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Antibody Reactivity to the Immunodominant Epitopes of the Caprine Arthritis-Encephalitis Virus gp38 Transmembrane Protein Associates with the Development of Arthritis

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High titers of antibodies to caprine arthritis-encephalitis virus (CAEV) envelope (Env) glycoproteins are found in infected goats developing a progressive arthritis. In order to identify linear B epitopes of the CAEV Env, which may be involved in the immunopathology of arthritis, we constructed a λgt11 Env expression library. By combining library screening with sera from naturally infected Swiss goats with an enzyme immunoassay with overlapping peptides (pepscan), four group-specific epitopes could be precisely defined in the transmembrane envelope proteins: TM1 to TM4, including a conserved structure (TM3) that corresponds to the immunodominant epitope of human immunodeficiency virus type 1 and other lentiviruses. A panel of 190 CAEV naturally infected goat serum samples, obtained from animals with defined clinical status, was tested for reactivity to synthetic peptides corresponding to the TM epitopes in an enzyme-linked immunosorbent assay. Antibody reactivity to two epitopes was highly associated (TM3, $P = 0.002$, and TM4, $P < 0.001$) with the presence of clinically detectable arthritis. Such an association is absent for anti-Gag antibody. Antibodies to the immunodominant structures of the TM glycoprotein could thus have an important role in the immunopathogenic process leading to disease.

Lentiviruses, typified by the human immunodeficiency virus (HIV), are animal retroviruses able to persist indefinitely in the infected host despite the presence of a specific immune response. The exact role played by the immune response in protection against viral dissemination or, conversely, in enhancement of disease is unclear. Indeed, data have been reported that suggested a deleterious effect of antibodies directed against viral envelope (Env) glycoproteins (21, 40). Because of important implications for understanding lentiviral pathogenesis and for the design of efficient vaccines against lentiviruses, including HIV (19), it is necessary to confirm the existence of deleterious antibodies in vivo, to determine their mechanism of action, and to identify the epitopes involved. Possible mechanisms involve enhanced entry and dissemination of virus in the presence of specific antibodies or other immunopathological processes, including inflammation or immune destruction of infected cells.

Caprine arthritis encephalitis virus (CAEV) is a lentivirus causing persistent infection in goats. Twenty to 30% of naturally infected goats show a progressive arthritis. The presence of an easily detectable and quantifiable clinical pathology such as arthritis makes CAEV infection of goats a convenient animal model to study the interplay between the development of the immune response and the intensity of disease.

In CAEV-infected goats, the inflammation of synovial spaces of the carpal joint is accompanied by a massive infiltration of lymphocytes, plasmocytes, and macrophages and by the local accumulation of antibodies to the viral envelope proteins (15, 20, 23, 44). Moreover, the severity of disease in infected animals correlates with the titers of anti-envelope antibodies in serum (17). In particular, high titers of antibody in serum to the transmembrane envelope subunit (TM), in its monomeric 38-kDa form or oligomeric form, have been found in goats with progressive arthritis (22).

For most lentiviruses, including HIV-1 and HIV-2, simian immunodeficiency virus from macaques, feline immunodeficiency virus, and equine infectious anemia virus, the major fraction of antibodies directed to the TM glycoprotein recognize a highly conserved structure of the TM ectodomain, containing two cysteine residues that are probably linked by a disulfide bridge (3, 4, 10, 24, 26). The antibodies directed against this epitope are not neutralizing. On the contrary, in the case of HIV-1, monoclonal antibodies to this epitope have been shown to enhance viral replication in a T-cell line (29, 30).

Here, we have mapped four immunodominant epitopes of the CAEV envelope, one of which corresponds to the conserved immunodominant loop present in other retroviruses. We monitored reactivity to the identified epitopes in a large panel of sera from asymptomatic or arthritic CAEV-infected goats. These experiments revealed a significant association between clinical manifestation of disease and reactivity to three epitopes.

MATERIALS AND METHODS

Animals and experimental infection. One hundred ninety goats from different geographical regions of Switzerland and from three different breeds were included in this study. Every

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goat was clinically examined before blood samples were taken. Arthritis was diagnosed by palpation, and the goats were scored on the basis of calculation of the difference between carpal circumference and metacarpal circumference. Animals with a score of >7 cm in at least one joint were considered clinically affected. Animals with a score of <5 cm were considered clinically healthy (asymptomatic). With few exceptions, all goats were female. One hundred eight goats with an average age of 2.8 ± 1.9 years were diagnosed with arthritis; the remaining 82 animals, with an average age of 2.9 ± 1.9 years, were infected but asymptomatic. Sixty-six serum samples were from Saanen goats, 58 were from Toggenburg goats, and 66 were from chamois-colored goats.

Twelve Saanen goats between 6 months and 24 months of age were experimentally infected. Six goats were infected with 1 ml of supernatant (10^7 50% tissue culture infective doses/ml) of goat synovial membrane (GSM) cells transfected with the CAEV-CO molecular clone (32). A total of 0.5 ml was injected intracarpally, and 0.5 ml was injected intravenously. The remaining six goats were mock infected with culture supernatant of nontransfected GSM cells.

Cloning of CAEV-CO Env. The Env gene was derived from an infectious clone of CAEV-CO (32). The nucleotide (nt) and amino acid (aa) positions defining different Env regions in this paper refer to the sequences published by Saltarelli et al. (32). The molecular clone of CAEV-CO consists of two plasmids, one containing almost the entire genome of CAEV-CO and the second encoding the carboxy terminus of Env and the 3′ part of the viral long terminal repeat. To generate a plasmid containing the entire Env coding region, we subcloned the *Sma*I-*Hin*dIII fragment of CAEV-CO in pUC18 and subsequently inserted the 321-bp *Hin*dIII-*Hin*dIII fragment. The new construct was named pUC18 Env 3.1.

Construction of the λgt11 expression library. To construct the Env expression library, we followed a previously described strategy (26), with minor modifications. Briefly, the pUC18 Env 3.1 plasmid was subjected to partial DNase I digestion at 15°C with 15 pg of DNase I per ml in Tris-HCl (pH 7.4)–1.5 mM $MgCl_2$ for 20 min to generate random DNA fragments. Fragments with an average length of 200 bp were isolated, blunt ended with the Klenow fragment of *Escherichia coli* DNA polymerase I, and ligated with *Eco*RI linkers. The fragments were subsequently cloned in EcoRI-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. To control the quality and the representativity of the library, we randomly isolated 40 phage plaques. The insert size was determined by PCR with λgt11 primers complementary to the β-galactosidase portion of the λgt11 template (see below). All phages contained inserts of different sizes, ranging from 130 bp to 250 bp. The hybridization of PCR-amplified inserts with three different radiolabelled *Nco*I fragments representing the entire sequence of CAEV-CO Env (probe 1, nt 6071 to 6456; probe 2, nt 6457 to 8071; probe 3, nt 8072 to 8712) indicated that the library was equally representative of the different parts of the Env gene.

Screening procedure to detect immunoreactive plaques. The library was screened with three serum samples from naturally infected Swiss goats, having a high titer in a CAEV Gag enzyme-linked immunosorbent assay (ELISA) (43). Approximately 3×10^4 PFU from the original, unamplified library was plated on *E. coli* Y1090 (5,000 PFU per plate) and incubated at 42°C for 4 h. Plates were then overlaid with nitrocellulose filters saturated with 10 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and further incubated for 3 h at 37°C to induce β-galactosidase fusion protein expression. Immunological screening of the filters was performed as described previously (26). Briefly, the filters were processed and incubated with the three goat serum samples separately (two filters per serum). 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 the supernatant was added to 85 μl of PCR mixture containing 50 pM (each) primer, 10 nM (each) deoxynucleoside triphosphate, and 0.5 U of *Taq* polymerase (Perkin-Elmer Cetus) in a solution containing 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′ GGTGGCGACGACTCCTGGA GCCCG3′; reverse, 5′TTGACACCAGACCAACTGGTAAT G 3′). PCR was carried out for 32 cycles (cycle 1, 94°C, 2 min; cycles 2 to 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 instructions. A total of 1 μl of dimethyl sulfoxide and 20 pM either forward or reverse primer were added in a final volume of 10 μl. This mixture was denatured at 100°C for 12 min and immediately transferred to a dry ice-methanol bath. Next, 1 μl of dithiothreitol (0.1 M), 0.2 μl of Labmix-dGTP (U.S. Biochemical Corp.), 0.5 μl of [^{35}S]ATP (600 Ci/mM), 0.6 μl of dimethyl sulfoxide, 3.8 μl of distilled water, and 0.2 μl of Sequenase (U.S. Biochemical Corp.) were added. The samples were incubated at room temperature for 1 min and subsequently were incubated at 37°C for 15 min.

Pepscan analysis. A combination of library screening with sera from naturally infected Swiss goats and an immunoassay with overlapping peptides (pepscan) was performed to precisely map the epitopes contained in the broadly reactive region spanning the ectodomain of TM previously identified in the library. Overlapping decapeptides covering the sequences from nt 8022 to 8327, Env aa 671 to 772 (Fig. 1), were synthesized in duplicate by Cambridge Research Biochemicals by using the pin technology system. The enzyme immunoassay was performed according to the manufacturer's instructions, with serum samples from seven goats (dilution 1:100). Five serum samples originated from goats infected with field strains of CAEV, one serum sample was from a goat experimentally infected with CAEV-CO, and one serum sample represented a pool of four serum samples from goats experimentally infected with CAEV-CO. Bound antibody was detected by incubation with peroxidase-conjugated rabbit anti-goat immunoglobulin (Sigma) diluted 1:20,000 or, alternatively, was detected by incubation with a peroxidase-conjugated anti-ruminant immunoglobulin monoclonal antibody (CHEKIT-CAEV/MVV; W. Bommeli, Liebefeld-Berne, Switzerland) diluted 1:200, followed by revelation with 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma).

ELISA. Peptides (TM1 to TM4) were synthesized and purified by Neosystem, Strasbourg, France. Peptide coating was performed as follows. Peptides TM3 and TM4 (100 μl of a 5-μg/ml solution in 0.1 M sodium carbonate [pH 9.6]) were adsorbed to each well of microtiter plates (Maxisorp; Nunc) overnight at 4°C. The TM1 peptide showed poor binding, and TM2 did not bind at all to these or several other ELISA plates tested. For this reason, plates that allow covalent binding of activated peptides (CovaLink; Nunc) were used for TM1 and TM2. The coupling of peptides on plates was performed as described by Sondergard-Anderson and colleagues (36). Briefly, the peptides were solubilized in water

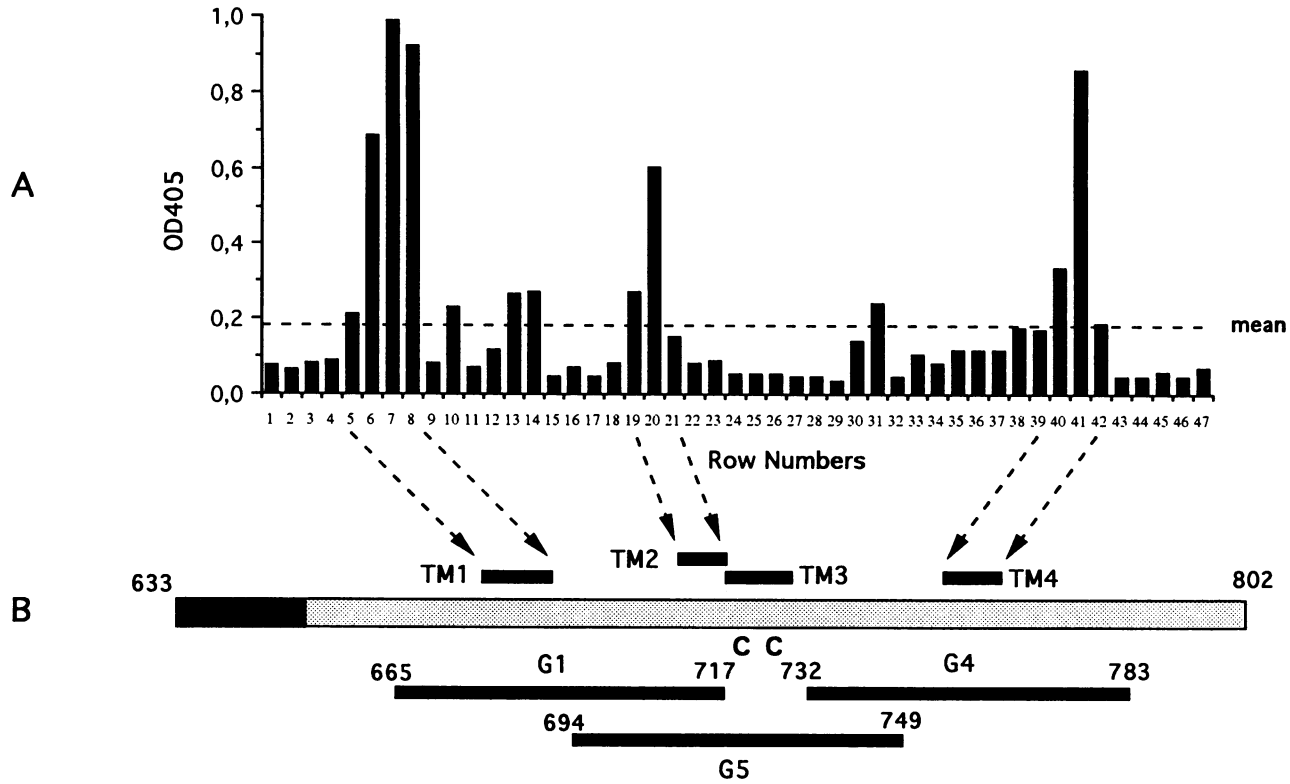

FIG. 1. Mapping of the immunodominant B epitopes of CAEV TM. (A) Pepscan analysis of overlapping decapeptides corresponding to sequence 671 to 772 of the TM protein. Represented is the pepscan performed with serum 4917 from a CAEV-infected goat diluted 1:100. Bound antibodies were detected by a peroxidase-conjugated, anti-ruminant immunoglobulin G monoclonal antibody (CHEKIT-CAEV/MVV). OD405, optical density at 405 nm. (B) Schematic representation of the TM ectodomain. The solid bars on top indicate the positions of the synthetic peptides used in this work. TM1, TM2, and TM4 correspond to the three sets of reactive peptides detected by pepscan (arrows); TM3 encompasses the conserved cysteine (C) domain. The solid bars on the bottom depict three fusion polypeptides isolated by screening of the Env epitope library (see Results).

(TM1, 1 mg/ml; TM2, 0.5 mg/ml). To 1 volume of peptide solution was added an equal volume of a freshly prepared aqueous solution of 0.1 M *N*-hydroxysuccinimide (Sigma) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl (Sigma). After 30 min at room temperature, the activated peptides were diluted with ice-cold 0.1 M carbonate buffer (pH 8.9) to 10 μg/ml and 100 μl of this solution per well was adsorbed to CovaLink plates overnight at 4°C. ELISA was performed as follows. After being coated, the plates were washed three times with phosphate-buffered saline (PBS). Residual adsorption sites on the plates were saturated at room temperature by incubation with 100 μl of PBS containing defatted milk powder (5%) and Tween 20 (0.1%) (ELISA buffer) for 2 h. After washing, 100 μl of goat serum, diluted 1:10 in ELISA buffer, was incubated for 2.5 h at room temperature. After being washed four times with ELISA buffer, peroxidase-conjugated protein G (diluted 1:5,000 in PBS–0.1% Tween 20) was added for 2 h at room temperature. After five washes with PBS, the adsorbed peroxidase conjugate was visualized with 0.2 mg of 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic) acid per ml in 0.6% acetic acid (pH 4.7)–0.01% (wt/vol) H_2O_2. Optical density was measured at a 405-nm wavelength. Assays were performed in duplicate. Serum reactivities were normalized with, as the standard, a pool of CAEV-positive goat sera calibrated in a diagnostic Gag–glutathione *S*-transferase (GST) ELISA (43). Normalization of the results (expressed as percentage of reactivity of the CAEV-positive pool of sera) allowed a direct comparison of ELISA results obtained at different times and on separate plates. A positive reaction was defined as having a reactivity >25% of that of the reference serum. The recombinant Gag ELISA was performed as described previously (43).

RIPA. CAEV-CO-infected or mock-infected GSM cells were labeled overnight with 100 μCi of [^{35}S]methionine and [^{35}S]cysteine (Trans[^{35}S] label; ICN, Flow) per ml in methionine-cysteine-deprived medium (ICN, Flow). Cells were washed with ice-cold PBS, harvested with a rubber policeman, and centrifuged at 700 × *g* for 10 min. Cell pellets were lysed in radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1% Triton X-100, 0.5% Na-deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride), centrifuged at 700 × *g* for 10 min, and then centrifuged at 17,000 × *g* for 1 h to eliminate nuclei and cell debris, respectively. Samples were precleared by incubation with preinfection serum for 1 h at 4°C and precipitation with protein G-Sepharose (Zymed, South San Francisco, Calif.) for 30 min, followed by centrifugation at 11,600 × *g* for 1 min. Supernatants were incubated with CAEV postinfection sera diluted 1:100 overnight at 4°C and precipitated with protein G-Sepharose. Beads were sedimented at 11,600 × *g* for 1 min, resuspended in 800 μl of RIPA buffer, and washed five times. Finally, pellets were resuspended in 40 μl of sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 5% 2-mercaptoethanol, 2.3% SDS), boiled, and ana-

lyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (8.5% polyacrylamide).

Western immunoblot analysis. Western blotting was performed to test the prevalence of antibody reactivity to the TM epitopes in naturally infected goats and to monitor the development of such antibodies in experimentally infected goats. Env antibody-binding domains G1 (nt 8003 to 8163; Env, aa 665 to 717), G4 (nt 8204 to 8360; Env, aa 732 to 783), and G5 (nt 8090 to 8259; Env, aa 694 to 749) (Fig. 1) were expressed as β-galactosidase fusion proteins in lysogenic *E. coli* Y1089 as described by Huynh and collaborators (12). Bacterial lysates containing fusion proteins were prepared from 4 ml of IPTG-induced lysogenic cultures as described previously (26). The lysates (40 μg per well) were resolved on SDS-PAGE gels (10% polyacrylamide) and transferred to nitrocellulose filters. Nonspecific sites were blocked by incubation in 3% defatted milk powder in Tris-HCl (10 mM), NaCl (150 mM), and Tween 20 (0.1%). The nitrocellulose strips were incubated overnight at 4°C with goat serum diluted 1:100 to 1:800 (depending on the serum). Antibody binding was revealed by incubation with peroxidase-coupled protein G diluted 1:5,000, followed by revelation with 4-chlor-1-naphthol (Sigma). A lysate of bacteria infected with nonrecombinant λgt11 phage was used as the negative antigen control. Sera collected before experimental infection and a pool of sera from CAEV-negative goats were used as antibody control. Additionally, the colocalization of the immunoreactive bands with the fusion proteins was verified with a monoclonal antibody to β-galactosidase (Promega). Western blotting with purified virus antigen was performed as described previously (42).

Statistical analysis. The reactivities of asymptomatic and affected animals were compared by using the nonparametric Mann-Whitney U test for independent groups (41).

RESULTS

Identification of immunodominant regions in CAEV TM. We chose to screen the epitope library derived from strain CAEV-CO with sera from naturally infected animals (heterologous sera) to focus directly on conserved, i.e., group-specific, immunodominant epitopes. Immunological screening of the λgt11 Env epitope library was performed by using three high-titer serum samples from naturally infected Swiss goats. Use of Swiss goat field sera to screen the epitope library prepared from a North American isolate of CAEV allowed a clear identification of immunodominant group-specific (conserved) epitopes. Five independent strongly immunoreactive clones were isolated and sequenced. The sequences of the five inserts [G1, nt 8003 to 8163 (Env, aa 665 to 717); G2, nt 8019 to 8264 (Env, aa 670 to 751); G3, nt 7991 to 8107 (Env, aa 661 to 698); G4, nt 8204 to 8360 (Env, aa 732 to 783); and G5, nt 8090 to 8259 (Env, aa 694 to 749)] are encompassed in the extracellular region of TM. All of these clones overlapped and did not allow a precise identification of reactive epitopes. Interestingly, they covered the immunodominant TM epitopes defined for other lentiviruses.

Prevalence of antibodies against immunodominant regions in naturally infected goats. The peptides G1, G4, and G5, chosen because they cover the entire immunoreactive region of TM, were expressed as β-galactosidase fusion proteins and used in Western blots to screen 60 serum samples of naturally infected goats originating from different Swiss flocks. Thirty-nine serum samples (65%) reacted to G1, 57 reacted to G4 (95%), and 57 reacted to G5 (95%).

Fine mapping of the immunodominant TM epitopes. By using pepscan technology, a fine mapping of the B epitopes contained in the identified TM ectodomain was performed. Three groups of overlapping peptides were reactive, defining three different epitopes (Fig. 1). The following synthetic peptides corresponding to these epitopes were synthesized: TM1, DVLEATYAMVQHVAK (nt 8049 to 8093; Env, aa 680 to 694); TM2, EAITDRIMLYQE (nt 8130 to 8165; Env, aa 707 to 718); and TM4, CTWQQWERELQGYD (nt 8256 to 8297; Env, aa 749 to 762). Surprisingly, the region containing two conserved cysteines, known to form an immunodominant and conserved structure in several lentiviruses, did not react with any of the sera tested. Despite the pepscan results, we decided to synthesize a peptide (TM3, QELDCWHYHQYCITS, nt 8160 to 8204; Env, aa 717 to 731) encompassing this region. A peptide with the same sequence but chemically cyclized by a covalent link between the two cysteines was also made (TM3c), in order to mimic a loop structure thought to exist in native TM (9). A set of 30 goat serum samples was tested by ELISA for reactivity to TM3 and TM3c. All sera recognized both peptides (not shown). No differences between TM3 and TM3c were observed; therefore, subsequent experiments were performed with TM3c. The failure of the pepscan assay to detect this epitope could reflect the inadequate conformation of peptides synthesized on a solid support or the formation of cross-links between peptides on the same pin blocking antibody binding. The requirement of a sequence longer than 10 aa for immunoreactivity cannot be excluded.

Genomic variability and epitope reactivity. Western blot analysis of 60 goat serum samples (with recombinant fusion proteins) and ELISA of 190 goat serum samples (with synthetic peptides) revealed differences in serum reactivities with the four group-specific TM epitopes. At present, the TM sequences of only two CAEV isolates have been determined (18, 32). However, we aligned the immunodominant TM sequences of the two CAEVs with three closely related maedivisna virus molecular clones (18, 28, 32, 33, 37) (see Fig. 5). The plot-similarity representation of these sequences (Fig. 5b) shows a high average similarity score of the TM3 region (1.38) compared with that of the entire Env (1.14). In fact, the broadest reactivity of the TM3 peptide, which is encompassed in the G5 fusion protein, correlates with a higher level of sequence conservation. TM4 also appears to correspond to a conserved region. Conversely, the G1 domain, which contains the TM1 and TM2 epitopes, seems to be more variable. About 35% of the animals failed to react to these epitopes in both tests.

Kinetics of anti-TM antibody development in experimentally infected goats. The evolution of the specific antibody response to the TM epitopes in comparison with the global humoral response to Gag and Env proteins was monitored for 32 weeks by Western blot, ELISA, and RIPA in six animals experimentally infected with CAEV-CO. The results are summarized in Table 1. All infected animals seroconverted rapidly to both Env and Gag. The sera of infected goats recognized Env glycoproteins 1 to 3 weeks postinfection and Gag proteins 2 to 3 weeks postinfection with RIPA on CAEV-infected cell lysate (Fig. 2) and with Western blotting on pelleted virus. The recombinant Gag fusion protein, Gag-GST, was also detected at 3 to 4 weeks postinfection by ELISA (Fig. 3B). In contrast, antibodies to TM epitopes were detected much later. Infected goat serum reactivity with the TM antibody-binding domains G1, G4, and G5 in the Western blot and with the TM1, TM2, TM3, and TM4 peptides in the ELISA was detected between 12 weeks and 32 weeks postinfection, varying with the epitope and the animal. The seroconversion to TM3 peptide was the first to appear and was detected in all of the animals (Fig. 3A).

TABLE 1. Evolution of specific antibody response to TM epitopes compared with global humoral response to Gag and Env proteins

Goat	Time of seroconversion (wk postinfection)										
	RIPA[a]		Western blot				ELISA				
	Env	Gag	Gag[b]	G1[c]	G4[c]	G5[c]	Gag-GST[d]	TM1[e]	TM2[e]	TM3[e]	TM4[e]
1	2	3	3	12	28	28	3	28	32	16	N[f]
2	2	2	2	28	28	28	3	28	28	28	32
3	2	3	3	28	12	28	3	28	N	12	N
4	1	3	3	N	N	N	3	N	N	28	N
5	3	3	3	28	12	28	4	N	32	12	28
6	1	3	3	N	28	N	3	N	N	28	32

[a] Env and Gag proteins were immunoprecipitated from CAEV-infected cell lysate.
[b] Gag proteins were detected by Western blotting of the whole virus pellet.
[c] β-Galactosidase fusion protein containing the TM antibody-binding domains.
[d] Glutathione *S*-transferase Gag fusion protein (39).
[e] Peptide ELISA.
[f] N, seronegative up to 32 weeks postinfection.

Association between antibody titer to Gag and Env and clinical status. One hundred ninety sera from CAEV-infected goats with defined clinical status were tested by ELISA for reactivity with the four epitopes and with Gag. As shown in Table 2, most sera reacted with the TM3 peptide and with Gag. TM4, TM2, and TM1 peptides reacted with the majority of sera, although less frequently than TM3. Few sera, although reactive in Western blot to p25 (Gag) with pelleted virus as antigen, were negative in the Gag ELISA and peptide ELISA. We observed some discrepancy between Western blot results with β-galactosidase fusion protein G4 and the ELISA results with TM4. Western blotting was more sensitive for this epitope, possibly because the peptide fused to β-galactosidase can assume conformations that are not present in the free peptide form. Alternatively, additional epitopes could be located in G4 sequence that escaped detection by the pepscan analysis.

To quantify the antibody titer present against each epitope, the optical density values obtained by the ELISA were expressed as percentages of reactivity compared with that of a reference serum consisting of a pool of CAEV-seropositive sera. ELISA reactivities of sera from asymptomatic and arthritic animals were then compared. A reactivity index was calculated for every serum tested as the percentage of reactivity to the TM peptides/percentage of reactivity to Gag. The results are summarized in Table 3. Sera from arthritic goats showed a higher reactivity to TM1, TM3, and, especially, TM4 than sera from infected asymptomatic goats. The U values calculated by comparing the two groups according to the nonparametric Mann-Whitney U test gave the following significant results: TM1, $U = 5{,}328.0$, $P = 0.014$; TM3, $U = 5{,}582.0$, $P = 0.002$; TM4, $U = 5{,}964.5$, $P < 0.001$. In contrast, there was no significant difference between the two groups of animals in the reactivity either to Gag or to TM2 (Gag, $U = 4{,}106.0$, $P = 0.391$; TM2, $U = 4{,}905.5$, $P = 0.198$). Box plot representations are shown in Fig. 4.

DISCUSSION

Immunodominant linear B-cell epitopes on the envelope of lentiviruses are thought to be involved in positive or negative control of viral dissemination. For example, in the case of HIV-1 infection in vitro, antibodies directed to the immunodominant domain of surface (SU) glycoprotein (V3) have a neutralizing activity, while antibodies against the immunodominant epitope of TM enhance viral production (7, 11, 29–31). In vivo, the neutralizing epitope V3 is hypervariable, as expected for a structure that impedes viral dissemination, whereas the enhancing epitope is highly conserved, as expected from a structure that favors viral dissemination.

However, the consequences of antibody-mediated neutral-

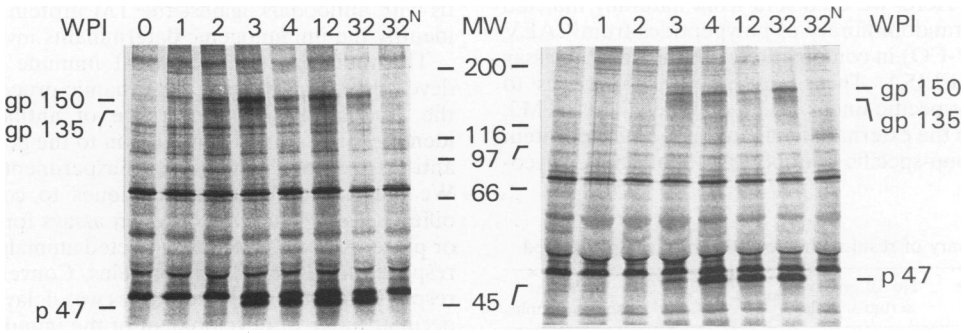

FIG. 2. Kinetics of anti-CAEV antibody development in experimentally infected goats determined by RIPA. Viral proteins were precipitated from CAEV-infected GSM cell lysate by using goat serum samples taken at 0, 1, 2, 3, 4, 12, and 32 weeks postinfection (WPI). 32^N, control RIPA performed with the 32-week serum on uninfected GSM cells. Two experiments are shown. Left panel, results from goat 4. The Env precursor gp150 was detected 1 week postinfection, and the SU precursor was detected 2 weeks postinfection, while the Gag precursor p47 was precipitated 3 weeks postinfection. Right panel, results from goat 5. Env and Gag proteins were detected at the same time (3 weeks postinfection). Sizes of the immunoprecipitated proteins correspond to the original description by Cheevers et al. (2). MW, molecular weight (thousands).

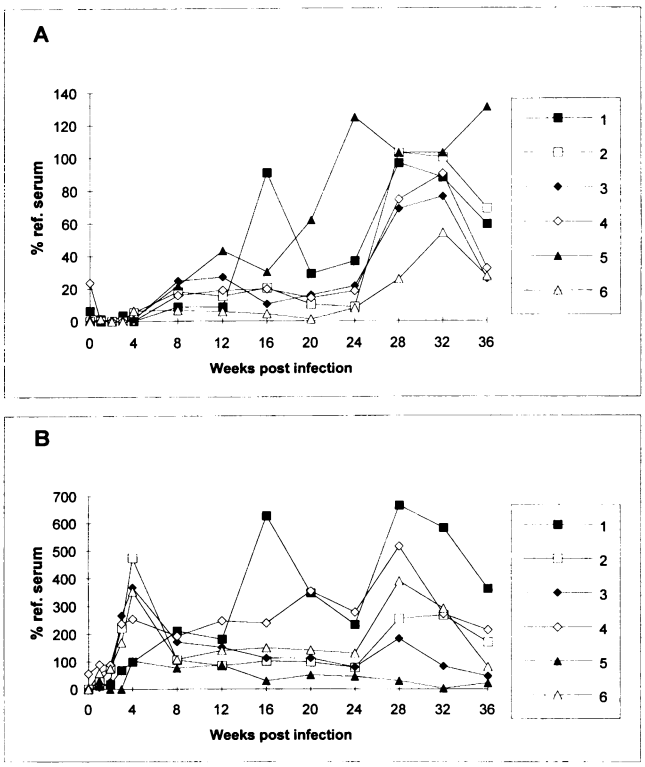

FIG. 3. TM3 and Gag ELISA. Sera from six goats (1 to 6) collected at different intervals before and after experimental infection with CAEV-CO were tested for reactivity in ELISA. (A) The goats developed anti-TM3 antibodies with different kinetics. Only 28 weeks postinfection, the reactivity of all sera were above 25% of that of the control serum. (B) Anti-Gag antibodies appeared early after experimental infection, and at week 4 postinfection, all goats were seropositive. Goat 5 turned seronegative 16 weeks postinfection, and goat 3 turned seronegative 36 weeks postinfection on Gag, but both remained clearly seropositive on TM3 during this period (A).

ization or enhancement for HIV-1 dissemination and pathogenesis in vivo remain to be elucidated.

In the case of CAEV infection of goats, the results of several studies have indicated that the development of arthritis involves antibody responses to the Env glycoproteins (13, 17, 22). The responsible epitopes and the mechanisms of pathogenesis remain unknown. Here, we used sera from naturally infected goats to screen a random library of polypeptides from CAEV Env (strain CAEV-CO) in combination with the pepscan assay and the peptide ELISA. These experiments allowed us to define four group-specific linear B-cell epitopes (TM1, TM2, TM3, and TM4) in the external domain of the TM glycoprotein of CAEV. No group-specific epitope was found in SU glycoprotein. We subsequently demonstrated a significant association between antibody titers to three of these epitopes and progressive arthritis. Similar association with pathology was not observed for antibody titers to CAEV Gag proteins.

TABLE 2. Summary of results for the 190 serum samples tested

Peptide	No. of positive[a] serum samples	% Positive[a] serum samples
Gag	175	93
TM1	122	64
TM2	136	72
TM3	174	92
TM4	145	76

[a] Positive, reactivity >25% of that of the reference serum.

TABLE 3. ELISA reactivity of asymptomatic versus affected animals

Peptide and clinical status of animal	% Reactivity		Reactivity index[a]
	Mean	Median	
Gag			
Asymptomatic	268	179	1
Affected	249	154	1
TM1			
Asymptomatic	148	14	1.57
Affected	189	80	2.14
TM2			
Asymptomatic	73	22	0.67
Affected	92	34	0.86
TM3			
Asymptomatic	147	77	1.79
Affected	205	160	2.51
TM4			
Asymptomatic	143	7	2.6
Affected	317	125	3.4

[a] Percentage of reactivity to TM peptides/percentage of reactivity to Gag.

The 190 goat sera analyzed by ELISA were taken from animals with defined clinical status and were representative of three breeds from different geographical regions of Switzerland. The observed association between antibody titers to some TM epitopes and arthritis is probably not explained by antigenic variation, given the high level of sequence conservation observed for these epitopes and, particularly, for the TM3 epitope (Fig. 5). On the other hand, the persistence of CAEV may permanently boost the immune response against these conserved regions, so that the antibody titers may simply reflect the viral load or the overall intensity of the immune response. However, the same correlation still held when we used serologic reactivity against Gag products, which are also conserved and highly immunogenic, to normalize the peptide ELISA titers against the TM epitopes. Thus, the results reported here extend previous observations correlating arthritis with antibodies against the TM protein (22) and precisely identify the immunogenic determinants involved.

The mounting of a humoral immune response and the development of arthritis are dynamic processes. We analyzed the kinetics of the appearance of antibodies to the four identified TM epitopes in relation to the general anti-Env and anti-Gag responses in a series of experimentally infected goats. We performed different techniques to control for possible differences in sensitivity between assays for different epitopes or proteins. Experimentally infected animals mounted an early response to Gag and Env proteins. Conversely, the antibody response to the four TM epitopes was delayed. This suggests a peculiar mode of development of the immune response to the immunodominant TM epitopes. Whether or not the slow appearance of both anti-TM antibodies and clinical disease (after more than 1 year on average) reflects a link between the humoral response to specific epitopes of the TM glycoprotein and arthritis development is an important question to address in further in vivo experiments.

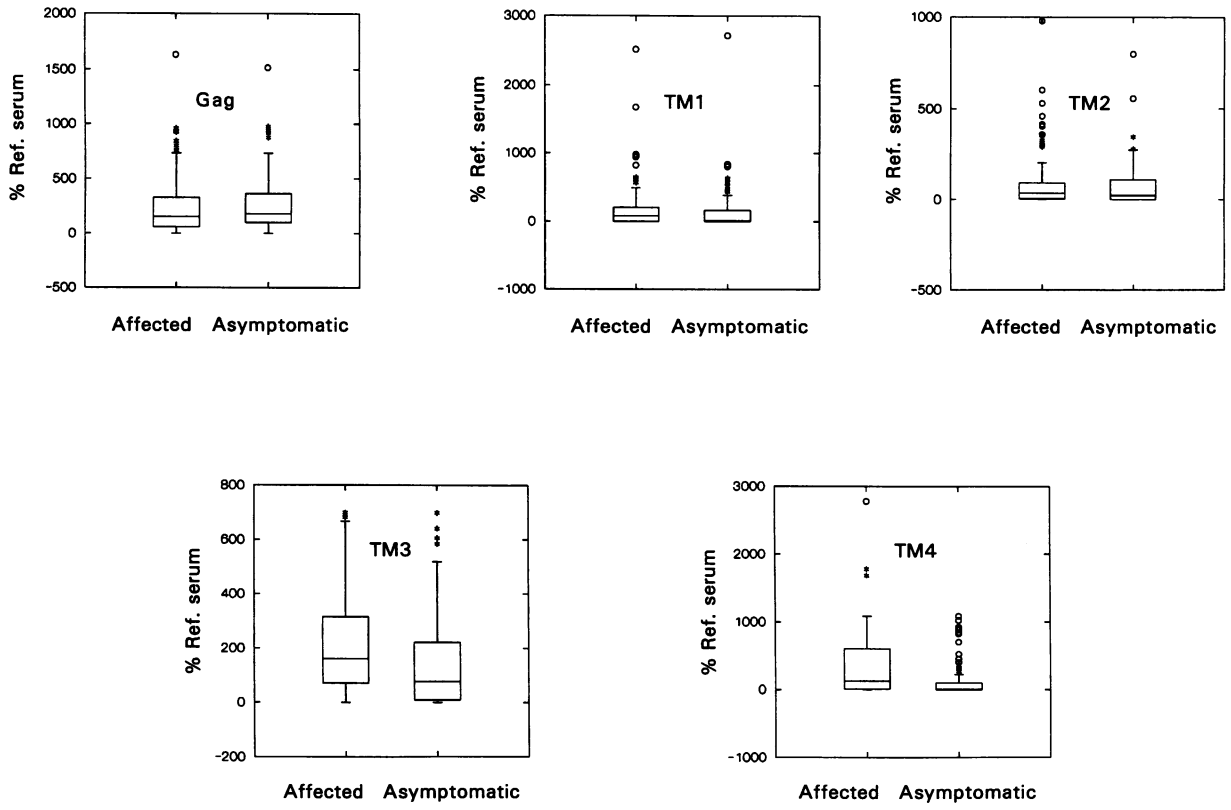

FIG. 4. Box plot representations of ELISA reactivity to Gag, TM1, TM2, TM3, and TM4 of sera from goats naturally infected with CAEV. The goats were divided into two groups, depending on their clinical status. Affected goats had clinical arthritis, and asymptomatic goats were infected but nonarthritic. Affected animals showed a significantly higher reactivity to TM1, TM3, and TM4 than asymptomatic animals did.

Several mechanisms could be proposed to explain the involvement of antibodies in pathogenesis. High-level viral expression has been found in inflamed tissues, mostly in macrophages (45), and it has been associated with joint enlargement in infected goats (16). It has been proposed that the inflammatory process depends upon constant antigenic stimulation in the synovial cavity, in which antibody and activated lymphocytes react with viral antigens and infected cells (16, 20, 45). This pathogenic mechanism is reminiscent of experimental antigen-induced arthritis in rabbits and mice, respectively (6, 38), and rheumatoid arthritis (46).

In light of our observation, it is necessary to explain why antibodies to a given TM structure only are involved in this process. It is possible that the immunodominant structure is preferentially exposed on the surface of infected cells or virions and/or allows strong immune stimulus and formation of stable immune complexes. However, current models of retroviral envelope structure indicate that the TM ectodomain is at least in part masked by the SU glycoprotein (9, 34, 35). We may also speculate that these antibodies belong to a specific isotype that mediates toxicity of the complexes through antibody-dependent cell-mediated cytotoxicity or complement activation, for example. However, no information is available about goat isotypes.

Interestingly, the TM3 epitope of CAEV TM has the same localization as the immunodominant epitope of other lentiviruses, such as HIV-1 or -2, simian immunodeficiency virus, feline immunodeficiency virus, and equine infectious anemia virus (reviewed in reference 27). This epitope is delineated by two cysteines, which are conserved in the external domain of the TM protein in all retroviruses (9). The two cysteine residues are probably linked by a disulfide bond, allowing the formation of a loop (25). The amino acid sequence within the loop differs from one retrovirus to another, although it is highly conserved between different viral isolates of the same species. It has been shown that mutations in the conserved loop region of HIV-1 dramatically impair the correct processing and cleavage of the Env precursor and result in the production of noninfectious virions (4, 9). Remarkably, the immunodominance of this structure is insensitive to antigenic or individual variation: in all lentiviruses in which it has been studied, this structure is recognized by nearly 100% of the serum samples from infected patients or animals and it can be used for serological diagnostic assays (1, 8, 10, 24, 39). Thus, both the structure and its immunodominance were highly conserved during evolution. The usual explanation for conservation of both structure and immunogenicity puts more weight on structure: the structure is essential and its immunogenicity is evolutionarily neutral because the epitope is not neutralizing. However, the remarkable conservation of immunodominance between different individuals and host-virus systems argues against evolutionary neutrality and may suggest that the immunogenicity itself provides some advantage to the virus in vivo. This hypothesis is supported by the observation of enhanced replication of HIV-1 in vitro in the presence of antibodies against the TM immunodominant epitope of HIV-1 (29, 30). In light of these data, we feel that it is reasonable to propose that antibodies to the immunodominant structure of the CAEV TM protein increase pathogenicity through enhancement of viral entry and dissemination in monocytes and

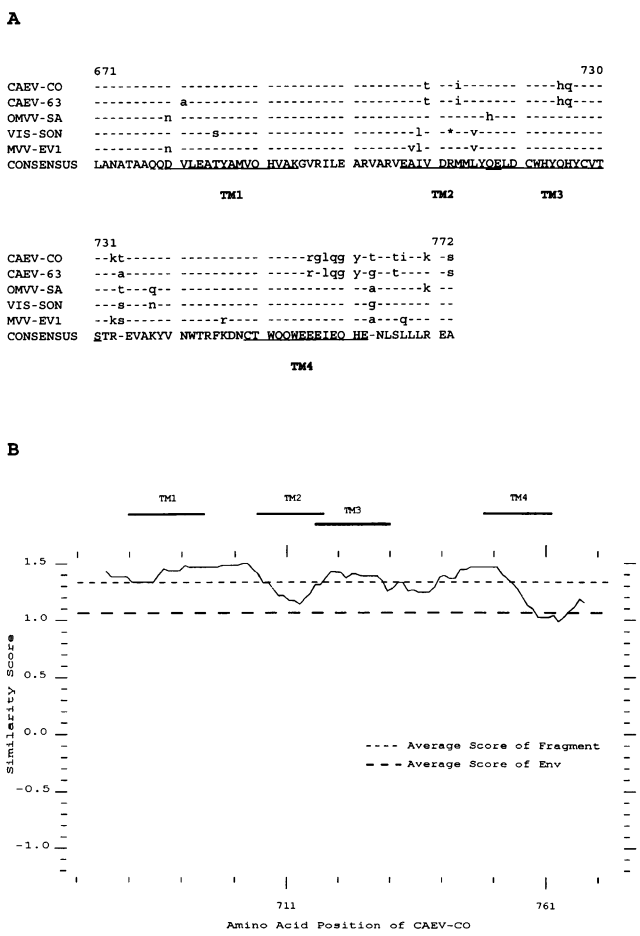

FIG. 5. (A) Alignment of amino acid sequences of two CAEV isolates and three related maedi-visna viruses in the region corresponding to the TM domain analyzed by pepscan (aa 671 to 772). Dashes represent identical amino acids and conserved substitutions (Dayhoff matrix, > 1) (5). The bars underlining the sequences indicate the positions of the synthetic peptides used in the ELISA. (B) Plot similarity of the region described in panel A. The average similarity for each amino acid position is shown (5). Bars above the plot indicate the position of the synthetic peptides used. Note that the similarity score of this TM region is higher than that of the entire Env.

macrophages in vivo. Indeed, an enhancement of viral binding and entry into sheep macrophages has been observed in vitro by using goat and sheep sera to CAEV and visna virus, respectively. However, surprisingly, no increased virus replication was found in this study (14). The production of monospecific or monoclonal antibodies to the immunodominant epitopes of CAEV TM will enable us to analyze more precisely the possible role played by anti-TM antibodies in virus-cell interactions by studies ex vivo with goat monocytes.

Furthermore, the potential involvement of antibodies to the immunodominant epitopes in the development of arthritis can be investigated in vivo. Synthetic peptides will allow modulation in vivo of the level of anti-TM antibodies by priming the immune response before experimental infection or by immunizing goats persistently infected with the virus.

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