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B-cell epitopes of the envelope glycoprotein of caprine arthritis–encephalitis virus and antibody response in infected goats

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Goats infected with caprine arthritis–encephalitis virus (CAEV) develop high titres of antibodies to Env. Not only is no consistent neutralizing response found but anti-Env antibodies have even been associated with disease in infected goats. To identify the continuous antigenic determinants involved in this atypical anti-Env response, we mapped CAEV-CO Env by screening an epitope expression library with infected goat sera. In addition to the four previously described epitopes, seven novel antigenic sites were identified, of which five were located on the surface (SU) and two in the transmembrane (TM) subunits of Env. The SU antibody-binding domains located in the variable regions of the C-terminal part of the molecule (SU3 to SU5) showed the strongest reactivity and induced a rapid seroconversion in six experimentally infected goats. However, the response to these immunodominant epitopes did not appear to be associated with any neutralizing activity. The pattern of serum reactivity of naturally infected goats with these epitopes was restricted, suggesting a type-specific reaction. Interestingly, the reactivity of peptides representing SU5 sequences derived from CAEV field isolates varied with the geographical and/or breeding origin of the animals. This suggests that peptides corresponding to the immunodominant SU epitopes may well be useful in the serotyping of CAEV isolates. Furthermore, the identification of the CAEV Env epitopes will permit us to functionally dissect the antibody response and to address the role of anti-Env antibodies either in the protection from or in the pathogenesis of CAEV infection.

Introduction

A hallmark of lentivirus infections is their persistence despite a robust immune response. In particular, a primary infection is followed by a humoral response to the envelope glycoprotein (Env) which is sustained during the entire course of the infection. This humoral response is directed against both continuous and conformational epitopes of the surface (SU) and transmembrane (TM) subunits of Env. The contrasting effects of anti-Env antibodies have been described as resulting in either neutralization or enhancement of infection. A neutralizing response is thought to be directed mainly against conformational epitopes, whereas continuous epitopes have been shown to be involved either in neutralizing or in enhancing responses. Neutralizing antibody has been mapped to linear epitopes in the SU and TM glycoproteins of human and feline immunodeficiency viruses (HIV and FIV) (Goudsmit *et al.*, 1988; Rusche *et al.*, 1988; Javaherian *et al.*, 1989; Lombardi *et al.*, 1993; Richardson *et al.*, 1996). Antibodies enhancing HIV-1 infection are mainly directed against the principal

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immunodominant domain of the HIV-1 TM (Robinson *et al.*, 1990, 1991), which corresponds to a conserved immunogenic structure in all lentiviruses (Pancino *et al.*, 1994; Bertoni *et al.*, 1994). Antibodies enhancing the infectivity of equine infectious anaemia virus (EIAV) have been shown to react with linear epitopes of the SU portion of Env (Grund *et al.*, 1996). The *in vivo* relevance of enhancing antibody is supported by the observation that vaccination against animal lentiviruses may have adverse clinical effects (Mascola *et al.*, 1993; Siebelink *et al.*, 1995; Wang *et al.*, 1994).

Caprine arthritis–encephalitis virus (CAEV) is a lentivirus that causes persistent infection in goats. Between 20% and 30% of naturally infected goats show symptoms of progressive arthritis. A relationship between anti-Env response and arthritis has been proposed (Knowles *et al.*, 1990). In particular, goats with progressive arthritis have been found to have high serum titres to the transmembrane envelope subunit TM (McGuire *et al.*, 1992). We previously described two immunodominant epitopes of CAEV TM that elicit an antibody response associated with the appearance of disease (Bertoni *et al.*, 1994). In the present study we performed an extensive analysis of the immunogenic domains of the CAEV Env in order to identify the targets of the natural antibody response to CAEV infection. In addition to the epitopes previously described (Bertoni *et al.*, 1994) we identified five antigenic sites in the SU and six in the TM (two new epitopes) subunit by screening a λgt11 random Env expression library and recombinant proteins. We followed the development of the antibody response to the SU epitopes in experimentally infected goats. Although we observed a strong and rapid response to three epitopes of the C-terminal part of the SU domain, we found no correlation between serum reactivity and neutralizing activity. Finally, screening of a panel of sera from naturally infected goats with recombinant peptides revealed a restricted, type-specific response to the immunodominant epitopes of the SU subunit consistent with their localization on variable regions. Our results will permit a further functional dissection of the humoral response to the continuous B-cell epitopes of CAEV Env to address the role played by antibodies in protection against CAEV infection or in its pathogenesis.

Methods

■ **Animals and experimental infection.** Goats 89G38, 91G27, 91G31, 91G33 and 91G34 were experimentally infected with biologically cloned CAEV-CO as described previously (Knowles *et al.*, 1991; Lichtensteiger *et al.*, 1991) and goat 89G50 was immunized with a CAEV-CO Env-expressing vaccinia virus (Cheevers *et al.*, 1994).

Twelve Saanen goats between 6 and 24 months of age were included in the experimental infection. Six goats were infected with 1 ml supernatant (10^7 $TCID_{50}$/ml) of goat synovial membrane (GSM) cells transfected with the CAEV-CO molecular clone (Pyper *et al.*, 1986); 0·5 ml was injected intracarpally, 0·5 ml intravenously. The remaining six goats were mock-infected with culture supernatant of non-transfected GSM cells.

■ **Env expression library.** Construction of the random Env expression library used for epitope mapping was described previously (Bertoni *et al.*, 1994).

The *env* gene was derived from an infectious clone of CAEV-CO (Pyper *et al.*, 1986). The positions of the nucleotides (bp) and amino acids (aa) defining different Env regions in this paper refer to the sequences published by Saltarelli *et al.* (1990).

Briefly, random DNA fragments of an average length of 200 bp were isolated, blunt-ended, ligated with *Eco*RI linkers and subsequently cloned in *Eco*RI-digested λgt11 DNA arms. Ligations were packaged and plated onto *E. coli* Y1090 to express the Env fragments as β-galactosidase fusion proteins from the recombinant phages. All phages contained inserts of different sizes, ranging from 93 to 285 bp. The representativeness of the library was demonstrated by hybridization with radioactively labelled fragments covering the entire sequence of CAEV-CO Env.

■ **Screening procedure to detect immunoreactive plaques.** Our previous mapping strategy (Bertoni *et al.*, 1994) was aimed at detecting group-specific epitopes and had been performed with sera obtained from goats infected with field strains of CAEV. In contrast, in this study we used sera of animals experimentally infected with the CAEV strain (CAEV-CO) that we had used to create the Env expression library. This strategy was chosen to warrant the detection of type-specific epitopes that can be missed when heterologous sera are used. The library was screened using serum 89G38 and a pool of four sera (91G27, 91G31, 91G33, 91G34) obtained from animals experimentally infected with a biologically cloned CAEV-CO. These sera and their neutralizing activity have previously been described (Lichtensteiger *et al.*, 1991). Approximately 3×10^4 p.f.u. from the original, unamplified library was plated onto *E. coli* Y1090 (5000 p.f.u. per plate) and incubated for 4 h at 42 °C. Plates were then overlaid with nitrocellulose filters saturated with 10 mM IPTG and further incubated for another 3 h at 37 °C to induce β-galactosidase fusion protein expression. Immunological screening of the filters was performed as described previously (Pancino *et al.*, 1993). Briefly, the filters were processed and incubated with the two sera, and the immunoreactive plaques were recovered from agar plugs and purified for two additional runs. The positive plaques were isolated and the PCR-amplified inserts were sequenced.

■ **PCR and sequencing.** A total of 10 µl of phage lysate was diluted with 60 µl of water and boiled for 10 min. After microcentrifugation, 15 µl of supernatant was added to 85 µl of PCR mixture containing 50 pM of each of the primers mentioned below, 10 nM of each deoxynucleoside triphosphate and 0·5 U of Taq polymerase (Perkin Elmer) in a solution of 10 mM Tris–HCl, 50 mM KCl and 2 mM $MgCl_2$. The λgt11 primers used were complementary to the β-galactosidase portion of the λgt11 template (forward, 5′ GGTGGCGACGACTCCTGGAGCCCG 3′; reverse, 5′ TTGACACCAGACCAACTGGTAATG 3′). PCR was carried out for 32 cycles (cycle 1: 94 °C, 2 min; cycles 2–31: 94 °C, 15 s/55 °C, 30 s/72 °C, 30 s; cycle 32: 72 °C, 3 min). For sequencing, the PCR products were run on a 1·8% agarose gel (FMC) and purified with QIAEX beads (QIAGEN) according to the manufacturer's protocol. The fragments were sequenced using the ABI PRISM fluorescent sequencing kits on an ABI PRISM 310 Genetic Analyser (Perkin Elmer) according to the manufacturer's protocol.

■ **Synthetic peptide.** A synthetic peptide of 17 aa (Rev-1: aa 21–37, bp 6072–6122) included in the first CAEV-Rev exon (6012–6123) was synthesized and purified by Neosystem, Strasbourg, France. This peptide contains numerous charged amino acids and was therefore predicted to be hydrophilic and exposed at the surface of the molecule. Sera of rabbit immunized with KLH-coupled Rev-1 peptide developed

high titres of antibody to the peptide and were used in a Western blot to detect the recombinant proteins SU1 and SU1-signal.

■ **Expression of recombinant proteins.** The SU antibody-binding domains SU1 to SU5, identified by screening the Env library, and two shorter peptides in the SU1 domain (SU1-signal and SU1-mature), as well as the unreactive regions located between the SU2 and SU3 (SU2–3) and the SU3 and SU5 (SU3–5) domains were expressed as recombinant proteins.

Two expression systems were used. Seven recombinant proteins (SU1, SU1-signal, SU2, SU2–3, SU3, SU4, SU5) were expressed using the pET14b vector, which allows the expression of proteins fused at the amino terminus either with 19 additional aa for the constructs cloned in the *Nde*I site, or with 23 aa for the constructs cloned in the *Bam*HI site. This amino-terminal tail contains six histidine residues in a row, which allow the expressed proteins to be purified using nickel-coupled Sepharose beads.

Three proteins (SU3–5, SU3–5 amino terminus and SU1-mature) could not be expressed in this vector but were successfully expressed using the pGEX-4T-1 plasmid, which allows the expression of proteins fused at their amino terminus with the 29 kDa glutathione *S*-transferase (GST) protein.

Fragments corresponding to the CAEV-CO Env regions to be expressed were amplified using a standard PCR technique, with primers containing the cloning sites (*Nde*I or *Bam*HI added to the 5′ primer, *Bam*HI added to the 3′ primer for the pET14b vector and *Eco*RI or *Xho*I for the pGEX-4T-1 vector), and were purified and cloned using standard techniques (Sambrook *et al*., 1989).

The 'wild-type' SU5-1163M, SU5-615 and SU5-1355 fragments were cloned by RT–PCR starting from total RNA isolated from infected GSM cells using TRIzol reagent (Gibco BRL) according to the manufacturer's protocol. cDNA synthesis was performed using Super-Script RNase H$^-$ RT (Gibco BRL) at 200 U/50 µl according to the manufacturer's protocol. The SU5 fragments were amplified by nested PCR using the external primer pairs #423 forward, 5′ GGAGCAG-AAATAATMCCWGAARSTATG 3′, and #425 reverse, 5′ TGCRG-CAGCKAYTATTGCCATGAT 3′, followed by amplification with the internal primer pairs containing in the 5′ extensions (in bold), the required *Bam*HI cloning sites and, in the reverse primer, a stop codon (TAA): #442 forward 5′ **TATGGATCC**GGGTAGGGTAAAGGCACAATA-TAGT 3′ and #443 reverse 5′ **TATAGGATCCTTA**AAGCACCAT-TACTAACCCTATTC 3′ for the isolate 1163M, and #444 forward 5′ **TATGGATCC**GACAAGAGTGAAAGCACAGTACAGC 3′ and #445 reverse 5′ **TATAGGATCCTTA**GAGCATTATGACCAAGCC-AACGC 3′ for the 615 and 1355 isolates. The heat-soaked PCR protocol described by Ruano *et al*. (1992) was applied. Finally, these 'wild-type' SU5 peptides were expressed using the pET14b vector as described above. The different plasmids were electroporated in the appropriate host cells [BL21(DE3) (Novagen) for pET14b and DH5α (Gibco BRL) for pGEX-4T-1 constructs] and immediately used for protein expression according to the manufacturer's protocol. Purification of the recombinant proteins was performed using protocols suggested by the manufacturers for the purification of denatured proteins.

■ **Western blot analysis.** Western blot was used to follow the development of antibodies against the SU epitopes in experimentally infected goats and to test this antibody response in naturally infected goats. Purified SU1, SU1-signal, SU2, SU2–3, SU3, SU4 and SU5 peptides expressed as 6 × His fusion proteins and SU1-mature and SU3–5 peptides expressed as GST fusion proteins were migrated onto 12% SDS–polyacrylamide gels and transferred to nitrocellulose filters. The nitrocellulose strips were incubated overnight at 4 °C with 1:50 to 1:800 diluted goat sera and further processed as described elsewhere (Bertoni *et al*., 1994). Sera collected before experimental infection and a pool of sera from CAEV-negative goats were used as antibody controls. Additionally, the co-localization of the immunoreactive bands with the fusion proteins was verified with alkaline phosphatase-labelled nickel (Ni–NTA–AP, QIAGEN) for the 6 × His recombinant proteins, and a murine monoclonal antibody to GST for the GST fusion proteins. A polyclonal rabbit serum to CAEV-Rev, obtained by immunizing two rabbits with a synthetic peptide of 17 aa (aa 21–37, bp 6072–6122) included in the first CAEV-Rev exon (6012–6123), was used for the recombinant SU1 and SU1-signal peptides.

■ **Neutralization assay.** Neutralization assays were performed using the virus reduction neutralization assay described previously (McGuire *et al*., 1988). Neutralizing antibodies were detected by mixing 0·4 ml of goat serum heat-inactivated for 30 min at 56 °C with 0·4 ml of MEM supplemented with 2% FCS and containing $10^{4.5}$ or $10^{3.5}$ $TCID_{50}$, respectively. To determine serum neutralizing antibody titres a constant quantity of virus ($10^{1.5}$ $TCID_{50}$) was incubated with a fourfold dilution of serum. Non-neutralized virus was detected on GSM cells (McGuire *et al*., 1988).

Results

Identification of antibody-binding domains by screening of the Env library

Screening of the epitope library derived from the CAEV-CO strain with sera from animals experimentally infected with the corresponding virus strain revealed a large number of positive plaques. A total of 326 immunoreactive plaques were isolated; 112 of them were purified and their inserts were amplified by PCR and partially sequenced. This first analysis permitted us to select, among the purified clones, only those containing distinct inserts and to discard the TM inserts whose partial sequence mapped to previously defined TM epitopes (Bertoni *et al*., 1994). A list of the completely sequenced inserts and their localization on the CAEV-CO genome is shown in Tables 1 and 2. Grouping the overlapping peptides permitted us to define four distinct regions containing B-cell epitopes. A single clone mapped to the first region. A large number of immunoreactive plaques had inserts that mapped to the second region. All these inserts started at base 6349, which corresponds to an internal *Eco*RI site in the viral genome, suggesting a positive bias in favour of these inserts in our random library of Env polypeptides. Several clones were shown to contain inserts that mapped to the third region and two clones had inserts mapping to the fourth region. The minimal overlapping sequence in each domain, 6349–6442 (aa 113–143) for the second region, 6890–6989 (aa 294–326) for the third region and 7761–7914 (aa 584–634) for the fourth region, define domains which contained epitopes. We cannot exclude that additional epitopes may be present on the longer overlapping immunogenic peptides and therefore, to monitor the development of antibody to these epitopes in experimentally infected goats, we expressed recombinant proteins larger than the minimal overlapping sequences (see subsequent sections). Previously, we had defined four immunodominant epitopes of

Table 1. List of the SU epitopes

Base pair and amino acid positions refer to Saltarelli *et al.* (1990).

Clone*	Nucleotide position	Peptide position	Region
20	6063–6348	18–112	First
7	6349–6522	113–170	Second
16	6349–6561	113–183	
17	6349–6512	113–167	
21	6349–6525	113–171	
24	6349–6442	113–143	
42	6349–6466	113–151	
68	6349–6492	113–160	
73	6349–6501	113–163	
84	6349–6549	113–179	
100	6349–6564	113–184	
120	6349–6573	113–187	
137	6349–6494	113–157	
32	6820–6997	271–328	Third
60	6776–6990	256–326	
149	6748–6993	247–327	
244	6875–7065	288–351	
256	6820–7014	271–334	
261	6890–7050	294–346	
264	6872–6990	288–326	
274	6824–6989	272–326	
323	7728–7914	573–634	Fourth
325	7761–7923	584–637	

* Clone number of the purified phage plaque. Bold and italic characters indicate the clone number of the phage with the shortest insert.

Table 2. List of the TM epitopes

Base pair and amino acid positions refer to Saltarelli *et al.* (1990). Previously described epitopes: TM1, bp 8049–8093, aa 680–694; TM2, bp 8130–8165, aa 707–718; TM3, bp 8160–8204, aa 717–731; TM4, bp 8256–8297, aa 749–762 (Bertoni *et al.*, 1994).

Clone*	Nucleotide position	Peptide position	Region
136	8330–8512	774–833	TM5
295	8330–8494	774–827	
327	8321–8466	770–818	
39	8491–8634	828–874	TM6

* Clone number of the purified phage plaque. Bold and italic characters indicate the clone number of the phage with the shortest insert.

the extracellular portion of TM (Bertoni *et al.*, 1994). Several immunoreactive plaques detected during this screening contained inserts mapping to these immunodominant regions. In addition, two new regions (Table 2; TM5 and TM6) containing

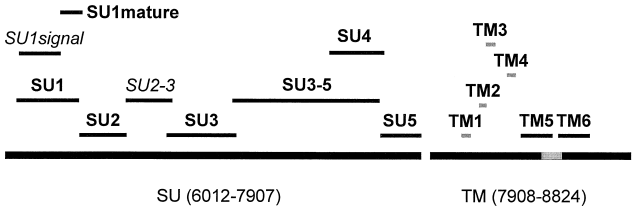

Fig. 1. Schematic representation of the CAEV Env. The black bars at the bottom represent the SU and TM subunits. The putative transmembrane portion of TM is shown in grey. Base pair positions refer to the sequence published by Saltarelli *et al.* (1990). The bars above the SU represent the different recombinant proteins produced and tested by Western blot: SU1, 6062–6348; SU1-signal, 6072–6263; SU1-mature, 6261–6362; SU2, 6349–6564; SU2–3, 6561–6773; SU3, 6748–7065; SU3–5, 7047–7718; SU4, 7485–7736; SU5, 7720–7907. Immunoreactive peptides are shown in bold, non-immunoreactive ones in italics. The grey bars above the TM represent the previously described epitopes TM1 (8049–8093), TM2 (8130–8165), TM3 (8160–8204) and TM4 (8256–8297) (Bertoni *et al.*, 1994). Black bars represent the two new antigenic domains TM5 and TM6 identified in this study.

B-cell epitopes were detected. Three clones (136, 295, 327) had overlapping inserts encompassing the extracellular and transmembrane portions of TM, and one immunoreactive clone (39) contained an insert which mapped to the intracytoplasmic tail of TM. The two newly identified antibody-binding domains of the TM were named TM5 and TM6 (Fig. 1). It is noteworthy that the TM5 epitope of CAEV is located in a region corresponding to a tryptophan-rich region of HIV-1 and FIV TMs. This domain is essential for the fusogenic activity and cell penetration of HIV-1 (Salzwedel *et al.*, 1999) and is the target of a broad-spectrum neutralizing monoclonal antibody (Muster *et al.*, 1993). A peptide corresponding to the FIV domain induced partial protection against FIV infection in cats (Richardson *et al.*, 1998). It will be of interest to evaluate whether the CAEV TM5 epitope may induce a protective response in goats.

Further mapping of SU epitopes

The first immunogenic region was represented by only one plaque in our library. This phage contained a large insert of 95 aa (bp 6062–6348) encompassing the signal peptide of Env (bp 6072–6260), which overlaps and partly shares the amino acid sequence with the first exon of Rev (bp 6012–6123), and the amino-terminal end of the mature Env (6261–6348). In order to map the epitope(s) present on this fragment we subdivided this region into two peptides, the first encompassing the signal peptide of Env and part of the first exon of Rev (Fig. 1; SU1-signal: aa 21–84, bp 6072–6263) and the second containing the amino-terminal end of the mature Env (Fig. 1; SU1-mature: aa 84–117, bp 6261–6362). None of the SU1-positive sera reacted with the SU1-signal recombinant peptide or the Rev-1 synthetic peptide in Western blot or ELISA, respectively. On the contrary, the sera from five of six experimentally infected goats and from three of six naturally

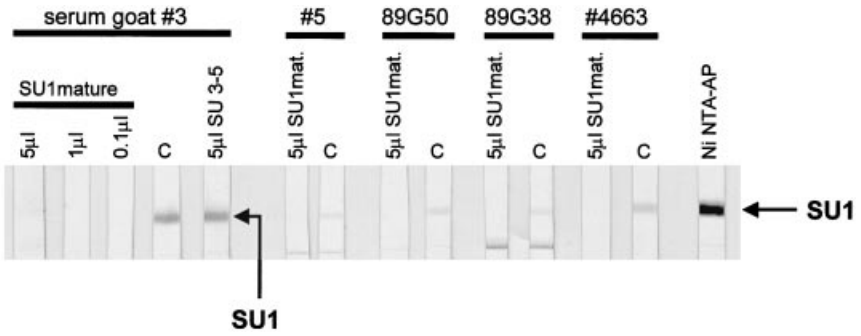

Fig. 2. Western blot performed using SU1 as antigen and SU1-mature as a competitor. Different amounts (5 µl, 1 µl, 0·1 µl) of the competing SU1-mature antigen were added to the serum of goat #3, which strongly reacted to SU1. A band is clearly visible in the control lane (C), without the addition of competing antigen; this reactivity is completely suppressed by the competing SU1-mature recombinant antigen, indicating that SU1 reactivity is restricted to the amino-terminal end of the mature SU. A control recombinant peptide (SU3–5) had no influence on serum reactivity to SU1. Competition experiments with four additional sera are shown. The respective donor goat is indicated above the lanes. In the last lane, the recombinant SU1 peptide was detected using Ni–NTA–AP.

infected goats reacted with the SU1-mature peptide expressed in the pGEX-4T-1 system. These results indicate that the SU1 epitope is located in the amino terminus of the mature SU. This was confirmed by competitive Western blot assays, where the reactivity to the SU1 recombinant protein was completely inhibited by adding to the test sera different amounts of SU1-mature recombinant protein but not by adding a control protein expressed in the same vector (SU3–5) (Fig. 2).

No reactive phages were found containing inserts located between the second and third and the third and fourth antibody-binding domains. In order to verify if additional undetected antigenic determinants were present in these regions we expressed the corresponding SU2–3 (aa 184–254, bp 6561–6773) and SU3–5 (aa 346–569, bp 7047–7718) peptides, respectively, and tested their reactivity in Western blot using the same panel of goat sera used for mapping the SU1 domain.

None of the sera reacted with the SU2–3 peptide, which confirms the results of library screening, suggesting that this region does not contain any continuous B-cell epitopes. Conversely, three out of six sera from the experimentally infected goats reacted with the SU3–5 peptide (Table 3 and Fig. 3). The other three sera could not be scored on this peptide because of their reactivity with the GST fusion partner. The sequence comparison of several CAEV and maedi–visna virus (MVV) isolates revealed the carboxy-terminal region of this peptide to contain a highly variable region (Knowles *et al.*, 1991; Valas *et al.*, 1997; Leroux *et al.*, 1997; Zanoni, 1998). We expressed this region as a recombinant protein using the pET14b expression vector (SU4: aa 491–575, bp 7485–7736) and tested its reactivity in Western blot. The sera from all six experimentally infected animals and six sera from naturally infected goats recognized this peptide (Table 3 and data not shown). Thus, this peptide contains the dominant epitope of the SU3–5 region. By expressing the remaining amino-terminal portion of the SU3–5 (SU3–5 amino terminus: aa 346–497, bp 7047–7502) as a GST-fusion protein we observed a weak reactivity with sera from two of six experimentally infected animals, with serum 89G50 from one animal immunized with a CAEV-CO Env-expressing vaccinia virus (Cheevers *et al.*, 1994) and with serum #4663 from a naturally infected animal. The additional 15 sera tested, which were selected on the basis of their strong reactivity to at least one of the SU epitopes, did not react with this peptide (data not shown). Serum 89G38, which was used to screen the peptide library, was negative on the SU4 peptide as well as on the SU3–5 and SU3–5 amino terminus peptides (data not shown), which explains why we were unable to detect this epitope in our expression library. The pool of sera used, in parallel with 89G38, to screen the Env library, was positive on the SU4 peptide. The higher background found with these sera, however, allowed us to select only the most reactive clones during the library screening.

In conclusion, by using different strategies, we succeeded in identifying five epitope domains in the SU and called them SU1 to SU5 (Fig. 1). The minor epitope(s) found in the SU3–5 amino terminus region were not considered in this list.

Development of antibody response to SU epitopes in CAEV infection

Recombinant proteins corresponding to the following regions of the SU were expressed and purified, SU1: aa 21–112, bp 6072–6348; SU2: aa 114–184, bp 6349–6564; SU2–3: aa 184–254, bp 6561–6773; SU3: aa 246–351, bp 6748–7065; SU3–5: aa 346–569, bp 7047–7718; SU4: aa 491–575, bp 7485–7736; SU5: aa 565–635, bp 7720–7907. We used rather large peptides and not the epitopes defined by the minimal overlapping sequences described in the first

Table 3. Kinetics of seroconversion to the SU epitopes (SU1 to SU5) of goats experimentally infected with the molecular clone CAEV-CO

Goat	Time post-infection	SU1	SU2	SU3	SU3–5	SU4	SU5
#1	2 weeks	−	−	+	+*	±	±
	4 weeks	−	−	++	+*	+	++
	12 weeks	−	−	ND	ND	ND	ND
	24 weeks	−	−	+++	ND	++	++
	52 weeks	−	−	ND	ND	ND	ND
	3·5 years	+	−	+++	++	++	+++
#2	2 weeks	−	−	+	−	±	+
	4 weeks	−	−	+++	++	++	+++
	12 weeks	−	−	ND	ND	ND	ND
	24 weeks	−	−	+++	ND	+	+++
	52 weeks	+	−	ND	ND	ND	ND
	3·5 years	+	−	+++	++	+	+++
#3	2 weeks	−	−	+	−	−	±
	4 weeks	−	−	+++	+	−	+++
	12 weeks	−	−	ND	ND	ND	ND
	24 weeks	−	−	+++	ND	−	+++
	52 weeks	−	−	ND	ND	ND	ND
	3·5 years	++	−	+++	++	+	+++
#4	2 weeks	−	−	+	−	−	+
	4 weeks	−	−	+++	−	+	+++
	12 weeks	−	−	ND	ND	ND	ND
	24 weeks	−	−	++	ND	++	++
	52 weeks	+	−	ND	ND	ND	ND
	3·5 years	+	−	++	+*	++	++
#5	2 weeks	−	−	−	−	+	−
	4 weeks	−	−	++	−	+	+
	12 weeks	+	−	ND	ND	ND	ND
	24 weeks	+	−	+++	ND	±	++
	52 weeks	++	−	ND	ND	ND	ND
	3·5 years	++	−	+++	−*	±	+++
#6	2 weeks	−	−	++	−*	−	++
	4 weeks	−	−	+++	−*	+	+++
	12 weeks	−	−	ND	ND	ND	ND
	24 weeks	−	−	+++	ND	±	+++
	52 weeks	−	−	ND	ND	ND	ND
	3·5 years	−	+	+++	−*	±	+++

* Not scored because of reactivity with the GST fusion partner.
ND, Not done.

section of the results because, in contrast to the phage expression library, these SU peptides are expressed fused to a minimal fusion partner (6 × His) and not to β-galactosidase. The addition of residues flanking the epitopes would preserve the reactivity of antibody binding to the edges of the epitopes. The kinetics of seroconversion to the epitopes contained in these recombinant proteins was monitored by Western blot from 2 weeks to 3·5 years post-infection using the sera of six goats experimentally infected with the CAEV-CO molecular clone whose Env was used to generate the epitope library. The results obtained at 4 weeks and at 3·5 years post-infection with sera of goat #3 are presented in Fig. 3 and the kinetics of seroconversion for all six animals are summarized in Table 3. ELISA was also used in order to detect potential additional reactivity to peptides in non-denaturing conditions. Although ELISA could not be performed on all the sera due to strong background reactivity to *E. coli* proteins contaminating the recombinant peptides, the results obtained in ELISA matched the Western blot data (data not shown).

These data can be summarized as follows. (1) SU1 appears to be a minor epitope inducing late seroconversions. Indeed, only five of six animals seroconverted to the SU1 epitope and this occurred a long time after infection. (2) SU2 reactivity was detected only in goat #6 and then only several months after infection. Three of six sera clearly reacted with the SU3–5 peptide, whereas the remaining three sera could not be scored due to their reactivity with the GST fusion partner (data not shown). (3) Seroconversion to the SU4 peptide was observed in all six goats, albeit at different time-points: goat #5 seroconverted 2 weeks post-infection, goats #1, 2, 4 and 6 seroconverted 4 weeks post-infection and goat #3 more than 1 year post-infection. The SU3 and SU5 epitopes were the most immunogenic. Five animals had antibody against SU3 and SU5 peptides as early as 2 weeks after experimental infection and all six goats developed a strong and sustained response to both epitope regions.

Sera from the six experimentally infected goats taken at 3·5 years post-infection were tested for neutralizing activity in order to establish a correlation between the response against the SU epitopes and the development of neutralizing antibodies. Sera were tested for neutralizing activity using the virus reduction assay. Four goats did not show any neutralizing activity and two goats (#1 and #5) showed low titre neutralizing antibody (1:4). These results confirm that in CAEV-infected animals the overall neutralizing activity is very low and that the strong reactivity observed with some SU epitopes (SU3, SU4 and SU5) in sera from experimentally infected goats is unlikely to be associated with neutralization.

Reactivity of field sera to SU3, SU4 and SU5

Thirty-two sera from Swiss and Italian goats naturally infected with CAEV, selected for their strong reactivity to at least one Gag protein (p28, p18 and p15) in Western blot (Zanoni *et al.*, 1989), were tested for reactivity to the SU3, SU4 and SU5 peptides. In summary, four sera reacted to SU3, 11 sera to SU4, only two sera reacted to SU5 and 20 sera did not react with any of these epitopes (data not shown). This suggests that these epitopes are type-specific, albeit at different stringency levels. SU5 showed the most restricted pattern of reactivity, whereas SU4 displayed a broader reactivity with this panel of sera.

The restricted pattern of reactivity with these SU epitopes may be due either to infections with viruses phylogenetically

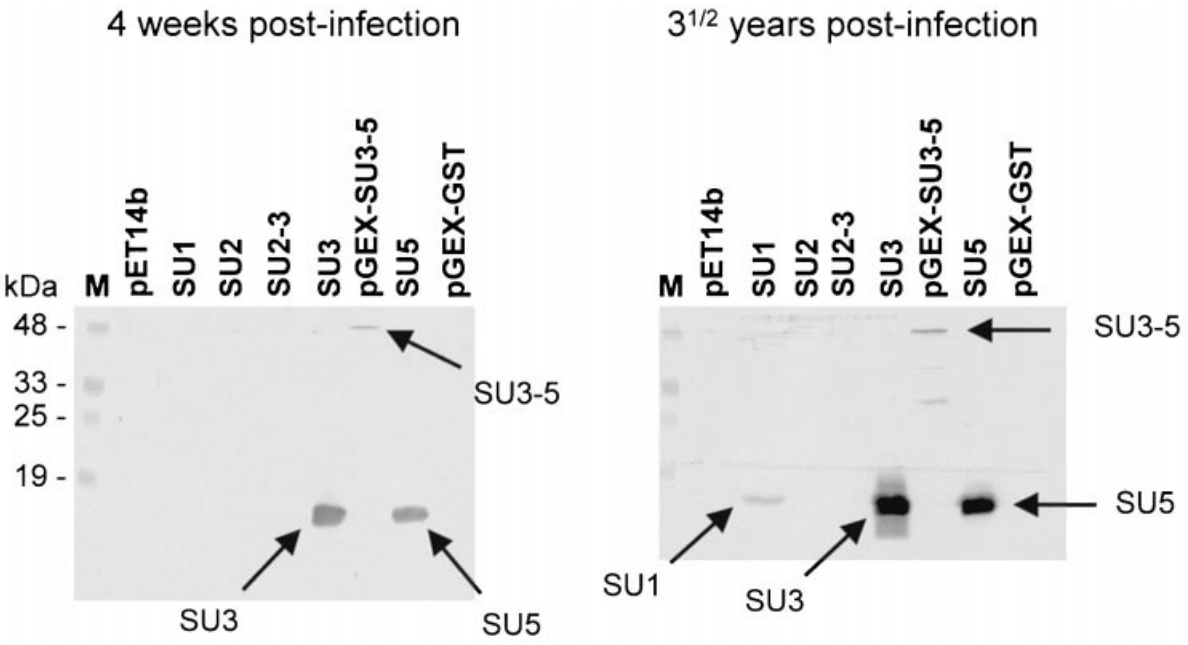

Fig. 3. Reactivity of sera from CAEV-CO-infected goat #3, taken at 4 weeks or 3·5 years post-infection. This goat seroconverted very rapidly to SU3 and SU5, showing a strong reactivity to these epitopes as early as at 4 weeks post-infection. A weaker signal was detected for the SU3–5 peptide as well. The seroconversion to SU1 was slower and was detected at 3·5 years post-infection.

distant from the CAEV-CO isolate or to different genetic backgrounds of the naturally infected goats.

To distinguish between these hypotheses we concentrated on the carboxy-terminal SU5 epitope, which showed the most restricted pattern of reactivity with the sera of the naturally infected goats tested. Four SU5-recombinant proteins derived from the CAEV-CO molecular clone and from three CAEV field isolates, CH-1355, CH-615 and CH-1163M, were cloned and expressed as recombinant peptides. Sixteen sera of naturally infected goats (Fig. 4*a*) were tested in Western blot on SU5-CO, -1355, -615 and -1163M peptides, respectively. As shown in Fig. 4(*a*), all control sera (P1 to P4) reacted with their specific epitopes. With few exceptions, the sera sampled from different geographical regions (Fig. 4*a*, A–G) showed a type-specific reactivity. Interestingly, the three Swedish sera (Fig. 4*a*, E) showed a weak but specific reactivity only to the SU5–1163M peptide.

The selective and partly type-specific reactivity of this epitope was confirmed by testing 14 additional sera from goats (sera #1 to #12 and #1163 from the Bernese Oberland and serum #4663 from the Canton of Ticino) on the SU5–1163M peptide, which had been derived from a field isolate of the Bernese Oberland, and the SU5-CO peptide. As shown in Fig. 4(*b*), 13 of 13 sera obtained from this region (#1 to #12 and #1163, which was obtained from the goat infected with the CAEV-1163M field strain) did not recognize the SU5-CO peptide, but all sera (serum #5 showed a weak but specific reaction on the original blot) reacted with the SU5–1163 peptide. These results strongly suggest that there are no genetic constraints blocking the reactivity with the SU5 epitope but that the antibody response to this epitope is, at least partly, type-specific. Indeed, a comparative analysis of the amino acid sequences encompassing the SU4 and SU5 domains of the CAEV-615, CAEV-1355, CAEV-1163M and CAEV-CO isolates showed a high variability consistent with the observed type-specific seroreactivity (Fig. 5). Compared to CAEV-CO, sequence divergence was 21% for CAEV-1355, 18% for CAEV-615 and 33% for CAEV-1163M. The divergence between the three Swiss field isolates varied between 22% and 33%. All the cysteine residues were preserved, suggesting a structural conservation in this variable region. However, 19 of 30 aa changes for CAEV-1355, 14 of 26 aa changes for CAEV-615 and 23 of 47 changes for CAEV-1163M were non-conservative, introducing new charges or changing a polar for a non-polar amino acid or vice versa. In particular, the alignment revealed the presence of insertions/deletions in the carboxy-terminal portion of the SU5 antibody-binding domain (Fig. 5, dotted box).

Discussion

CAEV infection of goats is characterized by high titres of anti-Env antibodies albeit without a consistent neutralizing response (Narayan *et al.*, 1984). In contrast, anti-Env antibodies and particularly antibodies reacting with continuous immuno-

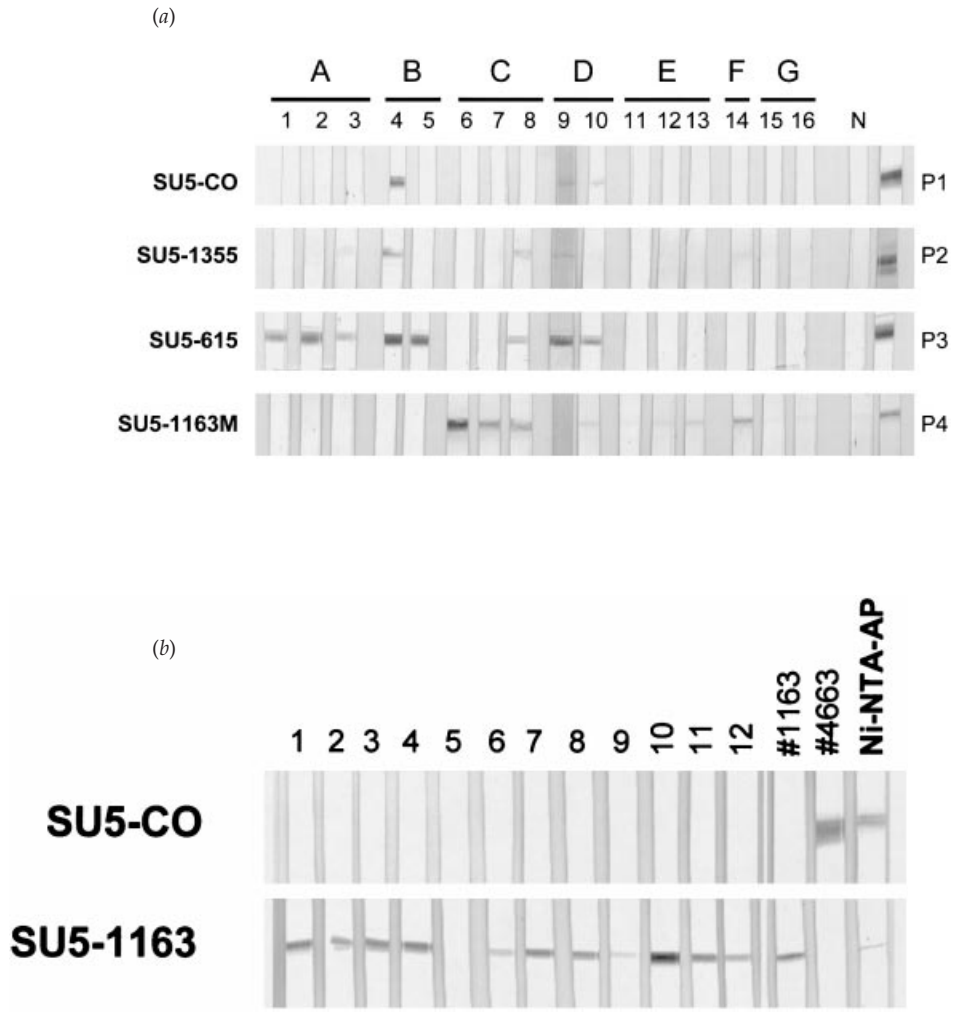

Fig. 4. (*a*) Sixteen goat sera, obtained from animals naturally infected with CAEV field strains, were tested in Western blot for a reaction to four recombinant SU5 peptides derived either from the CAEV-CO molecular clone or from three CAEV Swiss field strains, CH-1355, CH-615 and CH-1163M. In the last lane the recombinant peptides are revealed with control serum P1, obtained from a goat experimentally infected with CAEV-CO, or with control sera P2 to P4 from those animals from which the CAEV field strains CH-1355, CH-615 and CH-1163M, respectively, were isolated. The geographical origin of these sera is indicated by black bars: (A) Canton of Fribourg; (B) Canton of Ticino; (C) Bernese Oberland; (D) Italy; (E) Sweden; (F) Canton of Uri; and (G) Canton of Graubünden. (*b*) Sera from 14 naturally infected goats were tested for reactivity to recombinant SU5 peptides derived from CAEV-CO and CAEV CH-1163M, respectively. Sera 1 to 12 and serum #1163, obtained from goats of different flocks but from the same geographical region (Bernese Oberland), showed a strictly specific reactivity with the SU5–1163-derived peptide. Ni–NTA–AP results in the last lane show that a large amount of SU5-CO recombinant peptide was loaded onto the membrane and did not react with these goat sera. Serum from goat #4663 (Canton Ticino) strongly reacted to SU5-CO but not to SU5–1163M.

dominant epitopes of the TM have been associated with disease in infected goats (Knowles *et al.,* 1990; McGuire *et al.,* 1992; Bertoni *et al.,* 1994). The epitopes recognized by the caprine immune system in the course of infection must be identified in order to address the significance of this peculiar pattern of antibody response. In the present study we performed an exhaustive mapping of the continuous antigenic sites of CAEV-CO Env using an epitope expression library and recombinant peptides with homologous goat sera. In addition to the four TM epitopes we described previously (Bertoni *et al.,* 1994), we identified five sites in the SU- and two sites in the TM-portion of Env.

An analysis of the antibody response to the SU epitopes in experimentally infected animals revealed that the most immunogenic epitopes of SU (SU3, SU4 and SU5) are located in the C-terminal part of the molecule. These results are in agreement with those obtained in the analysis of the antibody response to SU epitopes in goats infected with field isolates (Valas *et al.,* 2000). While serum reactivity against the correspondent peptides developed early post-infection and

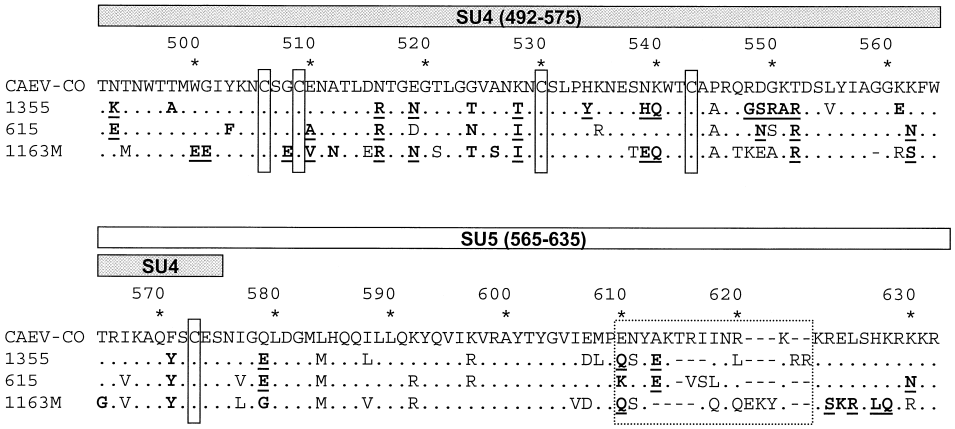

Fig. 5. Deduced amino acid sequences of the SU4 and SU5 overlapping antibody-binding domains (top bars) from three CAEV field isolates CH-615 (615), CH-1355 (1355) and CH-1163M (1163M) are aligned with the CAEV-CO prototypic sequence. Due to different deletions, the lengths of the four fragments analysed were 140 aa for the CH-615 and CH-1355 isolates, 139 aa for the CH-1163M isolate and 141 aa for the CAEV-CO clone. Conserved amino acids are depicted as dots and mutated amino acids are typed. Normal type indicates conservative changes; bold type indicates changes from polar to non-polar amino acid or from positive to negative charges and vice versa. Underlined bold indicates a change from charged to uncharged amino acid and vice versa. Boxes highlight conserved cysteine residues and the dotted box denotes a highly variable region of SU5.

was sustained during the 42 months of observation in infected goats, no significant neutralizing activity appeared to be associated with this response. Indeed, of the six experimentally infected goats, only goats #1 and #5 developed weak titres of neutralizing antibody against the homologous virus. The sera from these two goats showed antibody responses to the SU peptides that were indistinguishable from those of the animals that did not develop neutralizing antibody at all (Table 3). In particular, the reactivity to the SU4 peptide, corresponding to a highly variable region of the Env of small ruminant lentiviruses (Wain-Hobson *et al.*, 1995; Leroux *et al.*, 1997; Turelli *et al.*, 1997; Valas *et al.*, 1997) that was proposed to contain the putative counterpart of the V3 neutralizing domain of HIV-1 (Knowles *et al.*, 1991), did not appear to be the target of a neutralizing response. Recently, Skraban *et al.* (1999) described a type-specific, conformation-dependent neutralizing epitope in MVV. This epitope is localized between aa 525 and 552 and mapped in the MVV counterpart of our CAEV-SU4 peptide (aa 491–575). The authors proposed a model consistent with the existence of a conformation-dependent epitope centred on a cysteine loop highly conserved in MVV and in CAEV, which are closely related lentiviruses of sheep and goats, respectively. In MVV, this loop is flanked by two immunodominant linear epitopes not directly involved in neutralization which mapped to the CAEV-SU4 region described in this paper. Our results do not exclude the presence of such a conformation-dependent neutralizing epitope in the CAEV-SU4 region. However, as in MVV, the reactivity to linear epitopes in SU4 is unlikely to be involved in neutralization. Indeed, the sera of goats #2 and #4, which showed a strong reactivity to the SU4 peptide, did not have any neutralizing activity. Moreover, serum 89G38, which displays a strong neutralization activity in relation to other anti-CAEV sera (Lichtensteiger *et al.*, 1991; data not shown), did not bind to the homologous SU4 peptide.

Serum reactivity to the SU3, SU4 and SU5 domains appeared type-specific. Indeed, these antigenic domains correspond to variable regions of the SU with high rates of amino acid substitutions (Fig. 5; Leroux *et al.*, 1997; Turelli *et al.*, 1997; Valas *et al.*, 1997). Although this indicates that selective pressure for change exists, the absence of a consistent neutralizing response suggests that neutralizing antibody may not be the driving force accounting for the variability of these regions. This differs from other lentivirus infections, such as HIV-1 or EIAV infections, in which the neutralizing response drives the appearance of antigenic escape variants and, at least partly, accounts for the variability of immunodominant domains, like the principal neutralizing domains of HIV-1 gp120 or EIAV gp90 (Goudsmit *et al.*, 1991; Zheng *et al.*, 1997). In CAEV infection neutralizing antibody develops slowly, in only a minority of the infected individuals and at low titres. Although neutralization escape mutants have been described (Ellis *et al.*, 1987; McGuire *et al.*, 1988), neutralizing antibody does not appear to control virus replication *in vivo*: indeed, cell free virus was isolated from the synovial fluid of goats that had neutralizing antibody to the isolated virus (Cheevers *et al.*, 1993). Therefore, variability in these SU antigenic regions can depend on selection by immune mechanisms other than neutralizing antibody or it can occur independently of the immune response, as has been suggested for different RNA viruses (Domingo *et al.*, 1993). Small ruminant lentiviruses and especially CAEV are transmitted

from the mothers to the kids predominantly via infected colostrum. Vertical transmission and animal breeding in a relatively restricted pool of animals, as shown by pedigree analysis, favour the creation of geographical and/or breeding niches for variant viruses. Accordingly, the variability observed in certain Env regions, such as SU5, of CAEV isolates of different geographical provenance may be the result of the adaptation of viruses to genetically relatively homogeneous populations. The uniform reactivity to a region-specific SU5 peptide observed in the experiments performed on three field isolates from goats of different Swiss cantons (Ticino, Fribourg and Berne) (Fig. 4 *a*) supports this hypothesis, which has yet to be confirmed by molecular–epidemiological studies of the circulating viruses.

The relatively strict type-specific reactivity of the SU3–SU5 immunodominant domains (Fig. 4 and data not shown) suggests that peptides corresponding to these epitopes and particularly to different SU5 sequences may be used to serotype field viruses. Using peptides corresponding to the highly variable V3 loop region of HIV-SU, investigators have shown that in some instances serotyping can substitute the more cumbersome genotyping of virus strains (Barin *et al.*, 1996; Plantier *et al.*, 1999). The development of new methods for serotyping field isolates of small ruminant lentiviruses would be a useful tool to support the eradication programs of these viruses. Furthermore, the rapid seroconversion observed with the SU3 and SU5 peptides in experimentally infected animals makes these epitopes interesting candidates for a serological diagnosis of recent infections. This would complement a diagnostic method based on the broadly reacting epitope TM3, which induces a slower seroconversion in some infected goats (Bertoni *et al.*, 1994; unpublished results). Taking into account the type-specific reactivity of the SU epitopes, a diagnostic use would require a further definition of the reactive sequences and mixtures of different peptides (Baillou *et al.*, 1993).

The biological significance of the anti-Env antibody response in CAEV infection is unclear: the sustained non-neutralizing response against the immunodominant linear epitopes of the CAEV Env observed here could simply deflect the antibody response from domains more critical for virus replication. However, in CAEV infection non-neutralizing antibodies may be directly involved in the pathogenic mechanisms of chronic inflammation in infected goats, as suggested by the association of anti-Env antibodies with the degree of severity of arthritis (Knowles *et al.*, 1990; McGuire *et al.*, 1992). The identification of Env continuous epitopes represents a valuable tool for investigating the contribution of epitope-specific antibody to the pathogenesis of CAEV-induced arthritis. We are now in the process of immunizing goats with the recombinant peptides described here in order to produce monospecific sera for analysing the effect on the *in vitro* replication of CAEV and also to study the impact of the antibody response on CAEV infection of goats.

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