Genomic heterogeneity in the pol region of ovine lentiviruses obtained from bronchoalveolar cells of infected sheep from France

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In order to determine the genomic heterogeneity of ovine lentiviruses, we analysed eight isolates from naturally infected sheep from one geographical region of France. A 475 nt fragment in the region of the pol gene coding for reverse transcriptase was amplified by RT-PCR from RNA directly extracted from uncultured bronchoalveolar lavage cells. The resulting PCR fragments were analysed by restriction enzyme digestion, cloned in a TA vector and sequenced. Restriction enzyme analysis showed distinct patterns from the eight isolates, and sequencing showed them to be closely related in both nucleotide (2.3–8.1% variation) and deduced amino acid (0–6.2% variation) sequences. Their amino acid sequences differed from that of visna–maedi virus complete viral genome sequence K1514 by 12.5–15.3%, but from that of caprine arthritis encephalitis virus (CAEV) viral genome sequence Co by only 4.2–6.9%. Phylogenetic analysis showed that the French isolates form a group related to CAEV Co and distant from previously reported ovine lentivirus sequences from different origins.

Lentiviruses are retroviruses responsible for diseases involving the lungs, brain, mammary tissue, joints and, for some of them, the immune system in several mammalian species including humans. Lentiviral genomes are highly variable with elevated mutation rates due in part to the poor fidelity of their reverse transcriptase which has only a weak proofreading exonuclease activity (Preston et al., 1988; Roberts et al., 1988; Goodenow et al., 1989). Human immunodeficiency virus type 1 varies according to geographical location (Alizon et al., 1986), and the viral population within individual patients shows considerable genomic diversity and undergoes temporal fluctuations during the course of the disease (Meyerhans et al., 1989).

A distinct group of lentiviruses infect small ruminants (SRLV) causing neurological, pulmonary, articular and mammary symptoms in sheep (visna–maedi virus, VMV) and goats (caprine arthritis encephalitis virus; CAEV). These lentiviruses probably undergo genomic variation, because neutralization-resistant variants of VMV appear in infected sheep, although they do not completely replace the parental virus (Lutley et al., 1983; Cheever et al., 1993). Three complete viral genome sequences of VMV, K1514 (Sonigo et al., 1985), SA-OMVV (Quéré et al., 1990), EV1 (Sargin et al., 1991) and one of CAEV, CAEV Co (Saltarelli et al., 1990), from widely separated geographical locations show considerable variation. The sequences of partial (Zanoni et al., 1992) or complete genomes of SRLV have all been obtained from viruses extensively passaged in tissue culture, and cannot provide information about viral variability within geographical regions or in infected individual animals. The evolution of natural viral populations in the individual animal, the infected flock and differences between flocks in a given region may well influence the disease process.

In the present study, we describe the sequence variation in a 475 nt region of the SRLV pol gene from a population of naturally infected sheep from southern France. The amplified fragment of pol is part of the retroviral RT coding region. RNA was directly extracted from bronchoalveolar lavage cells with no culture step, retro-transcribed and amplified using PCR primers common to VMV and CAEV. Sequences of the cloned fragments were compared to each other and to the corresponding region of published SRLV genomes.

Intact heart-lung blocks were obtained at slaughter from eight adult ewes with clinical signs of respiratory disease. Two of these animals of Préalpes breed (663 and 664) were obtained from a commercial slaughterhouse, and the others were purchased from three different flocks of Lacaune sheep (flock 1, 676; flock 2, 679 and 684; flock 3, 680, 685 and 686), and slaughtered at the Lyon

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Veterinary School (French Ministry of Agriculture authorization no. 02133). All animals originated from southern France, a region of high sero-prevalence to VMV. Pathology of tissue sections from unlavaged segments of the lung showed typical lesions of maedi in seven animals and milder lesions in one (Mornex et al., 1994). Bronchoalveolar lavage was performed as described previously (Cordier et al., 1990), and the cells were pelleted and stored dry at −80 °C until RNA extraction. The presence of lentivirus was confirmed by co-culture with permissive ovine skin fibroblasts and assay of the supernatants for RT activity, as described previously (Mornex et al., 1994). All eight animals gave positive syncytia after 10–30 days coculture, and RT measurements confirmed the retroviral nature of the infection.

Total RNA was extracted from samples of 10^7 pelleted alveolar cells as described by Chomczynski & Sacchi (1987) by addition of 0.8 ml of denaturing solution (4 M-guanidinium thiocyanate, 25 mM-sodium citrate pH 7.0, 0.5% Sarkosyl and 100 mM-2-mercaptoethanol). Extracted RNAs were dissolved in 50 μl diethyl pyrocarbonate-treated water. RT–PCR using primers in the RT coding region of the pol gene was used to detect the presence of SRLV sequences in these RNAs. We chose the pol gene region for our preliminary analysis since it is usually considered to be taxonomically most significant, and because its conserved character makes it a region of choice for the detection of novel variant viruses (McClure et al., 1988). Polymerase genes have been recently used in a novel attempt to classify all viral sequences (Ward, 1993).

Total cellular RNAs (1 μl) were reverse transcribed using random hexamer primers (0.1 μg) and Moloney virus RT (50 units) in the presence of 20 units of ribonuclease inhibitor (RNasin; Promega) in a final volume of 20 μl (5 mM-MgCl2, 0.5 mM of each deoxyribonucleotide triphosphate, 10 mM-Tris–HCl pH 8.8, 50 mM-KCl and 0.1% Triton X-100). The mixture was incubated for 10 min at 20 °C, 15 min at 42 °C, 5 min at 99 °C and then 5 min at 4 °C. The cDNAs obtained were used as templates for PCR using a set of degenerate primers chosen in a conserved region of the pol gene of the known genomic SRLV sequences. Primer sequences corresponding to positions 2198–2215 and 2672–2650 in the known genomic SRLV sequences. Primer sequences corresponding to positions 2198–2215 and 2672–2650 in the published sequence of K1514 were 5′ DSAAGA-RAAATTARARGG 3′ (P1) and 5′ ATCATCCATRTATATBCCAAATTG 3′ (P2), where B = C, G or T; D = A, G or T; R = A or G; and S = C or G. The amplified product was 475 bp in length. A third primer, 5′ GATTTAACAGAGGCAACA 3′ (P3), was designed from sequences of the French isolates 663, 676 and 684, and gave a 303 bp PCR product with P2. The cDNA (5 μl), 20 pmol of each primer irrespective of degeneracy, and 1.5 units of Taq polymerase (Eurobio) in a final volume of 50 μl [1.5 mM-MgCl2, 40 μM of each deoxyribonucleotide triphosphate, 16.6 mM-(NH4)2SO4, 67 mM-Tris–HCl (pH 8.8) and 0.01% Tween 20] were initially denatured at 95 °C (10 min), then amplified by 35 cycles of 1 min at 95 °C, 1 min at 45 °C and 1 min at 72 °C, followed by a 10 min final extension step at 72 °C (Thermal cycler 480, Perkin Elmer Cetus). The resulting PCR products were analysed by electrophoresis through 1% agarose gels in the presence of ethidium bromide. The DNA fragments obtained were digested by FokI, HinfI and Rsal (Boehringer) following the supplier’s recommendations, then analysed by electrophoresis through 2.5% NuSieve 3:1 (Tebu) gels.

Lung cells recovered by bronchoalveolar lavage from all eight symptomatic sheep showed the presence of sequences related to a region of the pol gene of SRLVs using the RT–PCR technique. The DNA amplified using primers P1 and P2 had the expected size of 475 nt and was not produced when uninfected ovine skin fibroblasts were used as the source of RNA. Direct amplification of viral sequences from cells obtained by lavage from the lungs of the affected animals avoids the bias of selection of viral variants better adapted to propagation in tissue culture as described for HIV-1 (Meyerhans et al., 1989), and reduces the likelihood of contamination.

The 303 bp amplification fragments obtained using primers P3 and P2 contain restriction sites for FokI, HinfI and Rsal which should differentiate between K1514 and CAEV Co. Our eight isolates also showed length polymorphisms for these three enzymes, and the typical digestion pattern of each isolate was preserved after passage by coculture with susceptible ovine fibroblasts (data not shown), suggesting that little selection had occurred over one passage.

PCR products were purified using the QIA Quick-Spin PCR purification kit (Quiagen), and then 1 μl of product was ligated into a TA-vector (InVitrogen) according to the supplier’s instructions. Plasmids containing inserts were sequenced by the dideoxynucleotide chain-termination technique with the Sequenase version 2.0 DNA sequencing kit (United States Biochemical) using primers T7, SP6 (Promega) and internal primers chosen from sequences of amplified fragments. Sequence data were analysed using GeneWorks (Intelligenetics) and Gene-Jockey (Biosoft) software packages on a Macintosh computer. Phylogenetic trees were established using UPGMA (Unweighted Pair Group Method with Arithmetic Mean) with GeneWorks. Sequences of human immunodeficiency virus type 1 (HIV-1) Mal and Eli, HIV-2 Cam and Rod, simian immunodeficiency virus (SIV) smm and equine infectious anaemia virus (EIAV) were obtained from GenBank through CITI2, France.
Restriction sites consistent with the observed patterns were present on examination of the sequences. A continuous open reading frame with no stop codon was present in all cases. The 475 bp nucleotide sequences (Fig. 1a) and the 158 amino acid deduced peptide sequences (Fig. 1b) were aligned and compared with those from K1514 and CAEV Co. Most mutations occur in the third codon position, with 71% of 159 sites showing variation, compared with 24% and 11% of 158 first and second positions, respectively. This is in accord with the observed patterns.
with the amino acid replacement rate by site of 21.5%. The mutations observed are predominantly transitions between A and G (Fig. 1a) as observed in HIV-1 populations (Goodenow et al., 1989; Fitzgibbon et al., 1993).

To determine the genetic relationship of French ovine isolates to other ovine and caprine lentiviral strains, a phylogenetic tree was constructed from the deduced pol amino acid sequences (Fig. 2). It shows that the majority of our French isolates form a related group with affinities to CAEV Co on a clearly separate branch from the visna–maedi strains K1514, EV1 and SA-OMVV. Isolate 676 was as divergent from the VMV group as from the CAEV group. Interestingly, the divergence between members of the VMV cluster (SA-OMVV, EV1 and K1514) and those of the French and CAEV Co cluster is very similar, as shown by the nearly identical branch lengths. The most divergent sequence in the group (676) comes from a Lacaune sheep belonging to a separate flock located close to the others. A second clone from the same animal varies in only five nucleotide positions, and this isolate may represent a third cluster of SRLVs, at least in the pol gene.

Pairwise comparisons of typical sequences not including the degenerate primers, are shown in Table 1. The groups seen in Fig. 2 are represented by isolates 676, 679 and 685. The other five French isolates appeared closely related to 679 and 685, varying among themselves by 2.1–8.1% in nucleotides, and 0–6.2% in amino acids. They differed from the CAEV Co sequence by around 15% in nucleotides but only by some 6% in amino acids. They differed from K1514 by nearly 19% in nucleotides and by around 14% in amino acids. Sequence variation between these seven French isolates was considerably less than that between the same region of CAEV Co and K1514. Isolate 676 was as divergent from CAEV Co as from K1514 in its amino acid sequence, and a higher proportion of nucleotide substitutions resulted in amino acid changes. This isolate also diverged from our seven other isolates to a similar extent (Table 1).

The variations in the French isolates occur mostly at sites at which known strains of SRLV differ (Fig. 1a). Whenever we have sequenced a second clone from the isolates we have always found at least five to seven nucleotide changes, usually synonymous with respect to amino acid coding. This suggests that, like humans infected with HIV, sheep carry a varying population of SRLVs.

Whereas this comparison of nucleotide sequences shows only a moderate affinity between French isolates and CAEV Co as compared to the visna–maedi virus group, the amino acid sequences of the French group not comprising 676 are much more closely related to CAEV Co (Table 1). It is not clear whether this represents nucleotide divergence from a CAEV Co-like ancestor.

Table 1. Pairwise comparisons of virus sequences*

<table>
<thead>
<tr>
<th>Virus isolate</th>
<th>676</th>
<th>685</th>
<th>679</th>
<th>K1514</th>
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<tr>
<td>CAEV Co</td>
<td>21.7</td>
<td>15.5</td>
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<td>(13.2)</td>
<td>(4.9)</td>
<td>(6.3)</td>
<td>(13.9)</td>
<td></td>
</tr>
<tr>
<td>K1514</td>
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<td>18.7</td>
<td>18.7</td>
<td></td>
</tr>
<tr>
<td>(16.7)</td>
<td>(13.9)</td>
<td>14.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>679</td>
<td>21</td>
<td>4.1</td>
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<td></td>
</tr>
<tr>
<td>(16.0)</td>
<td>(4.9)</td>
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<tr>
<td>685</td>
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<td>0.6</td>
<td></td>
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</tr>
<tr>
<td>(13.9)</td>
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</table>

* Percentage divergence values were obtained by pairwise comparisons of nucleotide sequences and deduced amino acid sequences (in parentheses) between the representative French isolates 676, 679 and 685, and the reference strain K1514, and CAEV Co in the 433 nt amplified region of pol excluding the degenerate primer sequences.
with conservation of peptide sequence, or convergence on the CAEV Co peptide from a divergent ancestral nucleotide sequence.

Amplification of viral sequences by RT–PCR from suitable cells of infected sheep appears to be a useful method for the analysis of SRLV population structure. Studies of viral variation within a known pattern of sheep flocks should help us to understand how the viral population is generated and maintained. In addition, comparison of the French sheep isolates with those from goats from the same region will illustrate host species constraints on viral variability.

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