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Brief Communication

High Serum Folate Values in Lambs Experimentally Infected with *Anaplasma phagocytophilum*

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Folates are involved in nucleic acid, protein and amino acid synthesis. In human serum, it has been estimated that 60% to 70% of folates are bound to proteins (*Wagner* 1985). Folate binding proteins (FBPs) are therefore crucial to the assimilation, distribution and retention of the vitamin folic acid and have been identified in various cells, extracellular fluids and tissues from humans and several animal species. FBPs have different functions based on their biochemical properties and can be divided into 3 classes: high-affinity folate binding proteins (HFBP), membrane-associated folate-binding proteins, which function in the transport of folate compounds across cell membranes, and cytoplasmic-binding proteins with a high affinity for specific reduced-folate compounds (*Henderson* 1990).

Tick-borne fever (TBF) caused by the rickettsia *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*) is a common disease in domestic ruminants on *Ixodes ricinus* infested pastures in Norway (*Stuen* 1997, *Stuen & Bergström* 2001). During the acute infection, *A. phagocytophilum* are found in cytoplasmatic inclusions in blood leucocytes, mainly neutrophils. Up to 95% of the neutrophils can be infected. The rickettsemia is followed by a severe neutropenia lasting for 1-2 weeks (*Foggie* 1951, *Woldehiwet & Scott* 1993). The reason for this neutropenia is not known, but may be due to destruction and removing of infected cells (*Hudson* 1950, *Woldehiwet* 1983, *Rikihisa* 1991).

Neutropenia in the peripheral blood of lambs is seldom observed except for lambs infected with *A. phagocytophilum.* Recently, during a trace element deficiency study in Norway, some lambs grazing on *I. ricinus* infested pastures had both a severe neutropenia ($<0.7x10^9$ cells/l) and increased serum folate values up to 901.2 nmol/l (Kleppa, unpublished result). In contrast, the normal range of serum folate concentration in sheep has been estimated to vary from 3.2 to 6.6 nmol/l (*Branda* 1981). Unfortunately, EDTA-blood was not available for *A. phagocytophilum* examination. The aim of the present study was therefore to evaluate the variation in serum folate concentration in lambs experimentally infected with *A. phagocytophilum.*

Eight lambs, 4 to 5 months old, of the Dala and Rygja breeds were used. All lambs were kept indoors from birth and throughout the whole experimental period. The lambs were fed hay, silage and concentrates. Four lambs were inoculated intravenously on day 0 with 1 ml of a whole blood dimethyl sulphoxide stabilate of an ovine *A. phagocytophilum* variant. Sequencing result of the 16S rDNA gene of this variant was found identical with the DNA sequence of

GenBank accession number M73220. The stabilate contained $1.3x10^6$ *A. phagocytophilum* infected cells/ml. The 4 four lambs were left as uninfected controls. The infected lambs were followed for 42 days, while the controls were followed for 21 days. Rectal temperatures were measured daily. The incubation period was defined as the period between inoculation and the first day of fever (≥40.0 °C). The duration of fever was calculated as the number of days with elevated body temperature (≥ 40.0 °C) (*Woldehiwet & Scott* 1982).

Blood was collected in plain and EDTA- vacutainers (Venoject®, Terumo Europe) from all lambs on days 0, 1- 4, 6, 7, 8, 10, 12, 14, 16, 18 and 21. In addition, samples from the *A. phagocytophilum* infected lambs were collected on days 24, 28, 35 and 42. Blood samples collected in EDTA were analysed electronically (Technicon H1®, Miles Inc., USA) and haematological values including total and differential leukocyte counts were recorded. Blood smears were made, and stained with May-Grünwald Giemsa. Four hundred neutrophils were examined on each smear and the percentage of *A. phagocytophilum* infected neutrophils was calculated.

Whole blood samples were centrifuged within one hour, and sera were stored at ÷20 °C and later analysed for vitamin B_{12} and folate by Solid Phase No Boil Dualcount® kit (DPC) (Diagnostic Products Corporation, LA). As a quality control, 21 of the samples from both inoculated and control lambs were analysed in parallel by use of AutoDELFIA™ Folate® kit (Time-resolved fluoroimmunoassay kit B072-101) at the Division of Clinical Chemistry, Central Hospital in Rogaland, Norway.

Student´s 2 samples t-test and a linear regression analysis were used in statistical calculations (Software program JMP, version 3.1.6.2, SAS Institute).

All 4 *A. phagocytophilum* inoculated lambs reacted with high fever 3 to 4 days after inoculation (mean: 3.3 ± 0.43 days). Maximum temperature recorded in all lambs varied from 41.8 to 42.0 °C (mean: 41.95 ± 0.087 °C), and the duration of fever varied from 7 to 14 days (mean: 9.8 ± 2.68 days). The appetite in the infected lambs was generally depressed for 1 to 4 days during the fever period. In contrast, none of the controls developed fever or other clinical signs. *A. phagocytophilum* inoculated lambs reacted with neutropenia from day 10 to day 18. In this period, the absolute number of neutrophils in infected and contol lambs varied from 0.24 to $1.08x10^9$ and 1.70 to $3.18x10^9$ cells/l, respec-

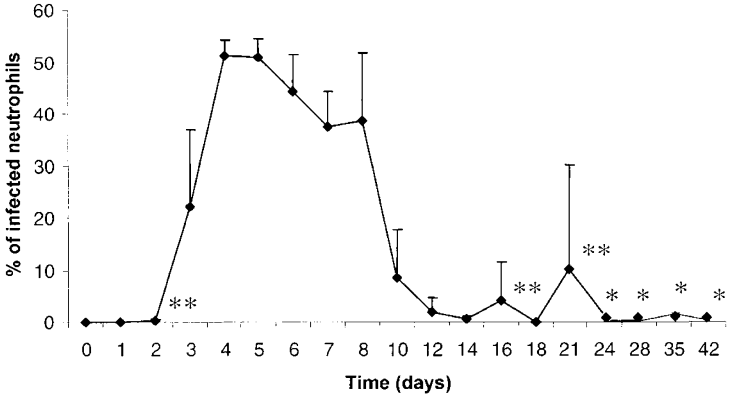

Figure 1. Percentage of infected neutrophils (mean + SD) in 4 lambs inoculated with *A. phagocytophilum* infected blood on day 0 and followed for 42 days. The percentage was less than 1% on days 2, 14, 24, 28 and 42

* one lamb was found infected; not the same lamb each time

** two lambs were found infected

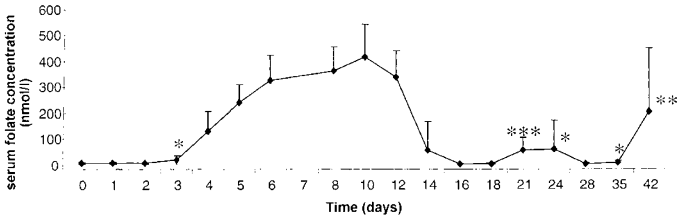

Figure 2. Mean (+ SD) serum folate concentration (nmol/l) in 4 *A. phagocytophilum* infected lambs
* increased value in one lamb; not the same lamb each time
** increased values in 2 lambs
*** increased values in 3 lambs

tively. The absolute number of neutrophils in these 2 lamb groups were significant different from day 8 to day 18 ($p<0.01$).

Inclusions in neutrophils (rickettsaemia) were first seen on days 2 and 3 in inoculated lambs (Fig. 1). The highest percentage of infected neutrophils was seen on day 4 in the inoculated lambs (mean: 51.3% ± 2.87%). Inclusions were not found in blood from the controls.

The median of 21 folate values analysed with the DPC assay system and the AutoDELFIA method was 4.4 and 4.5 nmol/l, respectively. The mean folate values measured by these 2 methods were not significantly different ($p>0.05$).

Mean serum folate concentration increased from day 4 in all infected lambs, except for an increased value (39.6 nmol/l) in one lamb on day 3, with a peak on day 10 (mean: 414.3 ± 130.2 nmol/l) (Fig. 2). In contrast, the mean serum folate concentration in the controls varied from 4.3 to 6.9 nmol/l. The mean folate values in *A. phagocytophilum* infected lambs were significantly different from the corresponding values in the control lambs from day 4 to day 12 ($p<0.05$).

Rickettsaemia and increased serum folate concentration were observed in all infected lambs from day 4 to day 14. Before and after this period, *A. phagocytophilum* infection was found in 5 of 8 samples in lambs with increased serum folate values marked with asterisks in Fig. 2.

The serum vitamin B_{12} concentration was above 300 pmol/l in both *A. phagocytophilum* infected and control lambs, and no significant difference between the 2 lamb groups was observed.

All inoculated lambs showed clinical signs consistent with a typical *A. phagocytophilum* infection (*Foggie* 1951, *Woldehiwet & Scott* 1993). Rickettsaemia were seen in all infected lambs on day 3, while the increase in serum folate concentration was observed on the following day. An exception, one lamb found infected on day 2 had already a high serum folate value the next day. Similarly, the observed rickettsaemia and high serum folate values lasted for around 9 and 10 days, respectively. The second and third peak of serum folate concentration was also observed in the same period when relapses of rickettsaemia were seen in blood smears, although not all lambs with detectable rickettsaemia had an increased serum folate concentration on the same day. This may be due to delay in serum folate response compared with blood rickettsaemia, as observed on days 2, 3 and 4. A direct day-by-day comparison of these two parameters may therefore be difficult. A regression analysis did not show a linear relationship ($p>0.05$).

Rickettsaemia is normally followed by neutropenia in *A. phagocytophilum* infected lambs (*Foggie* 1951). As mentioned earlier, the reason for this neutropenia may be due to destruction and removing of infected cells (*Hudson* 1950, *Woldehiwet* 1983, *Rikihisa* 1991). The high serum folate values in *A. phagocytophilum* infected lambs may therefore be caused by leak-

age of soluble and membrane-bound folate from neutrophils. This assumption is supported by the observation that soluble FBPs have been identified in specific granules of human neutrophils. These granules are released during phagocytosis and may play a role in the control of an infection (*Colman & Herbert* 1980). However, this theory has to be further investigated.

Changes in folic acid metabolism in vitamin B_{12} deficient sheep have been reported (*Gawthorne & Smith* 1974), but in the present study the serum vitamin B_{12} concentration was considered to be within normal variation (*Suttle* 1986).

Mantzos et al. (1974) examined the occurrence of specific binders of folic acid with high binding capacity in plasma from 16 healthy sheep and found only one sheep positive. This result indicates that HFBF are not normally present in the blood of sheep. Unfortunately, no further information about the sheep was available, since the blood samples were obtained from a slaughterhouse. In order to analyse the origin of the high serum folate concentration, it is necessary to know the exact nature of the folate components (*Wagner* 1985, *Henderson* 1990).

In conclusion, the present report is the first description of high serum folate concentration in experimentally *A. phagocytophilum* infected lambs. The present study indicates that *A. phagocytophilum* infected sheep can be used as a model in the study of soluble FBPs from neutrophils. However, the mechanism behind the elevated serum folate concentration in *A. phagocytophilum* infected lambs and the class of FBP involved have to be further investigated.

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