A Sequential Study of Virus Expression in Retrovirus-induced Arthritis of Goats

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SUMMARY

Persistent infection by the retrovirus caprine arthritis-encephalitis virus (CAEV) induces arthritis in goats which closely resembles rheumatoid arthritis. To examine the relationship between virus expression and development of clinical disease, ten goat kids were inoculated with CAEV and examined at successive intervals through 18 months post-infection. Virus was monitored in cell-free synovial fluid cells, serum and peripheral blood cells by titration, co-cultivation and immunofluorescent techniques. Virus was readily recovered from the synovial cavity of all animals during the first 4 weeks of infection, followed by a reduction and fluctuation in virus titres and ability to detect virus. Recovery of CAEV from peripheral blood cells occurred at low frequency while viraemia was rare. Results obtained over a period of 18 months indicate a positive association between virus expression in the synovial cavity and development of clinically detectable disease.

INTRODUCTION

Caprine arthritis-encephalitis virus (CAEV), of the family Retroviridae, subfamily Lentivirinae, is the aetiological agent of a chronic disease of the synovium and central nervous system of domestic goats (Crawford et al., 1980; Cheevers et al., 1981). A predominant feature of this syndrome is a progressive, proliferative synovitis of joints, closely resembling human rheumatoid arthritis. Histologically, the lesions are characterized by synoviocyte proliferation and massive infiltration of mononuclear cells with eventual cartilage and bone destruction (Adams et al., 1980). The central nervous system feature of this syndrome is usually manifested as leukoencephalomyelitis and is seen predominantly in young goats (Cork et al., 1974). CAEV can be isolated via explantation from many different tissues of infected goats including peripheral blood cells, although virus is most readily recovered from the synovium and central nervous system (Adams et al., 1980; Cork & Narayan, 1980; Crawford et al., 1980).

While conclusive evidence for an aetiological relationship is lacking, viral infection is currently considered a likely initiator of the inflammatory and immunological processes involved in rheumatoid arthritis (Marmion & McKay, 1978). Conventional viruses are known to produce arthritides of humans, but these are usually transient in nature and cause no tissue necrosis (Malawista & Steere, 1981). However, the isolation of a DNA parvovirus from human rheumatoid arthritic lesions has recently been reported (Simpson et al., 1984). In our continuing attempt to define the pathogenic mechanisms operative in the development of arthritis in CAE, the presence of virus in the synovial cavity and peripheral blood was monitored sequentially for 18 months in ten goats inoculated with CAEV. Concomitantly, clinical parameters of lesion development were followed.

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**Methods**

Experimental animals and virus inoculation. The prototype strain of CAEV (75-G63) was cloned and propagated in primary foetal goat synovial cell cultures as previously described (Crawford et al., 1980; Klevjer-Anderson & Cheevers, 1981). Five male and five female goats of the Sáen breed were obtained by Caesarean section (to prevent possible natural infection by CAEV) (Adams et al., 1983) and inoculated at 10 or 11 days of age with 1 ml CAEV (10<sup>6.0</sup> TCID<sub>50</sub>/ml); 0.5 ml intravenously and 0.5 ml into the left radiocarpal joint. Three males and two females were inoculated in the same manner with culture media from uninfected synovial cells. Control animals were housed separately from virus-inoculated animals.

Infectious virus isolation and assay. Peripheral blood and synovial fluid from the inoculated joint were collected from all goats weekly for 4 weeks, monthly, for a year, then at 15 and 18 months post-infection and assayed for CAEV. Synovial fluid samples were collected from the left radiocarpal joint by aspiration through a 20 gauge needle. The synovial fluid was diluted 1:10 with Dulbecco's modified Eagle's medium (DMEM) (necessitated by its viscosity), centrifuged in a Beckman microfuge for 5 min and the upper two-thirds of the supernatant removed for virus assay. Synovial fluid cells (SFC) pelleted from the centrifuged sample were washed twice in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free Hanks' balanced salt solution and resuspended in DMEM at 5 × 10<sup>5</sup> cells/ml. Cell-free synovial fluid and SFC were assayed for infectious virus by titration and co-cultivation techniques only. Samples of BCC taken after 6 months post-infection were purified as a mononuclear cell population on Ficoll–Hypaque gradients (Banks & Greenlee, 1982).

CAEV in cell-free synovial fluid was titrated as previously described using endpoint dilution with c.p.e. to detect virus infectivity (Klevjer-Anderson & Cheevers, 1981). For SFC samples, the term 'titration' was applied to the process of serial dilution and inoculation of the initial cell suspension onto synovial membrane microtitre cultures. This was done in order to compare quantitatively the relative proportion of the SFC population from which CAEV could be recovered. Virus titres recorded for SFC samples represent the 50% endpoint of the initial cell suspension which induced c.p.e. in indicator cultures.

Co-cultivation of synovial fluid and serum samples with indicator synovial membrane cells was performed by adding 0.5 ml of each sample to polybrene-treated (4 µg/ml) cells in 25 cm<sup>2</sup> flasks. SFC at 2 × 10<sup>5</sup> to 4 × 10<sup>5</sup> and BCC (or mononuclear cells) at 5 × 10<sup>6</sup> per flask were co-cultivated with indicator cells in a similar manner. Co-cultivated cultures were examined for c.p.e. weekly, passaged 1:2 every 2 weeks and discarded as negative at the end of 10 weeks. All cultures were examined by immunofluorescence (IF) for CAEV specificity as soon as c.p.e. was noted, or at the end of 10 weeks before discarding as negative.

Correlation of joint enlargement with infectious virus expression. Hair was removed from the legs of goats 2 cm above the carpus to the midshaft of the metacarpus. Each carpal circumference was measured in a transverse plane at the level of the accessory carpal bone with the leg extended. Circumferences of metacarpi were taken at the junction of the middle and upper thirds. A carpal/metacarpal (C/M) ratio was calculated by dividing the carpal joint circumference by the mean circumference of the two metacarpi. Measurements were taken monthly for a year, then quarterly. For purposes of correlation with joint C/M ratios, infectious virus expression for 6-month intervals was summarized as follows: +4 = virus recovered from 100% of co-cultivated samples and >75% had titratable levels of virus; +3 = virus recovered from >75% of co-cultivated samples and >50% had titratable levels of virus; +2 = virus recovered from >50% of co-cultivated samples and >15% had titratable levels of virus; +1 = virus recovered from >15% of co-cultivated samples and had no titratable levels of virus; +0 = no virus recovered. Half points were added if virus recovery was near the upper limit of a category.

**Results**

Expression of CAEV in the joint cavity

Synovial fluid withdrawn from the joint cavity at each collection interval was separated into fluid and cellular fractions and examined for CAEV. Fig. 1 summarizes the recovery of CAEV from synovial fluid of ten infected goats by titration assays. In general, all goats demonstrated early virus replication followed by undetectable or fluctuating levels of virus production. Titratable levels of extracellular virus in the synovial fluid during the first 4 weeks of sampling were present in nine of ten inoculated goats. The highest titres of virus in the majority of goats (six of ten) were obtained within the first 2 weeks post-infection, ranging from 10<sup>1.50</sup> to 10<sup>4.05</sup> TCID<sub>50</sub>/ml. However, in four of ten animals the presence of virus in the synovial fluid was greatest at 1 month post-infection or later. Viral titres were detectable in the majority of animals after 2 months post-infection, but the average titre had dropped. The number of goats with
quantitative levels of extracellular CAEV dropped dramatically (one of ten) at 8 months and remained low, varying from one to four goats throughout the 18 months post-infection period. The more sensitive technique of co-cultivation with indicator cells demonstrated that low levels of virus ($< 10^{2.0}$ TCID$_{50}$/ml) were present in cell-free synovial fluid of the majority of animals. Virus was recovered from 90 to 100% of co-cultivated synovial fluid samples from infected animals throughout 6 months post-infection (summarized in Table 1). In samples taken throughout 6 to 18 months post-infection CAEV was recovered from 55 to 90% of the synovial fluid samples. CAEV could not be isolated from the five goats injected with tissue culture media from uninfected synovial membrane cells.

The number and morphological classification of cells present in the synovial fluid at each sampling was compared in mock-infected and CAEV-inoculated animals. The average cell number present in the synovial fluid of mock-infected animals during the 18 month period was $2.8 \times 10^5$ cells/ml, ranging from $1.4 \times 10^5$ to $3.2 \times 10^5$ cells/ml. In contrast, the SFC number from CAEV-infected goats was consistently 13 to 15 times greater than the number found in mock-infected animals. Between 1 week and 6 months post-infection, the average cell number was $8.7 \times 10^6$ cells/ml, with a range of $7.0 \times 10^6$ to $11 \times 10^6$ cells/ml. After 6 months post-infection, the average cell number dropped to $3.6 \times 10^6$ cells/ml, ranging from $2.1 \times 10^6$ to $5.2 \times 10^6$ cells/ml. Viability of SFC was 90 to 95% as assessed by trypan blue dye exclusion. The SFC population was composed of variable proportions of macrophages and lymphocytes. This intense mononuclear cell infiltration of the joint cavity following CAEV inoculation is consistent with local antigenic stimulation and inflammation.
CAEV was also frequently recovered from SFC populations. The titration of SFC samples for CAEV is summarized in Fig. 1. Virus titres were expressed as the 50% endpoint of the original cell suspension which induced c.p.e. in indicator cultures. While these values cannot be directly compared to the titres of virus in cell-free synovial fluid, they were useful in comparing virus expression in individual goats at successive time points as well as detecting differences between animals. The highest titres of CAEV were recovered from SFC from the majority of animals during the first 4 weeks post-infection (Fig. 1). After this time, the number of goats from which virus could be recovered by titration of the SFC suspension decreased (0 to 50%), with only one sample containing titratable levels of virus from 8 to 12 months post-infection. Therefore, after 12 months SFC samples were no longer titrated but co-cultivated only to detect CAEV.

As with cell-free synovial fluid samples, CAEV was recovered from a greater percentage of SFC samples which were co-cultivated for several passages as compared to titration. CAEV was recovered from 70 to 90% of co-cultivated SFC samples up to 7 months post-infection, but from only 50 to 60% of the samples thereafter (Table 1). One goat consistently yielded CAEV from the SFC sample for 18 months post-infection. The presence of SFC-associated virus in most instances was accompanied by concomitant detection of virus in the synovial fluid. CAEV recovered from SFC may represent cells productively infected \textit{in vivo} and/or latently infected cells in which virus replication has been induced by co-cultivation \textit{in vitro}.

Cells present in the synovial fluid were also examined for the presence of viral proteins using a direct IF technique detecting primarily the viral core protein p28 (Adams \textit{et al.}, 1980; Roberson \textit{et al.}, 1982). While titratable levels of infectious CAEV were recovered from SFC samples from eight of nine goats at 2 weeks post-infection, only four of these contained IF-positive cells (ranging from 1 to 5% of cells fluorescing). Furthermore, there was no direct correlation between the amount of CAEV associated with SFC samples and the presence of IF-positive cells. No IF detectable virus antigen was seen in SFC after 4 weeks post-infection.

\textbf{Expression of CAEV in peripheral blood}

Peripheral blood was examined for the presence of CAEV as blood leukocytes often play a role in persistent virus infection, and it has been reported that CAEV can be recovered from these cells (Cork & Narayan, 1980). Infectious virus from BCC samples was first recovered by co-cultivation at 3 weeks post-infection (Table 1). Recovery of CAEV from BCC was infrequent during the next 7 months, with successful detection of virus in only 0 to 20% of samples co-cultivated. After 7 months post-infection, Ficoll–Hypaque-purified preparations of mononuclear cells (instead of BCC) were used for co-cultivation and CAEV was recovered with increased frequency (12 to 50% of samples positive). Virus was recovered at least once from the peripheral blood cells of each infected animal. IF techniques rarely detected viral proteins in BCC or mononuclear cells, indicating again the insensitivity of this technique for detecting virus-infected cells. CAEV was recovered once from serum samples examined during the first 7 months post-infection (Table 1). From 8 to 18 months post-infection, 0 to 30% of serum samples yielded virus. When virus was detected it required three or four passages of the infected indicator cell cultures before virus-specific c.p.e. appeared.

\textbf{Relationship between virus expression and joint enlargement}

Fig. 2 summarizes the relationship between infectious virus expression and joint enlargement during 6-month intervals after infection. Data accumulated monthly then quarterly were summarized in 6-month intervals due to the chronicity of lesion development. From 6 to 11 months post-infection seven of ten goats had significant enlargement of the infected joint. Furthermore, an association began to appear between joint enlargement and the presence of infectious virus in the joint cavity. A positive association was most obvious in goats with the maximum and minimum amounts of joint enlargement. An exception to this correlation was evident in one animal which had a very enlarged carpal joint (C/M ratio averaging 1.99 for the period) but with infrequent recovery of infectious virus (+1). However, residual swelling from the initial inoculation may be involved as this goat exhibited the greatest C/M ratio (2.18) of the study 2 months post-infection and has since shown continuous reduction in joint size. An
analysis of the data for the 6 to 11 month interval revealed, nonetheless, that the correlation coefficient of joint size and virus expression was not statistically significant (0.454, $P > 0.10$). By 12 to 18 months post-infection, eight of nine goats demonstrated significant joint swelling and the correlation coefficient between virus expression and lesion development increased to 0.723, significant at $P < 0.05$. Moreover, no virus was recovered from one goat during this sampling period and concomitantly its joint ratio (1.81) was not statistically different from control animals (1.79).

**DISCUSSION**

This study describes the infection of goats by CAEV, with particular emphasis on virus expression in the synovial cavity and its relationship to clinical disease. CAEV established a persistent infection of all ten goats for up to 18 months post-infection. Initially, virus was readily detectable in the joint cavity from both cell-free synovial fluid (titres of up to $10^4$ TCID$_{50}$/ml) and SFC samples. Subsequently, levels of virus in individual goats decreased and fluctuated markedly. Intrasyovial CAEV most likely results from the productive infection of synovial membrane cells [which begin to proliferate 2 weeks post-infection (Adams et al., 1980)] and/or the mononuclear cells which are found in the synovial cavity and which infiltrate the surrounding tissue. Virus particles have been observed in the synovial lining cells by electron microscopy between 18 and 45 days post-infection (at which time the experiment was terminated) (Brassfield et al., 1982). Using immunohistochemical techniques, Adams et al. (1980) reported the presence of a small number of CAEV-infected cells in the synovial membrane lining and in the monocytic cell population of the SFC during the first 2 weeks of infection. We confirm the latter finding in the present study. However, since CAEV can be recovered from the joint cavity for 18 months post-infection, the immunofluorescent assay appears to lack sensitivity as a technique for detecting virus expression in CAE. On the other hand, an extremely small proportion and/or the focal nature of the cells hosting productive infection during a given time period could also account for the inability to detect virus-infected cells by this means. In 1980, Cork & Narayan reported that virus could be consistently isolated from BCC beginning 1 week after infection. We did not recover CAEV from BCC as often; however, this discrepancy may be due to differences in our virus isolates, protocols or breed of goat. The slightly increased rate of recovery of CAEV from peripheral blood cells after 7 months post-infection may be associated with purification as mononuclear cells. This was done to
increase the percentage of macrophages after it was shown that these cells are capable of hosting a productive CAEV infection in vitro (Klevjer-Anderson & Anderson, 1982; Anderson et al., 1983a). However, on examining samples from individual goats, there was no direct correlation between the proportion of macrophages and measurable levels of CAEV in either mononuclear cell or SFC specimens.

Because viral proteins may initiate and provide for continued antigenic stimulation of the inflammatory process, we attempted to correlate virus expression and manifestation of clinical disease. Although the development of clinically measurable lesions requires many months, a positive relationship with virus expression began to appear after 6 months post-infection, particularly among the goats with very high or low virus expression. In the period 12 to 18 months post-infection, a relatively strong association between virus expression and joint enlargement was determined by correlation coefficients; however, this is regarded cautiously since the sample numbers are low (n ~ 9) and removal of 1 or 2 points from the extremes on the regression line would lower the significance markedly. Nevertheless, the association observed between carpal enlargement and expression of infectious virus is remarkable considering the host variables of residual swelling, individual inflammatory and immune responses to the virus, and anatomical structures in the vicinity of the radiocarpal joint that might contribute to enlargement of the carpus. Furthermore, chronic antigenic stimulation could be provided by the shedding of non-infectious particles, or expression of new antigens on infected cells (viral-coded or induced) without a corresponding productive virus infection (Crumpacker, 1980; Dubois-Dalcq et al., 1979).

The synovial lesion in CAE, as with rheumatoid arthritis, is consistent with an immunopathological process whereby antibody and sensitized lymphocytes react with antigens in the synovial cavity. Continual antigenic stimulation could be provided by virus proteins as a result of complete or partial expression of CAEV in the joint. Indeed, significant concentrations of antibody directed against CAEV surface glycoproteins gp125, gp90, gp70 as well as p28 are found in the synovial fluid of these animals (Johnson et al., 1983). Furthermore, lesion development is significantly enhanced in goats immunized with inactivated CAEV before challenge with infectious CAEV (T. C. McGuire et al., unpublished results).

Persistence of CAEV and the fluctuating nature of virus production in the joint cavity may be associated with a variety of factors. The most obvious of these is that CAEV, as a retrovirus, is capable of integrating its genomic information into host cell DNA (Cheevers et al., 1981; Klevjer-Anderson & Cheevers, 1981). Thus, fluctuating levels of virus may result from intermittent expression of virus genetic information, governed by host cell controls (Brahic et al., 1981). Secondly, although high titres of antibody directed to the virus surface glycoproteins were present in the synovial fluid, they were not effective in neutralizing virus infectivity in vitro (data not shown) (Klevjer-Anderson & McGuire, 1982). Persistence and fluctuating virus replication do not seem to involve the appearance of antigenic variants (Anderson et al., 1983b) as has been suggested for visna and equine infectious anaemia viruses, two other lentiviruses causing persistent infection (Scott et al., 1979; Kono et al., 1973). Continued virological and immunological studies are necessary to define more precisely the relationship between virus expression, immune response and lesion development in CAE.

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