INTERACTION OF VIRUSES WITH HUMAN JOINT MATERIAL

by

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We accept this thesis as conforming to the required standard:

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ABSTRACT

An in vitro cell and organ culture system for the study of human arthrotropic viruses was established to determine the permissiveness of joint cells and tissue to five strains of rubella virus (RV), adenovirus type 2 (Ad) and human parvovirus (B19). A semi-continuous line of fetal chondrocytes (FC) and primary synovial membrane/cartilage (SMC) cells were used to investigate virus/joint cell interactions. In addition, the SMC cells were used to determine the ability of a virus to establish a persistent infection in cells of joint origin. Intact joint tissue containing both synovial membrane and cartilage was also infected to determine the ability of the viruses to replicate in non-dividing cells and also their invasive capability in the presence of an extracellular matrix.

Results showed that all RV strains and Ad were able to replicate in FC, SMC cells and intact joint tissue. The only exception to this was that replication of RV strain Cendehill was not detected in joint tissue. In contrast, there was no indication, by either DNA-DNA hybridization or immunoperoxidase staining, that B19 was able to replicate in SMC cells. Most importantly, the behaviour of the rubella strains in this model was found to correlate with the clinical data concerning the incidence of complications associated with rubella infection or vaccination. This suggests that arthritogenicity is related to the ability of a particular virus to infect and persist in joint tissue and establishes the in vitro model as a useful system to evaluate the arthrotropicity of future rubella vaccines.
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I. INTRODUCTION

A. Arthritis Overview

Rheumatic diseases (disorders involving connective tissue) form a broad spectrum of illnesses which vary in duration and severity. The most prevalent symptom of the rheumatic diseases is arthritis which is simply defined as inflammation of a joint (46). The joint inflammation may be present on its own, affecting one or many joints, or be part of a more complex syndrome. In addition, the symptoms may be acute without sequelae or chronic and degenerative resulting in severe deformities. In its various manifestations, arthritis attacks one in every seven Canadians, regardless of age (15).

Because of its prevalence, a great deal of research has been committed towards the therapy, pathogenesis and of course, the cause of arthritis. Putative causes of arthritis which have been investigated include environment, genetics, immunological responses and diet (15). The potential involvement of infectious agents as the cause or trigger of arthritis has also received a great deal of attention. The latter is, on its own, an enormous field of research including the study of bacteria, fungi and viruses (74, 117, 126). As the research contained within this thesis is concerning the latter, the remainder of the introduction will be focused on the role of viruses in inflammatory joint disease.

B. Viral Arthritis

There are a variety of different viral infections in humans and animals that are accompanied by arthritis (43, 74, 82, 115, 117, 131). The incidence of joint involvement may be infrequent (mumps virus, herpesviruses, Coxsackie B virus, adenovirus, and enteroviruses) or common (rubella virus, human parvovirus, and hepatitis B virus). In general the arthritis is acute and without sequelae (115) but there are notable exceptions in which the joint inflammation has become chronic or recurrent, lasting for several years (3, 4, 111). There is ample evidence in the literature to indicate that viruses do play a role in the induction of arthritis but what is unfortunately lacking at this time is information regarding the pathogenic mechanisms involved. However, consideration of virus-host interactions leads to a number of different possibilities.
1. Mechanisms of induction of arthritis by viruses

   a. Direct tissue invasion

The most likely mode of access to the joint for any virus is via circulating lymphocytes during the viremic stage of the infection (43). Once within the joint, the most direct way for a virus to cause acute arthritis would be for it to infect and damage cells in the synovial lining causing inflammation as the host attempts to clear the infection and repair the damage (108, 131). However, depending on the nature of the virus, or if the host is unsuccessful at clearing the infection, the virus may set up a persistent infection (107). The result of a persistent infection within a joint may be to provoke a localized and chronic inflammatory response through the expression of foreign antigens or the deposition of immune complexes (131).

   b. Infection at an extra-articular site

Infection may also be at an extra-articular site such as in lymphoreticular cells. This would provide a virus with the opportunity to re-infect a joint and thus account for the recurrent nature of some viral arthritides. This latter possibility is examined in the following section concerning rubella virus. Alternatively, infection of another organ, such as the liver with hepatitis B virus, can give rise to immune complexes which may then settle in joints and cause inflammation (4, 131).

   c. Autoimmunity

Examples of autoimmune diseases with a suspected viral etiology include diabetes mellitus, multiple sclerosis, rheumatoid arthritis (RA), and systemic lupus erythematosus (SLE) (120). Two mechanisms through which a virus might induce autoimmunity are polyclonal B-cell activation and molecular mimicry (81, 120, 171).

It has been demonstrated that humans and animals normally possess low levels of autoantibodies (antibodies directed against self-antigens) in their sera (44, 87). During viral infection these antibodies often increase but usually subside when the infection is cleared (54, 73, 162).

Ahmed and Oldstone (119) proposed the following as possible mechanisms of B-cell activation. Several viruses have demonstrated mitogenic activity upon B-cells that only requires adherence to the cell surface such as vesicular stomatitis virus (65), adenovirus (62), influenza A virus (23), Sindbis virus (64) and African
swine fever virus (162). Herpes simplex virus and Epstein-Barr virus may also act as B-cell mitogens, but in­fection of the B-cell is required (137). Alternatively, a virus may lead to increased immunoglobulin synthesis by inactivating the suppressor T-cells responsible for switching off their synthesis. Finally, from studies with lymphocytic choriomeningitis virus in mice, the authors suggested that virus-specific helper T-cells may lead to B-cell activation by releasing non-specific B-cell growth and differentiation factors (BCGF and BCDF). They based this upon several observations. Many viruses induce strong T-cell responses that result in the for­mation of virus specific helper T-cells. These latter cells secrete non-specific B-cell stimulators which in turn affect primed B-cells (but not resting B-cells). Therefore B-cells with a variety of specificities may be stimu­lated including those which react with self-antigens. This in itself can cause direct damage to cells and tis­sues, especially in the event of a persistent infection. In addition, disease may be mediated by immune complex formation and deposition.

Autoimmunity may also be a result of antigenic similarity between an infectious agent and host epitopes (2, 81, 120, 137). Examples of such cross-reactivity have been found with bacteria (41, 49, 137, 171), protozoa (137) and viruses (59, 66, 85, 136, 137) and several of these have implications in rheumatic diseases.

The occurrence of HLA-B27 in association with reactive arthritis and ankylosing spondylitis is extremely high (approximately 75% in the former and 90% in the latter) (171). Cross-reactivity between this MHC class I molecule and both Yersinia enterocolitica and Klebsiella pneumoniae have been documented (171). Also anti-DNA antibodies from mice and humans with SLE have been shown to bind to mycobacteria (137).

The involvement of Epstein-Barr virus (EBV) in the development of RA through molecular mimicry has been reviewed (59). Patients with RA have decreased T-cell suppression of EBV-infected B-cells, increased num­bers of EBV-infected cells (approximately three times that of normal individuals) as well as higher titres of anti-RANA antibodies (rheumatoid arthritis nuclear antigen which is EBV specific). Fox et al. (59) have demonstrated that a 62 kd cellular protein shares an epitope with a virus encoded protein, EBNA-1 (Epstein­Barr nuclear antigen 1) but the significance of this in the pathogenesis of RA is still not clear.
2. Animal models of arthritis

To study any human disease, the presence of a suitable animal model is always desirable since it allows for the investigation of certain disease aspects that would be unethical in humans. This is especially true of infectious diseases. Unfortunately, there is no appropriate animal model for the study of the human arthrotropic viruses since almost all of them can only replicate in cells of human origin. However, there are a few examples of arthritis in animals, two of which are naturally occurring and have an identified viral cause.

a. Caprine arthritis-encephalitis

This is a multi-system disease of goats caused by a lentivirus, caprine arthritis encephalitis virus (CAEV) (35, 38). The clinical manifestations of this disease depend upon the age of the goat, with arthritis being the most common in adult goats (greater than one year) and leukoencephalomyelitis being the predominant complication in younger animals (88). The result of infection in young goats is often so severe that the animals either die or need to be destroyed within a week of infection (88). However, those that survive often go on to develop arthritis. Studies by Crawford et al. (38) clearly demonstrated the causal role of CAEV in the arthritis of goats by satisfying the postulates set forth by Koch over 100 years ago (24). Firstly, the virus was isolated from synovial membrane explant cultures from afflicted animals and grown in fetal synovial membrane cells. When this virus was inoculated into healthy goats they developed arthritis and the virus was again isolated from the affected tissue in culture.

One of the more interesting aspects of this disease is that afflicted animals are completely devoid of neutralizing antibodies to the virus. Experimental infection results in detectable antibody responses that remain for up to nine months after infection. Despite the fact that antibodies are produced for all major structural proteins, neutralizing activity could not be demonstrated (88). Lack of neutralizing antibodies in the presence of other virus specific antibodies, may allow greater dissemination of the virus by enhancing infectivity (119) but at present the mechanism of induction of arthritis is not understood.

b. Avian viral arthritis

The only other reported example of naturally occurring virus induced arthritis in animals is avian viral arthritis, which affects chickens (63). The etiologic agent is a reovirus that has been isolated from affected
birds. This disease has received little attention and this is probably due to the presence of a very effective immunization programme (104).

C. Arthrotropic Viruses
Many different viral infections may be associated with joint inflammation during the acute stage of disease. The frequency of arthritis following infection varies from a few isolated cases (human cytomegalovirus, adenovirus, Coxsackie virus B) to large numbers of cases described in population studies (human parvovirus B19 and rubella virus). The viruses included in the research of this thesis (rubella virus, adenovirus and human parvovirus B19) will be reviewed separately here and other arthrotropic viruses will be discussed together at the end.

1. Rubella Virus
Within the Family Togaviridae there are four genera: Alphavirus, Rubivirus, Arterivirus and Pestivirus (130, 158). All togaviruses possess positive sense, single-stranded RNA genomes that are approximately 10-12 kb in size (169). The genome is enclosed within a protein capsid which is in turn surrounded by a lipid envelope. All of these viruses have common structural and replicative characteristics but they differ with respect to host range (158). All the alphaviruses replicate in arthropods in addition to humans or animals, whereas arteriviruses and pestiviruses are only pathogenic in animals. Rubella virus (RV), the only member of the genus Rubivirus, is believed to only infect humans (169).

Rubella was recognized in 1881 by the International Congress of Medicine in London (18) as a distinct disease that mainly affected children, but it was not until 1941 that the association between RV infection in early pregnancy and congenital defects was observed (33, 72). This sparked a great deal of interest in this virus, resulting in the introduction of a live, attenuated vaccine in 1969 in the United States (18).

Also referred to as “German measles”, uncomplicated RV disease is marked by mild fever, rash, sore throat and arthralgia, but often the infection is subclinical (71, 135). When symptoms occur, they are usually transient and mild but post-rubella complications such as thrombocytopenia, encephalopathy and arthritis do occur (19, 164).
The association of arthritis in conjunction with RV infection was first noticed by Sir William Osler, a Canadian physician in 1906 (110). Since that time a tremendous amount of research has been focused on the arthritis following infection with both wild-type and vaccine strains. At present there are no definitive answers concerning the mechanism of joint inflammation elicited by RV, but the more recent reports suggest persistent infection (either within the joint or in circulating lymphoreticular cells) to be the cause of chronic forms of rubella-associated arthritis (RAA) (28, 29, 30, 67, 68, 155).

The arthritis associated with RV is usually of abrupt onset and appears one to five days following infection or two to four weeks post-vaccination (55, 126). The symptoms appear at, or near, the time of seroconversion, often coinciding with the rash (55, 82, 131). Most frequently, RAA is an acute, migratory polyarthritis that is symmetrical in distribution (82) and while it is most often transient, chronic or recurrent symptoms occur in a proportion of patients. The joints most commonly affected are (in descending order) hands, wrists, knees, ankles, elbows and feet (55). Factors predisposing individuals to RAA are sex and age; the highest incidence being in women over 25 years of age. The incidence of RAA is also higher following infection with wild-type strains as compared to vaccine strains but there is also variation between different vaccine strains. Since the time of Osler’s initial report, numerous publications have appeared which comment on these aspects, and these are summarized in a recent report by Tingle et al. in 1986 (154). These investigators had the unique opportunity to examine subjects during an epidemic of rubella in addition to subjects involved in a rubella vaccination programme. Their results confirmed earlier reports (97) of the increased incidence of RAA in women following wild-type infection (52.2% of women compared to 8.7% of men). In addition they demonstrated the reduced frequency of arthritis following immunization with RA27/3 as compared to natural infection (only 13.6% of vaccinated women experienced joint inflammation). No statement regarding gender differences following vaccination could be made, as all the vaccinees were female.

To examine the chronic or recurrent nature of RAA, all of the subjects from both groups were followed for at least 18 months. By doing this, Tingle et al. were able to conclude that increased severity of initial symptoms (objective arthritis compared to arthralgia) preceded joint symptoms of longer duration for both natural and vaccine infections. Of the women vaccinated 15.9% experienced two or more episodes of joint involvement, 9% were still experiencing arthralgia or arthritis six months post-immunization, and 4.5% were still
symptomatic 18 months later. As might be expected the incidence of prolonged symptoms increased in the group infected with wild-type RV, where 43.5% of females experienced two or more episodes of joint symptoms. By six months post-immunization 39.1% were still symptomatic and at the end of the study, 30.4% were still experiencing arthralgia or arthritis. The RA27/3 strain of RV used in this immunization programme is the strain currently in use throughout North America, and while it is associated with far fewer and less severe complications than wild strains or two of the older vaccine strains (HPV77/DE5 and HPV77/DK12) (118), there are at least 2 other strains (Cendehill and To336) that have demonstrated an even lower level of complications (22, 53, 55).

Since RAA is much less common in men and pre-pubescent females, the question of hormonal involvement is often raised with consideration to menstrual cycle or the use of oral contraceptives. Reports by Fox et al. (58) and Weibel et al. (165) suggested that the use of oral contraceptives offered protection from RAA but others (22, 34, 95, 102) disagreed. Similarly, there is controversy concerning the correlation between joint complications and the optimal time of vaccination during the menstrual cycle. Monto et al. (102) and Copper et al. (34) found no correlation at any given point, while others (22, 58, 103, 149) found that there were times within the menstrual cycle when vaccination resulted in decreased joint symptoms. However, the ideal time for vaccination was found to be different for each group.

The numerous observations that RAA may become chronic or recurrent led several groups to search for the involvement of RV in chronic arthritides such as RA and juvenile rheumatoid arthritis (JRA). In 1968 Martenis et al. (100) presented a case study of a woman diagnosed with definite RA, by American Rheumatology Association (ARA) (156) criteria, which began seven days after a typical rubella infection. The authors suggested either repeated exposure to, or persistent infection with RV to be the trigger of the RA due to the lengthy duration of high hemagglutination-inhibition titres. An earlier study (83) reported that 90% of RAA cases were rheumatoid factor (RF) positive compared to 28.6% of uncomplicated rubella infections. The authors did not indicate if their subjects met any other requirements for the diagnosis of RA other than the presence of serum RF and other reports (56, 75, 105) have failed to show a definite relationship between rubella infection and RA. However, there is good evidence to suggest a role of RV in JRA. In 1975 Ogra et al. (105) showed that patients with JRA below the age of 10 had anti-RV IgG titres four to eight times higher
than controls. In addition RV antigen was found in synovial fluid smears in nine out of 25 JRA patients but not in controls with RA, osteoarthritis or septic arthritis. The smears that were positive for RV were negative for mumps virus, polio virus and measles virus antigens. Ten years later Chantler et al. (28) were able to isolate RV from mononuclear cells in seven of 19 children with chronic arthritis (five with JRA and two with seronegative spondyloarthritis). In three cases RV was isolated from both peripheral blood and synovial fluid mononuclear cells (PBMC and SFMC), in two cases from PBMC, and in two cases from SFMC only. It is important to note that the diagnostic criteria for RA and JRA, as set forth by the ARA, are very specific. For this reason many cases of RAA may not be placed into these categories despite their chronic nature.

After an infectious agent is firmly associated with disease, the immediate questions which follow concern the mechanism of pathogenesis, which are crucial in order to design programmes of therapy and prevention. A few possible mechanisms were outlined at the beginning of this introduction.

The development of RAA often coincides, or immediately follows the typical rubelliform rash (34, 159) and since both these symptoms occur at the same time as RV specific antibodies develop, immune complexes have been suspected to cause both (77). The conclusion by Vergani et al. in 1980 (159) favoured the role of immune complexes since 100% of the vaccinees who developed joint symptoms possessed circulating immune complexes but only one asymptomatic patient was positive by the C1q binding assay. In contrast, Ziola et al. (172) examined the circulating immune complexes in 24 patients with acute rubella infection and found that the prolonged presence of circulating immune complexes in two patients was not associated with any symptoms. This was confirmed by Singh et al. (141) who found no correlation between increased levels of circulating immune complexes and the development of arthritis or arthralgia in patients infected with wild-type or RA27/3 virus.

All of these studies examined immune complexes in sera and therefore were unable to comment on the possibility of immune complexes localized to the joints. Given that synovial tissue mononuclear cells have been shown to secrete RV specific antibodies (80), it is conceivable that a persistent or recurrent infection within the joint could result in the localized production of immune complexes.
The ability of RV to directly invade the joint is provided by the virus' ability to infect lymphoreticular cells (26) and the passage of the latter into the synovial fluid (43). The ability of RV to establish persistent infections \textit{in vivo} in various cell-types (122), including cartilage (96, 144) has been documented and Cunningham et al. (40) established human synovial cells persistently infected with RV \textit{in vitro}. These reports, taken together with the numerous reports of RV isolation from PBMC and SFMC (27-30, 56, 67, 68, 78, 106, 145, 155) from patients with various forms of chronic arthritis, suggest that RAA is the result of persistently infected lymphoreticular cells which transport the virus to the joint. Once in a joint, it may then replicate and/or persist giving rise to inflammation.

2. Adenovirus

The human adenoviruses (Ads) belong to the genus \textit{Mastadenovirus} in the Family \textit{Adenoviridae}. This group of viruses has been extensively studied with respect to molecular biology and mechanisms of pathogenesis (for review see reference 80). Since the initial isolation in 1953 from the tonsils of a child, 42 distinct serotypes have been identified.

The virus particles of all Ads are non-enveloped icosahedrons 65-80 nm in diameter (116). Within the protein coat is a double-stranded DNA genome that is approximately 30 kb in size (116). Covalently attached to both 5' ends of the viral genome is a 55 kd terminal polypeptide which is required for viral DNA synthesis (80). There are approximately 11-15 different proteins constituting Ad virions and at least five other virus-encoded proteins that are not assembled into complete particles. These latter proteins are mainly involved in DNA synthesis (80).

There are several different classification schemes used to group Ads. Based upon hemagglutination patterns, this original classification yields three different groups. Ads within the same hemagglutination group were shown to have similar oncogenic potential in animals (all Ads show positive transformation capability in cell culture) and the oncogenicity may be related to the G+C content (143). After grouping by hemagglutination, oncogenicity and G+C content, Ads are assigned specific serotypes using monoclonal antibodies to specific epitopes found on two structural proteins (hexon and fiber).
Ads give rise to a variety of different illnesses in humans as a result of their ability to replicate in epithelial cells at different sites within the body (80). The more common Ad-associated diseases include acute respiratory disease, pneumonia, conjunctivitis, gastroenteritis, and cystitis (52). Several Ad serotypes are highly malignant in animals (143) but there has been no confirmation of Ad involvement in human tumors (20, 142).

Joint involvement is not a common complication of Ad infection but the literature contains a least four separate reports on seven individuals. A review of these separate cases indicates that when Ad is involved in the arthritic process it may do so via different mechanisms.

The earliest report of Ad-associated arthritis was presented by Panush in 1974 (112). Three weeks after vaccination with Ad types 4 and 7, a 19 year old military recruit developed a flu-like illness accompanied by swelling of the hands, wrists and elbows. Virus cultures from synovial fluid, rectal swabs and skin lesions were all negative but serum analysis indicated a 10-fold increase in Ad type 7 specific antibodies between acute and convalescent specimens. There was no similar increase in antibody titres to Ad types 3, 4 or 5. This patient fully recovered in four weeks and the final diagnosis was inflammatory synovitis due to systemic Ad type 7 infection. The increase in the serum complement component C3 and a decrease in C4 during the symptomatic period, together with the absence of virus in the synovial fluid, suggested that the immune complexes were responsible for the symptoms rather than direct invasion of the virus into the joints.

The next report described a nine year old girl who was repeatedly hospitalized during a 20 month period (121). The first diagnosis was recurrent viral pericarditis, when Ad type 7 was isolated from pericardial fluid. During this time there was joint pain, but true arthritis did not appear until eight months later. The arthritis lasted for approximately one year, after which she recovered and suffered no further sequelae. The authors suggested that the initial joint pain, which coincided with the virus isolation favoured direct invasion of the joint and that the subsequent arthritis was immune complex mediated but this is purely speculative since they report no evidence in support of either hypothesis.

The report by Utsinger in 1977 (157) of three adult women who developed arthritis shortly after an upper respiratory illness, is highly suggestive of immune complex mediated disease. Ad infection in these patients
was confirmed by direct isolation from throat swabs and complement fixation. All three patients possessed cryoglobulins (antibodies that precipitate at low temperatures) and immune complexes in both serum and synovial fluid. These cryoglobulins reacted with antiserum specific for Ad. In addition two out of three had decreased levels of serum C3 and C4, the classical indicator of immune complex mediated disease (166).

The most recent report of Ad-associated arthritis is also the most convincing. In 1985, Fraser et al. (60) reported a 29 year old male who was diagnosed as having acquired hypogammaglobulinemia. Clinical tests indicated rheumatoid-like arthritis involving fingers, wrists, elbows, shoulders and knees. His right knee required total replacement 16 months after a synovectomy was performed on the same joint. Tissue samples from both procedures were cultured and when these culture media were used to infect other cells, cytopathology appeared. This cytopathic effect was specifically neutralized by antibodies to Ad type 1. Similar cultures of peripheral lymphocytes were negative. It is quite likely that the immune deficiency of this patient contributed to his disease perhaps by allowing greater dissemination or recurrent infection with the virus. This is a valuable report because the direct isolation of a virus from affected joint tissue is not a common occurrence. What is also important is that the virus was isolated twice over a 16 month period of time, demonstrating that the synovial infection was not transient. Moreover, the absence of the virus in the circulation suggests a tropism for the joints.

Two conclusions to be drawn from these reports are that one, arthritis is not a common complication of Ad infection and two, when it does occur the mechanism of disease induction may vary depending on the condition of the patients immune system. When the patient has no underlying immunological dysfunction, the arthritis is more likely to be a result of immune complex deposition which appears to be acute and self-limited. However, when the infection is not impeded by the host’s immune defences, the virus may invade and cause complete destruction of the joint.

3. Human parvovirus, B19

The virus family, Paroviridae, comprises three genera: Parovirus, Dependovirus and Densovirus. Dependoviruses, (formerly adeno-associated viruses) gain their name from the fact that they require the “help” of a co-infecting adenovirus or herpesvirus for complete replication (21). Densoviruses, on the other hand, replicate autonomously in insects (21). Although several dependoviruses have been shown to infect humans,
they have not yet been associated with any disease (113) and so neither they nor the densovirdes will be dis-
cussed further.

Within the genus *Parvovirus*, only the one designated B19 has been firmly associated with human disease. There are reports of two other potential human parvoviruses but at present their identification is somewhat dubious (7, 140, 147). One of these, RA-1, was presumed to have been isolated from patients with RA, but there is still some debate as to whether the virus came from the joint material or from the mouse brains through which the joint extract was passaged for viral amplification. Stierle et al. (147) performed similar experiments but their results have further confused the matter. The first isolate by Simpson et al. (140), was shown to be antigenically distinct from other known parvoviruses; RA-1 did not react with antibodies to other parvoviruses nor did anti-RA-1 antisera react with other parvoviruses. The confusion arises when considering the second isolate (147) which was also antigenically distinct but was shown to be morphologically different. Electron microscopy indicated that the virus isolated by Stierle et al. was approximately half the size of the one isolated by Simpson et al. Further studies are obviously necessary to settle the controversy.

Human parvovirus B19 (hereafter called B19) is a relatively new virus with an interesting history. It was only identified 15 years ago and is unusual because it was isolated and identified before it was associated with disease. B19 was discovered by accident in 1975 by Cossart et al. (36) during antigen screening tests for hepatitis B virus (HBV). Unexpected precipitin lines from serum of HBV negative blood donors resulted in its detection. It was placed in the genus *Parvovirus* based on its physicochemical properties (37, 148). It is a small (20-25nm) (36), non-enveloped virus (21) containing a linear, single-stranded DNA genome of 5.5 kb (148). It is capable of complete replication without a co-infecting virus but, like all the autonomous parvoviruses, it is restricted to rapidly dividing cells, requiring the S-phase of the cell-cycle in order to replicate (124, 151).

Two diseases, aplastic crisis and erythema infectiosum, were suspected as having a viral etiology based upon epidemiological and clinical observations. Six years after the discovery of B19, Pattison et al. (114) discovered B19 in the sera of two children with hemolytic anemia who were undergoing aplastic crisis. Subsequent reports (13, 93, 134) confirmed that up to 90% of the cases of aplastic crisis were attributable to this virus due to the fact that it is cytotoxic to erythroid progenitor cells (111, 170). Two years later Anderson et
al. (11) confirmed that B19 was the infectious agent responsible for the pediatric disease, erythema infectiosum, (also referred to as Fifth disease or 'slapped cheek' syndrome due to the characteristic rash) (6). This is a mild exanthematous disease that may be clinically indistinguishable from rubella infection (153). Symptoms associated with adult infection tend to be more severe and often the rash is absent.

Many aspects of B19 disease were clarified by Anderson et al. (10) during examination of experimentally infected adults. Results from these investigations indicated that the route of transmission is via the upper respiratory tract and that viremia occurs one week after infection but only in IgG negative volunteers. The viremia lasts for approximately five days and may reach titres of $10^{11}$ genome copies per millilitre of serum and at the same time virus may detected in nasal washes. The clinical manifestations of B19 infection appeared in two phases. The first phase occurred at the time of viremia and was characterized by fever, malaise, myalgia, chills and itching. The second phase began 17 days post-inoculation and included a maculopapular rash on the trunk and limbs, with joint symptoms following 24 hours after the rash appeared. This study also confirmed that erythropoiesis was interrupted resulting in a slight drop in hemoglobin in normal individuals.

The most notable complication of B19 infection in normal individuals is arthritis. The literature contains numerous reports associating this virus with inflammatory joint disease and these are summarized below (1, 8, 10-14, 17, 32, 36, 45, 47, 48, 50, 70, 84, 89, 93, 94, 99, 113, 123, 128, 129, 133, 161, 163, 167).

Two studies (1, 161) in the late 1960's were of the first to report an association between joint involvement and erythema infectiosum, almost 10 years prior to the identification of the causative agent. Since then many similar case reports and larger epidemiological studies have followed which have defined many of the characteristics of B19-associated arthritis and shown that there are striking similarities to RAA.

Firstly, the frequency of B19 arthritis is much higher in adults than in children; one early study of erythema infectiosum indicated that only 5% of children less than nine years of age experienced joint pain whereas 77% of adults greater than 20 years old had similar symptoms (1). One study involving children (123) reported that acute arthritis was of a longer duration in children as compared to adults (average of 54 days in the
children and 13 days in the adults) but of the reported cases of chronic arthritis (greater than 2 months) all have been adult women with the exception of one 11 year old boy (123, 167).

A second generalization is that in adults, women are affected much more often than men and when it does occur in men it is usually less severe and of shorter duration. Regrettably not all the reports distinguished males from females with respect to symptoms, but of the reports including specific information concerning affected men, (47, 84, 128, 161) the average duration of joint symptoms was nine days compared to the average of 60 days seen in adult women.

B19-associated arthritis is usually symmetrical and may involve a variety of joints in different patients regardless of age or sex. The most frequently involved joints are, (in descending order) knees, fingers, hands, wrists and ankles (1, 13, 17, 45, 47, 48, 50, 70, 84, 99, 123, 128, 129, 133, 161, 167). Less commonly affected joints include elbows, back, feet, hips, shoulders and neck. In all the literature reviewed, there were no reports of joint destruction.

All of the above reports utilized serum antibody analysis to confirm B19 infection at or near the time of arthritis and collectively they have clearly demonstrated that B19 is involved in the pathogenesis of arthritis. With its role firmly established, more in-depth studies concerning pathogenic mechanisms are beginning to appear.

Induction of joint inflammation by B19 is largely believed to be immune complex mediated because the arthritis usually coincides with the increase in antibody titres in a manner similar to RAA. However, this is largely speculative since synovial fluid analysis for B19 antigen or DNA are lacking in all but two of the included studies.

The first of these reports (32) examined 76 patients with RA and inflammatory arthritis. They identified six patients with evidence of recent B19 infection (presence of IgM antibodies specific for B19) but did not find either B19 antigen or DNA within the synovial fluid. However, two years later, Dijkmans et al. (45) reported the detection of B19 DNA in the synovial fluid of a 33 year old woman with B19-associated arthritis. The negative results of the former group may be a reflection of sampling time. They examined samples taken up to five months after the onset of symptoms whereas the positive result was achieved with a specimen that was
taken only 24 hours after the arthritis appeared. Although the results reported by Dijkmans et al. do not distinguish between direct viral invasion and immune complex mediated disease, it is important to note that when arthritis does occur in association with B19 infection it is after the viremic phase (10).

Given the high incidence of arthritis associated with B19 infection, it is not surprising that several groups have attempted to link the virus to RA but the results of these efforts require confirmation. Cohen et al. (32) demonstrated that 4 out of 69 patients with RA experienced B19 infection at the onset of their disease but as there was no indication of chronic infection, they concluded that B19 was the trigger in a genetically predisposed population. Another study (94) concluded that B19 was not involved in the pathogenesis of RA because B19-specific IgG levels were similar between controls and patients. However, these latter findings may be the result of insensitive techniques and poor sampling times (113, 134, 153).

Several rheumatic diseases are associated with specific HLA haplotypes; HLA-B27 is present in up to 90% of patients with ankylosing spondylitis and approximately 75% of reactive arthritis cases (171). In addition, there is also a correlation between RA and the presence of HLA-DR4 (5, 126). It was therefore of interest to determine whether B19 arthritis was associated with a specific HLA haplotype. Unfortunately, these results, like those searching for a connection between B19 and RA, were ambiguous.

Dijkmans et al. (48) reported that out of five patients with B19 associated arthritis only one was DR4 positive and all five were B27 negative. In contrast, Klouda et al. (89) examined 18 patients with B19-associated arthritis and found that 67% were DR4 positive as compared to 36% of controls. Interestingly, all of these patients were negative for HLA-DR1 (compared to 20% of controls) suggesting that this antigen may provide resistance to B19 infection.

The current literature provides more than sufficient documentation to conclude that B19 is involved in the arthritic process, but the conflicting reports concerning its involvement in RA, HLA association and its mechanism of action make it clear that further investigation is required.

4. Other Arthrotropic Viruses
Arthritis is a symptom of infection by various other viruses including hepatitis B virus, herpesviruses, mumps virus, enteroviruses, alphaviruses and smallpox virus. At present there is no unifying theory for the
pathogenic mechanisms of viral arthritis. Because the arthrotropic viruses themselves, are so diverse, it would seem likely that their modes of induction of arthritis will also vary widely.

Hepatitis B virus (HBV) infection was one the first viral infections reported to be associated with joint inflammation (69) and this is probably one of the best examples of immune complex mediated disease (132, 138). Arthritis is not associated with either hepatitis A virus or hepatitis non-A non-B infections and due to diagnostic confusion between these three agents it is difficult to comment on the frequency of joint inflammation associated specifically with HBV (4, 82).

Five alphavirues (Family Togaviridae) are also strongly associated with arthritis (57, 152) but due to their geographic distribution (Africa, South America and the South Pacific) they have not received as much attention. These five viruses (chikungunya virus, o'nyongnyong virus, Mayaro virus, Ross river virus and Sindbis virus) are all transmitted to humans via mosquito vectors. The symptoms of alphavirus-associated arthritis are similar to RAA (57) and that the frequency of their association with joint inflammation is high is reflected in the names of two of them. In the language of East African tribes, chikungunya means “that which bends” and o’nyongnyong means “joint-breaker”. One report of arthritis following an outbreak of chikungunya fever (57) noted that four out of five of the patients with chronic arthritis were HLA-B27 positive.

The ability to stimulate B-cells (see Introduction), makes EBV a good candidate for an etiologic agent responsible for rheumatic diseases such as RA and SLE, due to the myriad of autoantibodies present in these diseases (42, 168).

The occurrence of arthritis has been documented for other human viruses (herpesviruses, enteroviruses, mumps virus and smallpox virus) but the numbers of reported cases are few. For more detail than may be included here concerning these viruses, there are numerous reviews (3, 4, 25, 43, 55, 74, 82, 86, 115, 117, 126, 131).

D. Purpose of the Study

The literature contains numerous accounts of the arthritogenic nature of many viruses but unfortunately the pathogenic mechanisms involved in the induction and perpetuation of joint inflammation are poorly understood. At least in part, this is due to the fact that there has been no model system to study the interaction of
viruses with joint tissue. An animal model would be ideal, as it would allow for the investigation of immunopathological mechanisms but as previously mentioned, the majority of human arthritogenic viruses are restricted to cells of human origin. However, the mechanism of direct invasion may be studied \textit{in vitro} assuming that if a virus is unable to replicate in joint cells under ideal conditions, (i.e., in the absence of an intact immune system) it is unlikely that it could do so \textit{in vivo}.

A culture system using cells and tissue of joint origin was therefore established to examine the replicative abilities of three different viruses, two of which are highly arthrotropic (RV and B19), and a third whose association with arthritis is much less frequent (Ad). In addition, the study of RV was expanded to a comparison of eight different strains (three vaccine and five wild-type) whose association with joint inflammation was known to vary. The permissiveness of both primary synovial membrane/cartilage cells and tissue to these viruses has been examined and the ability of the viruses to produce long-term persistent infection also assessed. The validity of the model system has been determined by comparing the results obtained with clinical data on the relative arthritogenicity of the various test viruses.
II. ABBREVIATIONS

Ad = adenovirus

ALCM = agar leukocyte conditioned medium

B19 = human parvovirus

BM = bone marrow

BSA = bovine serum albumin

CPE = cytopathic effect

DAB = diaminobenzidine tetrahydrochloride

DMEM = Dulbecco's minimal essential medium

dUTP = 2'-deoxyuridine 5'-triphosphate

EBV = Epstein-Barr virus

EDTA = ethylenediamine tetraacetic acid

EPO = erythropoetin

FBS = fetal bovine serum

FC = fetal chondrocytes

FFU = focus forming unit

fg = femtogram, $10^{-15}$ gram

HBSS = Hanks balanced salt solution

HCl = hydrochloric acid

Hep-2 = human epithelial cells

HIFBS = heat-inactivated fetal bovine serum
HLA = human lymphocyte antigen
IgG = immunoglobulin gamma
IP = immunoperoxidase
JRA = juvenile rheumatoid arthritis
kb = kilobase
kd = kilodalton
MHC = major histocompatibility complex
MOI = multiplicity of infection
PBS = phosphate buffered saline
PBS+ = phosphate buffered saline with calcium and magnesium
p.i. = post-infection
PF = paraformaldehyde
RA = rheumatoid arthritis
RAA = rubella-associated arthritis
RPMI = Roswell Park Memorial Institute
RV = rubella virus
SDS = sodium dodecyl sulphate
SLE = systemic lupus erythematosus
SMC = synovial membrane/cartilage
TAE = Tris, acetate, EDTA buffer
TCID = tissue culture infectious dose
TE = Tris, EDTA buffer
TES = Tris, EDTA, SDS buffer
TNE = Tris, NaCl, EDTA buffer
Tris = tris(hydroxymethyl)aminomethane
Vero = African green monkey kidney
III. MATERIALS

A. Materials

1. Cells

Vero (African green monkey kidney) cells, and Hep-2 (human epithelial) cells were obtained from American Type Culture Collection (ATCC). FC, (fetal chondrocytes) were a gift from Dr. L Sokoloff, New York, and SMC (synovial membrane/cartilage) cells were prepared from human fetal knee joints. Bone marrow (BM) cells from a patient with chronic myelogenous leukemia were kindly provided by Dr. K Humphries, Terry Fox Laboratories. See Methods for details of cell maintenance.

2. Viruses

   a. Rubella virus strains

      Cendehill - Röhm Pharma
      RA27/3 - Meruvax II Merck, Sharp and Dohme
      HPV77/DE5 - Meruvax I Merck, Sharp and Dohme
      M33 - ATCC wild type
      Therien - adapted in Finland
      Thomas - congenital rubella syndrome isolate
      1B2 - plaque purified patient isolate
      5209R - B.C. Provincial Health Laboratory wild-type

   b. Adenovirus type 2 - Dr. B Ziola, University of Saskatchewan

   c. Human Parvovirus B19 - Dr. P Tattersall, Yale University

3. Cell culture

All cell culture materials were obtained from Gibco (Burlington, Ontario) except for Biorich medium (Flow Laboratories, Mississauga, Ontario)

4. Antibodies

The mouse monoclonal antibody to adenovirus 5 was purchased from Biocan Scientific (Mississauga, Ontario) and recognizes a groups specific antigen. The rabbit polyclonal sera to B19 fusion proteins were kindly provided by Dr. P Tattersall. See Methods for rubella virus antiserum preparation.
5. Miscellaneous
All photographs were taken with Kodak 160 ASA Tungsten film.

B. Solutions

1. PBS pH 7.0 = 150mM NaCl
   7.5mM Na₂HPO₄
   2.5mM NaH₂PO₄·H₂O

2. PBS + pH 7.3 = 140mM NaCl
   2.7mM KCl
   8.3mM NaH₂PO₄
   1.5mM K₂PO₄
   0.9mM CaCl₂
   0.5mM MgCl₂

3. TNE pH 7.4 = 10mM Tris
   100mM NaCl
   1mM EDTA

4. TAE pH 8.5 = 40mM Tris-acetate
   2mM EDTA

5. PF = 4% (w/v) paraformaldehyde in 0.1M phosphate pH 7.4

6. Hematoxylin = 25% (v/v) 1,2 ethanediol
   0.2% (w/v) hematoxylin
   0.4mM NaI₀₄
   12.6mM Al₂(SO₄)₃·15 H₂O
   2% (v/v) acetic acid
7. Eosin = 0.05% (w/v) Eosin Y

0.25% (v/v) acetic acid

70% (v/v) ethanol

8. HBSS = Hanks Balanced Salt Solution

5.4mM KCl

0.44mM KH$_2$PO$_4$

136.9mM NaCl

4.2mM NaHCO$_3$

0.33mM Na$_2$HPO$_4$·12H$_2$O

5.5mM D-glucose

9. HBSS+ = 1.26mM CaCl$_2$

0.81mM MgSO$_4$

0.001% (w/v) phenol red

HBSS (as above)

10. Transport medium = HBSS +

300U/ml penicillin

300µg/ml streptomycin

0.75µg/ml amphotericin

11. Collagenase buffer = 1% (w/v) collagenase II (Worthington Biochemical Corp., Freehold, New Jersey)

= 0.1M Tris pH 7.5

= 0.1M CaCl$_2$
12. **DE buffer pH 7.2**

   - 32mM Tris
   - 25mM HCl
   - 50mM NaCl
   - 10mM NaN₃

13. **Luria broth**

   - 1% (w/v) tryptone
   - 0.5% (w/v) yeast extract
   - 0.5% (w/v) NaCl
   - 1mM NaOH

14. **glucose/Tris/EDTA**

   - 50mM glucose
   - 25mM Tris Cl pH 8.0

15. **TE**

   - 10mM Tris Cl (pH 7.5)
   - 1mM EDTA (pH 8.0)

16. **Lysis buffer**

   - 4ml TES buffer
   - 2ml 3M NaCl
   - 200ug/ml Proteinase K

17. **TES buffer**

   - 10mM Tris Cl (pH 7.5)
   - 100mM EDTA (pH 8.0)
   - 6% w/v SDS
18. Culture medium - maintenance of cells
   a. Vero cells - Biorich + 5% FBS
   b. Hep-2 cells - Biorich + 3% FBS
   c. FC - RPMI + 15% FBS
   d. SMC cells - RPMI + 9% FBS
   e. BM cells - Iscove's DMEM + 10% FBS

The concentration of antibiotic/antimycotic was the same for all cell types: 100U/ml penicillin, 100μg/ml streptomycin and 0.25μg/ml amphotericin B.

19. Culture medium - for infected cells

When the above cells were infected, the FBS was heat-inactivated (56°C for 60 minutes) and used at half the normal concentration. The antibiotic/antimycotic concentration remained the same.
IV. METHODS

A. Cell Maintenance

All cell and organ cultures were incubated in an atmosphere of 5% CO₂ unless otherwise indicated. The majority of cell lines used were adherent and therefore were passaged in the same manner. Confluent monolayers were washed with phosphate buffered saline (PBS) and incubated at 37°C in 0.25% trypsin (w/v in Hanks Balanced Salt Solution, HBSS) until the cells detached. The cells were then thoroughly resuspended in culture medium and distributed to new tissue culture flasks or plates. The split ratio varied from 1:2 for the fetal chondrocytes (FC) to 1:20 for the human epithelial (Hep-2) cells. African green monkey kidney (Vero) cells and Hep-2 cells were maintained as continuous lines and the FC were maintained until approximately passage 21 when their growth slowed dramatically. The synovial membrane/cartilage (SMC) cells were primary cells and were only used until passage number 3.

Bone marrow (BM) cells, stored in liquid nitrogen, were rapidly thawed and slowly added to approximately 10ml of Iscove’s DMEM without fetal bovine serum (FBS). The cells were pelleted (1000g for 10 minutes) and washed in Iscove’s DMEM without FBS. After centrifugation, the cells were resuspended in complete medium (Iscove’s DMEM, 20% FBS, 5% ALCM and 1.5 units/ml EPO) and incubated for 2.5 hours to allow dissociation of clumped cells prior to infection (see Viral Growth Curves).

B. Synovial Membrane/Cartilage Cell Production

Human fetal knee joints (ranging in age from 15-20 weeks gestation) were collected in transport medium from Anatomical Pathology at Vancouver General Hospital. Skin, muscle and fat tissue were aseptically removed to expose the joint. The entire joint was cut into 2-3 pieces and placed in RPMI, 4.5% HIFBS plus antibiotic/antimycotic and incubated for 15 hours. The medium was then removed and the pieces were incubated in collagenase for 3 hours at 37°C. The collagenase solution was replaced with 0.25% trypsin (w/v in 0.6mM EDTA) and the tissue was agitated with a magnetic stirrer for 15 minutes at 35°C in the absence of CO₂. The released cells were removed and placed in RPMI, 9% FBS and the tissue was treated again with trypsin/EDTA 3 times. The cells were pooled from each cycle, pelleted (1000g for 10 minutes) and washed in PBS. The cells were resuspended in RPMI medium, 9% FBS and allowed to settle overnight in 60 x 35 mm
tissue culture plates. The next day the cells were washed 2 times in PBS and incubated until confluent. At that time the cells were passaged 1:3 and this was referred to as passage 1.

C. Preparation of Intact Tissue

The fetal knee joints were collected and prepared as described above except that the joints were cut into 5-8 pieces depending on their size. These pieces were infected without further treatment.

D. Virus Stock Production

1. Rubella Virus (RV) Strains

All 8 strains of RV (see Materials for origins) were prepared by infecting a single subconfluent flask of Vero cells at a multiplicity of infection (MOI) of 1-10 for an adsorption period of 4 hours at 35°C. The inoculum was removed and the cells incubated in Biorich medium 2.5%, heat-inactivated fetal bovine serum (HIFBS) for 3 days or until 50% of the cells were exhibiting cytopathic effect (CPE). At that time the culture supernatant was distributed to 5 fresh flasks of subconfluent Vero cells. Again the cultures were incubated for 3 days or until 50% CPE was present. At that time the medium was removed, replaced with 10ml of fresh medium and incubated for 24 hours. The virus stock was prepared by clarifying the culture supernatant of cell debris by centrifugation (3000g for 10 minutes) and storage in aliquot parts at -70°C.

2. Adenovirus (Ad)

Subconfluent monolayers of Hep-2 cells were infected at an MOI of 1-5 for an adsorption period of 60 minutes at 37°C. The inoculum was removed and replaced with Biorich medium, 3% HIFBS. When 50-60% of the cells were exhibiting CPE, the culture medium was replaced with 10ml of fresh medium and 24 hours later, cell debris was removed from the culture medium by centrifugation (3000g for 10 minutes) and stored in aliquot portions at -70°C.

E. Virus Titration

1. Rubella Virus Strains

All strains of RV were titred in duplicate by infecting subconfluent Vero cells with serial 10-fold dilutions of virus in Biorich medium, 1% HIFBS. After a 4 hour adsorption period, the inoculum was replaced with an overlay of 0.5% agarose (Seakem, Mandel Scientific, Guelph, Ontario) in 199 medium. The focus forming
units (FFU) were counted 7-10 days post-infection (p.i.) for HPV77/DE5 and the wild-type strains. In the case of Cendehill and RA27/3, the monolayers received a second overlay containing neutral red on the third or fourth day following infection. These were counted the day after addition of the neutral red.

2. Adenovirus

Five and ten-fold dilutions of virus in Biorich, 1% HIFBS were distributed into 24-well plates (0.5ml/well) in quadruplicate. 4 X 10⁴ Hep-2 cells were then added in an equal volume of 3% HIFBS medium. Wells with CPE were marked 10 days p.i. and the virus titre determined by the Karber method (39) where,

\[
\log_{10} \text{TCID} = 0.5 + D \cdot S
\]

\( \text{TCID} \) = tissue culture infectious dose

\( D = \log_{10} \) of the highest dilution where all wells show CPE

\( S = \) sum of the proportions of the wells demonstrating CPE

Sample Calculation:

<table>
<thead>
<tr>
<th>Dilution</th>
<th>( \log_{10} )</th>
<th># Wells with CPE</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>10-4</td>
<td>-4.0</td>
<td>4/4</td>
<td>1</td>
</tr>
<tr>
<td>2X10-5</td>
<td>-4.7</td>
<td>4/4</td>
<td>1</td>
</tr>
<tr>
<td>10-5</td>
<td>-5.0</td>
<td>4/4</td>
<td>1</td>
</tr>
<tr>
<td>2X10-6</td>
<td>-5.7</td>
<td>4/4</td>
<td>1</td>
</tr>
<tr>
<td>10-6</td>
<td>-6.0</td>
<td>3/4</td>
<td>0.75</td>
</tr>
<tr>
<td>2X10-7</td>
<td>-6.7</td>
<td>1/4</td>
<td>0.25</td>
</tr>
<tr>
<td>10-7</td>
<td>-7.0</td>
<td>1/4</td>
<td>0.25</td>
</tr>
</tbody>
</table>

\[
\log_{10} \text{TCID} = 0.5 + (5.5) \cdot 1.25^* \\
= -6.25
\]

\( \text{TCID} = 10^{-6.25} \)

\( \text{TCID} = 1.78 \times 10^{6}/0.5\text{ml} \)

\( = 3.5 \times 10^{6}/\text{ml} \)

*Note: \( S \) is calculated using the proportions from dilutions separated by a factor of 10
F. Fixation of Cells and Tissue

1. Paraformaldehyde (PF) fixation

Cells to be fixed for immunoperoxidase (IP) staining were washed in PBS and covered with PF for 5 minutes at room temperature. The PF was replaced with 0.15M ethanolamine for one minute. This was removed and the cells were stored at 4°C under 70% ethanol until tested.

Pieces of tissue were washed in PBS and covered with PF for 3 hours on ice. The PF was replaced with 0.15M ethanolamine for 20 minutes and the tissue was stored at 4°C in PBS until sectioned.

2. Acid/alcohol fixation

Cells to be fixed were washed in PBS and covered in a solution of 5% acetic acid in 95% ethanol for 10 minutes at -20°C. The cells were washed four times in PBS and examined immediately for virus antigen by IP staining.

G. Viral Growth Curves

Subconfluent monolayers of cells (Vero, Hep-2 or SMC cells) in tissue culture plates (10 x 30 mm) were infected with virus (RV strains or Ad) diluted in PBS. An extra aliquot of virus was prepared at the time of infection so that the input virus titre could be determined. At various times (see Results) one plate of cells was harvested to prepare supernatant and cell-associated virus, and another was fixed with PF for subsequent viral antigen detection by IP staining.

Supernatant virus was prepared by removing the culture medium and freezing at -70°C. Cell-associated virus was obtained by trypsinizing the cells and washing the plates 3-4 times with PBS containing calcium and magnesium (PBS+) once the cells had detached. The cells were then pelleted (3000g for 10 minutes) and resuspended in 0.5ml of 0.02M Tris for 5 minutes. They were then sonicated for 5 seconds, mixed with an equal volume of TNE (Tris, NaCl, EDTA) buffer and stored at -70°C. Extra plates were established at the outset so that viable cell counts (trypan blue exclusion) could be performed at various times during the infection.
To infect the BM cells with B19, the cells were pelleted, resuspended in 0.5ml of Iscove's DMEM (no FBS) and divided into 2 tubes. Ten microlitres of B19 were added to one tube resulting in an MOI of approximately 20. The virus was allowed to adsorb for 1 hour at 35°C. After the inoculum was removed, the cells were washed in medium without additives, and finally resuspended in 9ml of complete medium (Iscove's DMEM, 20% FBS, 5% ALCM and 1.5 units/ml EPO). To harvest the viral DNA, the cells were gently resuspended with a pipette and 3ml was removed. This aliquot was centrifuged to separate the cells from the culture medium and the DNA was extracted from both (see Section I).

H. Persistent Infections

1. Synovial Membrane/Cartilage Cells

Two infected and two control cultures of SMC cells were established in RPMI medium, 4.5% HIFBS. Samples of supernatant medium were taken 3, 6, and 9 days p.i. On day 9, one infected and one control plate were fixed for viral antigen detection by IP staining and the other set was passaged 1:2 (passage 1) into RPMI, 9% FBS. The cells were passaged every week for 80 days, or as long as the cultures could be maintained.

2. Tissue

Pieces of intact joint tissue were infected in undiluted stock virus for 4 hours and then maintained in RPMI medium, 4.5% HIFBS. Uninfected pieces derived from the same tissue specimen were used as controls. The medium was changed every 3-4 days and the removed medium saved for virus titration. At the indicated times the tissue was fixed. Paraffin embedding and sectioning were done by Anatomical Pathology at Vancouver General Hospital. The sections were mounted onto glass slides that were acid cleaned (1% HCl in 70% ethanol for 20 minutes) and then dipped in a 0.5% solution (v/v in distilled water) of Kodak Photo-flo to improve tissue adherence (139).

I. Immunoperoxidase Staining

1. Cells

Endogenous peroxidase activity was eliminated by incubating the cells in 3% H₂O₂ in methanol for 30 minutes. Non-specific binding was then blocked by incubating the cells in a solution of 5% (w/v) bovine serum albumin (BSA) in PBS for 60 minutes. The cells were then incubated for 60 minutes in the primary antibody
(mouse or rabbit in origin) which was diluted in 0.5% NP40 in PBS. After 3 washes in PBS, the cells were incubated for 60 minutes with a biotinylated secondary antibody which was directed against the primary antibody (either goat anti-mouse or goat anti-rabbit). The avidin-biotin complex, ABC, (Vectastain, Mississauga, Ontario) was used at half the recommended concentration for 60 minutes after excess secondary antibody had been removed. A 10 minute incubation in a solution of 0.05% diaminobenzidine tetrahydrochloride (DAB), 0.001% \( \text{H}_2\text{O}_2 \) in PBS followed removal of excess ABC. Finally the cells were counterstained with hematoxylin and eosin, rinsed quickly in distilled water and air dried.

To ensure that observed staining was not a result of non-specific binding during the different incubations, two controls were included with every experiment. Uninfected cells were examined with the same primary antibody to control for specificity of the anti-viral antibody and infected cells incubated with all reagents but the primary antibody were examined to ensure that subsequent reactions were not due to non-specific binding. When either of these controls demonstrated stain (background) the appropriate reagent was absorbed and/or diluted until the controls were free of stain. In addition, the use of 0.5% NP40 in PBS as the diluent for the primary antibody helped to prevent background staining. Acid/alcohol was also used as an alternative fixative to PF for RV infected cells. However, it use was discontinued as it eliminated the antigen stain of Cendehill and RA27/3 strains.

2. Tissue Sections

Duplicate sections on slides were warmed in a 70°C oven to soften the paraffin. Without allowing them to cool, the sections were placed in warm (70°C) xylene (2 changes of 5 minutes each). The sections were then rinsed in 95% ethanol for 5 minutes and incubated in a solution of 3% \( \text{H}_2\text{O}_2 \) in methanol for 30 minutes. The sections were then hydrated by successive 5 minute incubations in 70% ethanol followed by PBS prior to the blocking step with 5% BSA in PBS (30 minutes at room temperature or overnight at 4°C). The remainder of the staining procedure was the same as described for the cells except that more rigorous washing procedures were used. Instead of 3 changes of PBS, the sections were washed in 0.1% SDS (sodium dodecyl sulphate) in PBS for 2 minutes followed by 2 washes in PBS for 5 minutes each. These more stringent washes were required to eliminate background staining in tissue. The duplicate sections on each slide were used to provide a test sample and a secondary antibody control on consecutive sections of joint tissue.
J. Preparation of Rubella Antiserum

New Zealand white rabbits were hyperimmunized with RV antigen which had been prepared by the ultracentrifugation of large volumes (10 litres) of virus derived from the culture fluid of 8-10 roller bottles of infected Vero cells. The rabbit serum was ammonium sulphate precipitated and passed through a column of DEAE-Sephacel (Pharmacia, Baie D'Urfe, Quebec) to enrich for IgG antibodies. The column was equilibrated and washed with DE buffer (see Solutions) and material eluted in the void volume was retrieved. The peak fractions were pooled and concentrated with polyethylene glycol to approximately half the volume and then dialyzed against DE buffer overnight. Following dialysis, the anti-sera were stored at -20°C in aliquot parts. These anti-sera were absorbed to uninfected cells fixed in PF or air-dried and the dilution of each preparation giving optimal IP staining was determined using RV-infected Vero cells.

K. Probe Preparation and Labelling

*E. coli*, strain HB101, transformed with the vector pAT153 containing a B19 specific sequence was kindly supplied by Dr. MJ Anderson (9). The procedure for large scale plasmid isolation (16) was slightly modified. The plasmid containing bacteria were grown overnight at 37°C in 40ml Luria broth containing 12ug/ml tetracycline. The cells were pelleted at 7800g for 10 minutes in a Sorvall SS-34 rotor using 30ml Corex tubes. After resuspension in 2.5ml glucose/Tris/EDTA, 5.0ml of 1% (w/v) SDS in 0.1M NaOH was added. After a 10 minute incubation on ice, 3.75 ml of potassium acetate (pH 4.8) was added and the solution was again incubated on ice for 10 minutes. Chromosomal DNA was pelleted by centrifuging at 7800g for 20 minutes. Two volumes of ice-cold absolute ethanol were added to the supernatant and the plasmid was allowed to precipitate overnight at -20°C. The plasmid was pelleted (7800g, 20 minutes), dried and resuspended in 1ml TE buffer. LiCl (0.5ml of 5M) was added to precipitate the RNA which was then pelleted by centrifuging at 14,000g for 5 minutes. One volume of isopropanol was added to the supernatant which was then incubated for 10 minutes at room temperature. The plasmid DNA was pelleted (14,000g, 5 minutes), dried and then resuspended in 100ul of Tris/EDTA buffer (TE), re-precipitated with 250ul absolute ethanol, pelleted, dried and resuspended in TE.

The plasmid preparation was restricted with Pst I (Pharmacia) and separated from the vector by electrophoresis in an agarose gel (1.2% w/v agarose in TAE with 1mg/ml ethidium bromide) at 60V. The in-
sert was isolated from a gel slice using GeneClean (BioCan Scientific, Mississauga, Ontario) which utilizes NaI to dissolve the agarose. In the presence of this salt, DNA binds to glass powder. The DNA is then washed to remove salt and then eluted into sterile distilled water by incubating at 50°C for 3-5 minutes (160). The purified insert was labelled via random priming with digoxigenin-11-dUTP according to the instructions supplied by Boehringer Mannheim (Laval, Quebec).

L. DNA Extraction and Dot Blots

B19 DNA was extracted from the infected cells and culture supernatants by a modified method of Hirt (127). The virus particles were precipitated from the culture media by the addition of polyethylene glycol (MW 3500) to a concentration of 10% (w/v) and incubated for 4 hours at 4°C prior to centrifugation at 7800g for 20 minutes. The precipitated pellets were treated in the same manner as the cells. After washing in cold PBS 3 times, 1ml of lysis buffer was added and the viscous solution was transferred to a 15ml Corex tube and incubated at 50°C for one hour. The samples were then stored at -20°C so that all samples could be extracted at the same time. After thawing, an equal volume of phenol/chloroform (the latter was 24 parts chloroform and 1 part iso-amyl alcohol) was added and the phases were separated by centrifugation (12,000g, 15 minutes at 4°C). The aqueous layer was carefully removed and again treated with phenol/chloroform. The DNA was recovered by ethanol precipitation (2 volumes absolute ethanol for 20 minutes at -20°C). The precipitated DNA was washed 3 times with 70% ethanol and transferred to a 1.5ml microcentrifuge tube. After vacuum drying, the DNA was dissolved in 100ul sterile distilled water.

The samples were denatured by heating to 70°C for 30 minutes in 250mM NaOH. After neutralization with an equal volume of 2M ammonium acetate, the samples were applied to Hybond N nylon from Amersham (Oakville, Ontario) using a 96-well dot blot apparatus from Schleicher and Schull (Toronto, Ontario). After air drying, the DNA was cross-linked to the membrane by exposure to U.V. light (312nm) for 5 minutes. Pre-hybridization, hybridization, blocking and development were performed according to the instructions supplied with the Boehringer Mannheim kit.
IV. RESULTS AND DISCUSSION

A. Rubella Virus

1. Single time-point Infections

Initially, experiments were carried out to compare the replication of eight strains of RV in Vero cells, FC and SMC cells. Each cell-type was infected for three or four days and a comparison was made of the degree of CPE and antigen expression and the yield of progeny virus.

a. Antigen expression

All strains of RV studied expressed viral proteins in all three cell types but variations were seen as shown in Figures 1-3. Considerable variability was found between the strains with respect to both the degree of CPE induced and antigen expression. Figure 1 represents Vero cells infected with eight strains of RV and it shows that while all strains expressed viral protein, those which induced the most severe CPE also expressed the most protein (HPV77/DE5, Therien, M33 and Thomas). Of particular note is M33 which induced CPE that is very characteristic of this strain. Heavily stained, star-shaped cells were observed in all cell-types (B2 in Figures 1-3). Figures 2 and 3 indicate that some strains, most notably the vaccine strains, were restricted in the cells of joint origin. Fetal chondrocytes infected with Cendehill, RA27/3, Thomas and 5209R demonstrated little or no CPE but the pattern of antigen staining was different. The two wild-type strains (Thomas and 5209R) exhibited a granular stain in contrast to the diffuse stain observed with the vaccine strains. In addition, this diffuse stain was located in the perinuclear region of the Cendehill and RA27/3 infected cells. The remaining strains all demonstrated moderate to severe CPE and granular staining patterns throughout the cytoplasm. The results for infected SMC cells (Figure 3) were generally similar. One difference is that HPV77/DE5 demonstrated more perinuclear stain in the SMC cells compared to the FC. If examined at earlier times, other strains also exhibited a predominantly perinuclear stain, but by 4 days p.i. antigen expression progressed throughout the cytoplasm (data not shown). This perinuclear staining pattern may represent the passage of certain viral proteins through the Golgi complex where membrane proteins are glycosylated, but double labelling for both RV antigen and Golgi markers would be required to confirm this. The difference found between the strains in the nature of the antigen expression may represent an alteration
Figure 1.

Antigen Expression of Rubella Virus Strains in Vero Cells

Subconfluent monolayers of Vero cells were infected at an MOI of 5-10 for 4 hours at 35°C and were then incubated for 4 days (except for M33 which was incubated for 3 days). The plates were fixed in PF and examined by IP staining using rabbit anti-Therien polyclonal antiserum as the primary antibody except for M33 and 1B2 which were examined with rabbit anti-RA27/3. Positive staining is achieved for all strains with either of the antisera.

A1 — Cendehill  A2 — RA27/3  A3 — HPV77/DE5
B1 — Therien  B2 — M33  B3 — 1B2
C1 — Thomas  C2 — 5209R  C3 — Uninfected
Figure 2.

Antigen Expression Rubella Virus Strains in Fetal Chondrocytes

Subconfluent monolayers of FC were infected at an MOI of 5-10 virus for 4 hours at 35°C. After incubating for 4 days, the cells were fixed with PF and examined by IP staining using rabbit anti-RA27/3 polyclonal antiserum as the primary antibody.

A1 — Cendehill  A2 — RA27/3  A3 — HPV77/DE5

B1 — Therien  B2 — M33  B3 — 1B2

C1 — Thomas  C2 — 5209R  C3 — Uninfected
Subconfluent monolayer of SMC cells were infected at an MOI of 5-10 for 4 hours at 35°C. After incubating for 4 days the cells were fixed with PF and examined by IP staining using rabbit anti-RA27/3 polyclonal antiserum as the primary antibody.

A1 — Cendehill  A2 — RA27/3  A3 — HPV77/DE5
B1 — Therien  B2 — M33  B3 — 1B2
C1 — Thomas  C2 — 5209R  C3 — Uninfected
in the glycosylation process in Cendehill and RA27/3 resulting in retardation of the growth cycle and lower viral yields.

b. Virus production
The culture media from the infections described above were assayed for infectious virus and the results are given in Table I. Again strain variation was distinctly seen. The titres from the Vero cells represent virus production under ideal conditions in a permissive cell line, with which the joint-cell yields can be compared. The results for both FC and SMC cells were similar for each RV strain and in most cases the viral yield was lower than that obtained in Vero cells. However, whereas only up to a 50-fold difference in virus titre was seen in FC and SMC cells infected with wild-type strains and HPV77/DE5, viral yields obtained with the RA27/3 and Cendehill strains were up to 2400-fold less in cells of joint origin compared to Vero cells. Indeed, the levels of virus seen at 4 days p.i. were so low for these 2 strains that they may be explained as residual virus from the inoculum.

2. Viral growth curves
A comparison of the growth curves of different strains of RV in SMC and Vero cells was carried out to determine whether virus replicated more slowly in joint cells but would eventually attain the same yield. In addition it was important to confirm whether Cendehill and RA27/3 replicated at all in these cells. If replication of virus is occurring, a lag period (eclipse), followed by an increase in supernatant virus should be observed. As the level of cell-association of a virus may have implications in disease pathogenesis, it was also of interest to examine intracellular virus titres. Figures 4 and 6 represent the supernatant and cell-associated virus levels for five RV strains in two cell types. Cendehill, RA27/3 and HPV77/DE5 were selected for study because they are the common vaccine strains, and clinical data are available concerning their arthritogenicity, and M33 and Therien were selected as examples of wild-type viruses. The primary SMC cells were used as they are heterogeneous cell populations that would more closely approximate the cells within an intact joint than the FC cell-line. The experiments were also done using Vero cells to provide a basis for assessing the level of viral restriction in the SMC cells.
Table I.

Rubella Virus Production in Cells of Joint Origin

The culture media from the infections described in Figures 1-3 were assayed for virus by microfocus assay (see Methods under Virus Titrations).
### Rubella Virus Production

#### In Cells of Joint Origin

<table>
<thead>
<tr>
<th>Rubella Strain</th>
<th>Vero Cells</th>
<th>Synovial Membrane Cartilage Cells</th>
<th>Fetal Chondrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cendehill</td>
<td>$1.0 \times 10^6$</td>
<td>$4.1 \times 10^2$</td>
<td>$1.8 \times 10^3$</td>
</tr>
<tr>
<td>RA27/3</td>
<td>$5.1 \times 10^6$</td>
<td>$7.5 \times 10^4$</td>
<td>$1.2 \times 10^4$</td>
</tr>
<tr>
<td>HPV77/DE5</td>
<td>$2.7 \times 10^6$</td>
<td>$6.3 \times 10^5$</td>
<td>$2.9 \times 10^5$</td>
</tr>
<tr>
<td>Therien</td>
<td>$4.6 \times 10^6$</td>
<td>$7.6 \times 10^5$</td>
<td>$1.8 \times 10^6$</td>
</tr>
<tr>
<td>M33</td>
<td>$1.5 \times 10^6$</td>
<td>$1.0 \times 10^6$</td>
<td>$1.4 \times 10^6$</td>
</tr>
<tr>
<td>1B2</td>
<td>$5.0 \times 10^6$</td>
<td>$5.2 \times 10^5$</td>
<td>$1.7 \times 10^5$</td>
</tr>
<tr>
<td>Thomas</td>
<td>$1.0 \times 10^7$</td>
<td>$6.3 \times 10^5$</td>
<td>$1.5 \times 10^5$</td>
</tr>
<tr>
<td>5209R</td>
<td>$1.0 \times 10^6$</td>
<td>$4.6 \times 10^5$</td>
<td>$2.0 \times 10^4$</td>
</tr>
</tbody>
</table>
Growth Curves of Supernatant Rubella Virus Strains in SMC and Vero Cells

Growth curve experiments were performed as described in Methods. The curves were drawn using the average values from replicate experiments and the vertical bars represent 1 standard deviation (σn-1). The value on Day 0 represents the level of input virus...
Growth Curves of Supernatant Rubella Virus Strains in SMC and Vero Cells

**Cendehill**

**RA27/3**

**HPV77/DE5**

**Therien**

**M33**

*Vero Cells*

*SMC Cells*

**X Axis = Time Post Infection (days)**

**Y Axis = Virus Titre (Log10 FFU/mL)**
The supernatant virus levels shown in Figure 4 indicate that within the time period of acute infection (seven days), each strain of RV was restricted in the SMC cells to varying degrees. In addition, the time at which this restriction became apparent varied. For Cendehill, there was a difference in virus titre of 60 fold between the different cell types and this was apparent by 2 days p.i. By the end of the experiment the difference had increased to over four orders of magnitude. Similarly, for RA27/3, the differences in viral titres between the two cell types became apparent by 2 days p.i. and the difference at the end of the experiment was 500 fold.

The other strains also showed differences but they appeared later and were not as pronounced. The two wild-type strains and HPV77/DE5 demonstrated the type of growth curve expected in a permissive system. In contrast, the curves for Cendehill and RA27/3 are such that de novo synthesis can neither be demonstrated nor excluded. For this reason the decay kinetics (fall in virus titre when incubated in the absence of cells) of these two strains were examined and the results are shown in Figure 5. For RA27/3 it can be seen that during the incubation, there is a steady decline in virus infectivity, whereas in the presence of SMC cells, the virus titre is stable for six days following an early decrease. This indicates that viral replication was occurring in these cells to a level that was equal to the rate of decay, resulting in a horizontal growth curve. For Cendehill, however, the situation was not as clear. The decay curve shows that a steady decline to the limit of detection (10 FFU/ml) occurs after five days of incubation. Likewise, the supernatant virus titre from infected SMC cells has fallen to this level by the same time p.i. However, the shape of the curve from SMC cells is different and together with subsequent results (see Persistence in SMC cells) indicates that very low levels of virus replication were occurring with this strain.

Similar strain variations were seen when levels of cell-associated virus were examined (Figure 6). The most apparent differences were again observed with the Cendehill and RA27/3 strains where no intracellular infectious virus above the limit of detection (10 FFU/ml) was observed. The cell-associated virus levels for the other three strains were also lower in the SMC cells as compared to the Vero cells but the differences were small and the fall in these titres with time was most likely due to cell death. The absence of detectable cell-associated Cendehill and RA27/3 may indicate that these two strains utilize a different mechanism for obtaining their viral envelope in the SMC cells compared to the Vero cells. If these two strains acquired their
Figure 5.

Decay Kinetics For Rubella Virus Strains Cendehill and RA27/3

Stock virus was incubated under the same conditions but in the absence of cells and aliquots were removed at the indicated times and titred.
Decay Kinetics for Rubella Virus Strains Cendehill and RA27/3

RA27/3

Cendehill
Growth Curves of Cell-Associated Rubella Virus Strains in SMC and Vero Cells

Growth curve experiments were performed as described in Methods. The curves were drawn using the average values from replicate experiments and the vertical bars represent 1 standard deviation (σn-1).
Growth Curves of Cell-Associated Rubella Virus Strains in SMC and Vero Cells

Cendehill

RA27/3

HPV77/DE5

X Axis = Time Post Infection (days)

Y Axis = Virus Titre (Log10 FFU/mL)

SMC Cells

Vero Cells
envelopes exclusively from the plasma membrane (rather than from an internal membrane) in the SMC cells, then detecting a cell-associated, infectious virion would be a rare event.

Figure 7 is a compilation of the data from Figures 4 and 6 to enable a direct comparison of RV strains in the SMC cells to be made. In the case of the cell-associated virus, the results split into two groups with Cendehill and RA27/3 showing no observable titre, and the other three strains exhibiting similar high viral yields. The supernatant virus titres also fell into the same groups with one representing the permissive viral strains (HPV77/DE5, Therien and M33) and those that were restricted (Cendehill and RA27/3). It is evident that although it is a vaccine strain, HPV77/DE5 behaved like a wild-type virus in this culture system.

3. Persistent infection in SMC cells

The ability of RV to induce chronic arthritis suggests that the virus may be capable of persisting in joint tissue. For this reason the ability of the five strains to establish persistent infections in the SMC cells was investigated. The results indicated that each strain was capable of persisting within these cells, but the characteristics of chronic infections were different.

a. Virus production

Viral yields obtained from SMC cells infected over a period of 80 days are shown in Figure 8. Similar to results obtained for the cell-associated virus during acute infections, there was a clear separation into two groups with HPV77/DE5 exhibiting a pattern like the wild-type strains although it showed much greater fluctuation than either M33 or Therien. The only infection that could not be maintained for 80 days was with the Therien strain despite careful treatment designed to favour the cells, such as increased concentrations of FBS and more frequent media changes. The titres of both Cendehill and RA27/3 were dramatically lower (at least 1000 fold) compared to the wild-type group but it is clear that these cells were producing virus, although at low levels.

b. Antigen expression

Approximately once a week, the persistently infected cells were examined by IP staining and the results are displayed in Figures 9-13. The results for Cendehill and RA27/3 demonstrate the lower limit of detection of viral antigen by this technique. A positive staining pattern is lost by the third passage in Cendehill infected
The data from Figures 4 and 5 (SMC cells only) has been compiled to enable a direct comparison of viral yields with different strains to be made.
Rubella Virus Strain Comparison in SMC Cells

**Supernatant Virus**

- Cendehill
- RA
- HPV
- Therien
- M33

**Cell-Associated Virus**

- Cendehill
- RA27/3
- HPV77/DE5
- Therien
- M33
Persistent Infection of SMC Cells with Rubella Virus Strains

Supernatant samples for titration were collected every 3-4 days when the media was changed or when the cells were passaged. The titre of the input virus is shown on Day 0.
Persistent Infection of SMC Cells

Virus Titre (log 10 FFU/ml)

Time Post Infection (days)

- Cendehill
- RA27/3
- HPV77/DES
- Therien
- M33
Figure 9.

SMC Cells Persistently Infected with Rubella Virus Strain Cendehill

Approximately every 7 days one control and one infected tissue culture plate of persistently infected cells were fixed for IP staining and rabbit anti-RA27/3 polyclonal antiserum was used as the primary antibody.

Antigen was not detected in cells from passages 4-8 and are therefore not shown.

A — Control P1  B — Cendehill P1

C — Control P3  D — Cendehill P3

P # = passage #

Synovial Membrane/Cartilage Cells Persistently Infected with Rubella Virus Strain Cendehill
Figure 10.

SMC Cells Persistently Infected with Rubella Virus Strain RA27/3

Approximately every 7 days one control and one infected tissue culture plate of persistently infected cells were fixed for IP staining and rabbit anti-RA27/3 polyclonal antiserum was used as the primary antibody.

Antigen was not detected in cells from passages 5-8 and are therefore not shown.

A — Control P1    B — RA27/3 P1
C — Control P3    D — RA27/3 P3
E — Control P4    F — RA27/3 P4

P # = passage #
Synovial Membrane/Cartilage Cells
Persistently Infected Rubella
Virus Strain RA27/3
Approximately every 7 days one control and one infected tissue culture plate of persistently infected cells were fixed for IP staining and rabbit anti-RA27/3 polyclonal antiserum was used as the primary antibody.

The appearance of the cells from passages 4-8 did not differ significantly from those presented.

A — Control P1       B — HPV77/DE5 P1
C — Control P3       D — HPV77/DE5 P3
E — Control P9       F — HPV77/DE5 P9

P # = passage #
Synovial Membrane/Cartilage Cells
Persistently Infected Rubella Virus Strain HPV77/DE5
Figure 12.

SMC Cells Persistently Infected with Rubella Virus Strain Therien

Approximately every 7 days one control and one infected tissue culture plate of persistently infected cells were fixed for IP staining and rabbit anti-RA27/3 polyclonal antiserum was used as the primary antibody.

A — Control P1    B — Therien P1
C — Control P3    D — Therien P3
E — Control P4    F — Therien P4

P # = passage #
Synovial Membrane/Cartilage Cells
Persistently Infected Rubella Virus Strain Therien
Figure 13.

**SMC Cells Persistently Infected with Rubella Virus Strain M33**

Approximately every 7 days one control and one infected tissue culture plate of persistently infected cells were fixed for IP staining and rabbit anti-RA27/3 polyclonal antiserum was used as the primary antibody.

The appearance of the cells from passages 4-6 were not significantly different from those presented.

A — Control P1  
C — Control P3  
D — Control P7  

B — M33 P1  
C — M33 P3  
E — M33 P7

P # = passage #
Synovial Membrane/Cartilage Cells
Persistently Infected Rubella
Virus Strain M33
cells and by the fourth passage in RA27/3 infected cells. In neither instance did the loss of antigen staining correlate with a decrease in virus titre, suggesting that at later times a greater number of cells were infected, each one producing very small numbers of virus particles, whereas in the beginning, there were fewer cells producing the same amount of virus thereby making antigen detection possible because it was concentrated in foci of infected cells. There was also no CPE induced by Cendehill during the 80 day infection, and RA27/3 only began to exhibit CPE after the seventh passage (data not shown). In contrast, the other three strains demonstrated considerable CPE from the first passage and this was maintained, with fluctuations, for the duration of the experiment.

4. Infection of joint tissue

Intact joint tissue was infected with the five strains of RV to determine if they were capable of replicating in the relatively quiescent cells of organ culture. This would be closer to the in vivo situation where an infecting virus would encounter non-dividing cells and also extra-cellular matrices which might inhibit viral attachment.

a. Virus production

Figure 14 shows the results of virus titrations performed every three or four days on the culture media of segments of infected joint tissue in organ culture over a period of 50-60 days. Once again HPV77/DE5 behaved like the wild-type strains Therien and M33, all three producing large amounts of virus for prolonged periods. The falling titres at later times p.i. were probably a result of cell death since the uninfected sections demonstrated that after 40 days in culture there were fewer viable cells (see Figure 15) as judged by the number of intact nuclei (91). There was no evidence that Cendehill was able to replicate at all (limit of detection, 1 FFU/ml) but RA27/3 showed that following a 16-day decline, low but stable titres were maintained for approximately 50 days.

b. Antigen expression

As expected, no antigen was detected in the joint tissue infected with the Cendehill strain (data not shown). Despite the low levels of virus produced by RA27/3, sections positive for antigen were observed (Figure 16) but the foci of infection remained small and discrete. In contrast, extensive staining and CPE was seen for
Figure 14.

Joint Tissue Infected with Rubella Virus Strains

The culture supernatants were assayed for virus every 3-4 days when the media was changed. For each infection control tissue derived from the same specimen was maintained in parallel. The media from all the controls was also assayed and found to be negative. The titre of the input virus is shown on Day 0.
Samples of control tissue were cultured, fixed, and examined in the same manner as the infected cultures. IP staining was performed using rabbit anti-Therien polyclonal antiserum as the primary antibody.

A — 0 days p.i. 
B — 7 days p.i.

C — 21 days p.i. 
D — 60 days p.i.

0 days p.i. = fixed at the time of infection
Uninfected Joint Tissue
Figure 16.

Infection of Joint Tissue with Rubella Virus Strain RA27/3

At the times indicated below, samples of infected tissue were fixed, sectioned and examined by IP staining using rabbit anti-Therien polyclonal antiserum as the primary antibody. A duplicate section treated in the same manner but without the primary antibody, is also presented.

A — 6 days p.i.  B — Control for A
C — 20 days p.i.  D — Control for C
E — 38 days p.i.  F — Control for E
Infection of Joint Tissue with Rubella Virus Strain RA27/3
HPV77/DE5, Therien and M33 (Figures 17-19). The antigen stain of these three strains was first observed in small foci on the edges of the synovial membrane (Figure 17A) which increased in size with time. All three strains demonstrated the ability to penetrate deeply into the synovial membrane, but they varied in the amount of tissue disruption produced, with M33 causing the most damage (Figure 19F). Variation was also observed regarding the ability of the different strains to express antigen within cartilage tissue. Even when the synovial membrane demonstrated extensive antigen stain, the cartilage was usually completely clear. In some cases a very few perichondrocytes lining the cartilage surface were found to express antigen. The exception to this was with the Therien strain. Figure 19A shows how this strain is unable to penetrate the cartilage at early times (7 days p.i.). Even by 28 days p.i. (Figure 20A) only perichondrocytes demonstrated Therien antigen. However, by 60 days p.i., a massive invasion of cartilage tissue had occurred (Figure 20C).

5. Summary and discussion
The experiments outlined have attempted to answer the question of whether RV strains can replicate in cells and tissue of joint origin, thereby providing evidence for one possible mechanism of induction of RAA. For six out of eight strains (Cendehill and RA27/3 excluded) the answer was clear; the virus titres from FC and SMC cells at 3 or 4 days p.i. were similar to those from Vero cells and this was accompanied by strong antigen stain and/or CPE in the cells. To clarify the question concerning the replicative capabilities of Cendehill and RA27/3, the more detailed growth curve experiments were undertaken. These results indicated a lack of growth of these two strains since the supernatant virus levels did not increase. However, when the decay kinetics of each virus were determined i.e., fall in titre when incubated in the absence of cells, it appeared that there was viral growth but at low levels.

The ability of RV strains to persist within joint cells and tissue is of interest because of the chronic nature of RAA. All strains (with the exception of Therien) were able to persist within the SMC cells for 80 days. However, M33 and HPV77/DE5, induced severe CPE and careful monitoring was required to maintain the cells for the duration of the experiment. In contrast, RA27/3 and Cendehill induced little or no CPE but continued to produce levels of virus so low that they were undetectable by IP staining. These results, on their own, might suggest that Cendehill and RA27/3 would be the ideal candidates for causing chronic arthritis because they were able to persist in a non-destructive manner that might elicit a weak but chronic inflammatory
Figure 17.

Infection of Joint Tissue with Rubella Virus Strain HPV77/DE5

At the times indicated below, samples of infected tissue were fixed, sectioned and examined by IP staining using rabbit anti-Therien polyclonal antiserum as the primary antibody. A duplicate section treated in the same manner but without primary antibody, is also presented.

A — 6 days p.i.  
C — 20 days p.i.  
E — 38 days p.i.  
B — Control for A  
D — Control for C  
F — Control for E
Infection of Joint Tissue with Rubella Virus Strain HPV77/DE5
At the times indicated below, samples of infected tissue were fixed, sectioned and examined by IP staining using rabbit anti-Therien polyclonal antiserum as the primary antibody. A duplicate section treated in the same manner but without the primary antibody, is also presented.

A — 7 days p.i.  
B — Control for A

C — 28 days p.i.  
D — Control for C

E — 60 days p.i.  
F — Control for E
Infection of Joint Tissue with Rubella Virus Strain Therien
Infection of Joint Tissue with Rubella Virus Strain M33

At the times indicated below, samples of infected tissue were fixed, sectioned and examined by IP staining using rabbit anti-Therien polyclonal antiserum as the primary antibody. A control section treated in the same manner but without primary antibody is also presented.

A — 7 days p.i.    B — Control for A
C — 14 days p.i.    D — Control for C
E — 60 days p.i.    F — Control for E
Infection of Joint Tissue with Rubella Virus Strain M33
Figure 20.

Penetration of Rubella Virus Strain Therien into Cartilage Tissue

At the times indicated below, samples of infected tissue were fixed, sectioned and examined by IP staining using rabbit anti-Therien polyclonal antiserum as the primary antibody. A duplicate section treated in the same manner but without the primary antibody, is also presented.

A — 28 days p.i.     B — Control for A
C — 60 days p.i.     D — Control for C
Penetration of Rubella Virus Strain Therien Into Cartilage Tissue
response. However, clinical data indicate there is a much higher incidence of RAA following wild-type infection compared with vaccination. In addition, the clinical data allow different vaccine strains to be placed in the following order of decreasing arthritogenicity: HPV77/DK12, HPV77/DE5, RA27/3, Cendehill and To336 (22, 53, 55, 118). HPV77/DK12 was one of the first rubella vaccines used in the United States in 1970 but its use was discontinued due to the high number of associated complications (53). To336 is a vaccine used in Japan that appears to be highly effective and arguably the best candidate for future vaccine use in adult women, as it induces protective immunity with the fewest complications (22).

The results of the experiments in joint tissue indicated that the different strains also varied in their ability to penetrate through the extracellular joint matrix.

The matrix of cartilage consists of collagen (types II, IX, X, and other unclassified types) which is secreted by chondrocytes. In addition, these cells manufacture proteoglycans and structural glycoproteins. These secreted materials form a three dimensional structure that gives cartilage its elasticity and shock-absorbing qualities (15, 51). One function of the synovial membrane is the production of synovial fluid which lubricates the joints which is accomplished by the B-type cells (126). These cells secrete some proteins and hyaluronic acid and proteoglycans surround the cells (91).

The wild-type strains and HPV77/DE5 demonstrated invasive capabilities accompanied by increased virus titres, whereas replication of Cendehill was not observed. RA27/3 antigen was restricted to foci near the surface of the synovial membrane and thus demonstrated some replication but very limited ability to penetrate the tissue. The amount of virus produced was much lower than the “wild” group, tissue invasion was slower and less extensive, and no tissue disruption was seen.

The five strains of RV that were studied in the most detail were selected because clinical data concerning their reaction in patients were also available. The results have shown that the behaviour of the three vaccine strains studied in joint tissue correlates with the reported incidence of complications with which they are associated. More specifically, those vaccine strains which are associated with the most frequent and severe joint complications exhibited more aggressive behaviour in this model system. HPV77/DE5 produced levels of virus comparable to the wild-type strains in all infections but the extent of CPE and tissue disruption were
not as extensive. Cendehill was less productive than RA27/3 in the growth curve experiments and in addition, it was unable to replicate in joint tissue. The inability of Cendehill to infect joint tissue may be explained in part by its restriction in SMC cells resulting in low viral yields incapable of overcoming the further obstruction provided by the extracellular matrix of joint tissue.

Infection with wild-type RV is associated with the highest levels of joint symptoms (55) and both wild-type strains studied demonstrated the most aggressive behaviour. SMC cells persistently infected with Therien could not be maintained beyond 45 days despite careful maintenance and both Therien and M33 caused considerable damage to joint tissue. It is known that there is variation between different wild-type strains with respect to the incidence of joint complications in separate outbreaks of rubella, but in general wild-type infection induces the highest level and most severe symptoms. Unfortunately, HPV77/DK12 and To336 were not available for study but it would be of interest to see if the correlation is maintained with these other vaccine strains.

In conclusion, this study has shown that direct infection of joint tissue is a possible mechanism of induction of arthritis by RV. Moreover, a model system to investigate the arthrotropicity of RV strains, which correlates well with known clinical data, has been established. This system should prove valuable in estimating the ability of future vaccine strains to cause joint damage.

B. Adenovirus

To determine the permissiveness of joint cells to adenovirus (Ad), initial experiments involved analysis of FC and SMC cells at a single time-point for viral antigen expression and yield. The cells were infected in the same manner as Hep-2 cells, incubated for four days and fixed with PF prior to IP staining. The results are represented in Figures 21 and 22. At first the FC and SMC were thought to be totally non-permissive to Ad as the culture appeared to express no antigen at all (Figures 21D and 22B). However, closer examination identified occasional cells, approximately 1 in 100, which expressed viral protein and exhibited a characteristic cytopathology (Figures 21B and 22C). By this time, in infected Hep-2 cells, every cell would be expressing antigen in addition to CPE involving rounding up and clumping of the cells prior to their detachment from the surface.
Subconfluent monolayers of FC were infected with Ad at an MOI of 5-10 for 60 minutes at 37°C. After incubating for 4 days, the cells were fixed with PF and examined by IP staining using a monoclonal antibody to an Ad group specific antigen as the primary antibody.

A — Uninfected

B — 4 days p.i.

C — 4 days p.i., no primary antibody

D — 4 days p.i.
Adenovirus type 2 Infected Fetal Chondrocytes
Subconfluent monolayers of FC were infected with Ad at an MOI of 5-10 for 60 minutes at 37°C. After incubating for 4 days, the cells were fixed with PF and examined by IP staining using a monoclonal antibody to an Ad group specific antigen as the primary antibody.

A — Uninfected

B — 4 days p.i.

C — 4 days p.i.
Adenovirus type 2 Infected Synovial Membrane/Cartilage Cells
1. Viral growth curves
Since the preliminary experiments demonstrated that Ad was restricted in SMC cells and FC, more detailed time course experiments were performed to examine antigen expression and virus production in the two cell types.

a. Antigen expression
SMC and Hep-2 cells infected with Ad were fixed for IP staining daily for seven days. Two early time-points, immediately following virus adsorption and 0.5 days p.i., were also examined so that the onset of viral protein expression would be detected.

Figure 23 depicts the progression of antigen expression and CPE in Hep-2 cells. By 2 days p.i. virtually every cell was expressing antigen, and 24 hours later early CPE was observed. By 5 days p.i. total destruction of the monolayer had occurred. In comparison, the restriction of Ad infection in SMC cells is shown in Figure 24. A low number of single cells expressing viral antigen could be detected 2 days p.i. Spread to surrounding cells occurred, forming foci of infection which slowly enlarged, but widespread infection of the monolayer did not occur. In addition, the CPE induced in the SMC cell cultures was very different from that seen with the Hep-2 cells which became more refractile, round and eventually detached. In contrast, the antigen positive SMC cells remained attached and many of these developed large vacuolated nuclei of unusual shape (Figures 21 and 22). This type of effect was also seen in the Hep-2 cultures, at early times p.i. (Figure 23).

b. Virus production
At the same time that cells were fixed for IP staining, supernatant and cell-associated virus titres were also determined. Virus titres from each cell type are depicted in Figure 25. This shows that SMC cells were capable of supporting complete Ad infection, but that they were not as permissive as the Hep-2 cells. The levels of supernatant and cell-associated virus did not vary appreciably for a single cell type but the titres were approximately 10-fold less in the SMC cells than the Hep-2 cells. Cell-associated virus titres beyond 5 days p.i. from the Hep-2 cells were not obtainable because the monolayer was destroyed by this time. However, since this virus is stable in culture media (Figure 26), supernatant virus titres were obtained for later times p.i.
Progression of Adenovirus Antigen Expression in Hep-2 Cells

Subconfluent monolayers of Hep-2 cells were infected with Ad at an MOI of 5-10 for 60 minutes at 37°C. At the indicated times, cells were fixed for IP staining using a monoclonal antibody to an Ad group specific antigen as the primary antibody.

A1 — 0 days p.i.  A2 — 0.5 days p.i.  A3 — 1 days p.i.
B1 — 2 days p.i.  B2 — 3 days p.i.  B3— 4 days p.i.
C1 — 5 days p.i.  C2 — 0 days p.i.  C3— 5 days p.i.

Control  Control

0 d.p.i. = cells were washed and fixed immediately following virus adsorption
Subconfluent monolayers of SMC cells were infected with Ad at an MOI of 5-10 for 60 minutes at 37°C. At the indicated times, cells were fixed for IP staining using a monoclonal antibody to an Ad group specific antigen as the primary antibody.

A1 — 0 days p.i.  A2 — 0.5 days p.i.  A3 — 1 day p.i.  A4 — 2 days p.i.
B1 — 3 days p.i.  B2 — 4 days p.i.  B3 — 5 days p.i.  B4 — 6 days p.i.
C1 — 7 days p.i.  C2 — 0 days p.i.  C3 — 3 days p.i.  C4 — 7 days p.i.

Control

0 d.p.i. = cells were washed and fixed immediately after virus adsorption
Adenovirus type 2 Antigen Expression
in Synovial Membrane/Cartilage Cells
Growth Curves of Adenovirus in Hep-2 and SMC Cells

Growth curve experiments were performed as described in Methods. The curves were drawn using the mean values of replicate experiments. Error bars represent 1 standard deviation (σn-1). The value on Day 0 for the supernatant virus represents the titre of input virus.
Growth Curves of Adenovirus in HEP-2 and SMC Cells

Supernatant Virus

Cell-Associated Virus
Figure 26.

Decay Kinetics of Adenovirus

Ad was incubated under the same conditions but in the absence of cells. At the times indicated, samples were removed and titred.
Adenovirus Decay Kinetics

Virus Titre
(log 10 TCID/ml)

Incubation Time (days)
An estimation of the productivity (infectious particles/cell) may be calculated by dividing the total number of infectious particles by the total number of infected cells. This assumes that a cell must be expressing detectable antigen to be producing virus because the number of infected cells is determined by counting positive cells following IP staining. To an approximation, it was found that at 4 days p.i. each infected Hep-2 cell was producing approximately 6000 infectious particles/cell whereas each infected SMC cell produced only about 400 infectious particles/cell.

2. Persistence in SMC cells

The restricted nature of the Ad infection in the SMC cells suggested that this virus would readily establish persistent infection in these cells. However, after the first passage (Figure 27) the infection became much more aggressive with every cell expressing antigen. The CPE continued to increase until the fourth and final passage (data not shown) and this was accompanied by an increase in virus titre (Figure 28).

3. Infection of joint tissue

Since the persistent infection in the SMC cells increased so dramatically after the first passage, it was concluded that Ad prefers cells that are mitotically active. For this reason it seemed very unlikely that this virus would be able to productively infect intact joint tissue since these cells are viable but not rapidly dividing. It was therefore surprising to observe the levels of virus produced in infected tissue (Figure 29) despite the fact that the culture media was changed every three to four days. There was considerable variability in the titres detected in separate infections which was likely due to variation between tissue specimens. In addition, the tissue from these experiments was sectioned and examined by IP staining for viral antigen using a monoclonal antibody. Even at late times p.i. (63 days) no Ad antigen was detected in any of the different tissues (data not shown).

4. Summary and discussion

The infection of Ad in cells of joint origin proved to be very different from that observed in the permissive Hep-2 cell line. Figures 21 and 22 represent preliminary experiments that were at first considered to demonstrate a lack of antigen expression. However, on careful examination, occasional cells (approximately 1 in 100) which were heavily stained by IP were seen. It appeared that there was a block at some stage of
SMC Cells Persistently Infected with Adenovirus

Approximately every 7 days one control and one infected tissue culture plate of persistently infected cells were fixed for IP staining using a monoclonal antibody to a group specific Ad antigen as the primary antibody.

A — Control P1   B — Ad 9 d.p.i.  
C — Ad P1       D — Ad P2

P# = passage #
Synovial Membrane/Cartilage Cells Persistently Infected With Adenovirus type 2
Figure 28.

Persistent Infection of SMC Cells with Adenovirus

When the media was changed or when the cells were passaged, samples of the supernatant were assayed for virus. The value on Day 0 represents the titre of the input virus.
Samples of the culture media from infected and control cultures were assayed for virus every 3-4 days. Control samples were all negative. The curves represent the results from separate infections where the value on Day 0 indicates the titre of the input virus.
Adenovirus Infected Joint Tissue

Virus Titre (log 10 TCID/ml)

Time Post Infection (days)
replication following viral protein translation which prevented the release of infectious progeny since the number of stained cells increased very slowly and mainly to adjacent cells suggesting spread by direct cell to cell contact. However, the growth curve experiments proved that infectious virus was produced and released in sufficient quantity to infect every cell. The long-term infections demonstrated that Ad could infect every cell but this did not occur until the cells were passaged. The procedure of trypsinizing, diluting and re-plating cells obviously causes changes in these cells that render them more permissive to infection. After any cells are passaged, there is a lag period of cell growth as they settle down and re-attach. During this time there is little or no increase in cell number but there are increases in metabolic activities such as DNA synthesis and cytoskeleton construction (61). All infections with Ad were initiated within 18 hours of passaging suggesting that infection is not cell-cycle dependent since at this time the cells are approximately synchronized (61). However, cell-cycle dependency cannot be entirely excluded as different cell types (SMC cells are a mixed population) are likely to have different cell-cycles. Another possible explanation for the rapid increase in infection following the first passage is that during trypsinization existing cell-surface proteins were altered or new ones expressed that functioned as the viral receptor. Even if this alteration or expression were only transient, as virus is present throughout the procedure, infection of the cells might occur. In order to determine if infection is truly cell-cycle dependent, more efficient methods of cell synchronization could be used such as DNA synthesis inhibition or nutritional deprivation (61), prior to infection. To more closely examine the role of trypsin, cells could be lightly trypsinized (not enough to cause them to detach) to determine if a viral receptor was being exposed by this treatment.

The fact that high titres of virus were produced from infections of intact tissue shows that at least a few cells susceptible to infection were present as with the SMC cell cultures. The inability to detect these infected cells in the tissue sections may be due to several factors. There may be a very low number of infected cells producing large quantities of virus and they may not have been detected since only a few sections were examined. If this were the reason, each piece of tissue would have to be completely sectioned and examined by IP staining in order to find the foci of infected cells. It is also possible that the antigenicity of the virus was altered by passage in joint tissue but this is unlikely since Ad antigen was undetectable even at early times before adaptation was likely to have occurred. In addition, when a piece of Ad infected tissue (63 days p.i.)
was co-cultivated with Hep-2 cells for several days, these cells exhibited the same antigen stain and CPE as was observed before (Figure 30). The most plausible explanation is that when the tissue pieces were processed for paraffin embedding and sectioning, the viral antigens were altered. Even a small alteration to the proteins, if located within the appropriate epitope, would be sufficient to prevent binding of the monoclonal antibody. If this were the case, then infected cells processed in the same manner as the tissue segments should also be negative when examined with the same antibody. In addition, the sections could be examined with a polyclonal antiserum which would be much less sensitive to small structural changes in individual proteins.

Adenovirus has clearly demonstrated that it can replicate within cells of the joint. The most likely mode of access to the joint for any virus is via the hematogenic route, possibly as passengers in circulating lymphoreticular cells. Adenovirus replicates in human lymphoid cell lines (92, 139) in a cell-cycle dependent manner (139). However, evidence to demonstrate its ability to replicate in primary human lymphocytes is scanty (79) and may explain why so few Ad infections are complicated by arthritis.

Once it has reached the joint, the results described above indicate that a low number of cells may be susceptible to infection. It would seem reasonable that in a normal host, this limited infection would be cleared quickly with a relatively short inflammatory response, but in an immunocompromised host the infection may spread, resulting in prolonged inflammation associated with joint damage as has been found in a clinical case study (60).

C. Human Parvovirus, B19

To date there is no in vitro system that will support complete replication of B19 (170), leaving serum from viremic patients as the only source of infectious virus. For this reason supplies of B19 are limited, and therefore only a few experiments with this virus were possible.

1. Virus growth

The ability of SMC cells to support the replication of B19 was examined by quantitating intracellular and supernatant viral DNA levels at three time-points p.i. by dot-blotting. Bone-marrow (BM) cells, infected in parallel were used as the positive control to ensure that the assay procedure was working.
Two pieces of joint tissue infected with Ad (63 days p.i.) were incubated with subconfluent Hep-2 cells for 3 days. The tissue was discarded and the cells were washed, fixed and examined by IP staining using a monoclonal antibody to an Ad group specific antigen as the primary antibody.

A — Uninfected

B — Co-cultivation
Co-cultivation of Adenovirus type 2 Infected Joint Tissue with Hep-2 Cells
Figure 31 represents the results of a time course of B19 infected SMC cells extending over a four day period and it shows that SMC cells do not support B19 DNA replication to a level that is detectable in this system (Columns A and B). The sensitivity of the detection system was determined by extracting DNA from a known number of virus particles and applying dilutions of the samples obtained to nylon filters. Strips of these standards were used in each test. The sensitivity was found to be approximately $5 \times 10^3$ virus particles (M-II c) which is equivalent to approximately 14.5 fg of DNA, indicating that while this is a sensitive system for DNA detection, it may not sufficient for detecting a virus with a small genome. A small amount of B19 DNA was seen in the cell-associated DNA preparation from SMC cells (Figure 31, B1). This may be due to the fact that virus particles were non-specifically adhering to the cell surface. Alternatively, it may represent virus which has been taken into the cells but is later degraded intracellularly as it is unable to replicate. The latter is more likely for 2 reasons. Firstly, the cells were washed once after the infection and twice before the DNA extraction. Secondly, if virus particles were attaching non-specifically to a cell it is likely that at least some of them would detach and be detected in the supernatant.

The positive control for infection in this experiment was DNA extracted from infected BM cells. The amount of B19 DNA was shown to increase in both the supernatant media and cell-associated samples (Columns E and F) between 8 and 24 hours p.i. but was more readily detected in the latter. The slight increase in the amount of B19 DNA observed in the supernatant media (Column E) may not represent the release of progeny virus, but rather input virus that was released into the culture medium because its initial attachment to the cells was of low affinity. No B19 DNA was detected in uninfected SMC and BM cells (Columns C, D, G and H) indicating that the probe was specific and the hybridization conditions correct.

2. Antigen expression

SMC cells infected with B19 for four days were also examined for antigen expression by IP staining after fixation in PF. Antisera to B19 capsid and non-structural proteins were used as primary antibodies and both required extensive absorbing to eliminate non-specific staining. Once this background staining was eliminated there was no evidence of antigen expression (data not shown).
Figure 31.

Detection of B19 DNA from Infected SMC and BM Cells

Experimental details are included in the Methods section.

The time-points for infected SMC cells were

1 = 0 h.p.i.
2 = 17 h.p.i.
3 = 96 h.p.i.

The time-points for infected BM cells were

1 = 8 h.p.i.
2 = 48 h.p.i.
3 = 96 h.p.i.

<table>
<thead>
<tr>
<th>Column A</th>
<th>SN B19 SMC</th>
<th>Column E</th>
<th>SN B19 BM</th>
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<tbody>
<tr>
<td>B</td>
<td>CA B19 SMC</td>
<td>F</td>
<td>CA B19 BM</td>
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<tr>
<td>C</td>
<td>SN Control SMC</td>
<td>G</td>
<td>SN Control BM</td>
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<tr>
<td>D</td>
<td>CA Control SMC</td>
<td>H</td>
<td>CA Control BM</td>
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SN = supernatant DNA, CA = cell-associated DNA

M–II = source of infectious B19. Dilutions of extracted DNA served to determine the limit of detection and were equivalent to the number of genomes indicated below.

\[ a = 5 \times 10^5 \]
\[ b = 5 \times 10^4 \]
\[ c = 5 \times 10^3 \]
\[ d = 5 \times 10^2 \]
\[ e = 5 \times 10^1 \]
Detection of Human Parvovirus B19 DNA

SMC

<table>
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<tr>
<th>B19</th>
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<tr>
<td>A</td>
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BM

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<th>B19</th>
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1

2

3

M-11

a b c d e
3. Summary and discussion

The above results suggest that the mechanism of B19 induced arthritis is not through direct invasion of joint tissue since under conditions designed to favour the virus, it failed to replicate. It is conceivable that B19 antigen was not detected because of the fixative used (see rubella virus results), but this is unlikely since the more sensitive technique of DNA hybridization was also negative. These results are not necessarily contradictory to those of Dijkmans et al. (45) who detected B19 DNA in the synovial fluid of a woman with B19-associated arthritis (see Introduction). Rather, when taken together, they serve to suggest immune complex-mediated joint inflammation as the mechanism of B19-induced arthritis.
VI. CONCLUSION

An *in vitro* system to examine the interaction of viruses with joint cells was established to investigate one of the postulated mechanisms of virus-induced arthritis, namely direct invasion and persistence of viruses in joint tissue. One of the advantages of using an *in vitro* system over an animal model, is that virus replication may be quantified. Also by examining infected tissue, the invasive capabilities of different viruses may be assessed and compared. The ability of a virus to replicate in cells and tissue of joint origin suggests that, given the opportunity (access to a joint), infection leading to inflammation may occur. An extension of the ability to infect, is the ability to persist which may be the cause of chronic viral arthritides. However, the ability to replicate does not negate the possible use of other mechanisms; a virus may utilize a variety of pathogenic pathways which may vary with the individual. If, on the other hand, a virus is completely incapable of replicating in a system designed to favour its growth in joint tissue, then it would be more likely that immunopathogenic mechanisms are involved in its induction of arthritis.

The results for RV strains indicated that the growth of each strain was restricted. The degree of restriction was quite pronounced for Cendehill and RA27/3 and present, but to a lesser degree for the other strains. This pattern of virus restriction according to strain is significant because it correlates with clinical data regarding the incidence of joint symptoms, suggesting that this may be a valuable tool for assessing the arthritogenicity of vaccine candidates. This model would then predict that RV strain HPV77/DK12 (a vaccine strain associated with severe side effects) would exhibit more aggressive behaviour in SMC cells or joint tissue than HPV77/DE5 and that To336 (the vaccine strain with the fewest reported clinical complications) would show a level of restriction equal to, or greater than, Cendehill. It would therefore be of great interest to examine the behaviour of these two strains to determine if the correlation is maintained.

The results indicate that B19 probably utilizes an immunopathogenic mechanism to induce joint inflammation since there was no indication of its replication in this system. It is interesting that RV and B19 behaved so differently *in vitro* when their clinical spectrums overlap so greatly. The most common result of infection with RV is an exanthematous diseases which may be clinically indistinguishable from that induced by B19. Likewise, the joint complications associated with both viruses share many clinical and epidemiological char-
acteristics. Given the tremendous biochemical differences between RV and B19, perhaps it is more surprising that they share anything in common at all.

It is conceivable that low levels of B19 replication went undetected due to the limit of hybridization assay. It would therefore be useful to either examine these cultures with more sensitive techniques such as polymerase chain reaction, or to attempt to amplify the virus with multiple passaging of the cells.

The behaviour of Ad in this model was intriguing but its significance in the in vivo situation is less clear. Its growth, which was initially restricted in the FC and SMC cells, seemed completely uninhibited after the cells were trypsinized suggesting that the virus receptor on these cells was either altered or exposed during the treatment. The literature reports of Ad-associated joint inflammation (see Introduction), together with the results presented here, suggest that Ad may cause arthritis by direct invasion of joint tissue particularly in immunocompromised individuals. However, if faced with an intact immune system, is more likely to be associated with the deposition of immune complexes. Given the prevalence of this virus in the community and the low incidence of joint involvement, it would seem that, in most cases, the host's defences are sufficient to prevent joint invasion.

The potential value of this model culture system extends beyond its uses here. Firstly, it could be used to examine the arthrotropicity of other viruses and secondly, the use of a heterogeneous cell population (SMC cells) may indicate if certain cells are preferentially infected. Thirdly, because it is a controlled system, the effects of viral infection may be assessed in ways that are impossible to do in animals. For example, now that the growth patterns of three different viruses have been established, it will be possible to examine other viral effects such as the functioning of joint cells. For example, the levels of inflammatory mediators (interleukins and prostaglandins) or changes in cell structure and function (proteoglycan composition and collagen secretion) may have subtle effects on the maintenance of joint structure with long-term pathological consequences.
REFERENCES


