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ADENOVIRUS INFECTION OF HUMAN LYMPHOID TISSUE: A DNA HYBRIDIZATION STUDY

by

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ABSTRACT

ADENOVIRUS INFECTION OF HUMAN LYMPHOID TISSUE:
A DNA HYBRIDIZATION STUDY

The aim of this study was to evaluate Southern blot hybridization as a means of studying latent adenovirus infection of human tonsils.

Latent adenovirus was isolated from 8 of 32 tonsils. While virus was not isolated directly from these eight tonsils, seven tonsils yielded adenovirus in explant fibroblast cultures and one tonsil yielded virus by culture of lymphocytes with HEp-2 cells. Seven of the 8 adenovirus isolates from latently-infected tonsils were adenovirus type 1 (subgenus C). The remaining isolate was an adenovirus type 3 (subgenus B) which is not known to latently infect tonsils.

Adenovirus was recovered directly from 3 of 32 tonsils, as well as from explant fibroblast cultures of these tonsils. All adenovirus isolates from non-latently infected tonsils were subgenus C members.

Southern blot hybridization detected adenovirus DNA in DNAs from the cultured fibroblasts of 5 latently-infected tonsils. Adenovirus DNA was detected in both cultured and uncultured lymphocytes from a single latently-infected tonsil; however, these lymphocytes did not yield infectious adenovirus in culture. Adenovirus DNA was not detected in uncultured total tonsil DNA at a detection limit of 1 adenovirus genome in 10 cells, on average, even those positive for virus by culture. However, hybridization with

adenovirus DNA probes detected cellular DNA sequences which show homology to distinct regions of the adenovirus genome. The identity and function of these cellular sequences is as yet unknown.

In this study, combined culture and Southern blot hybridization results have shown that the initial number of tonsillar cells harbouring latent adenovirus is small, and that other hybridization methods (eg. *in situ*) are required to study adenovirus latency at the molecular level.

Karl P. Fischer
March, 1989

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Abbreviations

Ad	Adenovirus
AINL	Adenovirus-infected non-latent
ALCD	Adenovirus-like cellular DNA
CPE	Cytopathogenic effect
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
EKC	Epidemic keratoconjunctivitis
HBSS	Hanks' balanced salt solution
HSV	Herpes simplex virus
kbp	Kilobase pairs
MEM	Minimal essential medium
PBL	Peripheral blood lymphocytes
PHA	Phytohemagglutinin
RE	Restriction endonuclease
RNA	Ribonucleic acid

I. INTRODUCTION

The first adenovirus to be recognized was isolated from explant cultures of adenoid tissues by Rowe *et al.*(1953). It was found that a proportion of these cultures underwent a spontaneous degeneration and this effect was attributed to a filterable infectious agent which is now known as an adenovirus. In retrospect, this was the first recorded instance of latent adenovirus infection.

It was not until the work of Evans (1956) that adenovirus latency was defined, albeit as an operational definition only. The phenomenon of adenovirus latency was defined as the recovery of infectious virus from explant cultures of infected tissue while infectious virus could not be isolated directly from said tissue. Since then, numerous reports have shown that adenoviruses are able to establish a latent infection in a variety of cell types from adenoids and tonsils - epitheliod cells, fibroblasts and lymphocytes. The adenoviruses recovered from latently-infected adenoid and tonsil tissues are exclusively from subgenus C (adenovirus types 1, 2, 5 and 6). Some of these studies, as well, indicate that the number of cells containing infectious adenovirus *in vivo* is very small; the reported frequency of cells harbouring infectious adenovirus ranges from one in 10^5 cells to one in 10^7 cells. While there are published studies to date where adenovirus DNA has been detected by DNA hybridization in tonsillar DNA, none of the studies are clearly interpretable with regards to adenovirus latency.

The phenomenon of adenovirus latency is poorly understood at the cellular and molecular levels. Unlike the well characterized herpesvirus latency model in which the herpesvirus DNA genome is present within sensory ganglia in the absence of infectious virus, many aspects are still unknown for the adenovirus system. At the cellular level it is still unclear as to which cell type or types are latently-infected with adenovirus *in vivo*. The actual state of

'latent' infection is unknown, as well; it is possible that adenovirus 'latency' is an abortive infection in the majority of cells within a tonsil or adenoid, with only a very small subgroup of cells producing infectious virus. Another possibility is that, as in the herpesvirus model, the adenovirus genome is present but unexpressed within a small number of cells until the conditions are conducive to the production of infectious virus. At the molecular level, it remains unclear as to the form of the adenovirus genome within latently-infected cells (*ie.* linear, integrated, episomal).

The objective of this study was to evaluate the usefulness of DNA hybridization for studying the phenomenon of adenovirus latency at the cellular and molecular level. While laent adenovirus has been demonstrated by explant culture of latently-infected tonsil tissue, explant culture and DNA hybridization using the Southern blot technique have never been directly compared. The rationale for using Southern blot hybridization ws that it might give an indication of what latency is at the molecular level. To this end both cultured and uncultured tonsil tissues were examined for the presence of infectious adenovirus and adenovirus DNA (by Southern blot hybridization). The use of culture would establish which tonsils in the study were latently infected according to the operational definition, while Southern blot hybridization was expected to allow some statement to be made regarding the identity of the infecting adenovirus and the form of the adenovirus genome. As well, Sothern blot hybridization would give an estimate as to the relative frequency of adenovirus-infected cells *in vivo*.

The study will show that while Southern blot hybridization was useful in the detection and identification of adenovirus DNA present in most latently-infected tonsil cultures *in vitro*, it was not sensitive enough in our hands to detect the small number of adenovirus genomes present in

what must be an extremely small number of tonsillar cells *in vivo*.

II. LITERATURE REVIEW

1. Adenoviruses

A. General Properties

Adenoviruses (family Adenoviridae) are DNA viruses which have been isolated from a variety of animal species - birds, amphibians, marsupials, and mammals (including man) (Wigand *et al.* 1982). All adenoviruses (Ads) have the same general structure: a linear duplex DNA genome, some 30-42 kilobase pairs (kb) in size, which is complexed with protein and is enclosed within an icosahedral, nonenveloped outer capsid. The mammalian Ads (genus Mastadenovirus), however, possess a common antigenic determinant (alpha) on the major structural protein of the virion, the hexon, which avian Ads (genus Aviadenovirus) do not (White and Fenner, 1986). While similar in structure, Ads do differ between the different animal species. One such difference is the property of host specificity. Human Ads are able to establish a productive infection in permissive cells of human origin but abortively infect non-permissive cells, such as those of rodent origin. These abortively infected cells do not produce virions though they may be induced to undergo malignant transformation. As well, human Ads themselves differ from one another in pathogenicity, oncogenicity in experimental animals, multiplication characteristics in cultured cells, G+C content and size of the DNA genome, and immunological properties of the virion.

B. Types of human adenovirus infection

Adenoviruses are able to productively infect permissive cell types (eg. human epithelium) in which a large number of

infectious viral particles are released with the death and lysis of the infected cell. As well adenoviruses are able to abortively infect nonpermissive cells (eg. hamster cells). In abortive infection early genes, representing functions required for the replication of the viral genome, are expressed but there is no virus multiplication and no infectious virus is released. During abortive infection the cells are either killed or, in a small percentage of cells, are transformed. Transformation results in morphological changes in the cell coordinated with a loss of contact inhibition and, in some cases, an enhanced oncogenicity when these transformed cells are inoculated into syngeneic infant animals or nude mice (Flint, 1980a). Cells which have been transformed by adenoviruses contain adenovirus DNA integrated into the host chromosome. The integrated viral DNA sequences are usually present in multiple copies but do not represent a complete genome. As well there seems to be no preferred location in the cellular genome of a specific cell type in which insertion of viral sequences occur. The leftmost 8-14% of the adenovirus DNA genome is conserved in all adenovirus-tranformed cell lines; the majority of this work has been done with Ad 2, Ad 5 and Ad 12 and these sequences represent the *E1* or transforming region of the adenovirus genome (Flint, 1980b).

In addition to these forms of infection, certain adenovirus types have been associated with latent infection of human adenoid and tonsil tissues (Rowe *et al.*, 1953). However, the term latency as applied to adenoviruses is a purely operational one; infectious adenovirus can be isolated from explant cultures of infected tissue but cannot be isolated directly from uncultured tissue. This is unlike herpes simplex virus latency where the viral genome, but no viral particles, is present within trigeminal and dorsal-root ganglia in an as yet unknown state (Roizman and Sears, 1987).

C. Separation of human adenoviruses into subgenera by DNA genome homologies

Prior to the advent of modern molecular biological techniques attempts had been made to group human adenoviruses on the basis of shared biological and structural properties such as oncogenicity in newborn hamsters, sizes of virion proteins, and differential hemagglutination of rat and rhesus monkey erythrocytes. However, in 1979 Green *et al.* proposed that the most definitive means of grouping adenoviruses is on the basis of DNA homology. By employing solution hybridization with *in vitro* labelled adenoviral DNA it was found that the 31 human adenovirus types known at the time could be separated into 5 groups or subgenera (A-E) on the basis of DNA homologies (Table 1). With the exception of subgenera A and F, there is a high level of homology between DNAs of members from the same subgenus (*ie.* intrasubgenic homology) while there is a low degree of DNA homology between the different subgenera (*ie.* intersubgenic). This grouping of adenoviruses on the basis of DNA homology was shown by Green *et al.*(1979) to correspond well with biological and structural properties previously mentioned.

By 1984, the number of known adenovirus types had risen to 41. Adenovirus types 32,33 and 36-39 were placed in subgenus D while types 34 and 35 were placed in subgenus B; the basis for these placements was DNA restriction enzyme pattern(Wadell, 1984). However, adenovirus types 40 and 41 were at this time placed in separate subgenera (F and G, respectively) on the basis of DNA restriction endonuclease fragment patterns (de Jong *et al.*, 1983). van Loon *et al.* (1985) showed, however, that DNAs from Ad 40 and Ad 41 showed 62-69% homology between themselves while only showing 15-22% homology with the DNA of Ad 5. On this basis Ad 40 and Ad 41 were both placed into subgenus F.

There are presently 47 human adenovirus types recognized (Hierholzer *et al.*, 1988) of which the prototype isolates of

TABLE 1

SEPARATION OF HUMAN ADENOVIRUSES INTO SUBGENERA ON THE BASIS OF DNA HOMOLOGY.

		DNA homology[1]	
Subgenus	Types	Intrasubgenic	Intersubgenic
A	12, 18,31	48-69%	8-20%
B	3, 7, 11, 14, 16, 21, 34[2], 35[2]	89-94%	9-20%
C	1, 2, 5, 6	99-100%	10-16%
D	8, 9, 10, 13, 15, 17, 19, 20, 22-30,32[2], 33[2], 36-39[2]	94-99%	4-17%
E	4	100%	4-17%
F	40, 41	62-69%	15-22%[3]

1. Adapted from Green et al., 1979.
2. Placement on basis of shared restriction endonuclease patterns (Wadell, 1984).
3. From van Loon *et al.*, 1985. Intersubgenic homology relates only to comparison with Ad 5 DNA.

the first 41 have been well characterized by DNA restriction analysis (Adrian *et al.*, 1986). While the determination of DNA homology between adenovirus types is the more definitive method of grouping, the ease, speed and amount of known adenovirus DNA restriction fragment patterns has made DNA restriction analysis the method of choice for initial classification of an adenovirus isolate to a subgenus and for tentative identification of the serotype.

D. Human adenovirus genome types

In the clinical laboratory, adenovirus isolates are typically typed by neutralization of infectivity with type-specific antisera. This procedure is, however, very labour intensive even if an isolate is only tested with antisera to the most commonly isolated adenovirus types (types 1-8). To overcome, this many groups have used DNA restriction endonuclease (RE) analysis to type uncommon clinical isolates (Hammond *et al.*, 1985) and evaluate adenovirus genome stability in a given population (Wadell, de Jong and Wolontis, 1981; Kemp and Hierholzer, 1986). As a consequence of RE analyses it was found that some Ad isolates, which react specifically with antiserum to a given adenovirus type, possess DNA RE patterns which are distinct and different from those of the adenovirus prototype. These variants have been called adenovirus genome types (Brukova *et al.*, 1980; Wadell *et al.*, 1981; Adrian *et al.*, 1985).

Adenovirus type 7 (Ad 7), a subgenus B adenovirus frequently associated with outbreaks of upper respiratory illness, is the best studied adenovirus with respect to genomic variation. Wadell and Varsanyi (1978) examined the use of RE analysis as an alternative to serological methods for examining Ad 7 isolates. RE digests of DNAs of Ad 7 prototype (Gomen) and the serological variant Ad 7a were compared to those of Ad 7 isolates from 3 patients showing severe systemic infections. Ad 7 (Gomen) and Ad 7a are

different from each other by DNA RE analysis. It was found that the Ad 7 DNA RE fragment patterns from the patients, while identical to each other, were distinct from those of Ad 7 (Gomen) and Ad 7a. These Ad 7 isolates were designated Ad 7b genomic variants. Wadell, de Jong and Wolontis (1981) showed that Ad genome typing was useful in establishing the alternating appearance of different Ad 7 genome types (Ad 7b and Ad 7c) during epidemic outbreaks in Europe. To date some 17 different Ad 7 genome types have been identified (Li and Wadell, 1986; Kannemeyer *et al.*,1988).

The evidence of genomic variation of those adenoviruses most often associated with latent infection of lymphoid tissues (subgenus C adenoviruses) is limited in the literature. Aird *et al.* (1983) found that a clinical isolate which was serologically Ad 2 showed different DNA RE patterns with 10 of 18 enzymes when compared to the Ad 2 prototype (strain adenoid 6). This isolate was designated Ad 2a. Fife *et al.* (1985) found that of 12 adenovirus type 1 and type 2 isolates, 8 demonstrated variation in DNA RE patterns compared to those of prototype strains. These isolates were designated Ad 1a-c and Ad 2 b-d. Genomic variants have been similarly described for adenovirus type 5 (Ad 5a, Bruckova *et al.*, 1980; Ad 5b-d, Webb *et al.*, 1987) and adenovirus type 6 (13 genome types, Adrian *et al.*, 1985).

E. Human adenoviruses and disease

Adenoviruses are able to cause a variety of diseases ranging from a mild upper respiratory infection to severe systemic infections in an immunocompromised host. Interestingly, the pathogenicity of the different adenovirus types follow their division into subgenera quite well (Tables 1 and 2).

Subgenus A members (Ad 12, Ad 18, Ad 31) are most commonly isolated from the feces of people showing no overt

TABLE 2

HUMAN ADENOVIRUS TYPES COMMONLY ASSOCIATED WITH SPECIFIC DISEASES[1]

Types commonly associated	Represented Subgenera	Disease
1, 2, 3, 5, 6, 7	B, C[2]	Acute febrile pharyngitis
3, 7, 14	B	Pharyngoconjunctivitis
3, 4, 7, 14, 21	B, E	Acute respiratory disease
1, 2, 3, 4, 7	B, C, E	Pneumonia
8, 11, 19, 37	B, D	Epidemic keratoconjunctivitis
11, 21	B	Acute hemorrhagic cystitis
40, 41	F	Gastroenteritis

1. Adapted from Horowitz, 1985.
2. Subgenus C members are associated with latent infection of tonsils and adenoids.

signs of illness although Ad 31 has been recovered from the stools of infants with gastroenteritis.

Subgenus B members are associated with a variety of diseases ranging from epidemics of upper respiratory infection and febrile pneumonia (Ad 3 and Ad 7) to acute hemorrhagic cystitis (Ad 11).

Subgenus C members (Ad 1, Ad 2, Ad 5, Ad 6) are most commonly associated with upper respiratory infections of children and infants. They are also associated with latent infection of lymphoid tissues; it is not known if these latent virus 'reactivate' to produce any pathogenic effects in humans.

Of subgenus D adenoviruses, Ad 8 and Ad 19 are known to be the etiological agents of epidemic keratoconjunctivitis (EKC). As well, the most recently discovered members of this subgenus (Ad 42 - Ad 47) are associated with patients who are immunosuppressed by AIDS(Hierholzer *et al.*, 1988).

The lone member of subgenus E, Ad 4, is responsible for epidemics of upper respiratory disease in military recruits as well as causing pneumonia.

Members of subgenus F, Ad 40 and Ad 41, are associated with mild to severe gastroenteritis and diarrhea in children (de Jong *et al.*, 1983; Wadell, 1984).

While particular adenovirus serotypes are associated with specific diseases, as illustrated in Table 2, other adenovirus serotypes (examples - serotypes 13, 14 and 18) are rarely isolated and the association of these viruses with a distinct illness has not yet been established.

2. Anatomy of selected lymphoid tissues

Palatine tonsils, often referred to simply as 'tonsils', are two ovoid masses of lymphoid tissue which thicken the lamina propria of the mucous membrane extending between the glossopalatine and the pharyngopalatine. Epithelium, of the stratified squamous nonkeratinizing type, overlies a thin

layer of connective tissue and invaginates to form 10-20 deep primary crypts. The epithelium lining the primary crypts may extend into the adjacent lymphatic tissue to form secondary crypts. Lymphatic nodules, with their prominent germinal centers, are embedded in a diffuse mass of lymphoid tissue and are usually arranged as a single layer under the epithelium. The epithelium crypts are partially separated from one another by thin partitions of loose connective tissue. In this connective tissue are numerous lymphocytes, mast cells and plasma cells. These lymphocytes are able to migrate directly through the crypt epithelium into the intraluminal space (Cormack, 1987).

The location of the tonsils at the crossover point between the respiratory tract and the digestive tract increases the likelihood that lymphocytes will encounter antigens of potentially infectious agents against which antibodies should be produced as rapidly as possible. Cells of the immune system present within tonsils are mainly of B-lymphocyte lineage (50-60%) with some T-lymphocytes (15-25%), macrophages and monocytes (0-2%)(Gelfand, 1976). M cells (membranelike epithelial) in the epithelium serve to transport samples of intraluminal antigens across their cytoplasms to these lymphocytes, macrophages and monocytes which reside in the lamina propria above the lymphatic nodules. This antigen sampling is thought to be important in the process of generating IgA-producing plasma cells. Secretory IgA produced then aids in the mucosal immunity of the respiratory and digestive tracts by decreasing the adherence of microbes to the epithelial surface, as well as neutralizing viruses and bacterially produced intraluminal toxins which may be present.

Pharyngeal tonsils (adenoids) project from the roof of the nasopharynx and are often present unpaired. Pharyngeal tonsils are covered by pseudostratified columnar epithelium but unlike palatine tonsils possess no epithelial crypts. As with palatine tonsils, pharyngeal tonsils have lymphoid cells

in the underlying connective tissue but owing to their thin dimensions (3 cm X 2 mm thick) the concentration of lymphoid cells does not approach those found in palatine tonsils.

Lymph nodes are small bean-shaped bodies which are encapsulated by fibroblastic connective tissue. As part of the body's lymphatic system, lymph nodes serve as one of the first lines of defence against bacteria. Resident macrophages line the sinuses, the space underlying the capsule of the node, and serve to engulf foreign material. These macrophage are thought to process the ingested antigens and present them to plasma cells and plasmablasts circulating through the node sinuses. By this means antibody production to a particular antigen is initiated (Han and Holmstedt, 1981).

3. Adenoviruses in naturally-infected lymphoid tissues - Detection by culture

The first human adenovirus was discovered by Rowe *et al.* (1953) during an attempt to establish cell lines from human adenoid tissues. It was found that fragment explant cultures of 33 of 53 adenoids examined underwent spontaneous degeneration after 8-28 days in culture. This cytopathogenic effect (CPE) could be passed in cell-free filtrates of culture supernatants to continuous cell lines (eg. HeLa). The agent responsible was subsequently identified as what is now known as an adenovirus. This was the first instance in which adenoviruses were shown to latently infect human lymphoid tissues.

A. Latent versus infectious virus

In order to properly establish whether or not virus recovered from a tissue is latent a distinction between what is and what is not adenovirus latency must be made. The definition of latent adenovirus infection, put forth by Evans (1958), is "... the viruses are present without evidence of

clinical illness, apparently [persisting] for long periods, and can only be demonstrated by growing the excised tissue *in vitro*." As such, latent virus can be distinguished from non-latent ('infectious') virus by direct examination of homogenates of tissue for virus in susceptible cell cultures (eg. HeLa, KB, HEp-2).

Evans (1958) attempted to recover infectious virus directly from adenovirus-positive tissues by i) inoculation of specimen collection fluid into HeLa cell cultures, ii) cocultivation of minced tissue with HeLa cell monolayers, and iii) inoculation of tissue homogenate into HeLa cell cultures. Even after carrying these cultures for 27-53 days Evans could not detect any infectious virus. However, Evans examined only 3 of 15 adenovirus-positive tonsil tissues by these methods; it is not inconceivable that if all these tissues had been examined one or more might have yielded infectious virus.

Strohl and Schlesinger (1965b) examined tissue fragments and suspensions of trypsinized cells derived from tonsils and adenoids for the presence of infectious virus. Trypsinized cell suspensions were subjected to five cycles of freezing and thawing then inoculated into KB cell cultures. Tissue fragments were homogenized, frozen and thawed five times, then likewise inoculated into KB cell cultures. Of twenty specimens examined by these methods, infectious virus was isolated only from one specimen. Unfortunately, the authors failed to indicate the duration of time for which these cultures were maintained, and it is therefore impossible to say if this single isolate represents a minimum or maximum recovery level.

Van der Veen and Lambriex (1973) examined total cell suspensions and purified lymphocytes derived from tonsils and adenoids for the presence of infectious virus. After the preparation of a cell suspension, an aliquot was subjected to cycles of freezing and thawing to disrupt the cells, clarified by centrifugation, and the supernatant was

inoculated into primary thyroid cell cultures. Cultures were maintained through two or more passages for a total of 30 days before a specimen was called virus-negative. By this method the authors were able to isolate adenoviruses from total cell suspensions of 7/55 (12.7%) adenoids and none of the three tonsils. Examination of purified lymphocytes from the same tissue specimens yielded only one isolate from the 55 adenoids examined and none from the three tonsils.

Snejdarova *et al.* (1975) examined the homogenates of 97 adenoid vegetations for infectious adenovirus. Homogenates were prepared by the method employed by Strohl and Schlesinger (1965b) and inoculated into LEP (*sic*) cell cultures. After 21 days in culture no virus was demonstrable in any of the homogenates. However, latent adenoviruses were recovered by explant culture from 30 of these 97 adenoid vegetations. This is the only report in the literature to date which shows the recovery of only latent adenovirus from tonsil or adenoid tissues (*ie.* total failure to recover infectious virus).

Andiman, Jacobson and Tucker (1977) reported the isolation of an adenovirus from mononuclear cells of a 5-month-old child three weeks prior to the development of Ad 2 lower-respiratory-tract disease. The mononuclear cell fraction was isolated from heparinized blood and co-cultivated with human placental fibroblasts. A total of 6×10^6 mononuclear cells (8 tubes with 7.5×10^5 cells per tube) were cultured in this manner for four weeks at which time a CPE was evident in one of the eight tubes. This isolate was subsequently identified by neutralization with type specific antiserum as Ad 2, the same adenovirus serotype recovered from tracheal aspirates taken during the patient's episode of lower-respiratory disease.

B. Method and frequency of adenovirus isolation

The most commmonly used method of culture for the recovery of adenoviruses from naturally-infected lymphoid tissues are i) explant cultures of tissue fragments and ii) cell suspensions of trypsin-dispersed tissue. Some researchers (Strohl and Schlesinger, 1965b; Snejdarova *et al.*, 1975) found that the recovery of virus from tonsils and adenoids by either method was comparable while Evans (1958) found that the rate of recovery was higher for suspension cultures than for explant cultures (20% versus 5%).

Recovery of adenoviruses from tonsil tissue ranged from 26% (Israel, 1962) to 57% (van der Veen and Lambriex, 1973) whereas recovery from adenoid tissue ranged from 31% (Snejdarova *et al.*, 1975) to 65% (van der Veen and Lambriex, 1973). Adenovirus recovery from mediastinal lymph nodes ranged from 0% (Evans, 1958) to 65% (Bell and Steyn, 1962).

C. Characteristics of tissue donors

Tonsils are removed if a patient has had a history of chronic tonsillitis (Evans, 1958). Surgical removal of adenoids aften accompanies tonsil removal if the patient is under 10 years of age (Israel, 1962). Maximum recovery of adenoviruses from tonsil and adenoid tissues is found if the donor is under 10 years of age (Evans, 1958; Israel, 1962; Snejdarova *et al.*, 1975). No information is available as to whether one sex is more predisposed to latent adenovirus infection than the other.

Mesenteric lymph nodes, appendices, and, in some cases, portions of the small intestine are removed if a patient was undergoing surgery for mesenteric adenitis, intussusception, or appendicitis (Bell and Steyn, 1962; Potter, 1964; Bonard and Paccaud, 1966). Adenovirus was isolated from a higher proportion of intussusception patients (typically less than 2 years old) than from patients with mesenteric adenitis

(typically 3-11 years old). As with tonsils and adenoids there is no evidence of predisposition to adenovirus infection due to gender.

D. Emergence and persistence of adenovirus in cultures of lymphoid tissues

The time of emergence and duration of virus release in cultures of tonsil and adenoid tissues have been examined in several studies. As a general rule, evidence of infectious adenovirus, either a CPE or virus in culture supernatants, can be found in most cultures from virus-positive adenoid or tonsil tissues after two to four weeks.

Evans (1958) found that degeneration (CPE) in tonsil explant cultures began after an average of 36 days (range 17-96 days). However, the first isolation of virus occured, on average, after only 20 days in culture (range 8-40 days). The average duration of virus release into the culture medium was 36 days (range 16-74 days) In the case of one tonsil, virus was first found in explant culture supernatants on day 40 and release of virus continued for 16 days; however, the fragment cultures did not show evidence of degeneration until day 96. These results indicate adenoviruses can be isolated from explant cultures of tonsil tissues on average 2 weeks before the appearance of a viral CPE. This illustrates that adenoviruses need not always cause a visible CPE in the cultures they infect.

Israel (1962) found that, of 45 tonsils yielding virus in suspended fragment culture, virus was isolated from 20 (44%) within 2 weeks of beginning culture. The remaining cultures only became virus-positive after a total of four weeks in culture. By comparison, virus was isolated from only 2 of 22 virus-positive tonsils after 15 days of explant culture. After an additional 15 days virus was detected in 17 (77%) of all Ad-positive tonsils. Virus recovery from the remaining 5 tonsils required more than 30 days of culture

before detection. By contrast, of 61 adenoid tissues ultimately yielding adenovirus in explant culture 69% were positive for virus after only 20 days in culture. After 25 days in culture the recovery level increased to 92% of all adenoids studied. This indicates that adenoviruses could be isolated earlier from adenoid culture than from tonsil explants. Whether this reflects a higher load of virus in the adenoid tissue, and increased permissiveness for adenovirus of cells cultured from adenoid tissue, or other factors associated with the particular type of tissue are questions which were not addressed.

Snejdarova *et al.* (1975) were able to detect virus in the supernatant from fragment cultures of adenoid vegetations more readily than in trypsinized cell cultures from the same tissues (62% versus 30%, respectively, after 2 weeks of culture).

Strohl and Schlesinger (1965b) found that CPE appeared, on average, earlier in cultures of trypsinized adenoid and tonsil tissue than in corresponding fragment cultures (2-5 days versus 2-20 days). Van der Veen and Lambriex (1973) reported similar findings for their explant cultures of tonsil and adenoid tissues (range 5-15 days). Neither group checked the time of appearance or duration of excretion of virus in these cultures.

As yet there is no explanation as to what accounts for the difference in adenovirus isolation frequency with the different tissue culture methodologies.

E. Cells involved in latent adenovirus infection

By examining the cell type or types which are present in explant cultures, researchers have attempted to identify which cell populations may be preferentially predisposed to latent infection by adenoviruses. Evans (1958) found that explant cultures of tonsil and mediastinal lymph node tissues resulted in cell outgrowths which were almost exclusively

fibroblastic in appearance. A proportion of cultures from each of these tissues was shown to harbour adenovirus. However, it was noted that small sheets of epitheliod cells were evident in some tonsil explant cutlures. Evans also showed that these epitheliod cells were as susceptible to adenovirus infection as HeLa cells, a cell line commonly used for detection and propagation of adenoviruses.

Strohl and Schlesinger (1965b) suggested that fibroblasts represented the major, if not only, susceptible cell type in tonsils and adenoids. This was based on their finding that the proportion of cells showing fibroblastic morphology in suspension cultures (0.3%-1.4%) was comparable to the maximum frequency of infectable cells (0.4-2%) as determined by infectious center assay. This, however, was found experimentally using cells exogenously infected with Ad 2. In latently-infected tissue it was found that the frequency of infected cells ranged from 10^{-7} to 10^{-9} and that these values could not be correlated to the presence of a particular cell type.

Van der Veen and Lambriex (1973) found that purified lymphocytes derived from both tonsils and adenoids yielded adenovirus in culture especially when cultured in the presence of phytohemagglutinin (PHA). However, they found that explant fragment cultures of the same tissues yielded adenovirus in a much higher proportion of cultures. Adenoviruses were isolated from 20 of 35 tonsils and 41 of 63 adenoids by fragment culture whereas these same tissues yielded adenovirus in PHA-stimulated lymphocyte cultures of only 2 of 27 tonsils and 6 of 63 adenoids. These same lymphocytes, when cultured without PHA, only yielded adenovirus in 4 of the 63 adenoids and in none of the 27 tonsils. It was also found that while tonsil explant cultures were almost excusively fibroblastic in morphology, similar explants from adenoid tissues showed a transition from initially epithelioid cells to fibroblastic cells with areas of epithelioid cells as the cultures aged. These

investigators hypothesized on the basis of these findings that lymphocytes may not be the principle cell type latently-infected *in vivo* but merely act as a courier of virus from a site of infection to the tonsil or adenoid where another cell type more permissive for the virus (fibroblastic, epithelioid) allows the establishment of a latent infection.

While Andiman, Jacobson and Tucker (1977) found that adenovirus could be recovered from mononuclear leukocytes a more definitive report of the cell preference for the establishment of latent infection has yet to be made.

F. Adenovirus serotypes isolated (Latent infection)

The majority of adenoviruses isolated from tonsil and adenoid tissues are members of subgenus C of which serotypes 1, 2, 5 and 6 are the only members. Evans (1958) found that of 15 adenoviruses isolated from tonsil explant cultures 10 were type 2, four were type 1, and one was type 5. Israel (1962) reported similar findings with explant fibroblast cultures of tonsil and adenoid tissues. Serotypes 1 and 2 together comprised 81% and 87%, respectively, of tonsils and adenoid isolates. The remaining isolates from both tissues were all adenovirus type 5. Strohl and Schlesinger (1965b) found that of 8 adenovirus isolates chosen at random for identification, 5 were type 2 with one isolate each of serotypes 1, 5 and 6. Snejdarova *et al.* (1975) reported identification of 9 serotype 1, eight serotype 2, nine serotype 5 and four serotype 6 adenovirus isolates from adenoid vegetations.

Van der Veen and Lambriex (1973) isolated adenoviruses from lymphocyte and fragment cultures from tonsil and adenoid tissues. Only adenovirus serotypes 1 and 2 were isolated from lymphocyte cultures. Adenoviruses isolated from corresponding fragment cultures of both tissue types yielded 25 serotype 1, 22 serotype 2, eleven serotype 5 and three serotype 6. As a general rule, based on this and other reports, the isolation

frequency of an adenovirus serotype from tonsil or adenoid tissues decreases as the serotype number increases.

Identification of adenovirus isolates from mesenteric and appendiceal tissues also follow this general rule. Bell and Steyn (1962) found that mesenteric lymph nodes from patients with intussusception and mesenteric adenitis yielded nine serotype 1, six serotype 2, two serotype 5 and one serotype 6. Adenovirus was isolated from the appendix of a single patient and was identified as serotype 2. As well, Potter (1964) and Bonard and Paccaud (1966) reported similar findings with the distribution of adenovirus types isolated from mesenteric lymph nodes and appendiceal tissues. No indication is made in any of these works as to whether the adenoviruses isolated were recovered directly from tissue homogenate, from tissue explant cultures, or both. This allows one to only note the association, and not latent infection, of adenoviruses with mesenteric lymph nodes and appendiceal tissues.

In addition to the isolation of subgenus C adenoviruses, some subgenus B adenoviruses were also recovered in these three studies albeit from a smaller number of tissues. Two adenovirus type 3 isolates were recovered from mesenteric lymph nodes of two patients in the study by Bell and Steyn (1962). The isolation of two adenovirus type 7 isolates and one each of adenovirus types 3 and 7 from mesenteric lymph nodes were reported by Bonard and Paccaud (1966) and Porter (1964), respectively. As yet there is no evidence that subgenus B adenoviruses are able to establish latent infection of any lymphoid tissues.

G. Adenovirus inhibitors in tonsil and adenoid tissues

It was suggested quite early in studies of adenovirus infection of tonsil and adenoid tissues that the presence of neutralizing antibody may be responsible for the phenomenon

which was, and still is, called adenovirus latency (Rowe *et al.*, 1955). It was thought that infectious adenovirus present from the outset of tissue culture was 'unmasked' (*ie.* no longer restrained) as neutralizing antibody was washed away by the routine of culture medium changes. Evans (1958) examined this possibility by incubating varying doses of an Ad 2 isolate with supernatant from cultured tissue that ultimately yielded this virus. The supernatant was taken at day 8, just prior to the first medium change for this culture. The adenovirus from this culture was first detected 15 days after the start of culture. By culture of these inocula in HeLa cells it was found that the supernatant did not possess any neutralizing activity against the isolate. Evans concluded, therefore, that neutralizing antibody did not play a role in the latent infection of this tissue specimen. However, he did not discount the possibility that neutralizing antibody may have a role in what is seen as latent adenovirus infection in some tonsil tissues.

Strohl and Schlesinger (1965b) tested homogenates of tonsil and adenoid tissues for the presence of adenovirus-neutralizing substances. This material was mixed with Ad 2, and in some cases also the virus isolated from the specimen, and remaining infectious virus was plaque titrated on KB cells (Strohl and Schlesinger, 1965a). Most homogenates demonstrated significant neutralizing activity as shown by a reduction in plaque number when compared to untreated virus preparation. Supernatants from frozen-thawed suspensions of trypsinized tissue failed to demonstrate any neutralizing activity. The authors concluded that failure to recover virus from trypsin-dispersed tissue cultures was not due to the presence of neutralizing substances.

Snejdarova *et al.* (1975) examined homogenates prepared from adenoid vegetations for the presence of adenovirus-neutralizing substances. The procedure employed was similar to that used by Strohl and Schlesinger (1965b). Tissue homogenate was incubated with 100 $TCID_{50}$ of virus for 2 hours

at 37°C followed by inoculation into cultures of LEP cells. Neutralization of virus was scored as positive if the onset of a CPE in LEP cell cultures was delayed or absent compared to untreated virus specimens. Of twenty-eight tissues yielding adenovirus in culture, homogenates of 22 tissues were shown to neutralize homotypic adenovirus (*ie.* the same adenovirus serotype as was isolated from the tissue). When eight of the homogenates were tested against heterotypic subgenus C adenovirus (*ie.* those subgenus C adenovirus serotypes not isolated from the tissue) no neutralization was evident. Of sixty-seven virus-free tissues, less than 10% possessed adenovirus-neutralizing substances. However, no indication was made as to whether the neutralizing substances from each of these tissues was specific for only a single subgenus C adenovirus serotype or was able to neutralize more than one subgenus C serotype.

The activity of the neutralizing substances present in these tissue homogenates was found not to be altered by heating at 60°C for thirty minutes. Also, the activity was not removed by centrifugation at 100,000 X g for 60 minutes. The activity could, however, be removed by absorption with concentrates of homotypic virus. These properties were reported as being consistent with those of specific neutralizing antibodies. However, since homotypic serum antibody titres were high in subjects whose adenoid vegetations were virus-positive, the origin of the antibodies present in these tissues has yet to be determined (*ie.* 'overflow' from the circulation, or locally produced). Regardless of its source, specific antibody in tissue was hypothesized to slow the spread of infection and thus control a low-grade persistent infection *in vivo* which is seen as a latent infection *in vitro*. Work to support or oppose this hypothesis has yet to be reported in the literature.

To date no studies concerning adenovirus-neutralizing or inhibiting substances have been reported for mesenteric lymph node or appendix tissues.

4. Detection of adenovirus sequences in naturally-infected lymphoid tissues by DNA hybridization

While explant culture of lymphoid tissue remains the standard method for detection of latent adenovirus, this technique is laborious, time consuming, and allows no insight as to the state of the viral genome within latently-infected cells. With the advent of molecular techniques, specifically DNA hybridization, a new means of studying adenovirus latency has become available.

Since subgenus C adenoviruses are ubiquitous in man and can transform non-permissive cultured cells *in vitro*, Green *et al.* (1979) examined human cancer DNAs and RNAs for nucleic acid sequences of subgenus C adenoviruses by solution hybridization. Positive controls for this study were tonsil DNAs and RNAs since the association of subgenus C adenoviruses with tonsil tissue had already been firmly established by explant culture studies. While subgenus C sequences could not be detected in cancer tissue DNAs or RNAs by solution hybridization at high stringency with a ^{32}P-labelled Hind III-G fragment of Ad 2 DNA, adenoviral sequences were detected in 14 of 52 (25%) tonsil DNAs. Of twelve tonsil DNAs further examined by Southern blot after Eco RI digestion, five were shown to hybridize with a ^{32}P-labelled Ad 2 genomic probe. Each positive tonsil DNA examined by Southern analysis showed all six Eco RI Ad 2 fragments; four of the five tonsil DNA blots also showed additional restriction fragments which differed in electrophoretic mobility from those of similarly restricted subgenus C adenovirus DNAs. The researchers, however, did not attempt to determine if these results represented an unusual form of the adenovirus genome (*eg.* integrated). Solution hybridization showed that in tonsils which contained adenovirus DNA that the complete adenovirus genome was present at greater than 20 copies per cell. This study,

however, does not address these findings in terms of adenovirus latency since neither culture of tissue nor virus isolation were performed.

Lord, Itzhaki, and Sutton, in a letter to Lancet in 1980, reported the comparison of cell culture with molecular hybridization for the detection of persistent adenovirus infection in tonsillar tissues. Of ten tonsils examined in this study, Ad nucleic acid sequences were detected in 6 tonsils (60%). In all six of these tonsils, adenovirus sequences were detected by *in situ* hybridization at high stringency with a subgenus C specific probe (^{3}H-labelled Ad 5 genomic DNA). However, when these same tonsils were examined by solution hybridization with the same probe at high stringency only DNAs from two of the six tonsils were shown to contain adenovirus sequences. The authors suggested that the disagreement between the two methods of hybridization might relate to the number of cells harbouring the virus genome. If only a small number of cells contain adenovirus sequences these may be detected by the *in situ* technique if a sufficient number of cells are screened whereas the same sequences might not be detected by solution hybridization.

Primary cell cultures of the ten tonsils were established in this study and 3 (30%) yielded adenoviruses, all of which were members of subgenus C (one each of adenovirus serotypes 1, 2 and 5). All three of these tonsils, as well, were positive for adenovirus sequences by either *in situ* or both hybridization methods.

While this study did compare isolation of Ads from primary tonsil cultures and detection of adenovirus sequences it made no attempt to recover adenovirus directly (*ie.* without culture of the tissue). Because of this deficiency, the study could conclude nothing regarding the virus status (latent or infectious) of the tonsil tissues from which adenovirus was recovered by primary culture. The hybridization techniques also failed to provide any information regarding the viral type detected though it can

be inferred from the stringency conditions used that the DNA detected belonged to an adenovirus of subgenus C.

Since adenoviruses have been shown to be associated with mesenteric lymph nodes in patients with mesenteric adenitis and childhood intussusception (Bell and Steyn, 1962; Potter, 1964; Bonard and Paccaud, 1966) a study was performed by Roche *et al.* (1981) in an attempt to determine whether or not adenoviruses may be a significant etiological agent in chronic inflammatory bowel disease (CIBD). Intestinal tissue DNAs from ten patients with CIBD and seven non-CIBD patients were examined by solution hybridization with ^{32}P-labelled genomic DNA probes representing five adenovirus subgenera (A-E). Intestinal tissues examined were colon, terminal ileum, sigmoid colon, cecum, and rectum. Under high stringency, and at a sensitivity level in which 0.2-1 virus genome equivalent per cell could be detected, no adenovirus sequences of any subgenus could be detected in either CIBD or non-CIBD patient tissue. The researchers did not, however, examine the tissues for adenovirus subgenus F sequences since the members of this subgenus had not been identified at the time of the study.

Horvath, Palkonyay and Weber (1986) screened human peripheral blood lymphocytes (PBLs), cord blood lymphocytes, and lymphoblastoid cell lines for the presence of subgenus C adenovirus DNA sequences since a direct demonstration of these viruses in uncultured lymphocytes had not been reported. PBL DNAs (from healthy adults) were digested with restriction endonucleases, electrophoresed, transferred to nitrocellulose then hybridized with ^{32}P-labelled Ad 2 DNA under conditions of high stringency. The cloned Ad 2 DNA consisted of an equimolar mixture of pBR322 plasmids containing all the Hind III fragments of Ad 2; the cloned DNAs span the entire Ad 2 genome. Thirteen of 17 samples were positive for adenovirus DNA sequences when examined in this manner. In addition to free viral DNA representing the entire adenovirus genome, high molecular weight bands were detected which did not comigrate with similarly digested subgenus C

DNA controls; the authors, however, did not examine this phenomenon further.

By employing the same probe for *in situ* hybridization, the authors examined PBL smears from positive donors to estimate the proportion of lymphocytes which carry Ad DNA. After examining 2000 cells per sample it was found that hybridization was restricted to 1 to 2% of cells.

The authors attempted to determine which cell population was responsible for carrying Ad DNA. After separation of T and non-T cell populations by the sheep rosette method, DNA was extracted from three samples and analyzed by Southern blot with the cloned probe. Both cell populations for each sample were positive for adenovirus DNA; as with the unfractionated PBL populations, additional bands were detected in addition to the genuine restriction fragments of Ad 2. Whether these originated from another adenovirus type, or from integrated sequences, was not determined.

Lymphocyte DNA from ten separate cord bloods from normal delivery of healthy pregnancies were examined by the Southern blot technique; only one of the ten was positive for adenovirus sequences. This positive cord blood gave a hybridization pattern similar to that of an adult PBL pattern, including a high molecular weight band, again suggesting possibly integrated sequences. These results compared with those of PBLs from healthy adults suggest that the infection of lymphocytes by subgenus C adenoviruses, as indicated by the presence of adenovirus sequences, is not likely due to vertical transmission but is more likely due to infection during childhood with subgenus C adenoviruses.

Neumann, Genersch and Eggers (1987) reported the only instance, to date, in which DNA hybridization and culture were directly compared for studying the phenomenon of adenovirus latency in tonsils. Using *in situ* hybridization at high stringency, cryostat thin sections of human tonsillar tissues were probed for subgenus C adenovirus sequences employing both a biotinylated cloned Hind III-G Ad 2 fragment

and a biotinylated Ad 2 full length genomic DNA probe. While positive hybridization was reported with both probes for a single tonsil no infectious virus could be demonstrated from the uncultured tissue nor explant cultures of said tissue even after propagation for 8 weeks. These cultures were themselves probed using *in situ* hybridization and again adenovirus sequences were detected. The *in situ* technique employed, however, does not allow any conclusion to be made as to whether i) the sequences being detected are RNA or DNA, ii) the sequences, if DNA, represent sequences other than those present in the Hind III-G Ad 2 fragment, and iii) the type of adenovirus whose sequences are present (though it can be inferred that the viral sequences belong to a member of subgenus C).

5. Permissiveness of human lymphocytes to adenovirus infection

Since it was found that adenoviruses could be isolated from purified lymphocytes of tonsil and adenoid tissues (van der Veen and Lambriex, 1973) many researchers began to examine the susceptibility of lymphocytes to adenovirus infection. This was examined so as to determine if virus-infected lymphocytes could play a role in initiating natural adenovirus infection.

Lambriex and van der Veen (1976) examined lymphocytes isolated from adenoid for the susceptibility to infection by adenovirus serotype 2 (Ad 2) and serotype 4 (Ad 4). Ad 2 was chosen since it represented one of the serotypes known to establish persistent infection of adenoid and tonsil tissues. Ad 4 was chosen, by contrast, because it has never been reported as an isolate from adenoid or tonsil tissues. Adenoid-derived lymphocytes infected with either Ad 2 or Ad 4 produced little virus after 8 days; the titre of cell-associated virus produced was less than ten-fold that of the titre of the virus inocula. However, if phytohemagglutinin

(PHA) was used to stimulate the lymphocytes at either the time of infection, or four days post-infection with Ad 2, the titre of virus produced increased 100-1000 fold. Little enhancement of virus production (less than 10-fold) was evident when these experiments were repeated with Ad 4. The researchers speculated on the basis of these findings that since Ad 2 could replicate in lymphocytes and Ad 4 could not that only those viruses capable of replicating in lymphocytes are able to initiate persistent infection of adenoid tissues.

Faucon and Desgranges (1980) found that lymphoblastoid cell lines derived from human cord blood leukocytes could allow the establishment of a persistent infection with Ad 5 but only after immortalization with Epstein-Barr virus (EBV). Ad 5 antigens were detected in 0.01% of cells 5 weeks after infection and this percentage reached a plateau 16 weeks post-infection at which time 3-5% cells were positive. The level of adenovirus production by these cell lines increased from 10^{-4} $TCID_{50}$ per cell 3 weeks post-infection to 32 $TCID_{50}$ per cell at 19 weeks post-infection; these findings, taken with those of the adenovirus antigen experiments, indicate that production of virus is restricted to only a small percentage of the total cells in culture. By contrast, Ad 5 infection of cord blood lymphocytes which were not co-infected with EBV resulted in cell death after 3 weeks without production of either Ad 5 antigens or infectious virus. Uninfected control cultures of these cells died out 4 weeks after isolation. Persistently-infected lymphoblastoid cell lines had undergone 27 weeks of culture without losing the ability to produce infectious Ad 5. The production of infectious virus by these cultures was found to be inhibited by the presence of Ad 5-specific neutralizing antibody in the culture medium. After 20 weeks of culture with Ad 5 antiserum the cells could be grown in normal culture medium for 4 months without detection of Ad 5 antigens or infectious virus. These results suggest that only a fraction of cord blood leukocytes are able to support

adenovirus multiplication and that the continual reinfection of susceptible cells results in the establishment of these persistent infections.

Andiman and Miller (1982) found that chronic infection with Ad 5 and Ad 6 could be established in EBV-transformed B-lymphocytes from human umbilical cord blood and woolly monkey blood. However, in contrast to the findings of Faucon and Desgranges (1980), adenovirus could be recovered for 8 weeks post-infection from infected cultures of primary mononuclear leukocytes from human umbilical cord blood. Using an infectious center assay it was shown that 20% of the EBV-transformed simian cells and 8% of the EBV-transformed human cells released infectious adenovirus at levels of 0.1-0.9 $TCID_{50}$ and 2-8 $TCID_{50}$, respectively. While Ad infection in cultures of EBV-transformed woolly monkey lymphocytes could be abolished by the presence of specific neutralizing antibody, EBV-transformed lymphocytes from human umbilical cord blood retained cell-associated virus for up to 6 weeks that restored infection in the culture when the antibody was removed. This finding contradicts those of Faucon and Desgranges (1980) who found that persistently-infected cells could be 'cured' of infectious virus by use of neutralizing antibody-containing culture medium; one possible explanation could be the relative lengths of cell culture in the presence of antibody (20 weeks versus 6 weeks). The authors thus concluded that chronic infection of lymphoid cells is maintained by at least two mechanisms: the intracellular persistence of infectious virus in the presence of neutralizing antibody and cell-to-cell spread of virus in the absence of antibody.

Faucon, Ogier and Chardonnet (1982) found that Ad 5 could replicate, albeit poorly, in both EBV-positive (Jijoye) and EBV-negative (Raji) Burkitt's lymphoma cell lines. Only a small fraction of each cell population was found to be able to support replication of Ad 5 (Raji 1-4%, Jijoye 1.6-12%). The production of infectious Ad 5 by these cell lines was

similar to results found previously (Faucon and Desgranges, 1980); Raji cells produced an average of 1.1 X 10^5 $TCID_{50}$ per 10^6 cells while Jijoye cells produced an average of 5.5 X 10^7 $TCID_{50}$ per 10^6 cells. Interestingly, virus particles produced by these cell lines differed from those produced in the fully adenovirus-permissive cell line HeLa. While the major viral components were synthesized, the virions produced by Raji and Jijoye cells exhibited a lower DNA content and variation in DNA restriction enzyme pattern. The significance of the changes and the method by which they arose was not addressed. However, the authors were able to conclude that while virus production by these cell lines was low compared to HeLa cells, EBV-transformation of the cell line was not required for the replication of infectious virus.

During a study examining human peripheral blood lymphocytes (PBLs), cord blood lymphocytes and lymphoid cell lines for subgenus C adenovirus DNA sequences, Horvath, Palkonyay and Weber (1986) found that adenovirus could be isolated from the PBLs of two healthy adult donors. After detection of Ad DNA in these specimens, different culture techniques were employed in an attempt to recover infectious virus. These PBLs were subjected to PHA- and IL-2 stimulation, co-cultivation with HEp-2 cells, transformation by EBV, and PBL fusion with 293 cells. Each of these treatments resulted in the recovery of adenoviruses from the PBLs of both donors; all the isolates were identified as subgenus C members. However, in both cases multiple subgenus C adenoviruses were isolated from a single donor's PBLs; all four subgenus C members were isolated from the PBLs of one donor while Ads 1, 2 and 5 were isolated from that of the second donor. These findings should be viewed with caution for, while not impossible, it is improbable that multiple adenovirus types, with all being subgenus C members at that, would be isolated from a single person's PBLs; one would be inclined to think that one adenovirus serotype would predominate. This conclusion is based on the work of van der

Veen and Lambriex (1973) which showed that adenoviruses isolated from tonsils and adenoid lymphocytes were of only single adenovirus types, *ie.* no multiple adenovirus types were ever isolated from a single specimen.

While this study does demonstrate the association of subgenus C adenoviruses with PBLs by culture, it has not examined whether or not infectious adenovirus could be detected directly (*ie.* without prior culture of the cells). Without this evaluation one can only speculate as to whether the adenovirus DNA sequences detected represented true latency or a chronic low-grade infection.

Productive infection of cultured human lymphoid cells by adenovirus serotypes 2 and 5 was demonstrated by Lavery *et al.* (1987). Both EBV-positive (Josh 7, SeD, 32A1) and EBV-negative (RPMI 8226, Manca, BJA-B) B-cell lines were shown to support adenovirus DNA replication as were three T-cell lines also examined (Jurkat, Molt-4, CCRF-CEM). Viral RNA synthesis, production of viral proteins, or assembly of infectious virions was also demonstrated for selected B- and T-cell lines. While the kinetics of infection were characteristic and different for each cell line there was no correlation apparent between the origin of the cell line and the kinetics of virus growth. The presence of the EBV genome was not found to be a prerequisite for adenovirus infection of the B-lymphoid lines. Unlike the findings of endogenous levels of adenovirus DNA in several established cell lines by Horvath *et al.* (1986), no discrete adenoviral DNA or RNA was detected in any of the lymphoid cell lines prior to infection with exogenous adenovirus.

Since adenoviruses had been previously shown to infect human lymphocytes *in vivo* (van der Veen and Lambriex, 1973) Abken, Butzler, and Willecke (1987) examined human lymphoid cell lines for the presence of adenoviral DNA sequences. These cell lines were established from human peripheral blood lymphocytes immortalized by fusion with cytoplasts from mouse L929 cells or by transfection with DNA isolated from L929

cytoplasts. Eight of 10 lymphoid cell lines of both T- and B-cell origin were found to contain Ad 5 DNA sequences. For these eight cell lines the Ad 5 genome was present at 40-70 copies per cell in an unintegrated episomal form. Transmission electron microscopic examination of these cell lines failed to detect adenovirions in the cytoplasm, prompting the authors to conclude that no adenovirus particles were produced. RNA transcripts of the adenoviral early regions E1A and E1B, as well as E1A proteins, could not be detected in any of these Ad 5-containing lymphoid cell lines. With detection of Ad 5 DNA sequences but the failure to detect RNAs and proteins of the transforming region (E1A/E1B) of the adenovirus genome these researchers concluded that the endogenous adenovirus was unlikely to be involved in the immortalization of the cells. There is, however, one error in the experimental procedure followed by these authors. They reported the failure to detect by EM the presence of adenovirions in the cytoplasm of the cells in culture. Since adenovirion assembly occurs in the nucleus of an infected cell, the nuclei should have been examined for the presence of virus particles, and not the cell cytoplasm.

Horvath and Weber (1988) reported that fresh peripheral blood lymphocytes (PBLs) were highly nonpermissive to exogenous infection with Ad 2 and that culture of PBLs with PHA could only marginally lift this nonpermissiveness. Adenovirus virions were found to bind to both T and non-T cells albeit to a lower percentage of cells (39% and 25%, respectively) than HEp-2 cells which are fully permissive to adenovirus infection. Adenovirus receptors were found to be present at approximately 780 per cell in unstimulated PBLs and this number increased to 980 per cell after culture in the presence of PHA for 24 hours. In comparison, receptors numbered 7,000 per cell for HEp-2 cells. Allowing for the difference in the cell size (the surface of a lymphocyte is approximately one-third that of a HEp-2 cell) it was concluded that virus adsorption was unlikey to be the cause

of lymphocyte nonpermissiveness to Ad 2 infection. However, virus uncoating was found to be much slower in PBLs than in HEp-2 cells and this uncoating process could not be enhanced by PHA treatment. As well,viral DNA synthesis was found to be approximately 460-fold lower in unstimulated PBLs than in HEp-2 cells. It was also found that, as previously described by other researchers, both the yield of infectious virus and the number of cells producing infectious virus were very low; compared to HEp-2 cells, virus production in resting PBLs was reduced 10^6-fold with only 0.35% of cells producing infectious virus (1.2 $TCID_{50}$ per infected cell). PHA stimulation of PBLs increased both virus yield and number of cells producing infectious virus (14-fold and 11-fold, respectively). Taken collectively these results demonstrate the high degree of nonpermissiveness of PBLs to Ad 2 infection.

In summary, established lines of lymphocytes have been shown to be permissive for adenovirus replication and the transformation of these cells by EBV is not required for this permissiveness. However, primary lymphocytes (either tonsil or PBLs) are almost non-permissive for adenovirus replication unless stimulated with a mitogen (eg. PHA). As well, this permissiveness is only present in a small percentage of cells in the total population of primary lymphocytes or cells of established lymphoid lines.

6. Adenovirus-like nucleic acid sequences in human tissues

Numerous groups have reported that uninfected human tissues have DNA or RNA which show homology to adenovirus DNA sequences.

Jones *et al.*(1979) found that RNA from human placenta and adult liver hybridize to four discrete regions of Ad 2 DNA. These four regions (0-7.5, 26-31.5, 37-58.5 and 70.7-75.9 mu) were also shown to hybridize with RNAs from gorilla organs, indicating that these homologous sequences are

possessed by at least one higher primate. Thermal stability analysis of the hybrids which were able to form showed that they were as stable as cellular DNA-cellular RNA hybrids.

Arrand *et al.* (1983) found that cytoplasmic RNA from human cell lines hybridized to similar discrete regions of the Ad 2 genome as Jones *et al.* (1979). It was also found, however, that cellular cytoplasmic RNA hybridized to discrete regions of Ad 3 and Ad 12 DNA. The regions of Ad 2 which were able to hybridize cytoplasmic RNA were similarly represented in Ad 3 while only a single region of Ad 12 DNA (spanning the *E1b* region) was able to hybridize. Thermal stability evaluation of the hybrids which formed was also examined and it was found that the level of mismatch between the cellular RNA and Ad 2 DNA was 7-11% echoing the results found by Jones *et al.*(1979).

Braithwaite *et al.* (1984) found that DNA, not RNA, from human white blood cells (adult and cord) and human placenta were able to hybridize to the *E1a* and *E1b* regions of Ad 2 and Ad 5. By employing cloned Ad 2 DNA fragments it was found that Ad 2 *E1a* and *E4* regions hybridized to numerous size classes (300 bp to 2.5 kbp) of Eco RI-digested human DNA. By reassociation kinetics it was found that these seemingly reiterated sequences were present at 10,000 to 100,000 copies per human diploid genome equivalent. In a subsequent work (Braithwaite *et al.*, 1986) it was found that the hybridizing Ad sequences could be localized to two small regions of the *E1a* and *E1b* genes. Computer comparison of *E1a* and *E1b* sequences demonstrated that these regions possessed no significant homology to each other nor to any known reiterated cellular DNA sequence (eg. Alu). Thus it was concluded that these regions are able to detect one or more as yet undescribed reiterated human DNA sequences.

Of these works the most consistent finding was that human cellular DNA and RNA sequences were able to hybridize to the *E1* region in the DNAs of several different adenovirus types. Whether this indicates that normal cells may contain

DNA (and RNA) homologous to the transforming region of adenoviruses (*ie*. a possible oncogene?) remains to be determined.

III. MATERIALS AND METHODS

1. Viruses

Prototype strains of adenovirus serotypes 1,2,3,4,5 and 7 were obtained from the American Type culture Collection (Rockville, Maryland). The adenovirus serotype 6 was a clinical isolate (strain 2533) kindly provided by the Cadham Provincial Laboratory, Winnipeg, Manitoba.

Herpes simplex virus type 1 (strain HSV-76) was a gift from Joseph Blondeau (Department of Medical Microbiology, University of Manitoba).

2. Viral and other DNAs

DNA was extracted from purified virions of adenovirus serotypes 2 and 3 by the procedure described in section III6. Adenovirus serotype 2 DNA was also purchased from Sigma Chemical Company (St. Louis, MD). Other adenovirus DNAs were extracted from infected HEp-2 cell cultures as total cellular DNA. It had been shown in this laboratory that restriction endonuclease patterns of adenovirus DNA can be clearly distinguished in total cellular DNA (Hammond *et al.*, 1985).

DNA from the human T-lymphoid cell line Jurkat was a gift from Dave Kristjanson (Department of Medical Microbiology, University of Manitoba).

Herring sperm DNA used in this study was purchased from Boehringer Mannheim.

3. Cell lines

HEp-2 (human epidermoid carcinoma) and Huff (human foreskin fibroblast) cell lines were generous gifts from the Cadham Provincial Laboratory, Winnipeg, Manitoba.

Stock cultures of both cell lines were propagated as monolayer cultures at 37°C in a 3% CO_2 in air atmosphere in

minimal essential medium (MEM) supplemented with 5% fetal calf serum and 5% Nu-Serum (Appendix C).

For detection and titration of adenovirus, HEp-2 cells were dispersed with trypsin (Appendix C), made into a cell suspension with MEM containing 5% fetal calf serum and 5% Nu-Serum and dispensed into disposable culture tubes at 120,000-200,000 cells per tube. After incubation at 37°C for 1-2 days with tubes tightly capped the cell monolayers were 70-80% confluent and ready for use.

Huff cells were processed for propagation of herpesvirus strains as described above except that 15 ml of each cell suspension (200,000 cells per ml) was used to seed each 75 cm^2 culture flask.

4. Plasmids and bacterial strains

The plasmid pCD2 (derived from pXC1) contained the leftmost 5% of the adenovirus serotype 5 genome and was a gracious gift from Frank Graham (Department of Biology, McMaster University). The plasmid was received as transformed *E. coli* strain C600 and was grown in LB broth or on LB agar, both supplemented with ampicillin (50 ug/ml) (Appendix C).

5. Propagation of virus

Adenoviruses were propagated in HEp-2 monolayer cultures. Medium was removed from 75 cm^2 monolayers and inoculated with 1 ml of supernatant containing 10^7 $TCID_{50}$ of virus. The flasks were incubated at 37°C for 1 hour to allow the adsorption of virus at which time the inoculum was removed. Fifteen millilitres of maintenance medium (MEM supplemented with 2% fetal calf serum and antibiotics - Appendix C) was added to each flask. Flasks were incubated at 37°C with caps sealed until an extensive cytopathogenic effect (CPE) was evident, usually after 2-4 days. Flask contents were subjected to 4-5 freeze-thaw cycles and then

clarified by low speed centrifugation (800 X g, 10 minutes). Clarified supernatants were stored at -56°C.

Herpesvirus strains in this study were propagated as described above but in Huff cell cultures rather than in HEp-2 cell cultures.

6. Purification of adenovirus particles

Ten to fifteen 150 cm^2 culture flasks of HEp-2 cells were frozen at -56°C when maximum CPE was first evident. The flasks were thawed at room temperature then subjected to four more freeze-thaw cycles. After the final thaw the supernatant was clarified first by centrifugation at 1500 rpm for 10 minutes, followed by a high-speed centrifugation at 10,000 rpm for 10 minutes. Virus present in the supernatant was collected by centrifugation at 30,000 rpm for 2 hours at 4°C in a Beckman Ti 42.1 rotor. Virus pellets were resuspended in DNAse buffer (Appendix D) and incubated at 37°C for 1 hour with DNAse I and RNAse A (final concentration of both enzymes - 50 ug/ml)(Appendix D). The solution was extracted twice with an equal volume of ice-cold 1,1,2-trichloro-1,2,2-trifluoroethane (Freon). The final aqueous phase was layered over pre-formed cesium chloride gradients (1.20 - 1.50 g/ml) and centrifuged at 27,000 rpm for 4 hours at 4°C in a Beckman SW 27 rotor. The virus band was collected by bottom-puncture fractionation, adjusted to a density of 1.34 g/ml with cesium chloride and centrifuged at 40,000 rpm for 24 hours at 4°C in a Beckman SW 40 rotor. The virus band was collected as described above, and the density of the virus fraction was verified to be 1.34 g/ml by refractometry (Bausch and Lomb refractometer, Model ABBE-3L). Virus was dialyzed against 4 litres of TE buffer overnight and processed immediately for DNA extraction.

7. DNA extraction from purified adenovirus particles

One-half millilitre aliquots of purified virus suspension were dispensed into sterile 1.5 ml microcentrifuge tubes and proteinase K (Appendix D) was added to each aliquot to a final concentration of 1 mg/ml. The suspension was mixed by inversion and incubated at 45°C for 1 hour. Sodium dodecyl sulfate (SDS) and 2-mercaptoethanol were added to final concentrations of 0.15% and 0.015% respectively, mixed and incubated at room temperature for 30 minutes. DNA was extracted with an equal volume of TSE-saturated phenol (Appendix D). After centrifugation for 3 minutes at 12,800 X g in an Eppendorf centrifuge (Model 5412), the aqueous phase was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1,v/v/v), then once with an equal volume of chloroform. DNA in the aqueous phase was precipitated with 1/25th volume of 5M NaCl and 2 volumes of absolute ethanol at -70°C for 30 minutes. The DNA was collected by centrifugation at 4°C for 30 minutes in the Eppendorf microcentrifuge. The DNA pellet was allowed to dry in air and was then dissolved in 100-200 ul of TE buffer (Appendix D). The DNA was then quantitated by UV spectrophotometry.

8. Quantitation of DNA

DNA was typically quantitated by UV spectrophotometry. An aliquot of DNA was diluted with 10 mM Tris-HCl (pH 8) or distilled deionized water such that it gave an absorbance at 260 nm of between 0.2 and 1.2. One A260 unit was taken to be equivalent to 48 ug/ml of double-stranded (ds) DNA (Wetmur, 1976). To evaluate DNA purity, the ratios of A260/280 nm and A250/260 nm were calculated. Pure ds DNA gives an A260/280 ratio of 1.9 and an A250/260 of 0.9 (Maniatis *et al.*, 1982).

When the volume of a DNA preparation was limiting and, therefore, UV spectrophotometry could not be employed, DNA was quantitated by the minigel method (Maniatis *et al.*,

1982). A known volume of sample was mixed 1:1 with tracking dye (Appendix D) and loaded into a 0.8% agarose gel. DNA solutions of known concentrations were likewise mixed 1:1 with tracking dye and loaded into the gel. The samples were electrophoresed until the dye had migrated 1 cm from the origin (15 minutes at 100 volts). The gel was stained with ethidium bromide (1 ug/ml) for 30 minutes. The gel was rinsed with distilled water and then examined with a UV transilluminator (Model TM-15, UVP Inc.). The DNA concentration of the sample was estimated by visual comparison with the DNA standards.

9. Processing of tonsillar tissue

A. Collection

Tonsils were obtained from children undergoing routine tonsillectomies at the Children's Hospital, Winnipeg, Manitoba. Immediately after surgical removal the tonsils were placed in cold HEPES-buffered Hanks' balanced salt solution (HBSS) supplemented with antibiotics (Appendix D), and were transported to the laboratory. Processing of the tonsils was initiated within 3 hours of surgical removal. The tonsils, received as pairs, were washed thrice in HBSS to remove blood clots and mucus, and were cut into 8-10 sections with sterile scissors. The tonsil washes were retained for virus isolation (see below). Care was taken to ensure equal representation of each tonsil of the pair for each form of tissue processing. The tissue sections were divided into four samples of 1-2 grams each. Samples were used for i) DNA extraction, ii) explant cultures, and iii) virus isolation. The fourth sample was frozen at -70°C and served as a tissue reserve.

B. Tonsil washes

Washing solutions remaining after initial cleaning of each pair of tonsils were pooled, clarified by centrifugation at 7000 rpm for 20 minutes then centrifuged in a Beckman Type 42.1 fixed angle rotor for 2 hours at 30,000 rpm at 4°C. All but 1 ml of the overlying supernatant was removed and the pellet was resuspended by trituration with a Pasteur pipette. After clarification by centrifugation at 1000 rpm for 10 minutes, the washings were examined for the presence of infectious virus in HEp-2 tube cultures. Washings were stored at -56°C if not examined for virus immediately.

C. Mechanical preparation of tonsil homogenate

Tonsil fragments were weighed and transferred to a 50 ml homogenization cup. For every gram of tissue, 10 ml of TE buffer (Appendix D) was added to the cup, which was then sealed and held on ice until homogenization. The tissue was homogenized on ice with two thirty-second high-speed bursts from a Sorval Omni-Mixer (Model 17150). To prevent overheating of the tissue slurry the homogenization bursts were separated by 1-2 minutes. After the final homogenization, the slurry was allowed to stand on ice for 5 minutes to minimize aerosol escape. A 1 ml aliquot of homogenate was transferred to a sterile 1.5 ml microcentrifuge tube for direct isolation of infectious virus. The remaining homogenate was placed in a 50 ml disposable centrifuge tube for DNA extraction. All steps in the preparation of tonsil homogenates were performed in a class II laminar flow hood (Baker, model B4000-1).

D. Manual preparation of tonsil homogenates

Tonsil tissue (0.5-1 gram) was finely minced with sterile scissors then transferred to a Ten-Broeck homogenizer. HBSS

was added to give a 20% suspension (w/v) and the suspension was homogenized for 5 minutes. The slurry was then centrifuged at 1500 rpm for 15 minutes to pellet solid debris. The 'raw' supernatant was carefully transferred to a sterile 5 ml bottle. Penicillin, streptomycin, and fungizone were added to give final concentrations of 1000 U/ml, 1000 ug/ml, and 40 ug/ml, respectively. Prior to inoculation of HEp-2 cell cultures for the detection of infectious virus, an aliquot of 'raw' supernatant was further clarified by centrifugation in an Eppendorf microcentrifuge (Model 4512) for 3 minutes. Both the 'raw' and remaining clarified supernatants were stored at -56°C.

10. Detection of infectious virus

Infectious virus present in culture supernatants or tonsil washings was detected by passage in HEp-2 cultures. The culture fluid of semiconfluent HEp-2 tube cultures was removed and replaced with MEM supplemented with 2% fetal calf serum and antibiotics (Appendix C). A 0.1 ml volume of specimen was then inoculated per tube and the tubes were incubated at 37°C with caps sealed for 6-10 days. All specimens were inoculated in triplicate, and uninoculated tubes served as negative controls. Specimens were examined daily for the appearance of a cytopathogenic effect (CPE), and the culture medium was replaced with fresh medium on days 3 and 5. If a CPE became evident the tube cultures were subjected to 3-5 freeze-thaw cycles and clarified by low speed centrifugation (800 X g, 10 minutes). Clarified supernatants were stored at -56°C until either used for verification of virus by re-passage through HEp-2 tube cultures, or for propagation of virus in HEp-2 flask cultures. If a specimen was negative for viral CPE the tube cultures were subjected to the freeze-thaw regiment described above, pooled and the clarified supernatant was blindly passed by the protocol described above. If after two blind

passages there was no evidence of a viral CPE the specimen was called virus negative.

For titration of virus, ten-fold dilutions of the virus-containing supernatant were prepared using HBSS or MEM supplemented with 2% fetal calf serum and antibiotics (Appendix C) as diluent. The specimens were then treated as above, with the exception that the number of tubes per dilution varied from 4-6.

11. Explant cultures of tonsil tissue

Explant cultures of tissue fragments and lymphocytes were prepared by modification of the methods described by Snejdarova *et al.* (1975) and van der Veen and Lambriex (1973).

A. Fragment culture

The sections of washed tissue were finely minced with scissors and forced through a fine mesh ($800/in^2$) stainless steel sieve with a glass pestle. The resulting fragments (1-2 mm diameter) were collected in 10 ml of HBSS, shaken for 5 seconds on a vortex mixer, and allowed to settle for 5-10 minutes. The supernatant, containing cells released from the fragments, was drawn off and set aside for lymphocyte culture. Washing of the fragments was repeated 2-3 times until the supernatant was visibly clear. The pooled supernatants constituted the crude lymphocyte preparation (see next section).

The washed fragments were resuspended in an equal volume of explant growth medium (Medium M199 supplemented with 15% fetal calf serum - Appendix C), and approximately 0.5 ml of the fragment suspension was dispensed into each of 10-12 $25cm^2$ plastic culture flasks in such a way as to ensure an even distribution of 30-40 fragments over each flask surface. The flasks were incubated at 37°C under 5% CO_2 in air for 24

hours to allow the fragments to attach. Five millilitres of explant growth medium was carefully added to each flask, and the flasks were incubated for up to 8 weeks under the conditions described above. Medium was changed at weekly intervals, with samples of spent medium being retained for detection of infectious virus. At intervals, cells from single or duplicate cultures were collected by trypsinization and the cell pellet was stored at -70°C for later DNA extraction, or for detection of infectious virus as described previously.

B. Lymphocyte cultures

Tonsillar lymphocytes were cultured both in the presence of phytohemagglutinin (PHA), and in its absence, using the 'crude' lymphocyte suspension as the lymphocyte source. A sample of lymphocytes was also co-cultivated with HEp-2 cells.

The evenly-dispersed cells in the 'crude' lymphocyte suspension were collected by centrifugation at 700 rpm for 10 minutes, washed once with 30 ml of explant growth medium (Appendix C) and finally resuspended in 30 ml of explant growth medium. The total and viable cell counts were performed with a hemocytometer using trypan blue as an indicator of cell viability. For 19 tonsils processed in this manner, the total cell yield averaged 5.7×10^9 (range - 0.6×10^9 to 32×10^9), of which an average of 91% (range - 85-98%) of the cells were scored as viable.

For culture the cell suspension was diluted in explant growth medium to contain 1×10^6 cells/ml and 6 ml (total - 6×10^6 cells) was dispensed in each of 12 $25cm^2$ culture flasks. Phytohemagglutinin solution (PHA-P, Difco Laboratories, Detroit, Mich.) was added to 6 flasks to a final concentration of 0.5%. The cultures were incubated at 37°C under 5% CO_2 in air for 8 days. At the end of the incubation period, the cells and supernatant were collected

from one flask in each set (PHA+, PHA-) and stored at -56°C for detection of infectious virus. The cells in the remaining 5 flasks in each set were pooled, collected by centrifugation, and the cell pellet was stored at -70°C for DNA extraction.

For co-cultivation with HEp-2 cells, a portion of the washed and counted 'crude' lymphocyte suspension was centrifuged and the cell pellet was resuspended in maintenance medium (MEM supplemented with 2% fetal calf serum and antibiotics - Appendix C) at a final concentration of 1 X 10^6 cells/ml. A volume of 1.5 ml was inoculated into each of 10-12 HEp-2 tube cultures which had been seeded 1-2 days previously and from which the medium had been removed. The HEp-2 cultures were examined daily for 8-10 days and medium was routinely changed after 4-5 days. If no cytopathogenic effect (CPE) had developed in the HEp-2 monolayers during the 8-10 days of observation, the cultures were frozen and thawed 5 times and the clarified supernatant was blind passaged twice more in HEp-2 cell cultures.

The surplus cells in the 'crude' lymphocyte suspensions were collected by centrifugation and the cell pellets were stored at -70°C.

12. Isolation of total tonsillar DNA

After the mechanical preparation of the tonsil homogenate, 10% sodium dodecyl sulfate (SDS) and protease (20 mg/ml - Appendix D) were added to final concentrations of 0.5% and 0.5 mg/ml, respectively. The homogenate was mixed by swirling and incubated at 37°C overnight.

The digest was transferred to a chromic acid-washed 30 ml Corex glass centrifuge tube and extracted with an equal volume of TSE-saturated phenol (Appendix D). After inverting the tube several times to homogenize, the phases were separated by centrifugation at 7,500 X g for 10 minutes in an IEC Model 320-A centrifuge. The aqueous phase was transferred

to a fresh 30 ml centrifuge tube, and the extraction was repeated with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) until no protein was visible at the phase interface. Phenol was extracted from the aqueous phase with an equal volume of chloroform-isoamyl alcohol (24:1, v/v) by the procedure described above. Nucleic acids were precipitated by the addition of 1/25th volume of 5M NaCl and two volumes of absolute ethanol. After 2 hours at -56°C the precipitate was collected by centrifugation at 10,500 X g for 20 minutes at 4°C. The ethanol was removed and the pellet was dissolved in TE buffer at 1/10th the original homogenate volume. RNAse A (Appendix D) was added to a final concentration of 1 mg/ml, and the solution was incubated at 37°C for 2 hours. The solution was thrice extracted with equal volumes of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v), extracted once with chloroform-isoamyl alcohol (24:1, v/v), and the DNA was precipitated and collected as previously described. The DNA pellet was dried in air and dissolved in TE buffer or distilled deionized water (1 ml per gram of tissue processed). DNA was quantitated and evaluated for purity by UV spectrophotometry.

13. Isolation of DNA from cultured cells

Cultured cells were removed from culture flasks by trypsinization and transferred to a disposable centrifuge tube. Cells were pelleted by centrifugation at 800 X g for 10 minutes at room temperature. After removal of the overlying fluid, the cell pellet was resuspended in TE buffer (Appendix D), 5 ml for every 75cm^2 equivalent of cell monolayer. SDS and pronase (Appendix D) were added to final concentrations of 1% and 1 mg/ml, respectively. The digest was incubated at 37°C for 2-6 hours. Upon completion of digestion the solution was distributed in 0.5 ml aliquots to sterile 1.5 ml microcentrifuge tubes and nucleic acids were extracted by the phenol method described previously. However, all

centrifugation steps during deproteinizaion were carried out for 3 minutes at 12,800 X g in an Eppendorf microcentrifuge (Model 5412). Similarly, nucleic acid and DNA precipitates were pelleted by centrifugation at 12,800 X g at 4°C for 15 minutes. After drying in air, the DNA pellet was dissolved in an appropriate volume of TE buffer or distilled deionized water. The DNA was quantitated by UV spectrophotometry or the minigel method.

14. Preparative isolation of plasmid DNA

Plasmid DNA was extracted and purified by a modified alkaline extraction procedure (Micard *et al.*, 1985) followed by Bio-Gel A-150 chromatography (Maniatis *et al.*, 1982).

Bacterial cells were collected from 500 ml of an overnight culture by centrifugation in an IEC Model 870 rotor at 5000 rpm for 10 minutes. The cell pellet ws resuspended in 4 ml of lysis buffer containing lysozyme (Appendix D) and chilled on ice for 10 minutes. Cells were lysed by addition of 8 ml of 1% SDS in 0.2 N NaOH. The mixture was gently mixed and placed on ice for 10 minutes. Chromosomal DNA and proteins were precipitated by the addition of 6 ml of 3M potassium acetate (pH 4.8). After gentle mixing and incubation on ice for 10 minutes, insoluble material was removed by centrifugation at 8500 rpm for 15 minutes at 4°C. RNA and plasmid DNA were precipitated from the aqueous phase by the addition of 2 volumes of absolute ethanol and incubation at -20°C for 1 hour. The precipitate was collected by centrifugation at 12,500 rpm for 15 minutes at 4°C. The pellet ws dissolved in 1 ml of 75mM NaCl: 50mM sodium acetate (pH 5.4), and twice extracted with equal volumes of phenol equilibrated with 50mM sodium acetate (pH 5.4). Nucleic acids were precipitated with ethanol and collected by centrifugation as previously described. The pellet was dissolved in 2.5 ml of TE buffer, adjusted to 2.5 M ammonium acetate by addition of solid salt, then held on ice for 20

minutes. High molecular weight RNA was removed by centrifugation at 8000 rpm for 10 minutes at 4°C. Plasmid DNA and low molecular weight RNA were precipitated with ethanol and collected as previously described. The pellet was dissolved in 0.2 ml of TE (pH 8)-0.1% SDS buffer and loaded on a 1 cm X 10 cm column of Bio-Gel A-150 (BioRad) equilibrated with TE-SDS buffer. Plasmid DNA was eluted using TE-SDS buffer and precipitated with 1/10th volume of 3M sodium acetate (pH 5.4) and 2 volumes of absolute ethanol. The plasmid DNA was collected by centrifugation and dissolved in distilled deionized water. DNA concentration was determined by UV spectrophotometry.

15. Restriction endonuclease digestion of DNA

DNA (100 ng - 10 ug) was placed in a sterile microcentrifuge tube and 2-3 ul of 10X reaction buffer, prepared to the specifications of the enzyme manufacturer, were added. Restriction endonuclease was added in a volume of 1-3 ul (representing 5-40 units) to the mixture and sterile distilled deionized water was added to bring the restriction buffer to 1X final concentration. The components were mixed by a brief centrifugation (10-15 seconds) in an Eppendorf centrifuge (Model 5412). The digest was incubated at the temperature recommended by the manufacturer for 3-6 hours. To terminate the reaction 1/5th volume of tracking dye (Appendix D) was added, mixed by a brief centrifugation, and subsequently heated at 42°C for 5 minutes. The sample was then loaded into a well of a preformed agarose gel.

16. Electrophoresis of DNA

DNA restriction fragments were separated by agarose electrophoresis employing a GNA-200 horizontal electrophoresis unit (Pharmacia, Dorval, Quebec) coupled to a LKB power supply (Model 3371D). Agarose gels (20 cm X 20 cm X

0.5 cm) were prepared with 1X TBE buffer (Appendix D) and either BioRad standard low -m_r agarose or SeaKem ME agarose. Agarose gels were prepared to a final concentration of 0.8%, and were run submerged in 1X TBE buffer. Electrophoresis was performed at 40 volts for 12-18 hours at room temperature. At the end of the electrophoresis, migration of the tracking dye (Appendix D) was typically 10-12 cm from the well origin. Gels were stained with ethidium bromide (10 ug/ml) for 30 minutes, followed by destaining against distilled deionized water for 30-60 minutes. DNA was visualized using a UV transilluminator (Model TM-15, UVP Inc.). Gels were photographed with a Polaroid MP4 camera using Polaroid Type 52 or Type 57 film.

17. Transfer of DNA to nitrocellulose

After DNA fragments had been separated by electrophoresis and examined by UV transillumination to verify that digestion was complete the DNA was transferred to nitrocellulose by the method of Southern (1975). The gel was submerged in 500 ml of 0.25 N HCl and gently agitated for 8-10 minutes. The solution was removed and the gel was washed with 500 ml of distilled deionized water. The gel was then submerged in 500 ml of denaturation solution (0.5 N NaOH-1.5 M NaCl) and gently agitated for 1 hour. This solution was removed, replaced with 500 ml of neutralizing solution (1 M Tris-HCl pH 8 - 1.5 M NaCl) and agitated for another hour. The gel was then placed well-side down upon a filter paper wick (Schleicher and Schuell #470) saturated with 10X SSC (pH 7). The filter wick extended over a glass plate support into a reservoir of 1 litre of 10X SSC (pH 7). A nitrocellulose membrane (Schleicher and Schuell BA85 or BioRad Trans-Blot) having the same dimensions as the gel was wet with 2X SSC (pH 7) and placed over the gel. Air bubbles trapped between the gel and the membranes were removed by gently rolling a glass pipette over the nitrocellulose. The nitrocellulose was overlaid with

3 pieces of filter paper saturated with 2X SSC (pH 7) and trapped air bubbles were removed as previously described. A stack of paper towels (15-20 cm thick) was placed over the filters and held in place with a glass plate and a 500 gram weight. DNA was allowed to transfer to the membrane overnight. After transfer was complete, the towelling and the filter papers were discarded, and the filter wick-gel-membrane 'sandwich' was inverted on a glass plate. The filter wick, now on top, was carefully removed and the gel wells were marked on the membrane with an ink pen. The gel was removed and discarded. The nitrocellulose membrane was placed in 6X SSC (pH 7) for 10 minutes, then sandwiched between sheets of filter paper and baked for 5 hours at 68°C. After baking, the membrane was stored at 4°C wrapped in plastic wrap until used for hybridization.

18. Radioactive labelling of DNA

DNAs to be used as hybridization probes were made radioactive by use of a commercial nick translation kit (Amersham, N5000). The kit reaction protocol was scaled down for the use of 50-100 ng of viral DNA as described by Hammond *et al.*,(1987).

One hundred nanograms of DNA, in a volume not exceeding 9 microlitres, was placed in a sterile 1.5 ml microcentrifuge tube held on ice. Four microlitres of solution A (nucleotides-Appendix D) were added to the DNA solution followed by 50 microCuries of dCTP alpha-^{32}P (3000 Ci/mmol) obtained form New England Nuclear or ICN Ltd.. Two microlitres of solution B (enzymes-Appendix D) were added to the reaction mixture, followed by sufficient sterile distilled deionized water to yield a final reaction volume of 20 microlitres. The reaction mixture was mixed by tapping the tube followed by a brief centrifugation to bring down recalcitrant droplets. The reaction mixture was incubated at 15°C for 90 minutes and the reaction was terminated by the

addition of prewarmed stop buffer (Appendix D) and subsequent incubation at 68°C for 10 minutes.

Incorporation of label and specific activity of the probe were evaluated by comparison of total and acid-precipitable (*ie.* DNA-associated) radioactivity. One microlitre of a 1:10 dilution in TE buffer (Appendix D) of stopped reaction mixture was spotted on a Whatman GF-C glass fibre filter. A second one-microlitre aliquot of the 1:10 dilution was added to 5 ml of ice-cold 10% trichloroacetic acid (TCA) and precipitated with 50 ug of herring sperm DNA (Boehringer Mannheim) as carrier. The solution was mixed briefly then held on ice for 10 minutes. The precipitate was collected on a Whatman GF-C glass fibre filter, and the filter was then rinsed twice with 5 ml of ice-cold 10% TCA and once with 5 ml of ice-cold 95% ethanol. This filter represented incorporated radioactivity while the first filter represented total radioactivity. Both filters were dried at 68°C for 5 minutes, then placed in separate scintillation vials and 5 ml of scintillation cocktail (Appendix D) was added to each vial. Radioactivity, as counts per minute (cpm), was measured in a beta liquid scintillation counter (Model 1215 RackBeta II, LKB).

Incorporation of label was calculated by the following formula:

$$\% \text{ incorporation} = \frac{\text{acid-precipitable cpm}}{\text{total cpm}} \times 100$$

Specific activity (cpm/ug) of the probe, taking into account the dilution of the reaction mixture, was calculated as follows:

$$\text{cpm/ug} = \frac{\text{acid-precipitable cpm/ul}}{\text{ng DNA/ul}} \times 1000$$

Incorporation of label ranged from 15-45% and specific activities varied from 1 X 10^8 - 1 X 10^9 cpm/ug of DNA.

Separation of unincorporated nucleotide from labelled DNA was achieved by Sephadex G-100 chromatography. The reaction mixture was layered on a 4.5 ml Sephadex G-100 column and DNA was eluted with TE buffer. One-half millilitre fractions were collected in sterile 1.5 ml microcentrifuge tubes which were monitored for radioactivity by use of a geiger counter (Model 3 Survey Meter, Ludlum Inc.). The fraction(s) with the highest radioactivity were evaluated for total and acid-precipitable counts by the method previously described. In this instance, however, the labelled DNA was not diluted prior to counting. When the activity was determined, 1-2.5 X 10^7 cpm of probe solution was placed in a microcentrifuge tube and the DNA was denatured by boiling for ten minutes followed by rapid cooling on ice. The now-denatured labelled DNA was held on ice until added to the hybridization solution for a time not exceeding 15 minutes.

19. Hybridization

Nitrocellulose membranes were wet with distilled deionized water and placed in heat-sealable bags. Thirty millilitres of prewarmed prehybridization fluid (Appendix D) were added to the bag and the bag was sealed with a Decosonic heat sealer after the removal of all air bubbles. The membrane was then incubated 4-6 hours at 68°C in a shaking waterbath. At this time the prehybridization fluid was removed and 30 ml of warm hybridization fluid containing 10% dextran sulfate (Pharmacia Fine Chemicals, Uppsala, Sweden - Appendix D) and 1-2.5 X 10^7 cpm of heat-denatured labelled DNA was added. Air bubbles were removed and the bag was resealed. The membrane was then incubated at 68°C in a shaking waterbath for 18-24 hours.

At the completion of incubation the hybridization fluid was drained into an aqueous radioactive waste container and

the membrane was washed twice for thirty minutes in 500 ml of 2X SSC-0.1% SDS followed by two washes with 500 ml of 0.2X SSC-0.1% SDS, again for thirty minutes. All washes were performed at 68°C in a shaking waterbath within a sealed plasticware container. After the final wash the membrane was blotted dry between two sheets of filter paper (Schleicher and Schuell #470) and then wrapped in clear plastic wrap. The membrane was then exposed to Kodak X-Omat X-ray film in the presence of Cronex II (Dupont) intensifying screens for varying lengths of time at -70°C before development.

20. Electron microscopy

Culture supernatants to be examined for virus by electron microscopy were centrifuged at 12,800 X g for 1 minute in an Eppendorf microcentrifuge (Model 5412). The clarified supernatant (45 µl) was loaded in a 50 µl sector cell of the Beckman EM-90 rotor in which a Formvar-coated grid was placed. The rotor was sealed after balancing the opposite sector cell and the rotor was centrifuged at 105,000 X g for 30 minutes. The grid was then removed and excess fluid drained off. The grid was stained for 1 minute with 25mM phosphotungstic acid (PTA - pH 7) after which the PTA was drawn off. The grid was examined using a Philips EM-201 electron microscope. Adenovirus and herpesvirus were identified by their distinctive morphology.

21. Identification of herpesvirus isolates

Herpesvirus isolates were identified using a commercially available indirect immunofluorescence assay kit (Herpes ID Kit, I.A.F. Production Inc., Laval, Quebec). Culture supernatants in which herpesvirus had been identified by electron microscopy were further examined by this method. Herpes simplex type 1 (strain HSV-76) was employed as a positive control for this assay.

Semiconfluent 25 cm^2 HEp-2 and Huff cell monolayers were each inoculated with 0.5 ml of culture supernatants from herpesvirus-containing isolates. Culture supernatant from HSV-1 (strain HSV-76) was inoculated in cultures of both individual cell lines as well. Uninoculated cultures served as negative controls. When a CPE was evident in 70-80% of cells in a culture, the supernatant was removed and the cells were collected by trypsinization. The cells were washed twice with phosphate-buffered saline (PBS - pH 7.3) and finally resuspended in 0.2 ml of PBS. Uninfected cells were treated as above. Twenty microlitres of each cell suspension were applied in triplicate to immunofluorescence slides supplied with the kit and allowed to air dry. Cells were fixed in ice-cold acetone (reagent grade, Fisher Scientific Co.) for 10 minutes and allowed to dry in air. To each specimen, 25 µl of either anti-HSV-1 specific, anti-HSV-2 specific, or anti-HSV(1 and 2) mouse monoclonal antibodies were applied. The slides were then incubated for 50 minutes in a humidified chamber at 37°C. Slides were washed twice in 50 ml of PBS, 5 minutes at room temperature for each wash. Twenty-five microlitres of goat anti-mouse IgG conjugated to fluorescein isothiocyanate was added to each specimen and the slides were incubated as indicated previously. The slides were then washed as described above and PBS-buffered glycerin was added to each specimen. The samples were covered with a #2 Corning glass coverslip and viewed at 400X magnification with a Nikon UV-microscope with appropriate filters.

Bright green cytoplasmic fluorescence was seen with the HSV-1 (strain HSV-76)-infected cells when treated with anti-HSV-1 or anti-HSV(1 and 2) monoclonal antibodies. If HSV-2 were present, nuclear fluorescence would be present (manufacturer's data). Uninfected cells showed minimal non-specific fluorescence. Results obtained with infected HEp-2 and Huff cells were comparable.

22. Isolation of adenovirus-like cellular DNA sequences

HEp-2 DNA was extracted from cells as total DNA as previously described. One hundred micrograms of HEp-2 DNA was digested to completion with Eco RI for 7 hours at 37°C. DNA fragments were separated by agarose electrophoresis as previously described. After staining with ethidium bromide, an agarose band corresponding to the calculated migration point determined from previous experiments was removed and placed in a dialysis bag with 2 ml of 0.5 X TBE buffer. DNA was electroeluted from the agarose for 8 hours at 50 volts. The polarity was reversed for 10 minutes and the retentate was transferred to a sterile microcentrifuge tube. After a brief centrifugation at 12,800 X g to remove agarose debris, the supernatant was transferred to a fresh tube and adjusted to 0.1 M with 5 M NaCl. Ethanol was added and the DNA was precipitated overnight at -20°C. The DNA was collected by centrifugation, dissolved in distilled water and extracted with an equal volume of phenol:chloroform. DNA was reprecipitated with ethanol and NaCl, as above, collected by centrifugation and dissolved in 50 µl of distilled water. DNA was quantitated by minigel as previously described. Total DNA yield was approximately 1.3 µg.

IV. RESULTS

1. Recovery of adenoviruses from tonsils

The objective of the study was to evaluate the usefulness of DNA hybridization for studying the phenomenon of adenovirus latency. The operational definition of adenovirus latency is the recovery of infectious virus from explant cultures of tissue from which infectious virus cannot be isolated directly.

In this study a total of 32 tonsils were examined by culture for the presence of adenovirus. Adenovirus was isolated directly from three tonsils (adenovirus-infected non-latent - AINL) as well as from the explant fibroblast cultures derived from these specimens. For 7 tonsils, adenovirus was isolated only from explant fibroblast cultures. A single specimen yielded an adenovirus only by co-cultivation of the tonsil lymphocytes with HEp-2 cells. Thus, a latent adenovirus was recovered from 8 of the 32 tonsils.

In addition to adenoviruses, herpesviruses were also isolated from two tonsil specimens. An explant fibroblast culture of one tonsil yielded a herpesvirus as the only virus recoverable, while the second herpesvirus was isolated from one of the 8 tonsils latently infected with an adenovirus, albeit from different explant fibroblast cultures.

The details of virus isolation experiments are described in the following pages. The overall results for virus isolation directly or by culture of the 32 tonsils are summarized in Appendix A. As well, the 8 tonsils that contained latent adenovirus are listed in Appendix B and Table 5.

A. Direct isolation of infectious virus

As a first step in the identification of tonsil specimens carrying latent adenovirus, all tonsils were examined to determine whether infectious adenovirus could be isolated directly.

A homogenate of each tonsil was mechanically prepared in a Sorvall Omni-Mixer and examined for the presence of infectious adenovirus by three consecutive serial passages in HEp-2 cell cultures. Virus was not isolated from any of the 32 tonsils. Pelleted washings available from 13 of the same tonsils were also examined for the presence of infectious virus and an adenovirus was recovered from each of three specimens, T280, T291, and T302. The identification of the isolated viruses is described later.

After having failed to detect infectious virus in any of the first twenty homogenates examined, it was considered as a possibility that the homogenization procedure was too harsh to allow survival of any virus that might have been present. Accordingly, a gentler method of preparing the homogenate was adopted in which tissue was manually homogenized in a Ten-Broeck tissue grinder. However, no virus was isolated from any of the 13 homogenates prepared in this manner.

The mechanical homogenization procedure does not, in itself, inactivate virus, as verified experimentally. An inoculum of adenovirus serotype 2 was added to the homogenization buffer prior to preparing a tissue homogenate from a tonsil previously shown to be free of both infectious and latent virus (T275 - Appendix A). A zero-time sample was then withdrawn and the tissue was homogenized as previously described. Samples were taken after 30, 60, and 120 seconds of homogenization. Virus was titrated for each specimen in HEp-2 cell cultures as described in Materials and Methods. The results of this reconstruction experiment (Table 3) show that the homogenization procedure did not inactivate virus. Therefore, it was deemed unnecessary to re-examine

TABLE 3

A RECONSTRUCTION EXPERIMENT TO TEST THE EFFECT OF MECHANICAL HOMOGENIZATION ON ADENOVIRUS INFECTIVITY

Duration of homogenization (seconds)	Virus titre[1] (log_{10} ID_{50}/0.1 ml)
0	5.0
30	5.0
60	5.0
120	5.5

1. Endpoint after 7 days in HEp-2 cell culture. Calculated by the Karber method.

homogenates of the first twenty tonsil specimens by the manual preparation method.

The failure to recover infectious virus from homogenates of tonsillar tissue brought forward the concern that there may be substances present in homogenates which inactivate virus. This possibility was examined once all latently-infected tonsils had been identified. Manually-prepared homogenates were made from latently-infected (T267, T298), uninfected (T294), and adenovirus-infected non-latent (AINL) tonsils (T291)(Tables 5 and 7). The homogenates were inoculated with known amounts of particular adenovirus serotypes (1 part virus plus 9 parts homogenate so as to contain $10^{2.0}$ - $10^{4.0}$ $TCID_{50}$ of virus per unit volume) and incubated at 37°C. At incubation times of 0, 1, and 2 hours an aliquot of homogenate was removed and immediately titrated for virus in HEp-2 cell cultures. By way of control for thermal inactivation, an equal amount of virus was incubated at 37°C in minimal essential medium (MEM) and titrated at the same time intervals. The results of these experiments are shown in Table 4.

Tonsil T267 was a latently-infected tonsil from which an adenovirus serotype 1 was isolated. As can be seen in Table 4, homogenate made from this tonsil decreased the titre of exogenous Ad 1 and Ad 2, another member of subgenus C. However, little effect can be seen on the titre of Ad 3 and Ad 4, members of subgenera B and E respectively. The second latently-infected tonsil, T298, yielded an adenovirus serotype 3 in explant culture. Homogenate made from this tonsil showed very rapid inactivation of exogenously added Ad 3 (homotypic virus). Homogenates from T291 and T294 showed no inactivating activity towards any adenovirus serotypes tested. The absence of adenovirus-inactivating activity in the homogenate of T291 is interesting since an adenovirus type 1 was isolated from tissue washings of this tonsil (Table 7).

TABLE 4

DETERMINATION OF ADENOVIRUS INACTIVATING ACTIVITY IN TONSIL HOMOGENATES

Virus	Time @ 37°C	Surviving virus (log_{10} ID_{50} per unit volume)[1]				
		Tonsil homogenates				Control
		T267	T291	T294	T298	MEM
Ad 1	0 hr	3.50	3.75	ND[2]	ND	3.75
	1 hr	2.50*	3.75	ND	ND	4.00
	2 hr	1.75*	3.50	ND	ND	3.75
Ad 2	0 hr	1.16	1.83	1.50	ND	2.50
	1 hr	<0.50*	1.83	1.50	ND	1.83
	2 hr	<0.50*	1.50	1.50	ND	2.17
Ad 3	0 hr	2.37	ND	ND	<0.50*	2.37
	1 hr	1.62	ND	ND	<0.50*	2.62
	2 hr	1.83	ND	ND	<0.50*	2.67
Ad 4	0 hr	4.00	3.50	ND	ND	4.00
	1 hr	3.70	3.50	ND	ND	4.00
	2 hr	3.70	4.00	ND	ND	3.70

1. Titrations done with 4-6 replicates per dilution.
2. Not done.

* $p < 0.05$ relative to MEM controls at the same time interval as calculated by the Karber method using 95% confidence limits.

While only a small number of tonsils have been examined in this manner, the results obtained concur with those of Snejdarova *et al.* (1975) in that some tonsils contain substances which inactivate adenoviruses. The identity of this inactivator is not known but the adenovirus type-specific (T298) and subgenus-specific inactivating (T267) activities suggest that it could be neutralizing antibody.

B. Explant fibroblast cultures

Tonsils were obtained from children undergoing routine tonsillectomies at the Children's Hospital, Winnipeg, Manitoba. The tonsils were placed in a buffered salt solution and processed for culture within 1-3 hours of surgical removal. The details of processing tonsil tissue for culture are specified in Materials and Methods.

The volume of washed tissue fragments derived from each tonsil was sufficient to yield from 8-12 individual cultures in 25 cm^2 flasks. Cellular monolayer outgrowths developed within 4-8 days and , in most cases, were fully confluent within 15-20 days. The monolayers were comprised almost exclusively of fibroblast cells, though in a few cases small patches of epitheliod cells were seen.

Explant fibroblast cultures (*ie.* primary cultures) were observed for the development of a cytopathogenic effect (CPE) at 3-4 day intervals. In most cases, samples of culture supernatant were taken at weekly or bi-weekly intervals, usually when medium was changed. In some cases, both supernatant and cells in a flask were subjected to cycles of freezing and thawing, clarification by centrifugation and subsequent inoculation into HEp-2 cell cultures for detection of infectious virus. Fibroblast cultures from the individual tonsils were maintained from 4 days to 141 days and the average time in culture was 40 days (Appendix B).

Of 32 tonsils examined for infectious adenovirus by explant fibroblast culture, 10 (31.3%) tonsils possessed

adenovirus as detected by either the development of a CPE in primary culture, or by up to three consecutive serial passages of culture supernatants in Hep-2 cultures, or by both (Table 5).

The development of a CPE was detected in primary cultures of 9 tonsils (Table 5). Six of the nine tonsils were latently-infected with adenovirus while the remaining three were adenovirus-infected non-latent (AINL) tonsils. Adenovirus was isolated also from two latently-infected tonsils (T267, T289) in which no CPE was detected in primary cultures. Adenovirus was recovered from the explant fibroblast culture supernatant of tonsil T267, whereas in the case of T289, adenovirus was isolated only from lymphocytes co-cultivated with HEp-2 cells.

In four latently-infected tonsils (T257, T277, T282, T298), adenovirus was isolated from primary culture supernatants after the appearance of a CPE in the culture (Table 5 and Appendix B). In the cases of tonsils T303 and T305, however, adenovirus was isolated from supernatants taken 7 and 34 days, respectively, before the appearance of a CPE. When taken cumulatively, a CPE developed in primary cultures from latently-infected tonsils after an average of 50 days (range 21-127 days) while adenovirus could be isolated after an average of 30 days in culture (range 19-130 days). If the results for tonsil T257 are excluded due to the unusually long time period (127 days - fourth serial passage) before the appearance of a CPE and detection of infectious virus, the average time for appearance of a CPE is 34 days (range 21-53 days) and the average time in culture before infectious virus could be detected is 25 days (range 19-39 days). These adjusted values are similar to those found by Evans (1958) in which the mean time to appearance of a CPE was 36 days (range 17-96 days) while the mean time in culture before detection of virus was 20 days (range 8-40 days).

Several companion cultures, if available, from each adenovirus-positive tonsil were examined for the presence of

TABLE 5

COMPARISON OF THE APPEARANCE OF A CYTOPATHOGENIC EFFECT (CPE) IN EXPLANT FIBROBLAST CULTURES AND FIRST DETECTION OF INFECTIOUS ADENOVIRUS.

Tonsils	Total days in culture	Day when first CPE seen	Sample day yielding first Ad isolate
Latent			
T257	141	127	130
T267	10	None	7
T277	65	29 (22)[1]	36 (24)
T282	44	22	25
T289[2]	54	None	None
T298	43	21	23
T303	53	46	39
T305	53	53	19
AINL[3]			
T280	59	17	17
T291	29	22	22
T302	36	15	8

1. Herpesvirus isolated from a culture from T277 exhibiting CPE at the day indicated.
2. Adenovirus was isolated from lymphocytes co-cultivated with HEp-2 cells.
3. Adenovirus-infected non-latent. Adenovirus was isolated from tonsil washes as well as from fibroblast cultures (see text).

infectious virus. Only a single culture was available for tonsils T267 and T289 before the fibroblast cultures were lost to bacterial contamination; infectious virus was detected in the lone specimen from T267 but not from that of T289 (Table 6 and Appendix B). Tonsils from which adenovirus was isolated directly (T280, T291, and T302 - Table 6) were found to have virus present in all companion cultures. For latently-infected tonsils, conversely, infectious virus was generally recovered from only some, but not all, of the companion fibroblast cultures, even when the cultures were maintained for as long as 50-60 days. The only exception to this generalization was tonsil T282 (Table 6 and Appendix B).

C. Explant tonsil lymphocyte culture

Since fibroblastic cells are not the only cell population present in tonsil tissue but are the primary cell population in explant monolayer cultures, suspension cultures of lymphocyte-enriched populations were also examined. Lymphocyte suspensions of 19 tonsils, prepared as described in Methods and Materials, were i) cultured in the presence of the mitogen phyohemagglutinin (PHA), ii) cultured in the absence of mitogen, or iii) co-cultivated with HEp-2 cells. After 8 days in culture, both supernatant and cell suspensions were examined for the presence of infectious virus.

No viruses were isolated from the lymphocytes of 19 tonsils cultured in the presence or absence of PHA (Appendix A and Table 9). As shown in Appendix A, latent adenovirus was isolated from explant fibroblast cultures derived from 5 of 19 tonsils, and adenovirus was recovered directly from 3 others. The finding that PHA stimulation failed to enhance the recovery of adenovirus from cultured tonsil lymphocytes is contrary to that reported by van der Veen and Lambriex (1973).

TABLE 6

ISOLATION OF ADENOVIRUSES FROM COMPANION EXPLANT FIBROBLAST CULTURES DERIVED FROM TONSIL EXPLANTS.

Latently-infected tonsil	Explant fibroblast cultures No. virus positive/No. examined
T257	2/13
T267	1/1
T277	4/7
T282	4/4
T289[1]	0/1
T298	1/8
T303	4/7
T305	3/6
AINL tonsils	
T280	2/2
T291	3/3
T302	5/5

1. Latent adenovirus was isolated from lymphocytes co-cultivated with HEp-2 cells.

Lymphocytes of 11 tonsils were co-cultivated with HEp-2 cells and only 1 (9.1%) yielded an adenovirus (Appendix A - T289). Since virus was not isolated directly from T289 this was regarded as evidence for latent infection of this tonsil.

D. Identification of virus isolates

When a CPE became evident in either primary culture or supernatant-inoculated HEp-2 cell culture, preliminary identification of the infectious agent was made on the basis of the characteristics of the CPE. The CPE seen in primary cultures subsequently shown to be infected with an adenovirus began as a focus (or foci) of rounded and clustered cells exhibiting granular cytoplasms. The CPE progressed until the majority of the cell monolayer showed this appearance and were becoming detached from the vessel surface. The average time from the detection of a focal CPE to destruction of the monolayer was 7 days. An adenovirus CPE in HEp-2 cell culture was identical in appearance to that seen in primary fibroblast culture, except that the total destruction of the cell monolayer took less time (an average of 4 days). The CPE due to virus subsequently identified as herpesvirus was characterized by a general, rather than focal, infection of the monolayer with affected cells being both rounded and enlarged. The CPE also progressed much quicker; total destruction of a primary culture monolayer took approximately 3 days while HEp-2 and Huff cell monolayers were destroyed after only 1-2 days.

In addition to the characteristics of the CPE most virus isolates were provisionally identified by electron microscopic (EM) examination of infected culture supernatants. Identification was made on the basis of size and morphology of virus particles, if seen. EM examination verified the presence of adenovirus in culture supernatants of tonsils T257, T267, T280, T282, T291, T298, and T302 (Figure 1). As well, EM revealed the presence of herpesvirus

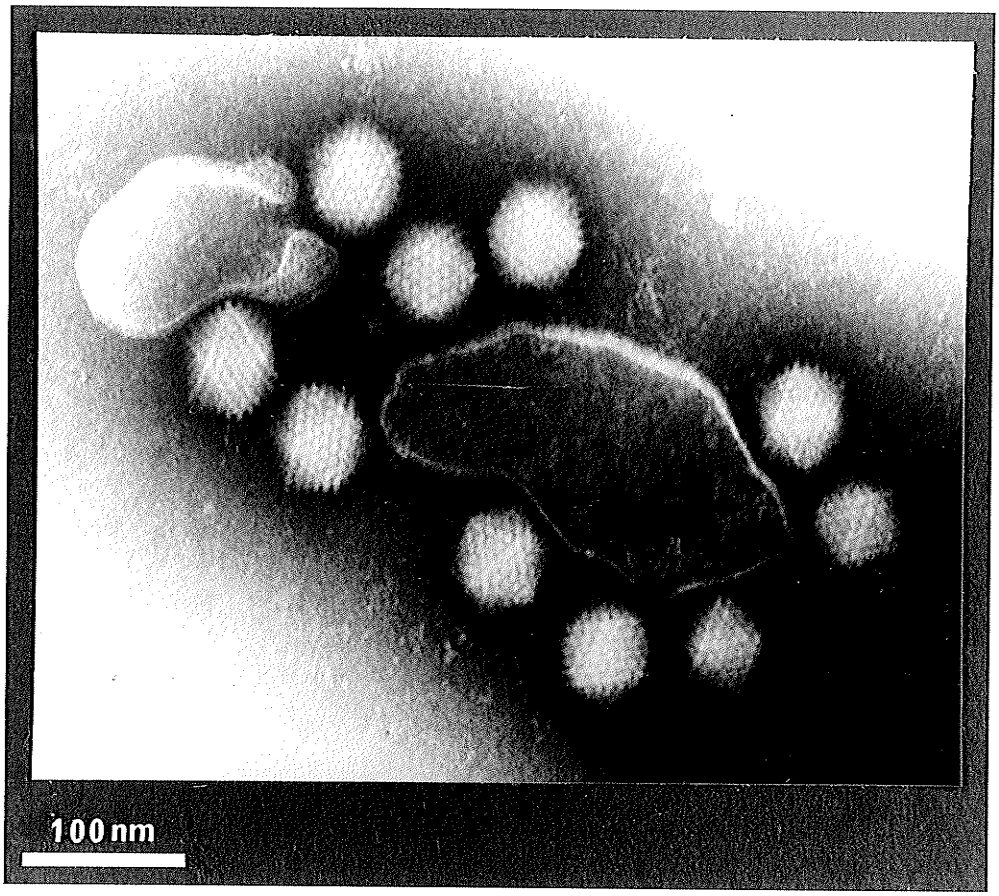

Figure 1 - Electron micrograph of adenovirus detected in fibroblast culture supernatant of T282.

in culture supernatants of tonsils T277 and T279 (Figure 2). Though adenoviruses were isolated from several companion cultures of T277, no adenovirus was seen in the supernatant of the explant fibroblast culture yielding the herpesvirus (Appendix B).

Once a virus isolate was identified as either an adenovirus or a herpesvirus, by its morphology, a more definitive identification was made by DNA restriction pattern analysis (for adenoviruses) or by indirect immunofluorescence (for herpesviruses) as described later.

Adenoviruses were isolated from eleven tonsils in this study. Each virus isolate was identified by its DNA restriction enzyme patterns in reference to similarly digested DNAs of prototype adenoviruses. For each isolate, adenovirus was amplified by a single passage in HEp-2 cell culture. The cells were harvested when CPE was maximal and total DNA was extracted. The DNA was digested separately with several restriction endonucleases and the resulting fragments were separated by agarose electrophoresis.

Eight of the eleven tonsils were latently-infected with adenovirus, as previously mentioned. Seven of the eight isolates were adenovirus type 1 and one was an adenovirus type 3 (Table 7). Two of these type 1 isolates (T282 and T289) exhibited DNA restriction patterns with Bam HI which varied from those of the prototype adenovirus (Figure 3). All remaining type 1 isolates and the type 3 isolate from latently-infected tonsils showed DNA restriction patterns identical to those of the prototype virus (Figure 4).

Adenovirus was also isolated from three AINL tonsils. Adenovirus type 2 was isolated from tonsils T280 and T302 whereas tonsil T291 yielded an adenovirus type 1 from tonsil washes and an adenovirus type 5 variant from cultured fibroblasts (Figure 5, Table 7). With the exception of the T291 isolates, adenoviruses identified from both tonsil washes and fibroblast cultures for a given tonsil were identical in DNA restriction pattern. However, the adenovirus

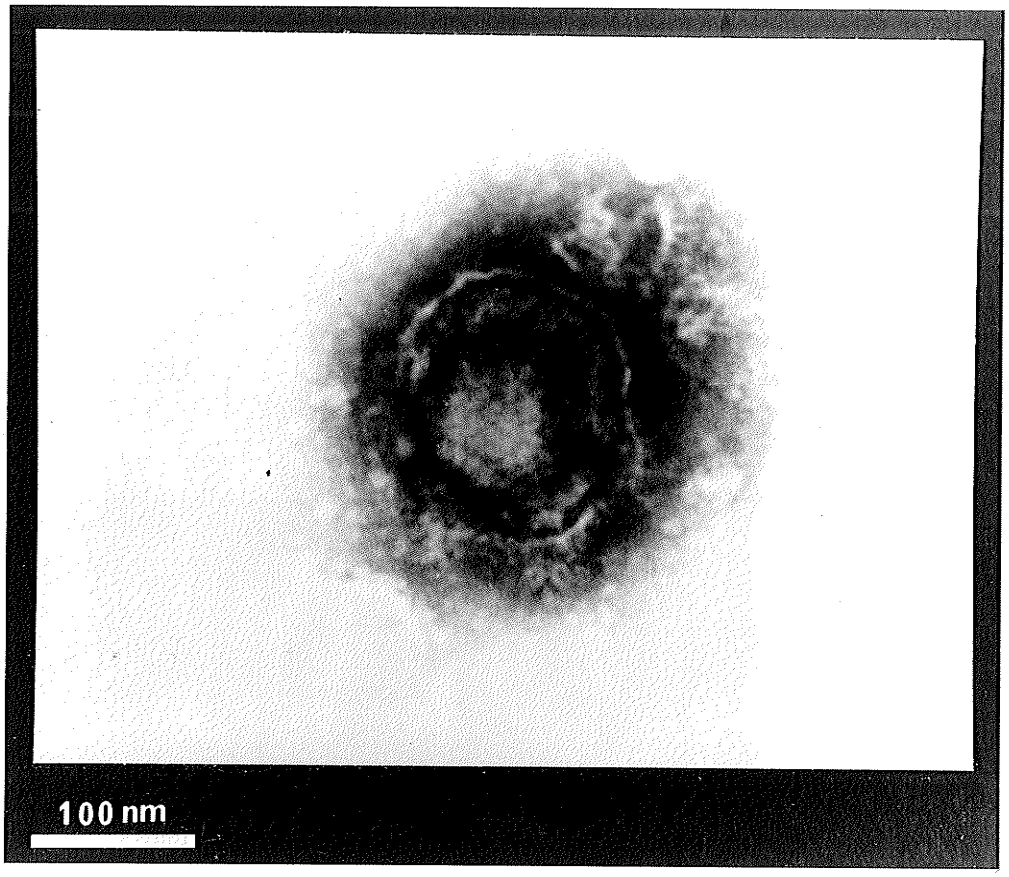

Figure 2 - Electron micrograph of herpesvirus detected in fibroblast culture supernatant of T277.

TABLE 7

IDENTIFICATION OF ADENOVIRUS ISOLATES

Latently-infected tonsils	Isolate source	Isolate identity
T257	fibroblast cultures	Ad 1 p *
T267	fibroblast culture	Ad 1 p
T277	fibroblast cultures	Ad 1 p
T282	fibroblast cultures	Ad 1 v
T289	lymphocyte co-cultivation	Ad 1 v
T298	fibroblast culture	Ad 3 p
T303	fibroblast cultures	Ad 1 p
T305	fibroblast cultures	Ad 1 p
Non-latent (AINL) tonsils		
T280	tonsil washes	Ad 2 p
	fibroblast cultures	Ad 2 p
T291	tonsil washes	Ad 1 p
	fibroblast cultures	Ad 5 V
T302	tonsil washes	Ad 2 v
	fibroblast cultures	Ad 2 v

* p = prototype, v = variant

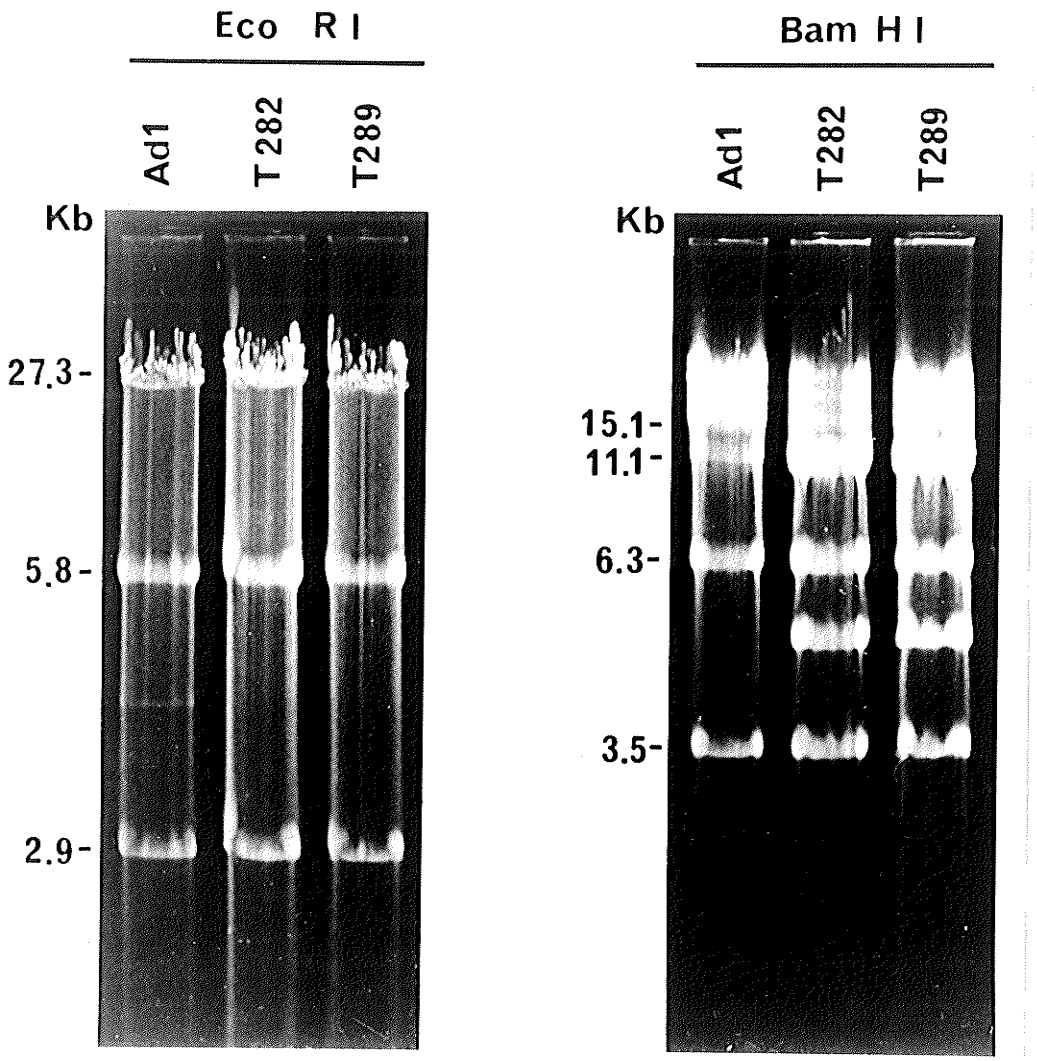

Figure 3 - DNA restriction pattern comparison of adenoviruses isolated from tonsils T282 and T289 relative to adenovirus type 1 prototype.

Note additional band in Bam HI digests of tonsil isolate DNAs.

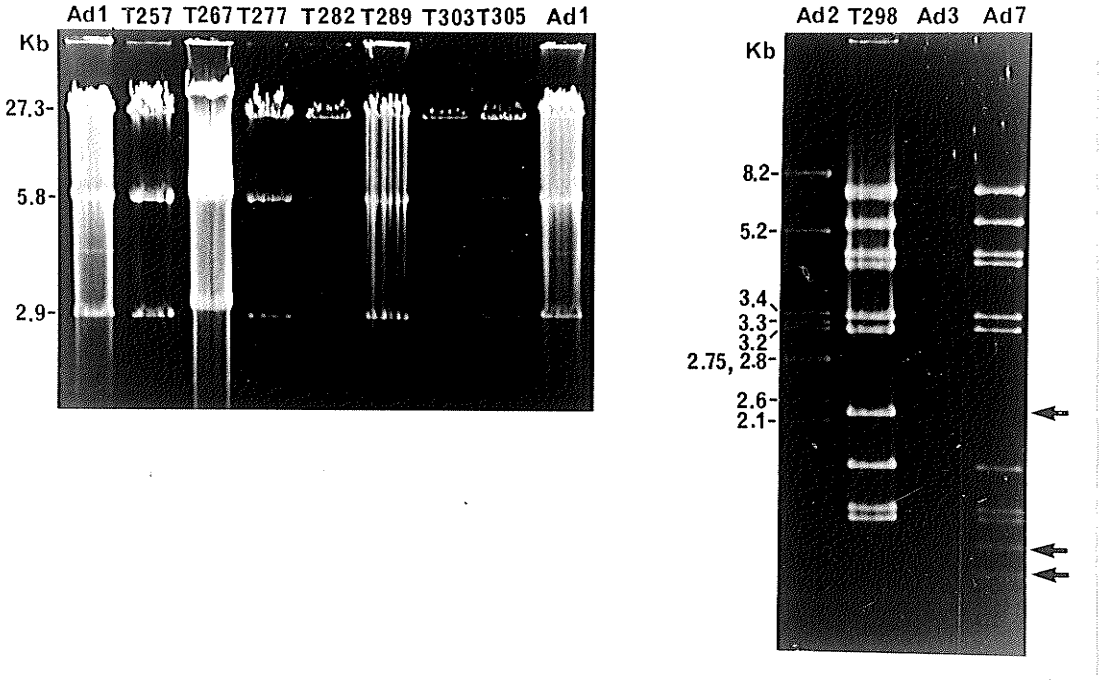

Figure 4 - DNA restriction pattern comparison of adenovirus isolates from latently-infected tonsils as compared to prototype adenoviruses.

Left panel - Eco RI digests of total DNA from prototype- or isolate-infected HEp-2 cells.
Right panel - Hind III digests of total DNA from isolate-infected HEp-2 cells and purified prototype DNAs. Arrows indicate DNA bands which are shared by T298 and Ad 3 and differ from Ad 7. Ad 2 DNA digested with Hind III serves as a molecular weight marker.

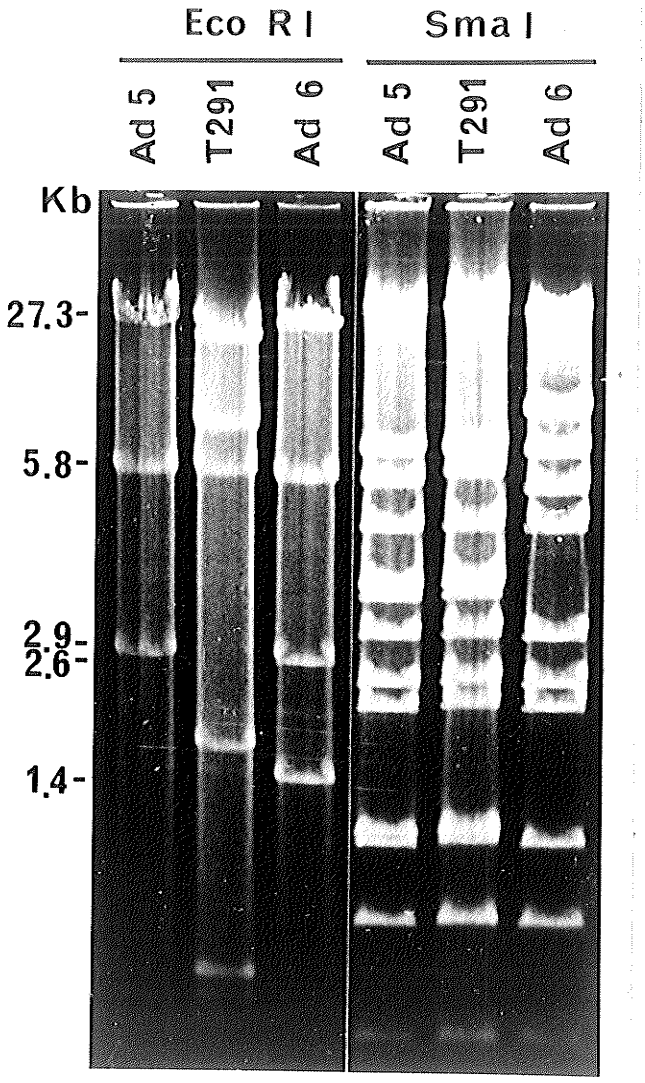

Figure 5 - DNA restriction pattern comparison of adenovirus isolated from fibroblast culture of T291 with Ad 5 and Ad 6.

Note Ad 5 pattern identity when isolate is digested with Sma I but variation when digested with Eco RI.

type 2 isolated from T302 exhibited variation from prototype when digested with Kpn I (Figure 6). Based on the fragments involved in this variation (Figure 6 - arrows) the isolate was tentatively identified as adenovirus type 2d described by Fife *et al.* (1985).

Adenoviruses were not the only viruses recovered from tonsils in this study. Herpesviruses were isolated from two tonsils (T277 and T279), one (T277) of which also yielded an adenovirus type 1. Initial identification was made by the characteristic CPE in culture. Subsequently, herpesvirus was identified by its morphology and size by EM examination of infected culture supernatants (Figure 2). Using a commercially available indirect immunofluorescence kit (I.A.F. Production Inc.) both isolates were subsequently typed as herpes simplex virus type 1.

E. Summary of culture results

Of 32 tonsils examined for the presence of infectious virus, both directly and by explant culture, eight tonsils were found to be latently infected with adenoviruses and three were adenovirus-infected non-latent (AINL). Fibroblast cultures yielded almost all adenovirus isolates, though a single adenovirus was isolated from co-cultivation of tonsillar lymphocytes with HEp-2 cells. All adenovirus isolates, save one, belonged to subgenus C. The exception was an adenovirus 3 isolate which belongs to subgenus B. Two herpesviruses (both herpes simplex type 1) were isolated from fibroblast cultures as well, one of which was also latently-infected with adenovirus type 1.

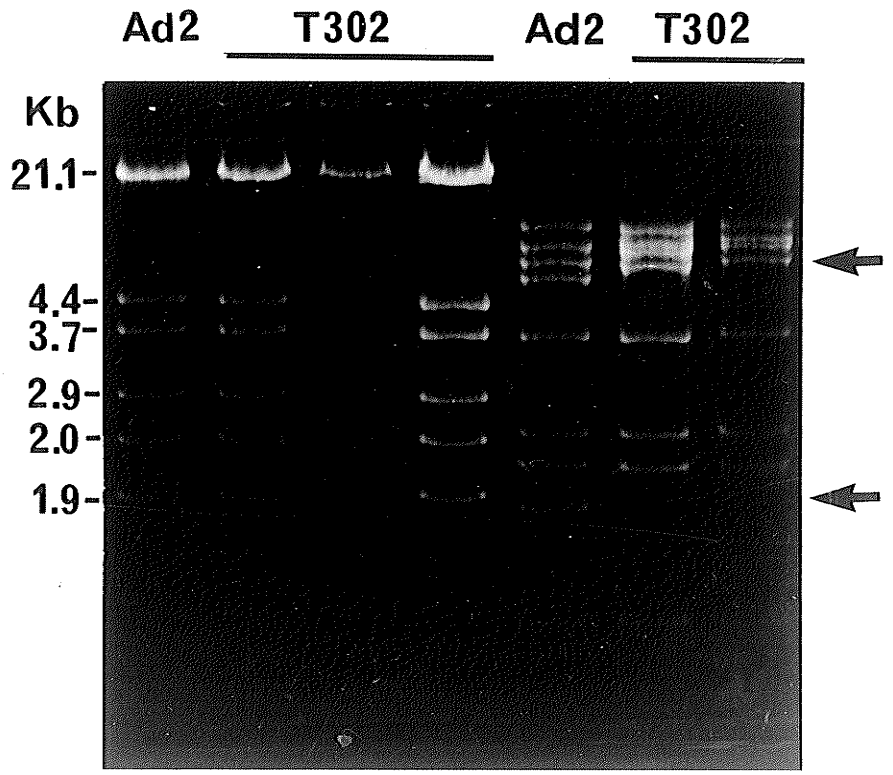

Figure 6 - DNA restriction pattern comparison of adenovirus isolated from tonsil T302 with adenovirus type 2 prototype after digestion with Eco RI (left 4 lanes) and Kpn I (right 3 lanes).

Multiple lanes of T302 DNA digests represent isolates from separate companion fibroblast cultures. Arrows represent DNA band variation of Kpn I-digested T302 isolate DNA relative to similarly digested Ad 2 DNA.

2. Adenovirus DNA sequences in tonsillar tissue

A. Sensitivity of hybridization for the detection of adenovirus DNA sequences in the presence of cellular DNA.

In order to interpret the detection, or absence thereof, of adenovirus DNA sequences, the sensitivity level of the hybridization procedure was examined. To this end, a reconstruction experiment was performed in which known anounts of adenovirus serotype 2 (Ad 2) DNA were mixed with 10 μg of HEp-2 DNA, digested with Eco RI, and DNA fragments were separated by agarose electrophoresis. The DNAs were then transferred to nitrocellulose and hybridized with radioactively-labelled Ad 2 DNA (specific activity - 5 X 10^8 cpm/μg). After washing to remove unbound probe, the membrane was exposed to X-ray film for varying lengths of time.

It was found that after 24 hours of exposure all six Eco RI fragments of Ad 2 DNA were detected at a level of 50 pg or 1.3 X 10^6 Ad 2 genome equivalents. By calculating that 10 μg of HEp-2 DNA is equivalent to 1.51 X 10^6 cells (*ie.* human diploid genome equivalents) this level of sensitivity is better expressed as detection of approximately 0.9 Ad 2 genome equivalents per cell. After three days of exposure the level of sensitivity was increased to detection of 10 pg (0.17 Ad 2 genome equivalents per cell), and after 15 days of exposure, all six Eco RI fragments of Ad 2 DNA were detected at a level of 5 pg(0.09 Ad 2 genome equivalents per cell). At this time the Eco RI-A fragment, representing the left 58.5% of the Ad 2 genome, was barely detectable at a level of 1 pg (Figure 7). Because detection of all restriction fragments of adenovirus DNA was desirable for identifying adenovirus types which may be present in tissues, we took the more conservative level of 5 pg of adenovirus DNA as our sensitivity limit. It was found from subsequent hybridization experiments that all Eco RI fragments of Ad 2 DNA were

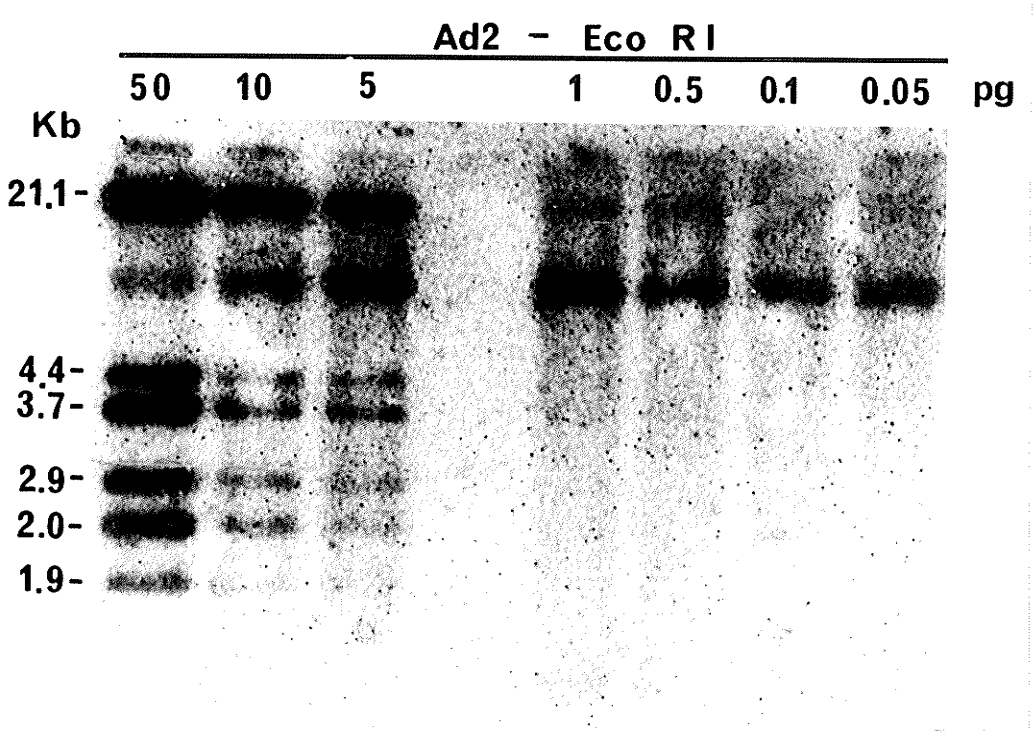

Figure 7 - Sensitivity titration of unlabelled Ad 2 DNA by Southern blot hybridization with an Ad 2 genomic DNA probe.

Varying quantities of unlabelled Ad 2 DNA were loaded in 10 µg of carrier HEp-2 DNA. All specimens were digested to completion with Eco RI. The probe was an Ad 2 genomic DNA probe (specific activity - 5 X 10^8 cpm/µg). Autoradiographic exposure for 15 days.

detectable at the 5 pg level after 7 days of autoradiographic exposure.

The band located between the Eco RI-A (21.1 kbp) and Eco RI-B (4.4 kbp) fragments seen in figure 7 is due to the hybridization of the labelled Ad 2 DNA probe to cellular DNA sequences, as explained in section 2E.

When radioactively-labelled Ad 3 DNA was used as a probe in some of the hybridization experiments it was found that an equivalent level of sensitivity in detection of Eco RI-digested homologous adenovirus DNA was attainable; *ie.* 5 pg of Eco RI-digested DNA was detectable after 7 days of autoradiographic exposure. As with Ad 2 DNA probes, a band due to the hybridization of labelled Ad 3 DNA to cellular DNA sequences was detected in some tonsil specimens (Figure 12 - specimens T267, T277 [weak signal], T282 [weak signal], T303, T305).

In order to prevent these adenovirus-like cellular DNA (ALCD) sequences from leading to additional hybridizing bands in adenovirus DNA controls, HEp-2 carrier DNA was replaced with herring sperm DNA. No ALCD sequences were ever detected in herring sperm DNA containing preparations with Ad 2 or Ad 3 DNA probes (examples - Ad DNA controls in Figures 8 and 12).

B. Detection of adenovirus DNA sequences in DNA from uncultured tonsillar tissue

While latent adenovirus has been demonstrated by explant culture of latently-infected tonsil tissue, no report is present in the literature in which explant culture and DNA hybridization using the Southern blot technique have been directly compared. In an attempt to add to our knowledge of adenovirus latency at the molecular level, both cultured and uncultured tonsil tissue were examined for the presence of adenovirus DNA.

As a first step, DNAs extracted from uncultured tonsil tissue were examined for adenovirus DNA sequences. Ten μg of DNA from each of the 32 tonsils examined by explant culture were digested to completion with Eco RI, transferred to nitrocellulose, and hybridized with a radioactively-labelled Ad 2 DNA probe (specific activity range : 1 X 10^8 to 1 X 10^9 cpm/μg). Adenovirus DNA sequences could not be detected in any of the 32 tonsil DNAs at a sensitivity level in which 5 pg of homologous Ad DNA could be detected. Even tonsils from which a subgenus C adenovirus was isolated failed to possess any detectable adenovirus sequences (Fig.8) though ALCD sequences were detected in all specimens. Because adenoviruses of subgenus C have 99-100% DNA homology with each other (Green *et al.*, 1979), the use of an Ad 2 DNA probe should be able to detect DNA from any subgenus C adenovirus, if it is present at or above the detection limit of the hybridization procedure.

With the isolation of adenovirus serotype 3 (Ad 3) from tonsil T298, DNAs from selected tonsils were re-examined with a radioactively-labelled Ad 3 DNA probe. DNAs from latently-infected (T257, T277, T298, T305), uninfected (T260, T284, T297, T300), and AINL (T280, T291) tonsils were hybridized with a ^{32}P-labelled Ad 3 DNA probe (specific activity - 3 X 10^8 cpm/μg) and, once again no adenovirus sequences were detected.

C. Detection of adenovirus DNA sequences in tonsil explant fibroblast cultures

DNA hybridization was used in an attempt to detect adenovirus DNA sequences in tonsil explant fibroblast cultures. The rationale is this : since adenovirus sequences could not be detected in DNA from uncultured tonsil tissue even when adenovirus was recovered from the tonsil, culturing the tissue would result in amplification of any adenovirus DNA which may be present.

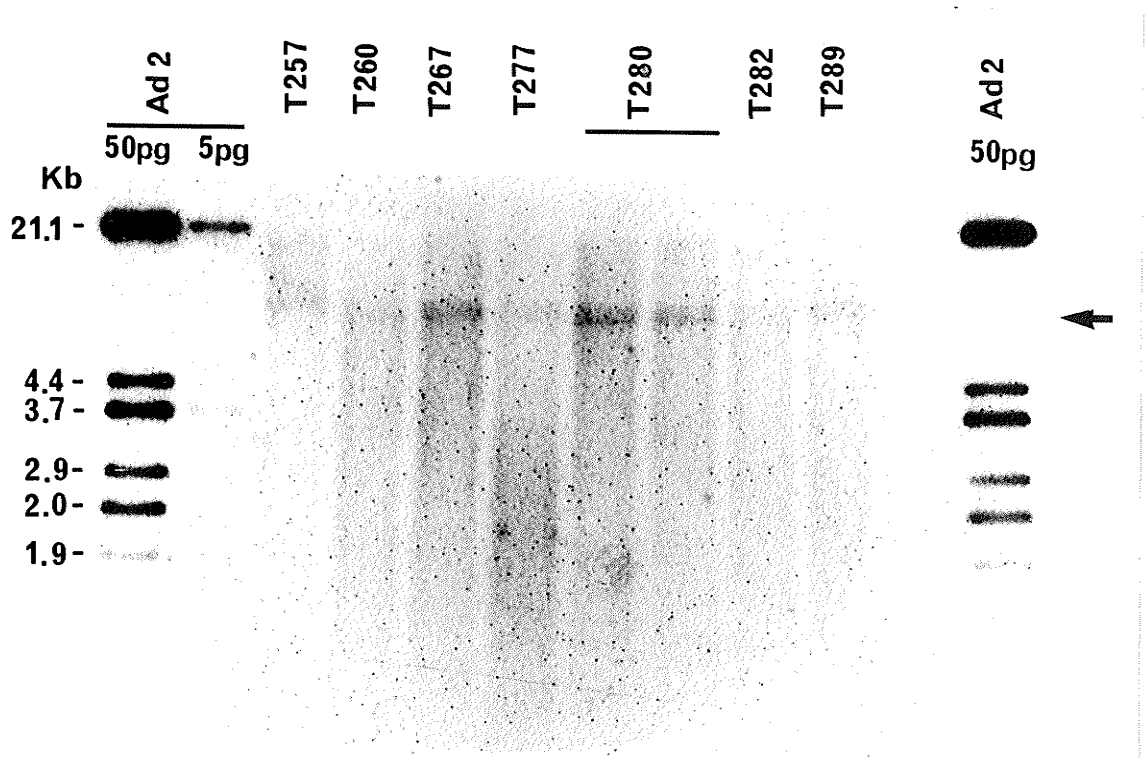

Figure 8 - Examination of uncultured tonsil DNAs for subgenus C adenovirus DNA sequences.

The probe used was an Ad 2 genomic DNA (specific activity - 4 X 10^8 cpm/μg). Ten micrograms of each tonsil DNA was examined. Control Ad 2 DNA in 10 μg carrier herring sperm DNA. All DNAs digested with Eco RI.Autoradiographic exposure for 15 days. Arrow represents adenovirus-like cellular DNA (ALCD) sequences detected with probe. Two lanes of T280 DNA represent two independent preparations of DNA from the uncultured tissue.

DNAs were isolated from tonsil fibroblasts after varying periods of time in culture, transferred to nitocellulose by Southern blot after digestion with Eco RI, and then hybridized with Ad 2 DNA probes. The results of these experiments, summarized in Table 8, demonstrate that DNA hybridization for detection of Ad DNA was as efficient as virus isolation. DNA hybridization detected Ad DNA sequences in 7 of 15 fibroblast cultures of 5 latently-infected tonsils whereas infectious adenovirus was isolated from the supernatants of six of these same fibroblast cultures. For latently-infected tonsils, Ad DNA sequences could be detected in DNA from fibroblast cultures which possessed infectious virus as detected in HEp-2 cultures. In one instance, however, DNA hybridization detected Ad DNA sequences in a fibroblast culture from which no infectious virus could be isolated (T303 day 16, Table 8 and Figure 9). In contrast to this there was a single culture from which infectious virus was isolated but in which no Ad DNA could be detected (T305 day 46). This case likely reflects the limit of sensitivity for the hybridization technique; if adenovirus DNA was present but at a level below 0.09 Ad 2 genome equivalents per cell the Ad DNA would not be detected. Conversely, only a single infectious adenovirus particle need have been present in the culture supernatant for detection in HEp-2 culture.

Adenovirus sequences were not detectable in DNAs from fibroblast cutures of tonsil T289 (Table 8). Supernatants from these same cultures, likewise, possessed no detectable infectious virus. These findings were not wholly unexpected since infectious virus was isolated only from lymphocytes co-cultivated with HEp-2 cells (Table 7).

The hybridization findings for the adenovirus-infected non-latent (AINL) tonsils were unremarkable; adenovirus sequences were detected in DNAs from all fibroblast specimens. In all cases adenoviruses were isolated from the

TABLE 8

DETECTION OF ADENOVIRUS DNA SEQUENCES IN CULTURED TONSIL FIBROBLASTS AS COMPARED TO DETECTION OF A CPE IN PRIMARY CULTURE AND ISOLATION OF ADENOVIRUS IN CULTURE SUPERNATANTS.

Tonsil	Days in culture	Hybridization[1]	CPE in primary culture	Virus isolated in HEp-2 culture
Latent				
T257	37	-	-	- d35 [2]
	122	+	-	NT[3]
	130	+	+	+
T277	65	+	-	+
T289	42	-	-	NT
	54	-	-	-
T303	8	-	-	-
	16	+	-	-
	32	-	-	-
	53	+	-	+
T305	8	-	-	-
	19	+	+	+
	32	-	-	-
	46	-	-	+
	53	+	-	+
AINL				
T280	51	+	+	+
	59	+	+	+
T302	8	+	-	+
	16	+	+	+
	25	+	+	+
	30	+	+	+
Uninfected				
T275	56	-	-	NT[4]
T276	56	-	-	NT[4]
T286	73	-	-	-
T294	49	-	-	-
T300	42	-	-	-

1. Hybridization with a radioactively-labelled Ad 2 DNA probe.
2. Supernatant of same culture sampled on day indicated.
3. Not tested.
4. Both cultures were virus negative after 26 days.

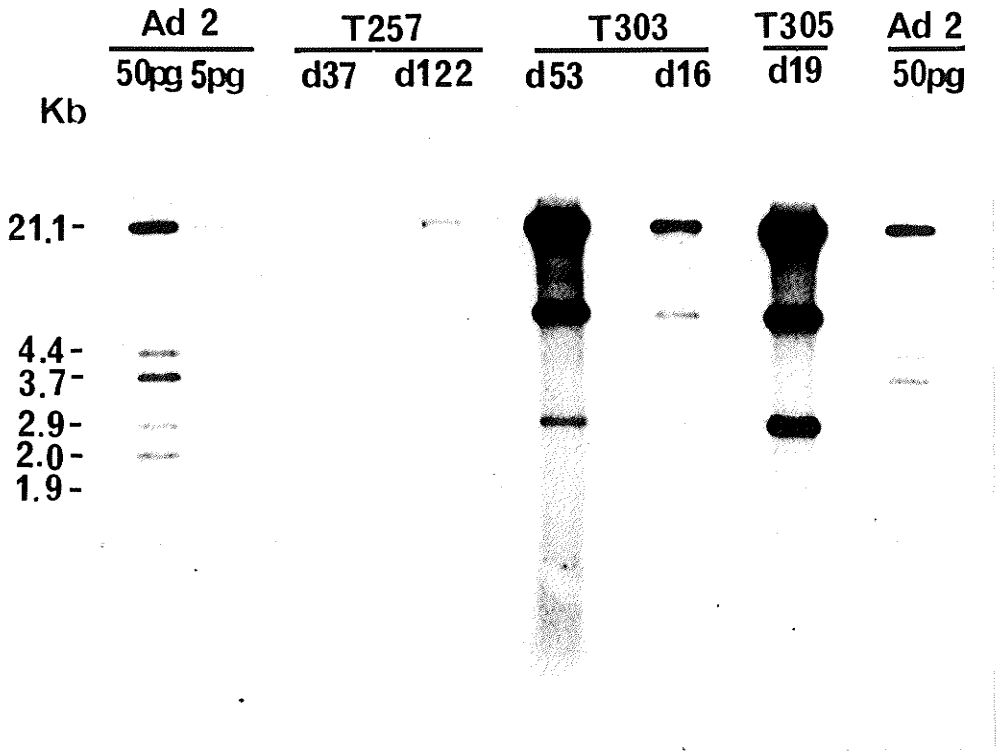

Figure 9 - Detection of subgenus C adenovirus DNA sequences in DNAs from cultured fibroblasts of latently-infected tonsils.

Ad 2 genomic DNA probe (specific activity - 6 X 10^8 cpm/µg. The letter 'd' indicates days in culture. Autoradiographic exposure for 22 hours (no additional sequences detected even after 7 days of exposure). Quantity of DNA - 150 ng (T303 and T305) and 200 ng (T257).

supernatants of these cultures. This was not surprising since direct isolation of virus from these tonsils indicate that high levels of infectious virus were present from the outset of culture. However, the level of Ad DNA present in the tissue before culture was still below the limit of sensitivity for the hybridization analysis. Culturing said tissue, even for only 8 days (Table 8 - T302) allowed sufficient replication of the adenovirus genome such that the level of Ad DNA was now at a detectable level.

For latently-infected tonsils the absence of a CPE in the fibroblast (*ie.* primary) culture was not always correlated with the absence of viral sequences or infectious virus (Table 8 - T277, day 65; T303, day 53; T305, day53). The same finding is true for the 8 day culture for the AINL tonsil T302. However, if a CPE was evident in the primary culture regardless of whether the tonsil was latently-infected or AINL, adenovirus sequences and infectious virus were detected.

The Eco RI restriction pattern of adenovirus sequences detected by DNA hybridization was identical, with one exception (T305 - day 53), to that of the adenovirus isolated from the tonsil. As can be seen in Figure 9, Ad 1 DNA was detected in tonsil fibroblast DNAs from latently-infected tonsils T257, T303 and T305. This finding correlates with the isolation of adenovirus type 1 from these cultures (Figure 4, Tables 7 and 8).The only exception was the 53 day fibroblast specimen from tonsil T305 (Figure 10) which showed 2 extra bands in addition to those characteristic for Ad 1, the adenovirus serotype isolated from cultures of that tonsil. The nature of these extra bands is presently unknown.

DNAs from cultured fibroblasts of latently-infected tonsils T267, T282, and T298 were unavailable for this part of the study. Likewise, DNA was not available from fibroblast cultures of the AINL tonsil T291.

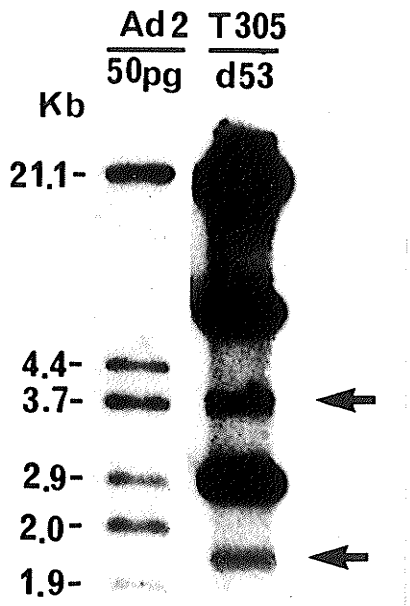

Figure 10 - Detection of variant restriction pattern in cultured fibroblast DNA of tonsil T305.

Ad 2 genomic DNA probe (specific activity - 5.2 X 10^8 cpm/µg). The letter 'd' indicates days in culture. Autoradiographic exposure for 7 days. Fibroblast DNA (25 ng) and control unlabelled Ad 2 DNAs digested with Eco RI. Arrows indicate additional bands.

Cultured fibroblast DNAs of several uninfected tonsils were examined for the presence of Ad DNA sequences (Fig. 11). The cultures examined represented those maintained longest for each tonsil. No adenovirus sequences were detected in any of these specimens (Table 8). However, as with the uncultured tonsil DNAs, bands of hybridization of the Ad 2 DNA probe to the cellular DNA were detected.

D. Detection of adenovirus DNA sequences in DNA from tonsillar lymphocytes

The isolation of adenoviruses from cultured tonsillar lymphocytes has been previously reported (van der Veen and Lambriex, 1973) and shown for one tonsil in this study (T289 - Table 7). However, nothing has been reported in the literature regarding the examination of tonsillar lymphocytes for adenovirus DNA sequences. In this study we examined DNA from both cultured and uncultured lymphocytes for the presence of adenovirus DNA sequences.

1. Uncultured lymphocytes

DNA from uncultured lymphocytes of 12 tonsils were examined by hybridization with a radioactively-labelled Ad 2 DNA probe for the presence of subgenus C Ad DNA sequences. As for DNA from uncultured tonsil tissue, DNA (5 µg) was digested to completion with Eco RI, electrophoresed to separate the DNA fragments, then transferred to nitrocellulose. Hybridization with the Ad 2 probe failed to detect adenovirus sequences in any of the uncultured lymphocyte DNAs including those from which an adenovirus was isolated (Table 9 and Appendix A). Adenovirus sequences were, however, detected in the uncultured lymphocyte DNA from T298 when these same DNAs were examined with a radioactively-labelled Ad 3 DNA probe (Figure 12). The DNA restriction

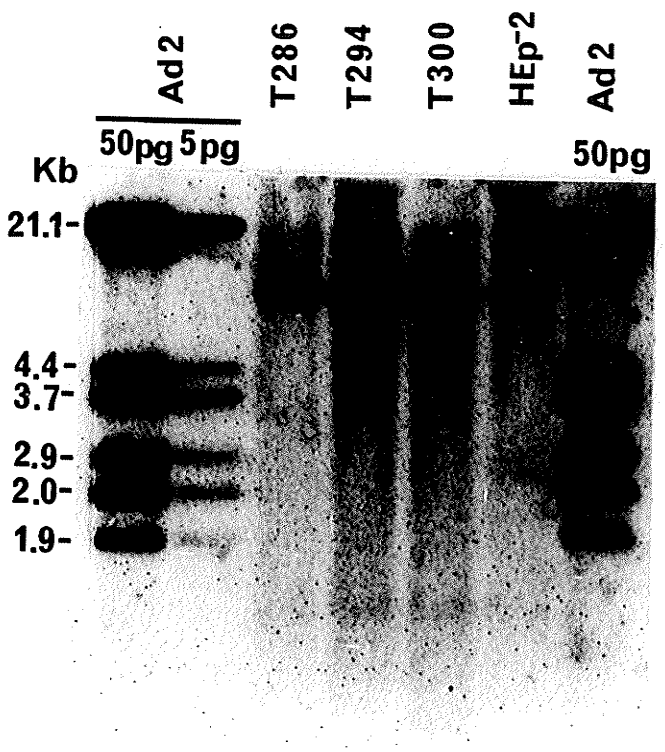

Figure 11 - Examination of DNAs from fibroblast cultures of infectious adenovirus-free tonsils for subgenus C adenovirus sequences.

Ad 2 genomic DNA probe (specific activity - 4.9 X 10^8 cpm/μg. Autoradiographic exposure for 8 days. Quantity of DNA (digested with Eco RI) - 500 ng (tonsil specimens), 10 μg (HEp-2).
Note band of hybridization between 21.1 and 4.4 kbp in cellular DNAs.

TABLE 9

DETECTION OF ADENOVIRUS DNA SEQUENCES IN CULTURED AND UNCULTURED LYMPHOCYTES FROM LATENT AND AINL TONSILS AS COMPARED TO ISOLATION OF ADENOVIRUS FROM TONSILLAR LYMPHOCYTE CULTURE SUPERNATANTS.

Tonsil	Lymphocyte Preparation[2]	Hybridization[1] Ad 2	Ad 3	Virus isolated in HEp-2 culture
Latent				
T267	u	-	-	NT [3]
T277	p-	-	NT	-
	p+	-	NT	-
T282	u	-	-	- [4]
	p-	-	-	-
	p+	-	-	-
T289	u	NT	NT	+
	p-	-	NT	-
	p+	-	NT	-
T298	u	-	+	-
	p-	NT	NT	-
	p+	-	+	-
T303	u	-	-	-
	p-	-	NT	-
	p+	-	NT	-
T305	u	-	-	-
	p-	-	NT	-
	p+	-	NT	-
AINL				
T280	p-	-	NT	-
	p+	-	NT	-
T291	p-	-	NT	-
	p+	-	NT	-
T302	u	-	-	-
	p-	NT	NT	-
	p+	NT	NT	-

1. Hybridization with radioactively-labelled Ad 2 or Ad 3 DNA probes.
2. Uncultured(u); cultured without PHA(p-); cultured with PHA(p+).
3. Not tested.
4. Cocultivated with HEp-2 cells.

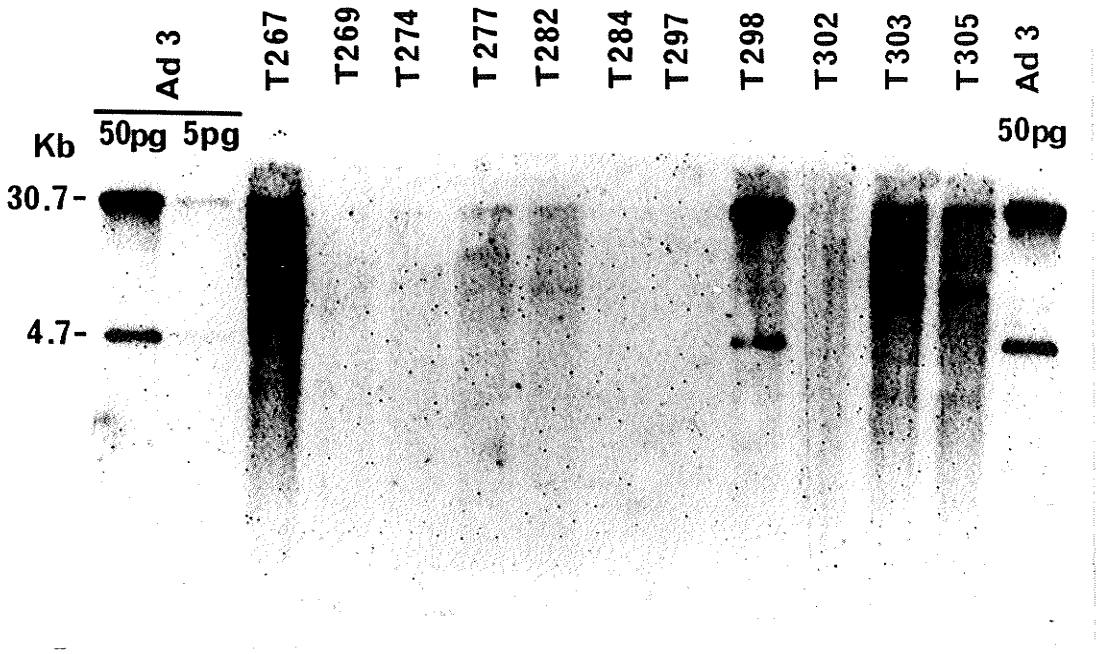

Figure 12 - Detection of subgenus B adenovirus DNA sequences in DNAs from uncultured tonsil lymphocytes.

Ad 3 genomic DNA probe (specific activity - 2 X 10^8 cpm/µg). Autoradiographic exposure for 7 days. Quantity of DNA - 5 µg (lymphocyte DNAs). All DNAs digested with Eco RI.
Note presence of bands of hybridization between 30.7 and 4.7 kbp in size in some tonsil lymphocytes lanes (examples - T277, T282, T303, T305).

fragment pattern seen by hybridization with this probe indicates that the viral sequences belong to an Ad 3. This was the adenovirus serotype isolated from explant culture of tissue from this tonsil. It is interesting to note that while DNA sequences were detected in the uncultured lymphocytes from this tonsil, no infectious virus could be detected in the cultured lymphocytes.

As with uncultured tonsil tissue DNAs, bands of cellular DNA sequences hybridizing to the Ad DNA probes were present in many of the specimens (Figure 12 - T267, T303, T305). However, while the ALCD sequences detected were of the same relative size with either Ad DNA probe (compare Figures 8 and 12), the hybridization signal using an Ad 3 DNA probe was considerably weaker. The reason for this difference in ALCD signal intensity is unknown.

2. Cultured lymphocytes

DNA from the cultured lymphocytes of 10 tonsils were examined for the presence of adenovirus sequences. In all but one case, DNAs from both lymphocytes cultured with and without the mitogen PHA were examined. Nine tonsils were examined with an Ad 2 DNA probe after Eco RI digestion and blotting to nitrocellulose. Because the amount of DNA isolated from each of the specimens was highly variable, the amount of DNA probed varied from 100 ng to 1 µg. Adenovirus sequences were not detected in any of the 9 tonsils; the tonsils examined were representative of adenovirus-free (T275, T279), latently-infected (T277, T282, T289, T303, T305), and AINL (T280, T291) tonsil groupings (Figure 13). The phenomenon of cellular DNA binding the Ad 2 DNA probe was once again present as seen in figure 13. However, this binding was not to a single size class of cellular DNA; DNAs of unstimulated cultured lymphocytes show two additional bands in excess of the size of that seen for DNAs of the

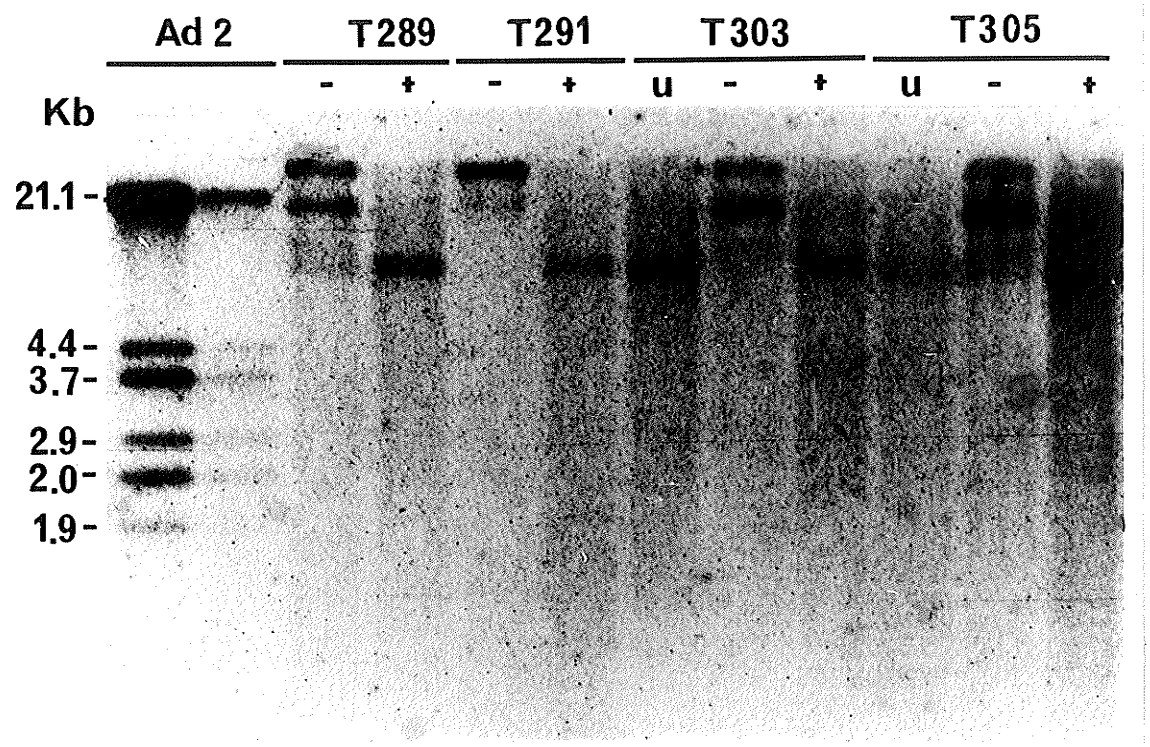

Figure 13 - Examination of DNAs from uncultured tonsil lymphocytes(u), tonsil lymphocytes cultured without PHA (-), and tonsil lymphocytes cultured with PHA (+) for adenovirus subgenus C DNA sequences.

Ad 2 genomic DNA probe (specific activity - 2.3 X 10^8 cpm/μg). Autoradiographic exposure for 7 days. Quantity of DNA (Eco RI-digested) - all cellular lanes contain 250 ng of DNA; Ad 2 DNA controls are 50 pg and 5 pg (left and right lanes, respectively).
Note varying bands of hybridization in excess of 4.4 kbp for all cellular DNAs.

uncultured and PHA-stimulated lymphocytes. The reason for this difference is unknown.

Since adenovirus sequences of Ad 3 were detected in the uncultured lymphocyte DNA of T298, DNA from lymphocytes cultured in the presence of PHA from this tonsil were examined with an Ad 3 DNA probe. As can be seen in figure 14, adenovirus sequences can be detected in this specimen and are identical in restriction pattern to those detected in the uncultured specimen.

E. Cellular DNA sequences show homology to adenovirus DNA

When cellular DNAs were digested with the restriction endonuclease Eco RI and hybridized with radioactively-labelled genomic Ad 2 or Ad 3 DNAs it was found that a band or bands of cellular DNA bound the probe DNA. These bands were called adenovirus-like cellular DNA (ALCD) sequences since they hybridize with adenovirus DNA yet did not comigrate with any Eco RI fragment of adenovirus serotypes 1, 2, 3, 5, or 6, the adenovirus serotypes isolated from tonsils in this and previous studies. ALCD sequences were detected regardless of whether the cellular DNA was extracted from cultured or uncultured tonsillar tissue, or of the presence or absence of adenovirus in the tonsil. ALCD sequences were also detected in blots of Eco RI-digested DNA from uninfected HEp-2 cells, a cell line of human origin used for the propagation of adenoviruses in this study (Figure 11).

In order to ascertain whether or not the ALCD sequences were, in fact, artifacts due to some technical event in the processing and subsequent hybridization of nitrocellulose-bound cellular DNAs, HEp-2 DNA was separately digested with both Eco RI and Hind III. This was done on the premise that if ALCD sequences were artifacts due to the processing procedure, hybridization of radioactively-labelled adenovirus DNA should take place at the same location on the DNA blot regardless of the restriction endonuclease used.

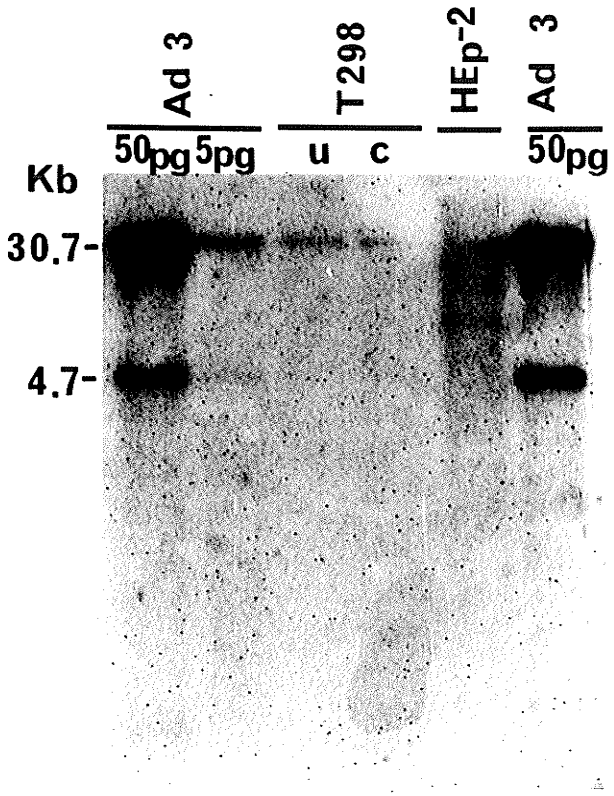

Figure 14 - Detection of adenovirus subgenus B DNA sequences in DNAs from uncultured tonsil lymphocyte (u) and PHA-cultured tonsil lymphocytes (c) of tonsil T298.

Ad 3 genomic DNA probe (specific activity - 3 X 10^8 cpm/μg). Autoradiographic exposure for 7 days. Quantity of DNA (Eco RI-digested) - 300 ng (lymphocyte DNAs) and 10 μg (HEp-2). Note band of hybridization between 30.7 and 4.7 kbp in size in HEp-2 lane.

Hybridization of a membrane prepared in this manner with ^{32}P-labelled Ad 2 DNA, however, demonstrated that Hind III digestion resulted in a change in the mobility of ALCD sequences (Fig. 15). In addition to HEp-2 DNA, DNA from Jurkat cells (a human T-lymphoid cell line) was included in this experiment to establish whether lymphoid cells of human origin possessed ALCD sequences; lymphocytes comprise 65-85% (T-lymphocytes - 15-25%) of all cells of the human tonsil (Gelfand, 1976). DNA from plasmid pCD2 was also included as a positive control since it contains the E1A region of Ad 5 and would hybridize with the Ad 2 DNA probe. As well, pCD2 possesses unique Eco RI and Hind III sites and, therefore, migrates to the same position in the agarose gel when cut with either enzyme; thus, pCD2 provides a constant size marker (6.0 kbp) for ready detection of any ALCD migration variation resulting from digestion with the different restriction enzymes. When Jurkat and HEp-2 DNAs were digested with Eco RI the ALCD sequences were found to be a broad band approximately 7.5 kbp in size while Hind III digestion resulted in a collection of bands having sizes in excess of 7.5 kbp (Figure 15). These results indicate that ALCD sequences are indeed not artifactual.

ALCD sequences have been detected by other researchers (see Literature Review, Section 6) and shown to have homology with E1A region of subgenus C adenoviruses. To determine if such homology existed between E1A sequences and the ALCD sequences detected in this study, bound probe DNA was removed from the membrane yielding the results seen in Figure 13 and hybridized with a pCD2 probe. The plasmid pCD2 is a pBR322 plasmid into which the E1A gene of Ad 5 was inserted. This membrane was chosen since multiple ALCD bands were present and it would be of interest to know if the bands equal to or larger than 7.5 kbp showed any homology to Ad 5 E1A sequences. The radioactively-labelled pCD2 DNA probe,

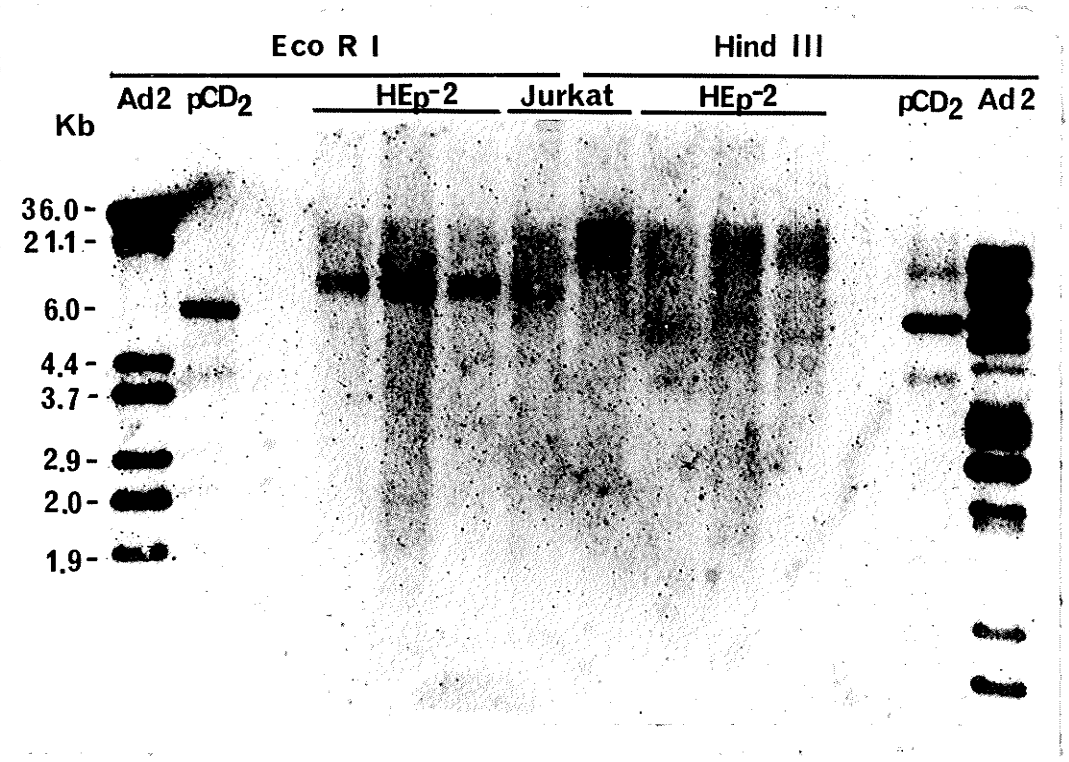

Figure 15 - Detection of ALCD variation in human cellular DNAs (HEp-2 and Jurkat) digested with Eco RI or Hind III by a labelled Ad 2 DNA probe.

Ad 2 genomic DNA probe (specific activity - 4.9 X 10^8 cpm/µg). Autoradiographic exposure for 8 days. Quantity of DNAs - 10 µg (HEp-2 and Jurkat); 50 pg (Ad 2 and pCD2).
The three lanes of HEp-2 DNA represent 3 different HEp-2 DNA preparations.
Note variation in bands of hybridization (cellular DNAs) with the differing restriction enzyme digests.

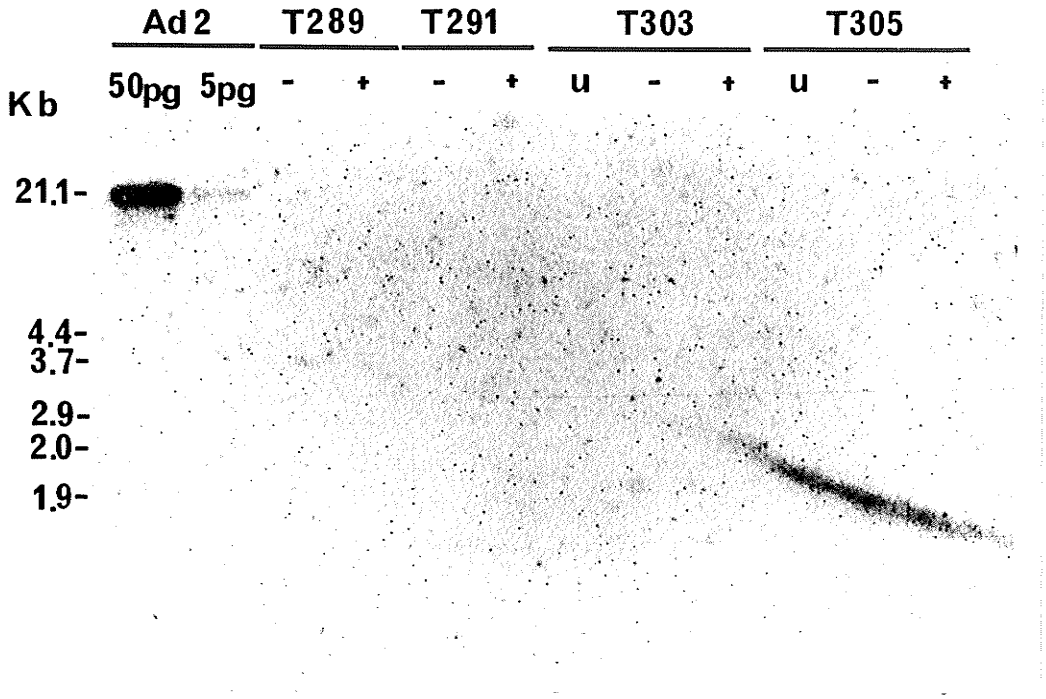

Figure 16 - Examination of DNAs from cultured and uncultured tonsil lymphocytes for ALCD sequences with a labelled pCD2 probe(see figure 13 for original hybridization pattern).

pCD2 DNA probe (specific activity - 4.5 X 10^8 cpm/µg).
Autoradiographic exposure for 7 days.
Quantity of DNAs (Eco RI-digested) - 250 ng (all lymphocytes).

however, did not hybridize with any ALCD sequences (Fig. 16); in fact, the pCD2 DNA probe hybridized only to the Ad 2 Eco RI-A fragment which comprises the leftmost 58.5% of the Ad 2 genome containing the E1A gene.

A reverse blot was performed in an attempt to determine which regions of the adenovirus genome show homology to ALCD sequences. ALCD was isolated as described in Materials and Methods, labelled with ^{32}P-dCTP by nick translation, and hybridized to a blot of unrestricted pCD2 DNA and restriction digests of HEp-2 and Ad 2 DNAs (Figure 17). The isolated ALCD sequences were also on this blot, but undigested, as a positive control. Both Eco RI-digested HEp-2 DNA and ALCD hybridized with the ^{32}P-ALCD probe. The probe also hybridized only to Eco RI-B and -C fragments, Hind III-A and -E or -F fragments, and Sma I-A and -G fragments of Ad 2 DNA. No hybridization was detected with other Ad 2 DNA fragments generated by the three enzymes. The DNA fragments which did hybridize map to two discrete regions of the of the Ad 2 genome. Hybridization was with the region from 58.5-70.7 map units (containing the Ad 2 E2A region and a portion of the L3 region) and the region spanning 80.6-100 map units (containing the Ad 2 E4 and L5 regions and a portion of the E3 region). As well, there was no hybridization of the ALCD probe to pCD2 DNA. This finding supports the results obtained when pCD2 DNA was used as a probe (Figure 16). It was therefore concluded that ALCD sequences do not possess homology with subgenus C E1A sequences under the highly stringent hybridization conditions employed.

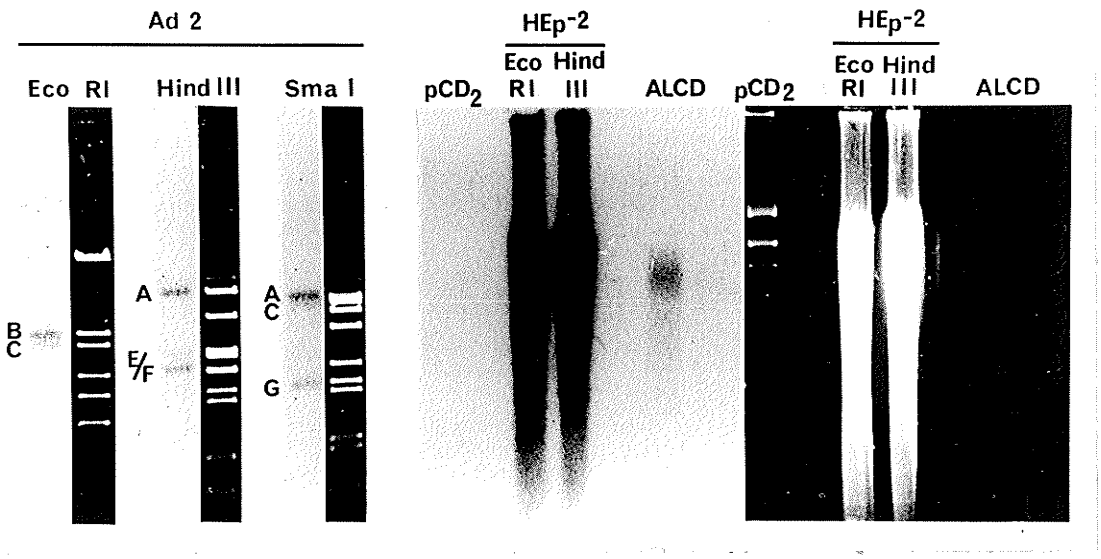

Figure 17 - Identification of adenovirus DNA regions which hybridize to labelled ALCD sequences.

Side-by-side comparison of hybridization results (left-hand lanes) with agarose gel results (right- hand lanes).
ALCD probe (specific activity - 2.5 X 10^8 cpm/µg)
Duration of exposure - 24 hours (Ad 2 lanes)
90 minutes (HEp-2, ALCD, and pCD2 lanes)
Quantity of DNAs - 500 ng (Ad 2 and pCD2)
50 ng (ALCD)
10 µg (HEp-2)
ALCD and pCD2 DNAs were undigested and the remaining DNAs were digested with the restriction endonucleases indicated.

Note - Extended exposure (24 hours) of membrane failed to detect any hybridization of ALCD to pCD2.

V. DISCUSSION

This study was undertaken to evaluate DNA hybridization for studying the phenomenon of adenovirus latency in tonsillar tissues. The operational definition of adenovirus latency is that infectious virus can be recovered from explant cultures of infected tissue while infectious virus cannot be isolated directly from uncultured tissue. In light of this definition and the aim of this study, tissue culture and Southern blot hybridization analyses were performed on 32 tonsils. The tissue culture examination allowed the identification of tonsils which fit this definition of latent infection while the Southern blot hybridization allowed the examination of the adenovirus genome at the molecular level within latently-infected tissues.

Latent adenovirus was isolated by explant culture from 8 of 32 tonsils examined in this study. Seven of these tonsils yielded adenovirus in explant fibroblast cultures (Tables 5 and 6) while one tonsil (T289) only yielded adenovirus by co-cultivation of tonsillar lymphocytes with HEp-2 cells. The isolation of adenoviruses from these 8 tonsils under these conditions was taken as evidence of latent adenovirus infection. As well, adenovirus was isolated directly from the tonsil washes of three of the 32 tonsils (T280, T291, and T302). Infectious adenovirus was likewise isolated from fibroblast cultures of these tonsils. These results were taken as indicating these tonsils were not latently-infected with adenovirus but rather productively infected and were termed adenovirus-infected non-latent (AINL) tonsils.

When DNAs from uncultured tissue of the 32 tonsils in this study were examined for the presence of adenovirus DNA, no sequences could be detected at a sensitivity level of 0.09 Ad 2 genome equivalents per cell. This finding, taken with the isolation of adenovirus from a total of 11 tonsils (both latent and non-latent [*ie.* AINL]), indicates that the initial

amount of virus (and, therefore, viral DNA) which was present prior to culture was less than 1 Ad 2 genome equivalent per 10 cells, on average. This finding is in accordance with those of Strohl and Schlesinger (1965b); these authors found by infectious centre assay of tonsil cells that the frequency of cells able to produce infectious virus was of the order of 1 per 10^7 cells. If each of these infected cells only possess a single copy of the adenovirus genome, the Southern blot hybridization would not be sensitive enough to detect this level of viral DNA; the limit of this hybridization technique is 10^5 genome equivalents of Ad 2 DNA. This would mean that the DNA from 10^{12} tonsil cells would need to be examined by this technique in order to detect the viral DNA. From these values it is easy to see that under the conditions used in this study Southern blot hybridization was not useful in the detection of latent adenovirus in uncultured whole tonsil tissue.

In light of the detection of adenovirus DNA sequences in uncultured lymphoid tissues by other researchers, notably Green *et al.* (1979) and Horvath *et al.* (1986), it remains unclear as to our inability to detect Ad DNA sequences in uncultured tonsil tissue. Both of these groups were able to detect adenovirus DNA sequences in uncultured lymphoid tissue using Southern blot hybridization. One possible explanation as to the striking difference between these studies and the results obtained in this study is the length of exposure for detection of these rare DNA sequences; this study used an average autoradiographic exposure of 15 days in attempting to detect Ad DNA in uncultured tonsil DNAs while no indication of autoradiographic exposure is made in either of these papers. These groups could have used extremely long autoradiographic exposures (> 1 month) which would have increased sensitivity. As well, these groups could have used lower stringency conditions to obtain their results. While both of these modifications could have potentially allowed the detection of these rare adenovirus DNA sequences in this

study, they also increase the likelihood of increased background which would make interpretation of the results more difficult. As well, the presence of ALCD sequences would likely interfere with interpretation of results since they are very prominent under condition of high stringency and would be increased even more with lower stringency and/or prolonged exposure.

Four to sixteen companion fibroblast cultures for each of 6 of the 8 latently-infected tonsils were examined for the presence of infectious adenovirus (Table 6). In only one instance (T282) was infectious adenovirus detected in all of the companion cultures. In the remaining 5 latently-infected tonsils only a fraction of the total number of fibroblast cultures for a given tonsil were adenovirus-positive. This likely reflects that the number of cells harbouring latent adenovirus within a tonsil is small. For the 2 remaining latently-infected tonsils, T267 and T289, only single fibroblast cultures were available. Adenovirus was isolated from that of T267 but not from that of T289.

It was also found that for some latently-infected tonsils, adenovirus could be detected prior to the appearance of a CPE (cytopathogenic effect) in fibroblast culture (Table 5 - T267, T303, T305). This indicates that some tonsil fibroblast cultures are able to release infectious virus for a long period of time before the appearance of a CPE (Table 5 - T305). This may indicate that, in some cases, what is seen as a latent infection may be a persistent infection of the 'carrier state' kind, *ie.* where the level of cell death due to viral infection is balanced by the rate of cell growth.

When tonsil fibroblasts were examined for infectious virus it was found that those of T257 could be maintained through 3-4 serial passages for 127 days before the appearance of a viral CPE and subsequent isolation of infectious virus on day 130 (Table 3, Appendix B). As illustrated in Figure 9, adenovirus DNA could be detected even earlier (day 122) than the appearance of a CPE. This

long lag period is unusual in that adenovirus was isolated from fibroblast cultures of other latently-infected tonsils between 7 and 39 days in culture. This, however, could be explained as a truly latent tonsil which required longer than other cultures for expression of the resident virus as infectious adenovirus. As well, an incubation period of 96 days for a latently infected tonsil has already been described (Evans, 1954). Thus it would seem that latent adenovirus is capable of expression at any time of explant culture. As to how this may apply *in vivo* is not known since it is not yet known if 'reactivated' latent adenovirus is responsible for any disease in man.

Having failed to detect adenoviral DNA sequences in the DNAs of latently-infected or AINL tonsils, the DNAs of explant fibroblast cultures were examined because it was felt that culture of said tissue could lead to an amplification of adenovirus DNA resident within the dividing cells and thus bring the level of Ad DNA to a detectable level. When these results were compared to the culture results (Table 8) it was found that DNA hybridization was as effective as culture in detection of virus. There was a single instance (out of 15) in which DNA hybridization was able to detect adenovirus DNA when no infectious virus could be detected in culture (Table 8 - T303, day 16). However, there was also one specimen where infectious virus was isolated while no adenovirus DNA could be detected (Table 8 - T305, day 46). Thus it was concluded that hybridization is as sensitive as culture for the detection of adenovirus. However, the Southern blot technique also has the added benefit of allowing the identification of the adenovirus serotype by the characteristic restriction fragment pattern.

In almost all cases when adenovirus sequences were detected by DNA hybridization, the pattern of hybridization was consistent with a linear adenovirus genome being present in its entirety. This was expected since the major source of the DNA is the virion or the replicating pool of viral DNA.

The only exception was found with the 53 day fibroblast specimen of T305 (Figure 10) in which, in addition to a prototype Ad 1 banding pattern, there were two extra bands which were of lower hybridization intensity and which did not comigrate with either Ad 1 or Ad 2 DNA fragments generated by Eco RI digestion. It is not known whether these extra bands represent integrated Ad 1 DNA, albeit in a smaller proportion of cells than those productively infected, or if they represent another configuration of the adenovirus genome. One fact which opposes the integration theory is that if integration had taken place the cellular DNA attached to the ends of the adenovirus genome would have decreased the electrophoretic mobility of the terminal DNA RE fragments. This was not the case, however, since it is the Eco RI-A and Eco RI-B fragments of Ad 1 which would show mobility change while the bands which appear to have shifted are the Eco RI-B and Eco RI-C fragments; fragments A and B are the terminal Eco RI fragments of Ad 1. The exact nature of these additional bands cannot be determined without further analysis. If mobility variation is retained when the full-length viral DNA is digested with a second enzyme, this would be consistent with the presence of an unusual form of the adenovirus genome.

Since fibroblasts are not the only cell type present in tonsil tissue, DNAs from tonsillar lymphocytes were also examined by DNA hybridization. No subgenus C adenovirus DNA sequences were detected when labelled Ad 2 genomic DNA was used to probe DNAs from uncultured lymphocytes and lymphocytes cultured in the presence and absence of PHA(Table 9). In light of the isolation of Ad 1 from the co-cultivated lymphocytes of tonsil T289, the failure to detect these adenovirus DNA sequences which must be present likely reflects the difference in sensitivity between Southern blot and culture; only a single infectious virion is required for detection by culture while hybridization requires at least 10^5 viral genome equivalents. However, the failure to isolate

any adenovirus from the lymphocyte-only cultures of T289 would also seem to indicate that infected lymphocytes within that tonsil are exceedingly rare as well; a total of 7.2 X 10^7 lymphocytes from T289 were cultured in the presence or absence of PHA. Thus it would seem that adenovirus infection of tonsillar lymphocytes is a rare event.

While subgenus C adenovirus sequences could not be detected in lymphocyte DNAs, reprobing these DNAs did show the presence of Ad 3 sequences within PHA-cultured and uncultured lymphocytes of tonsil T298 (Figures 12 and 14 and Table 9). As well, Ad 3 virus was isolated from one of 8 explant fibroblast cultures derived from this tonsil. The detection of viral DNA but the failure to isolate infectious virus from lymphocytes of this tonsil would seem to indicate that these cells were abortively infected (*ie.* the cells were not capable of producing infectious adenovirus) while the recovery of infectious virus from tonsil fibroblasts, albeit a single culture, would seem to indicate a productive infection of a small number of cells. From these results it is unclear as to whether this is a general phenomenon for 'latent' adenovirus or that this is unique to this uncommon 'latent' adenovirus isolate.

The adenoviruses isolated from both AINL and latently-infected tonsils were identified by restriction endonuclease (RE) analysis. All but one of the isolates from latently-infected tonsils were identified as adenovirus type 1 (Figure 4). The exception to this, tonsil T298, was identified as adenovirus type 3 by its RE pattern when digested with Hind III (Figure 4). This was an unusual finding since there has been no report in the literature in which a non-subgenus C adenovirus has been isolated from a latently-infected tonsil, though adenovirus types 3 and 7 have been isolated from other lymphoid tissues (Bell and Steyn, 1962; Potter, 1964; Bonard and Paccaud, 1966). It is unlikely that this adenovirus type arose from a lab contaminant since Ad 3 DNA was detected in

the DNAs from both cultured and uncultured lymphocytes from this tonsil (Figures 12 and 14).

All adenovirus isolates from AINL tonsils were found to belong to subgenus C. Isolates from tonsil washes and explant fibroblast cultures were identical in RE pattern for tonsils T280 and T302 but not for tonsil T291. The tonsil washes isolate of T291 was identified as having a RE pattern identical to that of prototype adenovirus type 1 while the fibroblast isolate had RE characteristics similar to that of adenovirus type 5. While this tonsil fits the criteria of what would be a productively infected tonsil the RE analysis would seem to indicate that the tonsil was latently infected with this second adenovirus. A possible explanation of these findings is that the adenovirus type 1 isolated directly from tonsil washes was resident on the surface of the tonsil and was removed by the washing of the tissue in preparation for the establishment of explant fibroblast cultures. The second adenovirus was probably latent within the tissue and only became detectable after a period of time in culture. The evidence to support this hypothesis is the complete absence of non-Ad 1 fragments present when the tonsil washings isolate was examined by Eco RI digestion (Figure 4). Had both viruses been present in the tonsil in an infectious state, both would have been amplified by HEp-2 culturing of tonsil washes and thus RE analysis would have yielded a mixed fragment pattern. As well, RE analysis of the fibroblast isolate shows that it is similar to Ad 5 but distinctly different from Ad 1 (compare Figures 4 and 5). This indicates that the Ad 1 was likely a surface contaminant of the tonsil which also possessed a latent adenovirus type 5 variant.

In some cases the adenoviruses isolated were shown to have RE patterns which differed from those of prototype subgenus C adenoviruses (Figures 3 and 6). The isolates from tonsil T282 and T289 were found to have the same DNA fragment variation when digested with Bam HI while showing the prototype pattern when digested with Eco RI (Figure 3). The

RE pattern for these Ad 1 isolates have not been previously described in the literature and likely represents a new variant adenovirus type 1. The adenovirus isolates from the AINL tonsil T302 also showed RE pattern variation; these isolates were identical to prototype adenovirus type 2 with Eco RI but showed a variation when digested with Kpn I. This variation is consistent with the adenovirus type 2d variant previously described by Fife *et al.* (1985). To date there is no known report of RE analysis of adenoviruses isolated from infected tonsils.

In addition to adenoviruses, herpesviruses were isolated from a single fibroblast culture from each of two tonsils (T277 and T279). Both of these isolates were identified as herpes simplex virus type 1 (HSV-1). Tonsil T277 is unusual in that latent adenovirus was recovered from 4 companion fibroblast cultures to that yielding the HSV-1 isolate (Table 5, Appendix B). Since tonsils are not innervated it is not known in which cell type these herpesviruses were resident though it may be inferred that it was fibroblasts on the basis of the cell type in the explant cutlure from which they were isolated. This is the second instance, to our knowledge, that HSV-1 has been isolated from tonsils under these conditions; the first isolation was reported by Israel (1962) but no indication was made as to whether this isolate was type 1 or type 2.

When it was found that adenoviruses could not be isolated directly from homogenates of tonsillar tissue it was hypothesized that an adenovirus-inactivating substance may be present. To examine this possibility, homogenates of tissue from AINL, latently-infected, and uninfected tonsils were incubated with known amounts of different adenovirus types and the surviving virus was titrated (Table 4). Homogenates prepared from latently-infected tonsils showed an adenovirus-inactivating activity towards the adenovirus type which was isolated from the tonsil in explant culture. This inactiviting activity also appears to be type specific (T298

- Ad 3) or at least subgenus specific (T267 - Ad 1 and Ad 2) since no significant inactivation was seen with heterotypic virus *ie.* with an adenovirus which was not isolated from that tonsil. Homogenates prepared from AINL and uninfected tonsils showed no significant level of adenovirus-inactivating activity.

The specificity of the adenovirus inactivation is consistent with the property of specific neutralizing antibody. The evidence that this inactivator is neutralizing antibody, while indirect, is none the less convincing in that if the inactivation were due to some non-specific inactivator one would expect inactivation to take place regardless of the adenovirus type used. Since this was not seen, the hypothesis that the inactivator may be neutralizing antibody is still valid. One means of determining whether the inactivator is neutralizing antibody would be to conduct experiments similar to those performed by Snejdarova *et al.*(1975) and Strohl and Schlesinger (1965b). If the inactivator can be removed by incubation of an inhibitor-positive tonsil homogenate with a high dose of homotypic virus but not with an equivalent dose of a heterotypic adenovirus this specificity would indicate that this could be specific neutralizing antibody. As well, the homogenate could be passed down an affinity column which contained anti-human IgG. If the eluted homogenate still retained adenovirus-inactivating activity then this would indicate that the substance responsible was not neutralizing antibody.

The presence of an adenovirus-inactivating substance in tonsils is a possible explanation of what is seen as adenovirus latency. Direct examination for infectious virus would be negative since resident adenovirus would only be detected if present in excess of the level of the inactivating substance. Explant cultures of the same tissue would result in the inactivating substance being diluted and eventually removed by culture medium changes. At this time infectious virus would be detectable and seen as being latent

according to the operational definition. However, the experimental evidence does not support or refute the possibility that 'masking' of infectious adenovirus by the presence of neutralizing antibody is responsible for what is seen as adenovirus latency

It was found in this study that labelled Ad 2 and Ad 3 DNAs were able to hybridize to cellular DNA sequences. These sequences, termed adenovirus-like cellular DNA (ALCD) sequences, hybridize to probe at a constant size of approximately of 7.5 kbp in Eco RI-digested DNAs of human cell lines (HEp-2, Jurkat), uncultured tonsils, uncultured tonsil lymphocytes, and tonsil lymphocytes cultured in the presence of PHA. However, DNA from tonsil lymphocytes cultured in the absence of PHA also showed bands but of a size greater than 7.5 kbp when hybridized with a genomic Ad 2 DNA probe. After establishing that these bands were not artifacts due to a step in the hybridization procedure (Figure 15), we examined the possibility that these may represent an unusual form of the adenovirus genome (eg. circularized, integrated). To this end we reprobed the membrane in figure 14 with labelled pCD2 DNA. The plasmid pCD2 contains the *E1a* region of Ad 5 and would hybridize to any subgenus C adenovirus DNA present since the *E1a* region is necessary for the replication of the adenovirus genome. However, no evidence of hybridization took place with any ALCD band (Figure 16) indicating that these sequences do not show homology to the *E1a* region of subgenus C adenoviruses.

A reverse blot experiment was performed in an attempt to identify the region or regions of the Ad2 genome which show homology to ALCD sequences. ALCD seqences were isolated from HEp-2 DNA, labelled with ^{32}P-dCTP, and used to probe a membrane containing restriction fragments of Ad 2 DNA. Hybridization was seen with homologous DNA controls (unlabelled ALCD and HEp-2 DNA) as well as to two discrete regions of the Ad 2 genome (58.5-70.7 mu and 80.6-100 mu) representing the genes *E2a, E4,* and *L5* in their entirety as

well as incomplete portions of *L3* and *E3*. As well, no hybridization was evident with pCD2 or any RE fragment of Ad 2 DNA containing the *E1a* . This indicates once again that there is no homology between subgenus C *E1a* regions and ALCD. This finding shows that there is one or more cellular DNA sequence present which shows homology to adenovirus DNA sequences. The regions of Ad 2 DNA which hybridize to ALCD found in this study are distinct from the regions reported previously. The only works which supports our findings are those of Braithwaite *et al.* (1984; 1986) who found that human cellular DNA showed homology to the *E4* region of Ad 2 and Ad 5 DNA. However, these and other studies (Arrand *et al.*, 1983; Jones *et al.*, 1979) consistently report homology between both human cellular DNA and RNA and the *E1a* region of subgenus C adenoviruses. The reason for these differing results is unknown as is the role of these sequences within human cells.

The variation in ALCD mobility in unstimulated cultured lymphocyte DNAs is an unusual phenomenon. When the lymphocytes were uncultured or cultured with PHA the characteristic 7.5 kbp band is evident but additional larger species become evident and prominent when the lymphocytes were cultured without PHA. A possible explanation for this phenomenon is that when the lymphocytes are cultured in the absence of PHA, the B-lymphocytes, which comprise the majority of the tonsil lymphocyte population (Gelfand, 1976), down-regulate the genes associated with the ALCD sequences by DNA methylation. Methylation, or more precisely undermethylation, is associated with control of gene activity (Naveh-Many and Cedar, 1981); active gene sequences have been shown, in general, to be undermethylated. In explaining this with regard to the ALCD phenomenon, methylation renders those sites in the genomic DNA resistant to Eco RI cleavage resulting in a concomitant increase in the fragment size. Since all cells in the lymphocyte population are not affected equally by the conditions of culture, some 7.5 kbp fragment

could still be detected in a hypomethylated (*ie.* Eco RI-sensitive) state.

As well, PHA stimulation of the lymphocyte population would target the stimulation of the T-lymphocytes in the cell mixture thus increasing the numbers of this cell type. These cells could act to increase or maintain the level of the activity of the numerically superior B cell population which may result, at the molecular level, in the activation of particular genes which would then be present in an undermethylated state. This status of B-lymphocyte activation would effectively maintain that level which was present in the unstimulated, uncultured lymphocyte population. This is a possible explanation as to why the pattern of ALCD hybridization is the same between these two populations. In the case of culture without PHA stimulation, the maintenance of this 'activated' state breaks down (possibly due to the lack of some cellular factor - lymphokines?) and the genes associated with ALCD become methylated and are resistant to Eco RI. Thus, according to this theory, one would expect to see bands of higher molecular weight which hybridize to the adenovirus DNA probes.

The ALCD probe was seen to hybridize to two separate Eco RI fragments of Ad 2 DNA; whether this represents two similarly sized ALCD species, each of which is homologous to a different region of the adenovirus genome, or whether it represents a single ALCD sequence which has regions homologous to both discrete regions of the adenovirus genome is as yet unknown.

An unexpected finding with regard to the ALCD is that fibroblast DNAs which were positive for adenovirus sequences never showed evidence of any ALCD signal, though ALCD sequences were readily detected in adenovirus-negative fibroblasts(Figure 11). While there is no experimental evidence for or against the following hypothesis, one possible explanation for this phenomenon is that a 'mopping up' of Ad DNA probe by the excess of homologous viral DNA

present in the fibroblast specimens takes place and that there is little, if any, left to hybridize with the ALCD. As well, the shorter duration of autoradiographic exposure for Ad DNA-positive specimens may have a role in the absence of detectable ALCD; however, ALCD was not seen in the adenovirus DNA-negative day 37 specimen of T257 (Figure 9) even after 7 days of autoradiography.

There is a notable difference between the ALCD detected by the Ad 2 and Ad 3 genomic probes. The level of ALCD detected with an adenovirus type 2 probe is far more prominent than that for the Ad 3 DNA probe as seen by comparing Figures 8 and 12. One possible explanation for this difference could be the homology differences between the probe DNAs. As was illustrated in Table 1, the level of homology between subgenus B and subgenus C is of the order of 9 to 20 % and this could be the reason why Ad 2 probes lead to stronger ALCD sequences; *ie.* ALCD sequences show a higher degree of homology with Ad 2 DNA than Ad 3 DNA. Along these same lines, there is also the possibility that there may be more than one species of ALCD, albeit of similar size, which vary in copy number. The Ad 3 homologous-ALCD would, according to this hypothesis, be present in smaller numbers than the Ad 2 homologous-ALCD judging from the differing levels of hybridization. As yet these possibilities have not been examined.

A possible explanation for the detection of ALCD is the possible contamination of the Ad 2 and Ad 3 DNAs with human DNA. This would likely to have occured as a consequence of the propagation and purification of virus for adenovirus DNA extraction. While this possibility cannot be ruled out entirely, the hybridization results presented in this study would tend to work against this hypothesis. Firstly, the sources of the adenovirus DNAs were different - the Ad 2 DNA was commercially prepared and, therefore, subject to quality control testing prior to sale while the Ad 3 DNA was isolated in this laboratory by two consecutive cesium chloride

isopycnic centrifugation runs. Neither Ad DNA preparation demonstrate any visible cellular DNA contamination by ethidium bromide staining after restriction endonuclease digestion (Figures 4 and 6). This ,however, only indicates that the level of human DNA which would be contaminating these preparations is below the DNA detection limit of ethidium bromide; this limit is approximately 5 ng of DNA (K. Fischer, unpublished observation). As well, if human DNA were contaminating the Ad DNAs used in hybridization and thus be responsible for the ALCD detected in tonsil, HEp-2 and Jurkat DNAs, one would expect an abberant hybridization signal(s) when the isolated ALCD was used to probe the three different restriction digests of Ad 2 DNA(Figure 17). There was, however, no abberant hybridization signal detectable with the ALCD probe; the hybridization signals detected corresponded with the Ad 2 DNA fragments visualized by ethidium bromide.

In order to examine the possibility of human DNA contamination of the Ad DNAs used in the hybridization portion of this study, one would need to use cloned Ad DNA representing the entire Ad genome; since any contamination would be by bacterial DNA which has little homolgy to human DNA, one would expect detection of ALCD only if DNA homology existed between the adenoviral DNA and the human DNA. Failure to detect ALCD, even with a cloned probe representing the entire Ad genome, would give support to the hypothesis that ALCD is due to Ad DNA contaminated with human DNA.

The study has shown that while Southern blot hybridization is a useful technique in the detection and identification of the adenovirus DNA sequences within latently-infected cultured tonsillar tissues it is not sensitive enough to detect the virus in the original uncultured tissue. This technique, while being sensitive enough for the detection of one adenoviral genome per every ten cells, is not sufficiently sensitive to detect latent virus which exist in a vanishingly small proportion of the total tonsil cell population.

One method which could be applied to the study of latent adenovirus infection of tonsils is *in situ* hybridization. While this technique would not allow the identification of the resident adenovirus nor allow determination of the state of the viral genome (*ie.* integrated or free linear) it would be useful in evaluating the relative distribution of tonsillar cells containing adenovirus sequences. The number of cells which harbour infectious adenovirus could be enumerated using an infectious centre assay (Strohl and Schlesinger, 1965a) and this value could be compared to the average number of cells which contain adenovirus DNA as determined by *in situ* hybridization. If both techniques yielded the same values this would seem to indicate that, based on previous infectious centre assay data (Strohl and Schlesinger, 1965b), there are very few cells present within a tonsil that harbour adenovirus and that these cells are productively infected. The finding that the number of cells harbouring Ad DNA exceed that able to produce infectious virus would indicate that the majority of cells are either latently or abortively infected. For the sake of illustration abortive infection will mean infectious adenovirus cannot be produced under any conditions, while latent infection, if we borrow from the example of herpesvirus latency, would be indicative of cells harbouring adenovirus DNA which have the potential for producing infectious virus if the proper conditions or stimuli are provided.

In order to distinguish between latent and abortive adenovirus infection, one would be required to perform both infectious centre assay and *in situ* hybridization on tonsil cells which had been cultured for varying lengths of time. If the ratio of virus-producing cells to Ad DNA-containing cells remained unchanged with time this would indicate that the majority of cells are abortively infected. On the other hand, if the ratio of adenovirus-producing cells to Ad DNA-contaning cells increases with time this would be consistent with latent infection; as time increases cells will have been

'reactivated' from the latent state to produce infectious virus. This hypothesis, however, assumes that the level of virus DNA present in latently-infected cells increases almost exponentially with virion production while in abortive infection the adenoviral DNA present would increase as a doubling trend since the Ad DNA is only replicated when the cells harboring them divide. The outcome of such experiments would further enhance our understanding of adenovirus latency. It is interesting to note that when Horvath et al. (1986) used *in situ* hybridization to examine human PBLs for the presence of adenoviral DNA, 1-2% of cells examined were shown to harbor subgenus C adenovirus sequences. This level of detection would seem to indicate that while infectious adenovirus may be only present in a vanishingly small proportion of cells, as shown by Strohl and Schleisinger(1965b), there may be a very significant proportion of cells which harbor adenovirus DNA. As to why the Southern blot procedure was not able to detect these sequences in this study, holding that this 1-2% level of adenovirus-containing cells may be an general phenomenon of lymphoid tissues, remains to be answered.

While this study did not show Southern blot hybridization to be the quintessential method for studying adenovirus latency at the molecular level, it did illustrate its strengths and limitations. This technique was able to detect adenovirus DNA in some cultured tissues where no infectious adenovirus could be detected and, at the same time, identify the adenovirus type infecting those tissues. As well, it showed that all detected adenovirus DNA sequences were present within tonsillar cells as the linear genome form. This study showed that while this technique allowed the detection of a small number of viral genome equivalents the sensitivity attainable was still not sufficient for the detection of adenovirus DNA in that small number of cells which must exist in the uncultured naturally-infected tissue.

VI. SUMMARY

i) DNA hybridization is as sensitive as culture in detection of latent adenoviruses in tonsillar fibroblast and lymphocyte cultures but not sufficiently sensitive to detect adenovirus DNA in uncultured whole tonsil tissues.

ii) The combined explant culture and hybridization results indicate that, *in vivo*, the number of virus-infected cells is very small in tonsils latently infected with adenovirus.

iii) Some tonsils contain a type-specific inactivator ,possibly neutralizing antibody, which may 'mask' the presence of infectious adenovirus and which may be responsible for what is seen as adenovirus latency.

iv) Tonsil-derived fibroblasts harbor latent adenovirus more often than tonsillar lymphocytes.

v) The adenovirus DNAs detected in fibroblast and lymphocyte DNAs are present as intact linear genomes.

vi) Adenoviruses isolated from latently-infected tonsils belong almost excusively to subgenus C.

vii) Present within human cells are DNA sequences which show homology to two discrete regions of the adenovirus type 2 genome.

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APPENDIX A

CUMULATIVE RESULTS OF ADENOVIRUS ISOLATION AND DETECTION OF ADENOVIRUS DNA IN TONSILS

Tonsil	Detection of infectious adenovirus			Detection of adenovirus DNA[1]		
	Direct	Fibroblast	Lymphocyte (-/+/co-c)[2]	Uncultured	Fibroblast	Lymphocyte (u/-/+)[3]
T250	-	-	NT/NT/NT[4]	-	NT	NT/NT/NT
T251	-	-	NT/NT/NT	-	NT	NT/NT/NT
T252	-	-	NT/NT/NT	-	NT	NT/NT/NT
T254	-	-	NT/NT/NT	-	NT	NT/NT/NT
T257	-	+	NT/NT/NT	-	+	NT/NT/NT
T258	-	NT	NT/NT/NT	-	NT	NT/NT/NT
T260	-	-	NT/NT/NT	-	NT	NT/NT/NT
T262	-	-	NT/NT/NT	-	NT	NT/NT/NT
T264	-	-	NT/NT/NT	-	NT	NT/NT/NT
T265	-	-	NT/NT/NT	-	NT	NT/NT/NT
T267	-	+	NT/NT/NT	-	NT	-/NT/NT
T269	-	-	-/NT/NT	-	NT	-/NT/NT
T271	-	-	-/NT/NT	-	NT	NT/NT/NT
T273	-	-	NT/NT/NT	-	NT	NT/NT/NT

APPENDIX A

Tonsil	Detection of infectious adenovirus			Detection of adenovirus DNA[1]		
	Direct	Fibroblast	Lymphocyte (-/+/co-c)[2]	Uncultured	Fibroblast	Lymphocyte (u/-/+)
T274	-	-	-/NT/-	-	NT	-/NT/NT
T275	-	-	-/-/NT	-	-	NT/-/-
T276	-	-	NT/NT/NT	-	-	NT/NT/NT
T277	-	+	-/-/NT	-	+	-/-/-
T279	-	-	-/-/NT	-	-	NT/-/-
T280	+	+	-/-/NT	-	+	NT/-/-
T282	-	+	-/-/NT	-	+	-/-/-
T284	-	NT	-/-/-	-	NT	-/NT/NT
T286	-	-	-/-/-	-	-	NT/NT/NT
T289	-	-	-/-/+	-	-	NT/-/-
T291	+	+	-/-/-	-	+	NT/-/-
T294	-	-	-/-/-	-	-	NT/NT/NT
T297	-	-	-/-/NT	-	NT	-/NT/NT
T298	-	+	-/-/-	-	+	+/NT/+

APPENDIX A

Tonsil	Detection of infectious adenovirus			Detection of adenovirus DNA[1]		
	Direct	Fibroblast	Lymphocyte (-/+/co-c)[2]	Uncultured	Fibroblast	Lymphocyte (u/-/+)
T300	-	-	-/-/-	-	NT	-/NT/NT
T302	+	+	-/-/-	-	+	-/NT/NT
T303	-	+	-/-/-	-	+	-/-/-
T305	-	+	-/-/-	-	+	-/-/-

1. Detected by hybridization with a labelled Ad 2 or Ad 3 DNA probe.
2. Cultured without PHA(-);with PHA(+);co-cultured with HEp-2 cells(co-c).
3. Uncultured(u);cultured without PHA(-);cultured with PHA(+).
4. Not tested.

APPENDIX B - ADENOVIRUS ISOLATION VERSUS CPE APPEARANCE IN FIBROBLAST CULTURES DERIVED FROM LATENTLY-INFECTED TONSIL TISSUE.

Tonsil	Fibroblast culture (days in culture)	CPE in primary culture	Adenovirus isolated
T257	1 flask (15)	-	-
	1 flask (22)	-	-
	pool-4 flasks (29)	- *	-
	2 flasks (32)	-/-	-/-
	2 flasks (35)	-/-	-/-
	1 flask (44)	-	-
	1 flask (50)	-	-
	pool-4 flasks (51)	-	-
	pool-3 flasks (56)	-	-
	1 flask (130)	+	+
	pool-2 flasks (130)	+	+
T267	pool-4 flasks (10)	-	+
T277	pool-7 flasks (7)	-	-
	1 flask (24)	+	- **
	1 flask (36)	+	+
	1 flask (56)	+	+
	pool-4 flasks (65)	-	+
	1 flask (65)	+	+
T282	1 flask (25)	+	+
	pool-7 flasks (25)	+	+
	pool-2 flasks (29)	+	+
	pool-2 flasks (35)	+	+
	pool-3 flasks (44)	+	+
T289	pool-2 flasks (54)	-	-
T298	1 flask (23)	+	+
	pool-2 flasks (23)	-	-
	pool-2 flasks (40)	-	-
	pool-3 flasks (43)	-	-
T303	pool-6 flasks (8)	-	-
	pool-3 flasks (16)	-	-
	pool-3 flasks (32)	-	-
	1 flask (39)	-	+
	pool-2 flasks (46)	-	+
	1 flask (46)	+	+
	pool- 2 flasks (53)	-	+
T305	pool-5 flasks (8)	-	-
	pool-3 flasks (19)	+	+
	pool-3 flasks (32)	-	-
	pool-2 flasks (46)	-	+
	1 flask (53)	-	+
	1 flask (74)	-	-

* - No evidence of CPE in any of the flasks from which the supernatant was taken.
** - A herpesvirus was isolated.

APPENDIX C - TISSUE CULTURE AND BACTERIOLOGICAL MEDIA

Tissue culture medium

Minimal essential medium (MEM) - Growth medium (10% serum)

450 ml	MEM (Earle's salts supplemented with 2 mM L-glutamine, Gibco)
7 ml	7.5% sodium bicarbonate (Gibco)
25 ml	Fetal calf serum (FCS) (heat-inactivated, Gibco)
25 ml	Nu-serum (Collaborative Research, Inc.)

Minimal essential medium (MEM) - Maintenance medium (2% serum)

As above except that serum concentration was reduced to 2% by addition of only 2.5 ml of each serum (FCS and Nu-serum).

Explant growth medium - M199 + 15% FCS serum

85 ml	M199 (Earle's salts, supplemented with 2 mM L-glutamine)
15 ml	Fetal calf serum (heat-inactivated, Flow)
1.2 ml	7.5% sodium bicarbonate
1 ml	penicillin-streptomycin (final concentrations- 400 U/ml and 400 µg/ml, respectively)
1 ml	fungizone (final concentration - 4 µg/ml)

Explant growth medium - M199 + 10% FCS serum

As above except that the serum concentration was reduced to 10% and the penicillin-streptomycin concentrations were reduced to 100 U/ml and 100 µg/ml, respectively.

Trypsin solution - (0.25%)

90 ml	Modified Hanks' balanced salt solution (HBSS, calcium- and magnesium-free, Gibco)
10 ml	Trypsin (2.5%, Gibco)

APPENDIX C

Trypsin solution - (0.25%) (continued)

2 ml	HEPES buffer (final concentration - 25 mM, Gibco)
0.8 ml	Sodium hydroxide (1 M)

Hanks' balanced salt solution (HBSS)

98 ml	Hanks' balanced salt solution (Gibco)
2 ml	HEPES buffer (final concentration - 25 mM, Gibco)
1 ml	penicillin-streptomycin (final concentrations- 400 U/ml and 400 µg/ml, respectively)
1 ml	fungizone (4 µg/ml - final concentration)

Bacteriological medium

LB broth

10 g	Bacto-tryptone (Difco)
5 g	Yeast extract (Difco)
10 g	NaCl (reagent grade, Fisher Scientific Co.)

Water was added to a final volume of 1 litre and the solution was sterilized by autoclaving. Ampicillin was added to a final concentration of 50 µg/ml.

LB agar

As above except agar (15 g/l, Bacto-agar, Difco) was added prior to autoclaving. After sterilization the medium was cooled to 45°C, ampicillin was added to a final concentration of 50 µg/ml and the medium was poured into disposable petri dishes.

APPENDIX D - CHEMICAL REAGENTS AND ENZYMES

Salt and Buffer Solutions

Tris-HCl (1 M, pH 8.0)

121.14 g Tris(hydroxymethyl)aminomethane (Boehringer Mannheim reagent grade)

800 ml distilled deionized water

After dissolving the Tris in water, the pH was adjusted to pH 8.0 with 12 N HCl (Fisher Scientific Co., reagent grade). The solution was brought to a volume of one litre with distilled deionized water, autoclaved and stored at 4 °C.

EDTA (0.2 M, pH 8.0)

74.44 g Ethylenediaminetetraacetic acid, disodium salt (Sigma Chemical Co.,reagent grade)

800 ml distilled deionized water

After dissolving the EDTA in water, the pH ws adjusted to pH 8.0 with 12 N HCl (Fisher Scientific Co., reagent grade). The solution was then brought to a volume of one litre with distilled deionized water, autoclaved and stored at 4 °C.

Magnesium chloride (0.6 M)

12.6 g $MgCl_2 \cdot 6 H_2O$ (Fisher Scientific Co., reagent grade)

Distilled deionized water to bring volume to 100 ml. The solution was autoclave and stored 4 °C.

Tris-HCl (10 mM, pH 7.4)

10 ml Tris-HCl (1 M, pH 8.0)

800 ml distilled deionized water

The pH was adjusted to 7.4 with 1 N HCl. The solution was brought to a volume of 1 litre with distilled deionized water, autoclaved and stored at 4 °C.

APPENDIX D

TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8.0)

1.0 ml Tris-HCl (1 M, pH 8.0)

0.5 ml EDTA, disodium (0.2 M, pH 8.0)

98.5 ml distilled deionized water

Autoclaved and stored at 4 °C.

TSE buffer (Tris-HCl, Saline, EDTA, pH 8.0)

11.69 g NaCl (Fisher Scientific Co., reagent grade) (200 mM)

20 ml Tris-HCl (1 M, pH 8.0) (20 mM)

0.74 g EDTA, disodium (Sigma Chemical Co., reagent grade) (2 mM)

800 ml distilled deionized water

Solid components were dissolved in the water and the Tris-HCl. The solution was adjusted to pH 8.0 with 1 N HCl, brought to a volume of 1 litre, autoclaved and stored at room temperature.

DNAse buffer

20 ml Tris-HCl (1 M, pH 8.0) (40 mM)

16.7 ml MgCl2 (0.6 M) (20n mM)

and distilled deionized water to bring to 500 ml. The solution was autoclaved and stored at 4 °C.

Enzymes

Protease (20 mg/ml)

1 g Protease (Sigma Chemical Co., Type VI)

50 ml Tris-HCl (10 mM, pH 7.4)

The solution was predigesed for 2 hours at 37 °C, dispensed in 1 ml volumes, and stored at -70 °C.

APPENDIX D

Enzymes (Continued)

Pronase (20 mg/ml)

1 g Pronase (Calbiochem, Grade B)

50 ml distilled deionized water

The solution was predigested for two hours at 37 °C, dispensed in 1 ml volumes, and stored at -20 °C.

Proteinase K (20 mg/ml)

0.1 g Proteinase K (Boehringer Mannheim)

5 ml Tris-HCl (10 mM, pH 7.4)

The solution was dispensed in 50 ul volumes and stored at -70 °C.

DNAse I (1 mg/ml)

		Final concentration
5 mg	DNAse I (Boehringer Mannheim)	1 mg/ml
0.15 ml	NaCl (5 M)	0.15 M
2.5 ml	Glycerol (Fisher Scientific Co., reagent grade)	50 %
2.35 ml	distilled deionized water	

The solution was dispensed in 100 ul volumes and stored at -70 °C.

RNAse A (20 mg/ml)

1.0 g RNAse A (Sigma Chemical Co., Type 1A)

50 ml distilled deionized water

The solution was boiled for 10 minutes, then rapidly cooled on ice. The solution was dispensed in 100 ul aliquots and stored at -20 °C.

APPENDIX D

Electrophoresis Solutions

TBE buffer (89 mM Tris-89 mM Borate-2.5 mM EDTA)

43.2 g Tris(hydroxymethyl)aminomethane (Boehringer Mannheim ,reagent grade)

22.0 g Boric acid (Fisher Scientific Co., reagent grade)

3.72 g Ethylenediaminetetraacetic acid, disodium (Sigma Chemical Co., reagent grade)

Add distilled deionized water to 4 litres final volume. Final pH of the solution was 8.0-8.4. The solution was stored at room temperature.

Tracking buffer

70 mg Bromphenol blue (George T. Gurr Ltd., reagent grade) (final concentration - 0.007%)

700 mg Sodium dodecyl sulfate (BioRad Laboratories, electrophoresis grade) (final concentration - 0.7%)

33 ml Glycerol (Fisher Scientific Co.,reagent grade) (final concentration - 33%)

67 ml distilled deionized water

The solution was stored at room temperature.

Ethidium bromide (10 ug/ml)

1.0 mg Ethidium bromide (Sigma Chemical Co., reagent grade)

100 ml distilled deionized water

The solution was stored at room temperature in a dark glass bottle.

APPENDIX D

Southern blotting solutions

Denaturation solution

150 ml 5M NaCl (final concentration - 1.5 M)

50 ml 5M NaOH (final concentration - 0.5 M)

300 ml distilled deionized water

Stored at room temperature.

Neutralization solution (1.5 M NaCl - 1 M Tris-HCl)

43.8 g NaCl (final concentration)

1 M Tris-HCl (pH 8.0) added to a final volume of 500 ml.

Nick translation reagents

The reagents were purchased as a kit (Nick translation kit N5000, Amersham Co.) and comprised solutions A and B.

Solution A

100 uM dATP
100 uM dGTP
100 uM dTTP
in a solution of Tris-HCl pH 7.8, MgCl2, and 2-mercaptoethanol. The solution was stored at -70 °C.

APPENDIX D

Solution B

500 units /ml DNA polymerase I
10 ng/ml DNAse I
in a solution of Tris-HCL pH 7.5, MgCl2, glycerol and bovine serum albumin. The solution was stored at -70 °C.

Stop buffer

5 ml Disodium EDTA (0.2 M, pH 8.0) (final concentration - 20 mM)

10 ml Sonicated herring sperm DNA (10 mg/ml) (final concentration - 2 mg/ml)

1 ml Sodium dodecyl sulfate (10%) (final concentration - 0.2%)

Distilled deionized water was added to a final volume of 50 ml. The solution was dispensed in 1 ml aliquots and stored at -70 °C.

Hybridization solutions

10 X SSC (Saline sodium citrate)

87.66 g NaCl (Fischer Scietific Co., reagent grade) (final concentration - 1.5 M)

44.12 g Sodium citrate (Fisher Scientific Co., reagent grade) (final concentration - 0.15 M)

The components were dissolved in 800 ml distilled deionized water. The pH was adjusted to pH 7.0 with 1 N HCl. Distilled deionized water was added to a final volume of 1 litre. The solution was dispensed, autoclaved and stored at 4 °C.

APPENDIX D

Denhardt's solution (50 X concentrate)

5 g Ficoll (Pharmacia Fine Chemicals Co., reagent grade) (final concentration - 1%)

5 g Polyvinylpyrrolidone (Sigma Chemical Co., reagent grade, MW 360,000) (final concentration - 1%)

5 g Bovine serum albumin (Sigma Chemical Co., fraction V) (final concentration - 1%)

Distilled water was added to a final volume of 500 ml. The solution was dispensed in 50 ml volumes and stored at -20 °C.

Prehybridization fluid		Final concentration
300 ml	10 X SSC	6 X
25 ml	10% sodium dodecyl sulfate solution	0.5% (v/v)
50 ml	50X Denhardt's solution	5 X
125 ml	distilled deionized water	

The solution was heated to 68 °C and filtered through a Millipore 0.45 um filter immediately before use.

Hybridization Solution		Final concentration
300 ml	10 X SSC	6 X
25 ml	EDTA, disodium (0.2 M, pH 8)	10 mM
50 ml	50X Denhardt's solution	5 X
25 ml	10% sodium dodecyl sulfate solution	0.5% (v/v)
50 g	Dextran sulfate (Pharmacia Fine Chemicals Co.)	10%

Distilled deionized water was added to a final volume of 500 ml. The solution was heated to 68 °C and filtered through a Millipore 0.45 um filter immediately before use.

APPENDIX D

Wash solution A (2X SSC - 0.1% SDS)

400 ml 10 X SSC

20 ml 10% SDS solution

1580 ml distilled deionized water

The solution was stored at room temperature.

Wash solution B (0.2X SSC - 0.1% SDS)

40 ml 10X SSC

20 ml 10% SDS solution

1940 ml distilled deionized water

The solution was stored at room temperature.

Miscellaneous reagents

10% Sodium dodecyl sulfate solution (10% SDS)

10 g Sodium dodecyl sulfate (electrophoresis grade, Bio-Rad Laboratories)

Add distilled deionized water to 100 ml final volume. Stored at room temperature.

TSE-saturated phenol ('phenol')

Phenol (Fisher Scientific Co., reagent grade) was distilled and stored at -70°C. The distilled phenol was melted at 68°C for 10-15 minutes and 8-hydroxyquinline (reagent grade, Sigma Chemical Co.) was added to a final concentration of 0.1%. An equal volume of 1 M Tris-HCl pH 8 was added and the phases were mixed by shaking. After phase separation was complete, the aqueous layer was removed. This was repeated thrice more with TSE buffer. After the final saturation step, all but 10 ml of TSE buffer was removed. Saturated phenol solutions were stored at 4°C until use.

APPENDIX D

Scintillation cocktail

42	ml	Liquiflor (New England Nuclear)
1	ml	Glacial acetic acid (reagent grade, Fisher Scientific Co.)
960	ml	Toluene (reagent grade, Fisher Scientific Co.)

Stored at room temperature in a dark glass bottle.

Lysis buffer containing lysozyme

			Final concentration
2.5	ml	Tris-HCl (1 M, pH 8.0)	25 mM
2.0	ml	EDTA, disodium (0.5 M, pH 8.0)	10 mM
10.0	ml	Glucose (500 mM)	50 mM
85.5	ml	Distilled deionized water	

Lysozyme (Sigma Chemical Co.) was added to a final concentration of 2 mg/ml immediately before use and the solution was used the day of preparation.