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Immune Responses of Goats Persistently Infected with Caprine Arthritis-Encephalitis Virus†

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Eight cesarean-derived goat kids were inoculated with caprine arthritis-encephalitis virus (CAEV), and proliferative responses of their peripheral blood mononuclear cells to mitogens and CAEV antigen were monitored for 9 months. Antibody specific for CAEV was measured by an enzyme-linked immunosorbent assay. Five cesarean-derived noninfected goats were tested simultaneously. Significant differences between the infected and control mononuclear cell proliferation reactions to CAEV began 14 days post-inoculation and continued in a fluctuant manner until 134 days post-inoculation. The magnitude of the proliferative reaction steadily increased in infected goats until the end of the experiment at 271 days post-inoculation. Responses to mitogens were not significantly different between infected and control goats. Virus-inoculated goats produced CAEV-specific antibody that reached a maximum level between 49 and 77 days post-inoculation and then declined to lower levels through 271 days post-inoculation. The virus-inoculated goats developed mild but characteristic clinical evidence of caprine arthritis-encephalitis, and CAEV was reisolated from four goats at 286 days post-inoculation. The five control goats developed neither an anti-CAEV immune response nor clinical disease, and CAEV could not be reisolated from them.

Caprine arthritis-encephalitis (CAE) is a disease of domestic goats caused by a retrovirus. It is most often manifested as chronic connective tissue disease involving the joints of adult goats (5a) and leukoencephalomyelitis of young goats (5). The relationship of the immune response to disease expression is unknown. The purpose of this study was to examine the competency of the cellular immune system by its ability to respond to mitogens and to assess the magnitude of the humoral and cellular anti-CAE virus (CAEV) responses during infection. Clinical criteria, including physical examination, radiology, and synovial fluid evaluations, were used to compare experimental CAE with the natural disease. Virus isolations were performed to confirm persistence of the infection in the experimental goats.

MATERIALS AND METHODS

Animals. Fourteen cesarean-derived CAEV-free goat kids were maintained separated from their mothers and deprived of colostrum. The details of the virus-free derivation and husbandry have been described previously (D. S. Adams, Ph.D. thesis, Washington State University, Pullman, 1979). Briefly, 12 of the kids received CAEV-free gamma globulin preparations that contained a small amount of anti-CAEV antibody, because gamma globulin without anti-CAEV activity was in short supply; the remaining kids (one infected and one control) received gamma globulin that had no anti-CAEV antibody. Kids that were infected with CAEV were housed separately from control kids. All experimental animals were between 4 and 28 days old at the beginning of the experiment.

Eight kids were inoculated intravenously with 1.0 ml of virus-infected medium, and 0.5 ml of the same medium was injected into both the left radiocarpal and the right tibiotarsal joints. Two kids received equal volumes of medium from uninfected cultures by the same routes. Three other kids served as uninoculated controls.

Virus inoculum. The prototype virus (75-63) used for inoculation was originally isolated from a goat with spontaneous arthritis (5a). The inoculum was medium from synovial cell cultures infected with passage 11 of the virus. The virus pool had been frozen in samples at −70°C and had an infectivity titer of $10^{6.2}$ 50% tissue culture infectious doses per ml.

Lymphocyte transformation. Venous blood was collected in an equal volume of Seligmann balanced salt solution, with 1 mg of ethylenediaminetetraacetic acid per ml added as an anticoagulant. This solution was layered over Ficol-Hypaque (specific gravity, 1.08) and centrifuged at 1,200 × *g* for 25 min at room temperature. Cells at the interface of separation medium and plasma were collected and washed at room temperature once in the plasma and twice in cold Dulbecco-modified minimum essential medium with

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2% fetal calf serum at 4°C. The cells were suspended in cold RPMI 1640 medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal calf serum, 2 mM glutamine, 20 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 100 μg of kanamycin per ml (Bristol Laboratories, Syracuse, N.Y.). The final cell concentration was adjusted to 2×10^6 cells per ml. Of these peripheral blood mononuclear cells, more than 90% were lymphocytes, 2 to 5% were monocytes, and the remainder were polymorphonuclear cells.

Cells were cultured in round-bottom microtiter plates (Cooke Engineering Co., Alexandria, Va.) with 0.2 ml of medium per well (4×10^5 cells per well). Cultures were stimulated with phytohemagglutinin (PHA; PHA-P, Difco Laboratories, Detroit, Mich.), lipopolysaccharide B (LPS; *Escherichia coli* O55:B5, Difco), or CAEV (75-63) antigen (described below). The concentrations of mitogens in the wells were 25 μg of PHA per ml and 150 μg of LPS per ml. The optimum CAEV antigen dilution of 1:25 in a volume of 0.02 ml was selected from preliminary experiments for use in the present experiments. All experiments, including background controls, were performed in quadruplicate. Peripheral blood mononuclear cells from a goat with chronic CAE were included with each plate as a positive control. Cultures were incubated at 37°C in 5 to 10% CO_2 for 72 h with mitogens and for 144 h with viral antigen. At 24 h before harvest, 1 μCi of [^{3}H]thymidine (34 Ci/mmol) was added to each well. The cells were harvested onto glass filters with a multiple-well cell harvester (Brandel, Rockville, Md.) and washed with phosphate-buffered saline. The dried filter disks were placed in scintillation vials with 8 ml of scintillation cocktail {12 mg of 2,4-bis-[2-(5-phenyloxazoyl)]benzene and 6 g of 2,5-diphenyloxazole per liter of toluene} and counted in a liquid scintillation counter (model LS 9000, Beckman Instruments, Inc., Fullerton, Calif.). Stimulation indices were calculated by dividing the mean counts per minute of wells with PHA, LPS, or viral antigens by the mean counts per minute of the appropriate control wells.

The CAEV antigen preparation was from virus that had been purified and concentrated approximately 1,000-fold (3; W. P. Cheevers, S. Roberson, P. Klevjer-Anderson, and T. B. Crawford, submitted for publication) and degraded by ether extraction (11). Cell culture medium containing approximately 10^6 tissue culture infectious doses of 75-63 virus per ml was centrifuged at 26,000 rpm for 90 min at 2°C, and the pellet was suspended in 0.01 M NaH_2PO_4–0.001 M ethylenediaminetetraacetic acid–0.01 M NaCl (PNE). This suspension was layered over an equal volume of 20% (wt/wt) sucrose in PNE, and the cell debris was sedimented from the sample at 20,000 rpm for 30 min at 2°C in an SW27 rotor. The supernatant was relayered over 20% sucrose, and the virus was pelleted at 26,000 rpm for 90 to 120 min at 2°C. The pellet was suspended in PNE and centrifuged in a 20 to 45% (wt/wt) sucrose gradient with either an SW27 (26,000 rpm, 16 h, 2°C) or an SW41 rotor (35,000 rpm, 16 h, 2°C). Fractions with buoyant densities of 1.14 and 1.16 g/cm^3 were collected, and the virus was sedimented from the sucrose. It was then suspended in 0.01 M tris(hydroxymethyl)aminomethane (pH 7.4)–0.1 M NaCl–0.001 M ethylenediaminetetraacetic acid (TNE) and stored at −70°C. This virus preparation was thawed, and an equal volume of ether was added. The solution was shaken vigorously at 2-min intervals for 10 min. The excess ether was removed with a Pasteur pipette, and the rest was evaporated by vacuum. For lymphocyte transformation, this solution was pooled, diluted with lymphocyte culture medium, aliquoted, and frozen at −70°C. The same pool was used for all experiments. Cell culture medium from uninfected synovial membrane cultures of identical origin and passage level was subjected to the same regimen of purification, concentration, ether degradation, dilution, and storage for use as a cell culture medium control.

Blood samples from virus-inoculated kids were collected for lymphocyte transformation 3 days before inoculation and at 7, 14, 21, 28, 35, 42, 49, 63, 77, 105, 134, 169, 203, 238, and 271 days post-inoculation (PI). Samples were collected from the five control kids within 2 days before collection from the virus-infected goats.

Enzyme-linked immunosorbent assay. The enzyme-linked immunosorbent assay (ELISA) was first used to measure specific antibody by Engvall and Perlmann (7) and later modified by Bullock and Walls (1). Rabbit anti-goat gamma globulin was conjugated with horseradish peroxidase (Sigma Chemical Co., St. Louis, Mo.) by the method of Nakane and Kawaoi (12). Antigen for the test was CAEV purified, concentrated, ether degraded, pooled, and stored at −70°C as described above for the lymphocyte transformation assays. Optimum dilutions for conjugate and antigen were chosen by checkerboard titrations.

A total of 60 wells, excluding the 36 outside wells of flat-bottom 96-well microtiter plates (Cooke Engineering Co.) were coated with CAEV antigen diluted in 0.1 M carbonate buffer, pH 9.6, for 3 h at 37°C. All plates were coated from a single pool of virus to ensure uniformity. After being washed three times for 3 min each with phosphate-buffered saline–0.05% Tween 20, the plates were either stored at 4°C or used immediately. Standard positive and negative control sera were run in each plate to assess the reproducibility of the assay. Sera were diluted twofold, starting at 1:4. First the sera and then the conjugate were incubated in the plates for 30 min at 37°C with three phosphate-buffered saline–Tween 20 washes after each. The enzyme substrate 5-aminosalicylic acid was added, the mixture was incubated for 1 h at room temperature, and the reaction was stopped with 1 M NaOH. After 10 min, the optical densities were determined spectrophotometrically at 450 nm. The serum titer was expressed as the reciprocal of the dilution nearest an optical density of 0.20. Sera yielding optical densities of less than 0.20 at 1:4 were considered negative.

Sera from the infected kids were collected on the same days as were the lymphocytes. Sera collected at 0, 75, and 203 days PI from five cesarean-derived control goats were tested to establish the range of nonspecific background.

Immunodiffusion. Anti-CAEV antibody was measured by the agar gel immunodiffusion method (4) with CAEV which had been purified, concentrated, ether extracted, pooled, and stored at −70°C as de-

scribed above for lymphocyte transformation. Uninfected cell culture medium which had been purified, concentrated, and ether extracted in the same way as infected medium was tested in the antigen well against known positive serum as a control.

Clinical evaluations. Physical examinations for evidence of arthritis and other manifestations of connective tissue disease or encephalitis were performed on the same days when lymphocytes and sera were collected. Leukocyte counts of synovial fluids from inoculated joints of infected kids and the corresponding joints from uninfected kids were determined at 286 days PI. Anterior-posterior and lateral radiographic views of carpi from each goat were taken at 240 days PI.

Viral isolation. Fetal goat synovial membrane cell cultures in 25-cm^2 plastic flasks were inoculated with 10^6 purified peripheral blood mononuclear cells at 238 and 271 days PI or with 50 μl of synovial fluid from left (inoculated) radiocarpal joints at 286 days PI. The cultures were observed for typical cytopathic effect and passaged three times before being considered negative.

RESULTS

Lymphocyte stimulation. Mitogen responses of peripheral blood mononuclear cells were measured to determine whether infection by CAEV caused either suppression or potentiation of the immune response. PHA and LPS were chosen as stimulants because of their relative specificity in other species for T and B cells (10, 13). Except for occasional differences, mitogen responses were essentially the same in the eight infected and the five control kids during the 9-month period (Fig. 1 and 2).

However, there was a highly significant difference between infected and control goats in the blastogenic response of peripheral blood mononuclear cells to CAEV antigen (Fig. 3). Because the variation in the stimulation indices with the *F* test was significantly greater in infected than in uninfected goats, it was necessary to use confidence intervals rather than Student's *t* test to compare the means of the two groups. The infected kids showed a significantly greater stimulation index at 14 days PI at the 5% level of significance. The difference continued to be significant throughout the 9-month period, except at 28 days PI, when the confidence intervals overlapped slightly. At 134 days PI, the mean stimulation index of the infected goats began to rise steadily, reaching a maximum of 11.2 at day 271. No concomitant rise was noted in the proliferative reaction of cells from the chronically arthritic goat used as a positive control. At 271 days PI, this goat had a stimulation index of 3.6, with a mean stimulation index of 4.2 ± 2.1 for the 9-month period of testing. This suggests that the rise in response of the inoculated kids was not a laboratory artifact. Peripheral blood mononuclear cells from the uninoculated control goats produced a mean stimulation index of approximately 1.0. The purified, concentrated, and ether-extracted control cell culture medium caused no stimulation of infected or control lymphocyte cultures.

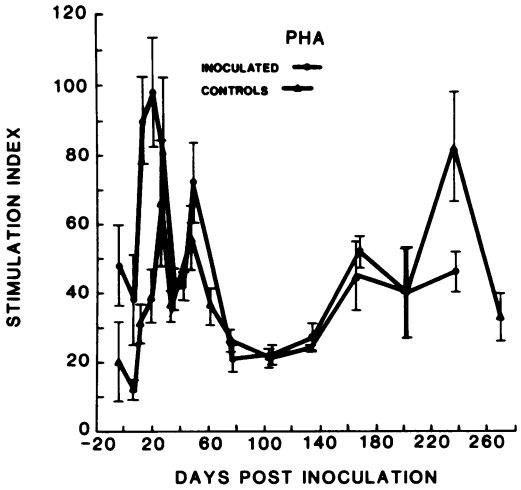

FIG. 1. *Mean PHA stimulation indices of peripheral blood mononuclear cells from eight CAEV-inoculated goats and five control goats from −3 to 271 days PI. Vertical bars represent ±1 standard error of the mean. No consistently significant differences were determined at the 5% level. Background counts are listed in Table 1.*

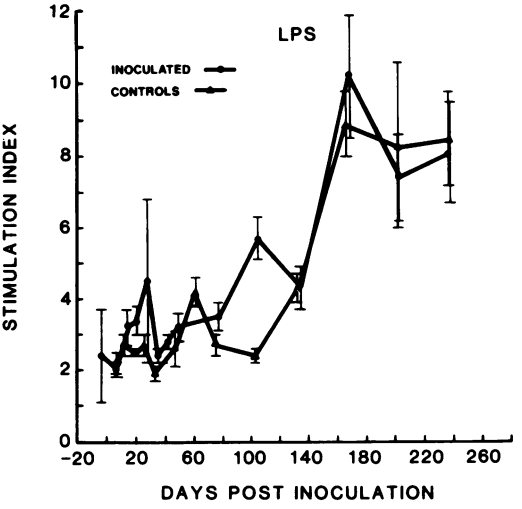

FIG. 2. *Mean LPS stimulation indices of peripheral blood mononuclear cells from eight CAEV-inoculated goats and five control goats from −3 to 271 days PI. Vertical bars represent ±1 standard error of the mean. No consistently significant differences were determined at the 5% level. Background counts are listed in Table 1.*

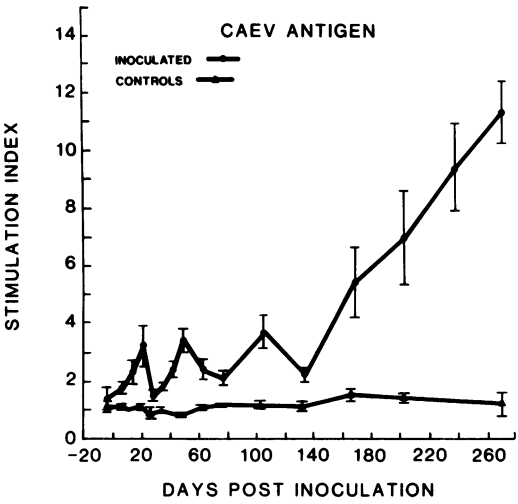

FIG. 3. *Mean CAEV antigen stimulation indices of peripheral blood mononuclear cells from eight CAEV-inoculated goats and five control goats from −3 to 271 days PI. Vertical bars represent ±1 standard error of the mean. With confidence intervals at the 5% level, significant differences between the two groups occurred at every day they were tested except −3, 7, and 28 days PI. Background counts are listed in Table 1.*

High background counts were obtained in unstimulated cultures, particularly in the CAEV antigen-driven experiments (Table 1). This finding may be explained by one or all of several possibilities: (i) the high specific activity of the [^{3}H]thymidine; (ii) the long length of time in culture; (iii) stimulation by something in the culture medium, for instance, fetal calf serum; or (iv) possible activation of the lymphocytes before culture for some unknown reason. Nevertheless, no significant difference (Table 1) was found in background counts between infected and uninfected goats, and it is clear in the case of the antigen-driven experiments that lymphocytes from infected goats responded to CAEV antigens to a much greater degree (Fig. 3).

Antibody response. The antibody response to CAEV antigens as measured by ELISA began with very low titers (1:4) in some goats probably because virus-specific antibody was present in the gamma globulin administered at birth. These titers declined to undetectable levels by 14 days PI. Antibody again became detectable in seven of eight infected kids at 21 days PI and in all eight by 35 days PI. The titers reached their maximum between 48 and 77 days PI, declined temporarily, and leveled off through day 271 PI (Fig. 4). The serum pool used as a

TABLE 1. *Mean background counts from lymphocyte cultures of five control and eight CAEV-infected goats*

Day PI[a]	cpm × 10^3 ± 1 standard error of the mean			
	PHA/LPS[b]		CAEV antigen[c]	
	Control	Infected	Control	Infected
−3	21.27 ± 12.51	7.48 ± 2.88	4.33 ± 2.55	5.62 ± 2.16
7	9.02 ± 3.76	6.27 ± 2.22	21.63 ± 9.01	12.02 ± 4.29
14	7.12 ± 3.23	1.60 ± 0.57	12.80 ± 5.33	2.44 ± 0.87
21	7.67 ± 3.20	3.65 ± 1.30	11.69 ± 4.87	9.20 ± 3.29
28	4.17 ± 1.74	14.34 ± 5.21	15.11 ± 6.29	69.78 ± 24.92
35	9.47 ± 3.95	8.39 ± 3.00	43.40 ± 18.08	47.54 ± 16.98
42	ND[d]	7.53 ± 2.69	ND	66.29 ± 23.67
49	6.91 ± 2.88	5.83 ± 2.08	18.53 ± 7.72	57.41 ± 20.50
63	9.79 ± 4.08	ND	11.36 ± 4.73	46.80 ± 17.43
77	16.23 ± 5.80	26.87 ± 9.60	63.21 ± 26.34	92.23 ± 32.94
105	18.18 ± 8.26	22.89 ± 8.17	66.26 ± 30.12	37.60 ± 13.43
134	15.51 ± 7.05	15.40 ± 5.50	76.66 ± 34.84	70.46 ± 25.17
169	3.71 ± 1.69	3.47 ± 1.24	9.02 ± 4.10	19.57 ± 6.99
203	4.21 ± 1.91	4.09 ± 1.46	19.44 ± 8.84	19.71 ± 7.04
238	2.47 ± 1.12	2.95 ± 1.05	13.10 ± 5.96	10.44 ± 3.73
271	5.56 ± 2.53	1.05 ± 0.38	6.50 ± 2.96	4.22 ± 1.51

[a] Days PI when infected goats were tested. Control goats were tested within 2 days before.

[b] LPS and PHA were both cultured with lymphocytes for 3 days and therefore had the same background wells. The mean ± 1 standard error of the mean was 9.42 ± 2.43 for control goats and 8.79 ± 2.27 for infected goats. No significant difference was indicated by Student's *t* test.

[c] Antigen cultures were 6 days. The mean ± 1 standard error of the mean background counts was 26.20 ± 6.78 for control goats and 35.71 ± 8.93 for infected goats. No significant difference was indicated by Student's *t* test.

[d] ND, Not determined.

positive antibody control on each plate consistently had a titer of 128 or 256. The mean optical density at 450 nm yielded by sera from the five cesarean-derived control kids after 14 days PI was less than 0.05; an optical density value of 0.20 was considered to be the minimum threshold for a reaction to be judged positive.

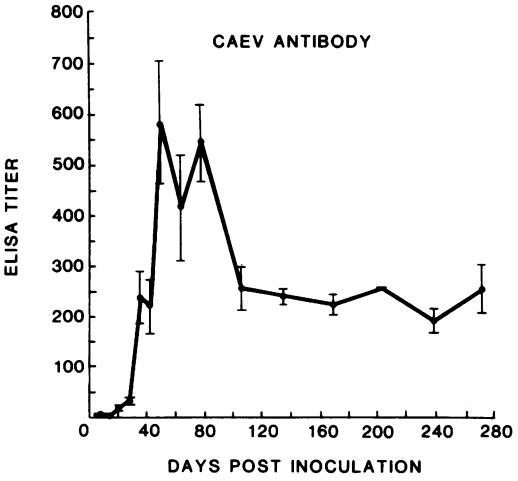

FIG. 4. *ELISA antibody titers from goats inoculated with CAEV from 0 to 271 days PI. Each point represents the mean of eight animals. Vertical bars represent ±1 standard error of the mean. No antibody was detectable in control goats.*

The immunodiffusion test was used in an attempt to give relative meaning to the ELISA test. Sera from two infected goats were tested by immunodiffusion at 0, 7, 14, 28, 35, 42, and 49 days PI. Both were negative by immunodiffusion at 0, 7, and 14 days PI, one turned positive and remained so at 21 days PI, and the other was positive by 35 days PI. All goats were tested at 105 days PI. The infected goats were uniformly positive and the control goats were negative at that time. With respect to sensitivity, sera that were hardly detectable by immunodiffusion had titers of 32 to 256 by the ELISA test.

Clinical evaluations. There was a transient increased distension of the inoculated joint in most of the virus-infected kids, with an insidious periarticular soft tissue swelling. Radiography of the carpi at 240 days PI revealed soft tissue swelling as well in most of the inoculated goats and soft tissue mineralization typical of natural CAE in two goats (Fig. 5). The synovial fluids from virus-inoculated joints had 350 to 14,150 mononuclear cells per mm^3, which is also typical of the natural disease. The five control kids had fewer than 250 cells per mm^3.

Virus isolations. Virus isolations from domestic goats with chronic disease and the high prevalence of specific antibody suggest that CAEV is a persistent infection. To confirm the persistent nature of the infection in the experimental goats, virus isolations were attempted.

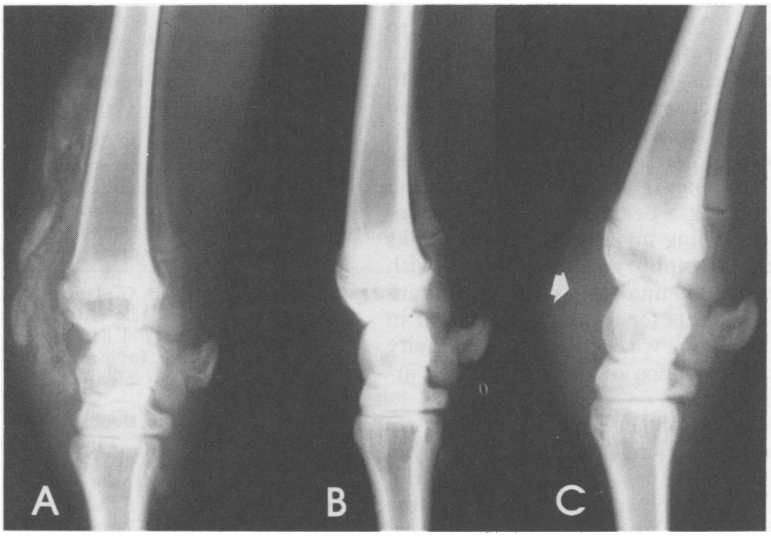

FIG. 5. *Radiographs comparing carpi of goats at 240 days PI. (A) Carpus of a goat inoculated intra-articularly and intravenously with CAEV-infected medium, showing anterior soft tissue swelling and considerable mineralization, periarticular bone remodeling, and reorganization of trabecular bone (inoculated joint). (B) Normal carpus from cesarean-derived goat inoculated intra-articularly and intravenously with control cell culture medium (inoculated joint). (C) Uninoculated carpus of a goat inoculated intra-articularly with CAEV in the contralateral carpus and intravenously. Notice the marked anterior soft tissue swelling and early mineralization (arrow).*

CAEV was isolated from peripheral blood mononuclear cells of two virus-inoculated kids at 238 and 271 days PI and from the synovial fluids of four virus-inoculated kids at 286 days PI. Attempts to isolate virus from peripheral blood mononuclear or synovial fluids of the five control kids failed.

DISCUSSION

The results of this study have shown that cesarean-derived goats infected with CAEV produce sustained, virus-specific cellular and humoral responses, whereas cesarean-derived control goats make no detectable response. No significant immune suppression or potentiation was demonstrated in either virus-inoculated or control kids. The virus-inoculated kids developed clinical signs and lesions compatible with those of natural CAE, including periarticular soft tissue swelling and mineralization (Fig. 5) and increased mononuclear cell concentration in synovial fluid. The virus could be reisolated from infected kids for as long as 9 months after infection, confirming persistence of the infection. Failure to isolate virus from the joints or peripheral blood mononuclear cells of the cesarean-derived control goats and a lack of specific immune responses to CAEV by these control goats further support the assumption that CAEV is not endogenous but is, in fact, a horizontally transmitted retrovirus infection.

The kinetics of the blastogenic response by goat peripheral blood mononuclear cells to CAEV appear to differ from the response of sheep to visna virus, but bear some similarity to the periodic responses seen in horses infected with equine infectious anemia virus. The anti-visna cell-mediated response of sheep resembles the response to typical acute nonpersistent viral infections, reaching a peak at 2 weeks after infection and becoming undetectable by 6 weeks (8). In contrast, lymphocytes from horses with equine infectious anemia respond to equine infectious anemia virus for a prolonged period in an episodic fashion, with peaks of reactivity shortly after emergence of new antigenic variants of the virus (9).

The significance of the marked increase in cellular responsiveness to CAEV antigens at between 4 and 9 months postinfection is unknown. The drop in background counts during that time (Table 1) may have been responsible in part. Hormonal or environmental factors may also have played a role, as this was the period of both sexual maturation and exposure to cold weather for these animals. In natural CAE, clinical arthritis is rarely seen in young goats, but whether the onset of disease is a function of age, hormonal status, environmental conditions, or duration of infection is unknown.

The kinetics and duration of the humoral immune response to CAEV antigen during the first 9 months of infection are not unusual for persistent retrovirus infections. The long latent phase of 21 days before antibody was detected, although seen in other persistent virus diseases (8), may have been artifactual, due to the reported failure of ELISA to detect early (2), low-affinity antibody (6). Antibody, when it began to appear, apparently increased quite rapidly since both the ELISA and immunodiffusion tests turned positive at the same time in the two animals tested; and although there was good correlation between the two tests qualitatively, ELISA was much more sensitive.

A primary objective of this study was to generate information about the immune competence and the antiviral immune response of goats during the early stages of infection with CAEV. Infected goats mount a vigorous response to CAEV in both the humoral and cell-mediated systems, but neither profound immunological deficit nor general immunological potentiation was detected. Variations in patterns of responses to CAEV among individuals are indicated by the variation bars of Fig. 3 and 4. Correlations, if they exist, between the magnitude of immune responses and the severity of lesions will be examined in the course of further studies.

ACKNOWLEDGMENTS

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