

## Priming with *tat*-Deleted Caprine Arthritis Encephalitis Virus (CAEV) Proviral DNA or Live Virus Protects Goats from Challenge with Pathogenic CAEV

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We previously reported that infection of goats with caprine arthritis encephalitis virus (CAEV) *tat*- proviral DNA or virus results in persistent infection, since the animals seroconverted and direct virus isolation from cultures of blood-derived macrophages was positive. In this study we wanted to determine whether goats injected with CAEV *tat*- proviral DNA or virus were protected against challenge with the pathogenic homologous virus and to investigate whether CAEV *tat*- was still pathogenic. All animals injected with CAEV *tat*- became infected as indicated by seroconversion and virus isolation. Challenge at 8 or 9 months postinfection demonstrated protection in four of four animals injected with CAEV *tat*- but did not in three of three mock-inoculated challenged goats. Challenge virus was undetectable in the blood macrophages of protected animals during a period of 6 or 10 months postchallenge. In two of four protected animals, however, we were able to detect the challenge wild-type virus by reverse transcriptase PCR on RNA directly extracted from synovial membrane cells surrounding the inoculation site. This result suggests that protection was achieved without complete sterilizing immunity. Animals injected with CAEV *tat*- and mock challenged developed inflammatory lesions in the joints, although these lesions were not as severe as those in CAEV wild-type-injected goats. These results confirm the dispensable role of Tat in CAEV replication *in vivo* for the establishment of infection and pathogenesis and demonstrate in another lentivirus infection model the efficacy of live attenuated viruses to induce resistance to superinfection.

Like other lentiviral infections, caprine arthritis encephalitis virus (CAEV) infection is characterized by viral persistence in the face of an immune response and the onset of slow and progressive degenerative diseases (20). Synovitis, mastitis, pneumonia, and encephalitis characterize the course of disease in CAEV infection (29). These inflammatory diseases are the result of viral infection of cells of monocyte/macrophage lineage, which are the main target cells *in vivo* (8–10). Infection of macrophages is a common feature of lentiviral infections and plays a central role in the development of associated diseases. The CAEV infection model thus provides a system to analyze the pathogenesis of diseases associated with macrophage infection and to develop vaccine strategies against macrophage-tropic viruses. The use of an infectious molecular clone of CAEV (34, 37) allowed us to investigate the role of different genes in the induction of infection and pathogenesis. We previously demonstrated the essential role of the *vif* gene for efficient CAEV replication *in vitro* and *in vivo* (13, 15), whereas the *tat* gene was shown to be dispensable in both instances (14). Goats inoculated with CAEV *vif*- virus or proviral DNA were not protected against CAEV wild-type (wt) challenge, due to the reduced level of CAEV *vif*- replication (15, 16). Similar results were obtained in the simian immunodeficiency virus (SIV) macaque model, in which an inverse correlation between the degree of virus attenuation and the

induction of protection was demonstrated (26, 42). In the macaque model, it is now clearly established that long-term protection against systemic challenge can be achieved by systemic immunization with live attenuated SIVmac239  $\Delta nef$  or  $\Delta 3$  (7, 42) or SIVmac32H C8 (31). This approach was also shown to be effective against mucosal challenge after either systemic SIVmac32H C8 (6) or mucosal simian/human immunodeficiency virus 89.6 (28) immunization. All these results were obtained with lymphocyte-tropic viruses. Since macrophage-tropic strains of human immunodeficiency virus type 1 (HIV-1) seem to be the transmitted viruses responsible for initial infection (36, 39, 44), they might be the initial targets to consider in vaccine strategies. Indeed, in the SIV model, immunization of macaques with attenuated macrophage-tropic SIV/17E-CI resulted in protective immunity against heterologous challenge (4).

In a previous study, we reported that proviral DNA of the CAEV Cork molecular clone with deleted *tat* sequences (CAEV *tat*-) produced persistent infection in goats, with an antibody response showing kinetics of appearance and a reactivity pattern against viral proteins that were similar to those in CAEV wt-infected goats (14). The present study was designed to evaluate the capacity of this live attenuated virus to induce resistance to superinfection and to investigate the pathogenic properties of CAEV *tat*- compared to those of CAEV wt. Protection against challenge with a high dose (>250 100% animal infectious dose [AID<sub>100</sub>]) of homologous CAEV wt was achieved in all goats inoculated with CAEV *tat*- without complete sterilizing immunity. A control animal inoculated with CAEV *tat*- and mock challenged developed mild inflammatory lesions in the joints, ruling out an essential role for the

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TABLE 1. Experimental design

Expt and goat no.	Priming with <sup>a</sup> :				Challenge <sup>b</sup>
	Live virus		Proviral DNA		
	wt	<i>tat</i> -	wt	<i>tat</i> -	
Expt 1					
9317	+				+
9319		+			-
9324		+			+
9327		+			+
9315					+
Expt 2					
306			+		+
307			+		-
303				+	-
311				+	+
312				+	+
308					+
C70					+

<sup>a</sup> Priming was carried out at day 0 by injection of  $10^5$  TCID<sub>50</sub> of virus or 100 or 350 µg of proviral DNA into the right carpus.

<sup>b</sup> Challenge occurred at day 228 p.i. (experiment 1) or at day 279 p.i. (experiment 2) by injection of  $>250$  AID<sub>100</sub> of CAEV wt into the left carpus.

CAEV *tat* gene in virus-induced pathogenesis. Although further attenuation of CAEV *tat*- is required to obtain a non-pathogenic live attenuated vaccine strain, these experiments demonstrated the efficacy of this vaccine strategy in an additional lentiviral animal model.

#### MATERIALS AND METHODS

**Viruses.** The infectious proviral DNA of the CAEV Cork strain was generated by ligation of the 9- and 0.5-kb *Hind*III CAEV fragments (34, 37). The *tat*-deleted mutant, containing a 153-bp in-frame deletion between positions 5677 and 5829, was previously described (14). Viral stocks were obtained by transfection of CAEV wt or *tat*- proviral DNA into primary goat synovial membrane cells or by infection of goat synovial membrane cells with transfection supernatants. These stocks contained  $10^5$  50% tissue culture infectious doses (TCID<sub>50</sub>) per ml. Preliminary in vivo virus titration with fivefold stock virus dilutions revealed that five of five goats that were given the last  $2.5 \times 10^{-2}$  dilution by the intratracheal route became persistently infected (data not shown). Therefore, the stock virus is estimated to contain  $>250$  AID<sub>100</sub> per ml.

**Immunization and challenge protocols.** Goats were obtained from two separate CAEV-free flocks and were included in two protocols summarized in Table 1. In the first experiment, four goats were inoculated intra-articularly in the right carpus with  $10^5$  TCID<sub>50</sub> of CAEV wt (goat 9317) or *tat*- proviral stocks (goats 9319, 9324, and 9327). Mock control goat 9315 was inoculated with culture medium. The goats were challenged on day 228 postinjection (p.i.) by inoculation of  $>250$  AID<sub>100</sub> of homologous CAEV wt in the left carpus and necropsied at day 185 postchallenge (p.c.). In the second experiment, five goats were inoculated in the right carpus with 100 µg of proviral CAEV wt DNA (goats 306 and 307) or with either 100 µg (goat 311) or 350 µg (goats 303 and 312) of CAEV *tat*- proviral DNA mixed with the cationic lipid DOTAP (Boehringer-Mannheim) as previously described (14). Mock control goat 308 was injected with 100 µg of plasmid DNA. Naive goat C70 was included as a positive control for infection at the time of challenge. These goats were challenged on day 279 p.i. by inoculation in the left carpus with  $>250$  AID<sub>100</sub> of homologous CAEV wt and necropsied on day 310 p.c. Blood samples were regularly drawn to purify macrophages for virus isolation and reverse transcriptase (RT)-PCR analyses and to determine the serological status by enzyme-linked immunosorbent assay (ELISA).

**Cells.** Primary goat macrophages were obtained from peripheral blood mononuclear cells purified on a Ficol-Paque density gradient (Pharmacia) and maintained for 10 to 12 days in Teflon bags, as previously described (14). Cells were cultured in RPMI 1640 containing 10% sheep serum, 1% glutamine, penicillin and streptomycin, 10 mM HEPES, and  $10^{-5}$  M β-mercaptoethanol. Macrophage culture supernatants were analyzed for RT activity as previously described (13). For RT-PCR analyses, blood-derived macrophages as well as necropsied tissues or lymph nodes were subjected to direct RNA extraction with the RNA<sup>B</sup> reagent (BioProbe) as described previously (13). A portion of the synovial membranes was frozen in liquid nitrogen for histological examination of tissue sections after hematoxylin and eosin staining.

**RT-PCR analyses.** RT-PCR analyses and Southern blot hybridization of the amplified products were performed as previously described (13). According to the CAEV Cork sequence published previously (37), the sequences and positions of the primers used were as follows: for VIF5 (sense primer), 5'-GACACAACGGGATACACGCA-3' (positions 5621 to 5640); for TAT (antisense primer), 5'-GATTATGTTCACCCACCCCGG-3' (5953 to 5934); for TATH (hybridization oligonucleotide), 5'-CAAGGCGCCTGTGATTAGG-3' (5891 to 5909); for ENV1 (antisense primer), 5'-CCCAGTTAAGCGCATGTATC-3' (6047 to 6028); for POLS (sense primer), 5'-GATAGGATAGGAGTGCATTG-3' (3721 to 3740); for POLH (hybridization oligonucleotide), 5'-TATTTCCGAAATATATTTGTC-3' (3801 to 3781); and for POLA (antisense primer), 5'-TGAGTCTATGATTCCTCT-3' (4020 to 4002).

To detect CAEV *tat*-specific sequences, a seminested PCR was performed after the nonspecific reverse transcription step with the VIF5/ENV1 primer pair used in the initial PCR, followed by another reaction with the VIF5/TAT primers. As an internal control for RT-PCR, the caprine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was amplified with the following primers: for CGAP1 (sense primer), 5'-GTTCCACTATGATTCCACCC-3'; for CGAP2 (hybridization oligonucleotide), 5'-CAGTCAAGGCAAGAGAATGGG-3'; and for CGAPR1 (antisense primer), 5'-TCCTCCACGATGCCAAA-3'.

**ELISA.** Antibody detection was performed by a whole-virus protein ELISA (Chekit CAEV/maedi-visna virus test; Hoechst Roussel Vet.) according to the manufacturer's instructions. Results were expressed as the percentage of positive controls with the following formula: (mean tests - mean negative controls)/(mean positive controls - mean negative controls) × 100. Sera with percentage values below 30% were considered negative, sera with percentage values between 30 and 40% were considered doubtful, and sera with percentage values above 40% were considered positive.

Anti-Gag antibodies were screened by a Gag-glutathione S-transferase ELISA as previously described (43). Anti-transmembrane 3 (TM3) and anti-TM4 reactivities were analyzed with TM3 (amino acids 717 to 731) or TM4 (amino acids 749 to 762) peptide ELISAs, respectively, as described previously (1). A positive serum sample was defined as having a reactivity  $>25\%$  of that of the positive control.

#### RESULTS

**Inoculation of goats with CAEV *tat*-.** Goats 9319, 9324, and 9327 received one intra-articular injection of  $10^5$  TCID<sub>50</sub> of CAEV *tat*- viral stock. Goats 303, 311, and 312 were inoculated once with 100 or 350 µg of CAEV *tat*- proviral DNA (Table 1). All six goats developed anti-CAEV antibodies detected by whole-virus protein ELISAs (Fig. 1), and no major difference was detected between animals inoculated with the CAEV *tat*- proviral DNA (Fig. 1A) and those inoculated with viral stock (Fig. 1B). Antibodies appeared between 3 to 8 weeks p.i. and persisted until the time of challenge. One goat in each group (goats 303 and 9327) exhibited a low level of antibody response, and the antibody response of goat 9327 dropped below the limit of positivity from day 102 p.i. Positive control goats inoculated with either CAEV wt proviral DNA (306 and 307) or viral stock (9317) seroconverted within 3 to 10 weeks (Fig. 1C and D, respectively). Compared to the three positive control animals, four of six goats inoculated with CAEV *tat*- developed similar levels of antibody response. These results confirmed that wt or *tat*- proviral DNA injection was as efficient as wt or *tat*- virus inoculation in inducing persistent infection (14, 15).

Virus production was monitored by measuring the RT activity of blood-derived macrophage culture supernatants. As shown in Table 2, virus isolation was infrequently positive for goats injected with CAEV wt or *tat*-. Blood-derived macrophages from two of three positive control goats (9317 and 306) allowed virus isolation in two of six and two of nine tests, respectively. The third positive control goat, 307, which exhibited the longest delay before seroconversion (day 70 p.i., Fig. 1C), remained negative for virus isolation in eight of eight assays. Among goats inoculated with CAEV *tat*-, virus isolation was positive in two of three goats injected with proviral DNA (311 and 312) and in only one of the three goats injected with the virus (9319). Goat 311, for which three of nine tests were positive, received one injection of 100 µg of DNA, whereas goats 303 and 312 were inoculated with 350 µg of

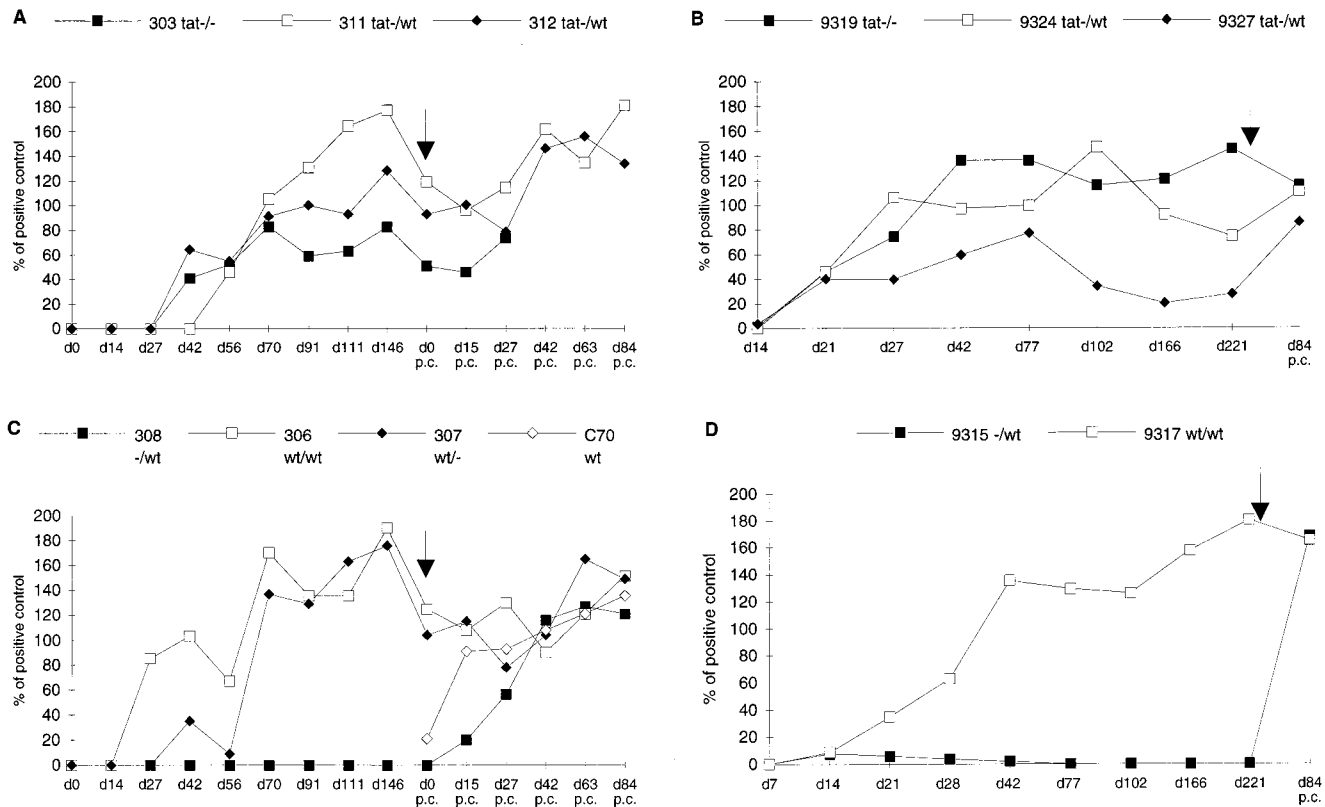


FIG. 1. Antibody response. Anti-CAEV antibodies to whole-virus proteins were measured by ELISA on different days (d) postinfection or p.c. Time of challenge is indicated by the arrows. (A) Goats inoculated with CAEV *tat*<sup>-</sup> proviral DNA and mock-challenged (303 *tat*<sup>-/-</sup>) or wt-challenged goats (311, 312 *tat*<sup>-/wt</sup>). (B) Goats inoculated with CAEV *tat*<sup>-</sup> and mock-challenged (9319 *tat*<sup>-/-</sup>) or wt-challenged (9324, 9327 *tat*<sup>-/wt</sup>) goats. (C) Mock-inoculated and wt-challenged goat (308 <sup>-/wt</sup>) or naive and wt-challenged (C70 wt) goat and CAEV wt proviral DNA-positive control animals mock challenged (307 wt<sup>-</sup>) or wt challenged (306 wt/wt). (D) Mock-inoculated challenged control goat (9315 <sup>-/wt</sup>) and positive control goat challenged with CAEV wt (9317 wt/wt).

DNA, suggesting that the inoculation dose did not affect the level of virus replication. Virus isolation remained negative for goats 303, 9327, and 9324, although they developed low to high antibody responses, respectively (Fig. 1A and B). These results probably reflect variation between animals in the ability to control viral replication, together with the difficulty of isolating virus from a small percentage of infected blood monocytes (9). No significant difference was observed in the frequency of isolation of viruses from animals injected with CAEV wt or *tat*<sup>-</sup> proviral DNA or virus-injected animals. RT-PCR analysis was performed on day 91 p.i. with RNA extracted from macrophage-produced viruses (Table 2, goats 306, 307, 303, 311, 312, and 308) and showed that the deletion introduced into the *tat* gene was still detectable in *tat*<sup>-</sup> virus particles (14), ruling out the possibility that a recombination event caused reversal to the wt phenotype, which would explain the lack of difference between goats injected with CAEV wt and those injected with *tat*<sup>-</sup>.

**Antibody response analysis.** In the CAEV infection model, a correlation was previously described between the severity of disease in infected animals and the titers of anti-Env antibodies (23), especially the anti-TM antibodies (27). Four immunodominant epitopes were delineated in the CAEV gp38 TM, and antibody reactivity against two of them, TM3 and TM4, was shown to be associated with the presence of clinical arthritis (1). To compare the ability of CAEV *tat*<sup>-</sup> proviral DNA or virus infection to induce such antibody specificities with that of CAEV wt infection, we tested the goat sera by ELISA with peptides TM3 (amino acids 717 to 731) or TM4 (amino acids

749 to 762) as antigens. In parallel, an ELISA specific for the recombinant Gag fusion protein glutathione *S*-transferase-Gag was used to detect anti-Gag antibodies. Sera were tested at day 146 p.i. for the group injected with CAEV wt or *tat*<sup>-</sup> proviral DNA and at day 221 p.i. for the group injected with CAEV wt or *tat*<sup>-</sup> virus. We chose to analyze late sera, since it was reported that the kinetics of anti-TM antibody appearance were around 12 to 32 weeks p.i. compared to 3 to 4 weeks p.i. for anti-Gag antibodies (1). Results are summarized in Table 3 (left-hand side). Sera were considered positive for values >25% of the ELISA for the positive controls. All positive control goats, 306, 307, and 9317, developed anti-Gag and anti-TM3 antibodies, whereas only goat 306 also produced anti-TM4 antibodies. In the groups injected with CAEV *tat*<sup>-</sup>, goat 311 (*tat*<sup>-</sup> proviral DNA) and goat 9319 (*tat*<sup>-</sup> virus) developed all three antibody specificities. Goat 312 (*tat*<sup>-</sup> proviral DNA) was weakly positive against Gag and TM4. Goat 303 (*tat*<sup>-</sup> proviral DNA) and goats 9324 and 9327 (*tat*<sup>-</sup> virus) were considered unreactive in all three ELISAs. Western blot analysis against whole-virus proteins, however, demonstrated that day 146 p.i. sera from goats 303 and 312 were weakly reactive against mature Gag proteins p28, p18, and p14.5 (14). This discrepancy between ELISA and Western blot results has already been observed with sera from naturally infected goats (1). Noticeably, three of six animals inoculated with CAEV *tat*<sup>-</sup> developed anti-Gag and anti-TM antibodies after inoculation (goats 311, 312, and 9319) compared to three of three goats injected with CAEV wt, which developed both reactivities.

TABLE 2. Virus isolation and RT-PCR detection in blood-derived macrophages

Day	RT activity/RT-PCR detection <sup>a</sup> in goat challenged as indicated											
	Expt 1					Expt 2						
	9317	9319	9324	9327	9315	306	307	303	311	312	308	C70
p.i. <sup>b</sup>	wt virus	<i>tat</i> - virus	<i>tat</i> - virus	<i>tat</i> - virus	Mock	wt DNA	wt DNA	<i>tat</i> - DNA	<i>tat</i> - DNA	<i>tat</i> - DNA	Mock	
0	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-	-	-
27	+	+	-	-	-	-	-	-	-	-	-	-
42	-	+	-	-	-	-	-	-	-	-	-	-
56						+			+	+		
70						-			+			
77	+	+	-	-	-							
91						+wt	-/-	-/-	+/ <i>tat</i> -	-/-	-/-	
102	-	-	-	-	-							
146												
p.c. <sup>c</sup>	wt	Mock	wt	wt	wt	wt	Mock	Mock	wt	wt	wt	wt
15	-/wt	-/-	+/ <i>tat</i> -	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/wt	-/-
27	-/-	-/-	-/-	-/-	+wt	-/-	-/-	-/- <sup>d</sup>	-/-	-/-	+wt	-/-
42	+wt	-/-	-/-	-/-	+wt	-/-	-/wt		-/-	-/-	-/wt	-/-
56	-/wt	-/-	-/-	-/-	-/-				-/-	-/-	-/-	-/-
63						-/-	-/-		-/-	-/-	-/-	-/-
84	-/-	-/-	-/-	-/-	-/-	-/-	-/wt		-/-	-/-	-/wt	+wt
118	-/wt	-/-	-/-	-/-	+wt				-/-	-/-	-/-	-/-
124						-/-	-/-		-/-	-/-	-/-	-/-
152						-/-	-/-		-/-	-/-	-/-	-/-

<sup>a</sup> Virus was isolated by RT activity measurement of 1 ml of macrophage culture supernatant at day 12, concentrated 100×/RT-PCR detection of RNA extracted from blood-derived macrophages cultured for 12 days with the VIF5/ENV1 primer pair. -, no fragment amplification; wt, detection of the 427-bp wt *tat* fragment; *tat*-, detection of the 274-bp *tat*-deleted fragment. Southern blots of the amplified fragments were hybridized with the <sup>32</sup>P-labeled TATH oligonucleotide probe.

<sup>b</sup> Right intracarpal inoculation of CAEV wt or *tat*- proviral DNA or virus.

<sup>c</sup> Left intracarpal challenge with pathogenic CAEV.

<sup>d</sup> Goat 303 died accidentally at day 30 p.c.

**Challenge of goats inoculated with CAEV *tat*-.** Challenge was performed with a high dose of homologous CAEV wt (>250 AID<sub>100</sub>) injected in the joint, which is one of the main targets of infection. Goats inoculated with CAEV *tat*- or wt virus were challenged on day 228 p.i., whereas goats injected with CAEV *tat*- or wt proviral DNA were challenged on day 279 p.i. A naive animal, goat C70, was included at that time as an additional positive control for infection (Table 1). Two of

three goats inoculated with CAEV wt, 306 and 9317, were challenged with the homologous CAEV wt, whereas goat 307 was mock challenged. Two goats inoculated with CAEV *tat*-, 303 (*tat*- proviral DNA) and 9319 (*tat*- virus), were mock challenged. Most additional results were obtained with goat 9319, since goat 303 died accidentally at day 30 p.c. The four other animals inoculated with CAEV *tat*-, 311 and 312 (*tat*- proviral DNA) and 9324 and 9327 (*tat*- virus), were chal-

TABLE 3. Antibody specificities<sup>a</sup> after inoculation with CAEV *tat*- or wt and pathogenic challenge

Goat no.	Type of inoculation	% of ELISA for positive control p.i.			Type of challenge	% of ELISA for positive control p.c.			Severity of lesions <sup>b</sup>	Protection
		Anti-Gag	Anti-TM3	Anti-TM4		Anti-Gag	Anti-TM3	Anti-TM4		
308	Mock	0	0	0	wt	27	530	254	+++	-
9315	Mock	0	0	0	wt	63	86	0	+++	-
306	wt DNA	66	418	39	wt	108	524	254	+++	-
307	wt DNA	78	67	0	Mock	37	446	4	+++	-
9317	wt virus	82	28	0	wt	88	20	0	+++	-
303	<i>tat</i> - DNA	11	13	2	Mock	ND	ND	ND	ND	
311	<i>tat</i> - DNA	91	42	43	wt	123	336	80	++	+
312	<i>tat</i> - DNA	25	18	26	wt	54	493	13	++	+
9319	<i>tat</i> - virus	31	398	70	Mock	19	314	47	+	
9324	<i>tat</i> - virus	21	24	0	wt	39	119	0	++	+
9327	<i>tat</i> - virus	6	0	0	wt	25	0	0	++	+

<sup>a</sup> Antibody reactivities were measured by ELISAs specific for a glutathione *S*-transferase-Gag fusion protein, the TM3 peptide (amino acids 717 to 731), and the TM4 peptide (amino acids 749 to 762). Sera were diluted 1:100, 1:60, and 1:20, respectively, and values higher than 25% were considered positive. Samples analyzed were taken at day 146 p.i. and day 152 p.c. for goats 308, 306, 307, 311, and 312 and at day 221 p.i. and day 84 p.c. for goats 9315, 9317, 9319, 9324, and 9327.

<sup>b</sup> Histopathological lesions observed in sections of synovial membranes were graded low (+), mild (++), or severe (+++). ND, not determined.

TABLE 4. Virus detection by RT-PCR analysis on necropsy tissues

Expt and animal no.	Priming	Type of challenge	Necropsy tissue <sup>a</sup>							BM
			SM		MS	RMLN		PSLN		
			Left	Right		Left	Right	Left	Right	
Expt 1										
9317	wt virus	wt	+	+	+	NT	+	NT	+	NT
9319	<i>tat</i> <sup>-</sup> virus	Mock	+	+	+	+	+	-	-	+
9324	<i>tat</i> <sup>-</sup> virus	wt	+	-	-	-	-	+	-	+
9327	<i>tat</i> <sup>-</sup> virus	wt	+	+	-	-	+	-	+	+
9315	Mock	wt	+	+	+	+	NT	+	NT	+
Expt 2										
306	wt DNA	wt	+	+	NT	-	+	-	+	-
307	wt DNA	Mock	+	-	+	-	-	-	-	-
311	<i>tat</i> <sup>-</sup> DNA	wt	-	+	+	-	-	-	-	-
312	<i>tat</i> <sup>-</sup> DNA	wt	+	+	+	-	-	-	-	-
308	Mock	wt	+	-	NT	-	-	-	-	-
C70 <sup>b</sup>		wt	+	+	+	NT	NT	NT	NT	+

<sup>a</sup> RT-PCR analysis on RNA directly extracted from tissues taken at sacrifice was performed with the POLS/POLA primer pair. Southern blots of the amplified fragments were hybridized with the <sup>32</sup>P-labeled POLH oligonucleotide probe. +, detection of the 299-bp *pol* fragment; -, no detectable amplified products; NT, not tested. Tissue samples analyzed were from left or right synovial membranes (SM), mammary secretions (MS), left or right retromammary lymph node (RMLN), left or right prescapular lymph node (PSLN), and bone marrow (BM).

<sup>b</sup> Goat C70 received no priming and was included at the time of challenge.

lenged with CAEV wt. All control animals became infected as shown by the seroconversion curves (goats 308 and C70, Fig. 1C and goat 9315, Fig. 1D). The antibody response level for goat 9319 remained high (Fig. 1B), suggesting continuous stimulation by viral antigens. An increase in antibody response was observed in the four wt-challenged animals inoculated with CAEV *tat*<sup>-</sup>, goats 311 and 312 (Fig. 1A) and goats 9324 and 9327 (Fig. 1B), as measured on day 84 p.c. Isolation of virus was possible from blood-derived macrophages of positive control goats (Table 2). Of the samples from the two animals challenged and inoculated with CAEV wt, only those from goat 9317 allowed positive virus isolation by RT activity measurement and RT-PCR on RNA from blood-derived macrophages (Table 2). Positive control mock-challenged goat 307 developed a persistent low-level infection as revealed by the late occasional RT-PCR virus detection on blood-derived macrophages (Table 2). Virus isolation was negative for all animals immunized with CAEV *tat*<sup>-</sup>, regardless of whether they were mock challenged or challenged with CAEV wt (Table 2). In only one case (goat 9324 on day 15 p.c.) was the immunizing *tat*<sup>-</sup> virus detected in blood-derived macrophages by RT-PCR with the VIF5/TAT primer pair (Table 2). This result suggests a transient reactivation of the CAEV *tat*<sup>-</sup> by the wt challenge virus. These observations indicate that prior immunization with the attenuated CAEV *tat*<sup>-</sup> induced protection or superinfection resistance against homologous pathogenic challenge at the peripheral blood level.

Protein-specific ELISAs revealed that the two positive control animals tested, 308 and 9315, developed both anti-Gag and anti-TM3 antibodies, but only goat 308 produced anti-TM4 antibodies (Table 3, right-hand side). An increase of anti-Gag, anti-TM3, and anti-TM4 antibodies was observed in sera from goat 306, whereas goat 9317 demonstrated no change in antibody specificity. The late development of anti-TM3 antibodies was detected in goat 307 (Table 3, right-hand side). Whereas a slight decrease was observed in the anti-Gag, anti-TM3, and anti-TM4 reactivities of goat 9319, all these antibody specificities were strengthened in the four wt-challenged animals that were inoculated with CAEV *tat*<sup>-</sup>, without the appearance of new reactivities (Table 3, right-hand side), suggesting that the

humoral response was stimulated by the wt challenge virus. RT-PCR analyses performed on RNA extracted from different tissues of positive control goats allowed the detection of challenge virus (Table 4). Virus was also detected in the synovial membranes and lymph nodes of goat 9317 as well as in the lymphoid tissues of goat 306 (Table 4). The infected status of goat 307 was confirmed by RT-PCR analyses on synovial membranes, mammary secretions, and lymphoid tissues (Table 4). Most of the necropsied tissues of goat 9319 were RT-PCR positive with the POLS/POLA primer pair (Table 4), suggesting a continuous replication of CAEV *tat*<sup>-</sup> in target tissues. RT-PCR analyses with the POLS/POLA primer pair on different tissues of wt-challenged goats inoculated with CAEV *tat*<sup>-</sup> gave positive reactions in the synovial membranes and mammary secretions of goats 311 and 312 and in the lymph nodes and bone marrow of goats 9324 and 9327 (Table 4). Since this reaction did not allow us to distinguish between the *tat*<sup>-</sup> immunizing virus and the wt challenge virus, further RT-PCR analyses were performed with the VIF5/TAT primer pair to amplify the *tat* gene.

**Viral RNA detection in necropsied tissues and histopathology.** As CAEV wt challenge virus was not detected at the peripheral level, we next examined whether sequestration of the challenge virus occurred in protected wt-challenged goats immunized with *tat*<sup>-</sup>. Tissue samples were taken at necropsy and analyzed by RT-PCR for the presence of deleted or wt *tat* viral RNA. Figure 2 shows results obtained from cells from mammary secretions of female goats. wt *tat* RNA could be amplified from negative control challenged goats 9315 and C70 as well as from positive control mock-challenged goat 307 or wt-challenged goat 9317. Only a deleted *tat* product was amplified from mock-challenged goat 9319 or wt-challenged animals 311 and 312 which had been inoculated with CAEV *tat*<sup>-</sup>. An RT-PCR specific for *tat* was negative for samples from wt-challenged goats 9324 and 9327, which had been injected with CAEV *tat*<sup>-</sup>. No wt challenge virus was detected in fluids secreted by animals immunized with *tat*<sup>-</sup>, confirming the results obtained with blood-derived macrophages.

Figure 3 shows results obtained with RNA extracted from synovial membranes surrounding the inoculation sites. A wt *tat*

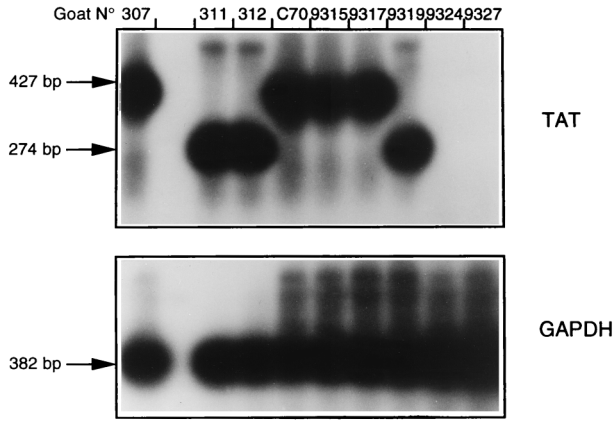


FIG. 2. Challenge virus detection by RT-PCR on RNA extracted from cells in mammary secretions. Amplification was performed with the VIF5/ENV1 primer pair, and a Southern blot of the amplified products was hybridized with the <sup>32</sup>P-labeled TATH oligonucleotide probe. The CGAP1/CGAP2 primer pair was used to amplify the caprine housekeeping gene GAPDH as an internal control. Molecular size markers are indicated at the left.

amplification product could be observed in all negative control challenged animals, in both the right and the left joints of goats 9315 and C70 but in goat 308 in only the joint inoculated with CAEV wt. Among positive control animals, mock-challenged goat 307 was negative for the *tat*-specific RT-PCR in both joints, whereas the *pol*-specific primer pair allowed viral RNA detection in the left joint (Table 4). For the two other positive control challenged animals, 306 and 9317, amplification of wt *tat* was positive in synovial membranes from both joints. *tat*-deleted amplification products could be observed in three of four wt-challenged goats inoculated with CAEV *tat*-, in goats 311 and 312 in the joint injected with CAEV *tat*-, and in both joints of goat 9327, although in this last case the *tat*- amplified product exhibited a slightly different size. Both wt *tat* and *tat*- amplification products were detected in the wt-injected joint of goat 312 and in both synovial membranes of goat 9327. Samples from goat 9324 were negative for *tat*-specific RT-PCR analysis, whereas parallel amplification performed with *pol*-specific primers allowed viral RNA detection (Table 4). This discrepancy, also observed for goat 307, could be due to the lower efficiency of the VIF5/TAT primer pair compared to that of POLA/POLS and/or to a lower viral load in these infected animals. We were able to detect the wt challenge virus at the inoculation site in two of four wt-challenged animals that had been immunized with CAEV *tat*-; this suggests that control of the wt virus replication occurred in these animals without complete sterilizing immunity.

The pathogenic properties of the *tat*- attenuated virus were then analyzed. A portion of the necropsied synovial membranes of the challenged animals was frozen, and tissue sections were examined for the presence of inflammatory lesions. Different tissue sections from each animal were examined independently by two or three examiners, and representative pictures for each group are shown in Fig. 4. Compared to the synovial membrane section of a naive animal (Fig. 4A), a tissue section from the wt-challenged goat inoculated with CAEV wt (goat 9317) (Fig. 4B) showed the characteristics of severe virus-induced inflammatory lesions with the high lymphocytic and monocytic infiltration that we and others have already described with similar experimental infections (14, 38). CAEV *tat*- injection resulted in lesions that were less severe than those resulting from the wt virus, as observed on a tissue sec-

tion from the mock-challenged goat injected with CAEV *tat*- virus (goat 9319) (Fig. 4C). Thickening of the synovial membrane as well as lymphocytic infiltration of the connective tissue was observed. Mild lesions were also observed in tissue sections of wt-challenged goat 9327, which was injected with CAEV *tat*- (Fig. 4D); these lesions were less severe than those in the positive control goat (Fig. 4B), indicating that wt-challenged animals immunized with CAEV *tat*- resisted the pathogenicity of the challenge virus, since lesions observed in these animals were most probably due to replication of the immunizing virus itself. Most of the animals developed either only anti-TM3 antibodies (9315, 307, 312, and 9324) or only anti-TM3 and anti-TM4 antibodies (308, 306, 311, and 9319) and lesions that ranged from low to severe (Table 3). Goats 9317 and 9327 developed severe and mild lesions, respectively, in the absence of either of these antibody reactivities (Table 3). In agreement with results previously obtained with a larger panel of sera (1), most sera in this study (80%) reacted with TM3 peptide and Gag and correlated with an arthritic condition.

DISCUSSION

The infectivity of retroviral DNA has been proved in several models, including SIV, bovine leukemia virus, CAEV, and feline immunodeficiency virus (14, 15, 24, 35, 41). This method prevents variation in infection efficacy due to the presence of various proportions of defective viruses in individual viral stocks and allows the evaluation of the infectious and pathogenic properties of genetically modified retroviral genomes. In this study, we extended our previous results (14) and demonstrated the reliability and efficiency of this infection procedure compared to those of infection with viral stocks obtained in vitro by transfection of the same infectious CAEV molecular clone, either wt or *tat*-. Overall, time to seroconversion and virus isolation were not significantly different between animals inoculated with CAEV *tat*- or wt proviral DNA and goats injected with CAEV *tat*- or wt virus. A more detailed analysis of the antibody response, however, revealed that infection with CAEV *tat*- was less efficient than injection with CAEV wt in inducing a high-level antibody response and stimulating the production of anti-Gag and anti-TM reactivities. The differences detected in the antibody response in animals injected with CAEV *tat*- compared to that in goats inoculated with CAEV wt could be due to an attenuated replication of CAEV *tat*- in vivo. We observed that goats 303 and 9327, with the

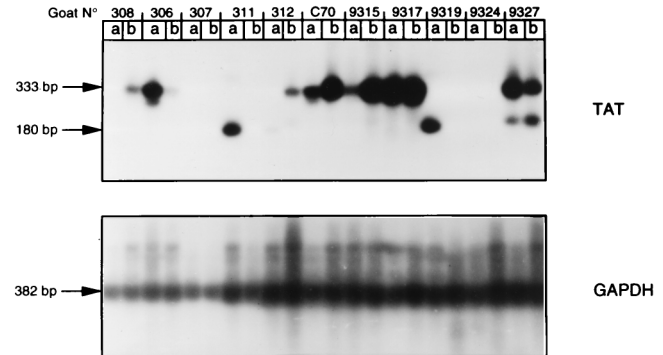


FIG. 3. Challenge virus detection by RT-PCR on RNA extracted from right (a) or left (b) synovial membranes. Amplification was performed by seminested PCR, first with the VIF5/ENV1 primer pair and then with primers VIF5/TAT, resulting in the production of a 333-bp fragment for the wt *tat* gene and a 180-bp fragment for the *tat*- gene. Amplification of the GAPDH control gene and Southern blot hybridization were as described in the legend for Fig. 3.

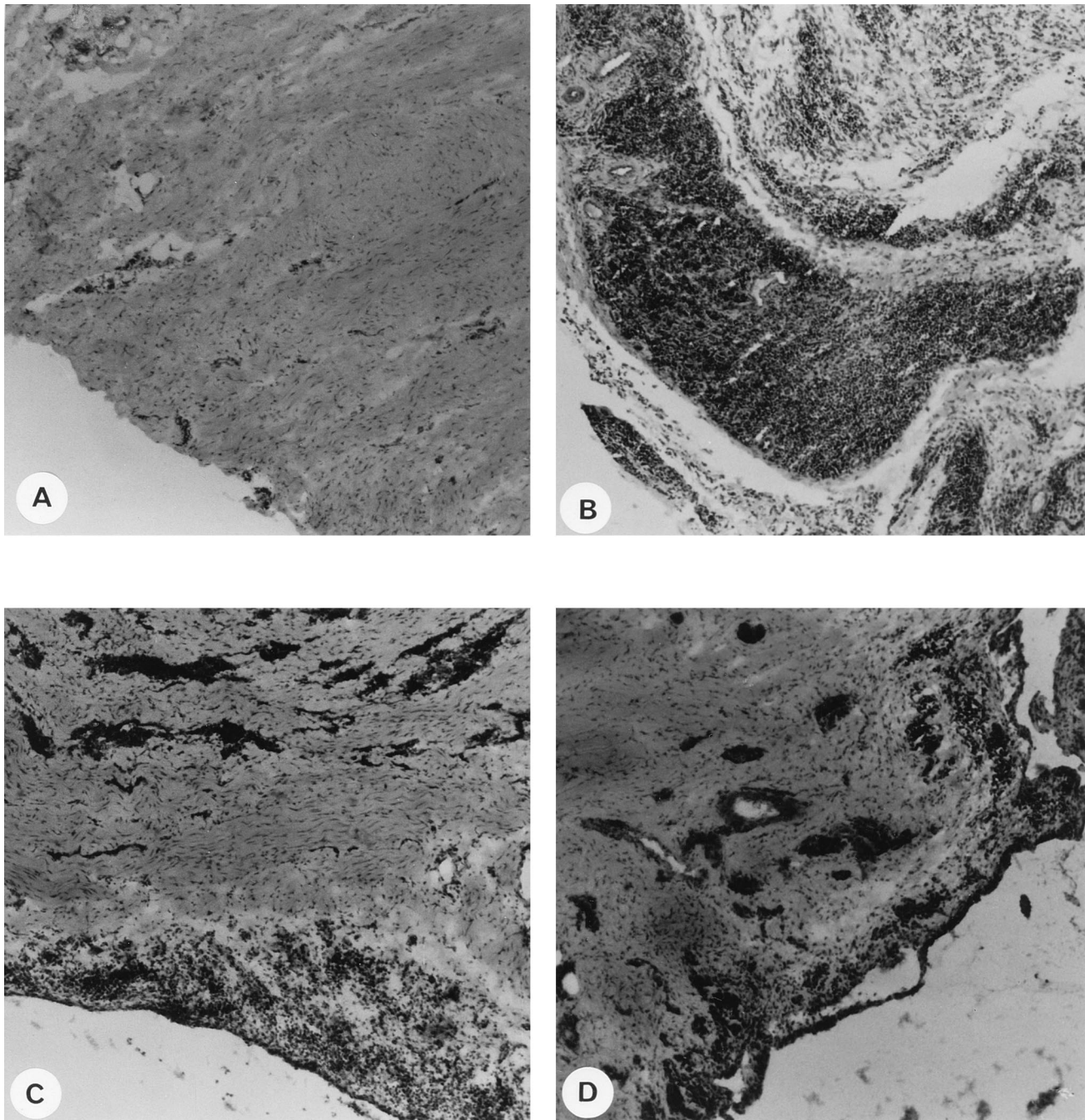


FIG. 4. Histopathology of synovial membranes from a naive goat (A), from positive control goat 9317, which was injected with CAEV wt (B), from mock-challenged goat 9319, which was inoculated with CAEV *tat*<sup>-</sup> (C), and from wt-challenged goat 9327, which was inoculated with CAEV *tat*<sup>-</sup> (D). Synovial membrane samples were taken at necropsy, and frozen sections were stained with hematoxylin and eosin. Magnification,  $\times 10$ .

lowest antibody response, had no detectable anti-Gag or anti-TM antibodies and were negative for virus isolation. Goats 312 and 9324, with a high antibody response, reacted weakly against Gag and TM epitopes before challenge and remained negative for virus isolation. Finally, goats 311 and 9319, with the highest antibody level, reacted strongly against the Gag and TM epitopes, and virus could be isolated from blood-derived macrophage cultures. Taking into account the variation between the animals' ability to control infection, these differences were not observed in the three positive con-

trol goats. In the CAEV infection model, there seems to be a correlation between the intensity of the immune response and the level of virus replication (15, 16); this correlation was also observed in the case of SIVmac32H C8 infection (6) but not in the SIVmac239  $\Delta nef$  (7, 22) or equine infectious anemia virus  $\Delta DU$  models (25).

The results demonstrate that infection with attenuated CAEV *tat*<sup>-</sup> via intracarpal inoculation can protect goats from homologous pathogenic challenge in the contralateral joint. Protection was defined as the absence of challenge virus de-

tection in peripheral blood-derived macrophages during the postchallenge period—185 days for the group injected with CAEV *tat*<sup>-</sup> proviral DNA and 310 days for the group inoculated with CAEV *tat*<sup>-</sup>. No anamnestic antibody response was detected; however, an increase in anti-CAEV response was observed in goats 311 and 312, suggesting limited exposure to CAEV antigens. As in some vaccination studies with attenuated SIV (6, 28, 31), we found no correlation between the level of antibody response and protection. Protection was achieved without complete sterilizing immunity, since RT-PCR analyses of different tissues obtained at necropsy allowed the detection of the challenge wt virus in the inoculated joints of two of four protected animals. In one of these two goats, 9327, CAEV wt RNA was present in both carpal joints, showing that the challenge virus may have diffused through the body before it was brought under control. The local immune response, either antibody or cell-mediated, may be responsible for this control mechanism, since the synovial fluid and synovium of infected goats are rich in plasmacytes, activated CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes, and macrophages (12, 21, 40).

In a previous study, we reported that immunization with the highly attenuated CAEV *vif*<sup>-</sup> failed to protect goats against homologous pathogenic challenge (15)—even when the goats were challenged after a long period of immunization (about 8 months p.i.)—whereas the slightly attenuated CAEV *tat*<sup>-</sup> immunization was protective. The fact that these two experimental groups were inoculated and challenged with the same protocol allows us to determine the impact of the degree of attenuation of the vaccine viruses on their efficiency to induce protection. In another lentiviral model, these results confirm the inverse correlation established with live attenuated SIVs between the level of vaccine strain replication and the induction of protection (26, 42). In addition to the replication efficiency of the vaccine strain, an important parameter of efficient protection is duration of the vaccination. In the macaque model, protection was obtained with live attenuated SIVs, and results demonstrate a clear trend toward increased protection with the time of vaccination (4, 7, 17, 31, 42). Most effective vaccination assays against lentiviral infection have failed to clearly demonstrate whether protection was immunity mediated or based on superinfection resistance due to interference. Further analyses are necessary to evaluate the maturation of the immune response, described in the SIV and equine infectious anemia virus models (4, 5, 11), as well as to investigate the induction of a cellular immune response to CAEV *tat*<sup>-</sup> immunization.

Examination of synovial membrane sections from a mock-challenged goat inoculated with CAEV *tat*<sup>-</sup> revealed mild histopathological changes compared to the severe inflammatory lesions observed in the joints of goats infected with CAEV wt. Since the CAEV *tat* gene is not strictly required to establish persistent infection and the onset of clinical signs, the function of Tat is still unknown. Several studies reported the correlation between the level of anti-Env (anti-SU and anti-TM) antibodies and the development of arthritic lesions in the CAEV infection model (our results and references 1, 23, and 27), together with the high level of virus expression in tissue macrophages (45) and the massive infiltration of the arthritic synovium by B lymphocytes, plasmacytes, and activated CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes (2, 12, 21, 40). Recent reports suggested the role of differential activation of CAEV-reactive T-helper subsets in virus expression control and disease outcome and associated the dominance of T-helper 2-like cells with arthritis (3, 33, 40). Our results suggest a role for CAEV Tat in the increase of the viral replication level, independently of its weak transactivation of the viral long terminal repeat (14,

18). As Tat of visna virus was reported to regulate the expression of cellular genes involved in activation pathways (30), one hypothesis is that Tat of small ruminant lentiviruses activates the infected macrophages, resulting in increased viral expression and thereby augmenting the reactivities of antibodies and activated lymphocytes to viral antigens and infected cells in the synovial tissue (2, 12, 21, 40). Nontranscriptional function of HIV-1 Tat in virion infectivity and immune activation of HIV-1-infected cells by Tat were recently described (19, 32) and may be a general feature of lentiviral Tat proteins.

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