PROPHYLAXIS AGAINST ADENOVIRUS PNEUMONIA: A LABORATORY INVESTIGATION.

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Declaration

I declare that this thesis represents my own work and that the opinions contained therein are my own and not necessarily those of the Technikon. This manuscript has not been submitted for examination at any other institution.

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Summary

Adenovirus type 7 (Ad7) is the sub-species of adenovirus most frequently associated with serious illness. In Cape Town, the strain predominantly responsible for infantile pneumonia due to adenovirus is adenovirus type 7c (Ad7c), and it has been the cause of several outbreaks of nosocomial infection in the respiratory unit at the Red Cross War Memorial Hospital for Children..

This study explored two strategies which could possibly assist in the prevention of the spread of adenovirus infection in the hospital environment.

The guanosine analog, 9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG) is a highly effective antiviral agent against herpesviruses. It has also been reported that this drug could have therapeutic potential with regard to adenovirus infections. Based on this postulate, it was decided to perform *in vitro* studies to evaluate the antiviral activity of DHPG against Ad7c.

DHPG was tested for it's inhibitory potential against the replication of Ad7c in cell culture. The virus was propagated in the presence of drug concentrations ranging from 2 μM to 150 μM and the manifestation of cytopathic effect was monitored. Ad7c replication in cell culture was not inhibited by the drug at those concentrations that were tested, although growth of herpes simplex virus, used as a control, was significantly impaired.

The value of passive immunization in the prevention or amelioration of disease has been well recognized. Commercial preparations of immunoglobulin are not routinely monitored for the presence of antibodies to Ad7c. In this study, 17 batches of intramuscular and intravenous globulin were tested for the presence of neutralizing antibodies specific for Ad7c. The batches, which were randomly selected, were all found to contain high levels of neutralizing antibodies to Ad7c.

The conclusions reached were that DHPG will not be an efficacious antiviral agent for treatment of Ad7c infections, but that the implementation of passive immunization to children who are infected or exposed to Ad7c in a hospital ward, may be of help to control the spread of nosocomial infections.

Opsomming

Die sub-spesie adenovirus wat meestal met ernstige siekte geassosieer word is adenovirus tipe 7 (Ad7). In Kaapstad is adenovirus tipe 7c (Ad7c) hoofsaaklik verantwoordelik vir longontsteking by kinders en is ook die oorsaak van verskeie hospitaal-verwante infeksies in die respiratoriese eenheid van die Rooi Kruis-Oorlogsgedenk Hospitaal vir Kinders.

Twee strategiëe wat moontlike bystand kan verleen om die verspreiding van adenovirus infeksie in die hospitaal omgewing te voorkom, is tydens hierdie studie ondersoek.

Die guanosien analoog, 9-(1,3-dihidroksi-2-propoksimetiel) guanien (DHPG) is 'n doeltreffende antivirale middel teen herpesvirusse. Daar is ook melding gemaak dat hierdie middel moontlike terapeutiese potensiaal met betrekking tot adenovirusinfeksies mag hê. Daar is dus besluit om *in vitro* studies uit te voer om die antivirale aktiwiteit van DHPG teen Ad7c te evalueer.

Die inhibitoriese potensiaal van DHPG teen die replikasie van Ad7c is in selkultuur getoets. Die virus is gekweek in die teenwoordigheid van verskeie konsentrasies DHPG (2 μM tot 150 μM) en die verskyning van 'n sitopatiese effek is aangeteken. Geen inhibisie van Ad7c replikasie is egter opgemerk nie, maar die groei van herpes simplex virus, gebruik as 'n kontrole, is beduidend benadeel.

Erkenning is reeds verleen aan die waardevolle rol wat passiewe immunisasie speel met die voorkoming van verskeie siektes. Kommersiele preparate van immunoglobuliene word nie roetineweg vir die teenwoordigheid van antiliggame teen Ad7c ondersoek nie. In hierdie studie is 17 lotte intramuskulêre en intraveneuse immunoglobulien preparate vir die teenwoordigheid van spesifieke neutraliserende antiliggame teen Ad7c ondersoek. Dit is vasgestel dat hierdie lukraak-geselekteerde lotte hoë vlakke Ad7c neutraliserende antiliggame bevat het.

Daar is tot die gevolgtrekking gekom dat DHPG nie as 'n doeltreffende antivirale middel vir die behandeling van Ad7c infeksies beskou kan word nie. Die instelling van passiewe immunisasie by gehospitaliseerde kinders wat ly of blootgestel is aan Ad7c, sal dalk help om die verspreiding van hospitaal-verwante infeksies te beheer.

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1. CHAPTER 1

1.1. INTRODUCTION

Although viruses had long been recognised as causative agents for disease, the identification of many remained elusive until the introduction of cell culture in the 1940's. Shortly after this time, Rowe *et al.*(1953), described an agent which caused the spontaneous degeneration of human tissue cultures that were grown from adenoids and tonsils. A year later, Hilleman and Werner (1954) reported that a similar virus was responsible for an outbreak of non-influenza respiratory disease among military personnel.

It soon became apparent that there were several serotypes of this respiratory virus and that they could be cultured, not only from adenoidal tissue, but also from nasal secretions, nasopharyngeal aspirates and conjunctival scrapings (Huebner *et al.*, 1954). This led to the recognition of a group of viruses that caused disease of the upper respiratory tract and conjunctiva and which had the following characteristics in common: they grew well in tissue culture, particularly HeLa cells (in which they produced a typical cytopathic effect) but when inoculated into laboratory animals, they were non-pathogenic; they all contained a group-specific antigen which could be identified in the Complement Fixation Test; and type-specific antigens could be demonstrated in the Neutralization Test (Enders *et al.*, 1956).

By this stage, the name for these viruses had changed from the original "adenoid-degenerating agent" to "adenoidal-pharyngeal-conjunctival agents". There was obviously a need for a less cumbersome terminology. In 1956, all the workers who had contributed towards the discovery of this new group of viruses met in New York City and decided upon the name of Adenoviruses (Enders *et al.*, 1956). Over the years, many more pathogens have been identified as belonging to the adenovirus family, and at present 42 serotypes are known to infect humans alone.

1.1.1. Clinical Features

Human adenoviruses may induce a variety of clinical symptoms, of which upper and lower respiratory tract illnesses and conjunctivitis are the more frequent. Most children are exposed to the common adenoviruses early in life and about half of these infections may be asymptomatic (Brandt *et al.*, 1972). In the cases with overt illness, the symptoms are usually indistinguishable from those of any mild cold. However, infections may be severe, particularly when the pharynx and lower respiratory tract are involved. Studies of ill children who required medical attention, suggest that 2-7% of all lower respiratory tract infections could be attributed to adenoviruses (Brandt *et al.*, 1972; Foy *et al.*, 1973).

Pneumonia due to adenovirus is a particularly serious disease, which may often be fatal (Benyesh-Melnick and Rosenberg, 1964; Similia *et al.*, 1971). It has also been suggested that some cases of severe pneumonia which occur as a complication of measles may in fact be due to superinfection with adenovirus, and not due to measles virus alone (Warner and Marshall, 1976). Children in hospitals are susceptible to adenovirus superinfection, especially if they have underlying respiratory disease (Staube *et al.*, 1983). If they survive adenovirus pneumonia, patients may be left with severe and possibly permanent lung damage, requiring them to be hospitalized for many months, and in extreme cases, for years. Adenovirus type 7 is the serotype most commonly associated with this illness (Similia *et al.*, 1971).

Epidemics of acute respiratory disease (ARD) due to adenovirus have been known to occur among military recruits (Hilleman and Werner, 1954; Dudding *et al.*, 1973). ARD is characterized by sore throat, fever and malaise and although not usually serious, the disease is responsible for high morbidity rates. The serotypes associated with these outbreaks have mostly been types 4 and 7 (Dudding *et al.*, 1973). Vaccine programmes have been implemented in the US military, in an endeavour to combat ARD (Top *et al.*, 1971).

Adenoviruses are particularly stable and may survive in swimming pools and waste water. They have, therefore, been the cause of many cases of pharyngoconjunctival fever (PCF), the symptoms of which are pharyngitis, conjunctivitis and a spiking fever. Epidemics of this disease, acquired mainly from swimming pools which have been inadequately chlorinated, have been well documented (D'Angelo *et al.*, 1979; Foy *et al.*, 1968).

Keratoconjunctivitis is a disease which is characterized by follicular conjunctivitis, oedema of the eyelids, pain, photophobia and lacrimation. Superficial erosions of the cornea may develop, and in some cases, deeper subepithelial corneal infiltrates can occur, causing permanent visual impairment. Epidemics of this illness have occurred, most often with adenovirus type 8, although types 3 and 19 have also been implicated. The source of infection has usually been attributed to contaminated ophthalmic instruments (Sprague *et al.*, 1973; D'Angelo *et al.*, 1981). Due to the hardiness of adenoviruses, sterilization of medical instruments with alcohol or ether is inadequate, and heat sterilization is required to inactivate the virus. Epidemic keratoconjunctivitis, also known as shipyard conjunctivitis, is caused by adenovirus-contaminated dust in industrial settings.

Enteric adenoviruses may cause gastroenteritis, mainly in children. Yolken *et al.* (1982), reported that most children who had adenovirus diarrhoea, presented with respiratory symptoms as well. These enteric adenoviruses, which belong to serotypes 40 and 41, are extremely fastidious and cannot be easily grown in conventional tissue culture systems. In this regard they are distinguishable from other adenoviruses.

Acute haemorrhagic cystitis (AHC) is a rare syndrome which is usually associated with adenovirus type 11 infection (Mufson *et al.*, 1973). Although AHC is a self limited disease, the diagnosis of AHC is important in order to differentiate it from serious renal disorders.

Illnesses most commonly associated with adenovirus infections are summarized as follows:

Disease	Principal serotype
Pharyngoconjunctival fever	3, 7, 14
Acute respiratory disease	3, 4, 7, 14, 21
Pneumonia	1, 2, 3, 4, 7
Epidemic keratoconjunctivitis	8, 11, 19
Gastroenteritis	40, 41
Acute haemorrhagic cystitis	11

Adenoviruses may persist in the host for many months after initial infection. They have the potential to form latent infections in lymphocytes (Abken *et al.*, 1987), and adenovirus nucleic acid has been demonstrated in lymphoid tissue in the absence of infectious virus (Neumann *et al.*, 1987). It has also been reported that an established T-cell line could be infected with adenovirus type 2 (Lavery *et al.*,1987). Some viruses, for example, Epstein Barr virus, can transform lymphocytes. When cells are thus immortalized, they have properties which could be precursors for malignancy. Because of the role that lymphocytes play in the maintenance of latent adenovirus infection, the association between human cancers and this virus group has been thoroughly investigated. In animal models, some serotypes have been highly oncogenic (Trentin *et al.*, 1962; Horwitz, 1990), particularly types 12, 18 and 31.

Although many cancers from both the respiratory system and digestive system have been examined for the presence of adenovirus DNA transforming sequences, none have been found (Green *et al.*, 1979a; Wold *et al.*, 1979).

Adenoviruses are species specific, therefore spread of the human viruses can only be from man to man. This may occur via droplet infections to the respiratory tract, or to the gastrointestinal tract by the faecal oral route. The ability of these viruses to survive on surfaces has contributed to their being responsible for many nosocomial infections (Staube *et al.*, 1983). Therefore, great care should be taken by health care workers to ensure that they maintain high standards of hygiene in order to prevent the spread of infection in the hospital environment. In addition, children with adenovirus pneumonia should be isolated as soon as possible, so that the infection may be contained.

1.1.2. Classification of human adenoviruses

Family: Adenoviridae

Genus: Mastadenovirus

Subgenera:(groups): A, B, C, D, E, F, G.

Species (serotypes): Human adenovirus (1-42).

The human adenoviruses belong to the genus Mastadenovirus in which there are 42 serotypes. These serotypes, or species, as they are now called, were classified into four subgroups, initially according to their haemagglutination pattern with rat or rhesus monkey red blood cells (Rosen, 1960). Haemagglutination patterns, which included species other than rat or rhesus monkeys, allowed for further classification to incorporate new isolates (Hierholtzer, 1973).

Molecular characterization of viral DNA from the human serotypes has shown that they can be divided into seven subgroups (Green *et al.*, 1979b). These groups have been designated A through to G (A-G). The degree of homology between the viral genomes belonging to the same group is about 90%, except for Group A, where the homology is only 60%. The different groups show a DNA homology of less than 25% (Garon *et al.*, 1973). It has recently been shown that there is a variation in the DNA pattern between certain serotypes although they are serologically indistinguishable (Wadell *et al.*, 1981). These strains with slightly different DNA electrophoretic patterns have been designated subtypes, e.g. adenovirus type 7c.

1.1.3. Structure

Adenoviruses (Figure 1) are non-enveloped virus particles with icosahedral symmetry and a diameter of 75-80 nm. The inner core of the virion contains double stranded DNA. The surrounding capsid consists of two hundred and fifty-two capsomers comprising two hundred and forty hexons, arranged so that each is surrounded by six other capsomers, and twelve pentons, each surrounded by five capsomers. The pentons are situated at the vertices of the capsid and a filamentous-like projection, known as the penton fibre, is attached to each penton base (Nermut, 1987). The penton fibre, which may vary in length according to the serotype, is glycoprotein in nature. All the members within each genus are related via a common group-specific antigen, which is situated on the inner surface of the hexon (Norrby, 1966). Each serotype also carries two type-specific antigens, which are located in the outermost parts of the hexons and fibres. These external antigens give rise to neutralizing and haemagglutinating antibodies (Rosen, 1960; Philipson *et al.*, 1975).

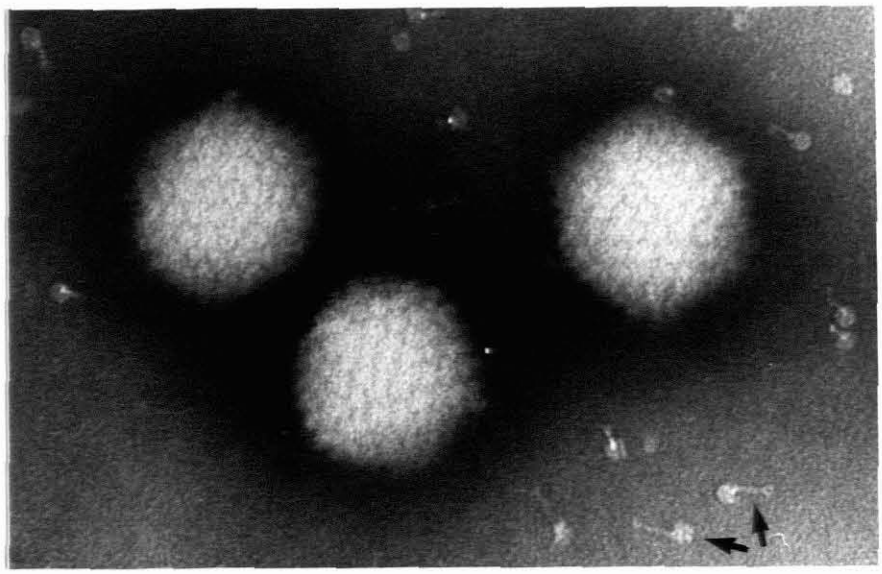

Fig. 1

Adenovirus particles viewed by electron microscopy after negative staining with 2% phosphotungstic acid, pH 6.0. Particles have icosahedral symmetry. Penton fibres, attached to penton bases, which are normally located at the apices, have become detached and are seen lying free in the background (arrows). Magnification x 300 000. {Micrograph supplied by Dr L M Stannard}.

1.1.4. Replication

Adenovirus infection is initiated by the attachment of virions via their penton fibres to receptors on the surface of susceptible cells. The mechanism by which the virus gains entry to the cell has not been clearly elucidated, although receptor-mediated endocytosis has been implicated (Pastan *et al.*, 1986). The endocytic vesicles, or receptosomes, have a low pH (about 5.5) which activates a process, as yet undefined, that enables the virus to disrupt the receptosome and be released into the cytoplasm (Pastan *et al.*,1986). By this stage the pentons and fibres have been shed (Morgan *et al.*, 1969; Philipson *et al.*, 1968) and the virion is transported to the nuclear membrane. The mechanism by which the virion moves through the cytoplasm is purported to involve the attachment of hexons to the cellular microtubules (Luftig and Weihing, 1975). DNA is released into the nucleus through the nuclear pores. Replication and transcription of viral DNA, and maturation of virions, takes place in the nucleoplasm. Six early transcription genes have been identified, which code for the non-structural proteins necessary for initiation of transcription and synthesis of viral DNA. This occurs about seven hours after infection. Five hours later, synthesis of structural proteins is initiated and these newly formed proteins are transported to the nucleus where they are assembled into mature virions. Approximately 10^5 virus particles may be found in each cell by 30 hours after infection. At this stage, cellular functions have ceased and the virions are released during lysis of the infected cell (Wadell, 1987).

1.1.5. Laboratory diagnosis of adenovirus

Adenoviruses replicate well in cells of human origin, and in most laboratories, continuous lines of epithelial cells, such as HEP2 and HeLa, are used to cultivate virus from patient specimens. It may take several days, and one serial passage, before cellular morphological change can be seen. Cytopathic effect is characterized by clustering and swelling of infected cells (Figure 2). When these cells are stained with haematoxylin and eosin, typical basophilic, intranuclear inclusions are demonstrable (Figure 3) (Appendix I). Adenoviruses increase glycolysis in continuous cell lines, which induces a colour change of the pH indicator used in the medium (Fisher and Ginsberg, 1957). This obvious change in pH of the medium has, in some laboratories, replaced microscopic examination (Horwitz, 1990).

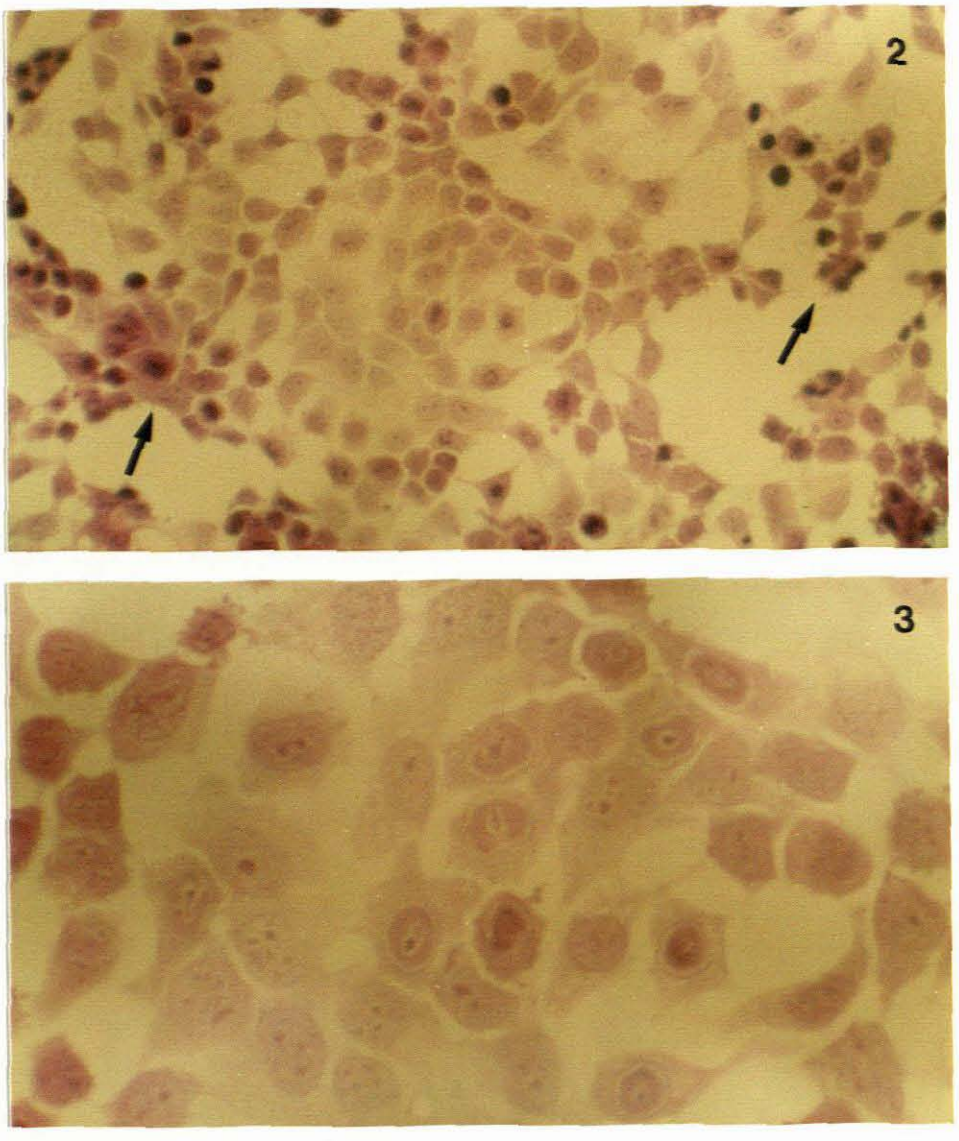

Fig. 2

HeLa cells infected with Ad7c, stained with haematoxylin and eosin. Foci of infected cells are visible as clusters of rounded and swollen cells (arrows).

Fig. 3

Higher magnification shows prominent intranuclear inclusions typical of adenovirus infection.

The introduction of the "shell vial" culture technique has accelerated the speed with which adenovirus isolates may be identified (Espy *et al.*, 1987). Utilizing this technique, the specimens are centrifuged directly onto cell monolayers. After incubation for 1 to 2 days, the cells are stained with fluoroscein-labelled monoclonal antibodies, which are directed against the group-specific antigen on the hexon.

Adenoviruses may sometimes grow in primary monkey kidney cells, but only if these cells are coinfected with the papovavirus, SV40 (Baum *et al.*, 1972).

The enteric adenoviruses are fastidious and do not replicate in conventional culture systems. Some strains have been cultured in 293 cells, a cell line that has been transformed by adenovirus type 5. It is postulated that early gene functions of adenovirus type 5 expressed in these cells may facilitate the replication of the enteric strains, although the exact mechanism has not been clarified (Takiff *et al.*, 1981). A latex agglutination test may provide a rapid diagnosis for diarrhoeal disease (Grandien *et al.*, 1987). In this procedure, latex particles coated with adenovirus antibodies, are used to detect viral antigen in stool samples.

It is possible to perform a rapid screening test for the presence of viral antigens in specimens taken from tracheal aspirates and conjunctival scrapings using an immunofluorescence (IF) technique. In this way, a positive diagnosis can be made in less than an hour, but only if very high concentrations of viral antigen are present in the specimen. Therefore, a negative result on a rapid immunofluorescence test does not exclude adenovirus infection. Gardner *et al.* (1972), showed that only 50% of proven cases of adenovirus were positive by immunofluorescence. The incidence of IF-confirmed adenovirus infection was higher for upper respiratory infections than for croup, bronchitis and pneumonia (Gardner *et al.*,1972). Rapid diagnosis of acute haemorrhagic cystitis has also been made by the IF detection of adenovirus antigen in exfoliated bladder epithelial cells (Belshe and Mufson, 1974).

1.1.6. Laboratory classification of adenoviruses.

Serological tests, using known antibodies, are used to characterize a viral isolate. Both complement-fixation and immunofluorescence will detect antibodies which bind to the group-specific antigen on the inner part of the hexon, which is common to all human adenoviruses (Norrby, 1966). To establish type-specificity, neutralization and haemagglutination-inhibition techniques are used. The species-specific antigens which are detected in these assays are situated on the penton fibres and the outer part of the hexon, and are particular to each serotype (Rosen, 1960; Philipson *et al.*, 1975).

Adenoviruses may also be classified according to their genome types, using restriction endonucleases (Wadell *et al.*, 1985). These enzymes cleave DNA at highly specific sites. The electrophoretic pattern of the resultant fragments will reveal a definitive profile for each group. This method is useful particularly for epidemiological studies, where genetic mutations need to be monitored.

1.1.7. The aim of this study

For several years, adenovirus type 7c has been the major cause of nosocomial infections in the respiratory unit at the Red Cross Children's Hospital. The children who have been affected most adversely are those with underlying respiratory disease. Following infection with adenovirus 7c several have died, while others have been left with permanent lung damage. Two patients have been hospitalized for longer than three years, and lung transplantation would be the only recourse to improve their condition. At present, they require permanent mechanical assistance with breathing (Dr. M. Klein, personal communication).

The laboratory identification of adenovirus pneumonia may take several days, by which time the virus may have spread to other patients. In order to control the nosocomial spread of this virus, the identification of effective antiviral compounds would be of great therapeutic value. Furthermore, the value of using prophylactic methods, such as passive immunization of susceptible individuals to prevent the spread of infection, needs to be evaluated.

This study explores the following:

The efficacy of the drug, ganciclovir (9-(1,3-dihydroxy-2- propoxymethyl)guanine) against adenovirus 7c. Previous reports have stated that this drug has an inhibitory effect on adenovirus replication both *in vitro* and *in vivo* (Wreghitt *et al.*, 1989; Taylor *et al.*, 1988). If this claim can be substantiated, the clinical implications will be significant, as the severity of adenovirus pneumonia may be abated.

Identification of laboratory techniques which will produce the most useful and reproducible results for drug evaluation in a cell culture system.

An assessment of accurate and rapid methods whereby specific adenovirus antibodies may be detected in human immunoglobulin preparations.

The feasibility of using normal human immunoglobulin to prevent or modify adenovirus infection.

The prevalence of adenovirus antibodies in commercial preparations of normal human immunoglobulin will be assessed to establish therapeutic potential.

2. Chapter 2

2.1. TYPING OF ADENOVIRUS BY DNA RESTRICTION ANALYSIS

2.1.1. Introduction

The differentiation of adenovirus subtypes is best achieved by the characterization of their DNA. This procedure is facilitated by the use of enzymes called restriction nucleases, which recognize specific sequences along the genome (4 to 6 nucleotides in length) and cleave both strands of the molecule at that site.

These restriction nucleases are derived from prokaryotic cells, where their function is to degrade foreign DNA, thereby protecting the organism from invasion. The bacterium which produces a particular restriction enzyme, will protect its own DNA by the methylation of cytosine and adenine residues within the relevant recognition sequence (Williams and Patient, 1988). The nomenclature for these enzymes incorporates letters from the name of the bacterial species from which they originate. For example, the enzyme *Eco*RI is derived from *Escherichia coli*, whereas *Hind*III is derived from *Haemophilus influenzae.*

A particular restriction nuclease will cleave a length of DNA (such as a viral genome) into a series of fragments known as restriction fragments. These fragments can be separated according to size, using gel electrophoresis. Nucleotide sequences of known length are included in each electrophoretic run, so that the size of the fragments in the test sample can be estimated. Ethidium bromide, which intercalates into nucleic acid, is incorporated in the gel, and thus the DNA bands can be visualized by ultra-violet light. By comparing the sizes of the DNA fragments obtained after treatment with a combination of restriction enzymes, a map can be constructed that shows the location of each cutting site in relation to its neighbours. Such maps, for two or more virus strains, will give an idea of the homology between them. Each serotype of adenovirus produces a distinctive digestion pattern, and identification can usually be achieved by using three enzymes; *Sma*I, *Eco*RI, and *Bam*HI.

Adenovirus type 7 has been shown to be the dominant serotype associated with serious disease, particularly respiratory illness. This virus has frequently been cultured in our laboratory from children with severe pneumonia. Enzyme restriction analysis has

identified the subtype 7c as the commonest isolate in Cape Town (Kannemeyer *et al.*, 1988).

Adenovirus 7c has been responsible for several episodes of nosocomial infection in the respiratory intensive care unit at the Red Cross Children's Hospital. As the aim of this study was to explore avenues by which nosocomial spread of infection with this virus may be controlled, a recent isolate from a child in that intensive care unit was considered an appropriate virus to use in our experiments. It was identified as type 7c by restriction enzyme analysis in the manner described in this chapter.

2.1.2. Materials

Virus (a): A prototype adenovirus type 7c previously characterized by restriction analysis using the enzymes *Eco*RI, *Sma*I and *Bam*HI.

Virus (b): An uncharacterized adenovirus (AdH), recently isolated from a patient with pneumonia in the intensive care unit at the Red Cross Children's Hospital.

1M Tris (pH 8.3)

Tris base	121.1 g
H_2O	100 ml

The pH was adjusted to 8.3 with concentrated HCl (approximately 42 ml), made up to 1000 ml with distilled water and autoclaved.

Hirt Buffer

10mM Tris (pH 8.0)

10mM EDTA

0.6 % sodium dodecyl sulphate (SDS)

Phenol

The phenol was melted at 56°C and 8-hydroxyquinoline was added to a concentration of 0.1%. The melted phenol was saturated several times with 1.0 M Tris (pH 8.3), and then with 0.1 M Tris (pH 8.3) until the pH of the aqueous phase was 8.3. Beta-mercaptoethanol (BME) was added at a final concentration of 0.2%. This was stored at 4°C.

Chloroform/Isoamyl alcohol (24:1 v/v)

This was stored at room temperature.

RNAse

Pancreatic RNAse (Boehringer Mannheim) was dissolved to a concentration of 10 μg/ml in 10 mM Tris.HCl (pH 7.5) containing 15 mM NaCl. The solution was heated to 100^{o}C and allowed to cool slowly. This was stored at -20^{o}C.

Tris-acetate EDTA buffer pH 8.0.(TAE)

Concentrated stock solution: (x50)

Tris base	242 g
Glacial acetic acid	57,1 ml
0.5 M EDTA	100 ml

Made up to 1000 ml with distilled water.

Working solution: A 1:50 dilution of the stock solution contained 0.04 M Tris-acetate and 0.001 M EDTA.

Stop/loading buffer (x10)

To TAE working solution buffer containing 50% glycerol, SDS was added to give a concentration of 1%, and two dyes, bromophenol-blue and xylene/cyanole were added at a concentration of 0.1%. For use, the solution was diluted 1:10.

Ethidium bromide (10mg/ml) - Stock Solution

Ethidium bromide	1 g
H_2O	100 ml

The solution was stirred for several hours to ensure that the dye had dissolved. The bottle was covered with aluminium foil to exclude light and stored at 4^{o}C.

Working Solution: The stock solution was diluted 1:100 in distilled H_2O to give a concentration of 100 μg/ml. For use, the final concentration of ethidium bromide incorporated into the gel, or added to the buffer, was approximately 0.5 μg/ml.

2.1.3. Method

Extraction of viral DNA

The technique used to extract adenovirus DNA from infected cell cultures was a modification (Eizuru *et al.*, 1984) of the Hirt method (Hirt, 1967).

A 150 cm tissue culture flask, containing a subconfluent monolayer of human embryonic fibroblasts (HEF), was inoculated with the adenovirus, and incubated at 37°C for several days, until most of the cells were infected. The medium was removed and the cells were gently washed with Phosphate Buffered Saline (PBS) (Appendix II). 4 ml of Hirt buffer was distributed evenly over the monolayer, which was then left at 37°C for about 15 minutes until the cells were lysed.

The lysate was transferred to a Greiner tube and NaCl was added to give a final concentration of 1 M. After overnight refrigeration, precipitated cellular DNA was removed by centrifugation at 10,000 rpm for one hour in a Sorvall centrifuge (HB4 rotor). Proteinase K (Boehringer Mannheim) was added to the supernatent fluid at a concentration of 100 μg/ml, and the mixture was incubated for 2 hours in a 56°C waterbath. Thereafter an equal volume of phenol (saturated with 0.1M Tris buffer, pH 8.3) containing 0.2% BME, was added to the tube, and mixed gently until an homogeneous mixture was formed. This was centrifuged at 10,000 rpm for 5 minutes to separate the phases.

The aqueous phase, containing the viral DNA was carefully transferred to a clean Greiner tube, and the phenol extraction was repeated. To remove residual phenol, a chloroform/isoamyl alcohol extraction was performed in the same manner.

The DNA was precipitated in the presence of 0.4 M lithium chloride and 2,5 volumes of cold ethanol. The mixture was left at -20°C for 18 hours.

The nucleic acid precipitate was sedimented by centrifugation at 10,000 rpm for one hour. To remove traces of RNA, the DNA pellet was carefully dissolved in 500 μl 1 M Tris buffer, pH 8.3, containing 5 μg of RNAse. The mixture was transferred to an Eppendorf tube, and incubated in a 56°C waterbath for 2 hours.

A second extraction with phenol and chloroform/isoamyl alcohol was performed to remove the RNAse. DNA was once more precipitated with lithium chloride and ethanol, then centrifuged to a pellet at 16,000 rpm for 10 minutes in an Eppendorf microcentrifuge. The final pellet was washed in 70% alcohol, whereafter it was dried in a dessicator and resuspended in 50 μl of deionized water. This was stored at 4^{o}C.

Digestion of DNA

Separate 10 μl aliquots of DNA were mixed with each of the three restriction enzymes (*Sma*I, *Bam*H1 and *Eco*RI) according to the manufacturer's instructions (Boehringer Mannheim). After incubation at 37^{o}C for one hour, the enzymatic activity was halted by the addition of 2 μl of stop/loading buffer. Lambda phage DNA ,which was used as a reference marker, had been cut with the enzymes *Hind*111 and *Eco*R1

Separation of restriction fragments

175 ml of 1% agarose in TAE buffer was melted and poured into a gel-casting mould. A Teflon comb was inserted at one end to provide channels for the samples. Once the gel had set, the comb was removed and the gel was submerged (in a horizontal position) in an electrophoresis tank containing TAE buffer. Samples were loaded into separate lanes, and electrophoresis was carried out at room temperature at 2 v/cm for 18 hours, or until the bromophenol blue had migrated to the bottom of the gel. Staining was achieved by immersion of the gel in TAE containing approximately 0.5 μg/ml ethidium bromide for 1 hour. The bands were visualised under ultra violet light and the patterns were photographed on Ilford FP4 film.

2.1.4. Results

Restriction patterns of adenovirus DNA were compared after digestion with the endonucleases *Bam*HI, *Eco*RI and *Sma*I. The Lambda phage DNA digest resulted in fragments of the following sizes: 21.2 kb, 5.1 kb, 4.9 kb, 4.3 kb, 2.0 kb, 1.9 kb, 1.6 kb, 1.4 kb and 947 bp, 831 bp, 564 bp and 124 bp. (Figures 4 and 5, lane 1).

*Bam*HI

This enzyme recognizes the nucleotide sequence GGATCC and digestion of adenovirus DNA resulted in 5 large fragments of approximately 9.6 kb, 8.4 kb, 6.3 kb, 4.6 kb, and 3.5 kb, plus smaller fragments <2kb. The presence of a 6.3 kb fragment is distinctive for Ad7c. The initial digestion with *Bam*HI did not yield bands of clear resolution (Figure 4, lane 3), and the experiment was therefore repeated (Figure 5).

*Eco*RI

The recognition sequence for *Eco*RI is GAATTC and cleavage of adenovirus DNA with this enzyme yielded two fragments of 32 kb and 4.8 kb respectively (Figure 4, lane 5) Ad7 subtypes which are cut at this site are Ad7a1-5, Ad7b, Ad7c, Ad7d, Ad7d1 and Ad7g.

*Sma*I

This enzyme recognises the sequence CCCGGG, and yielded 7 main fragments of the following sizes: 7.7 kb, 6.9 kb, 5.1 kb, 4.7 kb, 3.7 kb, 2.6 kb, and 2.5 kb, (Figure 4, lane 7). The fragment 7.7 kb is particular to Ad7c as well as Ad7a, Ad7a1-5 and Ad7e.

The uncharacterized adenovirus DNA gave identical patterns to the reference strain of Ad7c (Figure 4 lanes 2, 4 and 6; Figure 5, lane 2), confirming that our unknown isolate was indeed an Ad7c genome subtype.

Fig. 4

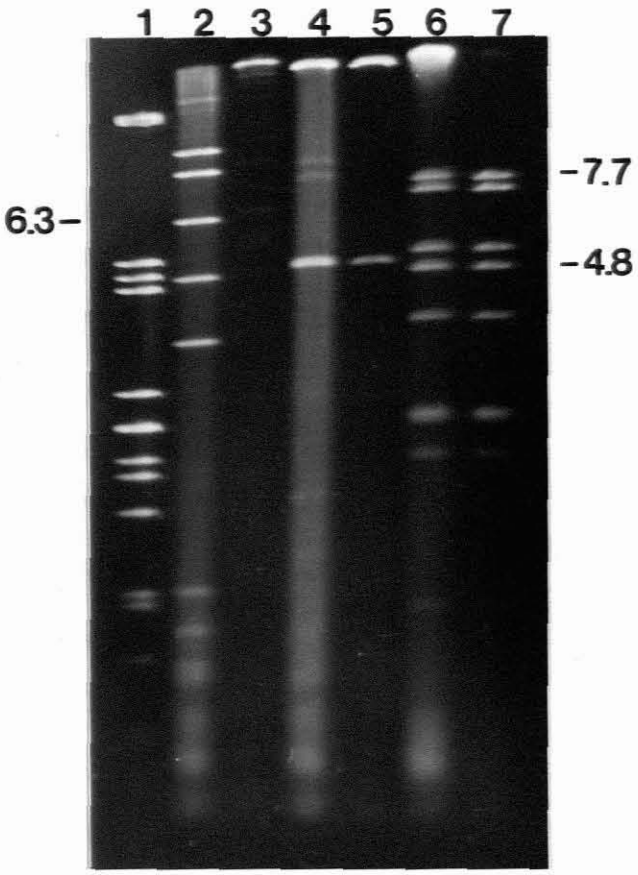

Fig. 5

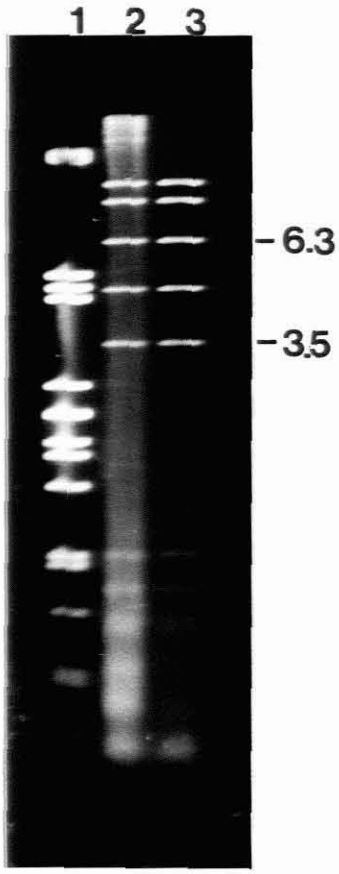

Fig. 4

DNA fragments from a known Ad7c (lanes 2, 4, 6) were run for comparison with adenovirus (AdH) (lanes 3, 5, 7) after digestion with restriction enzymes *Bam*HI (lanes 2 and 3); *Eco*RI (lanes 4 and 5); and *SmaI* (lanes 6 and 7). Lambda phage DNA, used as a size marker, was run in lane 1.

Fig.5

Restriction digestion with *Bam*H1 was repeated, and gave identical restriction patterns for the known Ad7c (lane 2) and AdH (lane 3).

2.1.5.Discussion

It is possible to classify adenoviruses in several ways, using different criteria. The subgenera have been formulated on the basis of (1) haemagglutination patterns ; (2) the oncogenic potential of these viruses in newborn hamsters; and (3) the ability of specific antisera to neutralize the replication of the virus. However, critical classification of viruses is based on the differences, or similarities, in their genomic structure. The application of the restriction enzyme analysis has allowed epidemiologists to map the various adenovirus genome types around the world and thereby establish their prevalence in the different regions (Wadell *et al.*, 1985; Adrian *et al*, 1989). Of the 42 serotypes, Ad7 has most frequently been associated with serious respiratory disease. Since Berge *et al.*, first isolated the prototype Ad7 in 1955, 6 additional genome types (Ad7a, 7b, 7c, 7d, 7e, 7f and 7g) have been identified (Wadell *et al.*, 1981, Wadell and Varsanyi, 1978; Wadell *et al.*, 1985; Li and Wadell, 1986). A number of variants within these subtypes has increased the total to at least 17 (Li and Wadell, 1986; Kannemeyer *et al.*, 1988).

The dominant genome type is not the same for different countries in the world. In South Africa, retrospective analysis of adenovirus isolates from Johannesburg showed that early strains (circa 1967) were Ad7b, whilst later isolates were Ad7c (Wadell *et al.*, 1985). Isolates from Cape Town have all been identified as genome type Ad7c (Kannemeyer *et al.*, 1988). In global terms, the prevalence of Ad7c in South Africa appears to be unique, as the genome type Ad7b is dominant in most other parts of the world, including the United States of America, Europe and Australia (Wadell *et al.*, 1985). So far, Ad7d and Ad7g have been found only in China, and the genome type Ad7e in South America and Australia. In Cape Town, two new variants of Ad7c have been isolated (Kannemeyer *et al.*, 1988). These differ from the prototype by exhibiting either one (Ad7c2) or two (Ad7c1) extra cleavage sites for *Eco*RI.

The genome of the Ad7c virus used for the present study showed no variation from the original reference strain when analysed with the three restriction endonucleases *Eco*RI, *Bam*HI and *Sma*I. It is apparent that the 7c subtype is still the major cause of adenovirus pneumonia in this part of the country, and laboratory investigations aimed at prevention or control of the disease have therefore been centred around this particular strain.

3. Chapter 3

3.1. CELL CULTURE

3.1.1. Introduction

To gain an accurate *in vitro* assessment of the extent to which certain agents, either drugs (Chapter 5) or antibodies (Chapter 6), can inhibit viral replication, it is first necessary to standardize optimal criteria for cell culture and viral replication.

Adenoviruses replicate readily in HeLa cells, where cytopathic effect (c.p.e.) is characterized by the formation of clusters of large, refractile cells which are very distinctive and can be easily distinguished. However, these rapidly dividing transformed cells are of limited use for studies that extend for a long period of time. After a few days, uninfected HeLa cells tend to round up spontaneously and the monolayer may become disrupted. These changes in cell morphology are difficult to distinguish from viral specific c.p.e.

Human embryonic fibroblasts (HEF) can be maintained for extended periods in culture. Adenoviruses do not grow as rapidly in this cell type as in HeLa cells, but once the infection is established, the characteristic c.p.e. of rounded up clusters of cells is easily discernible. For the drug study, it was important to be able to monitor the effect of the antiviral agent on viral replication from early until late stages of viral infection. It was therefore necessary to have a cell culture system that could be maintained for the duration of the study. For this reason, it was decided HEF cell cultures would be better suited to those experiments.

Primary requirements were to select a strain of adenovirus 7c which produced a clear c.p.e. in HEF, and to assess whether virus titres could be determined accurately and reproducibly. For this assessment, virus titrations performed in HEF were compared with those in HeLa cells.

3.1.2. Materials

Nutrient medium

Eagle minimum essential medium (MEM) was obtained in concentrated powder form from Highveld Biological [PTY] LTD.

MEM Powder	9.7 g
$NaHCO_3$	2.0 g
Distilled water	1000 ml

Filtered through a 0.2 micron filter and stored at 4°C.

Foetal calf serum (FCS)

Obtained from the State Vaccine Institute, Pinelands.

Antibiotics

Stock solutions of penicillin, streptomycin and neomycin (PSN) were prepared as follows:

Penicillin	1122.6 mg
Streptomycin	666.0 mg
Neomycin	2000.0 mg
Physiological saline (0.14 M NaCl)	100 ml

The solution was filtered through a 0.2 micron filter, dispensed in 0.5 ml amounts, and stored at -20°C.

5% Sodium Bicarbonate

$NaHCO_3$	100 g

This was dissolved in approximately 1500 ml distilled water.

When dissolved, 2 ml of 0,4% phenol red was added, and the volume was made up to 2 litres. The pH was adjusted by bubbling CO_2 through the solution until it became pale pink. The solution was sterilized using a 0.2 micron filter, dispensed into Bijou bottles and stored at 4°C.

Growth medium (10% FCS)

Prepared immediately before use.

MEM nutrient medium	85.5 ml
Foetal calf serum	10 ml
$NaHCO_3$	4 ml
PSN	0.5 ml

Maintenance medium (4% FCS)

Prepared immediately before use.

MEM nutrient medium	93.5 ml
Foetal calf serum	4 ml
$NaHCO_3$	2 ml
PSN	0.5 ml

4% Phenol Red

4 g of phenol red powder was dissolved in 60 ml of 0.05 N NaOH, using gentle heat to dissolve. The volume was made up to 100 ml with distilled water.

Trypsin Base (10x concentrated)

NaCl	80.0 g
KCl	2.0 g
KH_2PO_4	1.2 g
Na_2PO_4 (anhydrous)	9.1 g
Glucose	5.0 g
Distilled water	700 ml
Phenol red (0.4%)	25ml

2 g Ethylenediaminetetra-acetic acid (EDTA), also known as versene, was incorporated by first dissolving it in 100 ml of distilled water, and then adding it to the above solution while stirring.

The pH was adjusted to 7.8 using 1 N NaOH, and the volume was made up to 1000 ml with distilled water.

The solution was sterile filtered and dispensed in 20 ml amounts.

Stored at -20°C.

For use: Diluted 1:10 with distilled water.

5% Trypsin Stock Solution

Trypsin powder (Difco)(1:250)	5.0 g
Trypsin base	100 ml

Trypsin powder was sprinkled onto the trypsin base and allowed to dissolve slowly, without agitation.

Filtered and stored at -20°C.

Trypsin/EDTA (working solution)

The trypsin stock solution was diluted 1:100 in trypsin base, and the pH was adjusted to 7.8

Trypan Blue

Trypan blue powder	5.0 g
Normal saline	100 ml

This solution was filtered through Grade 1 filter paper and dispensed in 5 ml amounts.

Trypan blue will stain dead cells.

3.1.3. Methods

Preparation of Human Embryonic Fibroblasts

A single cell line of human embryonic fibroblasts (HEF) was used throughout this study. These cells, line no. $6/_{81}$, were obtained from the lung tissue of a twelve week old foetus from a pregnancy which was terminated at Groote Schuur Hospital. The lung tissue was washed thoroughly with sterile phosphate buffered saline to remove excess blood, then cut very finely into minute pieces using scalpel blades. Extreme care was taken to employ aseptic techniques throughout this procedure. Approximately 100 ml of MEM growth medium was added to the foetal tissue, which was then dispensed in 20 ml amounts into 75cm plastic cell culture flasks (Falcon). The flasks were left undisturbed for one week in a 37°C incubator, by which time confluent monolayers of cells were generated from the fragments.

Preparation of Stock HEF Cultures.

A culture flask containing a confluent monolayer of HEF was washed with phosphate buffered saline, then covered with a thin film of trypsin/EDTA solution. After incubation at 37°C for approximately five minutes, the cells were dislodged from the plastic flask by gentle agitation. The enzyme reaction of the trypsin was neutralized by the addition of 10 ml of growth medium which contained 10% FCS. A sample of this cell suspension was diluted 1:1 with trypan blue and viable cells were counted using a Neubauer counting chamber. 75cm plastic flasks were seeded with 25 ml of MEM growth medium containing $3x10^5$ viable cells per ml and incubated at 37°C in a CO_2 incubator. An additional amount of 2 ml of 5% $NaHCO_3$ solution was added per 100 ml of growth medium to counteract the acidyifying effect of the $C0_2$. Cells were grown to confluency and used as stock from which all subsequent cultures were made.

Subculturing of Stock HEF Cultures

Confluent monolayers were trypsinized as previously described, and using the same seeding rate of $3x10^5$ cells/ml, were dispensed into either 96 well polystyrene cell culture plates, test tubes containing coverslips, or 75cm culture flasks. A volume of 0.1 ml of cell suspension per well was used for the plates; 25 ml and 1 ml of cell suspension were dispensed into flasks and test-tubes respectively. These were incubated at 37°C in an atmosphere of 5% CO_2. When the cell sheets were 80% confluent, they were considered ready for use.

Preparation of HeLa Cells

An ampoule of HeLa cells was removed from storage in liquid nitrogen and allowed to thaw rapidly in a 37°C waterbath. A cell count was performed, using trypan blue to check the viability of the cells, whereafter they were seeded into tissue culture flasks and grown to confluency.

Subcultures were prepared in either flasks, test-tubes or 96 well cell culture plates using the same procedure as for HEF with the exception that the seeding rate was $1x10^5$ cells/ml.

Monolayers were considered ready for use when the cell sheets were 60% confluent.

Stock virus

Adenovirus 7c

Several laboratory isolates of adenovirus were grown from the tracheal aspirates of children in the intensive care unit at Red Cross Children's Hospital. These were compared for their ability to replicate in HEF. One strain of adenovirus designated H, was chosen, based on the criterion that it produced a clear and distinct c.p.e. in HEF. This virus was typed as adenovirus 7c using DNA restriction analysis (Chapter 2).

The original isolate of adenovirus H was removed from storage at -20°C and passaged three times in test tube cultures of HeLa cells. By the third passage, the virus produced an advanced c.p.e. within 18 hours. Four ml of virus suspension from the HeLa cultures were added to a flask containing a confluent monolayer of HEF, and allowed to adsorb for 1 hour at 37°C. Fresh growth medium was added, and incubation was continued until the cells showed an advanced stage of c.p.e. The contents were transferred to a

100 ml Schott bottle and subjected to three cycles of freezing and thawing. Finally, the harvest was aliquotted in 2 ml amounts in Nunc tubes and stored at -20°C.

Titration of adenovirus

An aliquot of stock virus was removed from -20°C and thawed rapidly. Ten-fold dilutions, starting at 10^{-1} through to 10^{-8} were made using MEM maintenance medium as diluent. A volume of 0.1 ml of each dilution was inoculated into 4 wells of a 96 well microtitre plate containing confluent HEF or HeLa cells. An additional volume of 0.2 ml of maintainance medium was added to each well. Four uninoculated wells were used as cell controls.

The plate was checked daily for c.p.e. for 6 days and the virus titre was calculated utilising the method of Reed and Muench (1938) to give a 50% tissue culture infective dose ($TCID_{50}$) endpoint (Appendix III).

Herpes simplex virus

A laboratory isolate from a trachael aspirate was used to inoculate a cell culture flask containing a confluent monolayer of HEF. The virus was allowed to adsorb for one hour at 37°C, whereafter fresh MEM maintenance medium was added and the flask was returned to the incubator. When advanced c.p.e. was visible, the contents of the flask were transferred to a 100 ml Schott bottle, and subjected to three cycles of freezing and thawing. This stock virus was aliquotted into 2 ml amounts and stored at -20°C.

Titration of Stock Herpes simplex virus

An aliquot of virus was removed from storage and thawed rapidly. Dilutions were made from 10^{-1} through to 10^{-8}, using maintenance medium as diluent. A volume of 0.1 ml of each dilution was inoculated into 4 wells of a 96 well cell culture plate containing confluent monolayers of HEF or HeLa cells. 0.20 ml of maintainence medium was added to each well. The plates were incubated at 37°C in a CO_2 incubator and monitored daily for c.p.e. over a period of 6 days. The $TCID_{50}$ was calculated according to the method of Reed and Muench (1938).

3.1.4. Results and Discussion

Adenovirus titration:

A virus strain of adenovirus 7c was selected on the criterion that it produced a good c.p.e. in HEF. This same virus strain was used for the assessment of antiviral activity of ganciclovir (Chapter 5), and in the neutralization assays for the detection of specific antibodies in the immunoglobulin preparations (Chapter 6).

Examination of the growth characteristics of the virus in two different cell lines, viz HEF and HeLa cells, allowed us to determine optimal culture conditions for virus growth and to establish confidence in the assay system to be used in these investigations.

In HeLa cells, the virus produced a rapid c.p.e. and titration endpoints were reached after three days. Monitoring of the cells was continued for 6 days, but the titre of the virus remained unchanged. However, because of the fast-growing characteristic of HeLa cells, confluent monolayers tended to become dislodged from the solid plastic surface by the end of the six day period. For this reason, the viral titres in HeLa cells were calculated after three days.

In HEF, adenovirus c.p.e. did not appear as rapidly as in HeLa cells. After three days, it was found that the titre in the HEF was approximately two logs lower than that in the HeLa cells. By six days, however, the titre in HEF was comparable to the endpoint obtained in HeLa cells after three days. When the HEF cultures were monitored for longer than six days, isolated foci of adenovirus c.p.e. could be discerned at higher dilutions, but the progression of the c.p.e. was slow and erratic, making it difficult to determine when the endpoint had been reached. This phenomenon has been described previously for adenovirus titrations (Horwitz, 1990). For consistency, it was therefore decided to use a kinetic end-point rather than an absolute endpoint for adenovirus titrations and the titre of the virus calculated after six days.

4. Chapter 4

4.1. MYCOPLASMA INVESTIGATIONS

4.1.1. Introduction

Mycoplasmas are a group of highly pleomorphic micro-organisms, ranging in size from <100 to >800 nm. The reason for their pleomorphism is that, unlike most bacteria, they lack a rigid cell wall, having instead a triple-layered membrane. They are resistant to antibiotics that specifically inhibit cell wall synthesis, such as penicillin, but are sensitive to tetracycline, which interrupts bacterial replication at ribosomal level (Razin, 1981). The mycoplasma genome is a circular double stranded DNA molecule.

The interest in these organisms from a virological aspect is due to the well established fact that mycoplasmas are common contaminants of cultured cell lines. As some strains do not have any overt effect on cell morphology, their presence may often remain undetected. Other mycoplasmas, however, may produce severe cytopathic effect, causing complete disruption of the cell sheet (Barile, 1981).

Many mycoplasma species attach to cell membranes, which allows them to interact closely with the affected cell and thus impede normal cellular functions. The biochemical properties of a mycoplasma species will often determine the effect which that organism will have on tissue culture systems; for instance, some mycoplasmas hydrolyze arginine, thus depleting the cell culture of an essential amino acid. As a consequence of this deprivation, cell metabolism and morphology may be profoundly affected. Cytolytic mycoplasmas release large amounts of acid metabolites as a result of glycolysis, thus causing the destruction of the cell sheet (Barile, 1981).

Although some mycoplasma species have no detectable effect on virus growth, others may increase or decrease virus yields. Arginine-hydrolyzing mycoplasmas will diminish viral growth but this effect can be reversed by the substitution of arginine in the nutrient medium. Mycoplasmas have also been known to reduce interferon levels in infected cells. This activity will consequently lead to enhanced virus yields (Barile, 1981).

Unless care is taken to ensure that the cell cultures that are being used in a viral study are free from mycoplasma, particularly those strains that produce occult infections, their presence may unwittingly influence the results of an experiment.

In general, primary cell cultures are less frequently contaminated with mycoplasmas than continuous cell lines. HeLa cell lines, particularly, are prone to infection with mycoplasma. Contamination usually arises from exogenous sources, such as cell medium additives. These may include bovine serum and trypsin. Laboratory workers are also a source of contamination and one study showed that 33% of mycoplasmas that contaminate cell cultures were of the human oral species (Barile, 1981).

As this study involved the extensive use of cell cultures, it was important to gauge the mycoplasma infection rate, if any, in the cell lines used. Fluorochrome staining, for the detection of DNA, is commonly used as a primary indication of mycoplasma contamination of cell cultures. Fluorochrome stains, such as Hoechst 33258, bind specifically to DNA molecules. If mycoplasmas are attached to cell membranes, they will be visualized as discrete, fluorescing bodies (Stanbridge, 1981). Intracellular mycoplasmas can sometimes also be seen in the cytoplasm. Cellular DNA should cause the nucleus to stain brightly, thereby serving as a control for the efficacy of the staining procedure.

4.1.2. Materials

Bisbenzamide Fluorochrome Stain

Stock concentrate (100 ml)

Bisbenzamide fluorochrome stain (Hoechst No.33258)	5 mg
Phosphate buffered saline	100 ml

Mixed thoroughly with a magnetic stirrer for 30 minutes at 22°C.

As the stain is light-sensitive, the bottle was covered with aluminium foil to exclude light.

Stored in the dark at 4°C.

Working dilution of stain

1 ml of stock concentrate was added to 100 ml PBS (in a bottle wrapped in aluminium foil) and, before use, it was mixed thoroughly with a magnetic stirrer at 22°C for 30 minutes.

The solution was checked carefully for microbial contamination and discarded if any discoloration or precipitate was evident. As the bisbenzamide fluorochrome stain is particulate, millipore filtration will diminish fluorescence. Merthiolate was added to both the stock and working solution to give a final concentration of 1:10,000.

Mounting fluid

0.1 M citric acid	22.2 ml
0.2 M disodium phosphate	27.8 ml
Glycerol	50.0 ml

Final pH 5.5

Fixative

One part glacial acetic acid to three parts methanol.

4.1.3. Method

Cell cultures were grown on glass coverslips in test tubes, and left for 5 to 7 days before staining. After the medium was removed from the test-tubes, the coverslips were exposed to glacial-acetic acid fixative for five minutes. This was aspirated, and replaced with fresh fixative for a further 10 minutes. The coverslips were allowed to air dry, and were then stained with benzamide fluorochrome for 30 minutes at 22°C. After staining, the coverslips were washed three times with deionized water, mounted onto glass slides and examined under a fluorescence microscope, using an ultra violet filter of 360 nm. HeLa cells known to be infected with mycoplasma were used as a positive control.

4.1.4. Results

Neither intracytoplasmic nor cell surface fluorescence was observed in the HEF cells that were examined (Figure 6). The nuclei were prominently stained. In the positive control cells, fluorescence was seen in both the cytoplasm and at the cell membrane (Figure 7). Faint intracytoplasmic fluorescence was discernible in the HeLa cells that were used for the adenovirus experiments (Figure 8). The intensity of fluorescence observed in the nuclei was much greater than that in the cytoplasm. The cytoplasmic inclusions were granular in appearance. Microscopic examination did not reveal the presence of either bacteria or fungi.

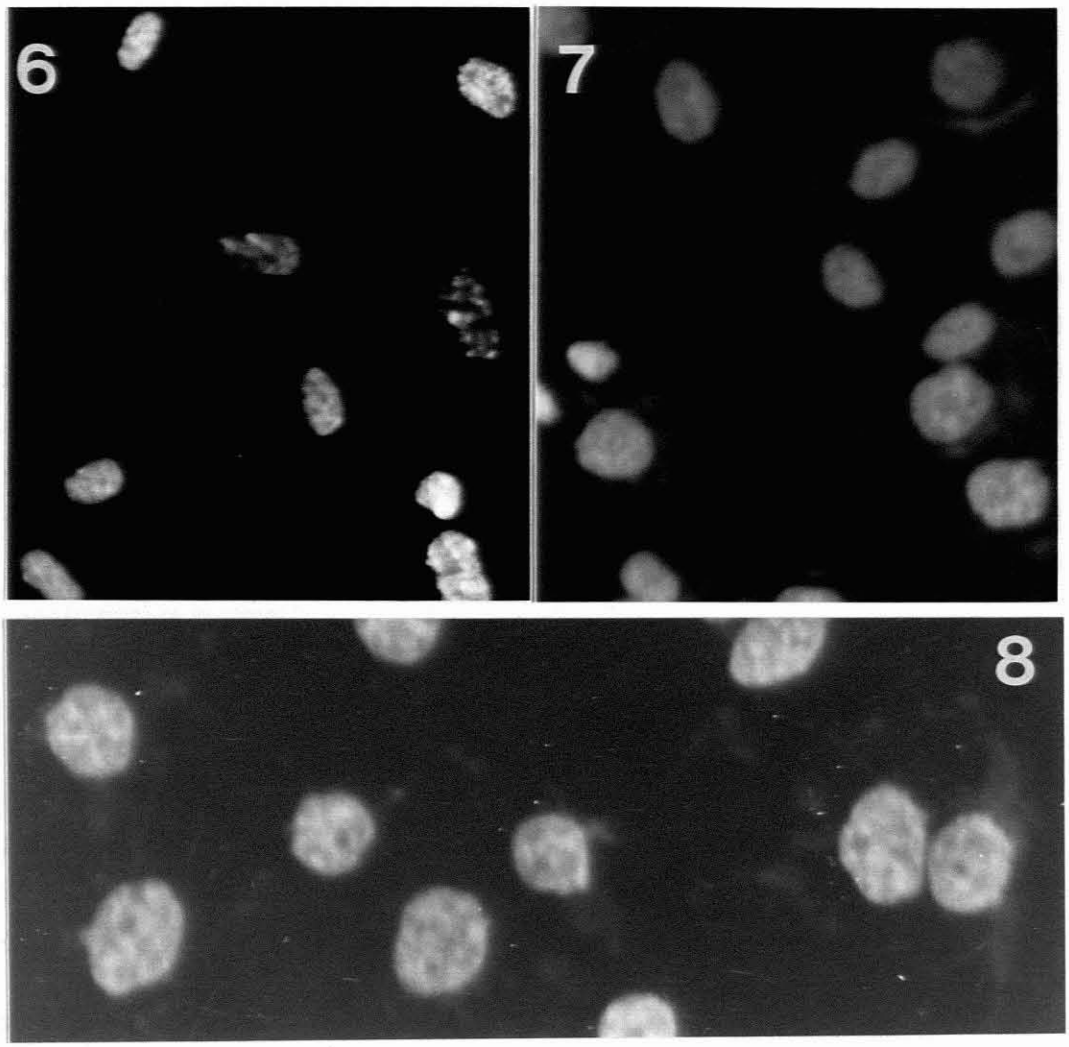

Fig. 6

Fluorescence staining for detection of DNA (Hoechst 33258) revealed that HEF cells were mycoplasma-free. Nuclei are prominently stained.

Fig. 7

HeLa cells, deliberately infected with mycoplasma showed fluorescence both in the cytoplasm and at the cell membrane.

Fig. 8

HeLa cells, stained with Hoechst 33258, showed faint intracytoplasmic fluorescence, indicating possible mycoplasma contamination.

4.1.5. Discussion

The HEF cells used in this study showed no microscopic evidence of contamination with mycoplasma. However, fluorochrome staining of the HeLa cells indicated the presence of DNA in the cytoplasm. The fluoresence observed in the cytoplasm could be indicative of bacterial, fungal or mycoplasma contamination. Confirmatory tests would have been necessary to establish an exact identification of the contaminant. However, we assumed that mycoplasma was the source of the infection, for the following reasons: Bacterial contamination in tissue cultures is usually overt; the nutrient medium becomes turbid and bacteria can easily be visualized under a light microscope. Moreover, broad spectrum antibiotics are added to each batch of medium to prevent bacterial infection. Likewise, fungal contaminants are also easily discerned, both macro- and microscopically, in infected tissue cultures. In view of these facts and the knowledge that most HeLa cell lines are infected with mycoplasmas, we decided not to continue with confirmatory testing.

The presumed mycoplasma contamination in the HeLa cell line was low grade, less than 15% of cells being affected. No disruption of the cell sheet occurred and adenovirus replication did not appear to be affected (see Chapter 3). In fact, when the virus was titrated in both HeLa cells and HEF, the virus yield was higher in the HeLa cells. The nutrient medium (MEM) that was used for the cell cultures incorporates arginine, which would have compensated for depletion of arginine by mycoplasma contaminants.

Since it was established that the replication of the virus used in this study (adenovirus type 7c), was not adversely affected by the mild infection with mycoplasma, it was decided that this HeLa cell line could be used for our experiments, without compromising the results.

5. Chapter 5

5.1. ASSESSMENT OF DHPG AS AN ANTIVIRAL AGENT FOR ADENOVIRUS 7C

5.1.1. Introduction

Many anti-viral compounds have proved to be highly toxic *in vivo*, often exacerbating an illness. For this reason, most anti-viral drugs are recommended only if the patient's life is threatened.

Viruses are themselves metabolically inert, but once they have invaded a susceptible cell they are able to utilise host cell metabolic processes to accomplish transcription of the viral genome and subsequent replication of progeny viruses. Early viral mRNA frequently codes for enzymes required for virus DNA synthesis. At this stage host cell DNA, RNA and protein synthesis may be halted and the biochemical machinery becomes redirected to produce components for new virus particles.

Ideally, an antiviral compound should interrupt viral replication without affecting normal host cell metabolism. This can usually only occur if the drug is administered in very low concentrations. At early stages of viral infection, such low concentrations may be therapeutic. However, when the infection in the host becomes advanced, higher concentrations of drug are needed to stop viral replication, which may result in host cell activity being deleteriously affected. The endeavour to find therapeutic drugs for the treatment of viral infections has been impeded considerably by this tenet.

Nevertheless, promising advances have been made, particularly with drugs directed against the herpes-virus group. These antiviral compounds are closely linked to the activities of virus-specific enzymes. Acyclovir, 9-(2-hydroxy ethoxymethyl guanine) a synthetic nucleoside analogue, has been used clinically with good effect for several years (Reviewed by Hirsch and Kaplan, 1990). This compound is phosphorylated by the thymidine kinase produced by herpes simplex virus (HSV), resulting in the formation of acyclovir triphosphate. The latter will inhibit the activity of herpes-specified DNA polymerase to a far greater extent than it will cellular polymerases. An additional action of acyclovir is that it may disrupt viral DNA synthesis by substituting for guanosine in the DNA chain (Derse *et al.*, 1981).

Other antiviral agents that are in use are vidarabine, amantadine and rimantadine.

Vidarabine (adenine arabinoside, ara A or 9-(β-d-arabinofuranosyl adenine) is also successfully used against the herpesviruses. It is phosphorylated by host cell kinases rather than viral thymidine kinase and is thus effective against thymidine kinase deficient mutants of HSV. Viral DNA synthesis is inhibited at very low concentrations of the drug, which leaves cellular DNA synthesis unaffected (Larder and Darby, 1986).

Amantadine and rimantadine are anti-viral compounds that have been used mainly against influenza A virus. Amantadine is a derivative of adamantane which is a 10-carbon cyclic compound; rimantadine is the alpha-methyl derivative of amantadine. These drugs also inhibit viral replication at concentrations low enough to have no effect on host cell mechanisms. Their inhibitory action is thought to occur at the early stages of infectivity (such as penetration or uncoating of the virus) although the exact mechanism has not, as yet, been elucidated (Hay *et al.*, 1986; Bukrinskaya *et al.*, 1982; Koff and Knight, 1979).

A recent addition to the above list is an acyclic nucleoside analogue of guanine; 9-(1,3-dihydroxy-2-propoxymethyl) guanine (DHPG). This drug has been given the generic name, ganciclovir. Both *in vitro* and *in vivo* studies have indicated that this agent is effective against all herpes viruses, particularly human cytomegalovirus (HCMV) (Field *et al.*, 1983); (Mar *et al.*, 1983). Intracellularly, the drug is phosphorylated to form ganciclovir-5'-triphosphate, (ganciclovir-TP) which blocks viral DNA polymerase activity. It has been shown that ganciclovir is a more efficient substrate for virus specific thymidine kinase than acyclovir (Field *et al.*, 1983) and 10 fold more ganciclovir-TP is formed than acyclovir-TP in HSV 1 infected cells. In spite of the fact that HCMV does not code for thymidine kinase, DHPG remains the most effective form of therapy for HCMV infections. It's mode of action is as yet unclear.

Reports have indicated that DHPG has some inhibitory effect on adenovirus replication *in vitro*. Taylor *et al.* (1988) described a reduction in virus titre of several strains of adenovirus in the presence of gancilovir. Wreghitt *et al.* (1989) reported that a strain of adenovirus 7, isolated from post mortem material from an immunosuppressed patient, was inhibited by ganciclovir.

In an endeavour to find a means of combating adenovirus 7c infections in the respiratory wards at Red Cross Hospital, it was decided to investigate the efficacy of DHPG against that virus with a view to it's potential use in adenovirus therapy.

This study examines the effect of DHPG on adenovirus 7c *in vitro* and compares it with that of equivalent amounts of the drug on herpes simplex virus growth. The adenovirus used was from a recent adeno 7c isolate from a patient in the intensive care unit at the Red Cross Children's Hospital. Herpes simplex virus, isolated from a trachael aspirate, was used as a control.

5.1.2. Materials and Methods

The effect of varying concentrations of DHPG on the growth of adenovirus 7 c

DHPG, manufactured by Syntex Pharmaceuticals, UK, and registered under the trade name of Cymevene, was obtained in lyophilized form. It was reconstituted in distilled water to give a concentration of 50 mg/ml. Appropriate dilutions were made, in MEM maintenance medium, to yield concentrations of 40 μM, 33,3 μM, 25 μM, 20 μM 15.3 μM, 10 μM, 4 μM, and 2 μM.

HEF monolayers were grown in 96 well cell culture plates as described in chapter 3. The medium was removed and replaced with 75 μl of maintenance medium containing DHPG at the above concentrations, using four wells per dilution. 25 μl of maintenance medium containing 100 $TCID_{50}$ of adenovirus 7c was then added to each well. To check for possible cell toxicity of the drug, the highest concentration of DHPG (40 μM) was added to four wells without the addition of virus. Both DHPG and virus were omitted from four wells used as cell controls. The plates were incubated at 37°C in an atmosphere of 5% CO_2, and monitored daily over a period of one week. The end-point was expressed as the highest dilution of DHPG that inhibited visible c.p.e. in 50% of the cultures inoculated (Reed and Muench, 1938).

The effect of varying concentrations of DHPG on herpes simplex virus

The same procedure was followed as for Ad7c, using instead 100 $TCID_{50}$ of herpes simplex virus.

Titration of adenovirus in the presence of DHPG

Ten-fold dilutions of stock virus were made in maintenance medium containing 40 μM DHPG. Duplicate dilutions were made in maintenance medium without DHPG. HEF in multi-well culture plates were inoculated with each set of dilutions, using four wells per dilution and 100 μl per well. These were incubated at 37°C in the presence of 5% CO_2, and monitored daily for c.p.e. over a period of one week.

5.1.3. Results

The effect of varying concentrations of DHPG on the growth of adenovirus 7c

Adenovirus cytopathic effect was observed in all the wells containing DHPG. There was no difference in the degree of c.p.e. between the lowest concentration (2 μM) of the drug and the highest concentration (40 μM). Table 1 illustrates the results of two separate experiments. Similar results were obtained in each case. Higher concentrations of DHPG were subsequently used (Table 2) but even in the presence of 150 μM of the drug, no inhibition of viral c.p.e. was discernible. Not unexpectedly, concentrations of DHPG lower than 2 μM did not affect the multiplication of adenovirus (Table 3).

No visible signs of drug toxicity were detected in the uninfected HEF exposed to 40 μM and 150 μM of DHPG.

The effect of varying concentrations of DHPG on the growth of Herpes simplex virus

Table 1 illustrates that the growth of herpes simplex virus was totally inhibited by DHPG, even at the lowest concentration used (2 μM). In order to establish the minimal inhibitory concentration of DHPG, the experiment was repeated using concentrations lower than 2 μM.(Table 3). It was found that concentrations of DHPG less than 0.2 μM were not completely inhibitory and no inhibition was detected at concentrations equal to or below 0.05 μM.

Adenovirus titration in the presence of 40 µM DHPG

No difference in titre was observed between the virus titrated in the presence of 40 μM DHPG and the control wells. The end-points obtained were comparable for both titrations, as was the degree of c.p.e. detected.

TABLE 1

EFFECTS OF DHPG ON VIRUS GROWTH

Concentration DHPG in μM	% Inhibition of viral c.p.e.		
	Ad 7c (a)*	Ad 7c (b)*	HSV-1 #
40	0	0	100
33	0	0	100
25	0	0	100
20	0	0	100
15	0	0	100
10	0	0	100
4	0	0	100
2	0	0	100

* Cpe visible in all wells by day 3

\# No c.p.e. visible by 8 days post inoculation

(a) & (b) Results of two separate experiments

TABLE 2

EFFECTS OF HIGHER CONCENTRATIONS OF DHPG ON ADENOVIRUS GROWTH

Concentration DHPG in μM	% Inhibition Ad 7c c.p.e.
150	0
120	0
100	0
80	0
60	0
40	0
20	0

Results at 6 days post inoculation

TABLE 3

MINIMAL INHIBITORY CONCENTRATIONS

OF DHPG ON HSV-1 C.P.E.

Concentration DHPG in µM	% Inhibition c.p.e.		
	HSV (a)	HSV (b)	Ad 7c
2.00	100	100	0
1.75	100		
1.50	100		
1.00	100		
0.75	100		
0.50	100		
0.40		100	
0.25	100	100	
0.20		100	0
0.10		75	0
0.05		0	0
0.025		0	0
0.0175		0	0

(a) & (b) Represent two separate experiments

5.1.4. Discussion

Although initially much hope was placed on the prospect of utilizing DHPG as a therapeutic agent for the treatment of Ad7c infections, results of this study have not been encouraging. Under laboratory conditions, the growth of Ad7c in cell culture was completely unaffected by ganciclovir, even at concentrations as high as 150 μM. By contrast, the growth of herpes simplex virus was totally inhibited in the presence of as little as 0.2 μM, indicating that the potency of the drug was not in question. In fact, our findings regarding the inhibitory concentrations of DHPG on HSV growth are in approximate agreement with those of Cheng *et al.*, (1983) and Field *et al.*, (1983), both of whom showed that HSV is inhibited by concentrations between 0.5 and 12 μM, depending on the strain and technique used. The techniques used by the latter included both plaque reduction assays and the inhibition of cytopathic effect in infected cells. In the present study, only microscopic observation of cytopathic effect was used to monitor viral inhibition. However, as it produced results comparable to those previously reported, it appears that this technique is as sensitive as the more cumbersome plaque reduction assay.

Having established the potency of the drug and the sensitivity of the assay system, it was evident that DHPG did not inhibit the growth of Ad7c in cell culture. This conclusion is contrary to previous reports which claim that DHPG might suppress the growth of adenoviruses. Wreghitt *et al.* (1989) reported that adenovirus 7 was inhibited by 35 and 97% in the presence of 10 μM and 100 μM DHPG respectively. Taylor *et al.* (1988) found the inhibitory ED_{50} range for known adenovirus serotypes to be between 4.5 μM and 33 μM.

A possible explanation for the discrepant results between this study and those of Wreghitt *et al.*, and Taylor *et al.*, is that different strains of adenovirus were used. Only one virus subtype was used in our experiments and results may not reflect the efficacy of DHPG for other adenovirus serotypes. Our interest in this drug was focused on it's potential therapeutic use for infections caused by the 7c subtype, which was responsible for the nosocomial infections at the Red Cross Children's Hospital. Unfortunately it was found to be ineffective against that strain.

The permissiveness of cell lines for viral growth is subject to variation. Adenoviruses replicate well in HeLa cells and to a lesser extent in HEF. As this study was based on the observation of cytopathic effect over several days, HEF were preferred because of

their superior stability over HeLa cells. If different cell lines were used in previous studies, this could possibly account for the contrast between our findings. However, in our experiments we used a cell type (HEF) similar to that used by Taylor *et al.*(1988).

It is possible that, in the presence of the drug, c.p.e. was produced by resistant variants of the virus. Such resistant strain(s) could have obscured any visible signs of viral inhibition due to the action of DHPG. This postulate is unlikely, however, as there was no delay in the development of c.p.e. in the presence of the drug when compared with the controls. Furthermore, if resistent strains can emerge with such rapidity *in vitro*, the same premise might apply *in vivo* and the clinical use of this drug would be of little value.

Concentrations of DHPG greater than 150 μM were not tested *in vitro*. Toxicological studies of DHPG in mice have shown that this drug has adverse side effects, the impairment of fertility being the most significant. In rabbits, doses of 20 and 60 mg/kg daily during gestation caused teratogenic effects and embryo death. In humans, the side effects have been mainly haematological in nature, with leucopaenia and thrombocytopaenia being the most common clinical manifestations (Syntex, 1988). Consequently, it has been recommended that this drug be used only for severely ill patients and that the maximum dosage of DHPG should not exceed 5 mg/kg body weight twice daily for 14 days. Even in these circumstances the occurrence of side effects is unavoidable. After a single intravenous dose, serum levels of DHPG may be >16 μM, but only for 2 hours, whereafter the levels drop to 4 μM for 8 hours. The half life *in vivo* is 2.9 hours (Information from the manufacturer's data sheet). This study has shown that the growth of adenovirus 7c cannot be inhibited by DHPG, even at maximal concentrations which are possible to achieve *in vivo* and therefore the drug has little potential as a therapeutic agent against adenovirus 7c pneumonia.

6. Chapter 6

6.1. ASSESSMENT OF NEUTRALIZING ANTIBODIES IN COMMERCIAL PREPARATIONS OF IMMUNOGLOBULIN

6.1.1. Introduction

Antiviral drugs have been of limited use as a means of therapy for viral illnesses. Health care workers have therefore concentrated on the prevention of disease rather than cure. The most effective form of prevention has been vaccination and many viral illnesses such as smallpox, polio and measles have been virtually eradicated from certain geographical areas as a result of efficient vaccination programmes.

Adenovirus types 4 and 7 have been used as vaccines. These respiratory strains (administered by mouth) were packaged in enteric capsules so that they would bypass the respiratory epithelium and only start replicating once inside the digestive tract (Couch *et al.*, 1962). However, this type of adenovirus vaccine has not been licensed for general use, mainly for the following reasons: It was found that the simian virus 40 (SV40), a common contaminant of monkey kidney cells, was present in the first batches of vaccine that were made. Not only is it most undesirable to administer a potentially tumorogenic simian virus to humans, but in addition, studies showed that SV40 genetic material is able to integrate into adenovirus DNA, resulting in an adenovirus-SV40 hybrid virus (Lewis *et al.*, 1966). In the process adenovirus sequences may be deleted, resulting in the production of defective virus (Rowe and Baum, 1965). It appears that SV40 was present in many vaccine strains of adenovirus propagated in monkey kidney cells, and attempts to eliminate the contamination have been unsuccessful. Subsequently vaccine strains were grown in human embryonic fibroblasts and seemed to be safe and effective when given to military recruits (Horwitz, 1990). A further complication arose when it was discovered that adenovirus types 12 and 7 induced tumours in rodents (Baum *et al.*, 1972). Although no association between human tumours and any of the adenovirus strains has been demonstrated, the acceptability of adenovirus vaccines for general use remains unlikely.

The major alternative strategy for the control or prevention of viral disease remains passive immunization with concentrated and purified immunoglobulin-G (IgG). In their role of counteracting viral disease *in vivo*, immunoglobulins have multiple functions (Mims and White, 1984). Firstly, they may attach directly to the virus particle and

thereby neutralize its infectivity. In these reactions, the neutralization depends on the inactivation of specific or critical antigenic sites on the virion, usually type-specific antigens. Antibodies can combine with other group-specific antigens on the virion surface, but this need not result in neutralization. Secondly, the attachment of antibodies to viral antigens expressed on the surface of infected cells can activate the complement cascade which will result in the destruction of the virus-infected cell. In addition, IgG bound to viral antigens will be able to interact (via their Fc regions) with effector cells of the immune system and thus stimulate cytotoxic T-cells, killer cells and macrophages.

Immunoglobulin preparations may be administered to patients for prophylactic, substitutional or therapeutic reasons. For example, susceptible individuals who are at risk of exposure to a given pathogen, may be given hyperimmune globulin in order to prevent disease; or patients who are immunocompromised may require passive immunization to substitute for the inability of their immune systems to mount an aggressive response. In some instances, during the course of a severe infection, the patient's own natural specific antibody pool may become depleted, and the administation of immunoglobulin may help to ameliorate the course of the illness.

Parenteral exposure to foreign proteins may induce anaphylactic reactions in certain individuals. Animal products are more likely to cause this reaction, therefore all blood products for clinical application are of human origin. Aggregates of IgG, albeit of human origin, may also give rise to anaphylactoid reactions by activating the complement system (Bochner and Lichtenstein, 1991). Consequently, the preparation of IgG for clinical use is a highly specialized and sophisticated procedure, aimed at reducing the chance of anaphylactic shock to a minimum. The antibodies in these preparations are intact, and their biological properties fully correspond with those of native IgG (Biochemie, 1989). Human immunoglobulin is prepared from a large pool of plasma (from at least 1000 donors) which ensures the presence of a high proportion of antibodies to most infectious agents in that geographical area. The IgG is concentrated to about 15 times the level found in normal plasma. To ensure biological safety, each unit of plasma is tested to exclude those which contain hepatitis B surface antigen and antibodies to human immune deficiency virus (HIV).

For globulin preparations to be most effective, it is necessary for them to contain high levels of specific activity against the incriminating pathogen, in our case adenovirus type 7c (Ad7c). On the assumption that locally produced products would provide the best source of specific Ad7c antibodies, it was decided to assay their levels of neutralizing

antibodies *in vitro*. In an attempt to gauge the immunogenicity of the Ad7c virus *in vivo*, parallel neutralization tests were performed on sera from members of staff who were currently working in the respiratory unit at Red Cross Hospital, and who would presumably have had repeated exposure to the virus. Assessment of these single sera could provide an indication of the variation in individual immune responses to the serotype of interest.

In order to gain an impression of the purity of the gammaglobulin preparations, and also to verify that we were indeed working with IgG, SDS polyacrylamide gel electrophoresis was performed on samples taken from both intramuscular and intravenous preparations. Sodium dodecyl sulphate (SDS) denatures proteins by wrapping around the polypeptide backbone, and at the same time confers an overall negative charge to the molecule. All the proteins in a given preparation will thus have the same charge per unit length, and their rate of migration through an electric field will be determined by their molecular weight. Standard proteins of known molecular weights were run concurrently with the polypeptides.

6.1.2. Materials

Human sera

Blood specimens were obtained from five members of staff in the respiratory intensive care unit at the Red Cross Children's Hospital.

Immunoglobulin

Samples of IgG from twelve batches of intra-muscular (IM) immunoglobulin and five batches of intravenous (IV) immunoglobulin were obtained from the Natal Blood Transfusion Service. The IM immunoglobulin solutions contained 16% protein in 0.3 M glycine. The IV immunoglobulin was in lyophilised form and when reconstituted, contained a protein concentration of 3%.

Virus

Adenovirus originally isolated from a patient in the respiratory unit at Red Cross Children's Hospital, and subsequently typed as Ad7c (Chapter 2).

Reagents used for polyacrylamide gel electrophoresis (PAGE)

1x SDS Gel-loading buffer

50 mM Tris Buffer (pH 6.8)

100 mM Dithiothreitol (DTT)

2% SDS

0.1% Bromophenol blue

10% glycerol

Stock acrylamide mix (30%)

Acrylamide	30 g
Bisacrylamide	0.8 g

Dissolved slowly in warm distilled water, and made up to a final volume of 100 ml.

Sodium dodecyl sulphate (SDS).

A 10% solution was prepared in distilled water and stored at room temperature.

TEMED (N,N,N',N'-tetramethylethylenediamine).

This solution is purchased ready for use, and serves as the catalyst for polymerization.

Ammonium persulphate

A 10% stock solution was prepared in distilled water, and stored at -20 C in aliquots of 100 μl. This substance is the initiator of the polymerization process.

Stock stacking gel buffer (0.5 M Tris with 0.4% SDS)

Tris-HCl	6.05 g
SDS	4 ml
Distilled H_2O	80 ml

The pH was adjusted to 6.8, and the solution was made up to 100 ml.

Stock separating gel buffer (1.5 M Tris with 0.4% SDS).

Tris-HCl	18.17 g
SDS	4 ml
Distilled H_20	80 ml

The pH was adjusted to 8.8, and made up to a volume of 100 ml.

Acrylamide stacking gel

Acrylamide mix	0.83 ml
Stacking gel buffer (stock)	1.25 ml
H_2O	3.92 ml

TEMED	10 μl
Ammonium persulphate	100 μl

Acrylamide separating gel

Acrylamide mix	2.7 ml
Separating gel buffer (stock)	2.5 ml
H_2O	4.8 ml
TEMED	20 μl
Ammonium persulphate	100 μl

Electrophoresis buffer (x10).

Tris-HCl	6.0 g
Glycine	28.82 g
SDS	20 ml

The solution was made up to 1000 ml, after the pH had been adjusted to 8.3

Protein staining solution

Coomassie Brilliant Blue	2.5 g
Methanol	900 ml
Glacial acetic acid	100 ml

Destaining solution

Methanol	300 ml
Glacial acetic acid	100 ml

6.1.3. Methods

Neutralization Tests of Serum Samples:

Dilutions of each serum sample were made in maintenance medium starting with 1:10, and then doubling dilutions to 1:1280. 0.1 ml of each dilution was added to an equal volume of 100 $TCID_{50}$ of adenovirus 7c. These virus/serum mixtures were incubated at 37°C for two hours, whereafter the total volume (0.2 ml) was inoculated onto monolayers of either HEF or HeLa cells grown in microwell plates. Four wells were used per dilution. The plates were monitored daily; the results in HeLa cells were determined after 3 days and in HEF after 6 days (see Chapter 3). A serum sample known to contain no antibodies to adenovirus 7c was used as a negative control.

Neutralization Tests of Immunoglobulin Preparations:

All samples of immunoglobulin were tested in the same way as the individual sera, except that doubling dilutions were continued as far as 1:2560, and only HeLa cells were used. Three of the batches were tested on two separate occasions.

Levels of neutralizing antibody were expressed as the reciprocal of the highest dilution which suppressed viral growth in 50% of the wells inoculated.

Polyacrylamide gel electrophoresis (SDS-PAGE).

A modified version of the Laemmli (1970) discontinuous system for SDS-PAGE was used.

The Mighty Small 1 SE200 (Hoefer) vertical slab gel electrophoresis unit was used. The apparatus was assembled according to the manufacturers instructions. The separating-gel acrylamide solution was poured into the chamber between the glass plates (approx. 10 ml) and a small volume of methanol was used as an overlay. After completion of polymerization, the overlay was poured off and the stacking-gel acrylamide solution was added (approx. 5 ml). A Teflon comb was inserted immediately. When polymerization was complete (after about 30 minutes) the comb was removed, and electrophoresis buffer was added to the reservoirs.

Two samples of IM globulin (Batch G135) were diluted 1:100; one in loading buffer which contained the reducing agent, DTT, and another was diluted in loading buffer which did not have DTT. After dilution, the approximate protein concentration was 0.16 gm/ml. Two samples of IV globulin (Batch GIV15) were diluted 1:15 in the same manner as the IM preparations, and yielded a protein concentration of approximately 0.2 gm/ml. These diluted samples were boiled for 5 minutes immediately before they were loaded onto the gel and 10 μl of each sample was loaded into separate lanes. Molecular weight markers (Appendices IV and V) were added to a separate lane on each gel. The proteins were electrophoresed at 15 mA until the dye front (bromophenol blue) had reached the bottom of the gel. The gels were removed from the apparatus and placed in glass dishes containing Coomassie Blue staining solution. After 18 hours, the gels were placed in destaining solution until the protein bands could be clearly visualized.

6.1.4. Results

Neutralization Assays

Serum samples:

Detectable levels of neutralizing antibodies to adenovirus 7c were found in all five serum samples. The titres were 1280, 320, 1280, 480 and 20 respectively. The negative control serum had no detectable neutralizing effect in the lowest dilution of serum tested ($1/_{10}$). These experiments were performed in both HeLa cells and HEF, with comparable results, except for one sample (serum 1) which showed a discrepancy of more than one serial dilution (See Table 4)

Immunoglobulin samples

All the batches of immunoglobulin (both IM and IV) had demonstrable levels of neutralizing antibody, the average titre being approximately 1280. Nine batches of IM globulin had antibody levels of $\geqslant$1280 (G119, G120, G123, G127, G128, G130, G131, G132, G134). Two batches had levels of 640 (G126 and G135). One batch (G129) had a titre of 160, which was much lower than the others. Three of the batches (G123, G127 and G132) were re-tested on a separate occasion and these demonstrated antibody levels of 640, 2000 and 1280 respectively (Table 5). Of the five batches of IV globulin tested, three had antibody levels $\geqslant$1280 (GIV 8, GIV9, GIV11). Batches GIV13 and GIV14 demonstrated antibody levels of 480 and 960 respectively (Table 6).

PAGE

In the unreduced samples of both IV (Fig. 9, lane 2) and IM (Fig. 9, lane 3) globulin, a large protein band was discernible at approximately 150 kDa, which is compatible with the size of intact IgG. In the IM preparation, a lesser band was seen at 97 kDa and a faint band at 47 kDa.

In the samples that were reduced (Fig. 10), two strong bands were observed at 50 kDa and 25 kDa respectively, in both IV (lane 2) and IM (lane 3) preparations. Reduction of IgG disrupts the disulphide bonds that bind the heavy and light chains of the IgG molecule; the band at 50 kDa represents the heavy chains, and the band at 25 kDa, the light chains.

The smaller band at 97 kDa in the unreduced IM sample, was not observed after reduction, but additional bands in the regions of 36, 45 and 66 kDa were apparent. This indicates that the preparations of both IM and IV globulin contained trace amounts of proteins other than IgG. The nature of the extraneous polypeptides was not explored in these experiments, as it was apparent that IgG was the predominant protein in both immunoglobulin preparations.

TABLE 4

NEUTRALIZING ANTIBODY TO Ad7c IN SERUM SAMPLES

Serum	Antibody levels* Cell Type	
	HEF	HeLa
1	1280	480
2	320	480
3	1280	1280
4	480	240
5	20	20
Negative control serum	< 10	< 10

* Reciprocal of the highest dilution of serum that neutralized virus growth in 50% of the wells inoculated.

TABLE 5

NEUTRALIZING ANTIBODY IN INTRAMUSCULAR IgG PREPARATIONS

Batch No.	Antibody levels *	
	First Test	Repeat Test
G119	1280	
G120	1280	
G123	>1280	640
G126	640	
G127	2560	2000
G128	1280	
G129	160	
G130	2000	
G131	2000	
G135	640	
G132	>1280	1280
G134	1280	

* Expressed as the reciprocal of the highest dilution on which neutralized viral growth in 50% of the wells inoculated.

TABLE 6

NEUTRALIZING ANTIBODY IN INTRAVENOUS IgG PREPARATIONS

Batch No.	Antibody levels *
GIV 8	1920
GIV 9	1280
GIV 11	1280
GIV 13	480
GIV 14	960

* Expressed as the reciprocal of the highest dilution which inhibited viral growth in 50% of the wells infected

Fig. 9

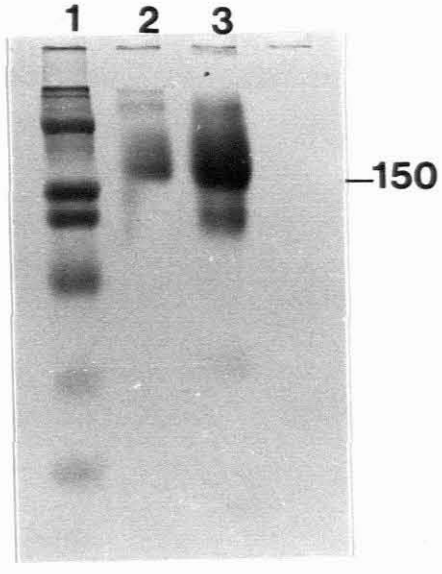

Fig. 10

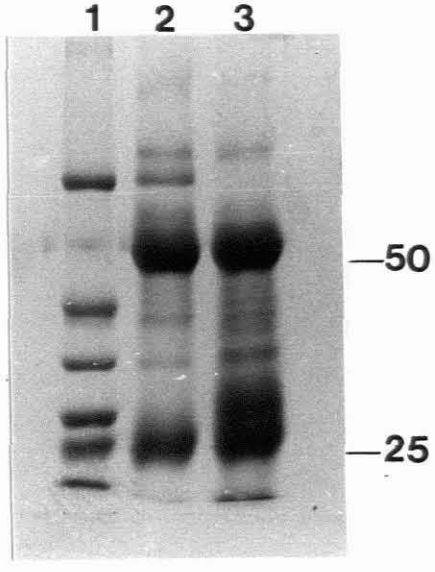

Fig. 9 and 10

Immunoglobulin preparations were subjected to SDS-PAGE under both non-reducing (Fig. 9) and reducing (Fig. 10) conditions. Protein bands were stained with Coomassie blue. Non-reduced samples showed prominent bands at 150 kDa (Fig. 9); and at 50 kDa and 25 kDa, after reduction (Fig. 10). Lane 2 contains IV globulin, and lane 3 contains IM globulin. High molecular weight markers were run in Figure 9, lane 1.and low molecular weight markers in Figure 10, lane 1. See appendix 1V and V.

6.1.5. Discussion

Neutralizing antibodies are the most specific antibodies produced against viral antigens. They persist for years and are responsible for immunity. Not all pathogens are equally immunogenic, and we had no prior knowledge of the expected *in vivo* immune response to type 7c adenovirus. However, by assessing the levels of neutralizing antibodies from medical personnel who were known to have been exposed to Ad7c, it was possible to conclude that the virus could induce a substantial antibody response. This gave strength to the expectation that locally produced immunoglobulin preparations might be a valuable source of neutralizing antibody for prophylactic use.

As adenovirus 7c is the dominant respiratory strain in South Africa (Wadell *et al.*, 1985), the presence of antibody to this virus in the human immunoglobulin could be assumed, but the assumption needed verification in the form of specific laboratory assays. These confirmed that high levels of neutralizing antibodies were present in both intramuscular and intravenous preparations of immunoglobulin. Each batch of globulin is prepared from a different donor pool and therefore may contain variable amounts of neutralizing antibody, as our results have demonstrated. Although there was considerable variation in antibody levels among the batches tested, the mean dilution of IgG that inhibited viral growth was in the region of 1:1280. These results were encouraging; the implication being that immunoglobulin preparations might indeed be of clinical use for the treatment of adenovirus 7c pneumonia.

The IM preparations have a higher gammaglobulin concentration (16%) than the IV (3%) and one might have expected the titres in the former to be higher. However, a five fold difference in globulin concentration would give only a 2-doubling dilution difference, which is only just within the sensitivity of the method. This fact, together with the batch variation previously mentioned, could explain why the IM and IV material yielded such similar results.

It is not possible to correlate antibody levels (determined by *in vitro* assays) in a given globulin sample, with the dosage required for effective antiviral activity *in vivo*. Furthermore, it would be impractical to assay each globulin sample for the presence of antibodies to adenovirus type 7c. Therefore, knowledge regarding the expected minimal level of specific antibody in a randomly selected vial of immunoglobulin, is of greater practical use than knowledge regarding the precise end-point of a number of individual batches of immunoglobulin. The present study has been of value in demonstrating that most (if not all) of the locally produced immunoglobulin products contain substantial levels of neutralizing antibodies to adenovirus 7c. They can therefore be used with confidence for the treatment of Ad7c infections.

Neutralization assays (as used in the present study) not only serve to identify the presence of specific antibodies to a select subtype of adenovirus, but also assess their biological activity. It is reassuring to have found that the infectivity of a standard dose of virus could be totally neutralized by a dilution of globulin in excess of three logs. The information gained from the PAGE analysis suggests that the immunoglobulin preparations are reasonably pure and that IgG is the predominant protein. Because the major component had the anticipated molecular size of 150 kDa in the unreduced state, and after reduction appeared as two main bands of 50 kDa and 25 kDa, it was concluded that the commercial preparations contained undegraded molecules of IgG

7.Chapter 7

7.1. CONCLUSIONS

The primary role of a clinical virology laboratory is to assist the clinician with diagnoses by establishing whether an illness has a viral etiology. In addition to this role, information gained through laboratory experiments may provide valuable guidelines for the control and prevention of viral illnesses.

Nosocomial infections due to adenovirus have occurred at the Red Cross Children's Hospital over several years. The outcome of these respiratory infections has been severe; some children have died whereas others have been left with permanent lung damage.

The projected aim of this study was to plan experimental studies that could give guidance regarding potential strategies whereby nosocomial infections of adenovirus pneumonia could be prevented or abated.

Laboratory experiments were performed to establish (1) the inhibitory potential of the drug, ganciclovir, against adenovirus type 7c replication in cell culture and (2) the presence of neutralizing antibodies to Ad7c in immunoglobulin preparations.

As adenovirus type 7c is the most common cause of juvenile pneumonia in the Cape Town area (Kannemeyer *et al.*, 1988), it was decided that all experiments should be focused on that strain. The virus used was cultured from a tracheal aspirate from a child in the respiratory unit, and using the restriction enzymes, *Eco*R1, *Bam*H1 and *Sma*I, the viral genome was characterized as that of Ad7c. Electrophoretic separation of the restriction patterns resulted in profiles which were identical to those of a previously established Ad7c.

A primary requirement was to select and standardize criteria whereby inhibition of virus growth could be monitored. It was decided to use c.p.e. as the indicator of active viral replication, and Ad7c growth was examined in both HEF and HeLa cells. The adenovirus strain that was selected not only replicated well in both HEF and HeLa cells, but also produced clear and definite c.p.e.. HeLa cells, however, were found to be unsuitable for experiments which required that cell cultures be maintained for protracted periods. After three days, spontaneous degeneration of the HeLa cell monolayers made

it difficult to distinguish viral c.p.e. In order to ensure reliable interpretation of the effects of low concentrations of antiviral agents, it was considered necessary to monitor the cells for as long as possible. Therefore, for the drug study, HEF was selected as the cell strain of preference. Moreover, immunofluorescence staining of HEF demonstrated that they were free from mycoplasma infection, thus providing greater confidence that cytopathic alterations would not be influenced by contaminating micro-organisms.

For the neutralization assays, prolonged culture was not required and HeLa cells were found to be optimal for obtaining more rapid (yet accurate) results.

Although previous studies had shown that adenovirus growth was inhibited by the antiviral agent ganciclovir (Taylor *et al.*, 1988; Wreghitt *et al.*, 1989), we could not support these findings. Ad7c was titrated in the presence of varying concentrations of gancilovir, ranging from 2 μM to 150 μM. As ganciclovir is known to have a significant effect on the growth of herpes simplex virus (Cheng *et al.*, 1983) parallel titrations of HSV were performed to monitor the potency and biological activity of the drug. We found that replication of herpes simplex virus in HEF was dramatically inhibited in the presence of low concentrations of ganciclovir (as little as 0.2 μM), but replication of Ad7c was not inhibited at all, even at concentrations of up to 150 μM. If ganciclovir were to have any inhibitory potential for adenovirus infections, it would be at concentrations higher than those that were tested and, given the toxic side-effects of the drug, such levels would be intolerable to the patient. It was concluded that for the treatment of Ad7c pneumonia, ganciclovir would not be an effective antiviral agent and that exposure of compromised infants to its potentially toxic effect, was not justified. In addition to the laboratory findings, clinical experience with the drug has not been encouraging. At the Red Cross Children's Hospital, the drug was administered to two children who were critically ill with adenovirus pneumonia, but in spite of treatment both of them died (Dr. M. Klein, personal communication).

It was apparent that in the absence of an antiviral drug with therapeutic potential for adenovirus type 7c infections, alternative strategies for virus control needed to be explored.

The therapeutic value of immunoglobulin preparations for the prevention or amelioration of infectious disease has been appreciated for many years. Although these commercial preparations are known to contain antibodies to certain notifiable infections such as measles and polio, they are not routinely assayed for the presence of adenovirus

antibodies. We tested locally produced human immunoglobulin preparations for antibodies to Ad7c in order to assess whether they might be useful for the control of Ad7c pneumonia.

Levels of neutralizing antibodies were measured in 17 batches of immunoglobulin; 5 intravenous and 12 intramuscular. The neutralization assays were performed mainly in HeLa cells, where an antibody titre could be calculated after three days. Although immunofluoresence staining of HeLa cells demonstrated the possible presence of mycoplasma, the replication of Ad7c in these cells was not impeded. High levels of Ad7c neutralizing antibodies were present in both intramuscular and intravenous immunoglobulin preparations. Significant neutralizing activity was demonstrated in all of the batches.

Neutralizing antibodies actively inhibit viral replication *in vitro*. Their presence in immunoglobulin preparations is therefore significant and lends support to the premise that passive immunization with these IgG products would be effective in the prevention or amelioration of Ad7c disease. All batches tested were selected on a random basis, and the finding that none was devoid of neutralizing antibodies, and that most had neutralizing activity at dilutions of greater than 1:1000, provides reassurance that any sample of immunoglobulin may be used with confidence without prior testing. Analysis by PAGE under both reducing and non reducing conditions, demonstrated that samples of both intramuscular and intravenous immunoglobulin contained predominantly IgG, and that the molecules were intact even after storage at $4^{o}C$ for prolonged periods.

Information gained from this study provides substantial data for the formulation of control measures against adenovirus infections. Already this knowledge has been brought into effect in the intensive care unit at the Red Cross Children's Hospital. In an endeavour to contain the spread of adenovirus infections in this ward, a protocol was introduced whereby intravenous immunoglobulin is administered to all patients who have a laboratory confirmed diagnosis of adenovirus pneumonia, and intramuscular immunoglobulin is given to the other children in the ward who may be susceptible. In recent years the number of laboratory-confirmed adenovirus infections in the intensive care unit at Red Cross Hospital has been significantly reduced. In 1988, 24 cases of adenovirus infection were diagnosed in our laboratory whereas in 1990 there were only 2 cases of adenovirus infection from that ward. We are confident that the administration of immunoglobulin according to the protocol previously described has contributed towards the control of nosocomial infections due to adenovirus type 7c.

Appendices

Appendix I

STAINING OF COVERSLIPS WITH HAEMATOXYLIN AND EOSIN

Rinse tubes in PBS, x2.

Fix in Bouin's fixative for 1 hour.

Wash in absolute alcohol for 30 minutes.

Leave in 70% alcohol until required for staining.

Wash very well in running tap water to remove fixative.

Stain with freshly filtered haematoxylin for 1 minute.

Rinse in tap water and then in Scott's tap water (0.04 M $NaHCO_3$; 0.16 M $MgSO_4$) for 15 to 30 seconds.

Wash in running tap water.

Counterstain with eosin for 15 seconds.

Dehydrate through 70%, 96% and 100% alcohol.

Soak in xylol for 2 minutes.

Mount coverslips onto glass slides using DPX as mounting medium.

Appendix II

PHOSPHATE BUFFERED SALINE (pH 7,5)

NaCl	8,0 g
KCl	0,2 g
KH_2PO_4	0,12 g
Na_2HPO_4 (Anhydrous)	0,91 g
Distilled H_2O	1 litre

Appendix III

CALCULATION OF THE LD_{50} OR $TCID_{50}$ TITRE BY THE REED-MUENCH METHOD.

$$\frac{(\%\text{ mortality at dilution next above 50\%}) - (50\%)}{(\%\text{ mortality at dilution next above 50\%}) - (\%\text{ mortality at dilution next below})} = \text{Proportionate distance}$$

Negative logarithm of $TCID_{50}$ endpoint titre = negative logarithm of the dilution above the 50% mortality + (the proportionate distance factor x log of the dilution steps employed.

Appendix IV

SDS (LOW) MOLECULAR WEIGHT MARKERS

	kDa
Lysozyme, Egg White	14,300
Beta-Lactoglobulin, Bovine Milk	18,400
Trypsinogen, Bovine Pancreas, PMSF treated	24,000
Pepsin, Porcine Stomach Mucosa	34,700
Albumin, Egg (Ovalbumin)	45,000
Albumin, Bovine Plasma	66,000

Appendix V

SDS (HIGH) MOLECULAR WEIGHT MARKERS

	kDa
Carbonic Anhydrase	29,000
Albumin, Egg, (Ovalbumin)	45,000
Albumin, Bovine Plasma	66,000
Phosphorylase B, Rabbit Muscle	97,400
Beta-Galactosidase, E.coli	116,000
Myosin, Rabbit Muscle	205,000

List of Abbreviations

Ad	adenovirus
BME	betamercaptoethanol
^{o}C	degrees Celsius
c.p.e.	cytopathic effect
DTT	dithiothreitol
DNA	deoxyribonucleic acid
DHPG	9-(1,3-dihydroxy-2-propoxymethyl) guanine
EDTA	ethylenediaminetetra-acetic-acid
FCS	foetal calf serum
Fig.	Figure
g	grammes
HEF	human embryonic fibroblasts
HCMV	human cytomegalovirus
HSV	herpes simplex virus
IF	immunofluorescence
IgG	immunoglobulin G
IgM	immunoglobulin M
IM	intramuscular
IV	intravenous
kb	kilobases

kDa	kilodaltons
M	molar
mA	milliamps
MEM	minimum essential medium
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
N	normal
nm	nanometre
PAGE	polyacrylamide gel electrophoresis
RNA	ribonucleic acid
PBS	phosphate buffered saline
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SV40	simian virus type 40
$TCID_{50}$	tissue culture infective dose 50% endpoint.
Tris	tris (hydroxymethyl) aminomethane hydrochloric acid
μM	micromole
μl	microlitre
μg/ml	microgrammes per millilitre
v/cm	volts per centimetre
w/v	weight per volume

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