Restricted mumps virus infection of cells derived from normal human joint tissue

Hans-Iko Huppertz† and J. K. Chantler*

Division of Medical Microbiology, University of British Columbia, Heather Pavilion 364, 2733 Heather Street, Vancouver, British Columbia V5Z 1M9, Canada

Mumps virus (MuV) is known to be associated with acute arthritis and may also have a role in chronic inflammatory joint disease. The mechanism of induction of joint inflammation is not known but may be associated with direct invasion of joint tissue. To investigate the possibility of persistent intra-articular infection, the interaction of MuV with primary cells from normal human joint tissue was examined. These mixed cultures of synovial membrane cells and chondrocytes were found to be semi-permissive to the virus; only a small proportion of cells (5 to 20%) were infected and produced low titres of progeny virions. In addition, little viral antigen was detected on the cell surface relative to that found on Vero cells. This restricted infection of synovial membrane cells was related to a severely decreased synthesis of the viral glycoproteins, fusion and haemagglutinin-neuraminidase, and the membrane protein in comparison to the levels found in Vero cells. Persistent infections were readily established and could be maintained for 2 to 3 months. During the first month, the infection remained highly focal and supernatant viral titres were low. Thereafter both the percentage of infected cells and viral titres increased until finally the cultures were killed. No evidence was obtained for the generation of temperature-sensitive mutants or defective interfering particles during long-term infection, but the persistent virus derived from the cultures gave cloudy plaques and induced no fusion in Vero cells until passaged. This study has shown that human synovial tissue cells have the intrinsic ability to support MuV replication and persistence which may be important in the pathogenesis of mumps arthritis.

Introduction

Arthritis is a significant complication of mumps occurring in up to 0.5% of patients (Gordon & Lauter, 1984). This acute joint inflammation may be the result of viral infection of synovial tissue or be associated with an immunopathological reaction (Butler, 1988). At present there is little information on the ability of mumps virus (MuV) to infect joint tissue, although the virus has been isolated from other organs, such as testis and brain, which may be involved in acute mumps infection (Gordon & Lauter, 1984). Persistence of the virus may occur frequently because MuV-specific IgM can be found in 50% of patients 5 months after infection (Benito et al., 1987). Rarely, this persistence of MuV has been associated with serious disease including chronic encephalitis (Julkunen et al., 1985) and otosclerosis (Arnold & Friedmann, 1988). In the case of arthritis, symptoms have been known to persist for at least 6 months and, although they normally resolve with time, an indirect association with rheumatoid arthritis has been suggested (Ford et al., 1988).

Several cell culture systems with persistent MuV infection have been established in vitro using animal cell lines or human carcinoma or transformed cells (Wolinsky & Server, 1985). Such systems have given insight into possible mechanisms of persistent infection including the generation of temperature-sensitive variants (Truant & Hallum, 1977) and defective interfering particles (McCarthy et al., 1981; Andzhaparidze et al., 1982), and the role of interferon (Ito et al., 1985). The model system described in this paper was developed to study the interaction of MuV with primary synovial cells (SMC) derived from normal human joint tissue. The possibility of MuV persistence in SMC and the mechanism of restriction of viral replication enabling long-term persistence has been investigated.

† Present address: Children's Hospital, University of Würzburg, Josef-Schneider-Strasse-2, D-8700 Würzburg, Germany.
Methods

Cells and virus. Normal human foetal joint tissue (synovial membrane plus attached articular cartilage) was digested with collagenase and trypsin and the liberated synovial membrane/cartilage cells (SMC) were grown at 37 °C in RPMI 1640 (Gibco) supplemented with 10% (v/v) foetal bovine serum (FBS), L-glutamine and antibiotics (complete RPMI). These cultures contained type A and type B synovial lining cells, mesenchymal cells and chondrocytes. Both type A and type B SMC are derived from the underlying mesenchymal layer but can be differentiated both morphologically and functionally as being macrophage-like or fibroblast-like. Intermediate type C cells have also been described suggesting that type A and type B cells may represent different functional stages of the same basic cell (Jee, 1983). These mixed cultures of SMC could be kept in cell culture for over 7 months, for up to 22 passages. During this time their appearance changed from a cell population of mixed morphology, including a large proportion of polygonal cells and smaller numbers of stellate and elongated cells, to a uniformly fibroblast-like or type B morphology. When not stated otherwise, only cells of the first few passages were used for the experiments. Vero cells (ATCC) were grown in Dulbecco's MEM (DMEM) or Biorich medium (Gibco) as described for SMC.

The Matti strain (wild-type) of MuV was grown in Vero cells at 37 °C in DMEM supplemented with 5% heat-inactivated FBS, L-glutamine and antibiotics. The virus was titrated by a plaque assay and the stock virus was found to have a titre of 10^6 p.f.u./ml. Intracellular virus titres were obtained by trypsinizing or scraping the cells, then carefully washing them prior to sonication and plaque assay. Neutralization assays were performed by incubating a virus preparation containing about 100 p.f.u./ml with 1:1000, 1:200 or 1:50 dilutions of a polyclonal rabbit anti-MuV antibody (Lee Biomolecular Research Laboratories) prior to performing the plaque assay.

To maintain the persistent infections, RPMI containing 5% FBS was further supplemented with 0.05 mm-mercaptoethanol, which helps to preserve the functions of macrophage-like cells (Chesnut & Grey, 1985). Preliminary experiments in acute MuV infections showed that in the presence of 2-mercaptoethanol, SMC morphology improved and c.p.e. was less marked. Its mechanism of action on MuV-infected SMC is not known.

Immunoperoxidase staining. Monolayers were fixed in 4% (w/v) paraformaldehyde in phosphate buffer. Fixation in methanol or phosphate-buffered 10% formaldehyde was also found to be effective. In contrast, 0.25% glutaraldehyde, 100% ethanol or ethanol with 5% glacial acetic acid destroyed, at least in part, the antigenicity of the viral proteins. After incubation in methanol containing 1.8% (v/v) hydrogen peroxide, indirect immunoperoxidase staining was performed using polyclonal rabbit anti-MuV antibody and an avidin-biotin development kit (Vectastain; Vector Laboratories). Diaminobenzidine was used for colour development, giving a cell-associated brown precipitate. Monolayers were counterstained with haematoxylin and eosin. Control dishes were incubated with an unrelated primary antibody or buffer only but thereafter were treated identically. To examine surface staining, unfixed monolayers were incubated with the antiviral antibody and then processed as above. Monolayers were fixed in methanol/H2O2 after incubation with the secondary antibody.

Electron microscopy. Cell monolayers were fixed in 2.5% phosphate-buffered glutaraldehyde and scraped into phosphate-buffered saline (PBS). Thereafter they were fixed in OsO4, stained in uranyl acetate and Epon-embedded. Ultrathin sections were observed under a Philips 400 electron microscope.

Radial labelling and immune precipitation of viral polypeptides. Cells were incubated in DMEM without methionine and cysteine for between 1 and 2 h and were then labelled by incubation in this medium supplemented with 70 μCi [35S]methionine/cysteine (ICN) per ml. Following the labelling period, the cells were washed in PBS, pelleted and then lysed in 1% sodium deoxycholate, 1% Triton X-100 and 0.1% SDS in 150 mm-NaCl, 10 mm-Tris-HCl and 1 mm-EDTA for 20 min at 4 °C. The lysates were centrifuged at 12000 g for 5 min prior to immune precipitation. In some experiments the commercial rabbit anti-MuV antibody was replaced by a locally made polyclonal rabbit antibody of similar specificity but lower potency. To identify individual viral polypeptides immunoprecipitation was carried out using monoclonal antibodies directed against the membrane (M) and fusion (F) proteins, and the nucleoprotein (NP), kindly provided by Dr J. S. Wolinsky (Wolinsky & Server, 1985). Immune complexes were precipitated by staphylococcal Protein A (Calbiochem) and were pelleted and stored at −20 °C prior to gel analysis.

SDS-PAGE and autoradiography. The immunoprecipitates were analysed by electrophoresis in 10% polyacrylamide slab gels using the Laemmli buffer system. M, markers (Bio-Rad) were used to determine the size of the virus-induced proteins. Gels were dried and exposed to Kodak OMEX film for about 2 weeks at 4 °C for autoradiography.

Results

Acute infection of SMC

Infection of SMC with MuV at an m.o.i. of 0.01 p.f.u./cell resulted in a low level of highly focal c.p.e., barely visible after 7 days. Small clumps of a few condensed nuclei with little cytoplasm attached could be seen scattered through the monolayer. In contrast, Vero cells showed extensive fusion, sometimes involving more than 15 cells, after 2 days of infection with MuV. These giant cells contained many nuclei clumped together or arranged in a ring-like manner within the cytoplasm. Cell fusion continued until nearly the whole monolayer was involved and after about a week almost all the cells were dead.

(i) Virus yield

The supernatant fluid of the SMC contained low but consistent titres of MuV (Fig. 1a) higher than those found for MuV in medium alone at 37 °C. In contrast, Vero cells produced high titres of MuV after 2 days which were three- to fourfold greater than those in SMC; this virus release continued until the cells died. In order to investigate whether this difference in extracellular virus titres was caused by a defect in virus release, resulting in accumulation of intracellular infectious virus in the SMC, supernatant and intracellular virus titres were compared in both cell types (Fig. 1b). In either cell type intracellular and extracellular titres were similar but the intracellular titres of SMC were reproducibly higher than the titres of released virus whereas the reverse was true for Vero cells. This may be due to less efficient budding from intracellular membranes in the SMC or...
(b) MuV restriction in synovial membrane cells

Fig. 1. Time course of virus production during acute MuV infection. (a) MuV titres in supernatant fluid of SMC and Vero cells, or after incubation in medium alone. □, Vero cells; ◼, SMC; ■, control. (b) Comparison of extracellular and intracellular titres in SMC and Vero cells. □, Extracellular SMC; ◼, intracellular SMC; ■, extracellular Vero cells; ◼, intracellular Vero cells.

Fig. 2. Indirect immunoperoxidase staining for MuV antigen in SMC (a and b) and Vero cells (c and d), 7 and 3 days post-infection, respectively. Cells in (a) and (c) are uninfected, (b) and (d) are infected with MuV. Note the limited spread of MuV in SMC. Bar markers represent 20 μm.

(i) Intracellular viral antigen
To examine intracellular compartmentalization of viral antigen in SMC in comparison with Vero cells, immunoperoxidase staining was carried out. After about 24 h viral antigen could be demonstrated in some Vero cells and after 72 h nearly all Vero cells were infected and multinucleate fused cells could be seen (Fig. 2). In contrast, only a fraction of the SMC were infected, even at 7 days post-infection. Antigen was detected in single cells or in small clusters of two to 10 cells, only some of which were fused. Inclusion bodies or a diffuse reticular pattern of staining of the cytoplasm was seen, similar to the staining pattern in Vero cells.

To investigate the possibility of diminished glycoprotein expression on the cell surface, unfixed monolayers were stained for viral glycoproteins using polyclonal anti-MuV antisera and the immunoperoxidase staining procedure. The percentage of Vero cells expressing either intracellular or surface antigen at 48 and 72 h was very similar (95 to 98% in each case); in contrast, 4 to 5% of SMC expressed intracellular antigen, whereas surface antigen was detectable on only 0.5% of cells, or on 10% of infected SMC.

(ii) Limitation in viral spread
The proportion of SMC infected during the first week varied from 5 to 20% from experiment to experiment. To determine whether this limited spread of the virus in the monolayer was due to lack of cleavage of the fusion protein precursor present on the surface of infected cells, monolayers were treated briefly with trypsin (0.25 mg/ml). However, this treatment did not alter the percentage of infected cells. When higher multiplicities of infection (1 p.f.u./cell instead of 0.01 p.f.u./cell) were used, c.p.e. appeared earlier but the percentage of cells infected and the titres of supernatant virus were of the same order of magnitude after 1 week (data not shown). Thus the limited spread of MuV in the monolayers could not be overcome by increasing the m.o.i.
(iv) Electron microscopical examination of infected SMC
To investigate whether the assembly of virions in the SMC was aberrant, electron microscopy was carried out on infected SMC and the observations were compared to those obtained with Vero cells. The different cell types in the SMC culture were readily distinguishable as fibroblast-like or macrophage-like, both by shape and the presence or absence of phagocytic vesicles. A proportion of both cell types was found to be infected and in Fig. 3 electron micrographs of SMC (type A cells) and Vero cells are shown, demonstrating the presence of nucleocapsids densely packed in intracytoplasmic inclusion bodies in both cell types. In addition, virus budding from the cell membrane could be seen in abundance in Vero cell cultures but was only rarely detected in infected SMC.

(v) Analysis of MuV polypeptide synthesis
The identification of MuV-specific polypeptides produced in Vero cells using polyclonal anti-MuV and monoclonal antibodies (Wolinsky & Server, 1985) against the F protein, M protein and NP is shown in Fig. 4. The commercial polyclonal anti-MuV recognizes the major MuV proteins with the exception of the M protein, which is only very faintly represented in lane 2. The monoclonal anti-NP antibody immunoprecipitates a double band at 68K and 72K, the reported Mr of this polypeptide, two forms of which are known to exist differing in their extent of phosphorylation (Naruse et al., 1981, Huppertz et al., 1977). In addition, a major broad band (NP') which migrates slightly faster than the F protein is recognized by this monoclonal anti-NP antibody. The identity of this species is not certain but it may represent a proteolytic cleavage product of NP which is known to be highly susceptible to proteases, similar to the NPs of other paramyxoviruses (Wolinsky & Waxham, 1990).

To compare polypeptide synthesis in SMC and Vero cells a time-course experiment was conducted between 12 and 48 h post-infection. Cells were labelled with
MuV restriction in synovial membrane cells

Fig. 5. Time course of MuV-specific polypeptide synthesis during acute infection of SMC (a) and Vero cells (b), analysed by SDS-PAGE. Cells were labelled for 6 h at 12 h (lanes 1 and 2), 24 h (lanes 3 and 4), 36 h (lanes 5 and 6) and 48 h (lanes 7 and 8) and virus-induced polypeptides were immunoprecipitated with polyclonal anti-MuV antibodies prior to gel analysis. Odd-numbered lanes are controls of mock-infected cells.

[35S]methionine for 6 h periods at 12 h intervals and the immunoprecipitated MuV polypeptides were analysed by SDS-PAGE and autoradiography. The results are shown in Fig. 5(a) and (b). Polypeptides of the expected size for the major MuV proteins were readily detected in Vero cells but only the double band at 69K and 72K representing NP (Naruse et al., 1981) was found in SMC. Further analysis using the F, M and NP monoclonal antibodies (Fig. 6) demonstrated that even strongly labelled samples, over-exposed with respect to the bands found in Vero cells, showed little detectable synthesis of F and M proteins in SMC. Only NP was identified but interestingly the heavy 55K to 60K band precipitated by the anti-NP monoclonal antibody in Vero cells was absent in SMC.

As both the M polypeptide and the surface proteins F and haemagglutinin-neuraminidase (HN) are necessary for assembly of infectious virus and budding, their severely decreased production in SMC would explain the limited spread of MuV in these cells, the low virus titres and the diminished staining of viral surface antigens. On the other hand the ample production of NP in SMC is in agreement with the finding of inclusion bodies in these cells which stained for viral antigen and contained nucleocapsids identifiable by electron microscopy.

Persistent infection of SMC

As a high proportion of SMC was not infected by MuV during the acute stage of infection, persistent infections were easily established and maintained for 2 and 4 months. Initially the supernatant virus titre was found to decrease to less than 10^3 p.f.u./ml. At later times (1 to 2 months post-infection) the titres increased slowly by 10- to 100-fold, although they never reached the levels normally found in Vero cells (10^6 p.f.u./ml). Throughout the persistent infection the amount of intracellular virus remained 10- to 50-fold higher than that found in the medium, as previously found during acute infection. The percentage of infected cells estimated by counting immunoperoxidase-positive antigen-expressing cells increased in parallel with the supernatant virus titres. For 2
Fig. 6. A comparison by SDS-PAGE of \(^{35}S\)-labelled polypeptides produced by MuV-infected SMC (lanes 4, 6, 8 and 10) and Vero cells (lanes 2, 5, 7 and 9) 43 to 48 h post-infection. Immunoprecipitation by polyclonal (lanes 1 to 4) or monoclonal antibodies against the viral polypeptides M (lanes 5 and 6), F (lanes 7 and 8) and NP (lanes 9 and 10) was carried out prior to PAGE. Lanes 1 and 3 are mock-infected controls.

Fig. 7. Indirect immunoperoxidase staining of MuV antigen during long-term persistent infection. Control cells (a, c and e), derived from the same joint tissue, or MuV-infected cells (b, d and f) were stained on day 17 (a and e), day 66 (c and d) and day 90 (e and f).

months, the virus remained focal and many uninfected cells were present in the culture. However, after 3 months almost all cells were infected and eventually died, thus terminating the persistent infection (Fig. 7). To investigate whether the SMC became more permissive during long-term passage, the control cells at the end of each experiment were infected with fresh MuV or MuV isolated from persistently infected cells (MuVpi). However, as before, only a low percentage of cells were infected and only low virus titres produced. Thus, although demonstrating an altered morphology, SMC after long-term passage in vitro restricted MuV replication in a manner similar to freshly cultured cells. Moreover MuVpi showed no adaptation to SMC. Persistent infections in SMC were established four times, similar results being obtained on each occasion.

Fig. 8. SDS-PAGE of MuV-infected synovial cells immunoprecipitated with polyclonal antibody after 3 (lanes 1 and 2), 64 (lanes 3 and 4) and 81 (lanes 5 and 6) days of infection. At day 64, 16% of the cells were infected and produced \(3 \times 10^2\) p.f.u. virus/ml, whereas at day 81, 99% of cells were infected and the supernatant viral titre had risen to \(7 \times 10^4\) p.f.u./ml. Note the increased synthesis of NP but limited expression of viral glycoproteins at day 81. Odd-numbered lanes are mock-infected controls.
(i) **PAGE analysis of persistently infected SMC**

When polypeptide synthesis during acute or persistent infection of SMC was compared (Fig. 8), the production of NP increased at later stages when higher virus titres were produced and a greater percentage of cells were infected. However the synthesis of the F and HN glycoproteins and M protein did not reach the levels found in Vero cells. Thus the SMC still showed some restriction of viral gene expression.

(ii) **Characterization of MuVpi**

A number of alterations in MuVpi were detected. Firstly, MuVpi produced cloudy plaques in Vero cells and these appeared several days later than the clear plaques of the original stock MuV. Secondly, neither MuV nor MuVpi were found to be temperature-sensitive, giving equal numbers of plaques at 37 °C and 39.5 °C (at 41 °C the cells did not survive). Furthermore, the pathogenicity of MuVpi for Vero cells was altered. A comparison of MuVpi with original stock virus, MuV, in an acute infection of Vero cells at 0.01 p.f.u./cell yielded smaller inclusion bodies (Fig. 9) and 10- to 100-fold less progeny with the MuVpi virus, although in both cases essentially all the cells were infected (Table 1). Much less fusion was found in MuVpi-infected Vero cell cultures unless the cells were passaged, in which case large multinucleate bodies appeared within 48 h. In addition, polypeptide analysis of Vero cells infected with MuVpi showed a similar pattern to that found in SMC, with NP being predominantly synthesized with only low levels of F, HN and M proteins (not shown).

Thus MuVpi could infect a high proportion of Vero cells but showed a diminished ability to cause fusion and cell death, associated with restricted expression of certain viral proteins. When freshly prepared SMC were infected with MuVpi, in comparison with stock MuV no adaptation to the synovial cells appeared to have occurred. A similar low percentage of cells were found to be infected with both viral strains (less than 5%) and no more fusion was seen with the virus adapted to SMC. These results concurred with those found in SMC after long-term culture described previously. Thus, limitation of MuV replication and spread in SMC appears to be a cell-dependent characteristic, whereas in Vero cells the restriction of MuVpi is a property of the virus isolated from long-term persistence in SMC. In a combined infection of equal amounts of MuV and MuVpi in Vero cells, no interference in the replication of MuV by the virus derived from persistent infection was seen. Progeny virus from the mixed infection showed both clear and cloudy plaques at a ratio of about 100:1.

<table>
<thead>
<tr>
<th>Virus titre (p.f.u. ml)</th>
<th>Day 2</th>
<th>Day 3</th>
</tr>
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<tbody>
<tr>
<td>Vero cells MuV</td>
<td>$2 \times 10^6$</td>
<td>$1.6 \times 10^7$</td>
</tr>
<tr>
<td>MuVpi</td>
<td>$4 \times 10^4$</td>
<td>$0.8 \times 10^6$</td>
</tr>
<tr>
<td>SMC MuV</td>
<td>$6 \times 10^4$</td>
<td>$4.0 \times 10^2$</td>
</tr>
<tr>
<td>MuVpi</td>
<td>$0.4 \times 10^1$</td>
<td>$0.6 \times 10^1$</td>
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Fig. 9. Indirect immunoperoxidase staining of viral antigen expressed in Vero cells by MuV (b) or MuVpi (c). Note small inclusion bodies and lack of fusion induced by MuVpi. (a) Uninfected control.
Discussion

The present study has shown that cells derived from human synovial tissue are partially permissive to the replication of MuV. Low viral yields (10^2 to 10^3 p.f.u./ml) were obtained and the infection remained highly focal for extended periods, indicating poor virus spread. However, within the cells large inclusion bodies filled with nucleocapsids were seen by electron microscopy, similar to those found in the highly permissive Vero cell line. This suggested that the block might be in viral assembly or in the expression of the envelope glycoproteins and, in agreement with this, surface expression of the viral glycoproteins was found to be greatly reduced as detected by immunoperoxidase staining.

To determine whether synthesis or processing of the viral glycoproteins was aberrant in SMC, polypeptide analysis was carried out and compared with that found in Vero cells. Normal quantities of NP were detected but little or no F, HN or M proteins. This may relate to a recent finding of the generation of polycistronic virus mRNAs in certain cell types (Afzal et al., 1990); these are only partially translated from the 5' start site required in eukaryotic systems. Further supporting this possibility, the gene order for MuV has been shown recently to be similar to that found for other paramyxoviruses, namely 3' N-P-M-F-SH-HN-L 5' (Elango et al., 1989). Thus, in the transcribed mRNA, NP would occur at the 5' end and be the predominant polypeptide translated from a full-length polycistronic mRNA. A small amount of transcription from internal initiation points would account for the low levels of synthesis of the other viral proteins. A similar restriction in expression of MuV envelope proteins in neurons of mouse brain inoculated with the Kihham or RW strains has been described previously (Kristensson et al., 1984). Analysis by immunofluorescence using monoclonal antibodies to viral structural antigens indicates that only NP and P antigen are detectable in infected neurons. In addition, limited expression of measles virus envelope protein transcripts during persistent infection associated with subacute encephalitis of Lewis rats has also been observed (Schneider-Schaulies et al., 1989).

The block in MuV replication in SMC is not complete and low titres of supernatant virus were continuously found in the medium during long-term persistent infection. Despite this, the spread of MuV through the monolayer was highly restricted for 6 to 8 weeks, indicating the importance of cell to cell fusion in this process as previously reported (Merz & Wolinsky, 1981; Waxham & Aronowski, 1988). Notably, in comparison, infecting Vero cells at low multiplicity (0-0001 p.f.u./cell) still resulted in complete infection of the monolayer in 7 days. Lack of cleavage of the F protein precursor did not appear to be a significant factor in SMC because trypsin treatment of infected cells did not increase the amount of fusion and, moreover, trypsinization of the cells during passage of the persistently infected culture did not result in a boost of viral progeny production. However, when MuVpi was used to infect Vero cells, significant fusion was not seen until the cells were trypsinized and passaged. This could be due to a trypsin effect, an alteration in the metabolic state of the cells by subculture, or adaptation of the MuVpi to Vero cells, for example by incorporation of different lipid components into the viral envelope.

The reason for the alterations in the permissiveness of persistently infected SMC after 4 to 8 weeks in culture is not known. Control SMC maintained for over 8 weeks were still found to restrict MuV replication in a manner similar to low passage cells. The increase in the percentage of cells infected and in virus titre which occurred slowly over a period of several weeks might best be explained by a gradual shift in the balance between virus and host cells. Uninfected cells divide more frequently than those carrying virus but as cell multiplication slows down with long-term passage the percentage of infected cells would be expected to increase inexorably until all cells in the culture were infected. Interferon production by macrophage-like cells present early in the culture might also have a role in restricting virus during the first 2 to 3 weeks. This is indicated by the reduced cytopathology in SMC cultured in the presence of 2-mercaptoethanol which helps to enhance the activity of macrophage-like cells in culture (Chesnut & Grey, 1985).

The intrinsic ability of MuV to infect and persist in human joint cells is of interest in relationship to the pathogenesis of mumps arthritis and the possibility that the virus is involved in chronic rheumatic disease in humans. The results reported here can be compared with a study of the permissiveness of organ cultures of human foetal joint tissue to this virus (Huppertz et al., 1989). MuV was found to be capable of infecting and penetrating deep within the synovial membrane and persisted in the tissue pieces for 2 to 3 months. The potential for MuV to have a significant role in chronic arthritis in certain patients is therefore clear.

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