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Induction of *Listeria monocytogenes* Infection by the Consumption of Ponderosa Pine Needles†

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An infectious microorganism, identified as *Listeria monocytogenes,* has been isolated from the bloodstream of pregnant mice fed a diet containing *Pinus ponderosa* needles. When the isolate was injected into pregnant mice, reproductive dysfunction and other changes, including speckled livers, spleen atrophy, and hemorrhagic intestines, appeared to mimic the signs of the disease in pregnant mice fed pine needles. Moreover, these pathological changes are similar to those observed in cattle and other mammals experiencing abortions or toxemia, or both, attributed to the ingestion of *P. ponderosa* needles, suggesting that *L. monocytogenes* may be a part of the etiology of "pine needle abortion."

For over 80 years, ranchers in Canada and the northwestern United States have claimed that ponderosa pine needles and buds were the causative agents of abortions in cattle (9). These reports were not seriously considered because for years the abortifacient activity had been attributed to brucellosis, phosphorus deficiency, or vitamin A depletion. In 1950, MacDonald undertook the first formal investigation that determined that the needles of *Pinus ponderosa* cause abortions in cattle (9).

The etiological agent in pine needles responsible for the abortifacient activity has been more intensely studied recently (2, 5); however, the exact nature is still not known. Theories range from the possibility of an antiestrogenic component to the potentially adverse involvement of microbial processes. Microorganisms were first implicated by Chow et al. (5) in studies designed to determine the effects of fungal metabolites from *P. ponderosa* needles on inducing reproductive dysfunction. Although the data of Chow et al. indicated that the mycotoxins contained abortifacient activity, the findings of Anderson and Lozano (2) suggest that toxins produced by fungi on the pine needles are of secondary importance.

Some clinical observations support the notion that microorganisms or their products (toxins) may be involved. Stevenson et al. (13), in summarizing their observations of cattle experiencing "pine needle abortion," reported excessive uterine hemorrhaging, a characteristic nauseating odor, septic metritis, and peritonitis as consistent entities in the problem. James et al. (7) reported that necropsy of fetal tissue expelled after the consumption of *P. ponderosa* needles revealed pronounced necrosis of proximal convoluted tubules of the kidney, pulmonary congestion, and excessive hemoglobin breakdown products. Similar phenomena have been induced in mice and rats fed a diet containing pine needles (1).

Infectious processes seem to be implicated in "pine needle-induced reproductive dysfunction"; however, it is unknown whether the pathological processes are part of a primary infection or whether the infection is secondary to yet another event. Thus, we considered it of great interest to determine whether an infectious microorganism could be isolated from the bloodstream of mice in the pre- and post-aborting time period when the animals were fed a diet of ponderosa pine needles and if the isolated microorganism could cause the same pathological abnormalities as the diet of ponderosa pine needles.

MATERIALS AND METHODS

Mice. Mice were of the Dub/ICR strain and were obtained from Flow Laboratories, Bar Harbor, Maine.

Adult mice weighing 28.0 g or more were put into four study groups: (i) females at day 9 of gestation; (ii) females at day 4 of gestation; (iii) nonpregnant females; (iv) males.

In all gestation studies, pregnancy was determined by examination of vaginal smears and detection of vaginal plugs. A fifth group of immature mice weighing 6 ± 1 g was also studied. They were weaned at 2 weeks of age and sustained on a diet of Wayne Lab-Blox and water for a period of 3 days to achieve total weaning before use in the study.

Pine needle chow. To insure consistency, all

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needles were obtained from the same location, 5 miles northeast of Greycliff, Mont. This area is particularly noted by local ranchers for its high incidence of pine needle abortion. The needles were stripped from their branches and stored at 4°C until prepared into chow. The pine needle chow was prepared by initially grinding the fresh needles and allowing them to air dry. The dried needles were then reground and mixed with ground Wayne Lab-Blox in a 3:2 ratio by dry weight. Five hundred grams of this mixture was combined with 400 ml of unsulfured molasses and 250 ml of 95% ethanol and mixed thoroughly. The chow was then stored at 4°C until used.

Wayne Lab-Blox was used as the control chow for the mice.

Medium. Tryptic soy (TSY) broth (Difco) was used to culture blood specimens. TSY agar (Difco) plates were used for isolation purposes.

Assay of the needles. The air-dried needles were washed with sterile water, and the washings were Gram stained. Dried needles were cultured in TSY broth to assay for the presence of microbial growth.

Isolation of microorganism. Ten mice from each study group were given the pine needle chow for 10 days. Five mice from each group were fed a regular diet of Wayne Lab-Blox as controls.

Blood samples were obtained by aseptically exposing the heart and suctioning blood directly from the right atrium via puncture with a 27-gauge needle attached to a 1-ml tuberculin syringe.

Gram and Wright stains were done on all samples. In addition, 0.10 ml of blood was inoculated in 5 ml of TSY broth. TSY agar plates were streaked for isolation. All inoculated media were incubated under increased CO_2 tension for 48 h at 37°C and examined for growth.

Preparation of challenge organisms. Isolated colonies on streak plates were transferred to TSY broth and incubated for 48 h at 37°C. The cultures were centrifuged at 9,500 × *g*, washed twice with 0.85% NaCl, and resuspended in 0.85% NaCl. The microorganism suspensions were diluted to 10^7 organisms per ml, and 0.5 ml of the suspension was injected intraperitoneally into six females at day 9 of gestation. The animals were sacrificed 5 days after injection. Control female mice on day 9 of pregnancy were injected intraperitoneally with 0.5 ml of 0.85% NaCl.

RESULTS

Course of the disease. The groups of mice varied in their response to the diet of pine needle chow (Table 1). Mice fed the pine needle chow starting on day 9 of pregnancy showed the greatest pathological changes, including decidual hemorrhaging (abortions), fetal death and resorbtions, blood-filled intestines, speckled livers and kidneys, and reddened adrenals. In addition, a severe loss of coordination, a substantial weight loss, and purulent exudate from the vaginal orifice were noted in the animals in this group. Upon exposure of the viscera, a characteristic pungent odor was detectable. Seven mice expired within 6 days of diet consumption. Mice fed pine needle chow beginning on day 4 of pregnancy differed in their response in that the toxic effects were observed only on the first 2 days of feeding the experimental rations. This group displayed some loss of coordination, lethargy, and weight loss; however, these signs subsided by the time of sacrifice at day 14 of gestation. There was a total loss of fetuses in all animals started on the pine needle chow at day 4 of pregnancy.

TABLE 1. *Susceptibility of various groups of mice to the effects of diet containing ponderosa pine needles*

Mouse group	Ration	No. of mice in group	No. surviving through test period (10 days)[a]
Immature mice	Pine needle	10	0
	Control	5	5
Mature females	Pine needle	10	10
	Control	5	5
Pregnant females			
Day 4 of gestation	Pine needle	10	10
	Control	5	5
Day 9 of gestation	Pine needle	10	3
	Control	5	5
Mature males	Pine needle	10	10
	Control	5	5

[a] Except in the case of immature mice (see text).

Adult males and nonpregnant females also showed lethargy and loss of coordination, which subsided during the remaining days on the pine needle chow. These animals showed no gross pathological changes upon dissection after 10 to 21 days on the pine needle chow.

Immature mice showed greater susceptibility than older mice to the effects of the ponderosa pine needles. The immature mice expired within the first 3 days of feeding. Prior to death, they showed loss of coordination, extreme lethargy, and starvation-like symptoms and were cold to the touch. Necropsy revealed hemorrhagic intestines and ulcerated stomachs.

All control mice remained healthy and showed no ill effects.

Gram stains and cultures of pine needles. Gram stains and culture of the pine needles often revealed fungal growth, but no other morphological forms were observed.

Isolation of the microorganism. Gram-positive coccobacilli were observed in Gram stains of blood specimens from pine needle-fed

mice of all groups, and TSY broth blood cultures were positive after 24 h of incubation. In the blood samples, the presence of organisms was accompanied by a substantial increase in circulating mononuclear cells. In contrast, little, if any, growth was obtained with samples taken from the control mice. A *Staphylococcus* sp. was isolated from one control male mouse, and a fungus-like form was observed in the sample taken from one immature control mouse.

Scant growth was obtained on TSY agar streak plates, although numerous coccobacilli were seen in Gram stains on TSY broth cultures of blood samples from pine needle-fed mice.

Identification of the isolated microorganism. The microbial species found in the bloodstream of mice fed the experimental pine needle chow was identified, using the biochemical and physical tests outlined in *Bergey's Manual* (12), as a strain of *Listeria monocytogenes*. The identification was confirmed by the Montana State Health Laboratory in Helena, Mont. The microbe was a gram-positive coccobacillus, was motile at 22°C and nonmotile at 37°C, and often was found to exist intracellularly. It produced acid but not gas from dextrose, lactose, maltose, and sucrose; it did not liquefy gelatin, reduce nitrate, or produce acid or gas from mannitol and dulcitol. The isolated organism was catalase positive and reduced litmus milk. Colonies were a characteristic black color when grown on potassium tellurite agar; colonies exhibited a blue-green hue when grown on TSY agar and illuminated with an obliquely transmitted light source. The injection of the organism into the conjunctiva of rabbits resulted in a positive Anton reaction. These observations are consistent with characteristics exhibited by *L. monocytogenes*.

The serovar of the isolate was type 4b, although this typing was not consistent and showed considerable cross-reactivity with other serovars. The Center for Disease Control, Atlanta, Ga., also confirmed the serovar of the isolate as type 4b.

TABLE 2. *Incidence of microorganism in blood samples taken from mice after 10 days of diet consumption*[a]

Group	Ration	No. of mice	Mice yielding positive cultures (%)	Estimated[b] no. of organisms/ml
Pregnant females				
Day 9	Pine needle	10	90.0	2.3×10^6
	Control	5	0.0	
Day 4	Pine needle	10	80.0	1.1×10^5
	Control	5	0.0	
Cycling females	Pine needle	10	60.0	4.0×10^3
	Control	5	0.0	
Adult males	Pine needle	10	100.0	5.7×10^3
	Control	5	0.0	
Immature mice	Pine needle	10	100.0	1.5×10^7
	Control	5	0.0	

[a] Except in the case of immature mice (see text).
[b] Estimates based on Petroff-Hauser counting technique.

DISCUSSION

L. monocytogenes is an apparently ubiquitous bacterium and is found commonly in small numbers in many healthy specimens, including several mammals, fowl, and fish (4, 8). *L. monocytogenes* and a nonvirulent variety apparently exist as saprophytic organisms in soil and on plants (14). It is characterized by its diphtheroid-like forms, gram-positive reaction, motility at room temperature, and tendency to occur in pairs. It is generally considered to be a faculative anaerobe and difficult to cultivate from natural sources.

The occurrence of *Listeria* infections is so sporadic as to lead one to believe that its distribution in nature is restricted in some unknown fashion and that the bacterium acquires pathogenic properties or that the susceptibility of the host varies only under specific conditions. Factors that cause and perpetuate listeriosis have not been fully delineated, and it is not known why the organism can cause such a variety of disease forms, including encephalitis, meningitis, septicemia, abortions, liver diseases, and papular skin lesions (11).

Infections by *L. monocytogenes* in ruminants are considered to produce encephalitis and "circling" in adults and septicemia in fetuses and neonates (3). *Listeria*-induced abortion in cows is a very serious and potentially fatal malady. Most abortions occur in the last trimester of pregnancy. Some calves are born alive but weak. Cows having late abortions frequently have pyrexia, depression, retained placentas, and purulent genital exudates. Abortions in a herd may be sporadic or multiple.

Stevenson et al. (13) reported that pine needle-induced abortions in cows are likely to occur within 48 h to 2 weeks after consumption of pine needles and have a number of similarities to *Listeria*-induced abortions. The abortion is characterized by weak contraction, incomplete

dilation of the cervix, and a nauseating odor. If the cow is near enough to term at the time of pine needle consumption, the calf usually is very weak at birth. A persistent retained placenta, atonic uterus filled with uterine fluid, placental debris and blood, and septic metritis which may be followed by peritonitis were constant findings.

In pregnant animals, *L. monocytogenes* appears to have a predilection for fetoplacental tissues, especially during the last trimester of pregnancy (14). Fetuses infected with *L. monocytogenes* may die and be retained in utero for 24 to 72 h before being expelled. Those affected near term may be aborted soon after death or may be born alive but weak.

Gray and Killinger (6) report that similar outcomes have been produced in mice by injecting *L. monocytogenes*. With few exceptions, infections have been characterized by a septicemia. If the mice are pregnant, as in the ruminants, they may abort. The pathology noted, including the liver speckling, adrenal color change and enlargement, hemorrhagic intestine, abortifacient activity, and loss of coordination in our animals, is consistent with pathological alternations induced by *L. monocytogenes*. Differences in responses to the effects of the pine needles between our test groups are significant in that pregnant and immature mice showed a considerably increased susceptibility to the effects produced by consumption of the chow, including extensive pathological changes and mortality. Adult males and nonpregnant females exhibited some signs of an adverse response to the experimental chow, which eventually subsided and yielded no gross damage to tissues. This is consistent with findings by Miller and Burns (10), who reported finding liver lesions in all mice tested on day 7 of oral feeding of *L. moncytogenes*. On day 14, there was little evidence in male and nonpregnant mice of any lesions or other pathological changes; however, in the pregnant mice numerous large lesions were detected. Although our observations appear to be similar, the course of the disease seemed to be accelerated in our studies.

The serovar typing of the isolated organism as type 4b is significant in regard to serovars found in ruminants. Gray and Killinger (6) report that 4b is the serovar most commonly found to be virulent.

The loss of coordination, lethargy, necrotic livers, spleen atrophy, and hemorrhagic intestines in mice when either intraperitoneally injected with isolated *Listeria* or fed pine needle chow substantiate the likelihood that *Listeria* is involved in the pathology induced by ingesting pine needles. Furthermore, the fetal loss observed when pregnant mice were injected with isolated *Listeria* or fed pine needle chow provides additional evidence that *Listeria* is intimately associated with pine needle abortion.

Current studies seek to characterize the pathogenesis of the *Listeria* infection in mice and include studies on the inductive factors which promote infectivity and those factors which promote morphological change in the host animal, particularly in the pregnant mouse fed pine needles.

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