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Laboratory Findings in Cows after Experimental Infection with *Ehrlichia phagocytophila*

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The goal of this study was to assess various hematological variables in 10 cows after experimental infection with *Ehrlichia phagocytophila*. Blood samples were collected at regular intervals for examination of leukocytes for *Ehrlichia* organisms and for determination of hematological and biochemical variables. In addition, PCR amplification was performed throughout the disease period on blood and milk samples for the detection of *E. phagocytophila* organisms. The time of seroconversion and the duration of serum titers indicating positivity were determined by indirect immunofluorescence. For all cows, *E. phagocytophila* organisms were first detected microscopically in leukocytes 5 to 8 days postinfection and could be demonstrated for a period of 6 to 14 days. For all cows, the appearance of *E. phagocytophila* organisms in leukocytes coincided with transient erythropenia, leukopenia, and thrombocytopenia and a decrease in hematocrit and hemoglobin concentration. For five lactating cows, *E. phagocytophila* organisms were identified in leukocytes of milk samples during the acute phase of the disease, which, we believe, has not previously been reported. *E. phagocytophila* DNA was detected in blood samples by nested PCR from 1 to 2 days before to 2 to 12 days after the organisms were identified microscopically. In milk samples, *E. phagocytophila* DNA was detected for an average of 11 days.

Bovine ehrlichiosis is a systemic disease in which pyrexia, decreased milk production, respiratory symptoms, and tick infestation are the predominant clinical signs (11). The agent, *Ehrlichia phagocytophila*, is an obligate intracellular bacterial agent that is found mainly in neutrophils and eosinophils but occasionally in monocytes during the acute stage of the disease. This results in severe leukopenia, characterized by neutropenia and lymphopenia. Other hematological changes include severe thrombocytopenia, a mild decrease in the hematocrit and concentration of hemoglobin, and mild erythrocytopenia (13, 16, 25, 26). To our knowledge, complete blood counts and serum profiles have not been determined during the course of disease in cows after experimental infection with *E. phagocytophila*. In humans, horses, and goats, granulocytic ehrlichiosis results in changes in hepatic and renal enzyme activities (20, 27).

Diagnosis of bovine ehrlichiosis is based on demonstration of *Ehrlichia* organisms in leukocytes during the acute phase of the disease; however, because they are present in low numbers, the organisms are difficult to detect during the early and the late phases of infection. Failure to demonstrate *Ehrlichia* organisms microscopically does not rule out disease. To overcome these diagnostic difficulties, a modification of the PCR, a sensitive and specific technique, was recently developed for numerous *Ehrlichia* species (1, 7). Barlough et al. (3) developed a nested PCR for identification of *Ehrlichia equi* in blood of horses after experimental infection. The PCR yielded positive results from 4 days before the first day of parasitemia until 2 days after the last day of parasitemia. Because of the 99.9% homology of the 16S rRNA genes of *E. equi* and *E. phagocytophila*, this method is also suitable for identification of *E. phagocytophila* in cattle (3).

Sheep and cattle produce antibodies to *E. phagocytophila* after natural or experimental infection. In sheep, antibodies were detected 9 to 11 days after initial infection and persisted for 6 to 10 weeks (28). For cattle such detailed data are not available. However, when naturally infected cattle were examined once a month, a maximum titer occurred after 60 days, and by day 120 the same animals had become seronegative (13).

The purpose of this study was to analyze hematological, biochemical, and serological data for 10 cows after experimental infection with *E. phagocytophila*. In addition, nested PCR was performed in an attempt to identify DNA from *E. phagocytophila* in blood and milk samples from these cows during the entire course of disease. The clinical examinations were previously published (17). Briefly, all cows became ill with symptoms of tick-borne fever after an incubation period of 5 to 9 days. The most important clinical signs were pyrexia, decreased milk production, respiratory symptoms, and mildly to moderately disturbed general condition. Clinical signs returned to normal in all cows without treatment after an average of 8 days.

MATERIALS AND METHODS

Cows. Ten clinically healthy Swiss Braunvieh cows aged 3 to 8 years (mean, 6.1 years) were used. Five of the cows were lactating, and the other five were dry and 6 to 9 months pregnant. All cows came from tick-free regions and had no antibodies to *E. phagocytophila*, as determined by indirect immunofluorescence.

Experimental infection of the cows was performed by administering 50 ml of whole blood from a cow with tick-borne fever from the central part of Switzerland. The blood contained 460 leukocytes infected with *E. phagocytophila* (Swiss strain) per μl. The possibility of bovine viral diarrhea virus, bovine leukemia virus, and bovine herpesvirus 1 contamination of the challenge inoculum in this experiment has been excluded.

Hematological, biochemical, and cytological examination. Blood samples were collected from jugular veins of all cows into evacuated glass tubes that contained EDTA or no anticoagulant (Becton Dickinson Vacutainer; Aichele Medico AG, Basel, Switzerland) on the day of experimental infection (defined as day 0) and every day thereafter for 3 weeks. Blood smears were prepared from each blood sample and stained with May-Grünwald Giemsa stain and examined at a magnification of ×1,000. Five hundred leukocytes were examined for *E. phagocytophila* organisms, and the percentage of positive cells was calculated. Blood samples collected into evacuated EDTA tubes were used for determination of leukocyte, erythrocyte, and thrombocyte counts; hematocrit; plasma protein and fibrinogen concentrations; and differential blood count. Blood samples without an anticoagulant were used to measure concentrations of urea nitrogen, creati-

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TABLE 1. Severity of leukocyte infection in cows after experimental infection with *E. phagocytophila*

Day	% Infected leukocytes									
	Cow 1	Cow 2	Cow 3	Cow 4	Cow 5	Cow 6	Cow 7	Cow 8	Cow 9	Cow 10
0	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
5	0	1	8.5	0	2.5	0	0	1	1	2
6	5	11	30	6.5	11	0	0	11	3.5	13.5
7	13.5	17	23.5	23	7.5	1	0	14.5	10.5	26.5
8	18	20	7.5	11	3.5	17	1.5	23.5	6.5	7.5
9	16.5	6	6.5	2	3	7.5	5	1.5	2	10.5
10	7	3	3	0	3	14.5	7.5	1	2.5	1
11	1	1	2	1.5	1.5	2.5	3	1.5	2	1
12	2	2	2	0	1	1.5	3	1.5	1	1
13	2	1.5	1	0	0	1	2	0	1	1
14	2	0	1	0	0	1	1	0	1	1
15	1	1	0	0	0	1	0	0	1	1
16	2	1	1	0	0	1	0	0	0	1
17	2	2	0	0	0	1	0	0	0	1
18	0	1	0	0	0	1	0	0	0	0
19	0	0	0	0	0	1	0	0	0	0
20	0	0	0	0	0	0	0	0	0	0
21	0	0	0	0	0	0	0	0	0	0

nine, total bilirubin, sodium, potassium, and chloride and activities of aspartate aminotransferase (AST), γ-glutamyltransferase (γ-GT), glutamate dehydrogenase (GLDH), sorbitol dehydrogenase (SDH), and creatine kinase (CK) in serum. The hematological and biochemical examinations were performed as previously described (4).

From each of the five lactating cows, a 30-ml milk sample was collected by using a sterile technique on the day of infection and every day thereafter for 4 weeks. The milk sample was collected from a quarter in which the milk had a negative California mastitis test and was bacteriologically sterile. The sample was centrifuged at 1,000 × *g* for 10 min. The pellet was washed twice with phosphate-buffered saline, and smears, made with a cytocentrifuge, were stained with May-Grünwald Giemsa stain. Five hundred cells were examined for intracellular *E. phagocytophila* organisms.

DNA isolation. Heparinized blood samples (10 ml) were collected from each animal on day 0 and every day thereafter for 4 weeks. The samples were centrifuged at 1,000 × *g* for 10 min. After removal of the plasma, 9.0 ml of deionized water was added to hemolyze the erythrocytes, and the procedure was repeated. During each wash, the isotonicity was restored 1 min after the addition of water by adding 1 ml of 9% NaCl solution. The resultant buffy coat was washed twice with phosphate-buffered saline and centrifuged at 1,000 × *g* for 3 min, after which a clean pellet of leukocytes was obtained. This procedure was used to obtain leukocytes from 30-ml samples of milk. Cell pellets from blood and milk samples were resuspended in 500 μl of DNA extraction buffer (10 mM Tris HCl [pH 8.0], 2 mM EDTA, 0.1% sodium dodecyl sulfate, and 500 μg of proteinase K per ml) and incubated at 50°C for 3 h. After phenol-chloroform extraction according to the method of Sambrook et al. (21), the DNA was precipitated with 0.7 volume of isopropanol by centrifugation (15,000 × *g* for 10 min at 4°C). The pellet was then washed with cold 70% ethanol. The ethanol was removed by vacuum centrifugation, and the DNA of the blood and milk samples was dissolved in 200 and 100 μl of sterile water, respectively. The DNA concentration was determined photometrically at 260 nm. For the PCR, the DNA concentration was set at 0.5 μg/μl.

Amplification by PCR and analysis of the synthesized DNA. The specific PCR primers for *Ehrlichia*, EE-1, EE-2, EE-3, and EE-4, described by Barlough et al. (3) were used with the following modifications: the reaction volume was reduced to 25 μl and the primer amounts were reduced to 10 pmol. In the first PCR (EE-1 and EE-2), 0.5 μg of DNA, 0.75 mM magnesium chloride, and a 0.1 mM concentration of each dNTP were used. Denaturation at 94°C for 5 min was followed by amplification for 35 cycles (94°C for 1 min and 72°C for 2 min) with *Taq* polymerase. For nested PCR, 1 μl of the product from the first reaction was used as a DNA template. After denaturation, amplification was performed in a DNA thermal cycler (Thermocycler 480; Perkin-Elmer Cetus, Rotkreuz, Switzerland) for 35 cycles (1 min each at 94, 60, and 72°C) with primers EE-3 and EE-4 (10 pmol) and a magnesium chloride concentration of 1 mM. Products from nested PCR were resolved on 1.2% agarose gels, stained with ethidium bromide, and examined under UV illumination. Direct solid-phase DNA sequencing of PCR products was performed to confirm the presence of PCR products and to identify *E. phagocytophila*. The band representing the amplicon that was 928 bp long was cut out of the gel, and the DNA was isolated. The amplicon was sequenced with an ABI 373A DNA sequencer (Perkin-Elmer).

Serological examination. Blood samples for the production of serum were collected from each cow on day 0, every day thereafter for 4 weeks, and then once a month for another 6 months. The serum was examined for *E. phagocytophila* antibodies by indirect immunofluorescence, and the cutoff titer for a positive serologic response was set to ≥1:20 as described previously (18).

Statistical analysis. The calculation of means and standard deviations (SDs) and the Student *t* test were performed by using the Statistix program for Windows (Analytical Software, Tallahassee, Fla.).

RESULTS

Hematological, biochemical, and cytological examinations. For all cows, *Ehrlichia* organisms were first observed in the cytoplasms of leukocytes at days 5 to 8 and were seen for between 6 and 14 days. During the phase of parasitemia, the percentages of infected leukocytes ranged from 1 to 30%. The maximum infection rates varied from 7.5 to 30% and occurred on the second (four cows), third (four cows), or fourth (two cows) day of parasitemia (Table 1). *Ehrlichia* organisms were identified predominantly in neutrophils but eosinophils and monocytes were also infected, to a smaller degree. Neutrophils and eosinophils were the only cell types infected during the acute phase of the disease. The infected cells at this stage were composed of 92 to 99% neutrophils and 1 to 8% eosinophils. On days 4 to 6 of parasitemia, infected monocytes also appeared at a low level (0.5 to 2.5% of infected leukocytes). The monocytes persisted until the end of the ehrlichemia and were often the only infected cell type.

In all cows, transient hematological and biochemical changes occurred with the onset of parasitemia (Table 2). The hematocrit decreased on days 5 to 8 and reached a minimum on days 8 to 14 in all cows. After that, it slowly increased again and reached preinfection values within approximately 1 week. From days 9 to 14, the hematocrit was significantly lower than on day 0 (Table 3). The erythrocyte count had a course parallel to that of the hematocrit. Five cows had erythrocyte counts below normal ($<4.8 \times 10^6/\mu l$) from days 6 to 10. From days 10 to 12, the erythrocyte counts were significantly lower than on day 0 (Table 3). The hemoglobin concentration was below the normal range in three cows from days 6 to 8. In the other cows, there was a mild decrease, yet the concentration remained within the normal range. In all cows, the hemoglobin concen-

TABLE 2. Hematological and biochemical results for cows after experimental infection with *E. phagocytophila* at the onset of ehrlichemia

Variable (unit)	Finding	No. of cows
Hematocrit (%)	Decreased (<28)	10
Concn of:		
Erythrocytes (10^6/μl)	Normal (4.8–6.8)	5
	Decreased (<4.8)	5
Hemoglobin (g/dl)	Normal (8–11)	7
	Decreased (<8)	3
Leukocytes (no./μl)	Decreased (<4,000)	10
Neutrophils (no./μl)	Decreased (<1,000)	10
Eosinophils (no./μl)	Decreased (<100)	10
Lymphocytes (no./μl)	Decreased (<2,000)	10
Monocytes (no./μl)	Normal (0–330)	10
Thrombocytes (10^3/μl)	Decreased (<100)	10
Plasma protein (g/liter)	Normal (60–80)	10
Fibrinogen (g/liter)	Normal (5–7)	10
Urea nitrogen (mmol/liter)	Normal (<7.5)	10
Creatinine (μmol/liter)	Normal (65–175)	7
	Increased (>175)	3
Bilirubin (μmol/liter)	Normal (0.8–8.6)	7
	Increased (>8.6)	3
AST (U/liter)	Normal (40–80)	10
γ-GT (U/liter)	Normal (0–30)	10
GLDH (U/liter)	Normal (4–26)	10
SDH (U/liter)	Normal (4–26)	10
CK (U/liter)	Normal (<200)	10
Sodium (mmol/liter)	Normal (135–155)	10
Potassium (mmol/liter)	Normal (3.5–5.5)	10
Chloride (mmol/liter)	Normal (96–110)	10

tration was significantly lower from days 9 to 12 than on day 0 (Table 3).

In all cows, the leukocyte count progressively decreased between days 4 to 7 and reached a minimum count of 1,200 to 2,600 cells/μl. The leukopenia lasted 8 to 17 days and was followed by a slow increase in cell numbers. However, on day 21 the values were still below those on day 0. There were significant differences between the leukocyte counts on day 0 and those on days 5 to 21 (Table 3). The leukopenia was characterized by marked lymphopenia, neutropenia, and eosinopenia. The lowest lymphocyte counts (299 to 961 cells/μl) occurred on days 6 to 9. Neutrophils (50 to 475 cells/μl) and eosinophils (12 to 65 cells/μl) reached minimum numbers on days 9 to 14 and 10 to 14, respectively. These cell counts were significantly different from those on day 0 (Table 3). Monocyte numbers decreased within the normal range until day 8 and were not significantly different from those on day 0. The thrombocyte count began to decrease on days 3 to 7 and reached minimum values (30,000 to 136,000/μl of blood) on days 7 to 13. The number then increased steadily, reaching normal values on day 19. There was a significant difference between thrombocyte counts on day 0 and those on days 6 to 14 (Table 3). In all cows the concentrations of plasma protein and fibrinogen remained within normal ranges and did not differ from values on day 0.

Changes in blood chemical constituents were limited to creatinine and bilirubin. In all cows, the creatinine concentration increased slowly starting on days 3 to 5. Three to 7 days later, maximum values from 132 to 235 μmol/liter were measured, after which the concentrations returned to normal. Mean concentrations measured on days 4 to 10 were significantly different from that measured on day 0 (Table 3). The bilirubin concentration increased slightly starting on days 5 to 7 and, in three cows, reached maximum values (11.4, 15.8, and 25.8 μmol/liter) on days 6, 9, and 10, respectively. After day 10, the concentrations slowly decreased to normal values. Blood urea nitrogen, AST, γ-GT, GLDH, SDH, CK, sodium, potassium, and chloride concentrations remained within normal ranges and did not differ from values on day 0.

Ehrlichia organisms were identified in 1 to 5% of neutrophils in milk of all lactating cows. The individual cell count in milk was between 70,000 and 110,000/ml, with a proportion of neutrophils of less than 25%. They were first seen microscopically from the second to the fourth day of parasitemia. In two cows, they were observed for 3 days (days 6 to 8 and 8 to 10), and in

TABLE 3. Hematological and biochemical findings for 10 cows after experimental infection with *E. phagocytophila*[a]

Day	Hematocrit (%)	Concn of:							
		Erythrocytes (10^6/μl)	Hemoglobin (g/dl)	Leukocytes (no./μl)	Lymphocytes (no./μl)	Neutrophils (no./μl)	Eosinophils (no./μl)	Thrombocytes (10^3/μl)	Creatinine (μmol/liter)
0	27.1 ± 1.5	5.2 ± 0.2	9.1 ± 0.6	5,580 ± 818	2,512 ± 319	2,239 ± 734	388 ± 210	368 ± 99	135 ± 24
1	28.1 ± 2.6	5.3 ± 0.2	9.1 ± 0.9	4,790 ± 831	2,336 ± 345	1,783 ± 1,111	406 ± 188	346 ± 78	137 ± 26
2	29.2 ± 2.3	5.5 ± 0.3	9.6 ± 0.8	4,890 ± 950	2,558 ± 420	1,535 ± 802	464 ± 303	346 ± 85	141 ± 26
3	27.8 ± 2.6	5.4 ± 0.3	9.4 ± 0.8	5,010 ± 1,244	2,483 ± 371	1,608 ± 892	448 ± 308	363 ± 83	141 ± 25
4	27.7 ± 3.0	5.4 ± 0.5	9.4 ± 0.8	4,760 ± 950	2,376 ± 452	1,497 ± 719*	465 ± 321	349 ± 72	145 ± 18**
5	27.1 ± 3.0	5.5 ± 0.5	9.2 ± 0.9	4,210 ± 1,304***	1,902 ± 659**	1,770 ± 1,284	323 ± 194	331 ± 72	149 ± 18**
6	27.5 ± 3.5	5.3 ± 0.4	8.7 ± 1.2	3,060 ± 1,419***	1,454 ± 983**	1,122 ± 331***	281 ± 140	253 ± 63**	159 ± 26***
7	25.7 ± 2.0	5.1 ± 0.4	8.9 ± 1.0	2,330 ± 715***	990 ± 486***	986 ± 325***	241 ± 141*	175 ± 91***	163 ± 31***
8	25.2 ± 3.2	5.0 ± 0.5	8.6 ± 1.1	2,130 ± 643***	1,064 ± 394***	783 ± 351***	150 ± 93**	163 ± 89***	158 ± 39**
9	24.6 ± 2.6*	4.9 ± 0.6	8.4 ± 0.9*	2,120 ± 796***	1,322 ± 768***	514 ± 232***	68 ± 35***	134 ± 65***	155 ± 33**
10	24.0 ± 2.4**	4.7 ± 0.5*	8.2 ± 0.9*	2,200 ± 800***	1,503 ± 594***	475 ± 202***	50 ± 33***	163 ± 70***	148 ± 22*
11	23.6 ± 2.9**	4.7 ± 0.6*	8.0 ± 1.0**	2,280 ± 642***	1,588 ± 529***	419 ± 199***	48 ± 27***	198 ± 64***	146 ± 16
12	23.7 ± 2.9**	4.7 ± 0.7*	8.1 ± 1.2**	2,390 ± 762***	1,556 ± 606**	500 ± 337***	42 ± 25***	231 ± 74**	137 ± 13
13	24.7 ± 3.1**	4.9 ± 0.8	8.2 ± 1.2	2.320 ± 964***	1,571 ± 701**	446 ± 412***	42 ± 31***	233 ± 74**	139 ± 16
14	24.3 ± 3.0**	4.9 ± 0.7	8.4 ± 1.0	2,500 ± 963***	1,802 ± 450**	590 ± 668***	48 ± 29***	249 ± 89**	135 ± 15
15	25.2 ± 3.1	5.0 ± 0.7	8.6 ± 1.1	3,310 ± 885***	2,179 ± 566	903 ± 710**	77 ± 55***	314 ± 81	136 ± 16
17	26.5 ± 3.2	5.4 ± 0.7	8.6 ± 1.2	3,690 ± 1,206***	2,109 ± 266	1,256 ± 591**	134 ± 98**	330 ± 44	136 ± 16
19	25.4 ± 2.7	5.2 ± 0.4	8.6 ± 0.9	3,880 ± 1,189**	2,282 ± 456	1,370 ± 845*	218 ± 161*	366 ± 68	135 ± 22
21	25.6 ± 2.7	5.4 ± 0.4	8.8 ± 0.9	4,480 ± 563**	2,585 ± 361	1,287 ± 682*	271 ± 169	405 ± 114	131 ± 17

[a] All values are means ± SDs. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

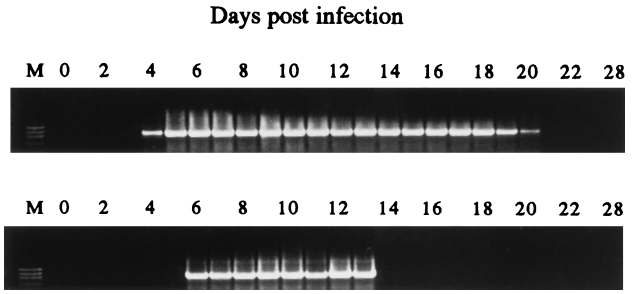

FIG. 1. Detection of *E. phagocytophila* by nested PCR of DNA from the buffy coats of blood samples (top) and milk (bottom) from a cow after experimental infection. M, molecular size standard (ϕX174/*Hae*III).

the other three cows, they were observed for 1 day only (days 7, 8, and 8).

Nested PCR. For all cows, a band representing 928 bp (Fig. 1) was first obtained from days 3 to 6 (mean ± SD, 4.2 ± 1.1 days [Table 4]). This was 1 to 2 days before *Ehrlichia* organisms were first identified microscopically in leukocytes of blood samples (mean ± SD, 5.7 ± 1.0 days). The last *Ehrlichia* identification by nested PCR occurred from days 17 to 26 (mean ± SD, 21.6 ± 2.9 days), which is 2 to 12 days after the last microscopic identification (mean ± SD, 15.1 ± 2.8 days). Parasitemia as demonstrated by nested PCR was detected from days 14 to 23 (mean ± SD, 18.4 ± 2.9 days) and lasted 3 to 14 days longer than parasitemia as demonstrated by microscopic identification of *Ehrlichia* organisms in leukocytes (mean ± SD, 10.4 ± 2.8 days).

For lactating cows, the band representing the 928-bp product was also obtained with neutrophils of milk samples (Fig. 1) and was first detected from days 5 to 6 (mean ± SD, 5.6 ± 0.5 days [Table 4]). This was 2 to 3 days before *Ehrlichia* organisms were first identified microscopically in milk neutrophils. The last of *Ehrlichia* identification by PCR occurred from days 13 to 16 (mean ± SD, 15.4 ± 1.3 days). Thus, products of 928 bp were amplified from *E. phagocytophila* DNA of milk samples for 8 to 12 days (mean ± SD, 10.8 ± 1.6 days).

The sequence of the amplicon was identified by sequence comparison as part of the 16S rRNA gene of *E. phagocytophila* (1, 7).

TABLE 4. Detection of *E. phagocytophila* by nested PCR amplification of DNA from buffy coats from blood samples and milk samples from experimentally infected cows

Cow no.	Day of:				Duration (days) of detection	
	First detection[a]		Last detection			
	Blood	Milk	Blood	Milk	Blood	Milk
1	4		21		18	
2	4		26		23	
3	4		21		18	
4	5		18		14	
5	3		17		15	
6	6	6	21	16	16	11
7	6	6	26	16	21	11
8	3	5	23	16	21	12
9	3	5	23	16	21	12
10	4	6	20	13	17	8
Mean ± SD	4.2 ± 1.1	5.6 ± 0.5	21.6 ± 2.9	15.4 ± 1.3	18.4 ± 2.9	10.8 ± 1.6

[a] Day 0 = day of infection.

Serological examination. Titers of antibody to *E. phagocytophila* of 1:20 occurred in all cows for the first time from days 6 to 11. The titers increased progressively until they reached a maximum of 1:320 to 1:5,120 from days 14 to 23. They remained at this level for 30 to 60 days, after which they began to decrease. Positive titers were determined for three cows until day 120, for one cow until day 150, and for six cows until day 180 postinfection. On day 210, all cows had negative titers (<1:20).

DISCUSSION

E. phagocytophila organisms were first identified microscopically 5 to 8 days postinfection and were seen for 6 to 14 days. This is in agreement with findings of other authors (13, 26). In most cows, the occurrence of *Ehrlichia* bodies corresponded to the occurrence of clinical findings (17); increases in rectal temperature were associated with the appearance of *Ehrlichia* bodies on the same day or 1 to 2 days earlier. In contrast, a decrease in rectal temperature was not accompanied by the disappearance of *Ehrlichia* bodies from the peripheral circulation. The percentages of infected leukocytes varied markedly among individual cows, but the course of infection over time was generally the same for all cows and was characterized by a rapid increase followed by a slow decrease in numbers of infected cells. In this study, the maximum percentages of infected leukocytes (7.5 to 30%), which occurred between the second and fourth days of parasitemia, were lower than those reported by Tuomi (26) (6 to 72%) and by Liz (13) (46 to 64%). The percentage of infected leukocytes did not appear to affect clinical, hematological, or biochemical findings. The most commonly infected leukocytes were neutrophils, followed by eosinophils and monocytes. Neutrophils were predominantly infected during the acute phase of the disease, whereas monocytes were infected towards the end. Streit (23) found that neutrophils and eosinophils are the cells in which the organisms primarily multiply, whereas monocytes and lymphocytes are secondary host cells for *E. phagocytophila.*

Hematological changes occurred on the same day as or a few days after *E. phagocytophila* organisms were identified in leukocytes. The first changes were a decrease in the hematocrit, erythrocyte count, and concentration of hemoglobin, similar to findings by Purnell et al. (16). These transient changes may have been the result of an increased rate of destruction or impaired erythropoiesis, similar to what has been described for disease caused by other species of *Ehrlichia* (5). The severe leukopenia was characterized by initial lymphopenia followed by neutropenia and eosinopenia. While lymphocyte numbers began to increase within a few days after the acute phase, the numbers of neutrophils and eosinophils continued to decrease and did not return to normal values until the end of infection. Monocyte numbers varied the least and suffered only a mild and short-lived decrease. These results are in close agreement with those of numerous authors (8, 9, 11, 16, 19, 25, 26). In all reports, a decrease in lymphocyte and neutrophil numbers, but not necessarily in eosinophil and monocyte numbers, appears to be a consistent finding. Leukopenia has been attributed to possible suppression of bone marrow production (10, 24) or to the destruction of infected cells (11). The number of thrombocytes also varied greatly during the course of the experimental infection. Although thrombocytopenia, which began prior to the microscopic identification of *Ehrlichia* organisms, was severe, it did not result in any clinical signs, such as spontaneous hemorrhage. In contrast to the granulocytes, the number of thrombocytes and lymphocytes returned to normal before day 21. We assumed that the thrombocytopenia was due to an

increased consumption of platelets and premature destruction in the spleen, as occurs in infections with other *Ehrlichia* species. Immunological and inflammatory processes play an important role in platelet consumption (12, 14, 15, 22). Changes in the concentrations of plasma proteins and fibrinogen were not observed in this study and have not been reported in the literature.

To our knowledge, biochemical changes in the blood of cows after experimental infection with *E. phagocytophila* have not been investigated. This study showed that the concentrations of creatinine and bilirubin increased slowly at the beginning of the infection and returned to normal near the end of the study. In humans and goats with granulocytic ehrlichiosis, increases in the activities of renal and hepatic enzymes in some individuals are often the result of a transient impairment of renal and hepatic functions (2, 6, 27). Although there were significant differences in the concentrations of creatinine and bilirubin between day 0 and the first few days of infection, possible renal or hepatic impairment would have been of minor importance.

To our knowledge, this is the first report on *E. phagocytophila* inclusions in milk leukocytes. There were smaller numbers of infected leukocytes in milk than in blood, and they appeared 2 to 4 days after the start of parasitemia. However, infection via ingestion of milk does not, at the moment, seem to be a likely possibility. Nevertheless, studies involving calves are currently under way in our clinic to investigate the possibility of this route of infection.

In recent years, the diagnosis of ehrlichiosis has been improved considerably with the development of PCR (1, 7). The method developed by Barlough et al. (3) for the detection of *E. equi* was used for the detection of *E. phagocytophila* based on the 99.9% homology of the 16S rRNA genes of *E. equi* and *E. phagocytophila*. *E. phagocytophila* DNA was present in leukocytes from blood as well as milk samples. *E. phagocytophila* was identified by PCR in blood samples from 1 to 2 days prior to until 2 to 12 days after the last day of microscopic detection of organisms. PCR products were obtained from *E. phagocytophila* DNA in milk samples for an average of 11 days. Thus, PCR was useful for the diagnosis of bovine ehrlichiosis, particularly during early and late stages, when the number of organisms was too small for diagnosis by microscopy.

Antibodies to *E. phagocytophila* were first detected from days 6 to 11, which is in keeping with the results of Webster and Mitchell (28), who reported that seroconversion occurred 9 to 11 days after infection. In our study, positive titers persisted for 120 to 180 days, which is in general agreement with the average duration of positive titers of 120 days observed after a single natural infection (13).

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