Intrauterine inoculation of *Mycobacterium paratuberculosis* into guinea pigs and cattle

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SUMMARY

Mycobacterium paratuberculosis was inoculated intrauterinely into guinea pigs and cattle near the time of insemination to assess the effect and subsequent distribution of the organisms. Guinea pigs inoculated intrauterinely with 1 of 3 concentrations of *M paratuberculosis* or not inoculated were caged with male guinea pigs for 10 days. Guinea pigs given the largest dose had the lowest rate of pregnancy when examined. At necropsy, tissues were cultured to determine extent of infection. Abortion followed hypersensitivity reactions to johnin in some lightly infected animals.

Mycobacterium paratuberculosis was recovered in culture from the body and horns of the uterus of cows necropsied 1, 2, 3, and 7 days after inoculation and from 1 of 3 cows necropsied 2 weeks after inoculation. The organisms were not detected in extrauterine organs of any cattle or in uterine specimens taken at 3 or 4 weeks after inoculation.

The evidence indicates that the small numbers of *M paratuberculosis* sometimes found in semen from paratuberculous bulls probably would be destroyed, rather than leading to systemic infection of the dam or to persistent hypersensitivity.

Paratuberculous cattle without clinical signs of Johne's disease have significantly higher cull rates because of breeding problems than noninfected cattle in the same herd.[1] The breeding problems might be due to delayed-hypersensitivity phenomena[2] or to active intrauterine infection. Also, *Mycobacterium paratuberculosis* sometimes can be isolated in small numbers from semen of paratuberculous bulls,[3] where its presence has caused concern that the recipient cows or the resulting calves might become infected. The effects of hypersensitivity and intrauterine infection were studied in guinea pigs and cattle intrauterinely inoculated with *M paratuberculosis* near the time of insemination.

Materials and Methods

Guinea pigs—Mycobacterium paratuberculosis was grown on mycobactin-egg yolk medium.[4] Inocula consisted of 0.25 ml of suspensions containing 10^{10}, 10^8, or 10^6 *M paratuberculosis*. Groups of 18 female NADC-strain English short-hair guinea pigs each were given 1 of the aforementioned inocula intrauterinely via transcervical catheter (while animal was anesthetized with CO_2). A group of 14 guinea pigs was used as noninoculated control. A lubricated glass tube (8 cm long and 8 mm in diameter) was used as a vaginal speculum. The inocula were administered via a 12-cm, 16-gauge needle tipped with 22-gauge Teflon tubing. One animal from each group was necropsied on postinoculation (PI) days 1, 2, 6, and 7. Specimens from liver, kidney, spleen, intestine (aggregated lymph nodules, Peyer's patches), uterus, and cervix were examined by microbiological cultural technique for *M paratuberculosis* by use of the benzalkonium chloride method,[4] but were not subjected to histopathologic examination.

Six to 8 weeks after inoculation, the animals were regrouped into 14 cages. Then, 1 of the remaining 14 females from each group given the different inocula, a male guinea pig, and a noninoculated female were put in each cage for 10 days. Five weeks after 1st placement of the females with the male guinea pigs, pregnancy was determined by palpation, and each guinea pig was given 0.25 ml of johnin IM in the right pelvic limb. Cages were examined several times a day during the following week to detect abortions. After abortion or normal parturition, the female guinea pigs were necropsied, and specimens were collected for microbiological cultural and histopathologic examinations. Tissues for histopathologic study were fixed in 10% buffered formalin. One section from each tissue specimen was stained with hematoxylin and eosin, and a duplicate section was stained for acid-fast organisms by the Ziehl-Nielsen method. Nonpregnant animals were necropsied after all pregnant animals had been examined at the termination of their pregnancies.

Cattle—Mycobacterium paratuberculosis was grown and prepared for inoculation as for the guinea pigs. Each inoculum consisted of 5×10^8 colony-forming units suspended in 5 ml of saline solution. Thirteen 3- to 4-year-old Holstein cows were bred by natural service or by artificial insemination, and 24 hours after breeding, each was given the bacterial inoculum—put in the uterus via plastic insemination tube. One cow was killed and necropsied on each of PI days 1, 2, 3, and 7, and 3 cows were killed and necropsied at 2 weeks, 3 at 3 weeks, and 3 at 4 weeks after inoculation. Specimens for microbiological cultural and microscopic examinations were obtained from liver, spleen, lung, cranial and caudal portions of both horns of the uterus, the body of the uterus, 5 pelvic lymph nodes, both supramammary lymph nodes, mediastinal lymph nodes, and 2 mesenteric lymph nodes. The benzalkonium chloride-egg yolk medium technique[4] was used for cultivation of *M paratuberculosis* from the specimens.

Results

Guinea pigs—Among the 12 guinea pigs necropsied on PI days 1 to 7, *M paratuberculosis* was cultured only from the reproductive organs of 7 and from both reproductive and nonreproductive organs of 4. Organisms were not isolated from 1 guinea pig given 10^6 *M paratuberculosis*. The

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ıber of organisms recovered from guinea pigs necropsied PI days 1 to 7 was variable, but organisms forming nies too numerous to count were recovered from the r, kidney, spleen, and aggregated lymph nodules (Peyer's hes) of each guinea pig given 10^{10} organisms.

Fewer organisms were recovered from all tissues after postpartum examinations than when recovery was attempted on PI days 1 to 7. *Mycobacterium paratuberculosis* detected culturally in 12 of the 14 guinea pigs given 10^{10} nisms, in all of the 14 given 10^8 organisms, and in 10 of 14 given 10^6 organisms; it was not found in the noninated guinea pigs.

Of 24 guinea pigs in which uterine infections were demrated at necropsy, 7 had become pregnant. Four of 12 ea pigs with infection detected only in organs other n the uterus became pregnant. Three of 6 inoculated ea pigs in which no *M paratuberculosis* was detected ame pregnant. None of these aborted or died.

At necropsy, adhesions were found in 1 uterine horn of the guinea pigs given 10^{10} organisms and in 2 given 10^6 nisms. The uterus of all others appeared normal, and lence of metritis was absent.

Perhaps because the male guinea pigs were defective, no ales at any dose level, including the noninoculated cons in 4 cages, became pregnant. In the remaining 10 cages, 2 of the guinea pigs given 10^{10} *M paratuberculosis* ame pregnant, and both of these died while pregnant. ee of the remaining 10 guinea pigs given 10^8 organisms ame pregnant, and 9 of those given 10^6 organisms became gnant. Six of the 14 noninoculated guinea pigs became gnant.

The 3 guinea pigs in the group given 10^8 organisms that ame pregnant did not respond to johnin and delivered young. Within the group given 10^6 organisms, 3 aborted r IM injection of johnin, 1 of which then died, and 1 gnant guinea pig died without aborting after IM johnin ction. The other 5 pregnant guinea pigs in this group vered live young.

Cattle—In the cow necropsied on PI day 1, lesions were n in the left horn and body of the uterus. The lumen was d with fibrin, cell debris, and inflammatory cells, mainly trophils. The uterine mucosa had large denuded areas, the exposed lamina propria had undergone coagulative rosis. Numerous neutrophils and eosinophils were presin the endometrial stroma. A similar cellular infiltrate observed in the endometrial stroma of the cow necropd at PI day 2, but uterine epithelium was not affected. cow necropsied 3 days after inoculation had diffuse ometritis characterized by the accumulation of numerlymphocytes in the uterine stroma. A few neutrophils eosinophils also were observed. The overlying epithen was intact and apparently normal. One cow necropsied 2 weeks after inoculation had a similar lesion in the rne body, and the uterine horns had numerous circumbed lymphocytic nodules in the endometrial stroma. ides lymphocytes, the nodules contained a few macroges and occasionally a neutrophil or eosinophil. The s necropsied at PI days 1, 2, 3, and 7 had similar phocytic nodules throughout the endometrial stroma of uterus, but such nodules were infrequent in sections n the other cows. Acid-fast bacilli were seen only in the necropsied at 1 day after inoculation. There were nerous organisms in the intraluminal exudate of the rus; however, none was seen in the uterine wall. Chorionic epithelium, loosely attached to the uterine epithelium, was seen in 2 cows necropsied at PI week 3 and in 2 cows necropsied at PI week 4.

Mycobacterium paratuberculosis was recovered in culture from the body and horns of the uterus of the cows necropsied at PI days 1, 2, 3, and 7 and from 1 of the 3 cows necropsied at week 2. A single colony was isolated from a pelvic lymph node of this last-named cow. *Mycobacterium paratuberculosis* was not recovered from mesenteric lymph nodes, ileum, liver, lung, spleen, or mammary lymph nodes of any of the cattle. It was not recovered from any specimens from the other 2 cattle necropsied at PI week 2 or from tissue from any of the cattle necropsied at 3 or 4 weeks.

Discussion

Mycobacterium paratuberculosis has been isolated from bovine semen specimens only sporadically and in small numbers. However, the need to detect the organisms at necropsy made it necessary to use a large inoculum both in guinea pigs and in cattle.

Breeding of the NADC strain of English short-hair guinea pig by caging 4 females with 1 male for 10 days has been shown to yield about 40% pregnancy.[5] The noninoculated group achieved that rate (6/14, or 43%), and those given the smallest dose of inoculum substantially exceeded it (9/14, or 64%). These rates would be even higher if the guinea pigs in 4 cages with apparently defective males were excluded from consideration. Pregnancy frequencies were greatly less, however, among the guinea pigs given the higher concentrations of organisms, and the 2 animals in the inoculation group given the largest dose that became pregnant died before parturition.

At necropsy, the uterus of all inoculated guinea pigs, except 4, appeared normal. Hence, the failure to become pregnant may not have been a result of gross physical alteration of the uterus, but rather on a heightened inflammatory response at the time of insemination. There was no evidence of an inflammatory response remaining at the time of necropsy.

Animals hypersensitive to mycobacteria may respond with either hypothermia or hyperthermia to systemic challenge injection of tuberculin. The abortions that occurred in some of these guinea pigs may have been due to a bodily temperature response or to a more localized inflammatory response within the uterus, but inflammatory cells were no more prevalent in infected than in noninfected animals, or in those that had aborted, compared with those that had not been pregnant.

Regardless of the mode of action, it is evident that intrauterine inoculation of guinea pigs with large numbers of *M paratuberculosis* before mating greatly reduced pregnancy frequencies and that some abortions followed hypersensitivity reactions to johnin in guinea pigs which had been given intrauterine inoculations of smaller numbers of *M paratuberculosis*.

The failure to detect *M paratuberculosis* in extrauterine specimens from cattle indicates that relatively fewer organisms were removed from the uterus of these sites in cattle than were obtained in guinea pigs. The failures to isolate any *M paratuberculosis* from 2 of the 3 cattle killed at PI week 2 and from all cattle killed at 3 and 4 weeks after inoculation probably indicate that the few organisms to be found in a semen sample would present only a small hazard for recipient cows.

On histopathologic examination, lesions were seen in the uterus only in cows necropsied in the 1st 2 weeks after inoculations were done. The initial lesion, necrosis of the superficial mucosa, apparently was transitory, because after the 1st PI day, we did not see any pathologic changes in uterine epithelium.

The cellular infiltrate during the period shortly after inoculation was granulocytic; however, we cannot be sure that it was a response to *M paratuberculosis* because neutrophils normally are present in the endometrial stroma of cows during the 1st 2 or 3 days after estrus.[6,7] We believe that the lymphocytic cell infiltration observed in 3 cows did represent an inflammatory response to the bacteria, because large numbers of lymphocytes are not characteristic of any stage of the bovine estrus cycle.[6] All of the cows necropsied within 7 days after inoculation had numerous lymphocytic nodules in the endometrial stroma. Because nodules rarely were seen after the 1st week, we concluded that they should be considered an inflammatory response to the *M paratuberculosis* inoculation. The presence of lymphocytic nodules in the uterus, although regarded as an abnormality,[6,8,9] is not thought to be a threat to pregnancy.[8]

In the present experiment, chorionic epithelium was examined in 4 of the 6 cows necropsied after the time at which implantation of the bovine blastocyst begins (about 20 days after conception). In these cows, the lymphocytic inflammatory response produced by intrauterine deposition of *M paratuberculosis* seemingly did not seriously interfere with normal conception or survival of the young embryo.

The number of *M paratuberculosis* isolated from bull semen has been small, but might be sufficient to establish hypersensitivity in a recipient cow. Such hypersensitivity conceivably could lead to abortions when such cattle are injected with johnin. However, results of the evidence in the present experiment indicate that small numbers of *M paratuberculosis* introduced into the uterus with semen probably would be destroyed, rather than leading to systemic infection or persistent hypersensitivity of the cow.

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