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Bactericidal Effect and Serological Response of Blood and Secretions in Bovine Vibriosis

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Udder infection of vaccinated heifers with *Vibrio fetus* var. *venerealis* led to an early and severe reaction consisting of local swelling, hyperthermia, and increased blood leukocyte counts. This reaction was absent or less pronounced in heifers not previously vaccinated. This was interpreted as an immediate hypersensitivity reaction elicited in the vaccinated heifers. Specific antibodies were found in udder exudate from vaccinated heifers. Immune serum and udder exudate were moderately bactericidal and had a strong immunosuppressive and opsonophagocytic effect in rabbits. Immune cervicovaginal mucus was neither bactericidal nor opsonophagocytic or immunosuppressive. This would suggest that antibodies found in cervicovaginal mucus are not protective. It appeared that the immediate hypersensitivity observed and the subsequent transfer of antibodies from serum to udder exudate would provide by analogy a possible explanation of the mechanisms of immunity to bovine vibriosis.

Bovine vibriosis is a venereal disease in cattle caused by *Vibrio fetus* var. *venerealis*. It has been known for a long time that the disease is self-limiting in the individual and in the herd (19). Although this convalescent immunity is well recognized in bovine vibriosis, its mechanisms are still poorly understood.

The disease is limited to the genital tract of cows (13). *V. fetus* does not usually invade the circulatory system, and lesions consisting of lymphocyte and plasma cell infiltration are confined mainly to the glandular epithelium of the uterine mucosa (7, 18). The infection does not usually stimulate the production of antibodies detectable in the serum (13, 15).

Agglutinating antibodies are formed locally in the uterus and in the vagina (12). Neither the nature of the cervicovaginal antibodies nor their role in immunity has been determined (3). They are elicited within a few weeks after infection and decrease after the elimination of the infection (21). High titers of these agglutinins are usually detected in the presence of *V. fetus* in the genital tract.

Immunity, artificially induced by the injection of live organisms or bacterins with adjuvants, has been successful in improving the reproductive performance in infected animals (2, 3, 6, 9–11). Also, intracervical inoculation of virulent strains has produced a minor degree of protection (9).

The mechanism by which parenteral injection of bacterins reduces the incidence of infection is not well understood. Serum antibodies are detectable after vaccination. There is no evidence of antibodies in the cervicovaginal mucus (CVM) after vaccination to parallel the antibody response in the serum (3, 13, 16, 20). Clark (2) found that the protection afforded by vaccination was equal to the immunity resulting from previous infection.

The objectives of this work were to elucidate some aspects of the mechanisms of immunity in bovine vibriosis. *V. fetus* was injected into the udder of vaccinated and nonvaccinated virgin heifers, and various reactions to infection were studied after vaccination and local exposure. Also, specific antibodies from secretions and from serum were compared on the basis of their bactericidal activity in vitro and their opsonophagocytic activity when injected with *V. fetus* into rabbits. Finally, the bactericidal activity of freshly drawn blood was measured in heifers after udder infection with *V. fetus* and subsequent vaccination.

MATERIALS AND METHODS

Animals. A total of 11 virgin Holstein heifers from 10 to 15 months old were used in these experiments. CVM and blood samples were collected prior to the experiments. No agglutinins were detected in the mucus, and very low titers were detected in the serum of some heifers.

A commercially prepared *V. fetus* adjuvant bacterin, Vibrin (Norden Laboratories, Lincoln, Nebr.), was given subcutaneously in the cervical region to six heifers assigned to be vaccinated. Two heifers in this group received a second injection of the vaccine, 4 months after the primary injection. Among the five heifers assigned to the control group, two received virulent cultures of *V. fetus* by intracervical deposition while in heat.

The udders were infected by inoculation of *V. fetus* through a small catheter into the left front quarter of the heifers at different times after vaccination or revaccination. Two heifers were infected intracervically and were inoculated into the udder 2 months after infection. *V. fetus* has been isolated periodically from the CVM of these two heifers. One purpose of udder infection was to determine the difference in local inflammatory and systemic reactions between six vaccinated and five control heifers. The rectal temperature was read for several consecutive days before and 3 days after udder infection. Blood samples were similarly taken and the total and differential white blood cell counts were determined. A second purpose of udder infection was to determine serologic differences between the udder exudates of vaccinated and control heifers. After udder infection, the heifers were "milked" every day until no more fluid could be secured from the inflamed quarter.

Four adult cows were infected intracervically with *V. fetus*. The CVM was sampled every week to provide mucus antibodies for the subsequent tests.

Vibrio fetus. Virulent *V. fetus* cultures were used for udder inoculation. They were kept in small samples in liquid nitrogen. For the serologic tests, *V. fetus* was subcultured every 2 or 3 days on cysteine heart blood agar plates (9).

Complement fixation test. The complement fixation (CF) test described by Fulton and Dumbell (8) was used in these investigations. Commercial guinea pig serum and hemolysin (Colorado Serum Co. Denver, Colo.) were used. The standardization of the antigen and the performance of the test were carried out by the method of Kramer and Hoerlein (13).

CVM agglutination. The extraction of these antibodies was performed by a method described previously (16). The mucus was diluted in saline, ground with a 7-ml Ten Broeck grinder and suspended in molten New Zealand Davis agar. The solidified agar was overlaid with saline and incubated overnight at 37 C. The antibodies were present in the supernatant in a standard final dilution of 1:15. Vibrio antigen of the UM strain (from A. Winter, Cornell University) was used for the agglutination test.

Bactericidal test of freshly drawn blood. The test was performed as described in an earlier publication (2). Four milliliters of blood was collected from the jugular vein and mixed with 0.1 ml of *V. fetus* standardized to 10^8 viable cells/ml. The results were expressed as percentage of survivals of *V. fetus* after 2, 5, and 10 min.

Bactericidal test on serum, udder exudate, and CVM. Pools of the different sources of antibodies were used. The tests were carried out as described previously (2) in various dilutions of the pools, using exogenous guinea pig complement.

Opsonophagocytic test. Rabbits of the New Zealand albino strain were injected with *V. fetus* opsonized with specific antibodies from serum, udder exudate, and CVM. The readings were done over a period of 2 hr (S. P. Carrier and T. T. Kramer, *in preparation*).

RESULTS

Reactions to udder infection with V. fetus. The group of six vaccinated heifers reacted severely to udder infection with *V. fetus*. The severity of this reaction was estimated by subjective and objective criteria, which consisted of local swelling, quantity and quality of inflammatory exudate, temperature rise, and rise in white blood cell counts.

An appreciable difference in the clinical reaction of vaccinated and nonvaccinated heifers was seen. In the former six heifers, inflammation was present after 24 hr and consisted of a warm and hard swelling extending approximately 15 cm around the inoculated quarter. In the latter five heifers, the reaction was considerably less pronounced.

The exudate from both groups was different. In the vaccinated group, at 24 and 48 hr, it con-

TABLE 1. *Mean values of white blood cell counts and rectal temperatures after inoculation of Vibrio fetus into the udder of vaccinated and nonvaccinated heifers*

Heifers	Days after udder inoculation											
	0			1			2			3		
	N/L[a]	WBC[b]	T[c]	N/L	WBC	T	N/L	WBC	T	N/L	WBC	T
Vaccinated	0.55	8.6	102.4	1.36	12.6	103.9	1.50	12.0	102.7	0.58	8.7	102.3
Nonvaccinated	0.36	8.6	102.4	0.83	10.5	101.9	0.51	9.5	102.4	0.48	8.4	102.7

[a] N/L, ratio of neutrophil counts over lymphocyte counts.
[b] WBC, white blood cells in multiples of 10^3.
[c] T, rectal temperature in F.

TABLE 2. *Reciprocal of complement fixation titers of serum and udder exudate after udder inoculation of Vibrio fetus var. venerealis in vaccinated and nonvaccinated heifers*

Days after udder inoculation	Vaccinated heifers												Nonvaccinated heifers									
	Days after revaccination				Days after primary vaccination								Days after intracervical infection				Not infected intracervically					
	7		14		53		53		63		78		60		60							
	S[a]	E[b]	S	E	S	E	S	E	S	E	S	E	S	E	S	E	S	E	S	E	S	E
0	32	—[c]	32	—	16	—	32	—	4	—	8	—	0	—	0	—	0	—	2	—	0	—
1	32	64	32	—	32	32	64	32	4	4	8	4	0	0	0	0	0	0	0	0	0	—
2	32	64	64	—	32	32	64	32	8	4	4	4	0	0	0	0	0	0	0	—	0	—
3	64	—	32	32	32	—	64	—	8	—	8	—	—	0	—	0	0	—	—	0	—	—
5	—	128	—	—	—	—	—	—	8	—	8	16	—	4	—	32	0	0	0	—	0	—
6	—	—	32	—	64	128	64	64	8	8	8	32	—	—	—	—	—	—	—	—	—	—

[a] S, serum.
[b] E, exudate.
[c] —, Not sampled or insufficient volume in the case of udder exudate.

sisted of an abundant serous fluid with few flakes which coagulated rapidly at room temperature. Afterwards, the exudate became thick and purulent with a yellowish creamy texture. In the nonvaccinated group, the exudate was thick and purulent throughout the sampling period, and the volume of exudate limited to 1 to 2 ml.

The white blood cell counts and differential counts in the vaccinated group showed increases of the polymorphonuclear series for a period of 48 hr or more. In the nonvaccinated group, the blood count did not change or changed only slightly in some heifers for a period of 24 hr. Hyperthermia was recorded only in the vaccinated group (Table 1).

V. fetus was isolated after 24 hr from 2/6 vaccinated heifers and from 5/5 nonvaccinated heifers.

The time elapsed between vaccination and udder infection of the vaccinated group did not seem to affect the degree of inflammatory reaction.

CF antibodies in serum and udder exudate. Similar CF antibody titers were found in the serum and in the udder exudate (Table 2). The CF titers of both serum and udder exudate dropped after 53 days postvaccination. Furthermore, the CF antibody titers of the exudate seemed to increase with the days postinfection in most vaccinated heifers. In the nonvaccinated, noninfected group, two heifers had a mild inflammatory reaction to the inoculation into the udder and no exudate or only a very small amount could be secured at 48 hr from the infected quarter. In the two cervically infected heifers, CF titers could be demonstrated in the udder exudate after 5 days from both heifers.

Bactericidal activity of freshly drawn blood. Two heifers were used in this experiment. The bactericidal activity of the freshly drawn blood varied in both animals (Fig. 1a, 1b). The bactericidal activity of one heifer was marked after udder inoculation (Fig. 1b), whereas this activity was minimal in the second heifer (Fig. 1a). The CF titers correlated with this observation since only heifer b had a CF titer when the bactericidal test was performed (Table 3).

The bactericidal activity was enhanced after vaccination, though individual variability was evident. However, complete elimination of *V. fetus* occurred in 10 min when blood was taken 3 and 4 weeks after vaccination.

Bactericidal activity in vitro. Bactericidal activity of serum antibodies was present at different dilutions (Table 4). This activity was demonstrated after an incubation of 1 hr at 37 C. Furthermore, it seems that an incubation of 3 hr did not modify the relative intensity of the reaction.

CVM antibodies did not have bactericidal activity at any of the dilutions tested (Table 5).

The antibodies found in the udder exudate had a considerably lesser bactericidal activity than sera of equivalent agglutination titers (Table 6). The increase in colony counts for each twofold dilution of immune serum was 0.175 to 0.200 $\times$ 10^7, whereas the corresponding increase for each twofold dilution of immune udder exudate was 0.050 $\times$ 10^7 colonies/ml. Semilogarithmic plots of twofold dilutions of serum or udder exudate versus colony counts gave a straight-line relationship.

Opsonophagocytic activity. When rabbits were injected with *V. fetus* opsonized with immune

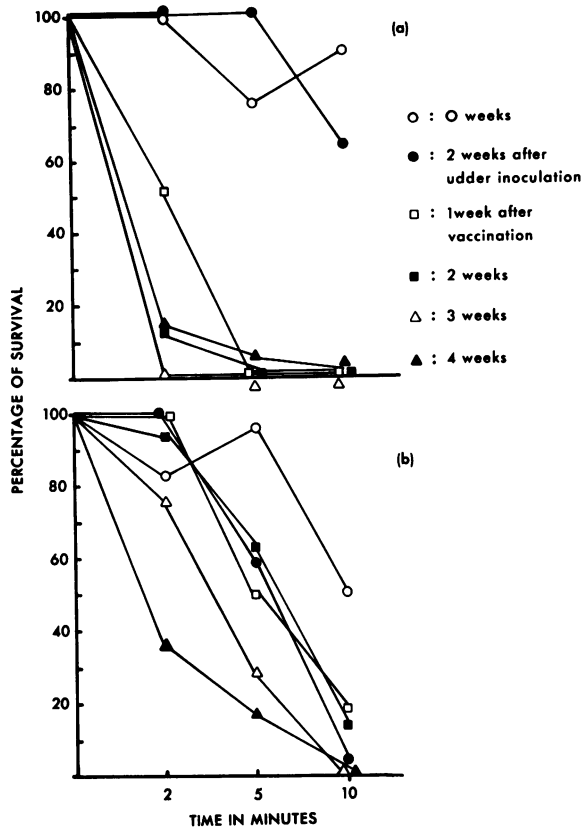

FIG. 1. *The percentage of survival of V. fetus var. venerealis in freshly drawn blood measured after udder infection and at weekly intervals after vaccination. The graphs represent the results obtained from two heifers separately.*

TABLE 3. *Reciprocal of complement fixation titers of serum taken at different intervals after udder inoculation and subsequent vaccination*

Heifers	Time in weeks					
	0	2[a]	1[b]	2	3	4
a	0	0	32	32	32	32
b	0	32	64	256	256	128

[a] Two weeks after udder inoculation.
[b] One week after vaccination.

serum and udder exudate, the rate of clearance was increased over that obtained when *V. fetus* was opsonized with nonimmune serum (Table 7).

In addition, the antibodies from serum and udder exudate proved to be immunosuppressive in the rabbits. The injection of *V. fetus* opsonized with immune serum or immune udder exudate did not elicit an antibody response in the injected rabbits. When *V. fetus* was opsonized with nonimmune serum (titer 1:20) or immune CVM (titers 1:120 and 1:240), the injection was followed by an agglutinin response (titers 1:640 to 1:5,120).

The antibodies from CVM were found to be neither opsonophagocytic nor immunosuppressive. However, a slight difference was observed between CVM antibodies sampled at estrus and midestrus. This difference was accompanied by a twofold difference in agglutination titers, probably insufficient to account for the difference in opsonocytophagic effect.

TABLE 4. *Bactericidal activity of immune pooled sera (agglutinin titer 1:640)*

Serum dilution	Incubation	
	1 hr	3 hr
1:20	3.9×10^{7}[a]	4.3×10^{7}
1:160	5.3×10^{7}	6.3×10^{7}
1:640	6.1×10^{7}	7.3×10^{7}
Controls	7.1×10^{7}	8.1×10^{7}

[a] Colony counts per milliliter.

TABLE 5. *Bactericidal activity of pools of immune cervicovaginal mucus (CVM) sampled at estrus and midestrus (agglutinin titer 1:120)*

CVM dilution	Pools CVM[a]	
	Estrus	Midestrus
1:15	3.6×10^7	3.8×10^7
1:120	3.8×10^7	4.0×10^7
1:480	3.6×10^7	3.8×10^7

[a] The control of this experiment gave a colony count of 3.5×10^7. Colony counts per milliliter.

TABLE 6. *Bactericidal activity of pools of immune and nonimmune udder exudates*

Udder exudate dilution	Agglutination titers of udder exudate pools[a]	
	1:320	1:20
1:20	1.3×10^7	2.5×10^7
1:160	1.7×10^7	2.6×10^7
1:640	1.9×10^7	2.6×10^7

[a] The control of this experiment gave a colony count of 2.9×10^7. Colony counts per milliliter.

DISCUSSION

V. fetus was injected into the udder of virgin heifers in order to evaluate the extent of local reactions and to gain easy access repeatedly to inflammatory fluids. Such observations could not have been gained from the female genital tract, the natural site of *V. fetus* infection in cattle. In addition, serologic data were compared, using serum, udder exudate, and CVM.

Udder infection of vaccinated heifers led to an early and severe reaction, consisting of local swelling, hyperthermia, and increased blood leukocyte counts. This reaction was absent or less pronounced in heifers not previously vaccinated (Table 2). The difference between vaccinated and nonvaccinated heifers was interpreted as an immediate hypersensitivity in the former group. *V. fetus* can elicit both an immediate and delayed hypersensitivity reaction upon intradermal inoculation in heifers. Hypersensitivity reaction may occur in the uterus of infected heifers (15), but such reactions are not easily observable. However, the large numbers of organisms injected into the udder may not allow us to draw any conclusions pertinent to natural infection with *V. fetus*.

The role of secretory antibodies in immunity is currently the subject of intensive investigations (22). The significance of vaginal agglutinins in immunity to bovine vibriosis is not well understood and has not been sufficiently studied. Vaginal agglutinins appear inconsistently in some animals (13, 16), due to changes in the composition of mucus during the estrus cycle.

Our data indicate that immune serum and udder exudate were moderately bactericidal and have a strong immunosuppressive and opsonocytophagic effect, using rabbits as test animals. Although Muschel (17) cautioned against using the bactericidal effect as a criterion of immunity, the in vivo opsonocytophagic effect was interpreted as a good indicator of immunity. A good correlation existed between serologic tests, bactericidal, and opsonocytophagic effects of serum and udder exudates. CVM mucus antibodies reacted quite differently. No bactericidal activity in vitro of CVM was demonstrated.

TABLE 7. *Mean rate of in vivo clearance in the rabbit of live Vibrio fetus var. venerealis opsonized with specific antibodies from blood, from udder exudate and from cervicovaginal mucus (CVM) of cows*

Opsonizing antibodies		Time in min						Serum agglutination titer 1 week after injection of opsonized *V. fetus*
Pooled samples	Agglutination titer	5	10	30	60	90	120	
Serum I (3 rabbits)	1:640	276[a]	107	31	17	13	2	0
Serum II (3 rabbits)	1:20	TNTC[b]	TNTC	348	301	262	217	1:640–1:5,120
Udder fluid (2 rabbits)	1:320	263	213	3	1	0	0	0
CVM (estrus) (3 rabbits)	1:120	TNTC	TNTC	TNTC	466	378	245	1:640–1:2,560
CVM (midestrus) (3 rabbits)	1:240	TNTC	TNTC	403	118	35	11	1:1,280–1:2,560

[a] Colony counts.
[b] TNTC, 500 or more colonies.

Furthermore, the opsonocytophagic effect of immune CVM was negligible in comparison to the effect obtained with immune serum or udder exudate (Table 7). Immunosuppression did not occur with CVM, an added indication that mucus agglutinins did not opsonize *V. fetus*. The above results suggest that antibodies found in CVM are not protective.

The killing effect of immune serum and udder exudate is a likely explanation of vaccinal immunity to bovine vibriosis. The rapid appearance of antibodies in the udder might parallel events occurring in the uterus of immune heifers. It was shown that circulating antibodies can be transferred to the uterine cavity of both pregnant and nonpregnant rabbits and guinea pigs. This passage of antibodies across the uterine wall does not depend on the presence of a placenta (1).

Snyder (M.S. Thesis, Colorado State University, 1967) observed the appearance of antibodies in deep inguinal lymph nodes in vaccinated heifers, 8 days after challenge; in contrast, antibodies were demonstrable in these lymph nodes only at 15 days after challenge in nonvaccinated heifers. The early appearance of antibodies in the mammary exudate is best explained by extravasation of circulating antibodies due to the inflammatory reaction. Such extravasation occurs in the mammary gland during colostrum formation prior to calving. Increases in colostral immune globulin were paralleled by a corresponding decrease in serum immune globulin (4, 5).

It has been demonstrated that specific immune globulin could be extravasated from the blood to the mucous membranes after stimulation with nonspecific agents, and it is thought to result from a local increase of tissue permeability caused by the inflammatory effect of these agents (22).

The transfer of antibodies is a likely explanation of the mechanism of immunity to bovine vibriosis. Our failure to find any significant immune effect of CVM upon *V. fetus* further suggests the role of systemic immunity in this disease.

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LITERATURE CITED

1. Brambell, F. W. R. 1958. The passive immunity of the young mammal. Biol. Rev. 33:488–531.
2. Clark, B. L., J. H. Dufty, and M. J. Monsbourg. 1968. Experimental *Vibrio fetus* (*venerealis*) infection in heifers. The immunizing properties of killed organisms injected subcutaneously. Aust. Vet. J. 44:110–114.
3. Clark, B. L., I. D. B. Newsam, M. J. Monsbourg, and J. H. Dufty. 1967. Experimental *Vibrio fetus* infection in cows. Studies on the immunizing properties of living organisms injected subcutaneously. Aust. Vet. J. 43:341–345.
4. Dixon, F. J., W. O. Weigle, and J. J. Vazquez. 1961. Metabolism and mammary secretion of serum proteins in the cow. Lab. Invest. 10:216–237.
5. Feldman, J. D. 1961. Fine structure of the cow's udder during gestation and lactation. Lab. Invest. 10:238–255.
6. Frank, A. H., J. H. Bryner, and P. A. O'Berry. 1967. The effect of *Vibrio fetus* vaccination on the breeding efficiency of cows bred to *Vibrio fetus*-infected bulls. Amer. J. Vet. Res. 28:1237–1242.
7. Frank, A. H., W. T. Shalkop, J. H. Bryner, and P. A. O'Berry, 1962. Cellular changes in the endometrium of *Vibrio fetus*-infected and non-infected heifers. Amer. J. Vet. Res. 23: 1213–1216.
8. Fulton, F., and K. R. Dumbell. 1949. The serological comparisons of strains of influenza virus. J. Gen. Microbiol. 3:97–111.
9. Hoerlein, A. B., and T. T. Kramer. 1963. Artificial stimulation of resistance to bovine vibriosis. Amer. J. Vet. Res. 24: 951–955.
10. Hoerlein, A. B., and T. T. Kramer. 1964. Artificial stimulation of resistance to bovine vibriosis: use of bacterins. Amer. J. Vet. Res. 25:371–373.
11. Hoerlein, A. B., E. J. Carroll, T. T. Kramer, and W. H. Beckenhauer. 1967. Bovine vibriosis immunization. J. Amer. Vet. Med. Ass. 146:828–835.
12. Hughes, D. E. 1953. A study of the diagnosis of bovine vibriosis with special reference to the detection of agglutinins in the vaginal secretions. Cornell Vet. 43:431–444.
13. Kramer, T. T., and A. B. Hoerlein. 1969. Serologic response of heifers vaccinated and exposed to vibriosis. Amer. J. Vet. Res. 30:1089–1098.
14. Laing, J. A. (ed.). 1960. *Vibrio fetus* infection in cattle. Food Agr. Organ. U.N. FAO Agr. Studies. 51:1–62.
15. Larson, K., and L. Ringen. 1967. Serologic analysis of bovine vibriosis. Amer. J. Vet. Res. 28:1231–1235.
16. Mitchell, D. 1968. Some effects of experimental *Vibrio fetus* (*venerealis*) infection on cattle inoculated with a commercial bacterin. Can. J. Comp. Med. 32:474–479.
17. Muschel, L. H. 1960. Serum bactericidal actions. Ann. N.Y. Acad. Sci. 88:1265–1272.
18. Peterson, J. E., and I. D. B. Newsam. 1964. The histopathology of genital vibriosis in virgin heifers. Brit. Vet. J. 120:229–245.
19. Smith, T., R. B. Little, and M. S. Taylor. 1920. Further studies on the etiological role of *Vibrio fetus*. J. Exp. Med. 32:683–689.
20. Te Punga, W. A. 1962. Vibriosis in cattle. Part 3. Studies on control by vaccination. Aust. Vet. J. 10:89–91.
21. Te Punga, W. A., B. W. Boyes, J. S. Young, and N. M. Wallace. 1964. Vibriosis in cattle. Part 4. Further studies on control by vaccination. Aust. Vet. J. 12:121–122.
22. Tomasi, T. B. Jr., and J. Bienenstock. 1968. Secretory immunoglobulins. Advan. Immunol. 9:1–96.