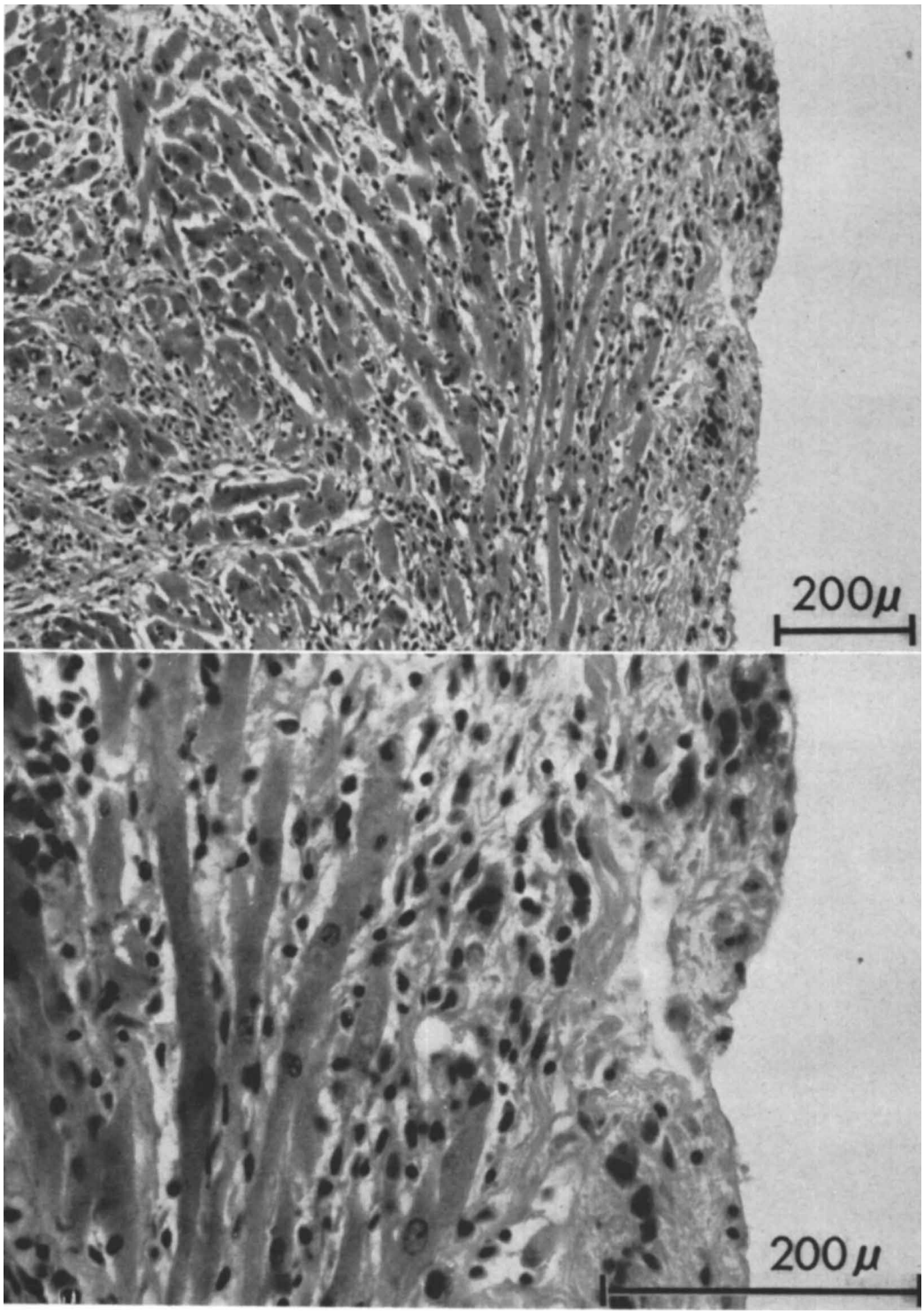
Although the cardiac lesion in most of the male mice was a marked chronic nonsuppurative inflammatory reaction with extensive fibrosis, areas of acute inflammation and necrosis were often present. Interstitial hemorrhage, hemosiderin, both free and within phagocytes, varying degrees of edema, and degeneration and fragmentation of myocardial fibers were also seen.

In addition to the myocardium, the epicardium, endocardium, atria and origin of the great vessels were affected. The endothelium of the endocardium and particularly the epicardium showed proliferation, thickening and accumulation of inflammatory cells. In some instances, when myocardial changes were not prominent, the epicardium was involved, usually at the base of the heart.

Treatment. The mortality before and after treatment with menadione is given in Table III.

Hematology. Comparison of the hemograms of 26 male SPF rats to those of conventional male rats showed a slight increase in mean corpuscular volume and mean corpuscular hemoglobin content. The 10 male SPF mice showed a mild normocytic anemia with hypochromasia, a small amount of basophilic stippling and a few Howell-Jolly Bodies.

The results of whole blood clotting time



FIGS. 1. (top) and 2. (bottom). Epicardium and myocardium of an SPF mouse dying of hemothorax; hematoxylin and eosin stain. Chronic inflammatory reaction with degeneration of myocardial fibers, cellular infiltration, hemosiderosis, and fibrosis.

TABLE IV. Whole Blood Clotting Times (WBCT) of Untreated Conventional and SPF Rats.

Animal type	No. of samples	WBCT (min)
Conventional	16	> 2 and < 3
SPF	17	> 2 and < 5
	3	> 5 and <10
	7	>10 and <30

determinations are given in Table IV and the prothrombin time determinations in Table V. In each case the SPF animals show remarkable differences from the conventional animals.

The results of the differential tests to determine the coagulation defects are shown in Table VI. These results indicate factors II and VII to be deficient. Factor X was also suspected to be deficient but deficient plasma was not available at the time to test specifically for this deficiency. Factors I and V were normal. Both the WBCT and PT_1 were prolonged, and only partial correction was obtained by adding aged serum. Full correction was obtained by the addition of 10% normal plasma to the tested plasma.

Treatment of 15 male SPF rats with menadione resulted in a significant decrease in PT_1 in 24 hours. ($T_{(26)} = 4.63$; $p<.01$) The PT_1 before treatment was 27.8 ± 3.73 sec;[4] 24 hours after treatment it was 12.3 ± 0.41.

Microbiology. α hemolytic streptococci, *Streptococcus fecalis, Escherichia coli, Paracolobactrum aerogenoides, Paracolobactrum intermedium,* and *Clostridium perfringens* were isolated from the ileal and colonic contents of the SPF mice. In addition, *Lactobacillus* sp., *Proteus mirabilis, Aerobacter aerogenes* and *Proteus rettgeri* were isolated from the SPF rats.

Analysis of bedding. The duplicate samples tested contained less than 0.015% ethylene glycol.

Discussion. Because all breeding animals affected and nonaffected, were being fed the same ration it initially seemed that a dietary factor could not be involved. The literature concerning hemorrhagic diseases in rodents, however, emphasizes nutritional problems, particularly vitamin K deficiencies. Factors II and VII, which were shown to be deficient, and also factor X, depend on vitamin K.

Numerous studies have been made on the vitamin K requirements of rodents and the role of the intestinal flora in synthesizing it. Rats receiving vitamin K-deficient diets have been reported to develop vitamin K deficiency and hemorrhagic lesions only when coprophagy was prevented (4,5). In another study (6), vitamin K deficiency and hemorrhage occurred in germfree but not in conventional rats when fed the same vitamin K deficient diet. Hemorrhages and deaths have also been previously reported in SPF rats fed commercial diets; the problem was prevented when vitamin K was supplemented (7).

In studies of factors influencing coprophagy (8–10) it was found that a diet can be depleted of vitamin K to a degree that coprophagy cannot supply adequate amounts of the vitamin because of limited synthesis in

TABLE V. One-Stage Prothrombin Times (PT_1) of Untreated Conventional and SPF Rats and Mice.

Species and animal type	No. of samples	PT_1 (sec)
Conventional rats	16	<15
SPF rats	25	>15 and < 60
	5	>60 and <300
	4	>300
Conventional mice	10	<15
SPF mice	4	>15 and < 60
	6	>60 and <300

TABLE VI. Effect of Reagents (Factors) on Abnormal One-Stage Prothrombin Time (PT_1) of SPF Rats.

Reagent added[a]	Results on abnormal PT_1
Oxalated plasma	Full correction
$BaSO_4$ adsorbed plasma	No correction
Aged serum	Partial correction
Aged plasma	Full correction
$BaSO_4$ adsorbed plasma + aged serum (1:1)	Partial correction

[a] Nine-tenths ml of abnormal plasma plus 0.1 ml of reagent.

[4] Mean and standard error. Does not include three animals with excessively high pretreatment PT_1 of 3 to 5 minutes.

the intestinal tract. The true dietary requirement of vitamin K when coprophagy is prevented, is in the order of 0.1 μg/gm of food (10). Gaunt and Lane-Petter (7) suggested that SPF animals might not carry species of bacteria capable of synthesizing adequate amounts of vitamin K. The gut flora of our SPF mice and rats included an abundance of vitamin K-producing organisms (11,12) and there is no reason to believe that they did not practice coprophagy. An hypothesis of altered gut flora as a predisposing influence is supported by the fact that the incidence of hemorrhage in both the mice and rats was high for the first 2 months, and gradually declined later. This could have resulted from the development of a more conventional intestinal flora over this period of time.

A marked difference in susceptibility to hemorrhage between male and female rats has been recognized (13) and is supported by our findings (Table II). It has been shown that estrogenic hormones have a protective effect against hypoprothrombinemia and hemorrhagic death during feeding of viatmin K-deficient diets (14). Similarly, estrogen injections and castration of male rats have a sparing effect on vitamin K requirements, while testosterone increases the requirements (15). Our observation that pregnant females were more commonly affected by the syndrome than nonpregnant females (Table II) can also be logically explained on an endocrinologic basis. Since castration of the female rat probably increases vitamin K requirements, the changes in endocrine balance associated with pregnancy could similarly affect the requirements and predispose the animal to hemorrhage. Unfortunately, studies of vitamin K requirements in rats and mice, or studies of differences in responses to deficiencies between males and females appear to have been always conducted on nonbreeding animals.

Dietary components can also influence vitamin K requirements. Overdosage with vitamin A leads to a hemorrhagic condition that can be prevented by vitamin K (16–19). Large doses were not required; physiologic levels of vitamin A (in the range of 0.5–5 IU/gm of diet) can reduce prothombin levels (20). Vitamin K deficiency is similarly influenced by the type of dietary protein used; soy and beef protein are less desirable than milk protein (21). Although it is difficult to comment on protein quality in our diet, vitamin A is added at the rate of 6.6 IU/gm of diet.

Meier *et al.* (3,22) described a hemorrhagic diathesis in mice which they ascribed to an intoxication with ethylene glycol produced by ethylene oxide sterilization of bedding. The syndrome was similar to that in our mice and there were either single or multiple deficiencies of factors II, VII, IX, and X. Our affected rats might have been in contact with ethylene oxide-treated bedding prior to the time we received them, but the mice were not because they were raised on steam-sterilized bedding at the laboratory. Chemical analysis of the bedding confirmed that no significant amount of ethylene glycol was present.

The lesions in the hearts of all male mice examined and in those of several of the male rats are particularly significant. Angevine and Furth (23) described a sporadic disease in adult male mice characterized by fatal pleural and pericardial hemorrhage. Although the heart lesions were similar to those in our mice, hemorrhages were restricted to the heart, spleen, and testes. Females were not involved but it was not clear if any were being bred. Myocardial lesions unaccompanied by hemorrhage have also been reported in mice on diets deficient in vitamin K (24), and the studies on hemorrhage associated with glycol intoxication described chronic myocarditis as the only consistent lesion (3, 22). It is possible that the glycol intoxication caused a suppression, inactivation, or increased requirement of vitamin K rather than direct inactivation of essential clotting factors.

The relationship of this disease to a vitamin K deficiency appears obvious but its predisposing factors are not clear. Perhaps the most provocative aspect of this disease is its frequent occurrence in the newly established colonies of SPF animals (W. R. Graham, personal communication). If some alteration in intestinal synthesis, absorption, or utilization of vitamin K is responsible for the onset of this problem, it might have serious implica-

tions for the use of SPF animals in experiments.

Summary. A disease characterized by spontaneous hemorrhage and death occurred in newly established breeding colonies of cesarean-derived, specific-pathogen-free, Sprague-Dawley rats and CF no. 1 mice. Male mice and rats were more commonly affected than females, and pregnant females were more commonly affected than nonpregnant females. In addition to a wide variety of hemorrhages, there was a high frequency of myocardial lesions in the decedent male mice. The clotting mechanism was studied and the defect was shown to be related to factors II and VII and possibly factor X. Menadione was effective in treating the disease but did not completely prevent it. The possible predisposing and etiologic factors, including the alteration of intestinal bacterial flora, are discussed, and the need to fully characterize animals used in experiments is stressed.

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