Differences in Clinical Manifestations and Hematological and Serological Responses after Experimental Infection with Genetic Variants of *Anaplasma phagocytophilum* in Sheep

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Five-month-old lambs were experimentally infected with two 16S rRNA genetic variants of *Anaplasma phagocytophilum*, variants 1 (GenBank accession no. M73220) and 2 (GenBank accession no. AF336220). Additional sequencing of the groESL heat shock operon gene indicated that these variants differ in three nucleotides at positions 782, 824, and 890. The variants were obtained by blood sampling of *A. phagocytophilum*-infected lambs from one sheep flock in Norway and were stored at −70°C with 10% dimethyl sulfoxide as a cryoprotectant before being inoculated intravenously into susceptible lambs. The infectious blood contained, per ml, approximately 0.5 × 10⁶ neutrophils infected with either of the variants. Six weeks after the primary inoculation, the lambs were challenged with the same infectious dose of the heterologous variant. The results of the study indicate a marked difference in clinical manifestation, neutropenia, antibody response, and cross-protection after experimental infection with the two variants of *A. phagocytophilum*.

Tick-borne fever (TBF), caused by the bacterium *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophila*), is a disease of sheep that is endemic in tick (*Ixodes ricinus*)-infested areas in Norway. Natural infection with *A. phagocytophilum* has been reported in humans and a variety of domestic and wild animal species (6). A serological survey of sheep indicated that *A. phagocytophilum* infection is widespread along the coast of southern Norway. However, TBF had been diagnosed in only half of these seropositive sheep flocks (15). The reason for this diagnostic deficit may be the existence of different variants of the agent that cause different clinical symptoms and immunological reactions (4, 18).

Although *A. phagocytophilum* variants are discriminated in the laboratory on the basis of 1 to 2 nucleotide differences in the 16S rRNA gene, biological and ecological differences, including variations in host pathogenicity, vectors, and geographical distribution, clearly exist (11). Sequences from the groESL operon have been shown to more clearly delineate genetic variants of *A. phagocytophilum* than have sequences of the 16S rRNA gene (17). Based on a 16S rRNA gene sequence study, it has recently been shown that different variants of *A. phagocytophilum* may exist simultaneously in the same sheep flock (16). The aim of the present study was to investigate further the clinical, hematological, and serological responses and the cross-protective abilities of two variants of *A. phagocytophilum* identified in the same sheep flock in Norway.

MATERIALS AND METHODS

Source of *A. phagocytophilum* and DNA sequencing. Blood samples were collected from a flock of Norwegian sheep with TBF. Both blood samples collected

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In the present study, the 1,256-bp sequences of the groESL heat shock operon of these two variants were described by Sumner et al. (17).

Animals, experimental design, and hematology. Twelve lambs, 5 months old, of the Dala breed were used in this trial. The lambs were unrelated and belonged to the experimental sheep flock at the Department of Sheep and Goat Research, Norwegian School of Veterinary Science. None of the lambs had previously been in *I. ricinus*-infested pastures, and they were kept indoors during the whole experimental period of 3 months. Four lambs were inoculated intravenously with 1 ml of a whole-blood dimethyl sulfoxide stablimate of *A. phagocytophilum* variant 1, and on the same day four lambs were inoculated with 1 ml of a whole-blood dimethyl sulfoxide stablimate of *A. phagocytophilum* variant 2. The infectious blood contained, per ml, approximately 0.5 × 10⁶ neutrophils infected with either of the variants. Four lambs left uninfected were used as controls. Six weeks after the primary inoculation, the lambs were challenged with the same infectious dose of the heterologous variant.

Rectal temperatures were measured daily in all of the lambs throughout the experimental period. 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 elevated body temperature (≥40.0°C). The magnitude of fever of each lamb was estimated from the area of plots of daily temperature on 5-mm² grids and calculated according to the trapazium rule (20). For this purpose, 40°C was taken as the baseline.

Blood samples were collected daily into EDTA tubes during the fever period following the inoculation of infected blood and on a weekly basis after the fever had subsided. In addition, EDTA-blood samples were later collected from individual lambs when rectal temperatures above 40.0°C were recorded. With these blood samples, hematological values, including total and differential leukocyte counts, were determined electronically (Technicon HI; Miles Inc., Tarrytown, N.Y.) and blood smears were prepared and stained with May-Grunwald-Giemsa stains. Four hundred neutrophils were examined on each smear by microscopy,
TABLE 1. Clinical variables for *Anaplasma phagocytophilum* variant 1- and 2-infected lambs

| *A. phagocytophilum* variant(s) | Incubation period (days) | Maximum temp (°C) | Duration of fever (days) | Magnitude of fever (mm²) | Nadir of neutropenia (<0.7 × 10⁸ liter⁻¹) | Duration of neutropenia (days) | Wt reduction (kg) |
|--------------------------------|--------------------------|-------------------|--------------------------|-------------------------|------------------------------------------|-------------------------------|------------------|
| 1                              | 3.3 ± 0.43*              | 4.195 ± 0.087*    | 8.8 ± 1.92**             | 1.677 ± 278***          | 0.34 ± 0.081                             | 9.3 ± 1.30                   | 3.5 ± 0.50**     |
| 2                              | 5.0 ± 0.71               | 4.98 ± 0.415      | 5.3 ± 1.09               | 0.64 ± 199              | 0.59                                     | 1.0 ± 0.71                  | 1.0 ± 0.71      |
| 1 and then 2                   | 6.0*                     | 4.0              | 1.0                      | 0                       | 0.051                                   | 1.0                          | 1.5 ± 0.50      |
| 2 and then 1                   | 3.8 ± 0.43               | 4.128 ± 0.083     | 4.5 ± 1.80               | 554 ± 195               | 0.33 ± 0.061                             | 7.3 ± 2.05                  | 3.3 ± 1.79      |

* Values are means ± standard deviations. Groups of four 5-month-old lambs were infected with one of the two variants. Six weeks after the primary inoculation, the lambs were challenged with the heterologous variant. Statistical differences between *A. phagocytophilum* variant 1- and variant 2-infected lambs are shown. *P < 0.05; **, *P < 0.01; ***P < 0.001.

† Weight reduction was measured 1 week after inoculation.

‡ Only one lamb developed neutropenia (this lamb did not scroconvert [Fig. 1]).

§ Measurements are based on plots of daily temperature on 5-mm² grids (see text for details).

and the number of cells containing *Anaplasma* inclusions was recorded. All of the lambs were weighed weekly during the whole experimental period.

**Serology.** Sera were collected on days 0, 7, 14, and 28 after each inoculation and analyzed by using an indirect immunofluorescence antibody assay to determine the titer of antibody to *Ehrlichia equi* antigen (1, 15). Briefly, twofold dilutions of sera were added to slides precoated with *E. equi* antigen (Protatec, St. Paul, Minn.). Bound antibodies were visualized by fluorescein isothiocyanate conjugated rabbit anti-sheep immunoglobulin (Cappel; Organon Teknika, West Chester, Pa.). Sera were screened for antibodies at a dilution of 1:40. If positive, the serum was further diluted and retested. A titer of 1.6 (log₂ reciprocal of 1:40) or more was regarded as positive.

**Statistics.** Statistical calculations were done by use of Statistix, version 4.0 (Analytical Software), and a two-sample t test was used to determine the hematological variables. A P value of <0.05 was considered significant.

**Nucleotide sequence accession number.** The groESL sequences found in the present study are available in the GenBank database under accession numbers AF548385 and AF548386.

**RESULTS**

**DNA sequence analysis.** The variants differ at positions 782, 824, and 890 in the groESL heat shock operon gene. Comparison with the GenBank database revealed that variant 1 (AF548385) and variant 2 (AF548386) each differ in a single nucleotide from *A. phagocytophilum* groESL sequences amplified from roe deer (*Capreolus capreolus*) in Slovenia (GenBank accession no. AF478558) and from sheep in Scotland (GenBank accession no. EPU96730), respectively. Furthermore, they were 99.6 and 99.4% similar to sequences ampliﬁed from *I. ricinus* and the number of cells containing *A. phagocytophilum* inoculated with *I. ricinus*.

**Clinical parameters, hematology, and serology.** All lambs inoculated with *A. phagocytophilum* developed fever. However, significant differences in clinical signs and hematological reactions were observed (Table 1). A 1- to 4-day period with reduced appetite was observed in lambs infected with *A. phagocytophilum* variant 1, while lambs infected with the other variant showed no signs of clinical illness. No hematological reactions were observed in the control lambs. A marked difference in onset of infection of neutrophils and percentage of infected neutrophils was observed between lambs infected with the two variants (Table 2). After the primary inoculation, the titers of antibody to *E. equi* antigen were absent in three of the four sheep infected with *A. phagocytophilum* variant 2 and only one lamb seroconverted. In contrast, all of the lambs infected with variant 1 mounted high antibody titers (Fig. 1). Neither clinical symptoms nor seroconversion was detected in the control lambs.

There was also a clear difference in clinical reactions between the groups of lambs infected with the two variants after challenge (Tables 1 and 2). No signs of clinical illness except fever were observed. The lambs infected with *A. phagocytophilum* variant 1 were almost fully protected against variant 2, since only one of the four lambs reacted with an elevated temperature. Furthermore, there was only a very modest and late infection of neutrophils in this group of lambs. In contrast, when the lambs infected with *A. phagocytophilum* variant 2 were challenged with variant 1, all of the lambs developed high temperatures and displayed hematological reactions similar to those seen following primary infection. In addition, the antibody titers in the two lamb groups were similar 1 week after challenge.

**DISCUSSION**

In the present study, a significant difference in clinical reactions between sheep infected with two variants of *A. phagocytophilum* was observed. Earlier investigations have shown that...
lams in the bacteremic and neutropenic periods of TBF are more susceptible to secondary infections (2, 5, 7, 19). The present study may indicate, therefore, that general resistance to other infectious agents is less affected in lambs infected with \textit{A. phagocytophilum} variant 2. This assumption is supported by the fact that this variant has been found mainly in a flock with no known disease problem due to tick-borne infections (16). However, \textit{16S} rRNA gene sequence analyses have shown that several variants of \textit{A. phagocytophilum}, including variant 2, are involved in fatal cases of TBF, and this indicates that other factors, such as climate, host condition, management, and other microorganisms, may be important in this context (S. Stuen, S. Nevland, and T. Moum, Abstr. Int. Conf. Rickettsiae Rickettsial Dis., p. 147, 2002.)

Only the \textit{16S} rRNA and \textit{groEL} heat shock operon genes of the \textit{Anaplasma} variants have been sequenced in this study. Whether sequence variations in these or other conserved genes can be used as molecular markers for pathogenicity has yet to be determined. Variations in genes coding for surface proteins are more likely to affect qualities such as virulence, host range, and interaction with arthropod vectors. Recently, it has been shown that antibodies specific for 44-kDa proteins may play a role in immunity against \textit{Anaplasma} infection and that the genes encoding the 44-kDa outer membrane proteins may be involved in the pathogenesis of, and the immunoresponse to, \textit{A. phagocytophilum} in mice (8, 9, 22). However, a recent study of mice indicates that the histopathological lesions observed in \textit{A. phagocytophilum}-infected animals may have an immunopathological basis (10). In order to evaluate the differences in pathogenicity observed in this study, virulence-related genes will have to be studied.

Only one of the four lambs inoculated with \textit{A. phagocytophilum} variant 2 reacted with a detectable antibody titer within the first 6 weeks of the infection. The sensitivity of the present antibody test might have been increased by the use of a more appropriate antigen. Strong serological cross-reactions between all members of the \textit{A. phagocytophilum} group have been reported, but the titer of the antibody to a heterologous variant is normally less than that to a homologous variant (3, 12). Unfortunately, \textit{E. equi} antigen was the only antigen that was available for use in the present study.

A clear difference in clinical reactions between the two variants was also observed after challenge. Similar results were found in an earlier cross-protection study of lambs that involved \textit{A. phagocytophilum} variant 1 and the \textit{A. phagocytophilum} prototype, earlier classified as the human granulocytic ehrlichiosis agent (GenBank accession no. U02521) (14). Earlier experimental studies have shown that full protective immunity to challenge with homologous \textit{A. phagocytophilum} variants lasts from a few months to more than 1 year (4, 21). Resistance to reinfection increases with increasing frequency of challenge (13). Unfortunately, no challenge with the homologous variants was done in the present work.

In conclusion, the results of the present study indicate marked differences in clinical manifestation, neutropenia, antibody response, and cross-protection after infection with variants of \textit{A. phagocytophilum}. Although the prevalences of \textit{Anaplasma} variants in sheep flocks are not known, the existence of such differences between two \textit{A. phagocytophilum} variants in sheep on the same pastures may explain the earlier observation that lambs on neighboring pastures may suffer differently from tick-borne infections. However, further analyses of fatal cases of TBF must be performed in order to elucidate differences in pathogenicity.

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