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Experimental Infection of Lambs with an Equine Granulocytic *Ehrlichia* Species Resembling the Agent that Causes Human Granulocytic Ehrlichiosis (HGE)

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Stuen S, Artursson K, Olsson Engvall E: Experimental infection of lambs with an equine granulocytic *Ehrlichia* species resembling the agent that causes human granulocytic ehrlichiosis (HGE). Acta vet. scand. 1998, 39, 491-497. – Five lambs were inoculated with a granulocytic *Ehrlichia* species originally isolated from a Swedish horse with granulocytic ehrlichiosis (EGE). The 16S rRNA gene sequence of the Swedish *Ehrlichia* sp causing EGE was identical to the sequence of the agent causing human granulocytic ehrlichiosis (HGE). After the inoculation, infected neutrophils and a low serologic response were seen in all lambs, but no clinical symptoms were observed. In one lamb 17% of the neutrophils were infected without a corresponding fever. Six weeks later the lambs were inoculated with an ovine isolate of *E. phagocytophila* After challenge with *E. phagocytophila* the lambs reacted with fever and infected granulocytes. The results presented herein show that the equine *Ehrlichia* isolate was infective for lambs but generated weak immune response and no distinctive protection from subsequent challenge with *E. phagocytophila.*

E. phagocytophila; antibodies; immunity.

Introduction

Tick-borne fever (TBF) in ruminants caused by *Ehrlichia phagocytophila* was first described in sheep (*MacLeod* 1932), and later in goats (*MacLeod & Gordon* 1933) and cattle (*Hudson* 1950). Granulocytic ehrlichiosis caused by *Ehrlichia equi* has so far only been reported from horses in USA (*Madigan* 1993), but similar granulocytic *Ehrlichia*-infection in horses has been described from Germany (*Büscher et al.* 1984), Switzerland (*Herman et al.* 1985), Sweden (*Björsdorff et al.* 1990), United Kingdom (*Korbutiak & Schneiders* 1994) and Denmark (*Eriksen et al.* 1997).

New strains of granulocytic *Ehrlichia* have recently been characterised, such as the agent causing human granulocytic ehrlichiosis (HGE) (*Chen et al.* 1994), and an unnamed *Ehrlichia* species isolated from dogs and horses in Sweden (*Johansson et al.* 1995) and from dogs and horses in USA (*Greig et al.* 1996, *Madigan et al.* 1996). The *Ehrlichia* isolates from both horses and dogs in Sweden are identical with respect to the 16S rRNA nucleotide sequence to the agent that causes HGE (*Johansson et al.* 1995), while it differs in only 2 and 3 positions from the 16S rRNA sequences of *E. phagocytophila* and *E. equi,* respectively (*Olsson Engvall et al.* 1996).

Ehrlichia spp. seem to have a preference for certain mammalian species in nature (*Rikihisa*

1991), although experimental infections have shown that it is possible to transmit granulocytic *Ehrlichia* spp. between different animal hosts. Thus, *E. equi* has been experimentally transmitted from horses to donkeys, sheep, goats, macaques, baboons and dogs (*Gribble* 1969, *Lewis et al.* 1975), and *E. phagocytophila* has been transmitted from sheep to splenectomized mice and guinea pigs (*Foggie & Hood* 1961) and reindeer (*Stuen* 1996). Furthermore, the HGE-agent has been transmitted from humans to horses (*Madigan et al.* 1995) and mice (*Telford et al.* 1996). Serological evidence of a granulocytic Ehrlichia infection in humans has recently been obtained in both Norway and Sweden (*Bakken et al.* 1996, *Dumler et al* 1997).

Observations on the immunity to TBF indicate that the immune response varies according to the strain of the organism, the type and age of the host and the time and frequency of challenge (*Woldehiwet & Scott* 1993). The aim of the present study was to examine whether an *Ehrlichia* species causing equine granulocytic ehrlichiosis (EGE) could infect lambs and induce immunity to TBF.

Materials and methods

Seven 6 months old lambs of the Dala and Rygja breeds were used. Five lambs were inoculated intravenously on day 0 with 5 ml of non-stabilated blood from a Swedish horse, known to be infected with a granulocytic *Ehrlichia* sp. (*Olsson Engvall et al.* 1996). The blood had been kept frozen at –70 °C and was thawed immediately before use. Two of the lambs inoculated with *Ehrlichia* were given prednisolone (Prednisolon®, Leo, Denmark) intramuscularly, 50 mg daily on days -2, -1, 0 and 1, respectively. Two lambs served as controls and received 5 ml of a defrosted non-*Ehrlichia* infected horse blood. All animals were kept indoors from birth and during the whole experimental period. EDTA-blood samples were collected on days 0, 2, 4, 6, 8, 10, 12, 13, 14, 16, 18, 21, 25, 28, 35, 42, 45-52, 54, 56, 58, 60 and 63. Hematological values, including total and differential leucocyte counts, were carried out electronically (Technicon H1®, Miles Inc., USA) and blood smears were prepared and stained with May-Grünwald Giemsa. Four hundred neutrophils were microscopically examined on each smear and the number of cells containing inclusions was recorded. The percentage of parasitised neutrophilic granulocytes was calculated. Rectal temperatures were measured once daily at the same hour in the morning. The incubation period was defined as the period between inoculation and the first day of fever (≥ 40.0 °C). The duration of fever was recorded as the number of days with body temperature of 40.0 °C or more. The magnitude of fever was calculated as the area under the temperature curve for each lamb as described by *Woldehiwet & Scott* (1982). All lambs were weighed weekly during the whole experimental period. To investigate if the *Ehrlichia* infected lambs had become immune to TBF, all lambs were inoculated intravenously with 1ml of a whole blood dimethyl sulphoxide stabilate of an ovine *E. phagocytophila* strain, which had been stored at –70 °C, on day 42 (*Stuen et al* 1992). Clinical and hematological changes were observed in these lambs for the next 3 weeks.

Serum samples were collected from the lambs on days 0, 7, 14, 21, 28, 42, 49, 56 and 66. An indirect immunofluorescence antibody assay (IFA) was used to determine antibodies to *E equi* (Protatek International and Organon Teknika) (*Madigan et al.* 1990, *Artursson et al.* submitted).

Results

No fever (≥ 40 °C) or other signs of illness were observed in the lambs during the first 42 days after inoculation. However, a variable percent-

Table 1. Percentage of infected cells in lambs inoculated with an *Ehrlichia* species originally isolated from a horse. The percentage was determined by examining 400 neutrophils in a May-Grünwald Giemsa stained blood smear.

Lamb no	Days after inoculation											
	0	2	4	6	8	10	12	14	16	18	21	25
1	–	–	–	–	–	–	–	0.25	–	–	–	–
2	–	–	–	–	–	–	–	0.25	1 75	–	0 25	–
3	–	–	–	–	0.25	0 5	1 75	1 5	0 5	–	–	–
4	–	0 25	–	0.25	2.5	6.75	6.0	0.25	–	–	–	–
5	–	–	–	0 5	13.5	17.0	5.75	–	–	0.25	–	–
6	–	–	–	–	–	–	–	–	–	–	–	–
7	–	–	–	–	–	–	–	–	–	–	–	–

Lambs 1-5 were infected with *Ehrlichia* sp on day 0
Lambs 4 and 5 were given 50 mg prednisolone i m. on days –2, –1, 0 and 1.
Lambs 6 and 7 were uninfected controls
– no inclusions were found.

age of infected neutrophils was registered in the blood of all lambs inoculated with the equine *Ehrlichia* isolate (Table 1). The absolute number of infected cells varied from 3.4×10^6 to 340×10^6 infected neutrophils/l, while the corresponding percentage of infected cells varied from 0.25 to 17.0, respectively. The 2 lambs given prednisolone had the highest number of infected granulocytes and showed a decrease in the blood neutrophil level on day 12 and day 14, respectively, but neutropenia (i.e. no. of neutrophils <0.7 G/l) was not observed (data not shown). No other hematological reaction was registered during this period.

After inoculation with *E. phagocytophila* on day 42 all lambs reacted with fever and infected neutrophils (Tables 2 and 3). The lambs also showed dullness, inappetence and coughing, which lasted for 3 to 4 days.

No difference in weight gain between the lambs was observed during the first 42 days of the experimental period. However one week after inoculation with *E. phagocytophila,* the mean weight dropped 1.6 (± 1.02) kg in the animals previously infected with the equine isolate and 3.5 (± 1.50) kg in the lambs not infected previously. Seven days later the lambs had gained weight again, 2.6 and 2.0 kg (mean values) in these 2 groups, respectively (data not shown).

After the first *Ehrlichia* sp. infection 2 of the lambs developed a positive antibody titre to *E. equi* (Table 4). After the inoculation with *E. phagocytophila* on day 42, positive fluorescent antibody titres to *E. equi* were found within 14 days in all lambs.

Discussion

The present study shows that the lambs only acquired a subclinical infection when inoculated with an equine granulocytic *Ehrlichia* sp. isolate. In spite of the lack of clinical signs, infected neutrophils were detected in the blood of all inoculated lambs. This is in accordance with an earlier experimental *E. equi* infection in sheep, where the sheep were afebrile after inoculation with infected horse blood, but inclusions were seen in granulocytes of some of the animals (*Stannard et al.* 1969). Similarly, horses do not seem to be susceptible to an *E. phagocytophila* infection, since no clinical signs, granulocytic inclusions or serologic responses were observed after infection with an

Table 2. Means (± SD) of different clinical variables in lambs challenged with *Ehrlichia phagocytophila* 42 days after being inoculated with an *Ehrlichia* sp. originally isolated from a horse

	Infected with *Ehrlichia* sp	
	Infected (n = 5)	Uninfected (n = 2)
Incubation period (days)	5.0 ± 0	4.0
Maximum temperature (°C)	41.4 ± 0.16	41.8
Duration of fever (days)	4 8 ± 2.71	8 5
Magnitude of fever (mm^2)*	336 ± 113.8	564
Minimum of neutropenia (G/l)	0.40 ± 0 06	0.41
Duration of neutropenia (days)	6.8 ± 1.92	7.0

* The magnitude of fever is calculated as the area uner the temperature curve with 40 °C as base line

Table 3. Percentage (mean ± SD) of infected neutrophils in lambs challenged with *E phagocytophila* 42 days after inoculation with an *Ehrlichia* sp. originally isolated from a horse. No inclusions were observed before day 4.

Infected with *Ehrlichia* sp	Days post inoculation with *E phagocytophila*								
	4	5	6	7	8	9	10	12	14
Infected (5 lambs)	12.4 ± 6.6	47.8 ± 5.4	33.2 ± 10.2	26.9 ± 7.8	19.1 ± 7.4	13.6 ± 5.0	2.6 ± 2.7	<1	<1
Uninfected (2 lambs)	19.5	54.5	41.3	43.8	46.0	27 5	3 0	2 0	<1

Table 4. Reciprocal antibody titres to *E. equi* in lambs inoculated with an equine *Ehrlichia* sp. on day 0 and challenged with *E phagocytophila* on day 42. A titre less than 40 was considered negative

Lamb no	Days after inoculation							
	0	14	21	28	42	49	56	66
1	–	–	–	–	–	80	5120	2560
2	–	–	–	–	–	80	10240	5120
3	–	–	–	80	40	–	160	160
4	–	–	–	–	–	–	640	320
5	–	–	–	–	320	320	1280	640
6	–	–	–	–	–	–	640	640
7	–	–	–	–	–	–	320	320

Lambs 1-5 were infected with *Ehrlichia* sp. on day 0 and with *E phagocytophila* on day 42.
Lambs 4 and 5 were given 50 mg prednisolone i.m. on days –2, –1, 0 and 1.
Lambs 6 and 7 were only infected with *E phagocytophila* on day 42.
– titre <40.

ovine strain of *E. phagocytophila* (*Stuen et al.* 1995).

The percentage of infected cells in the 2 lambs that were given prednisolone was more than 3 times higher than in the untreated lambs. The difference in the number of infected neutrophils could be due to a drug-induced immunosuppression in the lambs given prednisolone, mak-

ing them more susceptible to the infection (*Tizard* 1992). Immunosuppressive drugs have earlier been reported to induce relapsis in TBF-infected sheep (*Scott* 1978). These 2 lambs also showed a drop in the neutrophil level at the end of the bacteriemic period. However, a neutropenia, which is typical for a TBF infection in lambs, was not observed. No hematologic changes were observed in the other infected lambs during the same period.

After challenge with *E. phagocytophila* the lambs reacted with fever and infected granulocytes as described earlier in TBF-infected lambs (*Woldehiwet & Scott* 1993). The results indicate a difference between the 2 groups of lambs in the length of the incubation period, the temperature reaction, the weight loss and in the level of bacteriemia (Tables 2 & 3), suggesting some cross-protection between the ehrlichiae causing EGE and TBF. However, statistical calculations on the differences were not carried out due to the low number of controls. In another study it was shown that one horse, first experimentally infected with HGE agent, became resistant to a subsequent challenge with *E. equi* (*Barlough et al.* 1995).

In the present study no or a very low serologic response was observed during the primary infection, indicating a low antigenic stimulation of the immune system. One reason for this low antibody response could be that *E. equi* was used as antigen in the IFA test. Serological cross-reactions between *E. equi, E. phagocytophila* and the agent causing HGE have been reported, but the titre to a heterologous strain of *Ehrlichia* was normally lower than to the homologous strain (*Dumler et al.* 1995, *Nicholson et al.* 1997). Unfortunately, no antigen slides with *E. phagocytophila* or the Swedish *Ehrlichia* species were available for testing the sera in this study.

The low serologic antibody titre could also have been due to a low immunogenicity in sheep to the equine *Ehrlichia* isolate used. In contrast, the same agent could cause serious infections and give high IFA titres to *E. equi* when inoculated into dogs (*Egenvall et al.* 1998) and horses (*Olsson Engvall* unpublished observations).

When challenged with *E. phagocytophila,* the lambs in the present study showed an antibody response within 14 days. Except for 2 lambs which reacted with a high antibody titre, a moderat increase in the titre was registered. Again this could be due to the use of *E. equi* as antigen in the fluorescent test (*Stuen et al.* 1998).

Earlier studies of experimental infection with different isolates of *E. phagocytophila* have shown a variable degree of clinical manifestations and cross-immunity (*Foggie* 1951, *Tuomi* 1967a,b, *Scott* 1984). This could be due to variations in the genes coding for surface proteins (*Sumner et al.* 1997). Hopefully, more genetic information which will explain differences in the pathogenicity between isolates of granulocytic ehrlichiae infecting sheep, will be available in the near future. It is still a question if *E. equi, E. phagocytophila* and the agent that causes HGE, should be considered as separate species, subspecies, or different variants of one species. Both 16S rRNA and groESL sequence data indicate that these agents are very closely related (*Chen et al.* 1994, *Sumner et al.* 1997). Molecular characteristics such as DNA-DNA reassociation experiments have to be performed for a definite classification of these ehrlichiae, but also biological and ecological differences, e.g. host competence, between *Ehrlichia* isolates should be considered (*Dumler et al.* 1995, *Olsson Engvall et al.* 1996).

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Sammendrag

Eksperimentell infeksjon av lam med en equin granulocyttær Ehrlichia sp som ligner på det agens som forårsaker human granulocyttær ehrlichiose (HGE)

Fem lam ble podet med en granulocyttær *Ehrlichia* sp opprinnelig isolert fra en svensk hest med granulocyttær ehrlichiose (EGE) Etter podingen ble parasittemi og en lav serologisk respons påvist hos lamma, men ingen kliniske symptomer ble registrert Hos et lam var 17% av de nøytrofile infisert uten at feber ble observert Seks uker senere ble lamma podet med en ovin stamme av *E phagocytophila* Resultatet fra denne undersøkelsen viste at det equine *Ehrlichia* isolatet var infektivt for lam, men forårsaket en svak immunrespons og ga ingen reell beskyttelse mot en senere *E phagocytophila* infeksjon

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