AN INVESTIGATION INTO THE GROWTH AND ANTIGENIC PROPERTIES OF ADENOVIRUSES DETECTED BY ELECTRON MICROSCOPY IN THE STOOLS OF CHILDREN IN GLASGOW

BY

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A THESIS SUBMITTED FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
OF THE FACULTY OF MEDICINE,
UNIVERSITY OF GLASGOW.

September, 1980.

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ACKNOWLEDGEMENTS

I wish to thank:

Professor C.R. Madeley, for introducing me to electron microscopy, for much advice and encouragement during the work, and for reading the draft thesis.

Professor N.R. Grist, for advice and encouragement and for reading the draft thesis.

Dr. Eleanor J. Bell, for advice concerning isolation and identification of stool viruses, and for encouragement.

Mrs. Bonnie P. Cosgrove and Mr. I. Miller, who examined most of the original stool extracts by electron microscopy.

Miss Mary Gallacher, for technical assistance during work described in Chapter 2.

Miss Raewyn Brown (Mrs. McNiven), for technical assistance and comic relief.

Mr. G. Robinson, who performed the complement fixation tests described in Chapter 6.

Dr. D. Gilmore (Department of Physiology, University of Glasgow), for the supply of human embryo kidney, intestine and liver.

The sisters and nursing staff in the maternity/labour wards of the following Glasgow Hospitals: Eastern District Hospital, Duke Street; Redlands Hospital for Women; Southern General Hospital; Queer Mother's Hospital, Yorkhill; Stobhill General Hospital; - for the supply of human placentae.

Thanks are due to the staff of the Regional Virus Laboratory, Ruchill Hospital, Glasgow, who made my stay a pleasant one.

DECLARATION

The conclusions pertaining to practical work described in Chapters 1 to 7 were reached independendently of other workers.

Results from Chapter 7 have been accepted for publication by the Journal of Clinical Pathology.

SUMMARY.

Adenovirus particles which cannot be cultured have been found in the stools of children by various workers. To determine the extent of this problem, stools from children in Glasgow, sent to the Regional Virus Laboratory for electron microscopic examination over a 2-year period (1976-1977) and found to contain adenovirus particles, were put in culture. One third of 148 specimens containing adenovirus (from 28 of 69 children) failed to cause cytopathic effects in primary human amnion and secondary human embryo kidney (HEK) cell cultures. Variations in culture method such as rolling tubes or adsorption periods did not improve the adenovirus isolation frequency. There was no common factor apparent, concerning the patients or their stools, which might explain the non-growth of adenoviruses.

Attempts to 'reactivate' non-growing adenoviruses by culture attempts in human cells in the presence of either adeno associated virus (AAV), human adenovirus (type 8) or a canine adenovirus (ICHV) were unsuccessful. AAV type 1 did not multiply in the presence of non-growing adenovirus particles. An immunofluorescence test using group-specific guinea pig antiserum revealed that with some stool extracts a small number of KB cells become infected and produce adenovirus structural antigens. Fluorescence was limited to single cells, indicating that, at 3 days after inoculation, spread of any new infectious virus to adjacent cells did not occur. Attempts to detect an inhibitory substance to adenovirus replication, which might explain the lack of cytopathic effects, were unsuccessful.

Bovine cells, canine cells and various continuous human cell lines were tried for the detection of non-growing adenoviruses, and some success was achieved using Intestine 407 and Chang Conjunctiva cells maintained in Leibovitz' L15 medium. Two agents from different children caused cytopathic effects over 8 passages in Chang cells. Apart from these agents, the extent of the cytopathic effects declined in most cases with serial passage, but this decline was not so rapid if the cultures were incubated at 33°C.

Antisera prepared in rabbits against one strain of non-growing adenovirus showed group-specific reactivity by immunofluorescence and complement fixation tests to adenoviruses of mammalian origin. These sera at low dilution (1:20) did not neutralise the serotypes of adenovirus commonly isolated from stools, but neutralised all but one of the 'fastidious' (F) strains from 21 children. Strains from 9 of these children were neutralised by one antiserum at a dilution of 1:640 or greater. Three fastidious strains (from different children) were not neutralised by standard antisera to adenovirus types 1 to 33. It is suggested that they are a previously undiscovered serotype. Cultures of fetal human intestinal segments also appear to be capable of supporting the growth of these agents.

A limited study to screen the stools of children for fastidious adenoviruses by neutralisation indicated that F adenoviruses of the same or related serotypes may be very common. That some adenoviruses in stools produce neither group-specific antigens detactable by immunofluorescence in KB cells nor cytopathic effects in Chang cells remains to be explained.

The significance of these results is discussed and related to the recent work of others.

ABBREVIATIONS

The following abbreviations are used throughout.

Abbreviation	Meaning
ml	Millilitre.
g. mg. µg.	Gram / milligram / microgram.
g	Gravity units.
cm mm	Centimetre / millimetre
min	Minutes.
rev./min	Revolutions per minute.
TCD_{50}	50 per cent tissue culture infectious dose.
^{o}C	Degrees Celsius.
PBS	Phosphate buffered saline.
HEK	Human embryo kidney.
AAV	Adeno associated virus.
CPE	Cytopathic effect.
Ad	Adenovirus.
Ad 2	Adenovirus type 2.

Notation for Intermediate Adenovirus Strains

Strains of adenovirus showing the characteristics of more than one serotype are denoted by both type numbers, separated by a hyphen. For example, strains neutralised by antisera to both type 12 and type 31 are denoted as type 12-31 strains. This is in accordance with common practice (see Béládi, 1972). The word 'to' is used in place of the hyphen in the text when all serotypes between (and inclusive of) two numbers are being discussed.

Notation for Dilutions

Dilutions are denoted thus: 1:20, 1:40, to be read as 'one in twenty', 'one in forty'. Where the colon is used to denote a ratio of volumes rather than a dilution, the figure is followed by (v/v), meaning 'volume for volume'.

INTRODUCTION AND REVIEW OF THE LITERATURE

INTRODUCTION

The human adenoviruses have been regarded primarily as agents which infect the respiratory tract or conjunctivae (see Sterner, 1962). Individual serotypes have been associated with respiratory illnesses, keratoconjunctivitis (Jawetz et al., 1955), acute haemorrhagic cystitis (Numazaki et al., 1968; Numazaki et al., 1973) intussusception (Gardner et al., 1962) and other non-respiratory illnesses. However, apart from respiratory illness, keratoconjunctivitis and acute haemorrhagic cystitis, a causal relationship between the virus and the syndrome has not been fully proven. Only 12 of the 35 established serotypes have been implicated as agents causing acute disease in man (Chanock, 1974; Kasel, 1979).

Adenoviruses are frequently isolated in cell culture from throat swabs taken from children. In common with the enteroviruses and reoviruses, these agents can be isolated from rectal swabs or stools. Such isolations may be concurrent with or may follow a respiratory infection by the same serotype (Rosen et al., 1962b; Fox et al., 1969). There have been reports of diarrhoea accompanied by the shedding of adenoviruses in stools (Kjellén et al., 1957; van der Veen and van der Ploeg, 1958; Duncan and Hutchison, 1961; Sterner et al., 1961; Moritsugu, 1969) but isolations of adenoviruses from children without signs of infection are also common (Brandt et al., 1972; Fox et al., 1977). The adenovirus serotypes most commonly isolated from stools are types 1, 2, 3, 5 and 7, but many of the higher numbered serotypes have been isolated almost exclusively from faecal rather than respiratory specimens (Rosen et al., 1962b; Vargosko et al., 1965; Brandt et al., 1969). The shedding of

individual serotypes from the gut by children can persist over many months (Fox et al., 1969; Fox et al., 1977).

With the application of electron microscopy to the study of stool specimens in the early 1970's, virus-like particles which had never been detected in cell cultures were discovered in the stools of children. Some fitted the description of bacteriophages, with tail-like projections, but some had previously undescribed morphology. These are reviewed by Dolin (1979), Holmes (1979) and by Madeley (1979). In particular, 60-70 nm diameter reovirus-like particles with a distinct and very regular surface morphology (rotaviruses; Flewett et al., 1974) were discovered almost simultaneously in Melbourne, Birmingham and Toronto. These particles have been found to be ubiquitous and related to similar agents from the stools of mice, calves and other vertebrates. Until very recently, the human rotaviruses defied all attempts at isolation in cell culture. However, detection of intracellular antigens by immunofluorescence (Banatvala et al., 1975; Bryden et al., 1977) and direct identification of viral antigens by immunoelectroosmophoresis, complement fixation, radioimmunoassay or enzyme-linked immunosorbent assay (ELISA) have provided means of confirming their presence in stools and of investigating antigenic differences (see Holmes, 1979).

Pleomorphic coronavirus-like objects have also been seen in stools by electron microscopy (EM). These objects have a characteristic fringe 20-28 nm in diameter and have been seen in stools from cases of acute gastroenteritis (Caul et al., 1975).

Other virus-like particles in the size range 20-35 nm diameter have been detected in stools by EM (see Madeley, 1979). In particular, particles with indistinct surface morphology have been

found in stools from cases of diarrhoea and vomiting (for example, the Norwalk, Montgomery County and Hawaii agents; reviewed by Dolin, 1979). Astroviruses (Madeley and Cosgrove, 1975) with surface morphology appearing as 5- or 6- pointed stars on individual particles, and caliciviruses (Madeley and Cosgrove, 1976) with a 'star of David' configuration on the particles, were added to the list of 'small round viruses' which could be seen by EM but could not be cultured. Some of the smallest particles seen had a regular hexagonal profile (20-25 nm in diameter) and fitted the description of adeno associated viruses (Hoggan et al., 1966; C.R. Madeley, personal communication).

Adenoviruses were also found in stools by EM. Flewett et al. (1975) reported an outbreak of diarrhoea in a long-stay children's ward containing 19 children. 6 children and a nurse were affected. Adenovirus particles were detected by EM in stools from 4 children and the nurse but the viruses could not be isolated in cell culture despite repeated attempts over several months. Schoub et al. (1975) reported the detection of adenoviruses by EM in the stools of 3 children admitted to hospital in Johannesburg, with severe gastro-enteritis. The stool specimens produced typical adenovirus cyto-pathic effects in cell culture following initial inoculation, but the viruses could not be serially passaged or typed.

White and Stancliffe (1975) reported that of 60 stool specimens containing adenoviruses from children less than 5 years old, 27 isolates (all either adenovirus type 1 or type 2) were not seen by EM. This in itself is not surprising, since considerable quantities of virus particles have to be present in order to be detected by EM. However, 26 stcols known to contain adenoviruses by EM did not

cause cytopathic effect (CPE) in cell culture, and adenoviruses were both seen by EM and grown in cell culture in only 7 cases. Therefore there was an almost complete division between the strains that could be seen and those that could be grown. White and Stancliffe commented that replication in the host itself was the only logical explanation for the shedding of large numbers of adenovirus particles which could not be cultured *in vitro*.

Madeley and co-workers (1977) made a similar finding with stools taken from children admitted to hospital with diarrhoea. 21 of 183 children were found to be excreting adenoviruses (by EM and/or cell culture). Whereas adenoviruses from 9 children were isolated but not detected by EM, adenoviruses from 10 children were detected by EM only. Adenoviruses were both seen and cultured from 2 patients only.

The results of the above workers led to the work described in this thesis, which was an investigation into the nature and growth requirements of the non-growing adenovirus strains.

During this work, further reports appeared recording the authors' inability to isolate certain adenovirus strains from stools (for example, Kapikian *et al.*, 1976; Maass *et al.*, 1977; Middleton *et al.*, 1977; Whitelaw *et al.*, 1977; Appleton *et al.*, 1978; Brandt *et al.*, 1979; Gary *et al.*, 1979; Retter *et al.*, 1979; Richmond *et al.*, 1979). Brief details of these reports are given in Table 0.1. The comment has been made on at least 2 occasions that the chances of isolating adenoviruses from stools appear to be inversely related to the amount of virus detected by EM (Flewett, 1977; Madeley *et al.*, 1977). An explanation for this paradox might lead to a better understanding of the growth requirements of other viruses in stools which cannot be cultured at present.

TABLE 0.1 Recent studies reporting the presence in stools of adenoviruses that could not be cultured. Brief details of the culture results are given.

No. of Individuals / Specimens* From Which Adenovirus Was Detected				Brief Clinical Details	Reference
By EM Only	By Culture Only	By EM and Culture	Total		
5	0	0	5	associated with an outbreak of gastroenteritis	Flewett et al., 1975
3	NS†	0	3	3 out of 30 patients with gastroenteritis had adenoviruses in stools	Schoub et al., 1975
26*	27*	7*	60*	no details of illness given	White and Stancliffe, 1975
{14 infections detected by EM, 6 infections detected by culture}			16	from 143 children admitted to hospital with acute gastroenteritis	Kapikian et al., 1976
10	9	2	21	all cases (183) were admitted to hospital with diarrhoea	Madeley et al., 1977
7*	NS†	2*	9*	from 195 children with acute gastroenteritis	Mæss et al., 1977
27*	59*		86*	from children admitted to hospital with current or reoent gastroenteritis; 1751 specimens examined	Middleton et al., 1977

†only the results of culture with stools containing adenovirus particles (by EM) are given

TABLE 0.1 continued

No. of Individuals / Specimens* From Which Adenovirus Was Detected				Brief Clinical Details	Reference
By EM Only	By Culture Only	By EM and Culture	Total		
1	0	1 §	2	2 cases of gastro-enteritis, one of which died	Whitelaw et al., 1977
6	0	0	6	associated with an outbreak of gastro-enteritis	Richmond et al., 1979
3*	4*	1*	8*	from 84 children admitted to hospital; adenoviruses seen by EM only were from children with gastroenteritis	Appleton et al., 1978
36*	NS†	11*	47*	figures include specimens from children with gastroenteritis and those from controls	Brandt et al., 1979
176*	NS†	216*	392*	from 2606 specimens from patients with gastroenteritis	Retter et al., 1979
13	NS†	1	14	from 192 infants during the acute phase of gastroenteritis	Gary et al., 1979

§ adenovirus isolated from gut homogenate, untyped

†only the results of culture with stools containing adenovirus particles (by EM) are given

A. THE ADENOVIRUSES .

1. Discovery.

Adenoviruses were discovered by two groups of workers almost simultaneously. Hilleman and Werner (1954) reported the isolation of a cytopathogenic agent from the throat washings of army personnel with an acute febrile respiratory illness. This report was especially significant since there was already an indication from a 3-year clinical study during World War II that acute respiratory disease ('ARD') might be caused by a virus (Commission on Acute Respiratory Diseases, 1947). ARD was a recurrent problem in the training of military recruits.

The report by Hilleman and Werner was preceded by the reported isolation of a similar agent by Rowe et al. (1953) from fragments of human adenoids maintained in culture. The term Adenoid Degenerating agent (AD) was used by these workers. The agent could be passaged serially to fresh cultures of HeLa cells. In the following year, this group reported that from the study of 146 strains at least 6 types of the virus existed and all had a common (complement fixing) antigen (Huebner et al., 1954). The strains of types 1, 2, 5 and 6 had mostly been obtained from adenoid or tonsillar tissue taken from healthy children. Type 3 was obtained from nasal washings and throat, conjunctival or anal swabs from patients with a respiratory illness and/or conjunctivitis. The agent recovered by Hilleman and Werner (1954) was designated type 4.

Although Huebner et al (1954) used the term Adenoidal-Pharyngeal-Conjunctival (APC) agent to replace their term AD agent, the name adenovirus was agreed upon and gained general acceptance (Enders et al., 1956).

At least 35 distinct types of human adenovirus are currently known to exist, and there is a candidate type 36 (Wigand et al., 1980). The individual serotypes are distinguishable on the basis of serum neutralisation tests and (in most cases) by haemagglutination inhibition tests (see Section 2.e. below). However, wild strains with the characteristics of more than one serotype are occasionally encountered (Wigand et al., 1965; Rafajko, 1966). The prototype strains of adenovirus types 7 to 31 were first described in the years 1955 to 1965 and were from patients in U.S.A., Europe and Saudi Arabia (see Béládi, 1972). Brief details of the 35 prototype human adenovirus strains are given in Table 0.2.

In addition to the human adenoviruses, there are at least 50 serotypes of animal origin (see reviews by Andrewes and Pereira, 1972; Andrewes, Pereira and Wildy, 1978). Adenoviruses have been isolated from monkeys and chimpanzees, cattle, horses, sheep, pigs, dogs, mice and birds (Norrby et al., 1976). In general, the relationship between strains isolated in different laboratories has not been investigated as thoroughly as with human strains, and it is difficult to ascertain the precise number of serotypes discovered from individual species.

All mammalian adenoviruses have a common (group) antigen, with the exception of some bovine strains (Bartha, 1969; Bürki et al., 1978). Avian adenoviruses share an antigen which is distinct from the mammalian group antigen (McFerran et al., 1975). The absence of any immunologically cross-reacting structural protein in mammalian and avian adenoviruses has led to the taxonomic segregation of the

TABLE 0.2 The prototype strains of adenovirus.

Serotype	Prototype	Specimen	Country of Origin	Clinical Illness	Isolation System	Reference
1	Adenoid 71	adenoid	U.S.A.	hypertrophied tonsils and adenoids	adenoid	Huebner et al., 1954
2	Adenoid 6	adenoid	U.S.A.	hypertrophied tonsils and adenoids	adenoid	Huebner et al., 1954
3	G.B.	nasal washing	U.S.A.	common cold, volunteer	HeLa	Huebner et al., 1954
4	RI-67	throat washing	U.S.A.	primary atypical pneumonia	human tracheal epithelium	Hilleman and Werner, 1954
5	Adenoid 75	adenoid	U.S.A.	hypertrophied tonsils	adenoid	Huebner et al., 1954
6	Tonsil 99	tonsil	U.S.A.	hypertrophied tonsils	tonsil	Huebner et al., 1954
7	Gomen	throat washing	U.S.A.	pharyngitis	HeLa	Berge et al., 1955
8	Trim	conjunctiva*	U.S.A.	keratoconjunctivitis	HeLa	Jawetz et al., 1955
9	Hicks	stool	U.S.A.	rheumatoid arthritis?	HEK	Kibrick et al., 1957
10	J.J.	eye swab	U.S.A.	conjunctivitis	HeLa	Rowe et al., 1956
11	Slobitski	stool	U.S.A.	paralytic poliomyelitis	HEK	Kibrick et al., 1957
12	Huie	stool	U.S.A.	nonparalytic polio	HEK	Kibrick et al., 1957
13	A.A.	anal swab	U.S.A.	none	HeLa	Rowe et al., 1958a
14	de Wit	throat washing	Netherlands	acute respiratory disease	HeLa	van der Veen and Kok, 195
15	Ch.38	conjunctiva*	Saudi Arabia	early trachoma	Chang Conjunctiva	Murray et al., 1957

*conjunctival scraping

TABLE 0.2 continued

Serotype	Prototype	Specimen	Country of Origin	Clinical Illness	Isolation System	Reference
16	Ch.79	conjunctiva*	Saudi Arabia	early trachoma	Chang Conjunctiva	Murray et al., 1957
17	Ch.22	conjunctiva*	Saudi Arabia	early trachoma	Chang Conjunctiva	Murray et al., 1957
18	D.C.	anal swab	U.S.A.	Niemann-Pick disease	human embryo cornea	Rowe et al., 1956
19	AV-587	conjunctiva*	Saudi Arabia	trachoma	Chang Conjunctiva	Bell et al., 1960
20	AV-931	conjunctiva*	Saudi Arabia	conjunctivitis	Chang Conjunctiva	Bell et al., 1960
21	AV-1645	conjunctiva*	Saudi Arabia	trachoma	Chang Conjunctiva	Bell et al., 1960
22	AV-2711	conjunctiva*	Saudi Arabia	trachoma	Chang Conjunctiva	Bell et al., 1960
23	AV-2732	conjunctiva*	Saudi Arabia	trachoma	Chang Conjunctiva	Bell et al., 1960
24	AV-3153	conjunctiva*	Saudi Arabia	trachoma	Chang Conjunctiva	Bell et al., 1960
25	BP-1	anal swab	U.S.A.	none	HeLa/KB	Rosen et al., 1961
26	BP-2	anal swab	U.S.A.	none	HeLa/KB	Rosen et al., 1961
27	BP-4	anal swab	U.S.A.	none	HEK/KB	Rosen et al., 1961
28	BP-5	anal swab	U.S.A.	none	HEK/KB	Rosen et al., 1961
29	BP-6	anal swab	U.S.A.	none	KB	Rosen et al., 1962a
30	BP-7	anal swab	U.S.A.	none	KB	Rosen et al., 1962a

*conjunctival scraping

TABLE 0.2 continued

Serotype	Prototype	Specimen	Country of Origin	Clinical Illness	Isolation System	Reference
31	15/62	stool	England	none	HeLa	Pereira et al., 1965
32	H.H.	anal swab	U.S.A.	none	HEK	Blacklow et al., 1969
33	D.J.	anal swab	U.S.A.	none	HEK	Blacklow et al., 1969
34	Compton	urine	U.S.A.	renal transplant recipient	WI-38	Hierholzer et al., 1975
35	Holden	lung and kidney	U.S.A.	renal transplant recipient	lung and kidney/HEK	Stalder et al., 1977

adenoviruses into 2 genera: Mastadenovirus and Aviadenovirus (Norrby et al., 1976).

2. Properties.

a. Chemical Composition and Structure. (Reviewed by Taylor (1977) and Wold et al. (1977)). Adenoviruses are 70 to 80 nm in diameter and consist of a capsid of protein with a dense inner core containing DNA and protein. The overall shape of the capsid is icosahedral, which gives the particles a hexagonal outline when examined by negative staining EM. The capsid is made up of 252 morphological units or capsomers of protein arranged in 20 equilateral triangular faces (see Andrewes, Pereira and Wildy, 1978). The 240 non-vertex capsomers (occupying all faces and edges of the particle) have 6 neighbouring subunits and are called hexons, whereas the 12 vertex units of the particle have 5 neighbouring subunits and are called pentons (Ginsberg et al., 1966).

Each penton consists of a penton base (the vertex capsomer) and an outward-projecting fibre approximately 2 nm in diameter (see Valentine and Pereira, 1965). Visualisation of intact fibres in situ in unpurified preparations of virus by negative staining EM is very rare (C.R. Madeley, personal communication). The fibres of human adenoviruses appear to vary in length (9 to 31 nm) depending on the serotype (Norrby, 1969b). Fibres can be deliberately dissociated from the penton base by trypsin (Valentine and Pereira, 1965).

The core of adenovirus particles is 60 to 65 nm in diameter (Norrby et al., 1976) and consists of at least 2 proteins in addition to DNA.

Proteins. SDS-polyacrylamide gel electrophoresis of viral components has allowed the resolution of at least 10 structural polypeptides,

with molecular weights between 5,000 and 140,000 daltons (see Everitt et al., 1975; Wold et al., 1977; Wadell, 1979). The polypeptides designated II, III and IV have been identified as the structural subunits of the hexon, penton base and fibre respectively (see Table 0.3). Most of the other polypeptides appear to be located internally, or are only partially exposed on the surface of the virion (for references, see Wold et al., 1977). Wadell (1979) has suggested that a comparison of the molecular weights of polypeptides II, III and IV, and also a fourth structural polypeptide IIIa, may give an identifying fingerprint for each human serotype.

DNA. The genome consists of one piece of double-stranded DNA which is linear. The molecular weight of the DNA differs between serotypes (20 to 30 x 10^6 daltons), as does the guanidine and cytidine (G + C) content relative to the adenosine and thymidine (A + T) content (see Norrby et al., 1976; Wold et al., 1977). The homology of DNA from different human serotypes has been examined by molecular hybridisation techniques, and different subgroups of human adenovirus have been distinguished on this basis (see Green et al., 1979). The nucleotide sequence on different DNA molecules is not circularly permuted and duplex adenovirus DNA can be cleaved into specific fragments by different bacterial restriction enzymes (Pettersson et al., 1973). Resolution of these fragments by electrophoresis in agarose gels and examination of the cleavage products of different enzymes have given information about the organisation of the adenovirus genome (see Wadell and Varsanyi, 1978).

b. Biophysical Properties. The buoyant density of mammalian adenoviruses in caesium chloride gradients is 1.33 to 1.35 g./cm^3 and the sedimentation coefficient is 560 S (Norrby et al., 1976).

TABLE 0.3 The major polypeptides of adenovirus type 2 particles (from Everitt *et al.*, 1975). The molecular weights of polypeptides (separated by SDS-polyacrylamide gel electrophoresis) may differ considerably between different serotypes (Wadell, 1979).

Polypeptide	Molecular Weight	Location
II	120 000	Subunit of hexon
III	85 000	Subunit of penton base
IIIa	66 000	External, near vertex
IV	62 000	Subunit of fibre
V	48 500	Core protein
VI	24 000	Hexon-associated (internal)
VII	18 500	Core protein
VIII	13 000	Hexon-associated (internal)
IX	12 000	Associated with groups of nine hexons

c. Infectivity In Vitro. Adenoviruses from a particular species of animal usually produce the largest quantity of infectious progeny in cell cultures derived from tissues of that species. Cells which allow the production of infectious virus are said to be permissive: most non-human cells are non-permissive to human adenoviruses. However, rhesus monkey kidney cells can be used for the isolation and characterisation of some adenovirus strains (see Grist et al., 1979). Human adenoviruses of the established serotypes generally grow to highest titre in cultures of continuous human cell lines or in primary human embryo kidney (HEK) cell cultures (Jackson and Muldoon, 1975). Most adenovirus strains of established serotypes produce a characteristic cytopathic effect (CPE) consisting of cell rounding and the formation of grape-like clusters of cells. This is usually accompanied by increased acidity of the culture medium (Rowe et al., 1955).

Quantitation of adenovirus infectivity with susceptible cells can be by a standard plaque assay or a tube dilution ('quantal') assay (see Fenner et al., 1974a). Titres of 10^9 TCD_{50}/ml may be obtained with common established human serotypes, but other serotypes (in particular, type 8) will not give titres above 10^2 to 10^3 TCD_{50}/ml (Jackson and Muldoon, 1975).

The series of events in the replication of adenovirus type 2 and type 5 'laboratory' strains have been studied extensively (see Section 3.b. below). However, wild strains of adenovirus in clinical specimens may take several days or weeks to cause CPE in permissive cell cultures (van der Veen and Kok, 1957; Grayston et al., 1958). Strohl and Schlesinger (1965) suggested that the extended 'latent period' was analagous to the situation where adenoviruses persist in tonsils and adenoids and only become apparent after prolonged incubation of these tissues in vitro.

d. Stability. Unpurified suspensions of adenoviruses can be stored at -20°C or lower without loss of infectivity (see Kasel, 1979). Ginsberg (1956) originally reported the stability of adenovirus (types 1 to 4) which retained infectivity for at least 70 days at 4°C and for at least 7 days at 36°C (pH 7.4). However, infectivity was rapidly destroyed on incubation at 50°C. These serotypes were resistant to a wide range of pH (pH 6.0 to 9.0 at room temperature) and viral suspensions were not completely inactivated when held at room temperature for 30 min at pH 1.5 to 2.5.

Adenoviruses are resistant to ether, chloroform and fluorocarbons but selected serotypes have been found to be inactivated by acetone, sodium dodecyl sulphate (SDS; 0.25 per cent), chlorine and ultraviolet radiation (see Ginsberg and Dingle, 1965; Jackson and Muldoon, 1975; Kasel, 1979). The infectious adenovirus particle (type 5) was found to be resistant to trypsin (Pereira, 1958).

e. Haemagglutination. The human adenoviruses (except type 18; Wigand and Keller, 1978) cause agglutination of erythrocytes of at least one mammalian species. Different serotypes can be grouped according to their ability to agglutinate either rat or rhesus monkey erythrocytes (Rosen, 1960). The subgroups have been designated I to IV (see Table 0.4). Two forms of haemagglutination are observed: complete and partial (or incomplete), and partial haemagglutination can be enhanced by the addition of neterotypic antiserum (see Norrby, 1969b).

The subgrouping of human adenoviruses according to haemagglutination properties with rat and monkey erythrocytes is not a rigid classification since different workers have reported different results of haemagglutination with the same serotype. For example, types 12

TABLE 0.4 Subgroup classification of adenoviruses on the basis of haemagglutination (after Kasel, 1979). This follows the original classification by Rosen (1960).

Subgroup	Types	Haemagglutination	
		Rat Erythrocytes	Rhesus Erythrocytes
I	3, 7, 11, 14, 16, 21, 34, 35	No	Yes
II	8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24, 25, 26, 27, 28, 29, 30, 32, 33	Yes	Yes or No
III	1, 2, 4, 5, 6	Partial*	No
IV	12, 18, 31 †	No	No

* Group III members produce a complete haemagglutination pattern with rat erythrocytes in the presence of heterotypic antiserum

† agglutination of rat erythrocytes has been reported by some workers (see Wigand and Keller, 1978)

and 31 have been reported to show mild haemagglutinating activity with rat erythrocytes in some studies (see Wigand and Keller, 1978), but they were originally classified as having no haemagglutinating activity (see Wigand, 1970). Types 20, 25 and 28 have also been difficult to classify by Rosen's scheme (Wigand, 1970).

Hierholzer (1973) studied haemagglutination reactions between 33 prototype strains and erythrocytes from 14 animal species. With the use of human erythrocytes in addition to rat and monkey cells, 10 subgroups were distinguished. In a coded trial, over 90 per cent of wild strains tested were correctly subgrouped. (Haemagglutination can be used to narrow the possibilities as to the serotype of an adenovirus isolate but identification by type can only be achieved if this is followed by a haemagglutination inhibition test or neutralisation test with type-specific antisera).

Haemagglutination is due to fibre antigen and in particular to the determinant 'gamma' at the distal end (see Norrby, 1969b). The presence of more than one fibre on the virion allows the virus particle to form a bridge between erythrocytes. Complete haemagglutination by soluble components (that is, in the absence of virus particles) is said to be due to aggregates of pentons or of fibres alone, with gamma determinants exposed. The ennancement of haemagglutination due to soluble components by the addition of heterotypic antiserum (especially with adenoviruses of Rosen's subgroup III) is probably due to the bridging of monomers (pentons or fibres alone) already attached to erythrocytes by their gamma determinants (Norrby, 1969b). The phenomenon of haemagglutination inhibition is probably due to the blockage of gamma determinants on the fibre by type-specific antibodies.

f. Antigenic Characteristics. The positions of antigens on the adenovirus particle were extensively studied during the 1960s. Ginsberg and co-workers showed that by disrupting purified particles of adenovirus type 5 at high pH, group-specific ('L') and type-specific ('E') antigens (measured by complement fixation) were obtained. These corresponded immunologically to the soluble antigens that are produced in excess in virus infected cells (Wilcox and Ginsberg, 1963a). (E and L referred to the rate of elution of these antigens from DEAE-cellulose). The group-specific antigen was shown to correspond to hollow polygonal rods by electron microscopy (later called hexons), whereas fibres ('thread-like strands') from disrupted particles were shown to have type-specific antigenicity (Wilcox, Ginsberg and Anderson, 1963). Purified L and E antigens (hexons and fibres) were shown to give rise separately to neutralising antibodies in rabbits (Wilcox and Ginsberg, 1963b).

In order to explain the ability of hexons to act as type-specific antigens, Wilcox and Ginsberg (1963b) suggested that the L antigens (hexons) might be composed of different components which had either group- or type-specificity. The structure of the vertex units was described in detail by Valentine and Pereira (1965) and this was followed by the adoption of the terms hexon, penton and fibre (Ginsberg et al., 1966). Kjellén and Pereira (1968) prepared antisera in guinea pigs against purified hexons, pentons and fibres from adenovirus type 5. Although anti-hexon sera had group-specific antibodies by complement fixation and immunodiffusion, they also had much higher (type-specific) neutralising capacity than anti-penton or anti-fibre sera. Therefore the finding of Wilcox and Ginsberg (1963b) was confirmed and it was concluded that the production of neutralising antibody is elicited mainly by the hexon.

The immunological specificities carried by different structural

components have been summarised by Norrby (1969b). This remains the standard review. The specificities are discussed below and summarised in Table 0.5.

Hexons. The main group antigen, designated 'alpha', is located on the hexon and is demonstrable by complement fixation or immunodiffusion (see Valentine and Pereira, 1965). Norrby et al. (1969) observed that only homotypic anti-hexon sera associated with whole virus particles by immune electron microscopy and from this they suggested that the group-specific antigenic determinant alpha is located within the virus particle rather than being exposed.

The antigenic determinant against which the neutralising activity of anti-hexon sera is directed (Wilcox and Ginsberg, 1963b; Kjellén and Pereira, 1968; Norrby, 1969a) is termed 'epsilon'. Antigenic determinants on hexons with subgroup specificities are also known to exist (Norrby. 1969b).

Vertex Capsomers (Penton Base). Pentons (and in particular, penton bases) are responsible for the 'early' cytopathic effects seen in cell cultures infected with high-titre virus (Pereira, 1958; Valentine and Pereira, 1965).

One group-specific antigenic determinant is known to exist ('beta') from studies on the ability of heterotypic antiserum to enhance agglutination by free pentons (see Norrby, 1969b). Although antigens with subgroup specificities are known to exist, no antigens with type specificity have been demonstrated (Norrby, 1969a).

Fibres. The antigenic determinant 'gamma' elicits the formation of type-specific antibodies which inhibit haemagglutination by the same serotype. Antibodies directed against gamma (or rather, purified fibres) have shown neutralising activity in fluorescent focus

TABLE 0.5 Adenovirus structural components and their main immunological specificities (after Norrby, 1969b).

Structure	Antigen		Remarks
	Designation	Specificity	
Hexon	alpha	group	probably not exposed at surface of virion
	epsilon	type	available at surface of virion; reacts with neutralising antibodies
Penton base	beta	group*	(penton base carries toxin activity)
Fibre	delta	intra-subgroup*	situated at proximal part of fibre but not present in members of Rosen's subgroup I
	gamma	type	reacts with haem-agglutination inhibiting and, in some experiments, neutralising antibodies

* evidence for these antigens comes from haemagglutination enhancement experiments

inhibition tests (Pettersson and Höglund, 1969) but not in plaque inhibition tests (Kjellén and Pereira, 1968; Norrby, 1969a; Pettersson and Höglund, 1969). Norrby (1969a and 1969b) suggested that anti-gamma antibodies may only cause a delay in replication rather than causing complete inhibition. Wadell (1972) demonstrated that the degree of neutralising activity of antisera against purified fibres depended on the serotype: antibodies to type 3 fibres had some capacity to neutralise homotypic virus.

An antigen situated on the proximal part of fibres ('delta') is known to exist by haemagglutination enhancement reactions involving free fibres (see Norrby, 1969b). Unlike beta on the penton base, which is group specific, delta is common to members of the same subgroup (Norrby, 1969b). However, viruses with the shortest fibres (members of Rosen's subgroup I; Rosen, 1960) appear to possess only the gamma antigen.

g. Transforming Properties. The inoculation of non-permissive cells with adenoviruses at high multiplicity (that is, large numbers of infectious virus per single cell) can induce a state of transformation in a small proportion of cells (see Green, 1970). Transformed cells in culture lose contact inhibition and grow to high density. Human adenoviruses can transform rat, hamster and rabbit cells. There is substantial evidence from nucleic acid hybridisation studies that transformed cells contain more than one copy of some or all of the adenovirus genome (integrated in the cell DNA) (see Wold et al., 1977). The alteration of cell growth is thought to be due to the action of proteins coded by the integrated viral genes. Certain of the proteins produced in transformed cells are antigenic (the 'T antigens') and at least one virus-coded antigen is present on the cell surface. (The phenomenon of adenovirus DNA integration may not be peculiar to transformation since virus DNA has been found

to be associated with cell DNA in a number of different permissive cell cultures undergoing productive infection by types 3, 5 and 9-15 (Kümel and Hammer, 1980)).

h. Oncogenicity. An alternative classification of adenoviruses to that of Rosen (1960) was suggested by Huebner (1967) and was based on the different abilities of different serotypes to cause the development of tumours in hamsters. Types 12, 18 and 31 (subgroup A) induce tumours when injected into newborn hamsters, usually within 4 months. Types 3, 7, 11, 14, 16 and 21 (subgroup B) are weakly oncogenic, in that they induce tumours in a small proportion of animals and this generally takes longer (4 to 18 months). The remaining serotypes (subgroup C) do not cause tumours in newborn hamsters. The classification of adenoviruses by oncogenic properties corresponds to the relative G + C content of the virion (Pina and Green, 1965). Subgroup A adenoviruses have the lowest G + C content (47-49 per cent). Adenoviruses from other animals have been found to induce tumours (see Green, 1970).

Sera from hamsters with adenovirus-induced tumours usually react in complement fixation and immunofluorescence tests with T antigens produced in transformed cells and in productively infected cells before viral DNA synthesis (see Gilead and Ginsberg, 1965; Levirthal et al., 1966; Hayashi and Russell, 1968; Rhim et al., 1968).

i. Helper Function. Adenoviruses have a helper function in the replication of small 20-25 nm diameter DNA viruses called adeno-associated viruses (AAV). These were originally found as contaminants in adenovirus stocks. Atchison et al. (1965) were the first workers to report the dependence of AAV on adenoviruses for their replication and to show that the 2 viruses were otherwise unrelated.

Although adenovirus is required for AAV replication, AAV inhibits adenovirus replication (Hoggan et al., 1966), giving reduced yields of adenovirus complement fixing antigen and infectious adenovirus (Parks et al., 1968). Therefore the total number of adenovirus particles produced is altered rather than the proportion of infectious to uninfectious particles.

AAV appears to require the presence of helper adenovirus at different stages of its replication (Young and Mayor, 1979). Four distinct types (or groups) of AAV have been distinguished by neutralisation, immunofluorescence, complement fixation and immunodiffusion (see Hoggan, 1970).

j. Mutation. Host range and temperature-sensitive mutants of several human adenovirus serotypes have been isolated (see Taylor, 1977; Wold et al., 1977). Complementation analysis and recombination studies with these mutants have allowed genetic mapping of the adenovirus genome. However, genetic maps on the basis of recombination frequencies give a linear order only and not the physical location of the mutations on the DNA. Methods of correlating genetic maps with the physical organisation of the genome (as determined by DNA restriction site mapping) are now being investigated.

3. Replication.

a. Microscopic Observations. The site of replication of adenoviruses within the cell was studied initially by thin section electron microscopy, and by immunofluorescence and cytological staining methods (see Ginsberg and Dingle, 1965).

In haematoxylin and eosin-stained preparations of cells infected with the common prototype strains, sequential cytological changes were observed (Boyer et al., 1957; Boyer et al., 1959). The earliest change is the appearance of many eosinophilic Feulgen-negative bodies

in the nucleus. Later, the inclusion bodies enlarge and become basophilic, forming Feulgen-positive masses. The nuclear changes observed with cells infected by types 1, 2, 5 and 6 differed in detail to those described with types 3, 4 and 7, but these studies clearly implicated the nucleus as the main site of adenovirus replication. EM studies of thin sections of cells infected with the common serotypes, with the finding of crystals of virus particles in the nucleus, gave further evidence that adenovirus replication was predominantly nuclear (Harford et al., 1956; Morgan et al., 1956).

Boyer et al. (1959) studied the development of adenovirus antigens in HeLa cells infected with type 4, by the indirect immunofluorescence technique. (The antiserum used was prepared in rabbits and the test was apparently specific for type 4 antigens but not antigens of other serotypes). Viral antigens were located within the nucleus. The antigen distribution within the nucleus by immunofluorescence corresponded to that of granular aggregates seen by phase contrast microscopy. There was some diffuse cytoplasmic staining from 48 hours after infection by immunofluorescence, and this coincided with the onset of cytopathic effect.

The location of adenovirus antigens within the nucleus of cells undergoing productive infection by other serotypes has been reported by other workers. For example, Levinthal et al. (1966) described the development of type 12 structural antigens in various primate cells. Rabbit antisera were used which were directed against unpurified virus. The appearance of flecks and dots in the nucleus gave way to brightly staining masses 26-30 hours after infection. Beyond 30 hours, antigens were detected in the cytoplasm of some cells and this was interpreted as being due to breakdown of the nuclear membrane. KB and HEK cells showed the highest proportion of brilliantly stained nuclei in this study.

Hayashi and Russell (1968) used antisera directed against hexons, fibres and penton bases of adenovirus type 5 in a time study of the development of different antigens in HEK cells by immunofluorescence. Cells infected at high multiplicity showed fluorescent staining in the nucleus from 10-11 hours after infection, using any one of the three specific antisera. The brightness of staining increased after this time and filled the nucleus by 24 hours.

b. Events in Productive Infection. Most studies on the replicative cycle of human adenoviruses *in vitro* have used type 2 or type 5 virus and KB or HeLa cells. The events of the cycle at the molecular level are reviewed by Taylor (1977) and Wold *et al.* (1977) and only brief details are given here. The cycle takes 24 or more hours, but replication is more rapid when the multiplicity of infection is high. There is an early phase, during which early mRNA sequences are transcribed (before the onset of DNA synthesis 8-10 hours after infection) and a late phase which is dependent on the start of DNA synthesis.

Early Events. Uptake of virus particles may be by pinocytosis, or perhaps by direct penetration of the cell membrane. Uncoating of the virus particles takes place during migration to the nucleus, and the resulting viral cores release their DNA within the nucleus. The nuclear RNA transcripts are large precursors which are cleaved and transported to the cytoplasm. DNA sequences giving rise to early mRNA are probably transcribed by a cellular enzyme.

Translation occurs in the cytoplasm. The role of early products of translation (such as T and P antigens (Hayashi and Russell, 1968) and other infected cell-specific polypeptides whose appearance does not depend on DNA synthesis) is unknown.

DNA Replication. Viral DNA synthesis begins about 7 hours after infection and host cell DNA synthesis diminishes in parallel. The

enzymes involved in viral DNA synthesis are not known, although proteins have been found which bind to single stranded DNA.

Late Events. The shift from early to late gene expression at the onset of DNA synthesis results in the synthesis of structural polypeptides. This takes place on free cytoplasmic polyribosomes. After the combination of constituent polypeptides into structural proteins, these are transported into the nucleus and assembled into virions (Velicer and Ginsberg, 1970).

All major structural polypeptides are produced in excess of the quantity assembled into virions. Both complete and empty capsids are produced, the latter containing less DNA. The proportion of empty capsids produced may depend on the serotype (for references, see Wadell et al., 1973).

The production of infectious virus in vitro is inhibited if the medium lacks arginine (Rouse et al., 1963). The reason for the dependency of adenovirus replication on arginine is not yet understood (see Wigand and Kümel, 1978).

B. ADENOVIRUS INFECTIONS IN HUMANS.

1. Illnesses Associated with Infection by Adenoviruses.

a. General Comments. Adenoviruses *in vivo* appear to replicate best in mucous membranes and lymphoid tissue. They are often isolated from apparently healthy individuals and only about one third of the established serotypes have been associated with illness (Chanock, 1974). The illnesses which most commonly accompany infection by adenoviruses, together with the serotypes implicated, are shown in Table 0.6. The relationship between illness and infection by different adenovirus serotypes is summarised concisely by Taylor (1977): 'a particular serotype may give rise to a variety of clinical syndromes and conversely, a single disease entity may result from infection with any one of a number of serotypes'.

b. Respiratory Illness. Adenovirus infection of the respiratory tract is common in young children and often takes the form of an acute upper respiratory tract illness such as acute febrile pharyngitis. Pharyngitis may be accompanied by conjunctivitis, in which case the disease entity is referred to as pharyngoconjunctival fever (Table 0.6; see Jackson and Muldoon, 1975; Foy and Grayston, 1976).

In adults, adenovirus infection may cause an acute respiratory disease. Military recruits are well documented as targets for outbreaks of adenovirus-associated acute respiratory disease. Types 4, 7 and 21 have been isolated most frequently from such outbreaks (Huebner *et al.*, 1958; van der Veen, 1963).

Severe adenovirus-associated pneumonia can occur in children, and this may be fatal. Pneumonia may occur as a complication of

TABLE 0.6 Illnesses most commonly associated with infections by adenoviruses (after Kasel, 1979).

Disease	Individuals Most At Risk	Serotypes Commonly Involved
Acute febrile pharyngitis	Young children	1, 2, 3, 5, 6, 7
Pharyngoconjunctival fever	School-age children	3, 7, 14
Acute respiratory disease (ARD)	Military recruits	3, 4, 7, 14, 21
Pneumonia	Young children	1, 2, 3, 7
	Military recruits	4, 7
Epidemic keratoconjunctivitis	Any age group	8, 11, 19
Acute haemorrhagic cystitis	Young children	11
Pertussis-like illness	Young children	5

acute respiratory disease in adults (see Jackson and Muldoon, 1975). Adenoviruses have also been associated with illness indistinguishable from whooping cough (Table 0.6; see Nelson et al., 1975).

c. Eye Infections. Keratoconjunctivitis resulting from adenovirus infection has been reported widely (for references, see Taylor, 1977). Adenovirus-associated keratoconjunctivitis has been observed frequently in shipyard workers and has also been reported in opthalmic practices. Transmission from person to person is probably by finger to eye contact, or by the use of contaminated instruments. Type 8 is most frequently associated with this condition (see Jawetz, 1959; Dawson et al., 1963) but type 19 has also been implicated (Guyer et al., 1975).

Other serotypes of adenovirus may infect the eyes. This is emphasised by the fact that 11 of the 35 prototype adenovirus strains were isolated from eye swabs or conjunctival scrapings (Table 0.2).

d. Other Non-Respiratory Illnesses. Acute haemorrhagic cystitis may be accompanied by excretion (in urine) of adenovirus type 11 (Numazaki et al., 1968; Numazaki et al., 1973) or type 21 (see Mufson and Belshe, 1976).

Adenoviruses have been associated with several other illnesses, namely intussusception, meningitis, encephalitis, juvenile rheumatoid arthritis, orchitis, thyroiditis, appendicitis, neonatal sepsis (for references, see Kasel, 1979). However, there is insufficient evidence at present for a causal relationship between illness and virus infection in these cases.

Gastroenteritis. Some respiratory infections by common adenovirus serotypes have been reported to be accompanied by 'gastrointestinal symptoms' such as abdominal pain, vomiting and diarrhoea. For

example, Kjellén et al. (1957) reported that about one half of the cases during a type 3-associated epidemic of pharyngoconjunctival fever also had gastroenteritis.

A similar outbreak in the Netherlands was associated with adenovirus types 3 and 4, and about one third of the 57 patients had nausea, vomiting or diarrhoea (van der Veen and van der Ploeg, 1958). Stools appeared to be a better source of virus than throat swabs, and from this these workers concluded that infection was not restricted to the respiratory tract.

Gastroenteritis accompanied by acute respiratory illness apparently caused by type 7 was reported by Sterner et al. (1961). Isolations of type 7 in stools were significantly more frequent from children admitted to hospital with respiratory illness and gastroenteritis (53 out of 84 cases) than from children admitted with respiratory illness alone (36 out of 138 cases). No adenovirus infections were detected in a group of control patients with scarlet fever, and no bacterial pathogens were isolated from the study group.

There are relatively few reports of outbreaks of diarrhoea and/or vomiting associated with established adenovirus serotypes. Duncan and Hutchison (1961) reported the occurrence of such an outbreak involving 21 members of 5 neighbouring families. 11 members were studied, and adenovirus type 3 was isolated from 7. Moritsugu (1969) reported an outbreak of diarrhoea in an infant home in Tokyo. Types 6 and 31 were isolated. It is important to remember that several reports exist which emphasise that shedding of adenoviruses in stools is often asymptomatic (see Section 2.a. below). It is possible that the occurrence of gastroenteritis and spread of adenovirus infection in these cases were only coincidental and not causally related.

In prospective studies into the shedding of cultivable viruses by infants, conflicting reports have been published regarding the possible role of adenoviruses in causing diarrhoea. For example, Moffet et al. (1968) reported that of 167 infants in Chicago who were admitted to hospital with diarrhoea (sufficiently severe to require intravenous therapy), 17 per cent shed adenoviruses which could be cultured (from stools and/or throat swabs). Specimens from only 5 per cent of 95 control subjects yielded adenoviruses in culture. These workers considered that the increased frequency of adenovirus recovery in patients with diarrhoea could have been the result of diarrhoea rather than the cause (for example, by increased shedding of latent virus). However, the possible etiological association of adenoviruses with diarrhoea was considered to be an equally valid explanation.

In contrast, Yow et al. (1970) reported that in a study group of similar size in Texas, there was no significant difference between the frequencies of recovery of adenoviruses in culture from infants in the acute stage of gastroenteritis and from control infants. This was in agreement with the findings of Bell and Grist (1967) who reported that from the results of a 7-year study of gastroenteritis in Scotland, it was unlikely that adenoviruses (or enteroviruses) are significant causes of diarrhoea.

Brandt et al. (1979) studied the shedding of stool viruses (by EM and culture) in 604 gastroenteritis inpatients and 200 gastroenteritis outpatients at the Children's Hospital, Washington D.C. Stool adenoviruses which grew in cell culture were recovered less frequently from gastroenteritis patients than from control subjects. (Adenoviruses were rarely detected by EM in the stools from which adenoviruses were cultured). However, there was a highly significant difference between the frequency of adenovirus detection by EM

from gastroenteritis patients (3.9 per cent) and from the control subjects (0.6 per cent). This led Brandt and co-workers to suggest that 'adenoviruses detectable by EM are the prime enteric pathogens among the adenoviruses'. 26 of 31 specimens from gastroenteritis inpatients, containing adenoviruses detectable by EM, did not cause CPE. Therefore the antigenic status of these suggested enteric pathogens was left undefined.

Adenoviruses which grew poorly or not at all in cell culture have also been associated with outbreaks or incidents of gastroenteritis. The outbreak reported by Flewett et al. (1975) has already been described (see Introduction). Whitelaw et al. (1977) reported 2 cases of severe gastroenteritis (15-month old twins, one of whom died). Gut homogenate from the fatal case caused CPE typical of adenovirus in culture, but this was insufficient for identification of the virus by neutralisation (J. Parry, personal communication). Adenovirus particles were seen by thin section EM in the nucleus of a mucosal cell. Stool from the child who survived contained adenovirus particles by EM and caused CPE in cell culture but this did not redevelop on passage.

Richmond et al. (1977) reported an outbreak of gastroenteritis among 17 children on an RAF base in England. Adenoviruses were seen by EM in acute phase stool specimens from 6 children, but the viruses could not be cultured. Specimens obtained from these children more than 7 days from the onset of the illness were negative for adenoviruses by EM. The association of adenovirus excretion (in large quantity) with the acute stage of the illness was considered to be highly significant, and the incubation period for the adenovirus-associated illness was estimated to be 8-10 days.

The degree of intestinal dysfunction that results from adenovirus infection has not been examined in detail. Mavromichalis et al. (1977) reported that 3 patients in the acute phase of gastroenteritis who had adenoviruses present in small intestinal juice and stool, also had low blood xylose levels. It was suggested that the association between virus in the intestine and xylose malabsorption might indicate mucosal damage as a result of virus infection. The degree of success in culturing these adenoviruses was not stated.

2. Infection and Immunity.

a. Shedding of Adenoviruses. The shedding of adenoviruses by young children living together in a crowded welfare institution was reported by Bell et al. (1961). Throat and anal specimens were collected at least once a week. The study showed that adenovirus infection rates were high in the very young, and that infection by one serotype could be followed by infection with another serotype.

The 'Virus Watch' studies (see Fox et al., 1969; Fox et al., 1977), involving continued surveillance of families with young children in New York (1961-1965) and Seattle (1965-1969), have contributed much to the present knowledge concerning adenovirus excretion. The most important points concerning adenovirus shedding (from the two studies combined) were that:

1. Adenoviruses were isolated more frequently from faecal specimens than from respiratory specimens. (This finding confirmed the results of others (for example, Rosen et al., 1962b; Vargosko et al., 1965)).

2. Prolonged intermittent shedding of adenoviruses of one serotype over several months was common. This was observed with several different serotypes (but especially types 1, 2, 3 and 5).

3. The prolonged shedding of adenoviruses was observed in both children and adults.

4. The spread of adenovirus type 2 infection to new family members occurred, apparently as a result of prolonged shedding by older siblings.

5. There was a higher illness association in infections with both respiratory and faecal shedding than in infections where either respiratory or faecal shedding alone was detected.

Intermittent excretion of the one adenovirus serotype in these studies was interpreted as either recrudescence or reinfection. Fox et al. (1969) reasoned that if renewed shedding represented re-infection, it would probably result in a re-stimulation of homotypic antibody levels. Since almost all sera taken from New York patients in 43 instances of renewed excretion (with at least 3 months 'silent period') showed no increase in haemagglutination inhibition titres, it was suggested that renewed excretion was due to a recrudescent infection. However, in the Seattle study, 21 instances were recognised in which excretion by an immune individual coincided with an episode of family infection. In these cases, renewed shedding could have been due to re-infection via other family members. 3 of these individuals developed significant rises in serum neutralising antibody. 32 of 86 immune persons with renewed adenovirus excretion and/or a significant homotypic antibody rise were exposed in family infection episodes. Fox et al. (1977) interpreted this as representing possible reinfection.

Adenovirus excretion was found to occur throughout the year in the Virus Watch studies, but the lowest mean prevalence of excretion in healthy individuals was in September.

Brandt et al. (1969) reported a controlled study of respiratory tract disease in 18000 infants, which lasted 10 years (1957-1967). This was carried out at Children's Hospital, Washington D.C. These workers reported a decrease in the number of adenovirus infections in the autumn, with peaks in the warm weather as well as in winter (Brandt et al., 1972). Their conclusions on adenovirus shedding were as follows (Brandt et al., 1969):

1. The mucous membranes of the respiratory tract are the main affected tissues in early acute infection by serotypes 1 to 7.

2. Later, virus replication spreads to the intestine and may continue for longer periods. (This conclusion is in agreement with the results of experimental infection of adults with adenovirus type 4 (Couch et al., 1966). Infection by aerosol caused the volunteers to shed adenoviruses firstly and predominantly in respiratory specimens, followed by predominantly faecal shedding).

3. Illness does not always accompany the shedding of adenoviruses from the throat or gut.

4. Adenovirus serotypes above 7 (except types 14 and 21) probably pass to the intestine with little or no infection of the respiratory tract.

b. Immunity. Levels of immunity to adenoviruses are usually assessed by measuring group-specific serum antibody levels by complement fixation, or type-specific antibody levels by neutral-

isation or haemagglutination inhibition. Significant antibody responses to infection are not always detected, and are observed less frequently in children than in adults (Schmidt et al., 1966). From their 1957-1967 study, Brandt et al. (1969) reported that the complement fixation test seldom demonstrated rises in antibody levels in serum from children under 4 months. Moreover, patients with severe (lower) respiratory tract disease showed relatively higher rates of seroconversion than did patients with moderate (upper) respiratory tract disease. This was attributed to a greater production of virus antigens in severe infections.

Type-specific serological responses to adenoviruses in adults have been demonstrated, after experimental infection of volunteers via the respiratory tract (aerosol exposure; Couch et al., 1966) and conjunctivae (Lehrich and Kasel, 1966). As a result of efforts to control acute respiratory disease in military installations, immunity to infection has been successfully achieved in adults by administration of gelatin capsules containing infectious adenovirus (Edmondson et al., 1966; Gutekunst et al., 1967; Griffin and Greenberg, 1970).

By this method, adenovirus type 4 and type 7 produced a silent infection of the intestinal tract which stimulated the development of moderately high levels of serum neutralising antibody (Couch et al., 1963; Chanock et al., 1966). When types 4 and 7 were given simultaneously, neutralising antibodies to both viruses developed (Couch et al., 1963). However, the neutralising antibody response in adults, produced by experimental infection of the intestine with type 4, was found to be lower than the response produced by natural (respiratory) infection (van der Veen et al., 1968).

Schwartz et al. (1974) reported the administration of adenovirus types 1, 2 and 5 separately in gelatin capsules to adult volunteers. This resulted in rises in serum neutralising antibody levels in at least 70 per cent of subjects (irrespective of serotype administered). Antibody levels were maximal after 3 weeks. There was no correlation between the extent of faecal adenovirus shedding and the magnitude of the serum neutralising antibody response.

From the above results, it is well established that adenovirus infection of the intestine can lead to the development of serum neutralising antibodies (in adults) and these have a protective effect against respiratory infection by the homologous serotype. However, it is not known whether circulating neutralising antibodies can confer protection against infection on the gastrointestinal tract.

Hashimoto et al. (1970) investigated this question using the mouse and a mouse adenovirus strain (K87) as a model system. They found that mice re-challenged after 3 weeks showed resistance to intestinal infection. This resistance was not simply due to pre-existing serum neutralising antibodies, since animals given serum antibodies passively were not protected from intestinal infection. 18 days or more after challenge, K87 virus neutralising substances were detected in the intestinal wall and intestinal contents of the infected mice, but not in serum-transferred mice, though both groups had equal levels of serum antibodies. The neutralising substances were found to be antibodies, predominantly of the IgA class (Hashimoto et al., 1971). Therefore, by extrapolation, the control of human adenovirus infections within the intestine may rest with the secretory immunoglobulin system, with little or no protection from circulating antibodies.

C. RELEVANT ASPECTS OF STUDIES ON OTHER STOOL VIRUSES.

1. Culture.

Of the human viruses and virus-like particles shed in stools, only the enteroviruses, adenoviruses and reoviruses have been isolated frequently in cell culture. With some exceptions, Coxsackie A enteroviruses do not grow readily in the cultures of human and monkey cells used routinely to isolate viruses (see Melnick et al., 1979). However, a cell line is now available which supports the replication of a number of group A Coxsackie virus serotypes previously found to replicate only in suckling mice. This cell line (RD) is derived from a human rhabdomyosarcoma (Schmidt et al., 1975).

Although bovine and simian rotavirus strains have been successfully propagated in kidney cultures of the appropriate species (for references, see Kapikian et al., 1979), human rotavirus strains have been particularly difficult to grow. Intracellular antigens may be detected by immunofluorescence after the addition of stool extracts to cultures of pig kidney (IB-RS-2) or LLC cells, and this is enhanced by low-speed centrifugation of the cultures after inoculation (Banatvala et al., 1975; Bryden et al., 1977).

Limited success in the propagation of human rotavirus in HEK cells was reported by Wyatt et al. (1976). Improved results have been claimed by serial passage of a human rotavirus strain in gnotobiotic piglets prior to serial passage in primary cultures of monkey cells (Wyatt et al., 1980). Propagation in these cells was performed with trypsin treatment of the virus at each passage, a technique earlier found to enhance the infectivity of calf rotavirus (cell-adapted) and thus to allow the development of a plaque assay (Matsuno et al., 1977; see also Kapikian et al., 1979). Trypsin

appears to enhance bovine rotavirus infectivity by acting directly on the virus (Barnett et al., 1979). The reason for the enhancement of infectivity is unknown, but could be related to a similar finding with reoviruses on treatment with chymotrypsin or trypsin (Spendlove and Schaffer, 1965).

Immunofluorescence has been used successfully to detect intracellular astrovirus antigens in human embryo kidney (HEK) cells (Kurtz et al., 1977). However, these viruses and several other small round viruses and virus-like particles (including the Norwalk and similar agents) have not been successfully propagated in vitro (see Kapikian et al., 1979).

2. Immunity.

Infection of the human intestine by different viruses (including rotaviruses (Dolin, 1979)) can lead to an increase in serum antibodies to the agent. Work to investigate the possible value of inactivated polio vaccine showed that suppression of intestinal poliovirus infection was not achieved after parenteral immunisation (see Bodian and Horstmann, 1965). Moreover, the successful experimental infection of volunteers who apparently had pre-existing antibodies to Norwalk agent (Kapikian et al., 1972) indicated that circulating antibodies afford little or no protection. Therefore any protection against infection in the intestine probably depends to a large extent on the presence of secretory IgA antibodies in the lumen. This is borne out by the available evidence from studies of enteric animal virus infections (see Holmes, 1979).

Administration of immune human IgG to lambs by mouth gave passive protection against rotavirus infection (Snodgrass et al., 1977). Anti-rotavirus antibodies have been detected in human milk

by neutralisation test (Thouless et al., 1977) and this finding has been confirmed by others. Breast feeding appears to have a protective effect against rotavirus infection (Totterdell et al., 1976). However, anti-rotavirus antibodies may have little significance in this protective effect (Totterdell et al., 1980).

Very little is known about the specificity of the intestinal IgA response: that is, to what extent the response against one serotype or strain of virus protects against another. Similarly, the degree of memory shown by the IgA-producing system in combating an infection by the same or a similar strain is not known.

Results with poliovirus indicate that infection of one part of the gastrointestinal tract may only lead to stimulation of local secretory IgA production (Ogra and Karzon, 1969). Therefore it is possible that sequential infection of different parts of the intestine may occur before complete immunity is achieved. This may (in part) explain the prolonged shedding of virus particles observed in the case of adenoviruses, although it is not understood why a single serotype can override the secretory immunoglobulin system and continue to produce infectious progeny over several months. Mims (1974) suggested that one reason for persistent shedding could be the impairment of the immune system due to viral infection of immunocytes.

CHAPTER 1

CHAPTER 1 MATERIALS AND METHODS.

Note. The materials and methods described relate to the study as a whole. Where variations exist, these are indicated in the Methods Sections of the appropriate chapter.

1. Stool Specimens.

a. Source. Stools were obtained from children up to the age of 3 years during studies into viruses associated with infantile diarrhoea. The stools were obtained either from children admitted to hospital, or from home as part of a community study (Madeley et al., 1977; Scott et al., 1979). The stools selected for study were those found to contain adenoviruses by electron microscopy. The patients from whom stool specimens were taken in 1976, 1977, 1978 or 1979 were given a number. The patients from whom stool specimens were taken only in 1975 were given alphabetical designation.

b. Preparation of Stool Extracts. This is described in detail elsewhere (Madeley et al., 1977). The method was briefly as follows.

Approximately 8 g. of stool material was shaken with 14 ml of phosphate buffered saline (PBS) complete with calcium chloride and magnesium chloride (see Dulbecco & Vogt, 1954) and containing penicillin (100 U/ml) and streptomycin (100 µg/ml). The solid debris was allowed to settle. The supernatant was removed and spun in a bench centrifuge at 3000 rev./min for 30 min. The supernatant fluid was stored in 2 ml amounts at -20°C and 5 ml was retained for examination by electron microscopy (EM).

Usually preparation of stool extracts for EM and negative staining were carried out on the day the specimens were received.

2. Standard Virus.

a. Source.

Adenovirus type 1 (Strain Adenoid 71). ATCC No. VR-1, NIAID Catalogue number V201-001-014 (passaged 5 times in HeLa cells and 9 times in KB cells).

Adenovirus type 2 (Strain Adenoid 6). ATCC No. VR-2, NIAID Catalogue number V202-001-014 (passaged 15 times in HeLa cells and 10 times in KB cells).

Adenovirus type 3 (Strain GB). ATCC No. VR-3, NIAID Catalogue number V203-001-014 (passaged 7 times in HeLa cells and 3 times in KB cells).

Adenovirus type 5 (Strain Adenoid 75). ATCC No. VR-5, NIAID Catalogue number V205-001-014 (passaged 4 times in HeLa cells and 10 times in KB cells).

Adenovirus type 8 (Strain Trim). NIAID Catalogue number V208-003-014 (passaged in human embryo kidney and KB cells).

Adeno Associated Virus (AAV) type 1 (Strain 1H). NIAID Catalogue number V266-001-014 (passaged 3 times in human embryo kidney, twice in secondary monkey kidney and 3 times in KB cells).

The above virus strains were obtained from National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014.

Adenovirus type 7 (passaged in HeLa cells) obtained from Mr. R. Cornall Regional Virus Laboratory, Glasgow, who received it direct from the Central Public Health Laboratory, Colindale, London.

Infectious Canine Hepatitis Virus (ICHV) (passaged in GH cells) obtained from Dr. H.J.C. Cornwell, University of Glasgow Veterinary School.

b. Propagation. Adenovirus types 1, 2, 3 and 8 were passed once in human embryo kidney (HEK) tube cultures, frozen and thawed 4 times and passed to fresh HEK cultures in 50 cm^2 flat bottles. When CPE was complete, the cell culture fluids were frozen and thawed 4 times and centrifuged at 3000 rev./min for 30 min. The supernates were stored at -70°C in 1 ml amounts.

Adenovirus types 5 and 7 were propagated in an identical manner, but in primary human amnion cells. All types were found to be neutralised by their homologous antisera (see Section 5).

Infectious Canine Hepatitis Virus (ICHV) was passed once in continuous canine kidney cells (GH cells; Dr. H.J.C. Cornwell). When showing complete CPE, the culture was frozen and thawed 4 times, clarified and stored as for the other stock adenovirus strains.

c. Titration. Titration of human adenovirus serotypes was carried out using primary human amnion cells. ICHV was titrated using GH cells. Serial tenfold dilutions of freshly thawed virus were made in maintenance medium and 0.1 ml of each dilution was added to each of 4 tube cultures. The endpoints were read at 7 days and calculated by the method of Kärber (1931).

3. Cell Cultures.

a. Source.

(i) Tissue for Primary Cultures.

Primary human amnion. Placentae were obtained at caesarian section during births at 4 different Glasgow hospitals.

Human embryo kidney (HEK) and human embryo liver. Cell cultures were prepared from fetuses terminated at hysterotomy (8th to 20th week of gestation). The organs were obtained from Dr. D.P. Gilmore, Department of Physiology, University of Glasgow.

Bovine embryo kidney (BEK). Cell cultures were prepared from primary cell cultures in 120 cm^2 flat bottles obtained from Dr. D. Snodgrass, Moredun Institute, Edinburgh.

(ii.) Continuous Cell Lines.

KB cells (oral epidermoid carcinoma, ATCC No. CCL 17) were obtained at pass number 370 from Gibco-Biocult Laboratories Ltd., Paisley.

Chang Conjunctiva cells (ATCC No. CCL 20.2) were obtained at pass number 82 from Flow Laboratories Ltd., Irvine.

Intestine 407 cells (ATCC No. CCL 6) were obtained at pass number 282 from Flow Laboratories Ltd., Irvine).

Flow 4000 Clone 2 cells were obtained at pass number 28 from Flow Laboratories Ltd., Irvine.

Bristol HeLa cells (propagated in rabbit serum) were obtained from Dr. E.O. Caul, Public Health Laboratory, Bristol. Pass number unknown.

GH cells (continuous canine kidney cells) were obtained at pass number 90 (approx.) from Dr. H.J.C. Cornwell, University of Glasgow Veterinary School.

b. Media. The composition of culture medium used for growth and maintenance of the various cell types is given in Table 1.1.

In the methods described below, medium 'with antibiotics' refers to the incorporation of penicillin at 100 U/ml and streptomycin at 100 µg/ml. Unless otherwise stated, the phosphate buffered saline (PBS) used throughout was Dulbecco's Solution A (Dulbecco and Vogt, 1954).

TABLE 1.1 Composition of growth and maintenance media used for cell cultures.

Cell Type	Growth Medium		Maintenance Medium	
Bristol Hela	Lactalbumen hydrolysate 2.5 per cent	10 ml	Lactalbumen hydrolysate 2.5 per cent	10 ml
	Sodium bicarbonate 7.5 per cent	2 ml	Sodium bicarbonate 7.5 per cent	3.5 ml
	Antibiotics*	1 ml	Antibiotics*	1 ml
	Rabbit serum	8 ml	Deionised water	80 ml
	Deionised water	80 ml		
Chang Conjunctiva	Medium 199 (Hanks' salts) 10x	10 ml	Leibovitz' L15 medium**	100 ml
	Sodium bicarbonate 7.5 per cent	2 ml	Antibiotics*	1 ml
	Antibiotics*	1 ml	Fetal calf serum	2 ml
	Fetal calf serum	10 ml		
	Deionised water	80 ml		
Flow 4000 Clone 2	Eagle's MEM (Earle's salts) 10x	10 ml	Leibovitz' L15 medium**	100 ml
	Sodium bicarbonate 7.5 per cent	2 ml	Antibiotics*	1 ml
	Antibiotics*	1 ml	Fetal calf serum	0.5 ml
	MEM non-essential amino acids 100x	1 ml		
	Fetal calf serum	10 ml		
	Deionised water	80 ml		

*Antibiotics: penicillin 10^4 Units/ml and streptomycin 10^4 µg/ml
**see Leibovitz (1963)

TABLE 1.1 continued

Cell Type	Growth Medium		Maintenance Medium	
Human amnion	Eagle's MEM (Earle's salts) 10x	10 ml	Eagle's MEM (Earle's salts) 10x	10 ml
	Sodium bicarbonate 7.5 per cent	2 ml	Sodium bicarbonate 7.5 per cent	3.5 ml
	Antibiotics*	1 ml	Antibiotics*	1 ml
	Fetal calf serum	15 ml	Fetal calf serum	0.5 ml
	Deionised water	75 ml	Deionised water	80 ml
Human embryo liver	Eagle's MEM (Earle's salts) 10x	10 ml	Serumless medium (Neumann and Tytell, 1959)	100 ml
	Sodium bicarbonate 7.5 per cent	2 ml		
	Antibiotics*	1 ml		
	Fetal calf serum	10 ml		
	Deionised water	80 ml		
Intestine 407	As for human amnion cells		Leibovitz' L15 medium**	100 ml
			Antibiotics*	1 ml

*Antibiotics: penicillin 10^4 Units/ml and streptomycin 10^4 µg/ml
**see Leibovitz (1963)

TABLE 1.1 continued

Cell Type	Growth Medium		Maintenance Medium	
KB	Eagle's MEM (Earle's salts) 10x	10 ml	Eagle's MEM (Earle's salts) 10x	10 ml
	Sodium bicarbonate 7.5 per cent	2 ml	Sodium bicarbonate 7.5 per cent	3.5 ml
	Antibiotics*	1 ml	Antibiotics*	1 ml
	MEM non-essential amino acids 100x	1 ml	MEM non-essential amino acids 100x	1 ml
	Newborn calf serum	10 ml	Fetal calf serum	2 ml
	Deionised water	80 ml	Deionised water	80 ml
Kidney cells	Eagle's MEM (Earle's salts) 10x	10 ml	As for human amnion cells	
(Human embryo kidney,	Sodium bicarbonate 7.5 per cent	2 ml		
Bovine embryo kidney,	Antibiotics*	1 ml		
GH (canine kidney)	Fetal calf serum	10 ml		
	Deionised water	80 ml		

*Antibiotics: penicillin 10^4 Units/ml and streptomycin 10^4 µg/ml

c. Propagation.

(i.) Primary human amnion. Placentae were collected in 200 ml PBS and taken to the laboratory usually within 4 hours. The amnion was stripped from the placenta, washed in PBS containing antibiotics and cut into pieces about 6 cm^2. After blood clot and mucus had been removed with forceps, the amnion pieces were kept overnight at room temperature in 80ml Minimum Essential Medium (Eagle) containing 20 per cent fetal calf serum and antibiotics. Occasionally the amnion was put in trypsin on the day that it was collected.

The amnion pieces were put into a conical flask with 100 ml 0.25 per cent trypsin in PBS containing antibiotics for 30 min at 36.5°C. After this time, the trypsin was removed and fresh trypsin was added. The flask was agitated gently by hand at intervals of 30 min. After 3 hours the flask was shaken vigorously and the entire contents were removed.

The residual strands of amnion were taken from the cell suspension and put into a conical flask with 100 ml PBS. The flask was shaken vigorously and the contents removed. This second cell suspension was added to the first and the remaining strands of amnion were discarded.

The pooled cell suspension was centrifuged at 1000 rev./min for 10 min and the supernate was poured off. The pellet was resuspended in 20 ml human amnion growth medium. A 0.2 ml sample of cell suspension was diluted 10^{-1} in PBS containing 0.1 per cent trypan blue and the viable cell count was determined using a counting chamber. The cell concentration was adjusted to 3.5×10^5 cells/ml with growth medium. Glass tubes (100 mm x 13 mm) were seeded with 0.8 ml amounts. A single amnion yielded enough viable cells for 200 to 300 tubes on average.

After 2-3 days, growth medium was removed from the tubes and 0.8 ml fresh growth medium was added. When the monolayers reached confluence after 3-7 days, growth medium was removed and replaced by 0.9 ml maintenance medium.

(ii.) Human embryo kidney (HEK). The kidneys were removed and put into PBS. If taken from an afternoon termination they were stored overnight at 4°C in PBS containing antibiotics. Otherwise preparation of primary monolayers was carried out on the same day.

Preparation of primary monolayers. The kidneys were chopped finely using scissors and put in a screw-capped bottle containing 20 ml PBS with antibiotics. The tissue pieces were allowed to settle. The supernatant fluid containing blood was removed by pipette and the washing procedure was repeated until the fluid was clear. Depending on the volume of settled tissue pieces, 15 ml to 25 ml 0.25 per cent trypsin in PBS with antibiotics was added and the bottle was incubated at 36.5°C. The bottle was agitated by hand every 30 min.

After 3 hours, the container with dispersed tissue was shaken vigorously by hand and centrifuged at 1000 rev./min for 10 min. The supernatant fluid was removed by pipette and the pellet resuspended in 20 ml HEK cell growth medium. Depending on the size of the kidneys, primary cultures were set up in 50 ml amounts in 2 to 10 120 cm^2 flat bottles and incubated at 36.5°C.

Growth medium was removed and replaced by 50 ml fresh growth medium after one day. Primary cultures reached confluence in 3-7 days.

Preparation of secondary monolayers. To set up secondary cultures in tubes, the primary monolayers were washed twice with PBS and once with 5 ml 0.25 per cent trypsin in PBS. The trypsin was removed and the

bottles were incubated at 36.5°C until the cells became detached. HEK growth medium (3.5 ml) was added to the bottles and the cell suspension from each bottle was pooled. A 0.2 ml sample of pooled cell suspension was taken to determine the viable cell count as before.

The cell concentration was adjusted to 5×10^4 cells/ml with HEK growth medium and glass tubes (100 mm x 13 mm) were seeded with 0.8 ml amounts. On average 90 - 120 tubes were set up from the kidneys of one fetus.

After 2-3 days the medium was decanted from the tubes and 0.8 ml fresh growth medium added. At confluence growth medium was replaced by 0.9 ml maintenance medium.

(iii.) Bovine embryo kidney (BEK). Preparation of secondary tube cultures from primary monolayers was identical to that described for HEK.

(iv.) Human embryo liver. A primary culture of human embryo liver cells was set up as described for HEK. From this a semi-continuous line of fibroblast-like cells was derived by continuous sub-culture, as described in Section (v.).

(v.) Continuous cell cultures. All continuous cell cultures were propagated in 120 cm^2 flat bottles. Sub-culture was carried out at 3-5 day intervals.

Subculture. With the exception of Bristol HeLa cells, confluent monolayers were washed twice with PBS and once with 5 ml 0.25 per cent trypsin in PBS. The trypsin was decanted and the bottles were incubated at 36.5°C until the cells became detached. Growth medium (3.5 ml) was added to the bottles and a proportion of the resulting cell suspension from each bottle was added to fresh bottles containing 50 ml growth medium. The seeding ratio for each cell line is shown in

TABLE 1.2 Optimum seeding concentration for tube cultures and seeding ('split') ratio for flask cultures of continuous and semi-continuous cell lines.

Cell Type	Seeding Concentration	Split Ratio
Bristol HeLa	8×10^4 cells/ml	1 : 4
Chang Conjunctiva	8×10^4 cells/ml	1 : 4
Flow 4000 Clone 2	1.2×10^5 cells/ml	1 : 2
Human embryo liver	5×10^4 cells/ml	1 : 4
Intestine 407	8×10^4 cells/ml	1 : 8
KB	8×10^4 cells/ml	1 : 5

Table 1.2 .

Confluent cultures of Bristol HeLa cells were washed twice with PBS and 5 ml 0.02 per cent (w/v) versene (EDTA, disodium salt) added. The bottle was incubated at 36.5°C until the cells detached from the glass. The cell suspension was centrifuged in a screw-capped bottle at 1000 rev./min and the supernatant fluid was poured off. The pellet was resuspended in 4 ml Bristol HeLa cell growth medium. The suspension was divided into 4 aliquots and each aliquot was added to a fresh culture bottle containing 50 ml growth medium.

Storage. Cell suspensions prepared as above from several confluent bottle cultures were pooled and centrifuged at 1000 rev./min for 10 min. The supernatant fluid was poured off and the pellet resuspended in 10 ml storage medium consisting of 90 ml growth medium and 10 ml dimethyl sulphoxide. A 0.2 ml sample was taken for estimation of viable cells.

The cell concentration was adjusted to 2×10^6 cells/ml with storage medium and the cell suspension was added to screw-capped vials in 1 ml amounts. The cells were frozen to -70°C at 1°C per minute (Dougherty, 1962) in a Linde apparatus and stored in gaseous phase liquid nitrogen.

For recovery, the contents of one vial was thawed rapidly to 37°C and added to a 120 cm^2 flask containing 50 ml growth medium. The cells were dispersed by shaking and the bottle was then incubated at 36.5°C. On the following day the medium was removed and fresh growth medium was added.

All continuous cell cultures used were derived from stocks stored as above.

Preparation of tube cultures. Cells in bottle cultures were detached from the glass and cell suspensions were prepared in growth medium as described. Suspensions from more than one bottle were pooled, and

viable cells estimated.

The volume of cell suspension was adjusted to the optimum seeding concentration (see Table 1.2) and 100 mm x 13 mm glass tubes were seeded with 0.8 ml amounts. At confluence, growth medium was replaced by 0.9 ml maintenance medium.

4. Method For Virus Isolation.

a. Infection of Cell Cultures. Inoculation of cultures was carried out usually within 24 hours (at most 48 hours) of replacement of growth medium by maintenance medium. Where the maintenance medium was based on Leibovitz' L15, this interval was always 24 hours. The cultures received a further change of maintenance medium (0.9 ml) immediately before inoculation. 0.1 ml of each stool extract was added to each of 2 tubes and incubated stationary at 36.5°C. Uninoculated control cultures were always included.

b. Detection of Cytopathic Effect. The cultures were observed every alternate day for signs of degeneration. If cytopathic effect (CPE) was evident in specimen tubes, the effect was allowed to progress until more than 75 per cent of the cells were affected before the cultures were frozen to -20°C. If no CPE was detected in cultures containing specimens, the tubes were incubated until signs of non-specific degeneration were seen in both specimen and control tubes, at which time the cultures were frozen to -20°C.

c. Passage. Specimens which did not give CPE were maintained in culture for 3-4 weeks before being regarded as negative. This involved blind passage through 2 to 4 cultures, depending on the cell type.

All tubes were frozen and thawed at least 4 times before the culture fluid was introduced into fresh cultures. For passage the

contents of both initial tubes were pooled and mixed before 0.1 ml of this fluid was added to each of 2 tubes containing fresh cultures. The residual fluid was stored at -20°C. The contents of control tubes were passed to fresh cultures in the same way.

When a CPE was seen, the culture fluid was passed to fresh cultures at least once for confirmation.

5. Neutralisation Test for Adenovirus.

a. Antisera. Neutralising sera to adenovirus types 1 to 33 prepared in rabbits or goats were obtained commercially (Immunitalia, Rome) and from Dr. Eleanor J. Bell, Regional Virus Laboratory, Glasgow.

All antisera were used at optimum dilution in PBS (1:50 to 1:200; E.J. Bell, personal communication). Stock dilutions in PBS at ten times working dilution were stored at -20°C and working dilutions prepared as necessary.

b. Method. Neutralisation tests were carried out using culture fluid which had been frozen and thawed at least 4 times. For specimens giving CPE which developed quickly (2-3 days) on passage, culture fluid was diluted 10^{-1} in PBS. Dilutions of 10^{-2} or neat were occasionally used.

Mixtures of virus and antisera were made in the wells of UV-irradiated Linbro disposo trays (Flow Laboratories Ltd.) by adding 0.1 ml test antiserum to 0.1 ml culture fluid diluted as necessary. For each isolate tested 2 virus control mixtures were made with PBS in place of antiserum. The mixtures were allowed to stand at room temperature for 60 min. 0.1 ml from each well was added to a tube culture of susceptible cells to which fresh maintenance medium had been recently added. With isolates which showed low infectivity, the entire well contents were added to the cultures.

The cultures were incubated stationary at 36.5°C and examined daily for development of CPE. Any antiserum which held back development of CPE at least 2 days after the virus control tubes showed complete CPE was regarded as homologous with the serotype of the isolate.

Culture fluids were initially tested against antisera to adenovirus types 1 to 7 inclusive and also to type 17 (known to cross-react with type 1 by neutralisation test; E.J. Bell, personal communication). If none of the above antisera neutralised the isolate, the culture fluid was tested against antisera to adenovirus types 8, 9, 10, 11, 14, 15, 19 and 21. If these antisera also failed to neutralise the isolate, antisera to all remaining adenovirus serotypes were used. In all cases positive neutralisation results were confirmed by repeating the test with the appropriate antiserum.

c. Dual Infections. A dual infection was defined as the presence of more than one adenovirus serotype in an isolate from a single specimen. If two antisera to different adenovirus serotypes each caused partial neutralisation of an isolate, the possibility of a dual infection was investigated.

The isolate was mixed with antiserum of one serotype and put in culture. When breakthrough of CPE occurred the culture fluid was frozen and thawed, mixed with antiserum to the other serotype and put into fresh cultures. If serial exposure to antisera of both serotypes (irrespective of order) checked development of CPE completely it was concluded that a dual infection had occurred. If breakthrough continued then the isolate was regarded as a strain having characteristics in common with both serotypes. Triple infections by adenoviruses of established serotypes were not observed.

6. Electron Microscopy of Culture Fluids.

a. Concentration of Culture Material. Culture tubes (stored at -20°C) were thawed. Deionised water (4 ml) was added to each tube and the contents (4.5 ml approx.) put in a cellulose nitrate ultracentrifuge tube. The fluid was centrifuged at 35000 rev./min for 60 min in a Beckman L5-40 ultracentrifuge with SW 50.1 rotor. The supernatant fluid was poured off and the inverted tube was plugged with tissue and allowed to drain for at least 10 min. The pellet was resuspended in one drop of EM diluent (deionised water containing 0.1 per cent bacitracin as wetting agent) using a Pasteur pipette.

b. Negative Staining. Equal quantities of resuspended material and stain (2 per cent potassium phosphotungstate, pH 7.0) were mixed on a microscope slide and one drop of the mixture applied to the carbon formvar-coated side of a 400-mesh copper grid. Surplus liquid was drawn off at the edge of the grid using filter paper. The grid was allowed to dry in air.

c. Examination. No attempt was made to standardise the time between preparation and examination since such preparations of virus particles appear stable over a few days (C.R. Madeley, personal communication). The grids were examined on a Philips EM 301 electron microscope at a magnification of x50700. At least 4 widely separated areas of the grid were examined for the presence of virus particles. At least 15 mins was devoted to each grid.

A "+" system of scoring for numbers of virus particles was used as follows:

small number : less than 1 particle per 400 mesh grid square on average.

+	1-5 particles	"	"	"	"	"	"	"
++	5-20 particles	"	"	"	"	"	"	"
+++	20-100 particles	"	"	"	"	"	"	"
++++	greater than 100 particles	"	"	"	"	"	"	"

7. Fluorescence Microscopy.

The indirect fluorescent antibody staining technique was used throughout. The dilutions of antiserum used, the interval between infection and fixation of cells and controls used are described in Chapters 4, 6 and 7.

a. Cells.

(i.) KB Cells. Obtained from Gibco Bio-Cult Laboratories Ltd., Paisley, and propagated as in Section 3. Growth and maintenance media used were as described in Table 1.1.

(ii.) Secondary human embryo kidney (HEK). Cultures were prepared from primary cultures in bottles as in Section 3. Growth and maintenance media used were as described in Table 1.1.

b. Antisera.

(i.) Adenovirus-specific antisera. Preparation in rabbits and guinea pigs and testing is described in Chapter 4. Blood was collected from the ear vein (rabbits) or by cardiac puncture (guinea pigs) and stored at 4°C. On the following day the serum was separated from the clot, centrifuged at 2000 rev./min for 20 min and the supernate was stored at -20°C. All sera were heat-treated (56°C for 30 min) before use.

(ii.) Test antisera. Production of antiserum to adenovirus in stool extracts is described in Chapter 6, Section 2.

(iii.) Fluorescein isothiocyanate (FITC) conjugated antisera. These were directed against IgG of the species of origin of the serum used, and prepared in Swine (SWAR-FITC (Swine anti-Rabbit IgG), SWAGp-FITC (Swine anti-Guinea Pig IgG); Nordic Laboratories, Maidenhead).

The FITC conjugated antiserum was stored at -70°C and was thawed and diluted 10^{-1} in sterile PBS immediately before use.

c. Microscope. A Leitz Ortholux II Microscope with Ploemopak 2.2 incident light illuminator and filter system H2 was used. Preparations were examined using a x25 objective. The final magnification was x250.

d. Method. Except where otherwise stated, cells were seeded in 5 ml amounts in 9 mm x 35 mm coverslips within stoppered Leighton tubes. The seeding concentration was 1.2×10^5 cells/ml for KB cells and 5×10^4 cells/ml for HEK cells. At confluence (24 hours for KB cells) growth medium was replaced with 4.5 ml appropriate maintenance medium. The inoculum volume was 0.5 ml for standard virus but 0.1 ml for stool extracts in order to conserve material.

After the appropriate time interval following infection the coverslips were removed from the Leighton tubes and washed in 4 changes of PBS (pH 7.3), then dried for 15 min at 36.5°C. They were then fixed in acetone at room temperature for 2 min, followed by drying at 36.5°C for 15 min. The coverslips were stored at 4°C until stained (within 48 hours).

Rabbit or guinea pig serum diluted in sterile PBS (pH 7.3) was added to the monolayer (2 drops per coverslip) and incubated at 36.5°C for 30 min. After this time the coverslips were washed in 4

changes of PBS, followed by the addition of 2 drops of fluorescein-conjugated antiserum (diluted 10^{-1} in PBS). After incubation of the coverslips at 36.5°C for 30 min, excess conjugated antiserum was removed by washing in 4 changes of PBS. The coverslips were mounted on glass slides with PBS-glycerol (1:9 v/v) and examined under incident light UV illumination within 24 hours of staining.

CHAPTER 2

CHAPTER 2 ATTEMPTS TO ISOLATE ADENOVIRUS FROM STOOL EXTRACTS IN WHICH ADENOVIRUS PARTICLES WERE DETECTED BY ELECTRON MICROSCOPY.

A. CONFIRMATION OF PREVIOUS FINDINGS.

The work of Dr. Eleanor J. Bell in this laboratory, using stools from infants (Madeley et al., 1977) showed that in most cases where adenoviruses were detected in stool extracts by electron microscopy, these could not be isolated in cell culture.

The present study was begun by confirming these findings using the same stool extracts. Since some strains of adenovirus require prolonged incubation for isolation (van der Veen and Kok, 1957; Grayston et al., 1958) inoculated cultures of fetal human cells were maintained for 5 - 7 weeks with blind passage.

Method. 23 stool extracts from 16 children (collected during 1975 and stored at -20°C) were placed in primary human amnion culture with repeated blind passage at approximately 10 day intervals. Cultures were maintained for 42-48 days and observed on alternate days. The cultures which showed no evidence of CPE after this time were examined by electron microscopy for the presence of virus particles (one tube per specimen).

The stool extracts which did not cause CPE in cultures of human amnion cells were used to inoculate HEK cultures. Blind passage was carried out at approximately 7-10 day intervals. Specimens which did not cause CPE were maintained in culture for 34-39 days.

Results. As shown in Table 2.1, 22 of the 23 stool extracts were culture negative in human amnion cells. Electron microscopy of the

TABLE 2.1 Results of prolonged attempts to isolate adenovirus from 23 stool extracts known to contain adenovirus by EM, using human amnion cells and human embryo kidney (HEK) cells. Only those stool extracts negative by culture using human amnion cells were used to inoculate HEK cells.

Cell Type	No. of Patients from Whom:			No. of Stools Placed in Culture	No. of Stools Negative by Culture	No. of Stools Positive by Culture
	1 Stool Examined	2 Stools Examined	3 Stools Examined			
Human Amnion	11	3	2	23	22*	1**
Human Embryo Kidney	10	3	2	22	17	5***

* final culture fluids negative for virus by EM

** CPE at day 26; isolate identified as adenovirus type 1

*** stools were from 5 different patients. 3 stools caused CPE at day 22, and 2 stools caused CPE at day 4

final (fifth passage) culture fluids did not reveal virus particles. Adenovirus type 1 was isolated from one stool extract which caused CPE after 26 days in culture.

Of the 22 stool extracts added to cultures of HEK cells, 17 did not cause CPE. 2 stool extracts caused CPE typical of adenovirus at day 4 and this effect developed again when the culture fluid was passed to fresh cultures. 3 stool extracts caused CPE typical of adenovirus at day 22. In 2 cases the effects developed again when the culture fluid was passed to fresh HEK cells.

For the 5 cases where CPE was observed in HEK cells, the stool extracts were from different children. Attempts to identify the virus failed because CPE did not develop in neutralisation tests using HEK cells. Re-isolation attempts were not possible with the amounts of stool extract available.

Comment. The results confirmed the failure of the adenovirus particles detected in stool extracts by EM to cause CPE in cultures of human amnion cells. The isolation attempts were carried out over a longer period than is usual for routine virus isolation, but this proved to be of value in only one case.

The absence of CPE does not preclude the possibility that adenovirus replication occurred. However, it is unlikely that virus production occurred to the extent shown by culture-positive specimens. Cultures which show CPE typical of adenoviruses usually have detectable amounts of virus particles by electron microscopy (C.R. Madeley, personal communication). In this case, it is possible that virus particles were produced in amounts too small to be detected by EM or that limited amounts cf virus were produced at an earlier passage.

The use of HEK cells allowed detection of adenovirus by culture in a further 5 stool extracts. The HEK cell culture system was useful for recognition but not identification of these strains. Therefore

adenoviruses with low infectivity appeared to be present in the stools of some children for which the HEK cell culture system seemed barely adequate. This lack of sensitivity of the culture system might explain failure to detect CPE from the remaining stools.

It was important to determine whether poorly infectious isolates belonged to common serotypes, uncommon serotypes or new serotypes of adenovirus. If these strains belonged to serotypes which are commonly isolated the history of the virus or its stool environment would require examination to find an explanation for such low infectivity.

B. CULTURE ATTEMPTS USING FURTHER STOOL EXTRACTS.

1. Introduction.

The work was extended by examining a larger number of stool specimens in cell culture. There were 4 objectives.

1. To observe the characteristics of any agent which gave rise to a transient CPE and to identify it if possible.

2. To estimate the proportion of stools containing adenoviruses which could not be cultured and identified.

3. To increase the stock of stool extracts known to contain adenoviruses difficult to isolate in cell culture.

4. To assess the value of small variations in culture technique on the isolation of adenovirus from stool extracts.

In the virus isolation attempts to be described for the remainder of this chapter, all stool extracts used were known to contain adenoviruses by EM. The specimens and culture fluids were identified by laboratory number and the isolates were typed individually without information about the patient. In cases where adenovirus isolations

were attempted under 2 different culture conditions and CPE developed readily under both conditions, only one isolate was identified by neutralisation test.

In Section A., there was evidence that some strains of adenovirus which do not cause CPE with human amnion cells might be detected using HEK cells. Although it would have been preferable to use HEK cells throughout, the supply of this tissue was low. For some virus isolation attempts, human amnion cell cultures or a combination of both cell types (in series) was used. In these cases, specimens which did not allow adenovirus isolation or identification were re-tested by serial passage in HEK cultures.

2. Comparison of Stationary and Rolling Culture.

Some early workers favoured rolling culture for the isolation of viruses found in faecal specimens (Kibrick et al., 1955; Schmidt et al., 1964). However, stationary incubation has generally been considered adequate for the isolation of adenoviruses (Rose, 1969). With the application of EM to the examination of faecal specimens and the finding that not all adenoviruses can be cultured, a re-appraisal of rolling culture was considered worthwhile.

Method. 26 stool extracts (previously untested in culture) were added to cultures of fetal human cells. The extracts had been stored at -20°C and had been frozen once only.

Two isolation attempts were made with each stool extract: (a) using culture tubes incubated stationary at 36.5°C after inoculation, and (b) using tubes incubated at 36.5°C after inoculation on a roller drum revolving at approximately 10 rev./hour. A stationary adsorption period of 60 min at 36.5°C was given to all cultures on inoculation and at each passage, followed by the addition of 0.9 ml

fresh maintenance medium. For further details of the absorption technique, see Section 3. below.

Culture fluid was transferred to fresh cultures from a different fetus at each passage. Cultures from the same fetus were used at each corresponding passage for both stationary and rolling isolation attempts. The first passage was carried out using human amnion cells and HEK cells were used for subsequent passages. (First passage fluids from cultures incubated stationary were also passed to further human amnion cultures and incubated in the same way). Specimens which did not cause CPE were maintained in culture for at least 24 days.

Results. The 26 stool extracts were from 15 children (Table 2.2). There were single specimens from 8 patients, 2 specimens from 4 patients, 3 specimens from 2 patients and 4 specimens from 1 patient. The adenovirus serotypes readily isolated were types 1, 2, 5 and 7. Strains of adenovirus belonging to these serotypes were isolated without difficulty from 15 stool extracts, with simultaneous development of CPE in both stationary and rolled cultures.

Stool extracts from 5 of the 15 children failed to cause CPE in either stationary or rolled cultures. Specimens from 3 patients (numbers 5, 14 and 15) caused adenovirus CPE, but the agents were difficult to identify. The CPE did not develop again after passage if fluid from the first cultures to show CPE was diluted. Attempts to isolate virus using stationary cultures of HEK cells were successful in 2 cases, and the isolates were identified as adenovirus type 31.

Of those patients from whom more than one stool extract was placed in culture, the adenovirus serotypes isolated from successive stools were usually the same. However, patient no. 6 excreted adenovirus type 1 three months after the excretion of adenoviruses which did not produce CPE (stool date 12/2/76). Patient no. 5 excreted

TABLE 2.2 Result of culture with 26 stool extracts known to contain adenovirus particles by EM: Comparison of stationary with rolled culture, using cultures of fetal human cells. (Numbers in parenthesis refer to the approximate time taken for CPE to appear (days)).

Patient No.	Date of Stool	Other Virus by EM	Development of CPE		Virus Isolated
			Stationary	Rolled	
1	10/11/75		-	-	
2	1/12/75		-	-	
3	2/12/75		-	-	
4	30/ 1/76	SRV*	+ (6)	+ (6)	Ad2
	2/ 2/76	astrovirus	+ (8)	+ (6)	Ad2
5	30/ 1/76	AAV**	+ (3)	+ (3)	Ad untyped****
	29/ 3/76		+ (5)	+ (5)	Ad2
	31/ 3/76		+ (5)	+ (5)	Ad2
6	12/ 2/76	AAV	-	-	
	8/ 5/76		+ (5)	+ (3)	Ad1
	12/ 5/76		+ (5)	+ (5)	Ad1
7	9/ 3/76		+ (6)	+ (5)	Ad2
	9/ 3/76		+ (5)	+ (5)	Ad2
	12/ 3/76		+ (6/5)§	+ (5)	Ad2
	13/ 4/76		+ (5)	+ (6)	Ad1 + Ad2
8	16/ 3/76		+ (5)	+ (5)	Ad5
9	22/ 3/76		+ (6)	+ (5)	Ad7
10	12/ 4/76		+ (5)	+ (5)	Ad1
11	14/ 4/76		-	+(12)	Ad2
12	26/ 4/76		-	-	
	3/ 5/76		+(21/12)§	-	Ad2
13	27/ 4/76		+ (5)	+ (5)	Ad1
	28/ 4/76		+ (5)	+ (3)	Ad1
14	29/ 4/76		+(15)¶	+(14)	Ad17
	2/ 5/76		+(15)¶	+(11)	Ad untyped****
15	6/ 5/76		+(29/15)§	+(11)	Ad untyped***

\+ development of CPE characteristic of adenovirus
\- no development of CPE
* SRV: small round virus-like particles
** AAV: adeno associated virus-like particles
*** development of CPE characteristic of adenovirus, using only HEK cells in stationary culture; isolate untyped (2)
**** Ad31 isolated using only HEK cells in stationary culture (2; 5)
§ the first number refers to the time taken for CPE to appear using only human amnion cells in stationary culture
¶ no CPE using only human amnion cells in stationary culture

adenovirus type 2 two months after excretion of a virus strain with low infectivity (type 31, stool date 30/1/76). A dual infection by adenovirus type 1 and type 2 was apparent from the isolate of one stool extract from patient no. 7 (stool date 13/4/76), whereas only adenovirus type 2 had been detected in previous isolates.

Comment. Slow rolling of culture tubes has been especially beneficial for the isolation of rhinoviruses from respiratory specimens (Tyrrell and Parsons, 1960). This is possibly due to the even mixing of toxic products of metabolism or cell nutrients, or could be due to an increased opportunity for adsorption of virus to cells when out of contact with the pool of medium.

From Table 2.2 there is little evidence to suggest that rolling of tubes increases the rate of isolation of adenoviruses from stool extracts. That an adenovirus type 2 strain was isolated from one patient only in rolling culture is offset by the fact that another type 2 strain from a different patient was isolated only in stationary culture. These differences possibly reflect the chance event of detecting a viable virus dose in a poorly infectious specimen rather than the merit of one technique over the other. This emphasises the need for replicates in such an experiment, but limitations in the supply of tissue made this difficult.

In the case of 5 stool extracts which failed to cause CPE, rolling of the culture tubes offered no advantage. Where adenovirus strains were readily isolated, little difference was noted in the time taken for CPE to appear in stationary and rolled cultures. From these findings, stationary culture was considered adequate for further isolation attempts. (One disadvantage with rolling culture is that human amnion monolayers degenerate more quickly than with stationary

incubation).

Madeley et al. (1977) reported definite isolation of adenoviruses by culture from only 2 patients from a total of 12 children with diarrhoea whose stools were known to contain adenovirus particles by EM. In the present study, the proportion of children found to excrete adenoviruses which could be cultured and typed was 11 from a total of 15. (2 of the 11 children yielded both culture positive and culture negative stools by this criterion). Too much emphasis cannot be placed on this comparison since the numbers of children were small in both cases. Also, one might question the validity of comparing data for which clinical details are incomplete. Nevertheless, on the basis of previous reports (Madeley et al., 1977; Flewett et al., 1975; Schoub et al., 1975; White and Stancliffe, 1975) the relative ease with which 17 adenovirus isolates were obtained from 26 stool extracts led me to consider whether any particular culture condition used in these tests was responsible. 16 stool extracts from which common adenovirus serotypes were isolated in stationary human amnion/HEK culture also caused CPE in stationary human amnion culture alone. Therefore the relatively high isolation rate as compared with previous reports could not have been due to the sequential use of more than one cell type.

3. Comparison of Culture With and Without Adsorption Periods.

Since the adenovirus isolation rate was higher than expected in Section 2., the contribution made by using an adsorption period of 1 hour at each passage was investigated.

Method. 21 stool extracts, previously untested in culture, were added to cultures of fetal human cells. The extracts had been stored at -20°C and had been frozen once only.

Two isolation attempts were made with each stool extract:

(a) Stationary culture at 36.5°C, and adsorption at each passage with 0.1 ml volumes of stool extract or culture fluid from earlier passage. Adsorption was carried out using fresh cell cultures from which maintenance medium had been removed. After stationary incubation at 36.5°C for 60 min, 0.9 ml fresh maintenance medium was added to each tube.

(b) Stationary culture at 36.5°C and direct inoculation of tube cultures (already containing fresh maintenance medium) at each passage with 0.1 ml volumes of stool extract or subsequent culture fluid, as described in Chapter 1.

Each successive passage was made into a fresh culture from a different fetus. Cultures from the same fetus were used at each corresponding passage for both culture conditions. The first passage was carried out using human amnion cells and HEK cells were used for subsequent passages. Fluids were also passed through successive human amnion cultures. Specimens maintained in culture for at least 23 days without development of CPE were regarded as culture negative.

Results. The results are given in Table 2.3. The 21 stool extracts were from 13 children, with single specimens from 8 patients, 2 specimens from 2 patients and 3 specimens from 3 patients. The adenovirus strains isolated without difficulty were of types 1, 2, 5 and 9, and a strain neutralised by antisera to both type 14 and type 16. The stool extracts of one patient caused CPE typical of enterovirus. An isolate from one of these stools was identified as poliovirus type 2 by Dr. E.J. Bell.

In all cases where CPE arose, the time taken to appear was the same both with and without adsorption. Single stool extracts from 2

TABLE 2.3 Result of culture with 21 stool extracts known to contain adenovirus particles by EM: Comparison of culture with and without periods of adsorption at each passage, using cultures of fetal human cells.
(Numbers in parenthesis refer to the approximate time taken for CPE to appear (days)).

Patient No.	Date of Stool	Other Virus by EM	Development of CPE		Virus Isolated
			Adsorption	No adsorption	
5	6/ 4/76	SRV*	+ (6)	+ (6)	Ad2
	28/ 6/76	-	-	-	***
	8/ 7/76		+ (9)	+ (9)	Ad2 + Ad9
6	12/ 5/76		+ (2)	+ (2)	Ad1
	13/ 5/76		+ (6)	+ (6)	Ad1
	24/ 6/76		+ (17)**	+ (17)**	Ad9
7	5/ 4/76		-	-	****
8	31/ 5/76		+ (6)	+ (6)	Ad14-16
	9/ 6/76		+ (6)	+ (6)	Ad14-16
16	12/ 5/76	SRV*	-	-	*****
17	12/ 5/76		+ (12)**	+ (12)**	Ad untyped
18	17/ 5/76		+ (9)	+ (9)	Ad2
19	17/ 5/76		+ (8)	+ (8)	Ad1
20	26/ 6/76		-	-	
	28/ 6/76		-	-	
	1/ 7/76		-	-	
21	5/ 7/76		-	-	
22	7/ 7/76	rotavirus	+ (8)	+ (8)	Ad5
23	12/ 7/76		ent†(2)	ent (2)	Polio 2
	13/ 7/76		ent (2)	ent (2)	
24	13/ 7/76	SRV*	+ (8)	+ (8)	Ad1

\+ development of CPE characteristic of adenovirus
\- no development of CPE
† ent: development of CPE characteristic of enterovirus
* SRV: small round virus-like particles
** no CPE using only human amnion cells
*** Ad2 + Ad9 isolated using only HEK cells (no adsorption periods) (12)
**** Ad17 isolated using only HEK cells (no adsorption periods) (22)
***** development of CPE characteristic of adenovirus, using only HEK cells (no adsorption periods); isolate untyped (5)

patients (numbers 6 and 17) caused CPE only with the combined use of human amnion cells and HEK cells, but with or without adsorption. At least one stool extract from 5 of the 13 children (numbers 5, 7, 16, 20, 21) failed to cause CPE under either set of culture conditions. Attempts to detect CPE with these 7 specimens using HEK cells alone (and no adsorption period) were successful in 3 cases (patients No. 5, 7 and 16).

Of the 5 children from whom more than one stool extract was placed in culture, the serotypes of adenovirus isolated from successive stools were usually the same. However, adenovirus type 9 was isolated from one stool extract of patient no. 6, whereas adenovirus type 1 had been isolated from previous stools (see also Table 2.2). Adenovirus was isolated from one stool extract of patient no. 5 (dated 28/6/76) only by the use of HEK cell culture alone. As with an extract from a stool passed 10 days later, this revealed a dual infection by adenovirus type 2 and type 9.

Comment. Attachment of virus particles to susceptible cells will follow random contact between the virus and the cell surface, but only if free complementary sites are available on both (see Fenner et al., 1974b). Any treatment which increases the probability of collisions between the virus and the cell surface should increase the probability of attachment, and therefore the likelihood of infection. Decreasing the volume of cell overlay medium, inoculation with virus in a small volume, and incubation for a period (the 'adsorption period'), form a simple way of increasing the probability of attachment of virus to a cell monolayer. Isolation of adenoviruses from stool specimens might benefit from this technique, especially when the quantity of infectious virus in the stool extract is low.

From Table 2.3 it is apparent that for the 21 stool extracts tested under both conditions, adsorption periods of 60 min at each passage offered no advantage to the isolation of adenoviruses. Adenovirus CPE developed in culture with 12 stools (from 8 children) as readily with no adsorption. The use of an adsorption period did not increase the number of isolations. However, for 3 specimens a repeat isolation attempt using HEK cells alone (with no adsorption periods) allowed the development of CPE. Therefore the choice of cell type was probably of greater importance for detection of adenovirus than the use of an adsorption period.

The time given for adsorption or the increase in effective concentration of inoculum during this period may have been insufficient to alter significantly the probability of virus attachment. Both conditions represented a compromise. The volume of inoculum (0.1 ml) was small in order to conserve the stool extracts, but large enough to cover the monolayer. The time given for adsorption was not extended beyond 60 min because of the risk of water evaporation from the inoculum (resulting in osmotic damage to the cells) and also to minimise the risk of a direct toxic effect of the stool extracts on the cells.

Direct inoculation offers the practical advantage of eliminating one further exposure of the cultures to the atmosphere, thus reducing the risk of airborne contamination. Any technique which eliminates the replacement of medium in inoculated cultures will also reduce the risk of cross-contamination of specimens in culture. In all further culture experiments to be described, no adsorption period was used.

4. Comparison of Culture with Stool Extracts Stored Frozen or Unfrozen.

The stool extracts were stored for 1-7 days or longer until

examined by EM. All specimens previously tested for adenovirus infectivity had been stored at -20°C. However, it may not be correct to assume that all strains of adenovirus in stools have equal ability to survive storage at freezing temperatures or to survive the freeze-thaw process.

This experiment was designed to determine whether there is any advantage to adenovirus isolation in the storage of stool extracts at 4°C.

Method. Stool extracts were prepared and 5 ml taken for EM examination. The remainder was divided into 2 fractions. One 2 ml fraction was stored at 4°C while the other was stored at -20°C, until the result of EM examination was known. The first 26 stool extracts reported to contain adenoviruses by EM were used to inoculate human amnion cultures. Specimens stored at 4°C and those thawed from -20°C were added in parallel to tube cultures and isolation attempts were carried out independently.

Specimens maintained through 3 blind passages in human amnion cells (20 - 28 days) without causing CPE were regarded as culture negative.

Results. The extracts were from 12 children (Table 2.4). There were single specimens from 4 patients, 2 specimens from 3 patients, 3 specimens from 4 patients and 4 specimens from 1 patient. The adenovirus strains isolated were of types 1, 2 and 5.

From 19 of the 26 specimens (from 9 patients) no virus was isolated, whether from fractions of stool extract stored frozen or unfrozen. Stools from 4 patients (numbers 25, 37, 41 and 42) yielded adenovirus isolates which could be typed. For 3 patients, CPE arose simultaneously from stool extract fractions stored at both temperatures.

TABLE 2.4 Result of culture with 26 stool extracts known to contain adenovirus particles by EM: Comparison of isolation results from stool extract fractions stored at (a) $4^{o}C$, and (b) $-20^{o}C$ before inoculation of human amnion cells. (Numbers in parenthesis refer to the approximate time taken for CPE to appear (days)).

Patient No.	Date of Stool	Other Virus By EM	Date of Inoculation	Development of CPE		Virus Isolated
				$4^{o}C$	$-20^{o}C$	
11	7/12/76		24/ 1/77	-	-	
25	6/12/76		24/ 1/77	+ (7)	+ (7)	Ad1
	13/12/76		24/ 1/77	+ (11)	+ (9)	Ad1
28	17/ 1/77	SRV*	15/ 2/77	-	-	
35	10/12/76		24/ 1/77	-	-	
	11/12/76		24/ 1/77	-	-	
36	16/12/76		24/ 1/77	-	-	
	16/12/76		24/ 1/77	-	-	
	20/12/76	AAV**	24/ 1/77	-	-	
37	20/12/76	calicivirus astrovirus	24/ 1/77	+ (9)	+ (9)	Ad1
	22/12/76	calicivirus astrovirus	24/ 1/77	+ (3)	+ (3)	Ad1
38	18/ 1/77		12/ 2/77	-	-	
	19/ 1/77		12/ 2/77	-	-	
	20/ 1/77	calicivirus	12/ 2/77	-	-	
	21/ 1/77	calicivirus	12/ 2/77	-	-	
39	21/ 1/77		15/ 2/77	-	-	
40	27/ 1/77	AAV**	12/ 2/77	-	-	
	28/ 1/77	AAV**	12/ 2/77	-	-	
	31/ 1/77	AAV**	12/ 2/77	-	-	
41	3/ 2/77	SRV*	12/ 2/77	+ (24)	+ (22)	Ad2
	4/ 2/77		12/ 2/77	+ (22)	+ (22)	Ad2
	5/ 2/77	calicivirus AAV**	12/ 2/77	-	-	
42	7/ 2/77	SRV*	12/ 2/77	-	+ (25)	Ad5
43	8/ 2/77		12/ 2/77	-	-	
	8/ 2/77		12/ 2/77	-	-	
	9/ 2/77		12/ 2/77	-	-	

\+ development of CPE characteristic of adenovirus
\- no development of CPE
* SRV: small round virus-like particles
** AAV: adeno associated virus-like particles

the stool extract of patient no. 42 caused CPE, but only the fraction stored at -20°C.

As with patient no. 6 (Table 2.2), patient no. 41 excreted adenovirus particles in the stools on 2 occasions (3/2/77 and 4/2/77) from which adenoviruses could be isolated; but from one stool (dated 5/2/77) adenoviruses could not be isolated. Only one day separated excretion of viruses which could or could not be cultured.

Comment. There was no evidence that storage of specimens at 4°C before inoculation was of advantage. All subsequent isolation attempts to be described were made from stool extracts stored at -20°C. Storage at temperatures below -20°C was not possible due to space limitation.

The proportion of stool extracts which caused CPE (7 from 26) was low compared to the two previous experiments (Section 2. and Section 3.). Adenoviruses could be isolated from the stools of only 5 children from a total of 12. There are at least 3 possible explanations:

1. The presence of adenovirus particles which cannot be isolated from stools might be a feature of excretion in a proportion of infants in the population at any one time. (This proportion could be linked to such factors as signs of infection, age or sex of the child, or diet). The observed differences in this proportion might be the result of random variation from the true proportion produced by the low numbers of children sampled.

2. The proportion of children who excrete adenoviruses which cannot be cultured might vary with time. For example, if these were new serotypes, their incidence might fluctuate like other epidemic or endemic viruses.

3. If the proportion of relatively uncommon but recognised adenovirus serotypes was high in this collection of stools, the use of human amnion cells might not allow isolation of these strains. It was decided that for those stool extracts which did not cause CPE, isolation from the fractions stored at -20°C would be attempted using HEK cells.

5. Retrospective Attempt to Isolate Adenovirus Using HEK Cells.

Method. 19 stool extracts (from 9 patients) which were found to be culture negative with human amnion cells in Section 4 were used to inoculate HEK cell cultures. Only the fractions which had been stored frozen were used. Specimens which did not cause CPE were maintained in culture for 32-38 days with blind passage.

Result. The results are given in Table 2.5. For each stool extract from 4 patients (numbers 11, 28, 35 and 39) adenovirus CPE arose within 7 days. For the stool extracts of patient no. 40 CPE arose in 17 - 19 days.

Identification of the isolates proved difficult. Although CPE developed on passage to fresh cultures of HEK, the infectivity of the isolates was low. CPE did not develop in neutralisation tests if the culture fluids were diluted. Preliminary tests showed that these strains were not neutralised by antisera to the common adenovirus serotypes (types 1 to 7) and type 17. 3 isolates from 3 patients (numbers 28, 35 and 39) were partially neutralised by antiserum to adenovirus type 12. The isolate from patient no. 11 was identified as type 18 and one isolate from patient no. 35 was identified as type 31.

Comment. HEK cells allowed the detection of adenovirus CPE from the stools of 5 children although these specimens did not cause CPE with

TABLE 2.5 Result of culture in HEK cells with 19 stool extracts known to contain adenovirus particles by EM. These extracts did not cause CPE with human amnion cells.
(Numbers in parenthesis refer to the approximate time taken for CPE to appear (days)).

Patient No.	Date of Stool	Development of CPE	Virus Isolated
11	7/12/76	+ (6)	Ad18
28	17/ 1/77	+ (6)	?Ad12
35	10/12/76	+ (3)	?Ad12
	11/12/76	+ (4)	Ad31
36	16/12/76	-	
	16/12/76	-	
	20/12/76	-	
38	18/ 1/77	-	
	19/ 1/77	-	
	20/ 1/77	-	
	21/ 1/77	-	
39	21/ 1/77	+ (3)	?Ad12
40	27/ 1/77	+ (19)	Ad untyped
	28/ 1/77	+ (17)	Ad untyped
	31/ 1/77	+ (17)	Ad untyped
41	5/ 2/77	-	
43	8/ 2/77	-	
	8/ 2/77	-	
	9/ 2/77	-	

+ development of CPE characteristic of adenovirus
- no development of CPE

human amnion cells. For the stools of the other 4 patients, HEK cell culture was of no more advantage than human amnion cell culture for adenovirus isolation.

Although specimens were maintained longer in HEK than in human amnion cell cultures (32-38 days compared with 20-28 days) the greater success with HEK cannot be due to this. CPE in HEK cultures arose before the twentieth day in each case. Since the maintenance medium used for both cell types was the same, the difference in susceptibility to adenovirus must be a property of the cells.

In common with HEK cells, human amnion cells are of fetal origin and have epithelial-like morphology. However, the cells of the intact amniotic membrane are embedded in an intercellular glycoprotein matrix. This may persist after cell disaggregation and present a barrier to infection by covering a proportion of the virus receptors on the cell surface. This model was suggested by Chany et al. (1966) to explain the relative insensitivity of intact amniotic membranes and freshly trypsinised amnion cells to poliovirus.

The difference between HEK and human amnion cells in susceptibility to adenovirus may not be due to a different number of free receptors on the cell surface. The lower susceptibility of human amnion cells may be due in some way to the fact that amnion monolayers are derived from a tissue at the end of its functional life. Disaggregated amnion cells spread to form monolayers with little or no cell multiplication. The apparent arrest in or absence of some important metabolic functions may be related to the inability of the cells to support replication of some adenovirus strains.

One other difference existed between the conditions used with HEK and human amnion cell culture. The stool extracts had been frozen for a second time before inoculation of the HEK cell cultures. It is

possible that an inhibitor of adenovirus growth in the extracts became inactivated by the second freeze, thus allowing virus isolation. The high susceptibility of HEK cells to most adenovirus serotypes is generally recognised (Jackson and Muldoon, 1975) and appears a more likely explanation of the results. Further work on identification of the strains isolated is described in Chapter 3.

6. Further Culture Results.

This section describes the results of virus isolation attempts using 75 stool extracts known to contain adenoviruses but not previously tested in culture.

Method. Extracts of 75 stools (received between July 1976 and November 1977) were added to human amnion cultures. Specimens which did not cause CPE were maintained in culture for 22-32 days with blind passage. Those stool extracts which failed to cause CPE with human amnion cells were used to inoculate HEK cultures. These were maintained for 26-39 days with blind passage if CPE did not develop earlier than this. Culture fluids were examined by EM for the presence of virus particles if there was doubt as to the nature of cytopathic effects.

Results. As shown in Table 2.6, the 75 stool extracts were from 47 children. The common adenovirus strains isolated were of types 1, 2 and 3. The stools of 8 children caused CPE typical of enterovirus, supported by the finding of particles approximately 25 nm. in diameter in the culture fluids by EM in 6 cases. At least 1 stool extract from 18 children was negative by culture using either cell type.

One stool extract from each of 10 children did not cause CPE with human amnion cells but did so with HEK cells. Of those adenovirus strains in this category, only 2 could be identified with

TABLE 2.6 Result of culture with 75 stool extracts known to contain adenovirus particles by EM. Only those stool extracts which did not cause CPE in human amnion cell cultures were used to inoculate cultures of HEK cells. (Numbers in parenthesis refer to the approximate time taken for CPE to appear (days)).

Patient No.	Date of Stool	Other Virus by EM	Human Amnion Cells		HEK Cells	
			CPE	Isolate	CPE	Isolate
5	17/ 7/76		+ (22)	Ad2		
	2/ 8/76		+ (21)	Ad2		
	2/ 8/76		-		-	
	16/ 8/76		ent†(4)*	§	ent (3)	Echo 3
	26/ 8/76		+ (24)	Ad2		
6	23/ 7/76		ent (4)*	§		
7	26/ 5/76	SRV**	+ (14)	Ad1		
	29/ 7/76		+ (16)	Ad1		
	11/10/76		+ (24)	Ad1		
8	9/ 8/76		-		+ (8)	Ad14-16
12	29/11/76		-		ent (4)	§
	20/12/76		-		+ (6)	Ad2
14	2/ 8/76	SRV**	ent (2)*	§	ent (1)	Echo 11
	14/ 8/76		ent (2)*	§	ent (3)	§
	30/ 8/76		+ (16)	Ad2		
23	14/ 7/76		ent (6)*	§		
24	21/ 7/76	SRV**	+ (6)	Ad1		
25	19/ 7/76		-		-	
	21/ 7/76		-		-	
	24/ 7/76		-		-	
	25/ 7/76		-		-	
	27/ 7/76		-		-	
26	21/ 7/76		+ (4)	Ad1		
27	21/ 7/76		-		+ (3)	Ad untyped
28	21/ 7/76		-		+ (3)	Ad untyped
	7/ 2/77		+ (11)	Ad1		
29	24/ 7/76		-		-	
	26/ 7/76		-		-	
	26/ 7/76		-		-	
30	10/ 8/76		-		-	
31	19/ 8/76		+ (6)	Ad2		
	20/ 8/76		+ (4)	Ad2		

\+ development of CPE characteristic of adenovirus
\- no development of CPE
* EM of culture fluids revealed 30 nm diameter (approx.) particles, compatible with being enterovirus
† ent: development of CPE characteristic of enterovirus
§ presumed enterovirus isolate untyped
** SRV: small round virus-like particles
*** AAV: adeno associated virus-like particles

TABLE 2.6 continued

Patient No.	Date of Stool	Other Virus by EM	Human Amnion Cells		HEK Cells	
			CPE	Isolate	CPE	Isolate
32	4/11/76		-		-	
33	4/11/76		ent (2)	§	ent (3)	§
34	4/12/76		-		-	
	6/12/76		-		-	
	7/12/76	AAV***SRV**	-		-	
	8/12/76	AAV***calici-virus	-		-	
37	22/12/76	SRV** astro-virus	+ (7)	Ad1		
38	17/ 1/77	SRV**	-		-	
39	25/ 1/77		-		+ (3)	?Ad12
44	17/ 2/77	SRV** rota-virus	-		+ (4)	Ad untyped
45	21/ 2/77	AAV***	-		-	
46	24/ 2/77		+ (4)	Ad2		
	25/ 2/77		+ (4)	Ad2		
	25/ 2/77		+ (4)	Ad2		
47	28/ 2/77	SRV**	ent (4)*	§		
48	21/ 3/77		ent (4)*	Echo 13		
	23/ 3/77		ent (4)*	§		
	24/ 3/77		ent (4)*	§		
	25/ 3/77		ent (4)*	§		
49	30/ 3/77	SRV** calici-virus	+ (2)	Ad1		
50	30/ 3/77		+ (4)	Ad2		
51	24/ 2/77	SRV** rota-virus, calici-virus	-		+ (3)	?Ad12
52	14/ 3/77		-		-	
	15/ 3/77		-		-	
53	18/ 5/77		+ (15)	Ad2		
54	23/ 5/77		-		+ (4)	Ad untyped
55	3/ 6/77		+ (14)	Ad2		
56	28/ 6/77		-		+ (2)	Ad untyped
57	12/ 7/77		-		-	
58	18/ 7/77		+ (12)	Ad2		
59	1/ 8/77		-		-	
	2/ 8/77		-		-	
60	29/ 8/77		+ (12)	Ad2		
61	12/ 9/77		-		-	
62	8/10/77		-		-	
63	13/10/77		-		+ (2)	Ad untyped
64	17/10/77		-		-	
65	2/11/77	AAV***	-		-	
66	7/11/77		-		-	
67	15/11/77		+ (20)	Ad3		
68	21/11/77		-		-	
	21/11/77	AAV***	-		-	
69	30/11/77		-		-	

for key to symbols and abbreviations, see previous page

certainty. One strain (from patient no. 8) was neutralised by antiserum to type 14 and antiserum to type 16 (as in Table 2.3), and from patient no. 12 an adenovirus type 2 strain was isolated.

The other 8 strains which were detected by CPE only in HEK cell culture showed low infectivity. In each case CPE developed within 4 days and could be passed to fresh HEK cultures. However, the use of undiluted culture fluid from the first passage was necessary in order to ensure a CPE during neutralisation tests. Although it was established that these strains did not belong to common adenovirus serotypes (types 1 to 7) and type 17, final identification using HEK cells was not achieved. For 2 specimens (from patients no. 39 and no. 51) the isolates were partially neutralised by antiserum to adenovirus type 12. See Plates 1 to 3.

Comment. The adenovirus isolation attempts described were carried out after consideration of the results of Sections 2., 3., 4., and 5. The most important indication from the results of these Sections was the superiority of HEK over human amnion cells for detection of adenoviruses. Since it was probable that many isolates in this Section would belong to serotypes 1 and 2, human amnion cells (which appear susceptible to at least some strains of these serotypes) were used for initial isolation attempts. This screening procedure made the best use of the small amounts of HEK available. As predicted, HEK culture did give evidence of adenovirus CPE in cases where human amnion gave none.

Further work on the 8 unidentified strains is described in Chapter 3. One cannot exclude the possibility that the early CPE was the result of a toxic factor present in the stool extract. This might act at the cell surface to cause cellular rounding and detachment from the glass. A toxic substance could conceivably affect a second

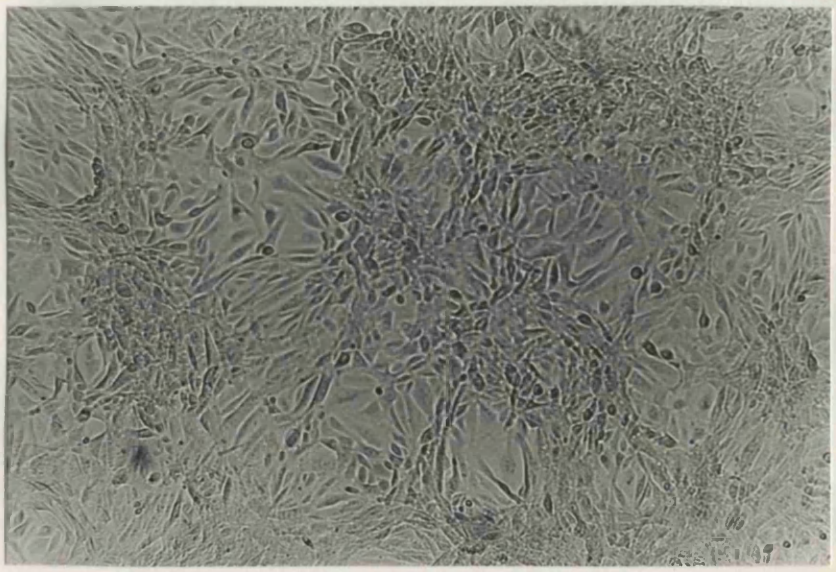

PLATE 1 Uninfected monolayer of human embryo kidney (HEK) cells.

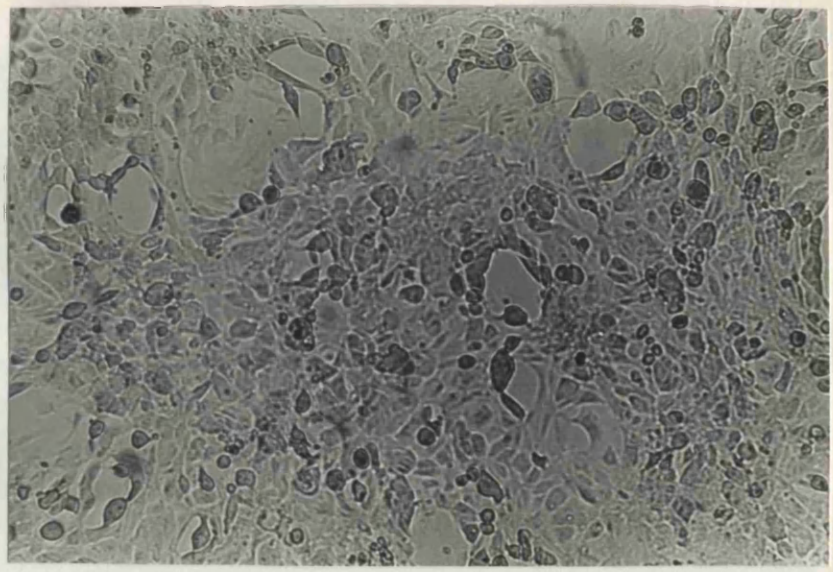

PLATE 2 Typical appearance of secondary human embryo kidney (HEK) cell monolayers after infection with wild strains of common adenovirus serotypes. In this case cell clustering and rounding is just beginning. Adenovirus type 3.

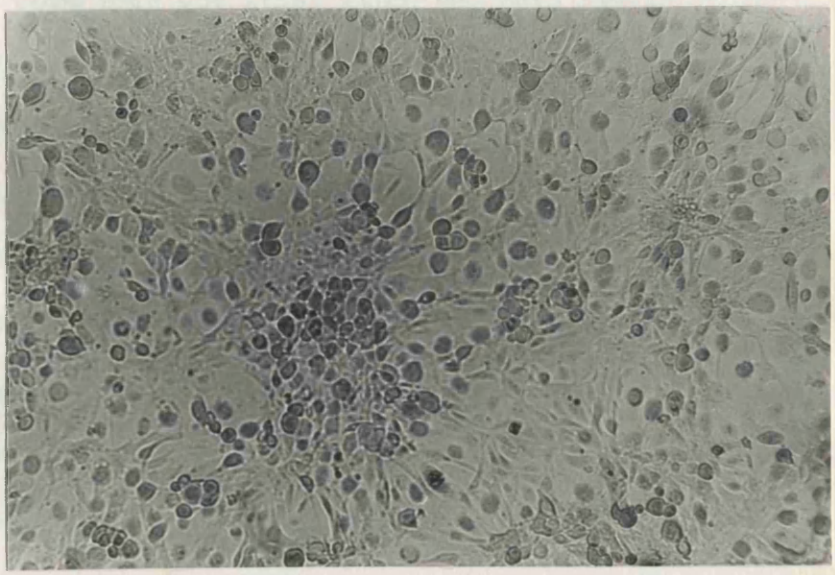

PLATE 3 Typical appearance of monolayers of secondary human embryo kidney (HEK) cells showing early cytopathic effects. The cytopathic effects in many cases were transient, but some were shown to be caused by wild strains of adenoviruses belonging to subgroup A. The nuclei are dense and prominent (compare with Plate 2). Cell rounding and clustering invariably followed from this stage.

culture on passage if originally present in large amounts. The presence of large quantities of adenovirus penton antigen or whole virus particles in culture fluid is known to have such a toxic effect (Pereira and Kelly, 1957; Rowe et al., 1958b; Valentine and Pereira, 1965).

That 18 of the 47 children showed evidence of excreting adenovirus particles which could not be cultured is important. The phenomenon is further reinforced by the finding that 6 of these children excreted more than one stool which was negative by culture. This is considered in detail in Section C.

Enterovirus CPE was detected from the stool extracts of 8 children, which possibly obscured the presence of any infectious adenovirus. Further isolation attempts for adenovirus from the culture fluids might have been possible if the enterovirus isolates had been neutralised by their homologous antisera. However, since eventual breakthrough of enterovirus CPE inevitably occurs (E.J. Bell, personal communication), further culture was not attempted.

7. Observation on the pH of Stool Extracts.

Since extremes in acidity or alkalinity within the intestinal lumen might inactivate some adenovirus strains, the pH of stool material was considered. Although adenoviruses of common serotypes are generally considered stable to extremes in pH (Ginsberg, 1956), this might not be the case for uncommon (or previously undescribed) serotypes.

During the attempts to isolate adenoviruses described in Sections 4., 5. and 6., note was taken of the colour of the phenol red incorporated in 72 stool extracts, immediately after thawing and before inoculation. This was later compared with the results of cell culture.

Results. Allowing for some discolouration of the red saline by pigments in the stools, 59 extracts of 72 examined had pH near neutrality (Table 2.7). 13 extracts had overcome the buffering capacity of the saline and were classed as acidic (yellow colouration) with a pH below 6 (by comparator). 4 (30.8 per cent) of these stool extracts did not cause CPE, whereas 22 (37.3 per cent) of 59 extracts with orange-red colouration were negative by cell culture. There were no alkaline stool extracts.

Conclusion. It is unlikely that deviation of pH from neutrality in stools which contain adenovirus is related to whether or not a particular specimen will cause CPE.

C. GENERAL DISCUSSION.

1. Aspects of the Isolation Technique.

a. Choice of Cell Type. Although HeLa cells (Scherer et al., 1953) have been used for isolation of adenoviruses (for example, Grayston et al., 1958) preliminary tests showed that HeLa cells presently available could not be maintained in tubes for more than 5 days. Consequently, isolation attempts would have required passage at frequent intervals. Evaluation of KB cells (Eagle, 1955) revealed the same problem. The growth properties of these continuous cultures have apparently changed with repeated subculture and are no longer convenient for the isolation of viruses which may require prolonged incubation.

Human embryo kidney (primary or secondary cultures) is generally regarded as the best system for isolation of adenovirus from either faecal or respiratory specimens (Brandt et al., 1969; Jackson and Muldoon, 1975). Since the supply of termination material for the

TABLE 2.7 Categorisation of 72 stool extracts according to the colour of the phenol red pH indicator.

	No. (per cent) of Stool Extracts Positive for Adenoviruses by EM but Negative by Cell Culture	No. (per cent) of Stool Extracts Positive for Adenovirus by EM
Yellow	4 (30.8)	13 (100)
Orange or Red	22 (37.3)	59 (100)
Purple	0	0
Total	26 (36.1)	72 (100)

preparation of HEK cultures was irregular and infrequent, much dependence had to be placed on primary human amnion cultures as second choice. An advantage of human amnion cells over HEK, HeLa cells or other heteroploid cancer cell types (KB, HEp2) is their ability to survive for up to 2 weeks in culture without a change of maintenance medium.

The advantage in using HEK cells rather than human amnion cells for the detection of adenovirus CPE has been demonstrated with 26 stool extracts from the 148 studied. In particular, detection of the strains apparently related to adenovirus type 12 was not achieved using human amnion cells.

Although HEK cells were more sensitive, their maintenance presented a problem. During the period May 1976 to December 1978 a change was noted in the ability of HEK cultures to survive in maintenance medium. Although the source of materials and the methods used were identical, the average survival time for HEK cultures in maintenance medium decreased form 8 days to 4 days. Survival could not be extended by regular replacement of maintenance medium. There was therefore a requirement for a culture system with sensitivity to adenovirus similar to HEK but which would survive as long as human amnion cultures.

b. Assumptions Made. This study has shown that more than one serotype of adenovirus may occasionally be isolated from one stool. It follows that one may not be justified in identifying a strain under one culture condition and assuming that it is of the same serotype as a strain isolated under another condition. Demand on available fetal tissue was so great that in most cases this assumption had to be made.

A second assumption, previously discussed, was that repeated freezing and thawing of stool extract fractions between culture

attempts would not alter appreciably the infectivity of specimens.

c. Choice of Method for Identification of Isolates. Apart from the neutralisation test, haemagglutination inhibition using type-specific antisera remains the only other widely accepted method for identification of adenovirus isolates by serotype (Rosen, 1960; Ginsberg, 1962).

Although more rapid to perform than neutralisation, the method of identification based on haemagglutination introduces a further variable, namely the reactivity of rat and monkey erythrocytes. The erythrocytes of individual animals may not be susceptible to haemagglutination by viruses of the appropriate group. Therefore each batch of cells received from suppliers must first be tested against standard virus (Rose, 1969).

A further disadvantage of the haemagglutination inhibition technique is that adenoviruses of at least one serotype (type 18) do not agglutinate either rat or rhesus monkey cells (Wigand and Keller, 1978) and must be typed by neutralisation. Furthermore, the haemagglutination of monkey erythrocytes by serotypes 20, 25 and 28 has been reported to be inconstant (Wigand and Fliedner, 1968). The neutralisation test was therefore preferred and used throughout.

2. Consideration of Culture Results Collectively.

a. Aims of Study. The adenovirus isolation attempts described in this chapter were performed in order to confirm previous reports (Flewett et al., 1975; Schoub et al., 1975; White and Stancliffe, 1975; Madeley et al., 1977) that adenoviruses present in the stools of some children have no apparent infectivity in cell culture. By examining a large number of stool extracts containing adenovirus particles, it was hoped that the extent of the problem would be more

clearly defined. In addition, it might be possible to discover any factors common to the children or their stools which might explain the phenomenon of non-growth.

It was important to establish the culture conditions under which the maximum number of adenovirus isolates would be obtained. This might lead to a clearer understanding of the significance of detecting transient cytopathic effects from some stool extracts. It was also important to determine whether the effects were due to previously unrecognized serotypes of adenovirus, and if so, whether these were related antigenically to the adenovirus particles in other specimens which did not cause CPE.

b. Definitions. It was convenient to consider the stool specimens under 3 categories according to the culture results of this chapter. These categories are defined below. The terms are used in the chapters to follow.

1. Stool extracts from which adenovirus could be isolated and identified using either HEK cells or human amnion cells were termed G specimens.

2. Stool extracts which caused cytopathic effect (typical of adenovirus) with HEK cells but not with human amnion cells, or stool extracts which caused adenovirus cytopathic effect in both cell types but the isolate gave difficulty in identification, were termed D specimens.

3. Stool extracts (containing adenoviruses by EM) which did not cause cytopathic effect in cultures of HEK cells and in cultures of human amnion cells were termed NG specimens.

Although adenoviruses could be isolated and identified from some stool extracts known to contain adenovirus particles by EM, the

serotypes of those strains isolated might not correspond to the serotypes of the particles seen by EM. Strains of common adenovirus serotypes (types 1, 2, 5 and 7) have been isolated from stool extracts which were negative for adenovirus by EM examination (Madeley et al., 1977). Therefore the common serotypes isolated in the present study may have been derived from viable adenovirus particles too few to be detected by EM. Some or all of the intact adenovirus particles seen (and probably present in quantities greater than 10^6 particles per ml of extract) may have been a different strain with more exacting culture requirements. It was therefore more correct to describe the stool extracts as adenovirus growth positive rather than the particles seen therein.

c. Frequency of G Specimens. Of 148 extracts tested in culture, 56 (37.8 per cent) did not present difficulty in the isolation and identification of adenovirus (Table 2.8). These isolations conformed to the results expected for specimens known to contain adenovirus.

The high proportion of strains identified as type 1 and type 2 (Table 2.9) is in agreement with the isolation frequencies for these serotypes from respiratory and faecal specimens reported by others (Brandt et al., 1969; Fox et al., 1969; Public Health Laboratory Report, 1970; White and Stancliffe, 1975).

Isolation of more than one adenovirus serotype from a single stool specimen is occasionally achieved (E.J. Bell, personal communication). In the present study, 2 dual infections in different children were observed (Table 2.9). Dual infections in vivo may provide opportunity for recombination between virus strains, leading to the production of new strains.

The strain isolated from patient no. 8 which was neutralised incompletely by antisera to both type 14 and type 16 is not unique.

TABLE 2.8 Classification of 148 stool extracts known to contain adenoviruses by EM, according to the results of virus isolation attempts in cultures of fetal human cells.

Reference	Number of Stool Extracts Classified as:				
	NG *	D **	G ***	ent****	Total
Table 2.2	5	5	16	0	26
Table 2.3	4	5	10	2	21
Table 2.4/2.5	11	8	7	0	26
Table 2.6	30	10	23	12	75
Total	50	28	56	14	148
(per cent)	(33.8)	(18.9)	(37.8)	(9.5)	(100)

*NG: stool extracts which did not cause CPE

**D: stool extracts which caused CPE in HEK cell cultures but not in human amnion cell cultures, or stool extracts which caused CPE in both cell types but the isolate presented difficulty in identification

***G: stool extracts from which adenoviruses were isolated and identified without difficulty

****ent: stool extracts which caused CPE characteristic of enteroviruses

TABLE 2.9 Serotypes of adenovirus isolated from 148 stool extracts known to contain adenovirus by EM, 84 of which caused cytopathic effects characteristic of adenoviruses.

Adenovirus Serotype	1	2	3	5	7	9	14-16	17	18	31	1 + 2	2 + 9	untyped	Total
No. of Isolations from D* Specimens	0	2	0	0	0	1	1	2	1	3	0	1	17	28
No. of Isolations from G** Specimens	21	26	1	3	1	0	2	0	0	0	1	1	0	56
Total	21	28	1	3	1	1	3	2	1	3	1	2	17	84

* stool extracts which caused CPE with HEK cells but not in human amnion cells or stool extracts which caused CPE with both cell types, but the isolate presented difficulty in identification. CPE characteristic of adenoviruses

** stool extracts from which adenovirus could be isolated and identified without difficulty

Adenovirus strains with characteristics of types 14 and 16 have been described previously (Parks et al., 1967; Wigand, 1975).

d. Frequency of D Specimens. Specimens which gave difficulty in the isolation and/or identification of adenovirus accounted for 28 (18.9 per cent) of the 148 stool extracts (Table 2.8). In 26 instances CPE developed when HEK cells were included in the isolation attempt but not when human amnion cells alone were used.

The low infectivity of most uncommon adenovirus serotypes (type 4 and high numbered serotypes from type 8 upward) apparent on initial isolation is well documented (see Rose, 1969). In the present study 8 of the 11 isolates from D specimens which were conclusively identified belonged to higher numbered serotypes (Table 2.9).

It is surprising that human amnion cells were not sensitive enough to detect 3 strains of adenovirus type 2. The quantity of infectious virus may have been so low as to require a more sensitive cell culture for isolation.

Of the 17 D specimens (from 13 children) which caused characteristic adenovirus cytopathic effect in HEK cells (Table 2.9) but from which the agent was not conclusively typed, CPE arose early (within 6 days) from 13 specimens (taken from 11 children). This finding agrees with the report of Madeley et al. (1977) in which Dr. E.J. Bell noted an early transient CPE from several stool extracts containing adenovirus particles. In the present study the CPE developed again when the fluids were passed to fresh cultures of HEK cells. However, with one exception, human amnion cells inoculated with these stool extracts showed no definite early cytopathic effect.

Adenovirus strains belonging to the 'oncogenic' subgroup A (containing types 12, 18 and 31) were isolated from 4 specimens and

implicated by neutralisation tests with 5 other isolates. Isolation of strains of these serotypes from anal swabs or stools of children has been reported by various workers (Pereira and MacCallum, 1964; Cramblett and Edmond, 1964; Pereira et al., 1965; Belian et al., 1968; Brandt et al., 1969; Moritsugu, 1969). However, any role for these viruses in the transmission of naturally acquired disease remains to be discovered.

Pereira and MacCallum (1964) reported that in a survey of children in England and Wales, 56 per cent of those tested between the ages of 1 and 5 years had neutralising antibody to adenovirus type 12. In the same year Cramblett and Edmond reported that 20 - 28 per cent of children tested who were over 3 years old had neutralising antibody to this serotype. Therefore infection in early childhood with type 12 or an antigenically related virus appears to be common. It is surprising therefore that reported isolations of this serotype are rare. Pereira and MacCallum suggested that this may be due to a requirement for HEK cell cultures which are not usually used routinely.

Moritsugu (1969) reported the superiority of HEK cells for the isolation of type 31 from an outbreak of infantile diarrhoea, whereas no CPE was observed with KB cells. Neutralising antibody to this serotype was found to be common in the sera from children near Tokyo. Pereira, Pereira and Clarke (1965) had earlier isolated the prototype strain(s) of adenovirus type 31 and reported its antigenic similarity to types 12 and 18 in neutralisation tests. Moreover, neutralising antibody to this serotype was found in at least two thirds of sera tested from all ages above one year.

Some adenovirus strains appear intermediate in neutralisation tests with antisera to type 12 and type 31 (Rafajko, 1966). This raises the possibility that the surveys on sera for neutralising activity

described above may have detected antibodies to more than one recognised serotype (12 and 31). It also indicates the possibility that the 5 isolates reported in this study which were neutralised by antiserum to adenovirus type 12 may also have been related to type 31.

e. Comparison of Isolations Over a Period. The isolations reported here confirm the findings of others that single serotypes of adenoviruses may be isolated from anal swabs or stools of individual children over several months (Bell et al., 1961; Fox et al., 1969). In the present study the longest period over which excretion of the same serotype by one child could be detected was 231 days (type 2 from patient no. 12; see Table 2.10). The prolonged excretion of adenoviruses in faeces is probably an important factor in the spread of infection among young children. Together with an apparent high infectivity, this may account for the widespread occurrence of some serotypes, notably types 1 and 2 which are endemic in most (if not all) countries.

From this study, one cannot say whether prolonged excretion of one adenovirus serotype was continuous or intermittent, since all the stool extracts tested were known to contain adenovirus particles. The knowledge that not all adenoviruses can be propagated in cell culture makes the interpretation of culture results concerning intermittent excretion difficult at present. One cannot assume that culture negative stools do not contain adenovirus particles. Although excretion of infectious adenovirus might be found to be intermittent by culture, the shedding of adenovirus particles in the stools might be continuous. A solution to this problem would only be found after the exact nature of adenoviruses which cannot be cultured is known.

Successive isolation of different adenovirus serotypes from stool specimens of individual children has rarely been described. In

TABLE 2.10 Patients found to excrete adenovirus of one serotype for longer than one month.

Patient No.	Adenovirus Serotype	Date First Detected	Date Last Detected	Interval (Days)
5	2	29/ 3/76	26/ 8/76	150
7	2	9/ 3/76	13/ 4/76	35
8	14-16	31/ 5/76	9/ 8/76	70
12	2	3/ 5/76	20/12/76	231

this study, demonstration of successive excretion of different serotypes by 6 children was achieved (Table 2.11). In 3 cases, this involved excretion of 3 different serotypes.

f. Frequency of NG Specimens. 50 specimens from 28 children did not cause CPE (Table 2.8). 5 of the children who excreted NG specimens (patients no. 5, 6, 12, 25 and 41) also excreted adenovirus which could be isolated. For example, 5 separate specimens taken from patient no. 25 over an 8 - day period were found to be negative by culture (although they contained large numbers of adenovirus particles). Less that 5 months later, adenovirus type 1 was isolated from each of 2 specimens from this child (Table 2.12). It would have been informative to study the serum antibody levels to adenovirus over this period, but serum was not available from any child.

Patient no. 5 excreted both G and NG stools on the same day (2/8/76) (Table 2.6). It is not known which stool was passed first. The reason for lack of infectivity in one specimen is not known. One possible explanation would be that the virus particles seen in both specimens were adenoviruses with special culture requirements, whereas the infectious virus isolated from one specimen (type 2) may have been present in amounts too small to be detected by EM.

From the culture results of the 5 children who excreted both G and NG stools, it appears that the excretion of non-growing adenovirus may be preceded or followed by excretion of growing adenovirus (Table 2.13). The culture results from consecutive stools from patient no. 41 (Tables 2.4 and 2.5) are compatible with the possibility of increased immunity in the gut, leading to virus neutralisation after a period of shedding infectious virus. If this is the case, type-specific immunity cannot be lasting since the excretion of a NG stool specimen by patient no. 5 was preceded and also followed (after 24 days) by

TABLE 2.11 Patients found to excrete adenoviruses of more than one serotype.

Patient No.	Adenovirus Serotypes Recovered from Stools (in Order of Appearance)
5	31, 2, 9
6	1, 9
7	2, 17, 1
8	5, 14-16
11	2, 18
14	17, 31, 2

TABLE 2.12 Results of adenovirus isolation attempts with stool extracts of patient no. 25.

Date of Stool	Virus Isolated
19/ 7/76	-*
21/ 7/76	-
24/ 7/76	-
25/ 7/76	-
27/ 7/76	-
6/12/76	Ad 1
13/12/76	Ad 1

* no virus isolated

TABLE 2.13 Patients found to excrete adenovirus particles in stools from which adenovirus was isolated in culture, but who also excreted at least 1 stool containing adenovirus which was negative by culture (NG specimen).

Patient No.	Serotype(s) Excreted Before NG Specimen	Serotype(s) Excreted After NG Specimen
5	31, 2, 9	2
6		1, 9
12		2
25		1
41	2	

excretion of the same serotype (type 2) (Table 2.6).

A list of patients who excreted more than one NG stool is given in Table 2.14. The measured interval of excretion of NG specimens did not exceed 8 days for any child, but may have been longer.

In no case was a child found to excrete adenovirus which could be isolated, during a period of NG stool excretion. Also, no child was found to have more than one episode of NG stool excretion with a period of weeks or months between. The data of Table 2.14 suggest that the phenomenon of adenovirus which cannot be isolated in cell culture is real and not simply due to poor infectivity of single specimens. However, one cannot deduce from the data that excretion of non-growing adenoviruses occurred in discrete episodes, since shedding of these viruses may have occurred at any time at a level too low to be detected by EM.

Table 2.15 shows the proportion of male and female children among the 69 patients studied, compared with the proportion among those children who excreted NG stools. There was no predominance of children of either sex in the numbers of patients from whom NG specimens were obtained ($\chi^2 = 0.74$; $P > 0.1$).

The ages of the children at the time of excretion of NG stools is given in Table 2.16. There was a fairly even distribution of ages at the time of excretion over the first year. Although over one half of those children who excreted NG stools were less than 5 months old, one child was 3 years old. Therefore it is likely that if some form of immune mechanism was responsible for the lack of infectivity apparent in these specimens, it would probably be an active influence rather than being the result of maternal antibody passively transferred in utero.

TABLE 2.14 Patients who excreted more than one stool positive for adenovirus by EM but negative by cell culture (NG specimens).

Patient No.	No. of NG Specimens	Interval Over Which NG Specimens Excreted (Days)
20	3	5
25	5	8
29	3	2
34	4	4
36	3	4
38	5	4
43	3	1
52	2	1
59	2	1
68	2	< 1

TABLE 2.15 Proportion of male and female patients.

	No. of Patients Whose Stools Were Positive for Adenovirus by EM But at Least One Was Culture Negative	Total No. of Patients Whose Stools Were Positive for Adenovirus by EM
Male	15	32
Female	11	33
Sex Unknown	2	4
Total	28	69

TABLE 2.16 Distribution of ages for 26 patients at the time of excretion of stools positive for adenovirus by EM but negative by cell culture (NG specimens).

Age (months*)	1-2	3-4	5-6	7-8	9-10	11-12	>12	Total
No. of Patients	8	7	4	1	3	3	2	28

* ages given refer to each whole month since birth

The presence of other viruses in the stools of those children found to excrete adenoviruses was of possible relevance, since these might inhibit replication of adenovirus. Adeno associated virus (AAV) has been reported to lower the yield of infectious adenovirus *in vitro* (Hoggan *et al.*, 1966). It is improbable that this would amount to total inhibition of replication, since AAV relies on adenovirus as a helper at each stage of its replication (Young and Mayor, 1979). 34 stool extracts in this study were noted to contain viruses other than adenovirus. However, any comparison between G and NG stools on this basis would be too uncertain, due to possible differences in interpretation by different workers as to which particles measuring 20-30 nm diameter are viruses, and as to which viruses are present. Moreover, adeno associated virus or any other virus type could have been present in all stool specimens in amounts too small to be detected by EM screening.

Several workers noted that adenoviruses which do not cause CPE in culture are usually present in stools in very large numbers (Bryden *et al.*, 1975; White and Stancliffe, 1975; Madeley *et al.*, 1977). It would therefore be relevant to compare the relative amounts of adenovirus detected by EM in G and NG specimens. However, to compare the amounts of virus in different specimens could be misleading since replicate preparations from one virus suspension can vary widely in virus content (personal observation).

The possibility of seasonal variation in the shedding of non-growing adenoviruses by the infant population has previously been suggested (Section B.4.). The number of NG specimens from different patients in this study was not large enough for conclusions to be made regarding the peak periods of excretion. However, NG specimens were received throughout 1976 and 1977.

g. Implications of Finding Adenoviruses Which Do Not Cause CPE. It is only with the recent application of electron microscopy to the examination of faecal specimens that failure to detect adenoviruses by cell culture was discovered. The phenomenon may be common to the detection of adenoviruses in respiratory specimens. In this case the problem might be complicated further by the low amounts of virus taken in throat swabs. Therefore the efficiency of detecting adenoviruses in respiratory specimens by cell culture could be very low relative to the number of adenovirus infections actually occurring.

Finding virus which cannot be isolated from stools but which is at least morphologically related to strains which can be isolated is of relevance to the problem of isolating other agents from stools. The reason for the apparent lack of infectivity of some adenoviruses, if discovered, might lead to a better understanding of the culture requirements of astroviruses (Madeley and Cosgrove, 1975), caliciviruses (Madeley and Cosgrove, 1976), rotaviruses (Flewett et al., 1974) and those agents associated with hepatitis A and B.

h. Suggestions to Explain Non-Growing Adenoviruses. One third of the stools examined in this study contained adenovirus particles which failed to cause CPE. Such a high proportion requires explanation. On the basis of the results presented thus far, the explanation appears to lie with the viruses or their environment rather than with the patients, some of whom excreted infectious adenovirus at other times.

In this section there are five suggestions which might explain the phenomenon. Different aspects of the culture results support the different possibilities. The work described in later chapters was

carried out to determine the relative likelihood of these suggestions.

Possibility No. 1. The adenovirus particles which do not cause CPE might be previously unrecognised serotypes, possibly requiring conditions for replication only to be found in vivo. Although adenoviruses commonly isolated from stools are of doubtful etiologic significance with regard to gastrointestinal disease, the adenoviruses in NG specimens might have a causative role.

Possible Support. Adenoviruses which do not cause CPE can be present in large enough amounts to be detected by EM examination of stools. This suggests free replication somewhere in the body. The period over which non-growing adenoviruses were found to be excreted (4 days or more for 6 patients in this study) also suggests ongoing production of virus.

Reports of others have associated outbreaks or incidents of gastroenteritis with the finding of apparently non-infectious adenoviruses in the faeces (Flewett et al., 1975; Whitelaw et al., 1977; Richmond et al., 1979). However, this may be due to the effect of flushing virus from the intestine and in itself does not imply a causative role for the viruses.

Possibility No. 2. The adenovirus particles which do not cause CPE might be strains of recognised serotypes for which HEK or human amnion cells are not a suitable culture system.

Possible Support. Some adenovirus strains of the established serotypes can be isolated only with difficulty in conventional cell culture systems (Vargosko et al., 1965). In particular, the present study has shown that adenovirus strains belonging to the subgroup of 'oncogenic' serotypes (12, 18 and 31) present problems in isolation. It is possible that less virulent strains of these serotypes exist

which produce no CPE in cell culture but require cells of the intestinal mucosa for replication.

Possibility No. 3. The adenovirus particles which do not cause CPE might belong to established serotypes and might be non-infectious due to a deficiency in virus structure and function.

Possible Support. There is little evidence for or against this possibility. Adenovirus particles in NG specimens appear morphologically identical to adenoviruses propagated in culture (Madeley, 1979). 'Empty' capsids which might lack some or all of the genetic material are uncommon for adenoviruses detected in stools by EM.

It is possible that the action of an enzyme could lead to inactivation of adenoviruses within the intestinal lumen. However, adenoviruses are resistant to both trypsin and papain (Pereira, 1958). Moreover, it is likely that viruses adapted to the faecal-oral route of transmission would have overcome sensitivity to enteric enzymes.

Extremes in pH or osmotic concentration might also influence adenovirus viability. From this study, there is no association between excretion of non-growing adenoviruses and excessively low or high pH of the stool extracts.

Possibility No. 4. The adenovirus particles which do not cause CPE might be established serotypes but non-infectious due to the action of some inhibitory substance or agent.

Possible Support. With the excretion of large numbers of adenovirus particles, one would expect the triggering of an immune response. Although viruses covered with specific antibody are said to have an indistinct 'feathery' appearance (Almeida and Waterson, 1969; Doane, 1974), the appearance of adenovirus particles in NG specimens is

distinct. Nevertheless, the amount of antibody required to neutralise the virus may be far less than is required to form visible cover.

In the present study, excretion of NG stools was found to be followed or preceded by excretion of adenoviruses which could be isolated, in different cases. In one case (patient no. 5; Table 2.6) shedding of non-growing adenoviruses was both preceded and followed by excretion of infectious adenovirus of one serotype. Therefore, if shedding of adenoviruses which do not cause CPE is due to the presence of antibody, any group-specific immunity (and possibly type-specific immunity) to gut infection appears short-lived.

Substances other than antibody might play a role in the inhibition of adenovirus replication, one possibility being interferon. This might be produced *in vivo* in response to extensive infection, and accompany the shedding of adenoviruses. However, adenovirus replication appears to have limited sensitivity to human interferon produced *in vitro* (Gallacher and Khoobyarian, 1971).

Possibility No. 5. The adenovirus particles which do not cause CPE might be strains of animal origin with the ability to replicate in the human intestine.

Possible Support. There is no direct evidence for this suggestion. Rhesus monkey kidney cells are not susceptible to these viruses (Madeley *et al.*, 1977), which makes a simian origin unlikely.

It is not unknown for adenoviruses to cross species barriers and cause infection, at least under laboratory conditions (see Cheville, 1975). There are very few reports of human infection by adenoviruses of other species. Existing reports give evidence of human infection by canine adenoviruses (Delage and Martin, 1955; Lanfranchi and de Jaco, 1959; Smith *et al.*, 1970). Smith *et al*

reported that 50 of 64 sera tested from normal individuals over 12 years old contained neutralising antibodies to Infectious Canine Hepatitis Virus (ICHV).

CHAPTER 3

CHAPTER 3 FURTHER ATTEMPTS TO ISOLATE NON-GROWING ADENOVIRUSES.

A. CELL CULTURE.

1. Introduction.

The virus isolation attempts described in Chapter 2 were carried out using primary or secondary human cells. However, adenoviruses in NG specimens might only replicate in continuous cultures of human cells or in cells of animal origin.

2. Continuous Human Cells.

One semi-continuous and 5 continuous human cell lines were tested for susceptibility to adenoviruses in stool extracts. Since the quantities of extracts were limited, it was not possible to test the same group of specimens in all cell lines tried.

a. KB Cells. Cells with this designation have been widely used for the isolation and propagation of adenoviruses (Jackson and Muldoon, 1975). There is evidence on genetic grounds that KB cells presently available are derived from the HeLa cell line (see Nelson-Rees and Flandermeyer, 1976). The cells lack a Y chromosome, have a complex of rearranged chromosomes or markers described for HeLa cells and exhibit fast mobility for glucose-6-phosphate dehydrogenase, in common with HeLa cells. Therefore a separate appraisal of HeLa cells for possible susceptibility to adenoviruses in NG specimens was considered unnecessary.

Method. 20 stool extracts containing adenovirus were incubated in cultures of KB cells. The specimens which did not cause CPE

were maintained in culture for at least 24 days with blind passage at 5-7 day intervals.

Results. 10 specimens designated NG with HEK cells also failed to cause CPE in KB cultures (Table 3.1). 3 specimens from which untyped agents were detected with HEK cells failed to cause CPE with KB cells. A specimen known to contain both adenovirus type 2 and type 9 did not infect KB cells. A specimen containing type 17 caused CPE but the identity of the agent could not be confirmed.

Comment. The use of KB cells did not result in an increase in the number of culture positive specimens. From the limited number of specimens tested, the susceptibility of this cell line to adenoviruses was no better than that of HEK cells and may have been slightly inferior. KB cells failed to detect the presence of 2 different serotypes of adenovirus in one specimen which had caused cytopathic effect in HEK cultures. All conditions in this comparison were not equivalent, for example, the serum content of the maintenance medium and the time of the isolation attempt. However, it was concluded that the KB cell system was not of value in the detection and isolation of adenoviruses in NG specimens.

b. Human Embryo Liver Cells. This semi-continuous liver cell line was the only culture with fibroblast-like cells used in the study. The cells could be maintained in serum-free maintenance medium for 15-19 days. Since some adenovirus strains may require long incubation times for isolation, the susceptibility of this cell line to adenoviruses in NG specimens was tested.

TABLE 3.1 Result of culture with 20 stool extracts known to contain adenovirus by electron microscopy, using KB and human embryo liver cells.

No. of Patients from Whom:				Total Patients
1 Stool Examined	2 Stools Examined	3 Stools Examined	4 Stools Examined	
16	0	0	1	17

	No. of Specimens Not Causing CPE	No. of Specimens Causing CPE			Total Specimens
		Agent Typed	Agent Untyped	Total	
HEK Cells*	10	6 †	4	10	20
KB Cells	14	4 §	2	6	20
human embryo liver cells	15	5 ¶	0	5	20

* results from Chapter 2
† adenovirus strains of types 1, 2, 5, 7, 2+9 and 17
§ adenovirus strains of types 1, 2, 5 and 7
¶ adenovirus strains of types 1, 5, 7, 17 and 18

Method. 20 stool extracts (those used to evaluate KB cells) were tested in cultures of human embryo liver cells. Those specimens which did not cause CPE were maintained in culture for at least 33 days with one blind passage.

Results. The 10 NG specimens did not cause CPE (Table 3.1). 3 of the 4 specimens from which untyped agents were detected using HEK cells failed to cause CPE in human embryo liver cells. The fourth untyped agent (HEK culture) was isolated and identified as a strain of adenovirus type 18. Of 6 isolates identified in HEK culture, 4 were detected and identified in human embryo liver cell cultures.

Conclusion. The use of this cell line did not result in an increase in the number of culture positive specimens. It was of no value for the detection of adenoviruses in NG specimens.

c. Bristol HeLa Cells. Most cell lines are adapted to growth in calf serum, although some can be maintained in serum from another species or in the absence of serum. However, factors may be present in calf serum which inhibit the replication of some strains of adenovirus. Therefore a cell line which is susceptible to adenovirus and which does not rely on calf serum might support the replication of adenovirus in NG specimens. The Bristol strain of HeLa cells is adapted to rabbit serum for both cell growth and maintenance. It was tested for susceptibility to adenovirus in 6 NG specimens.

Method. 6 NG specimens (from different patients) were added to cultures of Bristol HeLa cells in maintenance medium containing 4 per cent rabbit serum. The specimens were maintained for 21

days with blind passage at 5-6 day intervals.

Result. The 6 NG specimens failed to cause CPE with Bristol HeLa cells.

Comment. It was considered important that the cell line chosen should not only be maintained but also propagated in serum from another species. In this way, there would be no possibility of factors in calf serum being incorporated into the plasma membrane of the cells. No reports of natural infection of rabbits by adenoviruses could be found. Therefore it was unlikely that rabbit serum would contain anti-adenovirus antibodies. However, the use of the Bristol HeLa cell system was of no advantage in the detection of adenovirus in the NG specimens tested.

d. Flow 4000 Clone 2 Cells. These cells are reported to be derived from human embryo kidney (Flow Laboratories Ltd., 1978). It is generally accepted that cells of semi-continuous human embryo kidney cell cultures are fibroblast-like from about the fifth passage, with a decrease in susceptibility to adenovirus (E.J. Bell, personal communication). However, Flow 4000 Clone 2 cells are epithelial-like, which might indicate a susceptibility to adenovirus similar to primary or secondary HEK cell cultures. In addition, cultures of Flow 4000 Clone 2 cells could be maintained for 25 days without a change of maintenance medium. Therefore the longer incubation times possible at each passage relative to HEK cells might lead to further isolations from NG specimens.

Method. Samples of 28 stool extracts were incubated in cultures of Flow 4000 Clone 2 cells. All extracts contained adenovirus and 16 were NG specimens. Those specimens which did not cause CPE were incubated for at least 41 days with one blind passage.

Results. 3 stool extracts (NG specimens from patients no. 62, 64 & 68) caused CPE which destroyed the monolayer within 5 days. This effect at the first passage was repeatable but did not develop again on passage. The other cultures showed little or no signs of infection during incubation for 41-51 days (Tables 3.2 and 3.3).

Comment. The early destruction of the monolayer by 3 specimens may have been the result of a direct toxic effect, since it did not develop again on passage. The Flow 4000 Clone 2 cell system was not considered to be of value in the isolation of adenovirus from NG specimens or from specimens which had caused CPE with HEK cells. This was despite the long incubation times possible (3 - 4 weeks) relative to human amnion and HEK (1 - 2 weeks) and the supplier's claim that the cell line was derived from human embryo kidney.

e. Intestine 407 Cells. This cell line is reported to be derived from the minced pieces of jejunum and ileum of a human embryo of 2 months' gestation. The pieces had been embedded in chicken plasma clots, then subcultured directly on glass (Henle and Deinhardt, 1957). This is the only widely known cell line established from fetal human intestine in which the cells have epithelial-like morphology. It is similar to HeLa in appearance

TABLE 3.2 Result of culture with 28 stool extracts known to contain adenovirus by electron microscopy, using Flow 4000 Clone 2, Intestine 407 and Chang Conjunctiva cells.

No. of Patients from Whom:		Total Patients
1 Stool Examined	2 Stools Examined	
26	1	27

No. of Specimens Not Causing CPE	No. of Specimens Causing CPE			Total Specimens
	Agent Typed	Agent Untyped	Total	
HEK Cells* 16	2 †	10	12	28
Flow 4000 Clone 2 Cells 28**	0	0	0	28
Intestine 407 Cells 16***	7 §	5	12	28
Chang Cells 10****	8 ¶	10	18	28

* results from Chapter 2
** 4 specimens caused CPE at the first passage only
*** 7 specimens caused CPE at the first passage only
**** 1 specimen caused CPE at the first passage only
† adenovirus strains of type 31
§ adenovirus strains of types 12, 18 and five type 12-31 strains
¶ an adenovirus strain of type 12 and seven type 12-31 strains

TABLE 3.3 Presence/absence of CPE from 28 stool extracts known to contain adenovirus, when passaged in cultures of Flow 4000 Clone 2, Intestine 407 or Chang Conjunctiva cells.

Patient No.	Specimen*	Development of Cytopathic Effect														Virus Isolated:	
		Flow 4000 C2			Intestine 407				Chang Conjunctiva								
		P1	P2	P3	P1	P2	P3	P4	P1	P2	P3	P4	P5	P6	P7	Int. 407	Chang
20	NG	-	-		-	-	-	-	-	-	-						
21	NG	-	-		+	-	-	-	+	+	-						UT
25	NG	-	-		+	-	-	-	+	+	-						UT
29	NG	-	-		+	-	-	-	+	-	-						
36	NG	-	-		+	-	-	-	-	-	-						
38	NG	-	-		-	-	-	-	-	-	-						
43	NG	-	-		-	-	-	-	-	-	-						
45	NG	-	-		-	-	-	-	-	-	-						
57	NG	-	-		-	-	-	-	-	-	-						
59	NG	-	-		-	-	-	-	-	-	-						
61	NG	-	-		+	+	-	-	+	+	-					UT	UT
62	NG	+	-		+	⊕	-	-	+	⊕	+					UT	UT
64	NG	+	-		+	+	□		+	□	□					UT	UT
66	NG	-	-		+	-	-	-	+	+	-						UT
68	NG	+	-		+	□	-	-	+	⊕	+					UT	UT
69	NG	+	-		+	+	□		+	□	□					UT	UT
5	D	-	-		+	+	+		+	+	+	+	+	+	+	12-31	UT
14	D	-	-		+	-	-	-	+	+	+	+	+	+	+		12-31
27	D	-	-		-	+	-	-	+	+	+	+	+	+	+		12-31
28	D	-	-		+	+	+		+	+	+	-	-	-	-	12	12
28	D	-	-		-	-	-	-	-	-	-	-	-	-	-		
35	D	-	-		-	+	+		+	+	+	+	+	-	-	12-31	12-31
39	D	-	-		-	+	+	+	+	+	+	+	+	+	+	12-31	12-31
40	D	-	-		-	-	-	-	-	-	-						
44	D	-	-		+	-	-	-	-	+	+	+	+	+	+		12-31
51	D	-	-		-	+	+		-	+	+	+	+	-	+	12-31	12-31
54	D	-	-		+	+	+		+	+	+					12-31	12-31
56	D	-	-		+	+	+		+	+	+					18	UT

- \- no cytopathic effect
- + incomplete cytopathic effect (slow progression or late onset)
- + complete cytopathic effect (monolayer destroyed)
- UT untyped
- □ adenovirus by EM of culture fluid
- ○ no adenovirus by EM of culture fluid
- * results from Chapter 2

and handling requirements, and, for the reasons previously stated for KB cells, is probably contaminated with HeLa (Nelson-Rees and Flandermeyer, 1976).

Adenovirus in NG specimens may have an absolute requirement for cells of the intestinal mucosa of humans in order to replicate. It was important to determine the susceptibility of this cell line to adenovirus in NG specimens, in view of its reported origin.

Method. Samples of 28 stool extracts (those used to evaluate Flow 4000 Clone 2 cells) were incubated in cultures of Intestine 407 cells. Those specimens which did not cause CPE were maintained for at least 19 days with blind passage at 5-6 day intervals.

Results. The results are given in Tables 3.2 and 3.3. The total number of stool extracts which caused CPE (over more than one passage) was the same for Intestine 407 cells as with earlier culture using HEK cells (Table 3.2). However, 5 of the stool extracts which had been termed NG specimens did cause CPE with Intestine 407 cells (specimens of patients no. 61, 62, 64, 68 and 69; see Table 3.3).

The CPE with these 5 specimens was not typical of adenovirus but consisted of cell rounding and detachment without aggregation. In all cases the CPE was complete within 4 days of adding the specimen. The effect did not develop at the third passage in 3 cases. Adenovirus was detected by EM in the third passage culture fluids of the other 2 specimens. In all cases the agent(s) could not be identified.

Five of the specimens which had caused CPE with HEK cells

failed to infect Intestine 407 cells. Of the 7 isolates which were identified, each belonged to the so-called oncogenic subgroup A (Table 3.3). 5 of the isolates were neutralised by antisera to both type 12 and type 31.

Comment. Intestine 407 cells appear to be useful for detecting adenoviruses of subgroup A in stool extracts. However, like HEK cells, Intestine 407 cells did not survive more than 1 week as monolayer cultures. Therefore typing was difficult when CPE took several days to develop.

Since CPE was detected with 5 specimens previously designated NG, the cells may have been sensitive to an agent or agents not detected by HEK cultures. This could also be explained as a high sensitivity of these cells to toxic substances in the stool extract. However, this is unlikely since by the third passage the specimen has been diluted by 10^{-3}. If virus was responsible for the CPE, the adenoviruses detected by EM in 2 cases may have resulted from replication. However, it is possible that these particles were input virus. Further work with these specimens is described in Chapter 7.

f. Chang Conjunctiva Cells. The continuous Chang Conjunctiva 'D' cell line was derived from a continuous culture of conjunctival cells (Chang, 1954) by adaptation from growth in human and horse serum to growth in calf serum (Wong and Kilbourne, 1961).

Conjunctiva cell cultures might be expected to be sensitive to at least some adenovirus strains, since infections of the eye by some serotypes are common (see Foy and Grayston, 1976). Human conjunctival cells were used in the original isolation and

characterisation of 9 serotypes of adenovirus (Murray et al., 1957; Bell et al., 1960). Therefore the 'D' cell line was assessed for its susceptibility to adenovirus strains which were detected with difficulty or not at all using HEK cells.

Cultures of the Chang Conjunctiva 'D' cell line could be maintained for 10-14 days without a change of medium. They surpassed KB and HeLa cells in this respect. Since the appearance of the monolayer was also distinct from that of KB or HeLa cells, and matched the original description (Wong and Kilbourne, 1961), there was no evidence that the cell line had beer contaminated by HeLa. However, these cells were reported to have fast glucose-6-phosphate dehydrogenase mobility characteristic of HeLa cells (see Nelson-Rees and Flandermeyer, 1976).

Method. Samples of 28 stool extracts (those used to evaluate Flow 4000 Clone 2 and Intestine 407 cells) were incubated in cultures of Chang Conjunctiva 'D' cells. Those specimens which did not cause CPE were maintained for at least 27 days with 3 blind passages.

Results. 18 stool extracts caused CPE over more than one passage (Table 3.2). Of the 12 specimens which had caused CPE in HEK cultures, 10 also affected Chang cultures. In 8 cases the agents belonged to adenovirus subgroup A (Table 3.3). Repeated passage with these viruses led to loss of CPE in 2 cases. The specimens of patients no. 61, 62, 64, 68 and 69 caused CPE similar to that seen with Intestine 407 cells. Culture fluids from 2 specimens (of 4 examined) were found to contain adenoviruses by EM. The agents could not be identified by neutralisation test.

Comment. Repeated passage with 8 isolates of subgroup A adenoviruses showed that the original infectivity of these agents may not be sustained. In 2 cases the infectivity was lost completely (at the fourth and sixth passages). In other cases, development of CPE at the later passages was slower. Whereas wild strains of the commoner adenovirus serotypes adapt to cell culture with passage, the opposite was true of the subgroup A isolates. The same phenomenon was experienced previously with HEK cells, where the initial infectivity decreased so much on passage that typing was difficult. The Chang cell system can be considered a suitable (probably superior) alternative to the HEK cell system for the isolation of adenovirus strains related to type 12 and type 31. Moreover, it allowed the isolation of adenovirus type 12-31 from 3 specimens which were culture negative with Intestine 407 cells. This was probably due to the longer incubation times at each passage in Chang cells. Chang cells were especially useful in neutralisation tests where CPE took over 1 week to develop.

Eight NG specimens caused CPE with Chang Conjunctiva cells over more than one passage. As with Intestine 407 cells (which were affected by 5 of those 8 specimens) the effect may have been due to virus or a toxic agent. Investigation of this effect is described in Chapter 7.

3. Cells of Animal Origin.

a. Introduction. As stated in Chapter 2 adenovirus in NG specimens may be of animal origin. Other than the cells of human intestine, these viruses might also replicate in cells of a

particular animal species. Reports of human infection by canine adenovirus exist (Delage and Martin, 1955; Lanfranchi and de Jaco, 1959; Smith et al., 1970). Therefore a continuous culture of canine kidney cells was examined for susceptibility to the adenoviruses in NG specimens. Secondary cultures of bovine kidney cells were also used to determine whether they might be bovine strains.

b. Canine Kidney Cells.

Method. Samples of 21 stool extracts were added to cultures of GH cells (continuous canine kidney). 12 extracts were NG specimens. Specimens which did not cause CPE were maintained in culture for 22-27 days with blind passage at 7-10 day intervals. A strain of Infectious Canine Hepatitis Virus (ICHV) was titrated on the GH cell line and the titre compared with that achieved with the Madin-Darby line of canine kidney cells (MDCK).

Result. From Table 3.4 it can be seen that no specimen caused CPE over more than one passage. The titre of ICHV was $10^{9}TCD_{50}/ml$ using both GH and MDCK cell lines in parallel.

Comment. The susceptibility of the GH cell line to one strain of canine adenovirus compared favourably with that of the well-known Madin-Darby cell line. Although the ICHV strain tested was already adapted to growth in tissue culture, the very high titre achieved with GH cells probably reflected a general susceptibility of the cell line to canine adenovirus. It is therefore unlikely that the human specimens tested contained adenovirus of canine origin.

TABLE 3.4 Result of culture with 21 stool extracts known to contain adenovirus by electron microscopy, using Canine Kidney (GH) and Secondary Bovine Embryo Kidney Cells.

No. of Patients from Whom:			Total Patients
1 Stool Examined	2 Stools Examined	3 Stools Examined	
9	3	2	14

No. of Specimens Not Causing CPE	No. of Specimens Causing CPE			Total Specimens
	Agent Typed	Agent Untyped	Total	
HEK Cells* 12	7 †	2	9	21
GH Cells 21	0	0	0	21
Bovine Embryo Kidney Cells 18	3 §	0	3	21

* results from Chapter 2
† adenovirus strains of types 1, 5, 7, 2+9, 17 and two type 2 strains
§ adenovirus strains of types 1, 2 and 5

c. Bovine Embryo Kidney Cells.

Method. Samples of 21 stool extracts (those used to inoculate GH cells) were added to cultures of secondary bovine embryo kidney cells. Specimens which did not cause CPE were maintained in culture for 24-25 days with 3 blind passages. At each passage cells from a different fetus were used.

Result. 18 specimens did not cause CPE (Table 3.4). Human adenovirus types 1, 2 and 5 were recovered from 3 different specimens.

Comment. Although bovine adenoviruses of subgroup 2 replicate only in calf testis cells (Bürki et al., 1978), bovine embryo kidney cells would be expected to allow isolation of the common bovine serotypes (1, 2 and 3; subgroup 1) (see Andrewes, Pereira and Wildy, 1978). These viruses might be transmitted to humans by dairy produce.

The bovine embryo kidney cells did not allow the isolation of adenoviruses from NG specimens. However, the cultures were susceptible to 3 human strains of different serotypes. It is unlikely that bovine cells would be sensitive to human strains, yet fail to detect common bovine strains. Therefore it was considered unlikely that the adenoviruses in NG specimens were common bovine strains.

B. ORGAN SEGMENT CULTURE.

1. The System.

a. Introduction. Adenoviruses in NG specimens might require cells of the intact human intestinal mucosa in order to replicate. There

are many possible metabolic or architectural conditions found in the intestine which are not present in monolayer cultures and which may be necessary for productive infection. These conditions include the presence of microvilli in columnar cells, and other ultrastructural and chemical arrangements within these cells. The apposition of cells or cell types may be important for infection, as may be the presence of certain immunocytes or endocrine cells. The rate of cell division within the crypts, or the cell turnover time in the epithelium might also be critical for successful replication of these adenoviruses.

Incubation of stool viruses in the presence of pieces of human embryo intestine would resemble in vivo conditions, provided the culture could be maintained in a healthy condition for several days. However, conditions at the intestinal mucosa of an 8 - week embryo will not correspond exactly to those in the intestine of a child after birth. For example, continuous exposure to micro-organisms in the latter case would be expected to have increased the proportion of immunocytes within the mucosa (see Creamer, 1974).

Nevertheless, success in the culture of viral agents from stools using human intestinal segment culture has been reported, namely, the isolation of human rotavirus (Wyatt et al., 1974) and coronavirus (Caul and Clarke, 1975). However, attempts to propagate these agents in HEK cells met with limited success (Wyatt et al., 1976; Caul and Egglestone, 1977) and have not been confirmed by other workers. Therefore it was possible that adenoviruses in NG specimens might grow in intestinal segment cultures.

There are few reports that adenovirus can be propagated in intestinal segment cultures. One group which described a reliable

method for maintaining the cultures had propagated adenovirus type 21 over 18 days (Dolin et al., 1970). There was no decrease in virus titre in fluids taken and replaced every 2 days. The successful culture of adenovirus type 5 has also been reported (Rubenstein and Tyrrell, 1970).

b. Preparation of Cultures. The procedure was based on the method of Dolin et al. (1970). The cultures were prepared from the intestine of embryos of 8 - 12 weeks' gestation.

The intestine was removed within 8 hours of termination, and washed in PBS containing antibiotics. Transverse cuts were made to give segments 2-3 mm long. These segments were cut longitudinally using fine scissors. The resulting square or rectangular pieces were transferred, mucosa uppermost, to 4 cm diameter plastic Petri dishes (Sterilin Ltd.) containing 1.5 ml maintenance medium (Leibovitz' L15 medium (Leibovitz, 1963) containing 0.2 per cent bovine albumen and antibiotics). The inner surfaces of the dishes had been scratched in cross-hatch pattern to provide a rough substratum for the intestinal segments. 3 segments were placed in each dish.

All dishes were incubated in humidity chambers and were rocked 3.5 times per min at 36.5°C. On the following day the maintenance medium was replaced and inoculations were carried out. The medium was replaced on alternate days and all spent fluids were stored at -20°C. The cultures were examined regularly by dissecting microscope for signs of degeneration (loss of villous architecture and sloughing).

2. Attempts to Culture Prototype Strains of Adenovirus.

a. Serotypes 1 and 2. Prototype strains of these common serotypes were tested for their growth in cultures of human intestinal segments. These serotypes can be isolated in the stools of infants over long periods (see Chapter 2). They probably replicate in the intestine rather than being the result of downflow from a respiratory infection (Fox et al., 1977) and would be the serotypes most likely to infect intestinal segment cultures productively. Adenovirus types 1 and 2 were used in a preliminary study to determine whether the culture system had any susceptibility to adenovirus.

Method. For each serotype, segments of intestine from a different embryo were used. 4 dishes were inoculated with 0.1 ml virus (10^3 TCD_{50}/ml for type 1 and $10^{3.5}$ TCD_{50}/ml for type 2). Dishes without cultures but containing maintenance medium were also inoculated (4 dishes for each serotype). In addition, 4 uninfected control cultures from each embryo were maintained in parallel with the inoculated cultures.

The cultures were maintained until the mucosa no longer appeared intact. When all fluids had been collected and stored, a 0.1 ml sample from each fluid was added to a monolayer culture of human amnion cells. This gave information on whether or not infection had persisted. The extent of infection was quantified by titration using KB cells. Tenfold dilutions of the culture fluids were made in PBS and 0.1 ml of each dilution was added to each of 4 tube cultures. Endpoints were read at 4 days, and infectivity titres were calculated by the method of Kärber (1931).

Results. With each serotype, the infectivity titres increased after infection of the cultures (Figures 1 and 2). The decay of infectivity in the fluids from dishes with no cultures was rapid. In no case was infectivity detected after day 6. Virus was recovered from the dishes with cultures until these degenerated, at titres greater than 10^3 TCD_{50}/ml.

Conclusions. Although there were frequent changes of medium, the quantity of infectious virus in the culture fluids increased. There is no doubt that replication of adenovirus type 1 and type 2 took place, since the amount of virus recovered far exceeded the input. Therefore I concluded that human intestinal segment cultures are capable of supporting the growth of at least some adenovirus strains.

b. Serotype 8. Although many serotypes of adenovirus can be grown to very high titres, this serotype yields a maximum of 10^2 - 10^3 TCD_{50}/ml in HeLa cells (Jackson and Muldoon, 1975). Type 8 is the most frequent cause of epidemic keratoconjunctivitis in Western countries (Jawetz, 1959; Dawson et al., 1963). Therefore, although poorly infectious in monolayer cultures, the ability of this serotype to transmit infection in the wild is undoubted.

A prototype strain of adenovirus type 8 was used to infect human intestinal segment cultures. Measurement of virus infectivity by titration in monolayer cultures was unlikely to be achieved. However, I hoped to demonstrate the continued infection of intestinal segments by serial passage into fresh cultures. Recovery of this serotype in fluids taken after prolonged incubation

FIGURE 1 Average infectivity titres of culture fluids from intestinal segment cultures inoculated with adenovirus type 1. Open circles: fluids from inoculated dishes with cultures. Closed circles: fluids from inoculated dishes without cultures.

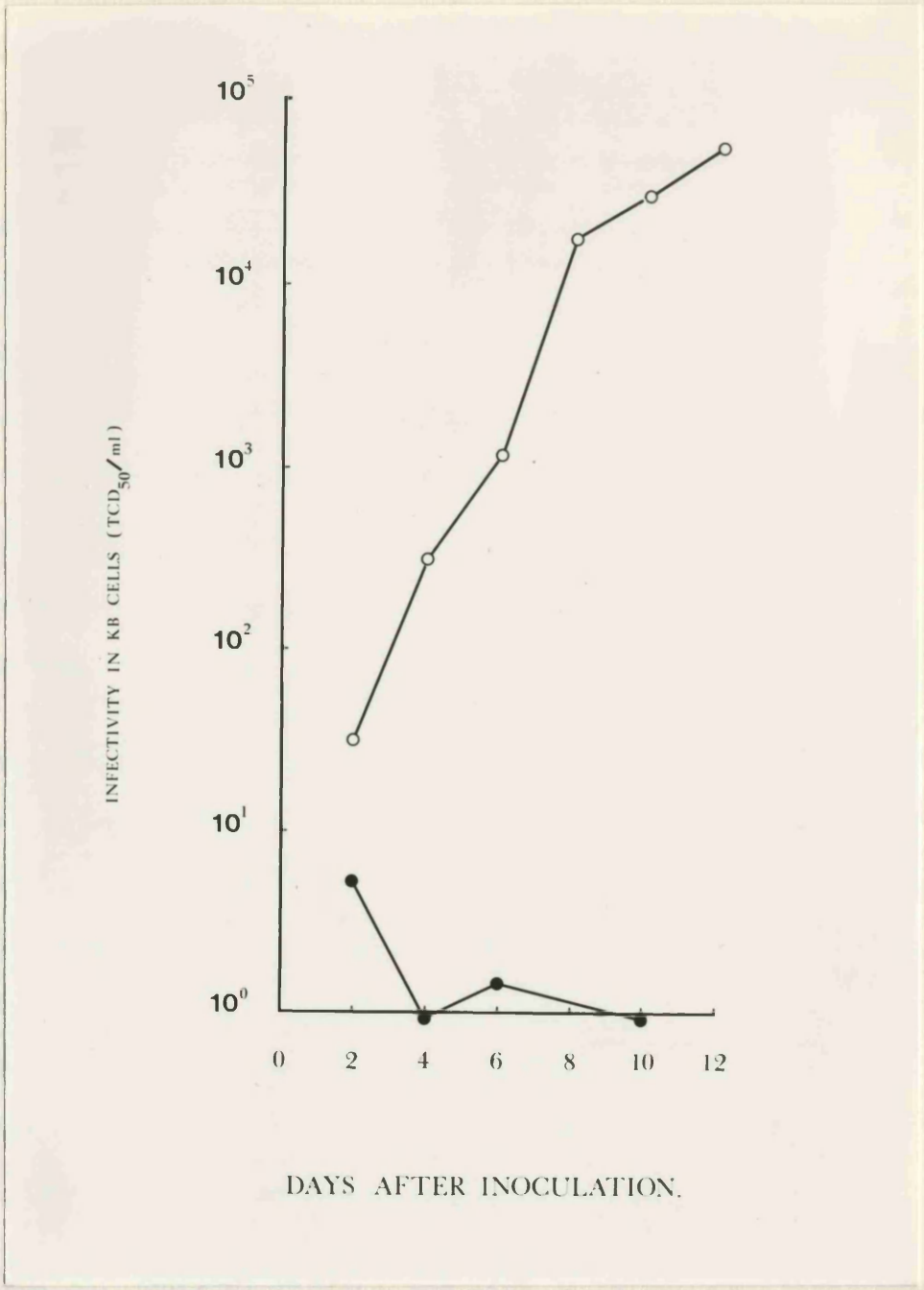

FIGURE 2 Average infectivity titres of culture fluids from intestinal segment cultures inoculated with adenovirus type 2. Open circles: fluids from inoculated dishes with cultures. Closed circles: fluids from inoculated dishes without cultures.

of these cultures would be regarded as proof of replication.

Method. Four dishes with cultures and 4 without each received 0.1 ml adenovirus type 8 ($10^{2.5}$ TCD_{50}/ml). They were incubated as above. Uninoculated dishes were included as controls. The fluids collected at 2 day intervals were stored at -20°C, then tested for infectivity with human amnion cells. Titration was attempted with KB cells. Medium collected at day 10 was also passed to HEK cultures. Fluid collected at day 10 (0.1 ml) from 2 of the cultures was passed each to a fresh intestinal segment culture. Incubation was continued for a further 16 days. The infectivity of day 12 and day 16 fluids from this second passage was tested by inoculation of HEK cell cultures.

Result. Human amnion cells allowed the detection of a cytopathogenic agent in day 8 and day 12 culture fluids, and not in the control fluids. HEK cells proved more sensitive to the day 10 culture fluids: adenovirus type 8 was identified from each (Table 3.5). This virus was also identified from the day 12 and day 16 culture fluids taken at the second passage using HEK cells. The KB titration system was not sensitive enough to detect any infectivity in culture fluids from the first passage.

Conclusion. Only $10^{1.5}$ TCD_{50} was used to infect each organ segment culture. Nevertheless, after 26 days with 11 complete changes of medium and transfer of fluid to a new culture, adenovirus type 8 was recovered. This could only have resulted from serial passage of the virus, since this serotype was not in use in the laboratory at the time of the second passage. Therefore propagation of adenovirus type 8 was considered proven.

TABLE 3.5 Results of cell culture using intestinal segment culture fluids as inoculum. The first passage fluids were from cultures inoculated with adenovirus type 8, uninoculated cultures and inoculated dishes without cultures.

No. of Dishes from Which Cytopathogenic Agents Were Detected (Actual/Possible)

First Passage

	Inoculated Cultures	Uninoculated Cultures	Control Inoculated Dishes
DAY 2	amnion 0/4	amnion 0/4	amnion 0/4
DAY 4	amnion 0/4	amnion 0/4	amnion 0/4
DAY 6	amnion 0/4	amnion 0/4	amnion 0/4
DAY 8	amnion 3/4	amnion 0/3	amnion 0/4
DAY 10	amnion 0/4 HEK *4/4	amnion 0/2 HEK 0/2	amnion 0/3 HEK 0/4
DAY 12	amnion 1/4	amnion 0/2	amnion 0/3

Second Passage (from DAY 10 fluids of first passage)

	Inoculated Cultures	Uninoculated Cultures
DAY 12	HEK *2/2	HEK 0/2
DAY 16	HEK *2/2	HEK 0/2

* agents typed individually as adenovirus type 8

c. Adenovirus in NG Specimens. Intestinal segment cultures were capable of supporting the replication of adenovirus which is poorly infectious in monolayer cultures. This system might also allow the replication of adenovirus in NG specimens.

It would have been informative to compare the efficiency of isolation of recognised serotypes from stools by organ segment culture and by cell culture. The frequency of isolation of serotypes might differ considerably by the 2 methods. However, the supply of intestine was limited and attempts could only be made to isolate adenovirus from NG specimens.

Method. 3 stool extracts from different patients were each used to inoculate 6 dishes with cultures and 6 dishes without cultures (0.1 ml per dish). 2 dishes with cultures were not inoculated, but were incubated in parallel. The culture fluids taken every 2 days were stored at -20°C.

Fluids collected at day 14 were examined by EM. Day 12 and day 16 fluids were used to inoculate HEK cell cultures. Culture fluids from day 16, and some from day 12, were passed to fresh intestinal segment cultures. Fluids taken at day 12 of this second passage were examined by EM.

Results. These are shown in Table 3.6. For each specimen, adenovirus particles were detected in day 14 fluid from at least one of the 6 cultures (Plates 4 and 5). From cultures inoculated with a specimen from patient no. 25, all day 14 fluids contained adenovirus.

No cytopathogenic agent was detected in the first passage fluids from any culture. When day 16 fluids were passed to fresh intestinal segment cultures, at least one of the fluids collected

TABLE 3.6 Results of attempts to detect virus in intestinal segment culture fluids, by EM and HEK cell culture. The first passage fluids were from cultures inoculated with NG specimens (extracts containing adenovirus which could not be isolated in cultures of HEK cells).

No. of Dishes (Actual/Possible) from Which:

First Passage

Specimen from Patient No.:	Adenovirus Detected in Fluid from DAY 14 by EM	CPE Developed in HEK Cells Using Fluid from DAY 12	CPE Developed in HEK Cells Using Fluid from DAY 16
5	2/6	0/6	0/6
25	6/6	0/6	0/6
30	1/6	0/6	0/6
uninoculated controls	0/2	0/2	0/2

Second Passage Specimen from Patient No.:	Adenovirus Detected in Fluid from DAY 12 by EM
from DAY 12 fluids of first passage	
25	6/6
from DAY 16 fluids of first passage	
5	1/6
25	3/5
30	1/6
uninoculated controls	0/2*, 0/4**

* controls with passaged fluids
** controls (second passage only)

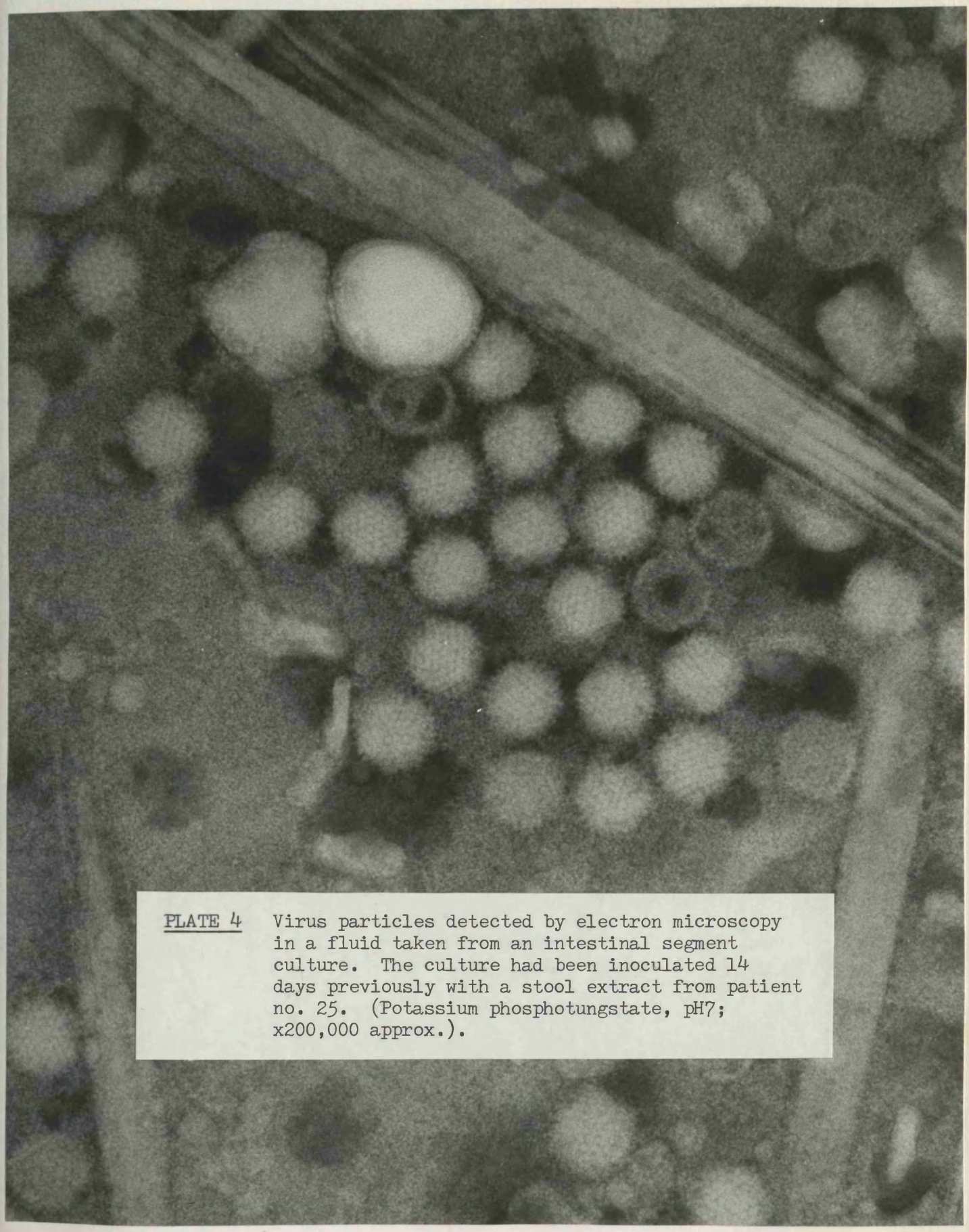

PLATE 4 Virus particles detected by electron microscopy in a fluid taken from an intestinal segment culture. The culture had been inoculated 14 days previously with a stool extract from patient no. 25. (Potassium phosphotungstate, pH7; x200,000 approx.).

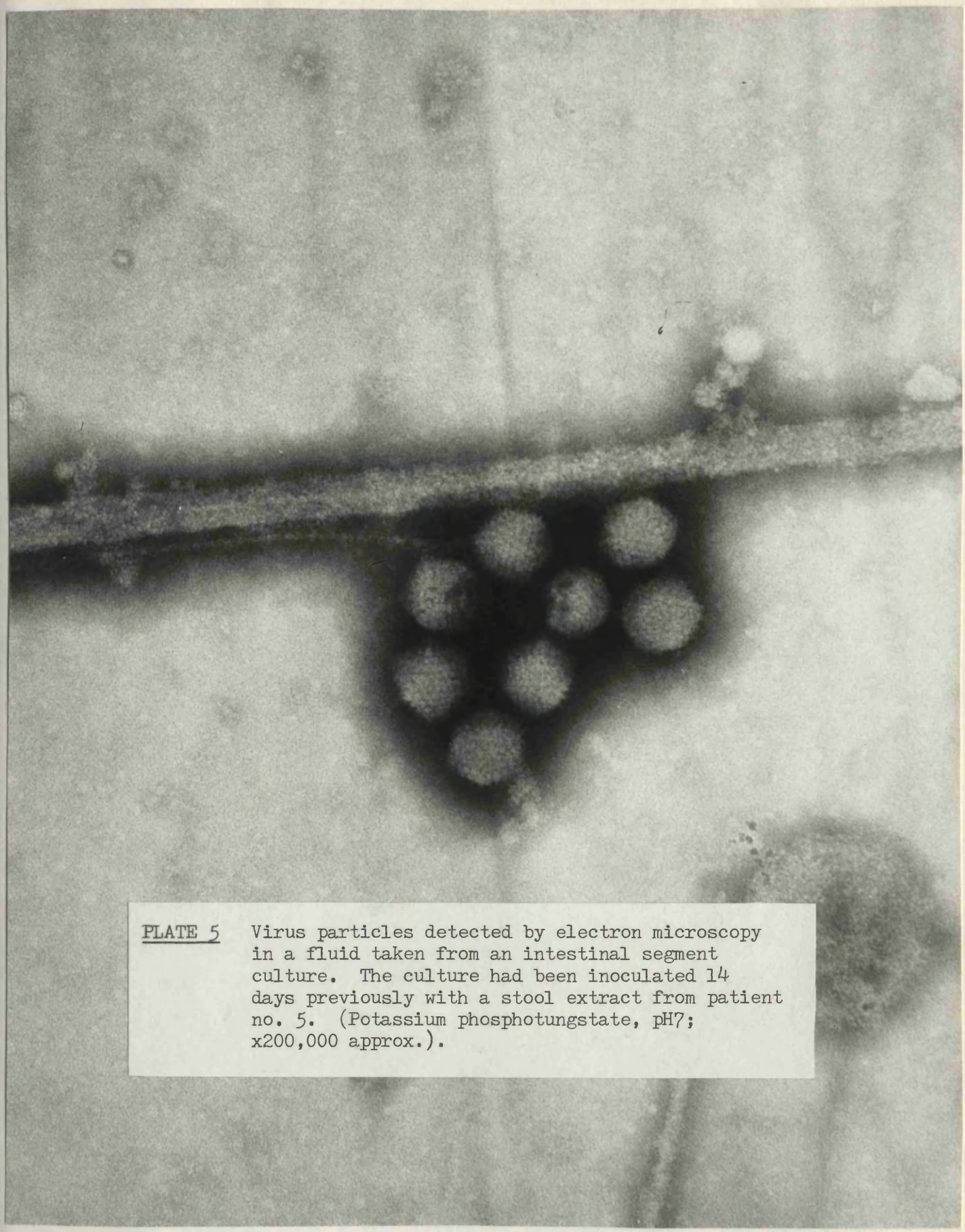

PLATE 5 Virus particles detected by electron microscopy in a fluid taken from an intestinal segment culture. The culture had been inoculated 14 days previously with a stool extract from patient no. 5. (Potassium phosphotungstate, pH7; x200,000 approx.).

on the twelvth day after passage contained adenovirus particles.

Comment. After 24-28 days with 10 or 12 complete changes of medium and passage to a fresh culture, it is unlikely that the adenovirus particles detected in fluids taken at day 12 of the second passage were input virus. It is probable that adenovirus replication took place, especially in the 6 cultures inoculated with an extract from patient no. 25. However, this was difficult to prove conclusively since titration of the virus was not possible even in cells recognised as being the most sensitive to known adenovirus serotypes.

The relatively insensitive technique of electron microscopy would not be expected to allow detection of virus particles in fluids in numbers less than about 10^6 particles per ml (C.R. Madeley, personal communication). Therefore, for adenovirus to be detected by EM after 28 days the degree of virus production may have been substantial, especially from the extracts of patients no. 5 and 30. In these cases the amounts of adenovirus detected in the original specimens by EM were small (less than 5 particles per grid square). Further investigation into the nature of the viruses from these cultures is described in Chapter 7.

C. VARIATIONS IN CULTURE TECHNIQUE AND SPECIMEN HANDLING.

1. Inoculation of Cell Suspensions Prior to Monolayering.

This technique has been reported to increase the number of adenovirus isolations and shorten the time for CPE to appear (Rose, 1964). Exposure of the cells to specimens apparently does not interfere with their ability to form monolayers. The technique

might allow detection of adenovirus in NG specimens.

Method. A suspension of HEK cells in growth medium was prepared as described in Chapter 1. The suspension was dispensed into tubes at a seeding concentration of 5×10^4 cells/ml. 8 NG specimens from different children were used to inoculate the cultures (2 tubes per specimen; 0.1 ml added per tube). All cultures (including uninoculated controls) were incubated at 36.5°C and observed daily for signs of degeneration. The growth medium was replaced with maintenance medium after 48 hours. On degeneration of the monolayers, the cultures were frozen and thawed at least 4 times. The contents of the appropriate tubes were pooled and passed (a) to each of 2 fresh tubes with HEK monolayer cultures in maintenance medium, and (b) to each of 2 fresh tubes containing HEK cell suspensions in growth medium. All cultures were incubated and observed as before. The (b) cultures received maintenance medium after 48 hours.

Results. One stool extract prevented monolayer formation but the effect did not show on passage. The other 7 stool extracts did not cause CPE after addition to cell suspensions, nor after passage into suspension or monolayer cultures. Each had undergone a total of 19-20 days in culture.

Conclusion. Apart from one stool extract (which may have had a toxic effect on the cells in suspension) these specimens did not have an effect on the monolayers once formed. Therefore the technique of inoculating HEK cell suspensions with NG specimens did not benefit the isolation of adenovirus, at least within a 3 week period.

2. Comparison of Different Methods for Releasing Virus from Cells.

Repeated freezing of cultures to -20°C and thawing at room temperature is usually used to release adenovirus from infected cells, presumably by rupturing the cell membrane (see Grist et al., 1979). However, this method may not be sufficient to release infectious virus if produced in small quantity. I considered the technique of sonication as an alternative which might cause more effective release of virus. The value of freezing culture fluids repeatedly at a lower temperature (-70°C) was also examined.

Method. 8 NG specimens from different children were used to inoculate HEK cells (6 tubes per specimen; 0.1 ml per tube). The cultures were incubated and examined as usual, until the monolayers began to degenerate. 2 cultures per specimen were frozen to -20°C 4 times, 2 cultures were frozen to -70°C 4 times, and 2 cultures were frozen to -20°C, thawed, then sonicated in a Brensonic bath sonicator for 30 seconds. All fluids were passed separately to fresh cultures, which were incubated and examined as above.

Results. Each specimen was in culture for 12 days before passage and 13 days after passage. CPE was not observed.

Comment. Neither sonication, nor repeated freezing to a lower temperature than usual allowed detection of infectivity from these NG specimens. There was no argument for adopting either of these techniques in future attempts to detect adenovirus in NG specimens.

3. Low Speed Centrifugation of the Cell Monolayer After Inoculation.

It has been reported that low speed centrifugation (3000 g) of cell monolayers after inoculation improves the efficiency

of detection of rotavirus by immunofluorescence (Banatvala et al., 1975). The reason for this remains unclear.

It may be that other viruses which cannot be cultured from stools might show infectivity by this technique. In particular, adenoviruses in NG specimens might cause CPE after this treatment. Therefore a pilot experiment was set up. Since the centrifugation of several specimens together required the use of tissue culture plates, a non-volatile buffer (HEPES; Williamson and Cox, 1968) was incorporated into the media for cell growth and maintenance.

Method. 24 - well tissue culture plates (Falcon Industries) were seeded with human amnion cells at a concentration of 5×10^5 cells/ml in growth medium containing 25 mM N-2-hydroxyethyl-piperazine-N'-ethanesulphonic acid (HEPES; Calbiochem). When confluent, the monolayers received maintenance medium containing 30 mM HEPES (0.9 ml medium per well).

Each of 5 NG specimens from different patients was used to inoculate 2 wells (0.1 ml per well) of one plate (A). Each of 3 G specimens from different patients was used to inoculate 2 wells of a second plate (B). Each plate was centrifuged for 30 min at 2000 rev./min in an MSE Magnum centrifuge, using MSE plate carriers (see Plate 6) and swing-out head. The same specimens were used to inoculate 2 other wells of the appropriate plate after centrifugation. A third uncentrifuged plate (C) was inoculated with 2 samples of each specimen. 4 uninoculated wells were included per plate. The plates were incubated at 36.5°C.

On the tenth day, the contents of each well were frozen and thawed 4 times, passed to 2 tubes of human amnion cells (0.1 ml

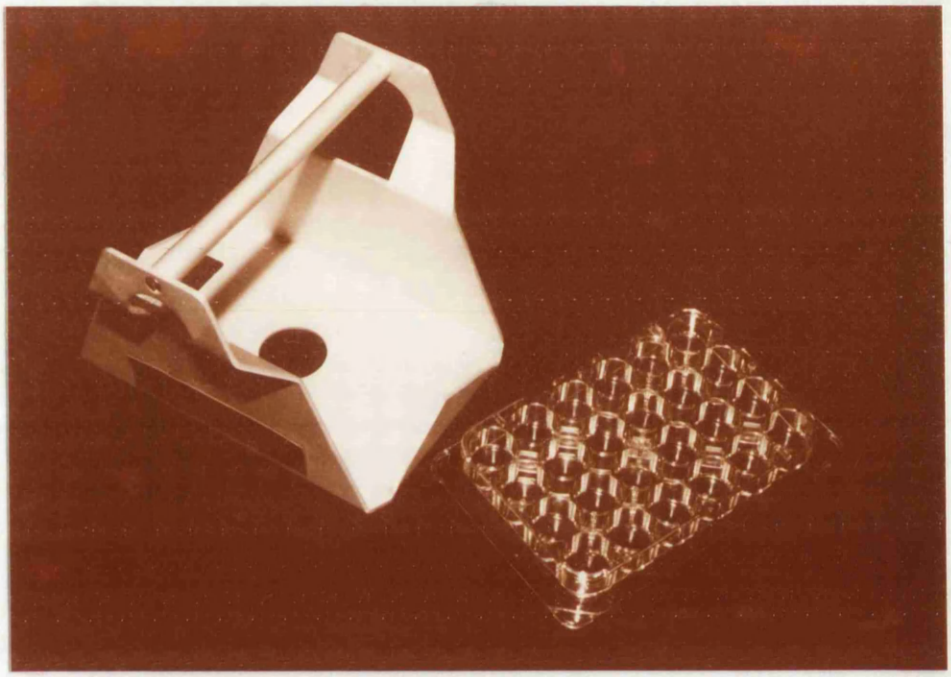

PLATE 6 24-well cell culture plate (Falcon) and plate carrier (MSE) used for centrifugation of plate cultures.

per tube) and incubation was continued at 36.5°C.

Results. The 3 G specimens caused CPE (Table 3.7). There was no appreciable decrease in the time taken for CPE to appear where inoculation preceded centrifugation. In one case (patient no. 7) the uncentrifuged controls took longer for CPE to appear. 2 NG specimens had an effect on the cells under all circumstances, but this did not show after passage.

Comment. Centrifugation of specimens onto monolayers of human amnion cells did not cause an increase in the number of positive isolations from NG specimens. The effect seen at the first passage with 2 specimens was probably a result of a toxic effect of the extracts. Since the 3 G specimens caused CPE, the presence of HEPES in the medium was probably not inhibitory to adenovirus replication, as reported previously (Williamson and Cox, 1968).

There was no argument for continuation of this method with observations of CPE. However, an assessment of the technique with detection of adenovirus antigen by immunofluorescence in cells inoculated with NG specimens is described in Chapter 4.

4. Incubation In Cultures At Different Temperatures.

Adenoviruses in NG specimens might be adapted to growth at a higher or lower temperature than is considered optimum for growing strains (around 37°C). An unusual temperature requirement (if it existed) might be the result of adaptation to replication at cool epithelial surfaces (of the respiratory tract), or epithelial surfaces located well within the torso (in the intestine) which might be at a slightly higher temperature

TABLE 3.7 Evaluation of the method of low-speed centrifugation of cell monolayers after inoculation, for the detection of adenovirus in NG specimens.

Patient No.	Specimen	No. of Days for Cytopathic Effect to Appear:			Virus Isolated
		Inoculation Before Centrifugation	Inoculation After Centrifugation	Inoculation (No Centrifugation)	
7	G	9	10	16	Ad2
31	G	16	16	16	Ad1
49	G	3	4	4	Ad1
5	NG	-	-	-	
25	NG	-	-	-	
29	NG	4*	4*	4*	
30	NG	-	-	-	
38	NG	6*	6*	6*	

\- negative result
* no cytopathic effect on passage

than the rest of the body.

Method. Single NG specimens from 4 different patients (numbers 70, 73, 74 and 75) were added (0.1 ml) to each of 6 cultures of secondary HEK cells. 2 cultures were incubated at 33°C, 2 were incubated at 36.5°C and 2 were incubated at 39°C. At degeneration of the cultures, the fluids from duplicate cultures were pooled and passed (as described in Chapter 1) to fresh cultures of HEK cells, in parallel with the fluids from cultures incubated at the other temperatures. All fluids were passaged for a third time in the same way. Cultures were observed on alternate days for CPE.

Results. The specimens of patients no. 73, 74 and 75 were incubated at each temperature for 35 days but no CPE developed in any culture. The specimen from patient no. 70 caused CPE after passage to fresh cultures. There was only a trace of CPE in the second passage cultures incubated at 39°C and 36.5°C but it was prominent in the cultures incubated at 33°C, and these cultures were frozen after 5 days since they showed complete CPE. On passage, only the cultures at 33°C showed CPE and this did not progress. Attempts to identify the cytopathogenic agent by neutralisation failed since CPE did not develop in the test cultures.

Comment. The partial success in detecting a cytopathogenic agent from one specimen at 33°C may simply have reflected the chance of detecting one viable virus dose in a series of replicate cultures either at the same or different temperatures. Therefore too much importance could not be attached to this result, especially since

33°C incubation did not allow detection of adenoviruses in 3 other NG specimens. There was little evidence to suggest that incubation at either 39°C or 33°C would allow isolation and characterisation of adenoviruses in NG specimens.

Further attempts to isolate adenoviruses in NG specimens at 33°C are described in Chapter 7.

D. DISCUSSION.

The work described in this chapter was done in an attempt to isolate adenovirus from NG specimens. The methods employed, with the exception of organ segment culture, were an extension of routine procedures for virus isolation. Electron microscopy and observation for CPE were the only monitoring techniques used; thus only productive infection was likely to be detected.

Only the use of 2 cell lines (Intestine 407 and Chang Conjunctiva) indicated the possible presence of cytopathogenic agents in NG specimens. However, this was not a CPE typical of known adenovirus serotypes; nor was it detected from all NG specimens. Since the effect on the cells appeared to diminish on passage, it could have been the result of a non-replicating factor in the specimens to which these cells were particularly sensitive.

The results of organ segment culture were equally equivocal. One factor which the cell lines above and the intestinal segment cultures had in common was the presence of Leibovitz' L15 fluid as maintenance medium, which may have allowed some replication of adenovirus in NG specimens. This is further discussed in Chapter 7.

CHAPTER 4

CHAPTER 4. USE OF METHODS TO DETECT PARTIAL REPLICATION OF ADENOVIRUS.

A. INOCULATION OF CELL CULTURES WITH A DIFFERENT VIRUS SIMULTANEOUSLY.

1. Introduction. Since there was no firm evidence for the viability of adenovirus in NG specimens in the work described in Chapter 2 and 3, the possibility that these agents might be defective in some function of replication was considered. Such defectiveness, although prohibiting the production of progeny virus, might not block the production of all enzymes or structural proteins. Therefore the adenoviruses in NG specimens might be capable of supplying a function required by another virus for infection. Alternatively, the presence of another virus might supply a function needed by the adenoviruses in NG specimens for replication.

2. Tests with Adeno Associated Virus.

a. Introduction. Adeno associated viruses (AAV) are parvoviruses which replicate only in the presence of multiplying adenovirus (see Young and Mayor , 1979). Since adenoviruses in some stool extracts failed to replicate in cell culture, the question of whether they could still supply a function to allow AAV replication was considered. If AAV was found to replicate for several passages after contact with adenovirus in NG specimens, this would suggest that either partial replication of adenovirus or adenovirus infection with low virus yield had taken place. Moreover, it was possible that adenoviruses in NG specimens might be adapted to replicate only in the presence of AAV, in which case the addition of AAV to the cultures might lead to productive adenovirus infection.

b. Determination of AAV Viability. To check the viability of the AAV strain to be used, it was serially passaged in culture both in the presence and in the absence of a prototype adenovirus strain. Electron microscopy of culture fluids was used to determine whether AAV particles were present after passage. The presence of these particles only in fluids derived from cultures which had also received adenovirus would indicate that the AAV was viable and depended on the presence of adenovirus for replication.

Method. AAV type 1 (strain 1 H) stored at -70°C was diluted 10^{-1} in human amnion cell maintenance medium. Samples of 0.1 ml were added to 8 fresh cultures of human amnion cells. Adenovirus type 2 ($10^{4.5}$ TCD_{50}/ml; 0.1 ml per tube) was added to 4 of the cultures and also to adenovirus control cultures (with no AAV). All volumes were standardised with maintenance medium and the cultures (including uninoculated controls) were incubated stationary at 36.5°C. Each fluid was passaged independently into a second, then a third culture of human amnion cells. At degeneration, all third passage culture fluids were frozen to -20°C and thawed. The fluids were prepared for EM examination as described in Chapter 1.

Results. The 3 serial passages lasted a total of 15 days. CPE developed at each passage in those cultures which had received adenovirus alone, or adenovirus and AAV. It did not develop in the cultures which had received AAV alone, or in control cultures (Table 4.1). Only those third passage fluids derived from cultures that had received both adenovirus and AAV contained detectable amounts of small virus-like particles less than 30 nm diameter with a hexagonal profile (Plate 7).

Comment. The small virus-like agents observed fit the description of

type 1 in the presence of adenovirus type 2: determination of AAV viability using human amnion cells, by electron microscopy.

Cultures Inoculated With:	No. of Cultures (Actual/ Possible) Showing Development of Cytopathic Effects			No. of Cultures (Actual/ Possible) Positive by EM of Pass 3 Culture Fluid	
	Pass 1	Pass 2	Pass 3	Adenovirus Particles	Smaller* Particles
AAV 1 + Ad 2	4/4	4/4	4/4	2/4	4/4
AAV 1	0/4	0/4	0/4	0/4	0/4
Ad 2	2/2	2/2	2/2	2/2	0/2
uninoculated	0/2	0/2	0/2	0/2	0/2

* the particles seen were less than 30 nm diameter with a hexagonal outline and often present in large clusters

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TABLE 4.2 Serial passage of AAV type 1 in the presence of NG specimens, with examination of culture fluids for the presence of virus by electron microscopy. Human amnion cultures were used.

Patient No.	No. of Culture Fluids (Actual/Possible) Positive for AAV Particles by EM							
	Pass 1		Pass 2		Pass 3		Pass 4	
	AAV	no AAV	AAV	no AAV	AAV	no AAV	AAV	no AAV
5			0/4	0/2			0/4	0/2
25			0/4	0/2			0/4	0/2
29			0/4	0/2			0/4	0/2
34			0/4	0/2			0/4	0/2
36			0/4	0/2			0/4	0/2
7 (Ad 1 previously isolated)			0/4	0/2			1/4	0/2
41 (Ad 2 previously isolated)			1/4	0/2			2/4	0/2
no specimen	6/6	ND*	0/6	0/4	0/6	ND*	0/6	0/4
Ad 2			4/4**	0/2***			4/4**	0/2***

* ND not done

**3/4 fluids contained Ad by EM

***2/2 fluids contained Ad by EM

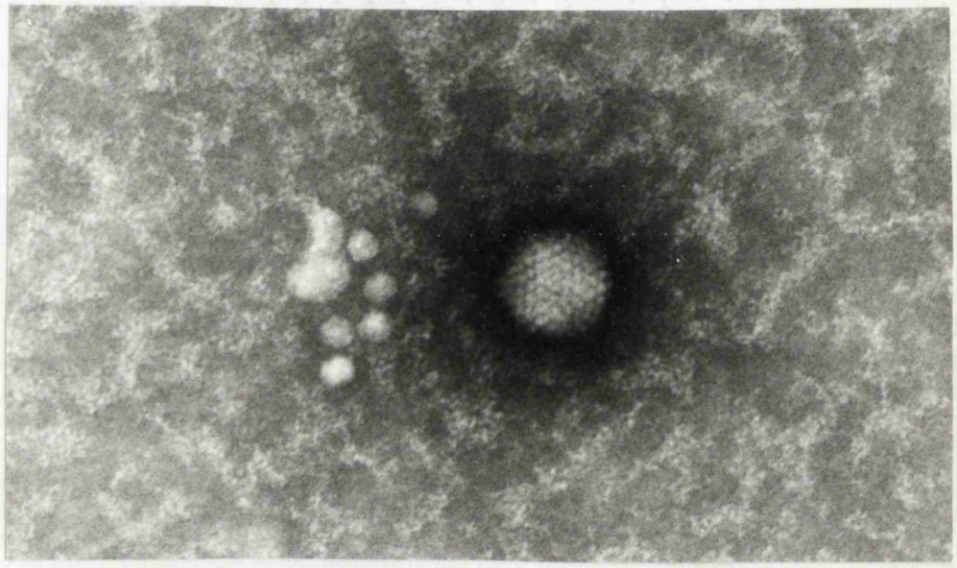

PLATE 7 Small virus-like particles with hexagonal profile found in cultures inoculated with adeno associated virus and prototype adenovirus. This micrograph was taken from a third passage culture fluid derived from a culture that had received adeno associated virus type 1 and adenovirus type 2. An adenovirus particle is also present. (Potassium phosphotungstate, pH7; x200,000 approx.).

adeno associated virus (Atchison et al., 1965). These particles were not present in fluids derived from cultures inoculated with AAV alone. Therefore, it was assumed that these particles were AAV which had undergone replication in the presence of adenovirus type 2.

c. Culture Attempts with AAV and Adenovirus in NG Specimens. To determine whether AAV would replicate with adenovirus in NG specimens as helper (or wice versa) these agents were incubated together over 4 passages in human amnion cells. Culture fluids were examined for the presence off virus by EM.

Method. 7 stool extracts from different children were added to fresh cultures of human amnion cells (6 tubes per specimen; 0.1 ml per tube). 5 of the specimens had been classed as NG (Chapter 2) and adenovirus type 1 or type 2 had been isolated from the other 2 specimens. 6 other cultures were inoculated with prototype adenovirus type 2 ($10^{4.5}$ TCD_{50}/ml; 0.1 ml per tube). 4 of the 6 tubes inoculated with a specimen or prototype virus also received AAV type 1 (0.1 ml of 10^{-1} dilution as before). AAV was added to 6 cultures not previously inoculated (AAV controls). All deficiences in volume were corrected with maintenance medium. The cultures (including uninoculated controls) were incubated stationary at 36.5°C and observed on alternate days for signs of degeneration. All fluids were passaged independently into a second, third and fourth culture of human amnion cells. The second and fourth passage fluids were prepared for EM examination as described in Chapter 1, except that the second passage fluids had been frozen 5 times rather than once. The culture fluids from the 6 AAV controls of all passages were examined.

Results. The 4 serial passages lasted a total of 31 days, except for those cultures containing the prototype strain of adenovirus type 2,

for which serial passage lasted 22 days. The 6 cultures inoculated with prototype adenovirus type 2 developed CPE repeatedly over the 4 passages. The G specimens containing either type 1 or type 2 caused CPE at both the third and fourth passages, but only in one and two cultures respectively. No CPE was detected in cultures with NG specimens.

The results of EM are given in Table 4.2. Whereas all fluids from the first passage AAV control cultures contained small hexagonal particles, these were not detected in fluids from later passages. However, these small particles were detected in all second and fourth passage fluids derived from cultures inoculated with AAV and prototype adenovirus type 2. Those cultures which developed CPE from G specimens were found to contain small hexagonal particles in the fourth passage culture fluids. However, no fluids derived from cultures inoculated with NG specimens were found to contain these particles.

Comment. It was assumed that the small hexagonal particles detected were adeno associated virus. They differed in appearance from the small round viruses found in culture fluids of some stool extracts, from which picornaviruses were identified by neutralisation test (Chapter 2). Adeno associated virus particles were detected only in culture fluids derived from cultures inoculated with both AAV and adenovirus. As in the previous experiment, AAV added to cultures without adenovirus appears to have been diluted on passage without replication. Not all of the cultures inoculated with G specimens showed CPE (which may have been due to a low number of adenovirus particles in the specimen). In the cases where CPE did develop, AAV was detected in very large numbers by the fourth passage. Therefore adenovirus of the commoner serotypes in stool specimens can support the replication of AAV type 1 in cell culture.

Adeno associated virus added to cultures together with adenovirus in NG specimens did not replicate to amounts detectable by EM. Therefore it is unlikely that the adenoviruses in NG specimens can supply the function required for AAV type 1 replication, although prototype adenovirus type 2 can.

AAV type 1 and adenovirus in NG specimens together did not cause CPE; nor was adenovirus production detected by EM. Therefore it is unlikely that this serotype of AAV can supply a missing function which might be required by these adenoviruses for productive infection. It is also unlikely that these adenoviruses depend on the presence of AAV for replication within the intestine.

3. Tests with Standard Adenovirus Strains.

a. Introduction. Adenoviruses in NG specimens might be common serotypes of human origin which lack some biochemical function. More than one virus-specified function could be missing, as would be the case if part of the genome was deleted. Although adenoviruses in NG specimens are shed in large quantity, this does not preclude the possibility that the parent viruses were also defective. For example, productive infection might result from the complementation of functions by more than one adenovirus strain within the intestinal cells.

Adenoviruses requiring complementation in order to infect cells productively might be able to have the missing function supplied by a non-defective strain as helper. To test whether adenoviruses in NG specimens can replicate in the presence of a helper adenovirus, 2 different categories of prospective helper virus were chosen:
(1) Adenovirus which can replicate productively in human cells. Adenovirus type 8 was chosen since it grows only to low titre and would not be expected to outgrow or mask the production of a reactivated strain of common serotype.

(2) Non-defective adenovirus of animal origin which does not replicate productively in human amnion cells. Infectious Canine Hepatitis Virus (ICHV) was chosen because there is evidence using complement fixation and immunofluorescence tests that ICHV interacts with human amnion cells to produce virus-specified antigens, but little infectious virus (Gehle & Smith, 1969). Furthermore, ICHV is reported to be capable of supplying a helper function for the replication of AAV in human cells (Smith & Gehle, 1967). ICHV produces little or no CPE in human cells. Therefore any development of CPE after mixing ICHV and adenovirus in NG specimens would indicate that either complementation of functions or genetic recombination had taken place.

b. Adenovirus Type 8.

Method. Prototype adenovirus type 8 was propagated in HEK cells as described in Chapter 1. To prepare antiserum to this serotype, 2 rabbits were inoculated intravenously 3 times at weekly intervals with 1.25 ml of clarified cell culture fluid. This contained adenovirus type 8 at maximum titre ($10^{2.75}$ TCD_{50}/ml). Serum from one rabbit, collected 2 weeks after the last injection, was used in the experiment below.

Nine NG specimens from 8 patients were added to cultures of primary human amnion cells (4 cultures per specimen; 0.1 ml per tube). 2 of the 4 tubes also received 0.1 ml adenovirus type 8 at 10^2 TCD_{50}/ml. Adenovirus type 8 was also added to 6 cultures which had not received stool extracts (prototype virus controls). All volume deficiencies were corrected with maintenance medium. The cultures (including uninoculated controls) were incubated at 36.5°C and examined on alternate days for CPE. At degeneration of the monolayers, the cultures were frozen and thawed 5 times and each

culture fluid was passed separately to 2 fresh cultures. Incubation was continued, with examination as before. At degeneration each culture was frozen 5 times and duplicate culture fluids were pooled.

Each stool extract control fluid (with no adenovirus type 8) was passed to a fresh culture. The specimen culture fluids containing adenovirus type 8 were mixed with a 1:50 dilution of antiserum to type 8 (0.2 ml culture fluid and 0.2 ml antiserum) and allowed to stand at room temperature for 60 min. The mixtures were added to fresh cultures (2 tubes per mixture; 0.1 ml per tube). The 6 second passage prototype virus control fluids were mixed with (a) adenovirus type 8 antiserum at 1:50 dilution, and (b) PBS. The mixtures were allowed to stand at room temperature for 60 min, then added to fresh cultures (2 tubes per mixture; 0.1 ml per tube). All third passage cultures were incubated at 36.5°C and observed daily for CPE.

Results. The serum prepared against adenovirus type 8 had a neutralising titre against homologous virus ($10^{1.5}$ TCD_{50}) of 1:128. Serum dilutions of 1:10 did not neutralise the stock strains of adenovirus types 1, 2, 3, 5 and 7 ($10^{1.5}$ TCD_{50}).

The first 2 passages in culture lasted a total of 13-15 days and all third passage cultures lasted 8 days. All first passage culture fluids which received adenovirus type 8 (with or without stool extracts) caused CPE when passaged (Table 4.3). Those first passage fluids which received stool extracts alone did not cause CPE.

After exposure to adenovirus type 8 antiserum, the second passage prototype virus control fluids did not cause CPE. After exposure to this antiserum, fluids derived from the cultures which received both adenovirus type 8 and NG specimens failed to cause CPE.

Comment. The antiserum prepared against adenovirus type 8 was considered suitable for the test since it had no measurable neutra-

TABLE 4.3 Serial passage of adenovirus type 8 in the presence of NG specimens: examination of the cultures for the development of cytopathic effect before and after exposure to adenovirus type 8 antiserum. Human amnion cultures were used.

Patient	No. of Cultures (Actual/Possible) Showing Cytopathic Effect			
	Pass 2		Pass 3	
	Ad 8	no Ad 8	Ad 8	no Ad 8
M	4/4	0/4	0/4*	0/2**
T	4/4	0/4	0/4*	0/2**
T	4/4	0/4	0/4*	0/2**
2	4/4	0/4	0/4*	0/2**
3	4/4	0/4	0/4*	0/2**
6	4/4	0/4	0/4*	0/2**
12	4/4	0/4	0/4*	0/2**
20	3/3	0/4	0/4*	0/2**
21	4/4	0/4	0/4*	0/2**
no specimen	12/12	0/10	0/12* 12/12**	0/3**

* the inocula for these cultures (second pass) were mixed with antiserum to adenovirus type 8

** the inocula for these cultures (second pass) were used without mixing with antiserum

lising activity to the commonest serotypes found in stools. Therefore any reactivation of these serotypes would be unhampered by the presence of this antiserum, unless there was a continuous requirement for the presence of helper virus. There is no evidence from the above results that replication of the adenoviruses in NG specimens can be helped by the presence of viable adenovirus, to the extent that these agents could replicate independently thereafter.

It is possible that adenoviruses in NG specimens might be strains of type 8. Any replication of such strains would have stopped on exposure to adenovirus type 8 antiserum. Type 8 is rarely isolated from stools, but this does not preclude the possibility that it could be shed in NG specimens. The arguments that (a) infectious helper virus may have to be continuously present, and (b) antiserum may be neutralising any reactivated virus, made the assessment of an animal virus as helper more attractive. The use of a viable adenovirus strain which does not infect human cells productively would eliminate the need for antiserum to a human serotype.

c. Infectious Canine Hepatitis Virus (ICHV).

Method. Nine NG specimens from 8 children (those used in Section b. above) were used to inoculate fresh cultures of human amnion cells (4 tubes per specimen; 0.1 ml per tube). 2 of the 4 cultures also received 0.1 ml of clarified GH cell culture fluid containing 10^8 TCD_{50}/ml ICHV. 6 other cultures received ICHV (ICHV controls). All deficiencies in volume were corrected with maintenance medium. All cultures (including uninoculated controls) were incubated at 36.5°C and examined on alternate days for CPE. At degeneration the cultures were frozen 5 times and 0.1 ml of each fluid was passed to 2 fresh cultures. These cultures were incubated and examined as before.

Results. The first passage lasted for 6-8 days and the second for 8 days. The control cultures which received only ICHV did not develop CPE. There was no CPE in cultures which received NG stool extracts (with or without ICHV). No second passage culture fluid developed CPE.

Comment. After 14-16 days, there was no evidence that the presence of ICHV reactivated a cytopathic agent in NG specimens. If indeed these adenoviruses are defective, then ICHV (in common with adenovirus type 8 and AAV type 1) was incapable of supplying the necessary function under the conditions used.

B. IMMUNOFLUORESCENCE.

1. Introduction.

The immunofluorescence technique is commonly used in clinical virology for the detection of virus-specific antigens within cells (Nairn, 1969). The cells examined can be from specimens or from cultures inoculated with specimens. The widely used indirect method depends on (1) the attachment of virus-specific antibodies to virus antigens within infected cells, followed by (2) detection of the virus-specific antibodies using an anti-species immunoglobulin labelled with a fluorescent tracer substance, for example fluorescein isothiocyanate. This fluoresces green when stimulated by ultraviolet light. In the presence of appropriate controls to indicate any non-specific fluorescence, the method can allow the detection (and often intracellular location) of virus antigens.

The immunofluorescence technique has been used to detect antigens of virtually all virus types that can be propagated in cell culture. It has also been used successfully to detect antigens of

viruses which cause little or no CPE, for example rotavirus (Banatvala et al., 1975; Bryden et al., 1977) and astrovirus (Kurtz et al., 1977). It might therefore allow detection of adenovirus antigens in cell cultures inoculated with NG specimens. If adenovirus-specific fluorescence could be detected, this would indicate that the adenoviruses had undergone at least part of the replicative cycle.

The antigenic composition of adenoviruses in NG specimens is unknown. These agents could have type-specific or subgroup-specific determinants not possessed by the established strains. However, they would be expected to share group-specific determinants with other adenoviruses. Antisera raised in animals to whole adenovirus particles are likely to contain antibodies to group-specific, subgroup-specific and type-specific determinants. With a serum displaying broad specificity to adenovirus in the immunofluorescence test, production of group-specific antigens by these uncharacterised adenoviruses might be detected. Therefore it was necessary to ensure that the antisera used for immunofluorescence would allow detection not only of the serotype to which the antiserum was raised, but also of other human serotypes.

The reading of an immunofluorescence test is subjective. Interpretation of what does and does not represent the presence of specific viral antigens will vary between individuals. It is important to eliminate as many sources of error as possible by including controls in the test which would indicate (a) non-specific reaction between conjugated antibody and uninfected cells, (b) non-specific reaction between conjugated antibody and infected cells, (c) non-specific reaction between test antiserum and uninfected cells, and (d) non-specific reaction between test antiserum and infected cells. The controls (a) and (b) involve substitution of

from the animal before immunisation (pre-inoculation serum). In practice, it is reasonable to assume that if control (d) is negative for fluorescence, control (b) is unnecessary, since there can not have been a reaction between the conjugated antibody and infected cells.

A detailed description of the general method used for immunofluorescence is given in Chapter 1.

2. Preparation and Testing of Rabbit Antisera.

a. Preparation. Antisera for use in immunofluorescence tests were prepared by inoculating rabbits with either adenovirus type 3 or type 5.

Method. Prototype strains of adenovirus type 3 and type 5 were each propagated for 3 passages in Bristol HeLa cells with maintenance medium containing 4 per cent rabbit serum (see Chapter 1). The third passage fluids from 50 cm^2 flask cultures were frozen 4 times to -20°C, centrifuged at 3000 rev./min for 30 min, and the supernatants were stored at -20°C. The culture fluid containing adenovirus type 3 (10^5 TCD_{50}/ml) was given intravenously (1.5 ml) 3 times at weekly intervals to each of 2 rabbits. Another 2 rabbits were inoculated with fluid containing adenovirus type 5 ($10^{5.5}$ TCD_{50}/ml) by the same procedure. Blood was collected from the ear vein 1 and 2 weeks after the last injection.

The sera were tested on a preliminary basis by neutralisation and immunofluorescence tests.

b. Testing of Rabbit Antisera by Neutralisation.

Method. The sera collected 1 and 2 weeks after the last inoculation were diluted 1:25, 1:125, 1:625 and 1:1250 in PBS and mixed (0.1 ml + 0.1 ml) with 10^3 TCD_{50} homologous virus (4 wells per dilution). The

1:25 dilutions of sera were also tested against $10^{2.5}$ TCD_{50} adenovirus type 1 and $10^{2.5}$ TCD_{50} adenovirus type 2. After 60 min at room temperature, the mixtures were added to fresh cultures of human amnion cells.

Result. All virus titres were adjusted to ensure development of CPE in the virus controls at the sixth day after inoculation. The serum titres (taken as the highest dilution to protect at least 50 per cent of the cultures at the seventh day) are given in Table 4.4. All sera showed neutralising activity to homologous virus. However, the antisera to adenovirus type 3 or type 5 at 1:25 dilution did not protect cultures from infection by adenovirus types 1 and 2.

Comment. The serum from rabbits inoculated with adenovirus type 3 had higher levels of homologous neutralising antibody. The homologous neutralising titre of the antisera to type 5 declined between the first and second week after the last inoculation. This further confirms the relatively weak immune response in the rabbits inoculated with adenovirus type 5. Assuming that antibody levels assessed by neutralisation test and immunofluorescence correspond, it was likely that the antisera prepared against type 3 would be the most useful for detecting adenovirus antigens within infected cells.

c. Testing of Rabbit Antisera by Immunofluorescence.

Part I. Antiserum to adenovirus type 3 from the rabbit which showed the highest neutralising antibody response was tested for the ability to react as adenovirus-specific antiserum in the indirect immunofluorescence test, using KB cells infected with common serotypes.

Method. The serum was diluted from 1:20 to 1:320 in doubling dilutions. These dilutions were applied in duplicate to coverslip

TABLE 4.4 Results of tests on sera from rabbits injected with adenovirus type 3 or type 5: neutralising activity against homologous virus. Tests were performed using human amnion cells.

	Highest Dilution to Protect 50 Per Cent or More of the Cultures from Infection With Homologous Virus	
Time After Last Injection:	1 Week	2 Weeks
Rabbit 1 * (Ad 3)	1:625	1:625
Rabbit 2 * (Ad 3)	$\geqslant$ 1:1250	$\geqslant$ 1:1250
Rabbit 3 * (Ad 5)	1:125	1:25
Rabbit 4 * (Ad 5)	1:125	1:25

* sera at 1:25 gave no protection against adenovirus type 1 and type 2

cultures of HEK cells infected with adenovirus type 3 ($10^{}$ TCD_{50}/ml; 0.5 ml per culture) fixed 30 hours after infection. Serum at 1:20 dilution was also applied to fixed coverslip cultures previously infected with adenovirus type 1 ($10^{4.5}$ TCD_{50}/ml; $50^{/ml)}$ type 2 ($10^{4.5}$ TCD_{50}/ml). Fluorescence staining was carried out as described in Chapter 1.

Result. The highest serum dilution to allow fluorescence with adenovirus type 3 infected cells was 1:80 (Table 4.5). 1:20 dilutions of serum did not allow fluorescence in cells infected with adenovirus type 1 or type 2. Fluorescence with type 3 infected cells was located in the nucleus.

Comment. Infection of the cells with the homologous serotype was detected by immunofluorescence, but infection by heterologous serotypes was not detected. Therefore the requirement for a group-specific serum was not satisfied by the result of this test.

Part II. Since fixation in the above test was carried out relatively soon after infection (30 hours), it was possible that the antigens of adenovirus types 1 and 2 were not detected because of variations in maturation times with different serotypes. Therefore the above test was repeated using the antiserum at 4 times titre, with the cultures fixed at different times after infection.

Method. Cultures of HEK cells infected with adenovirus type 1, type 2, type 3 and type 5 (10^{4} TCD_{50}/ml; 0.5 ml per culture) were fixed at (a) 30 hours, (b) 48 hours, and (c) 72 hours after infection. Duplicate cultures infected with each virus serotype and fixed at the different times were treated with test serum at 1:20 dilution and were stained as described in Chapter 1. Controls with pre-inoculation serum were prepared from cultures fixed at 72 hours.

TABLE 4.5 Results of immunofluorescence tests with serum from Rabbit No. 2 (see Table 4.4). Titration was performed using HEK cells infected with the homologous virus (adenovirus type 3). 1:20 dilutions of serum were also applied to cultures infected with adenovirus type 1 and type 2.

Infecting Virus:	Fluorescent Staining When Serum Used at 1:20 Dilution				Highest Dilution to Give Fluorescence With Cells Infected by Adenovirus type 3
Serum	Type 1	Type 2	Type 3	U.I.	
Pre-inoculation	-	-	-	-	
Post-inoculation	-	-	+	-	1:80

- negative by immunofluorescence
+ positive by immunofluorescence (cell nuclei stained)
U.I. uninfected control cultures

Result. Only those cultures inoculated with adenovirus type 3 showed fluorescent staining (Table 4.6).

Comment. Accumulation of viral antigens within infected cells would be expected to have occurred by the third day for all serotypes. However, only antigens of the homologous serotype (type 3) were detected, despite the longer incubation times used. Therefore the antiserum prepared against adenovirus type 3 would be of no use in assessing the infectivity of unknown strains by immunofluorescence.

Part III. An antiserum from one rabbit inoculated with adenovirus type 5 was evaluated as in Part I above. This serum might also allow detection of only the homologous virus antigens.

Method. Serum with the higher neutralising antibody levels (Table 4.4) from one rabbit was tested. This was diluted 1:20 to 1:160 in doubling dilutions. These dilutions and pre-inoculation serum at 1:20 were applied in duplicate to coverslip cultures of HEK cells infected with adenovirus type 5 (10^4 TCD_{50}/ml) fixed 48 hours after infection. 1:20 dilutions of serum were also applied to cultures infected with type 2 (10^4 TCD_{50}/ml) and type 3 (10^4 TCD_{50}/ml). Fluorescence staining was carried out as before.

Result. Cells infected by adenovirus type 5 showed fluorescence with all dilutions of post-inoculation serum tested. This fluorescence was spread generally within the cells. No fluorescence was detected in cells infected with type 2 or type 3.

Comment. As with the antiserum to adenovirus type 3, this rabbit antiserum would not be useful for the general detection of adenovirus infections by immunofluorescence. The type-specificity shown could be the result of accumulation of type-specific antigens within the cell

TABLE 4.6 Results of immunofluorescence tests using serum from Rabbit No. 2 (see Table 4.4). HEK cells infected with adenovirus type 1, type 2, type 3 or type 5 and fixed at various times after infection were treated with this antiserum at 1:20 dilution.

	Time of Fixation				
	30 Hours	48 Hours	72 Hours		
	1:20 Serum			1:20 pre	PBS
Adenovirus Serotype					
1	-	-	-	-	NT
2	-	-	-	-	NT
3	+*	+**	NT	-	NT
5	-	-	-	-	NT
U.I.	NT	NT	-	NT	-

U.I. uninfected control cultures
- negative by immunofluorescence
NT not tested
+ positive by immunofluorescence
pre pre-inoculation serum
* less than 1 fluorescing focus per field (only cell nuclei stained)
** 5 fluoresceing foci per field (only cell nuclei stained)

far in excess of group-specific antigen production. More likely, these results could reflect the particular antibody content of the sera (that is, the proportion of type-specific to group-specific antibodies). Antiserum giving type-specificity by immunofluorescence could be treated by immunoadsorption methods to remove the type-specific antibodies. The remaining antibodies to group-specific determinants could then be concentrated. However, immunofluorescence tests using heterologous serotypes showed no group-specific reactivity at low dilutions of the serum. Therefore it might be preferable to change the means of immunisation or the inoculum in order to obtain group-specific sera for immunofluorescence. For example, injection of rabbits with purified capsid components (in particular, hexons) might lead to an immune response against group-specific antigenic determinants (in particular, alpha) not exposed on the intact virion (Norrby, 1969a and b).

3. Preparation and Testing of Guinea Pig Antisera.

a. Introduction. Antiserum prepared against adenovirus is issued by the Standards Laboratory for Serological Reagents, Colindale, London for immunofluorescence work in Public Health Laboratories. It is intended for use in the diagnosis of adenovirus infections irrespective of serotype, and is prepared by the intranasal inoculation of guinea pigs (C.M.P. Bradstreet, personal communication). This method of raising antiserum offered the possibility of obtaining a group-specific reagent without the need for antigen or antibody purification.

b. Preparation.

Method. Adenovirus type 3 (prototype strain) was propagated for 3 passages in Bristol HeLa cells. The third passage fluid was frozen 4 times to -20°C and centrifuged at 3000 rev./min for 30 min. The supernatant fluid was centrifuged at 24000 rev./min for 150 min in a Beckman L5-40 ultracentrifuge with SW 27 rotor. The supernatant fluid

was discarded and the pellet was resuspended in 20 ml PBS. Each of 5 guinea pigs was inoculated intranasally with 0.1 ml approximately ($10^{5.25}$ TCD_{50}/ml). The animals had been bled by cardiac puncture on the previous day. Blood was taken again by cardiac puncture 15 days after inoculation.

c. Testing of Guinea Pig Antisera. The sera were tested by immunofluorescence.

Part I. The 5 sera were tested for group-specificity to adenovirus.

Method. 1:20 dilutions of each post-inoculation serum were tested in duplicate using KB cell cultures infected with adenovirus (a) type 1 ($10^{4.5}$ TCD_{50}/ml), (b) type 2 ($10^{4.5}$ TCD_{50}/ml), (c) type 3 (10^{4} TCD_{50}/ml, and (d) type 5 (10^{4} TCD_{50}/ml). The cultures were fixed 36-42 hours after infection. Cultures infected with type 2 virus were also treated with the post-inoculation sera at dilutions of 1:40, 1:80 and 1:160 and pre-inoculation sera at 1:20. The preparations were stained as described in Chapter 1 using FITC-conjugated Swine anti-Guinea Pig IgG. The serum which gave the brightest fluorescence at the higher dilutions was retested in an identical manner using secondary HEK cells.

Results. Of the 5 sera tested, 2 allowed identification of adenovirus infection by all 4 serotypes (Table 4.7). All dilutions of these 2 sera that were tested allowed infection by type 2 virus to be detected. Sera from other 2 guinea pigs allowed the detection of infection by the homologous serotype (type 3) but not by heterologous serotypes. The fifth serum did not give fluorescence under any circumstances. All 5 test sera gave negative results with uninfected cells. All 5 pre-inoculation sera at 1:20 gave negative results with type 2 infected cells. The serum which was re-tested using HEK cells gave the

TABLE 4.7 Results of immunofluorescence tests on 5 guinea pig sera for group specificity to adenovirus, using KB cells. 1:20 dilutions of each serum were tested in duplicate using cultures infected with adenovirus types 1, 2, 3 and 5. Higher dilutions of each serum were tested in duplicate using cells infected with type 2. Pre-inoculation serum from each guinea pig was tested in duplicate using cultures infected with type 2, and did not give fluorescent staining.

Infecting Virus:	Type 1	Type 2	Type 3	Type 5	U.I.	Highest Dilution to Give Fluorescence With Cells Infected by Adenovirus type 2
	Fluorescent Staining When Serum Used at 1:20					
Serum						
1	+	+	+	+	-	≥1:160
2	+	+	+	+	-	≥1:160
3	-	-	+	-	-	<1:20
4	-	-	-	-	-	<1:20
5	-	-	+	-	-	<1:20

- negative by immunofluorescence
+ positive by immunofluorescence

U.I. uninfected control cultures

same results as with KB cells.

Comment. This method of preparing antiserum to adenovirus allowed the detection of infection by adenoviruses of a different serotype and subgroup (types 1, 2 and 5; subgroup C) to that used as immunogen (type 3; subgroup B), in 2 out of 5 cases. Therefore the 2 positive sera would be suitable for detecting replication of the commonest, if not all, adenovirus serotypes. Since inoculation of the guinea pigs was by the respiratory route, it is likely that the animals which gave positive sera underwent some degree of infection, either at the respiratory or the intestinal epithelium. It is not understood why some sera showed type-specificity and others did not. This may reflect the extent of infection in some way.

At the dilutions tested, the sera showed no non-specific reaction with uninfected cells. Therefore any kind of absorption procedure for the removal of antibodies to cellular or calf serum components was unnecessary. However, at dilutions lower than 1:20 a slight increase in background staining of the cultures was seen, using either pre-inoculation or post-inoculation serum. Therefore 1:20 was the dilution chosen for later work. The guinea pig serum tested using HEK cells again allowed intracellular antigens of all 4 serotypes to be detected. Therefore it might be possible to test NG stool extracts in both primary-derived and continuous cell cultures using this serum, with some chance of success in one or other culture.

Part II. The guinea pig serum chosen for testing stool extracts was tested for possible reactivity to agents other than adenovirus that are found in stools.

Method. 2 stool extracts containing rotaviruses (by EM), 2 containing astroviruses and 2 containing caliciviruses (all from different children) were added separately to coverslip cultures of KB cells.

Poliovirus type 1 ($10^{-7.5}$ TCD_{50}/ml at 4 days in KB cells) and coxsackie virus type 1 ($10^{1.75}$ TCD_{50}/ml at 4 days in KB cells) were also added to KB cultures. Adenovirus type 7 ($10^{2.5}$ TCD_{50}/ml) was used as a positive adenovirus control. Each stool extract or culture fluid was added (0.5 ml) to each of 4 coverslip cultures and incubated at 36.5°C for 48 hours. After fixation in acetone (see Chapter 1) 2 of the 4 coverslips received post-inoculation serum from one guinea pig and the remaining coverslips received either PBS or the corresponding pre-inoculation serum. Uninfected cultures were treated in the same way. The coverslip preparations were stained as described in Chapter 1 using FITC-conjugated anti-Guinea Pig IgG.

Results. Only those cells infected with adenovirus type 7 and treated with post-inoculation guinea pig serum showed fluorescence.

Comment. The serum tested here was prepared for the possible detection of adenovirus specific antigens in KB cells inoculated with NG specimens. It was important that the serum should be specific for intracellular antigens of adenoviruses and that antigens of other agents which might also be present in cells should not be detected.

The serum did not give positive immunofluorescence results using KB cells inoculated with specimens containing rotaviruses, astroviruses or caliciviruses. Therefore, although these agents might be present in stools together with adenovirus particles, my interpretation of positive immunofluorescence results being due to adenoviruses alone appeared valid. (However, it is possible that the serum contained antibodies to rotaviruses, astroviruses or caliciviruses with different antigenic determinants to those tested, and with those types positive immunofluorescence results might be possible. No reports of natural infection of guinea pigs by such agents could be found but the possibility that antibodies to these agents were present

in the serum cannot be excluded entirely).

Enteroviruses are commonly isolated from stools. With over 70 different known serotypes, it was not possible to test the guinea pig serum for specificity to all individual types. There was no reactivity apparent with the 2 serotypes tested. At the time of fixation the cultures infected with each type showed mild CPE. Therefore virus antigens were almost certainly present in the KB cells, though undetected by immunofluorescence. Since there was no evidence for reactivity to agents other than adenoviruses in these tests, the serum was considered suitable for detection of adenovirus antigens.

Part III. It was noted in Part I that cells infected with adenovirus type 3 showed either nuclear or general fluorescence, whereas cells infected with types 1, 2 and 5 showed either general or cytoplasmic fluorescence. It could be argued that the pattern of fluorescence seen with any adenovirus serotype should be the same if a group-specific serum is used. However, differences may occur in the rate and extent of accumulation of viral antigens with different serotypes, although the basic replicative cycle may be the same. In this case there might be a higher concentration of structural proteins within the nucleus with type 3 at the time of fixation. To investigate this further, cultures infected with either type 2 or type 3 were fixed at various times after infection and were treated with group-specific guinea pig antiserum.

Method. Coverslip cultures of KB cells infected with adenovirus type 2 (10^4 TCD_{50}/ml) and type 3 (10^4 TCD_{50}/ml) were fixed at 24 hours, 30 hours, 40 hours and 50 hours after infection. Uninfected cultures were treated in a similar manner. The coverslips were treated with 1:20 dilutions of either pre-inoculation or post-inoculation serum as used in Part II. The preparations were stained and mounted as

described in Chapter 1.

Result. The preparations from cultures infected with type 3 and fixed 24 hours after infection failed to show fluorescence (Table 4.8). All other infected cultures that were treated with post-inoculation serum showed fluorescence. In cultures infected with both serotypes there was a notable difference between the location of fluorescence detected early (30 hours after infection) and fluorescence detected later (50 hours after infection). However, the patterns of fluorescence for the two serotypes were different. In the case of type 2, general cellular fluorescence was noted in cultures fixed at 24 and 30 hours, but fluorescent staining of the nuclei was absent at later times. With type 3, fluorescence was mainly confined to the nuclei at 30 hours after infection, but cultures fixed at later times showed general cellular fluorescence.

Comment. It is apparent from this test that, with the reagents and methods used, location of fluorescence within the cell depends not only on the serotype of adenovirus but also on the time allowed for infection before fixation. Therefore, in testing unknown specimens for the presence of adenovirus, any pattern of fluorescence could be expected. To make the tests repeatable and allow comparisons of results with different specimens, the time allowed for infection should be standardised.

4. Evaluation of the Method Using Culture Positive Specimens.

a. Introduction. The time taken for antigens to accumulate in cells infected with prototype adenovirus at high multiplicity can be measured in hours rather than days. This may not be the case with wild strains of even the commonest serotypes. Such strains often take several days to produce CPE, which presumably reflects delayed

TABLE 4.8 Results of immunofluorescence tests using KB cells fixed at various times after inoculation with (a) adenovirus type 2, and (b) adenovirus type 3. Tests with post-inoculation (anti-Ad) sera were carried out in duplicate.

	Time of Fixation							
	24 Hours		30 Hours		40 Hours		50 Hours	
	pre	anti-Ad	pre	anti-Ad	pre	anti-Ad	pre	anti-Ad
type 2	-	+G	-	+G,C	-	+C	-	+C
type 3	-	-	-	+N,G	-	+G	-	+G
U.I.	NT	-	NT	-	NT	-	NT	-

U.I. uninfected control cultures
- negative by immunofluorescence
NT not tested
+ positive by immunofluorescence
pre pre-inoculation serum
anti-Ad post-inoculation guinea pig antiserum (adenovirus group-specific)
G general staining of cells
N staining of nuclei (not cytoplasm)
C staining of cytoplasm (not nuclei)

initiation or blockage of the replicative cycle. It was necessary to determine whether KB cells, which are difficult to maintain for more than 3 days without over-growth, would allow detection of adenovirus from culture positive specimens within this interval. It was decided that if over 50 per cent of culture positive specimens tested could be classed as immunofluorescence positive also, there would be grounds for testing NG specimens by the same method.

b. Incubation Time. To determine whether incubation of cultures for 2 or 3 days after inoculation with G specimens would allow the detection of adenovirus antigens, 6 specimens from different children were tested. The inoculum volume for stool extracts was 0.1 ml in this and subsequent tests because the quantity of each specimen was limited.

Method. The 6 specimens were added to coverslip cultures of KB cells. Infected and uninfected cultures were fixed at 48 hours and 72 hours after infection. Cultures infected with prototype strains of adenovirus type 2 ($10^{4.5}$ TCD_{50}/ml), type 3 (10^{4} TCD_{50}/ml) and type 7 ($10^{2.5}$ TCD_{50}/ml) were included as positive controls (0.5 ml per culture). The fixed cultures were treated in duplicate with guinea pig antiserum and stained for immunofluorescence as before.

Result. All prototype strains caused fluorescence in cultures fixed after 48 hours, whereas 3 of the 6 specimens were positive with general or cytoplasmic staining (Table 4.9). With the cultures fixed after 72 hours, one further specimen was positive.

Comment. Since there was a marginally better result with 72 hours incubation before fixation, it would be best to incubate all further specimens with KB cells for this length of time: this is the maximum

TABLE 4.9 Result of immunofluorescence tests using KB cell cultures fixed at 48 and 72 hours after inoculation with G specimens. Tests using cultures infected with prototype strains of adenovirus types 2, 3 and 7 were positive and all other controls were negative.

Time of Fixation	No. of Specimens Positive (serotypes)*	No. of Specimens Negative (serotypes)*	Total No. Tested
48 Hours	3 (1, 1, 2)	3 (2, 5, 14-16)	6
72 Hours	4 (1, 1, 2, 5)	2 (2, 14-16)	6

TABLE 4.10 Result of immunofluorescence tests using KB cell cultures fixed 72 hours after inoculation with G specimens. Tests using cultures infected with prototype adenovirus type 7 were positive and all other controls were negative.

No. of Specimens Positive (serotypes)*	No. of Specimens Negative (serotypes)*	Total No. Tested
10 (1, 1, 2, 2, 2, 2, 3, 12-31, 12-31, 12-31)	4 (2, 5, 17, 12-31)	14

* serotypes given refer to isolates identified previously (Chapter 2)

incubation time possible with these cells. Failure to detect infections with G specimens may have been due in part to the greater dilution factor on inoculation (1:46) than was the case when the specimens were tested for cytopathogenic agents previously (1:10 dilution). If the number of viable virus particles in the specimen is low, extra dilution might limit the chance of a culture receiving an infectious dose. Also, prototype strains at terminal dilutions can take several days for the first signs of plaque formation to occur (personal observation). Therefore, even with moderately infectious specimens, it is probable that dilution would delay the time taken for the infection to be detected by immunofluorescence.

c. Testing of Further Culture Positive Specimens. G specimens from 13 children were tested to determine the proportion of positive results that could be expected using KB cells fixed 3 days after infection.

Method. 14 stool extracts were tested as in Section b. above, with fixation at 72 hours after inoculation. The specimens chosen for test had given a range of adenovirus serotypes in culture (types 1, 2, 3, 5, 17 and 12-31). Cultures infected with adenovirus type 7 ($10^{2.5}$ TCD_{50}/ml) were included as positive controls.

Result. 10 of the 13 specimens were positive by immunofluorescence (Table 4.10). The location of the fluorescence within the cells was nuclear for one specimen (type 3 previously isolated) and general or cytoplasmic for the others. The adenovirus strains previously isolated from these specimens included representative serotypes of subgroup A (type 12-31), subgroup B (type 3) and subgroup C (types 1 and 2).

Comment. The proportion of culture positive specimens which were also positive by immunofluorescence was again approximately two thirds of

the number of samples tested. This ratio of positive to negative results was considered high enough to justify the testing of NG specimens by immunofluorescence.

5. Testing of NG Specimens For Adenovirus Specific Immunofluorescence.

a. Introduction. The immunofluorescence method used allowed the detection of infection by adenoviruses of different serotypes and subgroups in stool extracts. Therefore detection of adenovirus infection in cultures inoculated with NG specimens might be possible by this group-specific method provided that (a) these viruses share group-specific determinants with established serotypes, and (b) the viruses undergo at least part of the replicative cycle to the stage of structural protein production.

b. Tests Using KB Cells.

Method. 28 stool extracts from 24 children were tested, all of which were negative by culture using HEK cells. The inoculated and uninoculated cultures were fixed after 72 hours. For each specimen or uninfected control, 2 cultures were treated with adenovirus-specific guinea pig antiserum, one culture was treated with PBS and one culture was treated with pre-inoculation serum. The coverslips were stained for immunofluorescence as before. Cultures infected with adenovirus type 3 (10^4 TCD_{50}/ml) and/or type 7 ($10^{2.5}$ TCD_{50}/ml) were included as positive controls.

Results. Fluorescence was detected in cultures inoculated with 15 of the 28 NG specimens (Table 4.11). In all cases there were less than 10 fluorescing foci per field (at x250 magnification) and in 2 cases there was an average of less than 1 fluorescing focus per field. In

TABLE 4.11 Results of immunofluorescence tests on 28 NG specimens, using KB cells.

Patient No.	Date of Stool	Serum		Location of Fluorescence	No. Fluorescing Foci Per Field
		pre	anti-Ad		
1	10/11/75	-	+	general	1-10
2	1/12/75	-	-		
3	2/12/75	-	-		
12	26/ 4/76	-	+	general	less than 1
20	26/ 6/76	-	-		
20	28/ 6/76	-	-		
25	21/ 7/76	-	+	general	1-10
29	26/ 7/76	-	+	general	less than 1
29	26/ 7 /76	-	-		
59	1/ 8/77	-	-		
62*	8/10/77	-	+	general	1-10
65*	2/11/77	-	+	general	1-10
66*	7/11/77	-	+	general	1-10
75	15/ 3/78	-	-		
78	14/ 5/78	-	-		
79	1/ 8/78	-	-		
80	8/ 8/78	-	-		
81	21/ 8/78	-	+	nuclear,general	3
82*	29/ 8/78	-	+	nuclear,general	2-3
83	31/ 8/78	-	+	nuclear,general	4
84	21/10/78	-	+	nuclear,general	3-4
85	18/ 9/78	-	-		
86	3/10/78	-	+	general	1
87*	7/ 2/79	-	+	nuclear,general	3
87	12/ 2/79	-	-		
88	5/ 3/79	-	+	general	3-4
89	6/ 3/79	-	+	nuclear	5
89	9/ 3/79	-	-		
Ad 7 control		-	+	general	3
U.I.		NT	-		

U.I. uninfected control cultures
- negative by immunofluorescence
NT not tested
+ positive by immunofluorescence
pre pre-inoculation serum
anti-Ad post-inoculation guinea pig antiserum (adenovirus group-specific)
* these specimens were found to be positive when re-tested. Specimens from patients no. 82 and 87 caused fluorescent staining in cells fixed 72 hours after inoculation, but not in cells fixed 24 or 48 hours after inoculation

all positive cases fluorescence was limited to single cells and was confined to the nucleus or distributed generally within the cell (see Plates 8, 9,10 and 11). Stool extracts from 5 children were re-tested and were again found to be positive. In 2 of the 5 cases infected cultures were fixed at 24, 48 and 72 hours but only those fixed at 72 hours showed fluorescence. There was no fluorescence in any infected culture treated with pre-inoculation serum or PBS.

Comment. The results indicate that at least some NG specimens contain adenovirus which is capable of inducing antigen production in KB cells. The fluorescence seen with NG specimens was bright and stippled. It was similar to that seen with G specimens, except that in the former case staining was always limited to single cells without any indication of spread of infection to adjacent cells (Plates 8, 9 and 10). As with the G specimens, it is possible that longer incubation times would have allowed a greater number of NG specimens to be scored as positive. Results in the cases where 2 specimens were taken from the same child emphasise this point, since in 3 cases only one of these extracts was positive (Table 4.11). Incubation of the negative specimen in culture for another day or more might have allowed detection of adenovirus antigens as with the other specimen. Results with 2 specimens suggested that 72 hours might be the minimum period required for NG specimens to cause the formation of intracellular antigens. Therefore HEK cells, which survive in culture for up to 10 days, might be preferable for detection of these agents by immunofluorescence.

c. Tests Using HEK Cells. NG specimens by definition did not cause CPE in HEK cells. It was of interest to determine whether these specimens would allow adenovirus-specific immunofluorescence in HEK cells similar to that seen in KB cells.

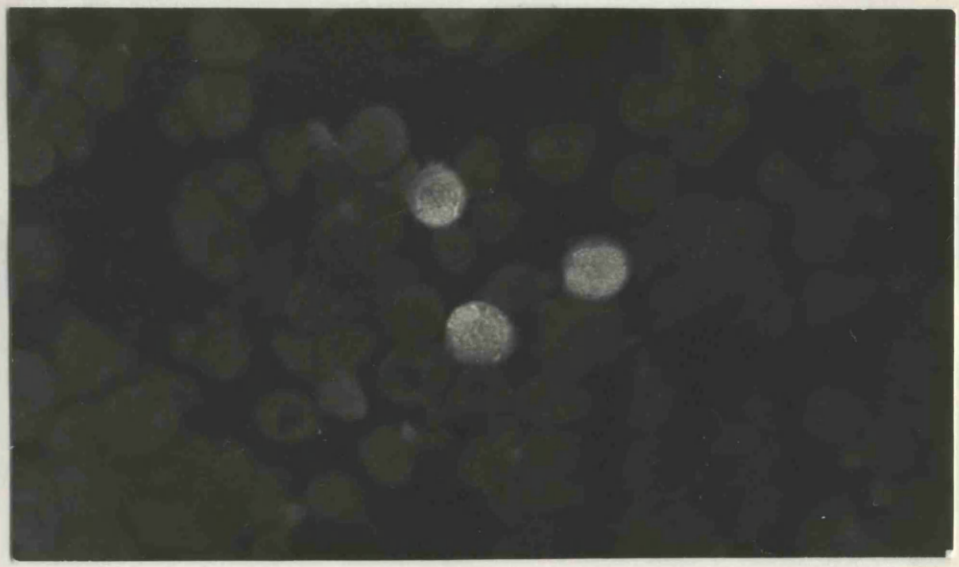

PLATE 8 KB cells inoculated with a NG specimen from patient no. 84 and tested for the presence of adenovirus antigens by indirect immunofluorescence test using group-specific guinea pig antiserum. The cells were fixed 3 days after inoculation. (x25 objective).

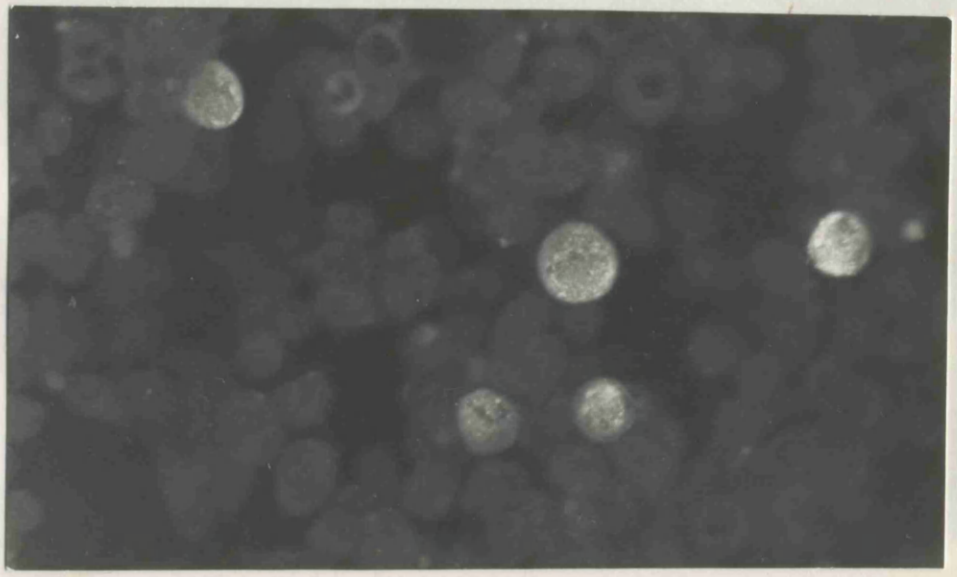

PLATE 9 KB cells inoculated with a NG specimen from patient no. 86 and tested for the presence of adenovirus antigens by an indirect immunofluorescence test using group-specific guinea pig antiserum. The cells were fixed 3 days after inoculation. (x25 objective).

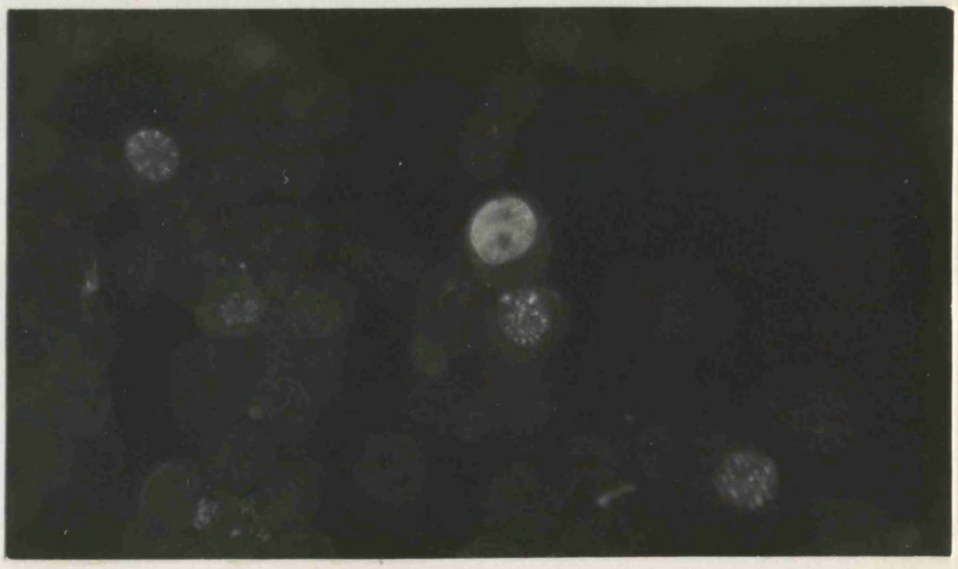

PLATE 10 KB cells inoculated with a NG specimen from patient no. 89 and tested for the presence of adenovirus antigens by an indirect immunofluorescence test using group-specific guinea pig antiserum. The cells were fixed 3 days after inoculation. (x25 objective).

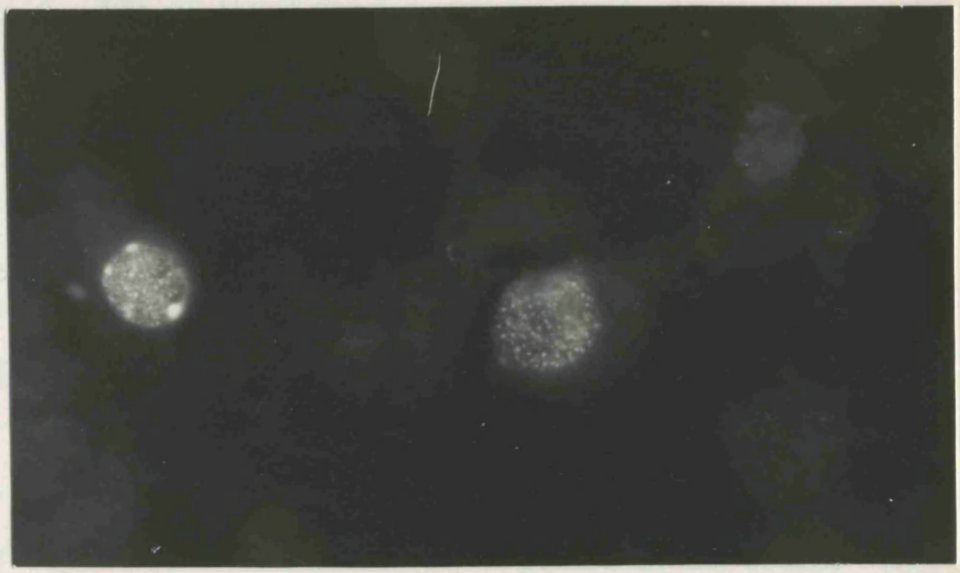

PLATE 11 KB cells inoculated with adenovirus type 7 and tested for the presence of adenovirus antigens by an indirect immunofluorescence test using group-specific guinea pig antiserum. The cells were fixed 3 days after inoculation. (x40 objective).

Part I.

Method. 8 NG stool extracts from 6 children (including 4 specimens from different children which were positive by immunofluorescence using KB cells) were tested with HEK cells. Infected and uninfected cultures were fixed after 72 hours. Duplicate cultures were treated with guinea pig serum and stained for immunofluorescence as before. Adenovirus type 7 was used as a positive control.

Results. One of the 8 stool extracts was positive (Table 4.12). The other 3 specimens which were positive with KB cells were negative using HEK cells. The stool extract scored as positive by immunofluorescence gave general staining of the cells, but only 3 cells per coverslip were affected.

Comment. Since 3 of the 4 specimens which were positive with KB cells were negative with HEK cells, accumulation of virus antigens appears to have taken place more readily within KB cells. This may be due to a requirement for a longer incubation period in HEK cells. Therefore HEK cells inoculated with NG specimens and fixed at times greater than 3 days might allow the detection of adenovirus antigens.

Part II. To determine if longer incubation times would make a difference to the detection of adenovirus antigens in HEK cells, 2 NG specimens negative by the above test were tested again with longer intervals before fixation of the inoculated cultures.

Method. The 2 NG specimens tested were from patients no. 25 and 29 (stool date 24//7/76; see Table 2.7). Inoculated and uninoculated cultures were fixed at 2, 5 and 10 days after the addition of maintenance medium. Duplicate cultures were treated with guinea pig serum

TABLE 4.12 Results of immunofluorescence tests on 8 NG specimens using HEK cells.

Patient No.	Date of Stool	Serum		Location of Fluorescence	No. Fluorescing Foci Per Field
		pre	anti-Ad		
2	1/12/75	-	-		
25*	21/ 7/76	-	-		
25	24/ 7/76	-	-		
29	24/ 7/76	-	-		
29*	26/ 7/76	-	-		
65*	2/11/77	-	-		
66*	7/11/77	-	+	general	less than 1
67	15/11/77	-	-		
Ad 7 control		-	+	nuclear,general	less than 1
U.I.		-	-		

U.I. uninfected control cultures
- negative by immunofluorescence
+ positive by immunofluorescence
pre pre-inoculation serum
anti -Ad post-inoculation guinea pig antiserum (adenovirus group-specific)
* these specimens were found to be positive by immunofluorescence using KB cells (Table 4.11)

and stained for immunofluorescence as before. Adenovirus type 7 was used as a positive control.

Results. Fluorescence was not observed in cultures inoculated with NG specimens and fixed at any time.

Comment. The stool specimens contained large amounts of adenovirus by electron microscopy, yet were negative by immunofluorescence with HEK cells. Prolonged incubation of these specimens was of no advantage for the detection of intracellular antigens. The limited quantity of stool material did not permit the testing of these specimens with KB cells; therefore the relative suitability of the 2 culture types cannot be compared from this result. However, from Part I above, KB cells were more sensitive for detection of antigens in cells inoculated with NG specimens. Here it has been shown that HEK cells can remain insensitive to NG agents even with extended incubation.

Part III. Banatvala et al. (1975) reported the detection of human rotavirus in pig kidney cultures by immunofluorescence if the inoculated cultures were centrifuged at low speed (3000 g) before incubation. This method was adapted to determine whether low-speed centrifugation could improve detection of adenovirus antigens in HEK cells inoculated with NG specimens.

Method. HEK cultures were prepared by seeding cells from primary bottle cultures onto 1 cm diameter coverslips in 24-well plates (Falcor) at a concentration of 8×10^4 cells/ml. The growth medium contained 25 mm HEPES (Calbiochem). After 2 days, growth medium was removed from each well and replaced with 0.9 ml maintenance medium containing 30 mm HEPES. 6 NG specimens were tested as follows.

Stool extract (0.1 ml) was added to each of 3 wells containing

coverslip cultures, in each of 3 plates. Adenovirus type 8 ($10^{3.5}$ TCD_{50}/ml) was used as positive virus control and was treated in the same way. 3 cultures per plate were left uninoculated. The 3 plates were treated as follows. (1) Plate 1 was centrifuged at 1800 rev./min for 30 min after inoculation, (2) Plate 2 was centrifuged at 1800 rev./min for 30 min before inoculation, and (3) Plate 3 was inoculated but not centrifuged. Centrifugation was carried out in an MSE Magnum centrifuge with swing-out rotor and MSE plate carriers. The cultures were incubated at 36.5°C and at 2 days the coverslips were washed and fixed as before. For each specimen or uninfected control, 2 cultures were treated with adenovirus specific antiserum (guinea pig) and one culture was treated with pre-inoculation serum. The coverslips were stained and mounted as described in Chapter 1.

Results. Only one NG specimen caused fluorescence (Table 4.13). This specimen was scored as positive whether centrifugation was carried out or not. This was also true of the positive virus control (type 8). In both cases, the intensity or extent of fluorescence was no different after either of the centrifugation treatments from the result of the uncentrifuged controls.

Comment. There was no advantage to the detection of adenovirus antigens in the centrifugation of cultures after inoculation by the method used. Those specimens which were negative by immunofluorescence with untreated cells were also negative after centrifugation of the monolayers. There was little argument for investigating this method further, although tests with a range of incubation times or centrifugation speeds might have given improved results.

C. DISCUSSION.

The work of this chapter was carried out to determine whether

TABLE 4.13 Results of immunofluorescence tests on 6 NG specimens using HEK cells, with evaluation of the method of low-speed centrifugation of the cell monolayers after inoculation.

Patient No.	Date of Stool	Inoculation Before Centrifugation		Inoculation After Centrifugation		Inoculation (No Centrifugation)	
		Serum		Serum		Serum	
		pre	anti-Ad	pre	anti-Ad	pre	anti-Ad
29	26/ 7/76	-	-	-	-	-	-
45	21/ 2/77	-	-	-	-	-	-
57	12/ 7/77	-	-	-	-	-	-
59	1/ 8/77	-	-	-	-	-	-
61	12/ 9/77	-	-	-	-	-	-
62	8/10/77	-	+*	-	+*	-	+*
Ad 8 control		-	+**	-	+**	-	+**
U.I.		NT	-	NT	-	NT	-

U.I. uninfected control cultures
\- negative by immunofluorescence
NT not tested
\+ positive by immunofluorescence
pre pre-inoculation serum
anti-Ad post-inoculation guinea pig antiserum (adenovirus group-specific)
* less than 1 fluorescing focus per field
** 1-10 fluorescing foci per field

adenovirus in NG specimens might undergo at least part of the replicative cycle. There was no evidence that AAV replication in culture can be supported by these adenoviruses. The presence of AAV, viable human prototype virus, or viable adenovirus of canine origin along with these viruses did not lead to CPE in cultures of human cells. Immunofluorescence tests using a group-specific antiserum allowed the detection of intracellular adenovirus antigens in KB cells. In the work described thus far, this is the only firm evidence that the adenoviruses in NG specimens have at least partial infectivity.

Not all NG specimens were positive by immunofluorescence, but this was also true of the G specimens tested. The proportion of specimens which were positive was over 50 per cent of the total number tested in both cases. Results with the limited number of NG specimens tested with HEK cells suggest that this culture system may be less useful for the detection of these agents by immunofluorescence, despite its acknowledged superiority in detecting strains of many established adenovirus serotypes by CPE.

The limited extent of fluorescence with KB cells with no spread to neighbouring cells may reflect an abortive infection with production of structural antigens but with defective virus assembly or release from the cell. This would explain the failure of these agents to cause CPE. In this case, passage from culture to culture would only dilute out the semi-infectious inoculum. The abortive infection postulated need not arise from a genetic defect in the virus. This would be unlikely on consideration of the numbers of particles shed in stools. The lack of replication may result from incompatibility between the cells and the virus at the biochemical level. Alternatively, the virus and cell may be compatible for replication but prohibited from interacting efficiently because of some external

influence. This could be the absence of the correct physical or chemical conditions, or the presence of an inhibitory influence.

CHAPTER 5

CHAPTER 5 EXAMINATION OF NG STOOL EXTRACTS FOR THE PRESENCE OF AN INHIBITORY SUBSTANCE.

1. Introduction.

Adenoviruses in NG specimens from 15 children were shown to be capable of antigen production in KB cells by immunofluorescence test (Chapter 4). No CPE was observed with these specimens at the time of fixation, nor with 10 other NG specimens incubated in KB cell cultures (Chapter 3 Section A.2.a.). The reason for limited infection by these adenoviruses could be the presence of an inhibitory substance in the stool. An inhibitor might prevent the spread of infectious virus, for example by blocking virus maturation or entry to adjacent cells. Such an inhibitor could act at one of 3 possible levels:

1. It might have an effect on the replication of viruses in general. A single inhibitor with a similar effect on the replication of viruses from different groups could not be antibody, since other virus groups do not have virion antigen in common with adenoviruses. Interferon in stools has not been described but might be a possible candidate as a general inhibitor. The possibility of a general inhibitor is relevant to the problem of other stool viruses which do not undergo productive infection *in vitro* (rotavirus, astrovirus, calicivirus and other agents including hepatitis A and B viruses).
2. The inhibitor might prevent the replication of adenoviruses in general, but not other viruses. Such a group-specific inhibitor could be a free component of the stool, acting on some stage of adenovirus replication. Alternatively, the inhibitor could be antibody which might attach to group antigens and prevent entry or

uncoating of the virus particles.

3. The inhibitor in a particular stool might be specific for one adenovirus serotype. A substance showing this degree of specificity would probably belong to one of the recognised classes of antibody. Since adenoviruses in NG specimens are of unknown serotype, the type-specificity of an inhibitor in one NG specimen might not be the same as the type-specificity of an inhibitor in another NG specimen.

The following sections describe experiments to test NG specimens for the presence of inhibitory substances. The experiments fall into 2 categories: those designed to test for the action or presence of an inhibitor (Section 2.), and those designed to detect productive infection after treating the specimens in ways which might remove an inhibitor (Section 3.).

2. Examination of Virus-Free Supernates For the Presence of an Inhibitory Substance to Adenovirus Replication.

a. Effect of Mixing NG Supernates with Good and Poor Grower Serotypes of Adenovirus. To determine if NG stool extracts contain a substance inhibitory to the replication of adenoviruses in general, prototype strains of types 3 and 8 were incubated separately in the presence of NG specimens from which debris and virus particles had been removed by ultracentrifugation. In case inhibition of infectivity should depend on the combination of inhibitor with virus particles, the virus and supernates were incubated together before inoculation of cell cultures. Adenovirus type 8 was chosen as an indicator virus because it replicates in human cell cultures with a poor yield of infectious virus particles, in contrast to type 3.

Since it appears less adapted to growth in cell culture, type 8 might show a more marked decrease in infectivity in the presence of an inhibitor than would a serotype giving a better virus yield.

Method. Supernates were collected after ultracentrifugation of the stool extracts for electron microscopy at 35000 rev./min (see Chapter 1 Section 1.). These supernates were collected from all stool extracts intended for EM examination over 5 months, and were stored at 4°C until the pelleted material was examined by EM. If the extracts were found to contain adenovirus particles, the supernates were stored at -20°C to await the result of culture with the complete stool extract in HEK cells.

Supernates from 7 NG specimens (from patients no. 12, 20, 21, 34 and 36) were tested. Mixtures of adenovirus type 3 (0.3 ml) and supernate (0.3 ml) were made in bijoux and incubated at room temperature for 1 hour. The titre of virus after 1:2 dilution was 10^3 TCD_{50}/ml. Control mixtures, adenovirus type 3 with PBS (virus controls) and supernate with PBS (supernate controls), were made and tested in parallel. Each mixture was added to each of 4 tubes of human amnion cells (0.1 ml per tube) and the cultures were incubated at 36.5°C. The procedure was repeated using the same supernates, but with adenovirus type 8 (titre at final dilution (1:2): $10^{1.75}$ TCD_{50}/ml) and HEK cells.

Results. CPE arose in 4-6 days in the virus controls. The controls containing supernate and PBS caused no CPE. In all cases where supernate and adenovirus were added together, CPE arose at the same time as in the virus controls.

Comment. The time taken for CPE to appear was used as an indicator of infectivity in this test. The replication of 2 prototype strains

in cell culture was not affected by mixing them with NG stool extract material. There was no evidence from the above results that NG stool extracts contain a free (unbound) inhibitor which prevents development of adenovirus CPE. A plaque-reduction assay would have been a more sensitive test for the presence of an adenovirus inhibitor. However, if a free inhibitor was responsible for the apparent lack of viability in the large numbers of adenovirus particles seen by EM, one would expect its inhibitory effect to have been easily detected.

b. Effect of Mixing NG Supernates with the Common Serotypes of Adenovirus Found in Stools. Adenovirus types 1 and 2 are the most common established serotypes isolated from stools of children in Glasgow (Chapter 2; E.J. Bell, personal communication). Their presence in the intestine may induce production of type-specific antibody in the mucosa. Therefore adenoviruses which cannot be cultured from stools might be common serotypes associated with antibody. To determine if NG stool extracts contain free (unbound) antibody to the most common known serotypes found in stools, prototype strains of types 1 and 2 were incubated separately in the presence of NG supernates prepared as above.

Method. Supernates from 3 NG specimens (from patients 41, 43 and 52) were tested. Mixtures of adenovirus type 1 (0.3 ml) and supernate (0.3 ml) were mixed in bijoux in duplicate and incubated at room temperature for 1 hour. The titre of type 1 virus after 1:2 dilution was 10^3 TCD_{50}/ml. Virus and supernate control mixtures were prepared as in Section a. and treated as above. Each mixture was added to each of 4 tubes of human amnion cells (0.1 ml per tube) and the cultures were incubated at 36.5°C. The same supernates were mixed with type 2 virus (titre at 1:2 dilution: $10^{3.5}$ TCD_{50}/ml) and the mixtures were

treated as above.

Results. CPE developed at 5 days after inoculation in the virus controls. The supernate controls did not cause CPE. In all cases where supernate and adenovirus were added to the cultures together, CPE arose at the same time as in the virus controls.

Comment. Development of CPE caused by adenovirus types 1 and 2 was not inhibited by the presence of NG supernates. There was no evidence from these results that the NG specimens tested contained free (unbound) neutralising antibody to the two most common adenovirus serotypes isolated from stools.

c. Effect of Pre-treating Cells with NG Supernates Prior to Infection with Prototype Adenovirus. An inhibitor might affect virus metabolism after the initial stages of infection. Inhibition was not detected when prototype adenovirus and NG supernate were added to the cultures together (Sections a. and b.). However, pre-treatment of the cells with NG stool material might make the cells refractory to productive infection. This pre-treatment of cultures with NG supernates might be a closer parallel to inoculation of cultures with NG stool extracts. Wild strains of established serotypes usually take several days to show infectivity in culture. This delay could allow time for an unbound inhibitor in NG stools to diffuse into the cells and condition them in some way. The tests below were carried out with NG supernates added to HEK cells one day before the addition of prototype adenovirus.

Method. Supernates of 4 NG specimens (from patients no. 38, 43, 45 and 52) were added (0.1 ml) to each of 10 cultures of HEK cells. Sixteen control tubes received PBS in place of supernate. After

incubation at 36.5°C for 20 hours, each set of 10 tubes containing one supernate was inoculated as follows: (a) adenovirus type 2 ($10^{3.5}$ TCD_{50}/ml; 0.1 ml per tube) to each of 4 tubes, (b) adenovirus type 8 ($10^{1.75}$ TCD_{50}/ml; 0.1 ml per tube) to each of 4 tubes, and (c) PBS (0.1 ml per tube) to each of 2 tubes (supernate controls). Eight of the 16 virus control tubes received 0.1 ml adenovirus type 2 and the other 8 received 0.1 ml type 8. All cultures (including uninoculated cell controls) were incubated at 36.5°C and observed daily for development of CPE.

Result. CPE developed in 3 days in the virus controls. The supernates alone did not cause CPE. In all cases where addition of supernate was followed by addition of adenovirus, CPE developed at the same time as in the virus controls.

Comment. Development of CPE caused by types 2 and 8 was not delayed after treatment of the monolayers with NG supernates. There was no evidence to suggest that the NG supernates contained a factor which makes HEK cells refractory to adenovirus infection. However, such a factor could have been present, which either required longer than 4 days to have an inhibitory effect, or was specific for some other serotype(s) of adenovirus.

3. Treatment of NG Specimens to Remove a Possible Inhibitor to Adenovirus Replication.

a. Effect of Diluting NG Specimens. The adenoviruses in NG specimens are present in numbers probably greater than 10^6 particles per g. of stool (C.R. Madeley, personal communication). If an unbound inhibitory factor is present in these specimens, serial dilution of the extracts might remove the effect of the inhibitor

but leave sufficient viable adenovirus particles to cause CPE.

Method. 6 NG stool extracts (from patients no. 2, 3, 6, 25, 29 and 45) were diluted in tenfold steps from 10^{-1} to 10^{-7} in PBS with antibiotics. Each dilution (including 10^{0}) was added (0.1 ml) to each of 3 cultures of human amnion cells. The cultures were incubated at 36.5°C and observed for signs of degeneration. On the ninth day the cultures were frozen and thawed 4 times. For each specimen at each dilution, the contents of each of 2 first passage tubes were passed separately (0.1 ml) to each of 2 fresh cultures. These cultures were incubated for a further 8-9 days at 36.5°C.

Result. None of the 6 specimens at any dilution caused CPE.

Comment. The human amnion cultures were apparently not susceptible to the adenoviruses in diluted NG specimens. It is unlikely that these particular cultures were especially insensitive to adenoviruses since replicate cultures were used to isolate and propagate strains of types 1 and 2 in other tests. Therefore, there was no indication from dilution of the inoculum that an unbound inhibitor is present in NG specimens. Although used here as a preliminary test for the presence of an inhibitor, dilution is an unsuitable method of removing such a substance for 2 reasons: (1) dilution is not an effective way of removing a soluble component (although its effect might be reduced), and (2) dilution will reduce the number of adenovirus particles per inoculum and therefore the probability of detecting infectious virus. A more effective means of removing soluble stool components while retaining the virus was then tried.

b. Effect of Removing the Soluble Components of NG Stool Extracts. It might be possible to free adenoviruses from an unbound inhibitor

by ultracentrifugation. The viruses in NG specimens might therefore cause CPE after repeated washing in PBS.

Method. 4 NG stool extracts (from patients L, 3, 12 and 21) were tested, together with 2 G specimens from which adenovirus type 1 and type 2 had been isolated previously (from patients no. 5 and 6). 4.5 ml of each specimen was centrifuged at 35000 rev./min for 1 hour in a Beckman Model L5-40 ultracentrifuge with SW 50.1 rotor. The supernates were discarded and the pellets were resuspended in 4.5 ml PBS with antibiotics. The specimens were centrifuged as before and the supernates were again discarded. The pellets were resuspended in 4.5 ml PBS with antibiotics. Cultures of human amnion cells were inoculated with these specimens as described in Chapter 1 Section 4. Specimens which did not cause CPE after 3 blind passages at 36.5°C (26 days in culture) were considered negative.

Results. The treated specimens from which types 1 and 2 had been isolated (separately) caused CPE after 8 days in culture. The identity of these agents was confirmed by neutralisation test. The treated NG specimens did not cause CPE.

Comment. The infectivity of wild strains of adenovirus in the G specimens was demonstrable after pelleting the virus twice by ultracentrifugation; therefore the viability of the most common serotypes in stools would probably not be affected by this treatment. However, the removal of soluble components from NG specimens did not allow development of CPE. It is unlikely therefore that adenoviruses in NG specimens are common serotypes which fail to grow because there are unbound inhibitors in the stools. However, NG adenoviruses might not be common serotypes. In this case human amnion cells might not show CPE whether or not an inhibitory factor was present. The scarcity of

fetal material did not allow repetition of this work using HEK cells.

c. Effect of Treating NG Specimens with Trypsin. To determine if a protein in stools might be involved as an inhibitor of adenovirus replication, NG stool extracts were mixed with a proteolytic enzyme (trypsin) before being added to cell cultures. Any CPE caused by NG specimens after this treatment would suggest that a proteinaceous inhibitor had been destroyed. (Treatment of bovine rotavirus with trypsin has been found to enhance plaque formation (Matsuno et al., 1977). It might therefore enhance the infectivity of other stool viruses which are difficult to culture).

Method. 11 specimens were tested: 7 NG specimens (from patients L, T, 2, 3, 21, 36 and 43), 2 D specimens (from patients no. 5 and 12) and 2 G specimens (from patients no. 8 and 9; adenovirus type 5 and 7 previously isolated). Each stool extract (0.1 ml) was mixed with 0.1 ml 0.2 per cent trypsin (crystalline; BDH Chemicals Ltd.) prepared in PBS, in the wells of a Linbro disposo tray (Flow Laboratories Ltd). Each stool extract (0.1 ml) was also mixed with 0.1 ml PBS (specimen control). 2 separate wells which had received trypsin also received 0.1 ml PBS (trypsin controls). The mixtures were incubated at 36.5°C for 1 hour. All wells then received 0.2 ml 0.1 per cent trypsin inhibitor ovomucoid (BDH Chemicals Ltd.), except one trypsin control well which received 0.2 ml PBS. These mixtures were left at room temperature for 15 min and 0.1 ml from each well was added to each of 2 cultures of human amnion cells. The cultures were incubated at 36.5°C. At degeneration of the monolayers (8 days), the tubes were frozen and thawed 4 times. The culture fluids were passed separately to fresh cultures and incubated at 36.5°C for up to 12 days. All cultures were examined on alternate days for CPE.

Result. The monolayers which received trypsin without the ovomucoid inhibitor were disrupted by the third day after inoculation. The trypsin control cultures which received trypsin inhibitor were unaffected. The cultures inoculated with the 2 G specimens developed CPE at the second passage (after 14 and 17 days in culture). This arose at the same time for both trypsin-treated and untreated specimens and the isolates were identified as adenovirus types 5 and 7. None of the cultures inoculated with D specimens or NG specimens developed CPE, whether inoculated with trypsin-treated or untreated specimens.

Comment. The concentration of trypsin used was sufficient to disrupt the cell monolayers, and the concentration of inhibitor used was sufficient to prevent this effect; therefore both reagents were working adequately. Treatment of the specimens with trypsin and trypsin inhibitor resulted in a 1:4 dilution of the stool extract before inoculation of the cultures. However, this would not be expected to alter the probability of detecting infectious adenovirus significantly since all specimens contained large numbers of adenovirus particles. Trypsin treatment of the G specimens did not affect the time taken for CPE to appear. This result is in agreement with an early report that adenovirus type 5 is resistant to trypsin (Pereira, 1958). Trypsin did not appear to alter the infectivity (or lack of infectivity) of adenoviruses in stools. It did not reduce the infectivity of G specimens, nor did it enhance the infectivity of D or NG specimens. Therefore it is unlikely that any inhibitor of adenovirus replication which may be present in stools is sensitive to trypsin.

d. Effect of Treating NG Specimens with Heat. Adenovirus in NG specimens might be inhibited from replication by a heat-labile factor in the stool. This was tested by exposing NG specimens to the highest temperature reported which would still allow persistence of infectivity ($50^{\circ}C$) (Ginsberg, 1956).

Method. 6 NG specimens were tested (from patients no. 30, 34, 41, 43, 45, 52). Each specimen (0.3 ml) was incubated at $50^{\circ}C$ for 30 min, while the remainder was stored at $4^{\circ}C$. 0.1 ml from each heat treated and untreated extract was added to each of 2 HEK cultures and the cultures were incubated at $36.5^{\circ}C$ for 14 days. To determine the loss of infectivity caused by this treatment, prototype adenovirus type 2 was included as a positive control. A sample of clarified culture fluid from HEK cells infected with type 2 (0.4 ml) was incubated at $50^{\circ}C$ for 30 min, while the remainder was stored at $4^{\circ}C$. Tenfold dilutions (10^{-1} to 10^{-7}) of heat treated and untreated fluids were made in PBS and 0.1 ml of each dilution was added to each of 4 HEK cultures. The endpoints were read after incubation at $36.5^{\circ}C$ for 7 days and infectivity titres were calculated by the method of Kärber (1931).

Results. Prototype adenovirus type 2 without heat treatment had a titre of $10^{7.5}$ TCD_{50}/ml. After heat treatment, the titre was 10^{5} TCD_{50}/ml. No CPE was observed in the cultures inoculated with NG specimens, whether heat treated or untreated.

Comment. Less than 1 per cent of the infectivity remained after incubation of prototype adenovirus type 2 at $50^{\circ}C$. This is in agreement with the results of Ginsberg (1956), when the infectivity of a type 4 strain was found to decrease almost 2 log units in 20 min. Whether or not an inhibitor was destroyed by heat treatment

in the NG specimens, this treatment may have destroyed any residual adenovirus infectivity that was present. Since these adenoviruses were present in large numbers in the specimens, it is probable that there would still have been sufficient viable particles to cause CPE after the destruction of a heat-labile inhibitor. Therefore, the presence of a heat-labile inhibitor of adenovirus replication was possible, but unlikely.

e. Effect of Lowering the pH of NG Specimens. Ginsberg (1956) reported that suspensions of adenovirus types 1 and 4 retain some infectivity when held at pH 1.5 to pH 2.5 for 30 min. It might be possible to destroy an inhibitor or dissociate it from adenovirus particles by exposing the NG specimens to low pH conditions. This treatment has been successful for the dissociation of antibody from poliovirus particles (Mandel, 1961; for review see Mandel, 1971). A method was required by which the hydrogen ion concentration could be increased with little or no dilution of the virus. It was necessary to return the specimens to neutral pH before testing for infectivity in cell culture, to prevent damage to the cells. This return to neutral pH had to be achieved quickly, to reduce the risk of any re-association between inhibitor (possibly antibody) and virus. The methods chosen were: (1) dialysis to reduce the pH, and (2) ultracentrifugation to remove the low pH medium and return the specimens to neutral pH.

Part I. Before testing NG specimens, it was necessary to determine whether prototype adenovirus can be dissociated from antibody at pH 2.5. Adenovirus type 3 was mixed with neutralising antibody and treated by the method outlined above. Due to the shortage of fetal material, the continuous line of Chang Conjunctiva was used for titration of infectivity.

Method. Clarified HEK cell culture fluid containing prototype adenovirus type 3 (1.5 ml; $10^{5.25}$ TCD_{50}/ml) was mixed with (a) a 1:100 (recommended) dilution of homologous antiserum from the Standards Laboratory for Serological Reagents, Colindale, London (1.5 ml), and (b) PBS (1.5 ml; virus control). The mixtures were allowed to stand at room temperature for 1 hour. The virus-antibody mixture was then treated in 3 ways: (1) 1 ml was dialysed against 100 ml PBS for 1 hour at room temperature (treatment A), (2) 1 ml was dialysed against 100 ml 0.01 M TRIS (tris-(hydroxymethyl) amino methane), adjusted to pH 2.5 with HCl, for 1 hour at room temperature (after Kjellén, 1966) (treatment B), (3) 1 ml was kept at 4°C (treatment C). The virus control fluid was dialysed (1 ml) against 0.01 M TRIS (pH 2.5) as above (treatment D) and the remainder was kept at 4°C (treatment E).

After 1 hour, the pH of each mixture (A, B, C, D and E) was checked using universal pH paper. Each mixture (0.6 ml) was transferred to a 3/16" x 1 21/32" cellulose nitrate tube (Beckman) and centrifuged at 35000 rev./min in a Beckman model L5-40 ultracentrifuge with SW 50.1 rotor. The supernate was removed and the pellet was resuspended in 0.6 ml PBS. The resuspended material was immediately diluted in tenfold steps in PBS at 4°C and 0.1 ml of each dilution (10^{-1} to 10^{-6}) was added to each of 4 cultures of Chang Conjunctiva cells. Cytopathic effects were read at 7 days after inoculation and infectivity titres were calculated by the method of Kärber (1931).

Results. The method of titration did not allow detection of infectivity below 10^{2} TCD_{50}/ml because the resuspended fluids were not tested undiluted. The results are shown in Table 5.1. The infectivity recovered from the virus control exposed to pH 2.5 (treatment D) was less by tenfold than the infectivity recovered from the virus control kept at pH 7.2 (treatment E). However, the infectivity recovered

TABLE 5.1 Infectivity values (TCD_{50}/ml) obtained from an adenovirus type 3 and homologous antibody mixture after treatment at pH 2.5 or pH 7.2, compared with the infectivity values obtained with virus alone treated at pH 2.5 or pH 7.2. The titrations were performed using Chang Conjunctiva cells.

Mixture:	virus-antibody			virus alone	
Treatment:	A	B	C	D	E
Dialysis:	YES	YES	NO	YES	NO
Temperature:	RT	RT	4°C	RT	4°C
pH:	7.2	2.5	7.2	2.5	7.2
INFECTIVITY RECOVERED (TCD_{50}/ml)	$<10^2$	$10^{3.5}$	$<10^2$	$10^{3.5}$	$10^{4.5}$

from the fluids B (with antibody) and D (without antibody) was the same after low pH treatment. The virus-antibody mixture held at pH 7.2 had no detectable infectivity.

Comment. From this limited study using a prototype strain of adenovirus, it appears that the infectivity of an adenovirus-antibody mixture can be enhanced by treatment at low pH (2.5). The infectivity was improved by more than tenfold after low pH treatment and was equal to the infectivity of virus without antibody after low pH treatment. However, the treatment itself caused a tenfold decrease in virus infectivity. Therefore, in testing NG specimens for infectivity after treatment at low pH, it should be remembered that release of any inhibitory substance from the adenoviruses leading to infectivity might be offset by a reduction in the proportion of viable virus particles at low pH.

The above test was carried out once only. With no replicates, the results were only indicative. However, they were sufficiently encouraging to justify investigation into the low pH treatment of adenoviruses in NG specimens.

Part II. NG specimens from 4 different children were treated by the above procedure and examined for infectivity in Chang Conjunctiva cell cultures.

Method. 4 NG specimens (from patients no. 12, 20, 25 and 29) were dialysed (0.8 ml) against 100 ml 0.01M TRIS, adjusted to pH 2.5 with HCl, for 1 hour at room temperature. Samples of stool extract were also kept at 4°C. Each treated stool extract (0.6 ml) was centrifuged at 35000 rev./min as in Part I. The supernates were removed and the pellets were resuspended in 0.6 ml PBS at 4°C. The resuspended material from each stool extract was added (0.1 ml) to each of

5 cultures of Chang cells. The untreated stool extracts were each added (0.1 ml) to each of 2 cultures. The cultures were incubated at 36.5°C and observed daily for CPE. At degeneration of the cultures, the fluids were passed to fresh Chang cultures.

Results. The specimens were maintained in culture for 13-17 days. The untreated specimen from patient no. 29 caused CPE which developed again within 2 days of passage. However, the 5 cultures inoculated with treated stool extract developed only mild CPE. The 3 other specimens (treated or untreated) did not cause CPE.

Comment. Treatment of these specimens at pH 2.5 did not give enhanced infectivity. Therefore it is unlikely that they contained an inhibitor which could be inactivated or dissociated from the adenovirus particles by low pH. Since NG adenoviruses have not been characterised, their degree of resistance to extremes in pH compared with growing strains is unknown. It is possible that these viruses are especially susceptible to low pH conditions, in which case the probability of detecting infectivity in culture after this treatment could be even less than with the untreated NG specimens. The possibility still stands that antibody was present on these particles and that dissociation of antibody at low pH was concomitant with inactivation.

Low pH treatment of a specimen from patient no. 29 reduced the amount of CPE seen. This may have resulted from the destruction of a toxic factor in the stool at low pH, or from the inactivation of a replicating agent. The sensitivity of Chang Conjunctiva cells to agents in NG specimens is further discussed in Chapter 7.

4. Discussion.

The methods used in this chapter to detect a possible inhibitor

in NG stools or remove its effect were uniformly negative. The tests to detect unbound inhibitor (Section 2.) were not the most sensitive possible. There was no indication of inhibitory effects, which might otherwise have led to deeper investigation. The possibility of a type-specific inhibitor is a real one but the existence of 35 established serotypes of adenovirus made the task of testing the extracts against all prototypes a daunting one. The most common serotypes (1 and 2) were not apparently inhibited.

The treatments to remove a possible inhibitor from NG stool extracts (dilution, ultracentrifugation, trypsin, heat and low pH; Section 3.) might have affected a bound or unbound inhibitor if present, but did not. Therefore the reason for lack of complete infectivity with these specimens was still unknown. The remainder of the investigation was directed towards characterising the adenoviruses in NG specimens and further assessment of the Chang Conjunctiva cell line for detection of these agents.

CHAPTER 6

CHAPTER 6. ATTEMPTS TO IDENTIFY NON-GROWING ADENOVIRUSES: I. PREPARATION AND TESTING OF ANTISERA TO NON-GROWING ADENOVIRUSES.

1. Introduction.

I hoped to detect an antibody response to adenoviruses in NG specimens after inoculation of animals with these agents. Positive identification of group-specific antibodies in post-inoculation sera but not in pre-inoculation sera would indicate that the NG adenoviruses share group-specific antigenic determinants with other adenoviruses of mammalian origin. By testing the sera for any neutralising activity to common serotypes of adenovirus, it might be possible to type the adenoviruses in NG specimens.

2. Preparation of Antisera.

a. Rabbit Serum. One NG stool extract from patient no. 25 was used to inoculate 2 rabbits. This extract (from 27/7/76) was the fifth NG specimen taken from this child, and was reported to contain large numbers of adenovirus particles when originally examined by EM. 9 ml of stool extract was centrifuged at 5000 rev./min for 20 min in a Beckman model L5-40 ultracentrifuge with SW 50.1 rotor. The supernate was passed through a 0.22 μm (12.5 mm dia.) filter (Millipore Corp.). The filtered extract was negative by bacteriological culture when incubated aerobically and anaerobically on blood agar. Samples of the filtered extract were stored at -20°C and 2 rabbits (coded 90 and 96) were inoculated intravenously 3 times at weekly intervals with 1.25 ml amounts. Blood was taken from the ear vein before the first inoculation and 1, 2 and 3 weeks after the last inoculation. The blood was stored at 4°C. The sera were separated on the following

day, centrifuged at 2000 rev./min for 20 min and stored in 2-3 ml amounts at -20°C. All sera were heat-treated before use (56°C for 30 min).

b. Guinea Pig Serum. The method used for preparing antisera in guinea pigs was that used by Dr. C.M.P. Bradstreet (Standards Laboratory for Serological Reagents, Colindale, London). Three NG stool extracts (from patients no. 25 (25/7/76), 29 (26/7/76), and 59 (2/8/77)) were each used to inoculate 2 guinea pigs. All 3 specimens were reported to contain large numbers of adenovirus particles when originally examined by EM. 2 ml (approximately) of each extract was passed through a 0.22 μm filter as above. The filtered specimens were stored at -20°C, and each was given intranasally (2 drops; approx. 0.1 ml) by Pasteur pipette to each of 2 guinea pigs after collection of pre-inoculation blood by cardiac puncture. The animals were bled again on day 7 and day 14 after inoculation. One guinea pig died after the inoculation, and one died after the first post-inoculation bleed (day 7). The sera were separated and stored as for the rabbit sera, and were heat-treated (56°C for 30 min) before use.

3. Testing of Antisera.

a. Reaction of Sera from Rabbits Inoculated with NG Adenoviruses in the Indirect Immunofluorescence Test. Pre-inoculation and post-inoculation (day 7) sera were tested for the ability to react as adenovirus-specific antiserum in the indirect immunofluorescence test, using KB cells infected with common serotypes.

Part I. The sera were first tested at 1:5 dilution using cells infected with either type 2, type 3 or type 5.

Method. Cultures of KB cells were prepared in Leighton tubes as

described in Chapter 2. Sets of 9 replicate cultures were infected with type 2 (0.5 ml; $10^{4.5}$ TCD_{50}/ml), type 3 (0.5 ml; 10^{4} TCD_{50}/ml) or type 5 (0.5 ml; 10^{4} TCD_{50}/ml). 8 cultures were not inoculated. All cultures were incubated for 48 hours, then washed with PBS and fixed in acetone (see Chapter 1). For each set of cultures infected with a different serotype, 2 of the 9 replicate cultures each received 2 drops of rabbit 90 serum (1:5 dilution), 2 of the cultures received rabbit 96 serum (1:5), and 2 received guinea pig anti-adenovirus serum (1:20) as prepared and used in Chapter 5. The 3 remaining coverslips received the corresponding pre-inoculation sera. The uninoculated cultures received (in duplicate) rabbit 90 serum, rabbit 96 serum, guinea pig anti-adenovirus serum (all post-inoculation sera) or PBS. After incubation at 36.5°C for 30 min, the coverslips were washed in PBS and the appropriate species-specific fluorescein isothiocyanate-conjugated antibody was applied (either Swine anti-Rabbit IgG or Swine anti-Guinea Pig IgG). After incubation at 36.5°C for 30 min, the coverslips were washed and mounted as described in Chapter 1.

Results. See Table 6.1. The pre-inoculation sera from rabbits 90 and 96 did not allow fluorescent staining of infected cells, but the post-inoculation sera did cause fluorescence in cells infected by each of the 3 serotypes. The fluorescence was mainly cytoplasmic.

Comment. The rabbit sera taken 7 days after the final inoculation with a NG specimen caused fluorescence in adenovirus-infected cells, whereas pre-inoculation sera did not. Therefore it is likely that there was an antibody response to NG adenoviruses. The post-inoculation sera allowed detection of intracellular antigens of 3 different serotypes belonging to 2 different subgroups. Therefore it

TABLE 6.1 Results of immunofluorescence tests using sera from 2 rabbits (90 and 96) inoculated with a NG specimen from patient no. 25. Tests were performed using KB cells infected with adenovirus type 2, type 3 and type 5. The positive control serum was the group-specific anti-adenovirus serum (1:20) (guinea pig) as used in Chapter 4. The rabbit sera were tested at 1:5 dilution.

Serum	PBS	pre-90	90	pre-96	96	PBS	pre-GP	GP
FITC-Antibody	Swine anti-Rabbit IgG					Swine anti-Guinea Pig IgG		
Infecting Serotype								
2	NT	-	+	-	+	NT	-	+
3	NT	-	+	-	+	NT	-	+
5	NT	-	+	-	+	NT	-	+
U.I.	-	NT	-	NT	-	-	NT	-

U.I. uninfected control cultures
- negative by immunofluorescence
NT not tested
+ positive by immunofluorescence
pre pre-inoculation serum

is probable that adenovirus group-specific antibodies were present in these sera. The extent of this group-specific response (i.e. the level of group-specific antibodies after inoculation) could be determined by titration of the antisera.

Part II. To determine whether there was an even antibody response to intracellular antigens of more than one established serotype and subgroup, the sera from rabbits 90 and 96 were tested at various dilutions using KB cells infected with either type 2 or type 3.

Method. KB cultures infected with adenovirus type 2 or type 3 were prepared as in Part I. They were fixed 2 days after infection. The day 7 sera from both rabbits at dilutions of 1:5, 1:20, 1:40, 1:80, 1:160 and 1:320 (in PBS) were each applied to single coverslip cultures infected with (a) type 2, and (b) type 3. Uninfected cultures were treated with 1:5 dilutions of these sera in duplicate, and the corresponding pre-inoculation sera (1:5) were applied to single coverslip cultures infected with each serotype. The coverslips were incubated at 36.5°C for 30 min, washed in PBS and treated with fluorescein isothiocyanate-conjugated antibody (Swine anti-Rabbit IgG). After incubation at 36.5°C, the coverslips were washed and mounted as described in Chapter 1.

Results. See Table 6.2. The tests using pre-inoculation serum and infected cultures were negative. The tests using uninfected cultures and post-inoculation serum were negative. Of the two post-inoculation sera, serum 96 had higher levels of antibody to intracellular antigens of both serotypes. However, both post-inoculation sera had higher levels of antibody to intracellular antigens of type 2 than of type 3. In all cases the post-inoculation sera had antibody levels at least 4 times greater than those of the pre-

TABLE 6.2 Results of immunofluorescence tests using post-inoculation (day 7) serum from 2 rabbits (90 and 96) inoculated with a NG specimen from patient no. 25: assessment of antibody levels to intracellular antigens in KB cells infected with adenovirus type 2 and type 3. Tests using pre-inoculation sera at 1:5 dilution with type 2- and type 3-infected cultures were negative. Tests using uninfected cultures and post-inoculation sera at 1:5 dilution were also negative.

Highest Dilution to Give Fluorescent Staining in Cultures Infected with:-

Adenovirus	type 2	type 3
Serum 90	1:80	1:20
Serum 96	$\geq$ 1:320	1:80

inoculation sera.

Comment. Since this test was not carried out with replicates, the levels of antibody detected are only indicative of the true values, rather than being absolute. Since pre-inoculation sera at 1:5 dilution had no detectable antibody, the elevated antibody levels detected in the post-inoculation sera were probably the result of immunisation with NG adenoviruses. The fluorescence may have resulted from the interaction between group-specific antibodies in the serum and group antigens in the cells. This is most likely, but it is also possible that the NG specimen used to immunise the rabbits contained adenoviruses of serotypes 2 and 3. In this case, the antigen-antibody interaction may have been predominantly type-specific. To affirm that the positive immunofluorescence results were due to a group-specific antibody response, the sera were tested by a technique which is used widely for detecting group-specific antibodies (complement fixation).

b. Reaction of Sera from Rabbits Inoculated with NG Adenoviruses in the Complement Fixation Test. This test was performed by Mr. G. Robinson using a standard complement fixation test employed in this laboratory (see Grist et al., 1979). Sera from rabbits 90 and 96 were tested in parallel with routine tests on human sera. The pre-inoculation and post-inoculation sera (from day 7) were tested together, using adenovirus antigen prepared by the Standards Laboratory for Serological Reagents, Colindale, London. Only the results of the tests are given here.

Results. The pre-inoculation sera from both rabbits had a titre of less than 1:8. The day 7 serum from rabbit 90 had a titre of 1:64 and that from rabbit 96 had a titre of 1:256.

Comment. The complement fixation technique allows the detection of

group-specific antibody in serum (see Jackson and Muldoon, 1975). By this test, both rabbits had developed significant antibody levels to adenovirus antigens (greater than a 4-fold increase in titre). As in the immunofluorescence test, the post-inoculation serum of rabbit 96 had antibody levels 4 times higher than that of rabbit 90. Since there is agreement between the results of immunofluorescence and complement fixation, the immunofluorescence with type 2- and type 3-infected cells probably resulted from the presence of group-specific antibody.

The soluble group antigens of mammalian and avian adenoviruses are distinct by complement fixation test (see Andrewes, Pereira & Wildy, 1978). Since antibodies to adenoviruses of mammalian origin were detected by complement fixation in the day 7 sera of rabbits 90 and 96, it is probable that the NG adenoviruses used as immunogens were of mammalian rather than avian origin.

c. Effect of Sera from Rabbits Inoculated with NG Adenoviruses on the Replication of Common Adenovirus Serotypes. Since the sera from rabbits 90 and 96 had developed group-specific antibody to adenoviruses, there might also be antibodies present specific for the serotype(s) of NG adenoviruses. The presence of type-specific antibody might explain the higher levels of antibody to adenovirus type 2 intracellular antigens in Table 7.2. Such type-specific antibodies might be detected by neutralisation test against the common serotypes of adenovirus.

The sera from rabbits 90 and 96 were tested for neutralising activity against serotypes 1, 2, 3, 5 and 7. The lowest dilution of serum which would not have a toxic effect on the cells (1:20) was used in all tests.

Method. Sera from each rabbit (pre-inoculation sera and sera taken at day 7 and day 14 after the last inoculation) were tested at 1:20 in PBS. Each serum (0.1 ml; 1:20) was mixed in each of 4 replicate wells of a Linbro disposo tray with 0.1 ml adenovirus of a particular serotype (types 1, 2, 3, 5 and 7 all at 10^3 TCD_{50}/ml). The virus control mixtures were also prepared in quadruplicate (0.1 ml virus + 0.1 ml PBS). All mixtures were kept for 1 hour at room temperature, then added to fresh cultures of Chang Conjunctiva cells. The cultures were incubated stationary at 36.5°C and observed daily for CPE.

Result. In all cultures, CPE arose in 2-4 days, depending on the serotype of adenovirus. In all cases where a particular serotype was mixed with a particular serum, CPE arose at the same time as in the virus controls.

Comment. These sera at 1:20 did not have detectable neutralising activity against the commonest adenovirus serotypes isolated from stools. There are at least 2 explanations for this: (1) the NG adenoviruses used to inoculate the rabbits were not of these common serotypes, or (2) the NG adenoviruses might have type-specific determinants blocked in some way (possibly by antibody) and failed to act as type-specific immunogens. That the post-inoculation sera did not neutralise types 2 and 3 is a further indication that the serum antibodies were reacting with either subgroup- or group-specific determinants in the immunofluorescence tests.

d. Reaction of Sera from Guinea Pigs Inoculated with NG Adenoviruses in the Immunofluorescence Test. Pre-inoculation and post-inoculation (day 7 and (where applicable) day 14) guinea pig sera were tested for ability to react as adenovirus-specific antisera in

the indirect immunofluorescence test, using KB cells infected with common serotypes.

Method. All sera were tested in an identical manner as follows. KB cell cultures in Leighton tubes were infected with either adenovirus type 1 (0.5 ml; $10^{4.5}$ TCD_{50}/ml), type 2 (0.5 ml; $10^{4.5}$ TCD_{50}/ml), type 3 (0.5 ml; 10^{4} TCD_{50}/ml) or type 5 (0.5 ml; 10^{4} TCD_{50}/ml) and fixed 2 days after infection. Day 7 serum at 1:20 dilution in PBS was applied in duplicate to cultures infected with type 1, type 2, type 3 and type 5, and to uninfected cultures. Other cultures were treated with day 14 serum in the same way. In addition, dilutions of serum from day 7 and day 14 (1:40, 1:80 and 1:160) were applied in duplicate to cultures infected with adenovirus type 2. Pre-inoculation serum at 1:20 was applied to single coverslips infected with each serotype. The preparations were treated as described in Chapter 1. The fluorescein isothiocyanate-conjugated antibody used for staining was Swine anti-Guinea Pig IgG.

Results. Neither the pre-inoculation nor the post-inoculation sera allowed detection of adenovirus-specific intracellular antigens in this test (Table 6.3).

Comment. The negative results here contrast with the results of intranasal inoculation of guinea pigs with adenovirus type 3 (Chapter 5), in which 2 of 5 animals showed a marked group-specific response to adenovirus. There are at least 2 possible explanations for the negative result: (1) guinea pigs may not always develop a local infection with resulting antibody response, following intranasal inoculation. Therefore the negative result may simply have been due to the small number of animals tested. (2) the NG adenoviruses may be less infectious for guinea pig cells than common serotypes of

TABLE 6.3 Results of immunofluorescence tests on 5 guinea pig sera for group-specificity to adenovirus, using KB cells. Each guinea pig was inoculated intranasally with one of 3 NG specimens. 1:20 dilutions of each serum from day 7 and day 14 after inoculation were tested in duplicate, using cultures infected with adenovirus serotypes 1, 2, 3 and 5. Higher dilutions of each serum were tested in duplicate using cells infected with type 2. Immunofluorescence tests using cells infected with the above serotypes and pre-inoculation serum from each guinea pig were negative.

Infecting Virus:	Type 1	Type 2	Type 3	Type 5	U.I.	Highest Dilution to Give Fluorescence With Cells Infected by Adenovirus type 2
	Fluorescent Staining When Serum Used at 1:20					
Serum						
1* Day 7	-	-	-	-	-	< 1:20
Day 14	-	-	-	-	-	< 1:20
2** Day 7	-	-	-	-	-	< 1:20
Day 14	-	-	-	-	-	< 1:20
3** Day 7	-	-	-	-	-	< 1:20
Day 14	NT	NT	NT	NT	NT	
4*** Day 7	-	-	-	-	-	< 1:20
Day 14	-	-	-	-	-	< 1:20
5*** Day 7	-	-	-	-	-	< 1:20
Day 14	-	-	-	-	-	< 1:20

- negative by immunofluorescence
NT not tested
+ positive by immunofluorescence
U.I. uninfected control cultures
* serum from guinea pig inoculated with a NG specimen from patient no. 25
** serum from guinea pig inoculated with a NG specimen from patient no. 59
*** serum from guinea pig inoculated with a NG specimen from patient no. 29

adenovirus grown in cell culture. The latter explanation is quite feasible, since NG adenoviruses do not (by definition) infect human cells in culture productively. Since there was no group-specific antibody response by immunofluorescence test, the presence of type-specific antibodies in the post-inoculation sera was unlikely.

e. Effect of Sera from Guinea Pigs Inoculated with NG Adenoviruses on the Replication of Common Adenovirus Serotypes. The sera from the 5 guinea pigs were tested for neutralising activity against serotypes 1, 2, 3, 5 and 7.

Method. Sera from each guinea pig (pre-inoculation sera and sera taken at day 7 and day 14 after inoculation) were tested at a dilution of 1:20. Each serum (0.1 ml; 1:20) was mixed in each of 2 wells of a Linbro disposo tray with 0.1 ml adenovirus of a particular serotype (types 1, 2, 3, 5 and 7 all at 10^3 TCD_{50}/ml). The virus control mixtures were also prepared in duplicate (0.1 ml virus + 0.1 ml PBS). All mixtures were left at room temperature for 1 hour, then added to fresh cultures of Chang Conjunctiva cells. The cultures were incubated stationary at 36.5°C and observed daily for CPE.

Results. In all cultures, CPE arose in 2-4 days. In all cases where a particular serotype was mixed with a particular serum, CPE arose at the same time as in the virus controls.

Comment. As with the rabbit sera tested in Section c., the sera from guinea pigs inoculated with NG adenoviruses showed no neutralising activity against the most common serotypes of adenovirus isolated from stools. With the guinea pig sera, this result was to be expected, because there was no detectable antibody to intracellular antigens of the common serotypes by immunofluorescence. There is no

evidence from this result that the NG adenoviruses present in the specimens used to inoculate the guinea pigs were common serotypes.

4. Conclusions.

The mechanism whereby common serotypes of adenovirus can cause an antibody response in guinea pigs after intranasal inoculation is unknown. It is likely that (at least) a localised respiratory infection occurs, of sufficient severity to cause an increase in serum anti-adenovirus antibody levels in some animals. The extent of infection after administration of NG adenoviruses was probably low or negligible, either because of special conditions required by these viruses for replication, or because of an inhibitory factor in the stool. It is unlikely that antibodies in the sera were present but undetected by immunofluorescence because NG adenoviruses have group antigens distinct from established serotypes. Although NG adenoviruses in one specimen from patient no. 25 did not cause an antibody response in guinea pigs, another NG stool specimen taken 2 days later caused an antibody response in rabbits to adenoviruses of mammalian origin.

The anti-adenovirus antibody response by rabbits 90 and 96 was not apparently caused by common serotypes found in stools. The NG adenoviruses could be less common serotypes, new serotypes, or could be any serotype with type determinants masked by antibody. Therefore, even if the sera were tested against prototype strains of all established serotypes, demonstration that these lacked neutralising ability would still not prove that NG adenoviruses were a new serotype. They might contain no detectable neutralising antibodies at all, only subgroup- and group-specific antibodies. Therefore it was necessary to prove that these rabbit sera did

possess neutralising ability against one serotype of adenovirus found in stools.

CHAPTER 7

CHAPTER 7 ATTEMPTS TO IDENTIFY NON-GROWING ADENOVIRUSES: II. USE OF CHANG CONJUNCTIVA CELLS.

A. INVESTIGATION OF CYTOPATHIC EFFECTS FROM FOUR NG SPECIMENS.

1. Introduction.

In the work described in Chapter 3 (Section A.1.) 5 NG specimens from different children caused CPE over more than one passage in both Chang Conjunctiva cell cultures and Intestine 407 cell cultures (see Table 3.3). This phenomenon required further investigation, and for this Chang cells were chosen because they could be maintained twice as long as Intestine 407 cells in Leibovitz' L15 medium. It was necessary to determine whether the CPE caused by the NG specimens was the result of a toxic factor in the stools, or the result of multiplication of adenovirus or some other agent. If adenovirus replication was responsible for the CPE, attempts to identify the agent(s) by neutralisation tests might establish whether these strains were recognised serotypes or new (previously undescribed) serotypes.

2. Passage of Cytopathic Effects.

NG specimens from patients no. 62, 64, 68 and 69 which caused CPE in Chang cell cultures (Table 3.3) were re-tested. If a toxic factor was responsible for the CPE, its action would probably be diluted out with repeated passage in culture.

a. Titration of Apparent Infectivity. The second passage fluids were titrated to determine the degree of apparent infectivity (cytopathogenicity). The relative importance of repeated freezing and thawing for development of CPE was also investigated.

Method. The specimens were passaged twice in Chang cell cultures as described in Chapter 1. The second passage fluids were frozen

once, thawed and diluted 10^{-1} and 10^{-2} in PBS. The remainder of the fluids was frozen and thawed twice and 10^{-1} and 10^{-2} dilutions were again made in PBS. The remainder of the fluids was frozen and thawed twice and 10^{-1} and 10^{-2} dilutions made. The 10^{0}, 10^{-1} and 10^{-2} dilutions of fluid prepared after the first, third and fifth freeze were added to each of 2 cultures of Chang cells. The cultures were incubated at 36.5°C and examined on alternate days for CPE. The duplicate third passage culture fluids derived from the cultures inoculated with undiluted second passage fluids that were frozen 5 times were examined for the presence of virus particles by EM.

Results. CPE developed in cultures inoculated with undiluted culture fluids derived from the specimens of all 4 patients (Table 7.1). For the specimens of patients no. 64, 68 and 69, CPE was complete by the sixth day after inoculation. The 10^{-1} dilutions of fluids derived from the specimens of these patients also caused CPE, but that of patient no. 62 did not. No 10^{-2} dilution of any second passage culture fluid caused CPE. Where CPE arose, the time taken to develop was the same whether the second passage fluid had been frozen once, 3 times or 5 times. All third passage fluids examined by EM were found to contain adenovirus particles.

Comment. The time taken for CPE to develop was not reduced by repeated freezing of the second passage fluids. Therefore repeated freezing to release virus from the cells was probably not critical for the development of CPE (although it may have made a difference over several passages). The factor responsible for CPE was apparently diluted out at a 10^{-2} dilution of the second passage fluid (10^{-1} for one specimen). The results of EM suggest that this factor may have been replicating adenovirus. However, the virus particles seen may

TABLE 7.1 Development of CPE in Chang cell cultures inoculated with second passage culture fluids derived from the specimens from patients no. 62, 64, 68 and 69. The second passage culture fluids were tested at dilutions of 10^{0}, 10^{-1} and 10^{-2}, after freezing once, 3 times and 5 times

No. Times Culture Fluid Frozen	Dilution of Second Passage Culture Fluid	Development of Cytopathic Effect Caused by the Specimen of: Patient No. 62	64	68	69
1	10^{0}	±	+	+	+
	10^{-1}	-	±	±	±
	10^{-2}	-	-	-	-
3	10^{0}	±	+	+	+
	10^{-1}	-	±	±	±
	10^{-2}	-	-	-	-
5	10^{0}	± *	+ **	+ **	+ **
	10^{-1}	-	±	±	±
	10^{-2}	-	-	-	-

\+ complete CPE (monolayer destroyed)
± incomplete CPE (slow progression or late onset)
\- no CPE
* less than 5 particles per grid square (EM)
** greater than 5 particles per grid square (EM)

have been input virus carried over from the specimen. If the cytopathic effects were caused by replicating adenoviruses, the effects would be expected to develop over several more passages, representing dilutions of the second passage fluid (10^{-2} and higher) which caused no cytopathic effects in the above titrations.

b. Repeated Passage. The 4 second passage fluids used above (a.) were passaged repeatedly in Chang cell cultures (6 times, or until CPE was no longer apparent). As a result of work carried out in parallel with these tests (Section C. below), passages no. 4, 5, 6, 7 and 8 were carried out with incubation of the cultures at 33°C.

Method. 50 cm^2 flask cultures of Chang cells containing 20 ml maintenance medium were inoculated with 0.5 ml of second passage fluid. When the monolayers had degenerated, the cultures showing CPE were frozen and thawed 4 times. The fluids were divided into 2 ml amounts and stored at -20°C. For each specimen from each patient, a 2 ml sample of third passage fluid was added to a 120 cm^2 flask culture of confluent Chang cells containing 50 ml maintenance medium. The flasks were incubated at 33°C. Cultures showing CPE were frozen and thawed 4 times. The fluid from each culture was divided: half was stored unclarified at -20°C in 2 ml amounts, and half was centrifuged at 3000 rev./ min for 30 min. The supernatant fluid was stored in 2 ml amounts at -20°C. The fourth passage culture fluids thus prepared were used as 'stock' preparations in later tests.

The passages no. 5, 6, 7, and 8 were carried out with tube cultures as described in Chapter 1. The contents of one tube from each specimen after the seventh passage was examined by EM.

Results. The culture fluids from the specimens from patients no. 62 and 68 did not cause CPE beyond the third and fourth passage respectively.

The CPE from the specimens of patients no. 64 and 69 developed repeatedly over the 8 passages. It was similar to that seen in all cultures at early passages. In the tube cultures (fifth to eighth passage) the CPE took 6-7 days to develop fully. Adenoviruses were detected in the seventh passage culture fluids by EM (5 particles or more per grid square).

Comment. 8 passages represent a 10^{-9} dilution of the original specimen. It was found in Section a. that a 10^{-4} dilution of the original specimens (that is, a 10^{-2} dilution of the second passage fluid) was sufficient to prevent development of CPE. Therefore it is likely that the cytopathic effects caused by the specimens of patients no. 64 and 69 were due to the presence of replicating agents and (from the results of EM) that these agents were adenoviruses.

3. Attempts to Identify the Cytopathogenic Agents.

a. Sensitivity to 5-BUdR. 5-bromo-2'-deoxyuridine (5-BUdR) is a thymidine analogue which increases the frequency of copy errors when incorporated into newly synthesised DNA (see Stent, 1963). Kjellén (1962) found that the presence of 5-BUdR at a concentration of 20 µg/ml was sufficient to prevent the replication of adenovirus type 5 in human cells.

To determine whether normal DNA synthesis was required for the development of CPE caused by these NG specimens, the third passage fluids of Section A.2.b. (from the specimens of patients no. 64, 68 and 69) were incubated in Chang cell cultures in the presence of 5-BUdR. Any inhibition of cytopathic effects would suggest that correct DNA replication (possibly viral) was necessary for development of CPE.

Method. Each of the third passage fluids from the specimens of the

patients above were added separately (0.1 ml) to duplicate tube cultures of Chang cells containing either (a) 0.9 ml Chang cell maintenance medium, or (b) 0.9 ml maintenance medium containing 5-BUdR (Sigma Chemical Co.) at 50 µg/ml. The cultures were incubated at 33°C and examined on alternate days for CPE.

Results. CPE from the specimens of patients no. 64 and 69 developed in untreated cultures and also in those treated with 5-BUdR. Although the CPE in untreated cultures was complete by the seventh day after inoculation, the CPE in treated cultures only affected about 25 per cent of the cells by the fourth day and did not progress. For the specimen of patient no. 68, CPE did not reach completion in the untreated cultures before degeneration and did not develop in the cultures treated with 5-BUdR.

Comment. The development of CPE appears to have been dependent on correct DNA replication. The results of this test do not prove that CPE resulted from adenovirus replication, but it is unlikely that a toxic factor in the stool caused it. However, if the CPE was caused by adenoviruses of established serotype(s), its development should be inhibited by standard neutralising antiserum prepared against the relevant serotype(s).

b. Neutralisation Tests Using Standard Antisera to Types 1 to 33.
Culture fluids derived from the specimens of patients no. 64, 68 and 69 were tested for neutralisation by standard antisera prepared against the established serotypes of adenovirus.

Method. The tests were performed as described in Chapter 1 using antisera provided by the Standards Laboratory for Serological Reagents, Colindale, London. These were used at the recommended dilutions. The antisera to type 30, 31, 32 and 33 were obtained from Immunitalia

Diagnostics, Rome, and used at a 10^{-2} dilution. (From the details given, this dilution would be capable of neutralising at least 100 TCD_{50} of homologous virus). The clarified and unclarified fourth passage culture fluids (undiluted; see Section A.2.b.) of specimens from patients no. 64 and 69 were tested separately. A first passage fluid of the specimen from patient no. 68 was tested at 1:2 dilution.

Results. In all tests where culture fluids and antisera were mixed, CPE affected more than 25 per cent of the cells by the seventh day. Although there was slight variation in the time taken for CPE to appear, there was no appreciable difference in this respect between the individual test cultures and the virus control cultures.

Comment. No antiserum at the dilution used was capable of preventing or inhibiting the development of CPE caused by the 3 specimens. In view of the low infectivity and similarity of cytopathic effects in all cultures, it was considered unlikely that the test fluids contained a mixture of adenovirus serotypes. As previously stated, it is possible that the cytopathic effects were not caused by adenoviruses. Alternatively, they may have been caused by adenoviruses of serotypes other than 1 to 33.

c. Immunofluorescence. The possibility that the cytopathogenic agents might be adenoviruses was further tested by immunofluorescence using third passage culture fluids and group-specific antiserum. Detection of intracellular adenovirus antigens in KB cells after inoculation with these fluids would be a further indication that these agents were adenoviruses undergoing at least partial replication.

Method. The tests were carried out as described in Chapter 1. Each of 4 coverslip cultures of KB cells was inoculated with a third passage

Chang cell culture fluid (0.1 ml) derived from the specimen of patient no. 64, 68 or 69 (Section A.2.b.). The cultures were fixed 3 days after inoculation. Group-specific guinea pig antiserum (1:20 dilution; prepared and tested as described in Chapter 4) was applied to 2 cultures and the other 2 cultures received either pre-inoculation serum (1:20) or PBS. The fluorescein isothiocyanate (FITC) conjugated antiserum used was Swine anti-Guinea Pig IgG (Nordic). Uninfected cultures were treated in the same way, and cultures infected with adenovirus type 7 ($10^{2.5}$ TCD_{50}/ml) were included as a positive control.

Results. Adenovirus antigens detectable by immunofluorescence were formed in cultures inoculated with the third passage fluids from the specimens of patients no. 64 and 69 (Table 7.2). The average number of fluorescing foci per field was 10 (at x250 magnification) and the staining was both nuclear and cytoplasmic. The third passage fluid from the specimen of patient no. 68 was negative by immunofluorescence.

Comment. The 2 third passage fluids which gave a positive result by immunofluorescence had caused CPE when passaged repeatedly in Chang cell cultures (Section A.2.b.). The third passage fluid from the specimen of patient no. 68 had caused mild CPE when passaged further, and did not cause detectable adenovirus antigen production in KB cells. Since the fluids capable of causing distinct CPE also caused adenovirus antigen production, it is probable that the cyto-pathogenic agents from patients no. 64 and 69 were adenoviruses. The serotype(s) of these viruses required further investigation.

TABLE 7.2 Results of immunofluorescence tests using KB cells inoculated with third passage Chang cell culture fluids derived from 3 NG specimens taken from 3 children

Patient No.	Serum			Location of Fluorescence	No. Fluorescing Foci Per Field
	PBS	pre*	anti-Ad **		
64	-	-	+	general	10
68	-	-	-		
69	-	-	+	general	10
U.I.***	-	-	-		
type 7 §	-	-	+	general	3

- negative by immunofluorescence
+ positive by immunofluorescence
* pre-immune guinea pig serum
** post-inoculation guinea pig serum (adenovirus group specific)
*** uninfected cultures
§ cultures infected with adenovirus type 7 (positive control)

d. Neutralisation Tests Using Sera from Animals Inoculated with NG Specimens.

Part I. Sera from rabbits inoculated with NG adenoviruses from patient no. 25 had antibodies to adenovirus group antigens by immunofluorescence and complement fixation (Chapter 6). Although these sera at 1:20 dilution did not neutralise the commoner serotypes of adenovirus found in stools (types 1, 2, 3, 5 and 7) they might neutralise the cytopathogenic agents from NG specimens. The sera from rabbits 90 and 96 were tested for neutralising activity against the agents from patients no. 64 and 69. The sera from the guinea pigs inoculated intranasally with NG adenoviruses were also tested.

Method. 1:20 dilutions of pre-inoculation and day 7 (post-inoculation) sera from rabbits 90 and 96 were tested. 1:20 dilutions of pre-inoculation, day 7 and day 14 sera from guinea pigs no. 1 - 5 (Chapter 6) were also tested. All sera at this dilution were mixed (0.1 ml + 0.1 ml) in duplicate with undiluted third passage culture fluids of specimens from patients no. 64 and 69. After 1 hour at room temperature, the mixtures and virus controls (with PBS in place of antiserum) were added to fresh cultures of Chang cells and incubated at 36.5°C.

Results. By the eighth day the virus controls and all cultures which had received guinea pig serum developed CPE, with at least 25 per cent of the cells affected. The cultures which had received pre-inoculation serum rabbit 90 or 96 showed CPE, whereas cultures which had received post-inoculation serum did not. The 1:20 dilutions of post-inoculation sera from the rabbits had apparently protected the cultures from the cytopathogenic agents in the specimens of patients no. 64 and 69.

Comment. The sera taken from the rabbits after inoculation with NG

adenoviruses were tested at low dilution (1:20). Prevention of CPE may have been due to non-specific inhibitors in the serum. However, this is unlikely since these inhibitors would also have prevented development of CPE in the cultures which received pre-inoculation serum. A more likely explanation is that the serotype(s) of adenovirus to which the antisera were prepared were antigenically related to the cytopathogenic agent(s) in the specimens of patients no. 64 and 69.

Part II. The neutralising activity of the sera from rabbits 90 and 96 was detected only in sera taken after inoculation of the rabbits with NG adenoviruses. Since there was an increase in adenovirus group-specific serum antibodies after inoculation (measured by immunofluorescence and complement fixation), it is probable that neutralisation of CPE above was due to type-specific neutralisation of adenoviruses. To determine the extent of neutralising activity in the day 7 sera, they were titrated against the cytopathogenic agents from 3 patients.

Methods. Test 1. Serum from rabbits 90 and 96 (taken at day 7 after the last inoculation with NG adenoviruses) were prepared in doubling dilutions from 1:20 to 1:640 in PBS. These dilutions, and also 1:20 dilutions of pre-inoculation sera, were mixed in duplicate with first passage culture fluids (diluted 1:2) of specimens from patients no. 68 and 69. After 1 hour at room temperature, the mixtures (including virus controls with PBS in place of serum) were added (0.2 ml) to fresh cultures of Chang cells and incubated at 36.5°C.

Test 2. Serum from rabbit 96 was prepared in doubling dilutions from 1:20 to 1:2560 in PBS. These dilutions, and 1:20 dilutions of pre-inoculation serum, were tested in quadruplicate against undiluted fourth passage culture fluids of the specimens from patients no. 64

and 69. After 1 hour at room temperature, the mixtures (including virus controls) were added to fresh cultures of Chang cells and incubated at 33°C.

Results. Test 1. The CPE in the virus controls affected at least 50 per cent of the cells by the seventh day. Cultures which had received pre-inoculation serum were not protected. Rabbit 96 post-inoculation serum at dilutions up to and including 1:640 protected the cultures. Rabbit 90 post-inoculation serum at 1:640 protected the cultures from the cytopathogenic agent of patient no. 68, but dilutions 1:320 and 1:640 failed to protect the cultures from the cytopathogenic agent of patient no. 69.

Test 2. The CPE in the virus controls affected at least 50 per cent of the cells by the seventh day. The cultures which had received pre-inoculation serum were not protected. Rabbit 96 post-inoculation serum at 1:2560 failed to prevent breakthrough of CPE but dilutions up to and including 1:1280 were protective.

Comment. The infectivity of the cytopathogenic agents from patients no. 64, 68 and 69 was very low and could not be standardised in the tests. Therefore it would be incorrect to make quantitative comparisons of the neutralising activity shown by the sera to the cytopathogenic agents from different patients. However, it is clear that the high levels of neutralising activity shown by the post-inoculation sera (1:160 to 1:1280) cannot be explained as non-specific inhibition. From the appearance of the CPE, the virus content of the stool extracts, and the results of immunofluorescence with the third passage culture fluids, it is likely that the cytopathogenic agents were adenoviruses. Adenovirus neutralisations are generally type-specific. From the high levels of neutralising activity of the

rabbit sera, it is probable that these adenoviruses were neutralised by a type-specific antigen-antibody reaction. The neutralising activity of the serum was probably directed against the NG adenoviruses of patient no. 25. Therefore, the adenoviruses passaged from the specimens of patients no. 64, 68 and 69 were probably of the same serotype as the NG adenoviruses from patient no. 25.

B. EVALUATION OF CHANG CELLS FOR THE ISOLATION OF ADENOVIRUSES IN GENERAL.

1. Introduction.

Chang cells allowed the detection and identification of adenoviruses in 3 NG specimens (Section A.). They were also shown to be more useful than HEK cells for the isolation and identification of adenoviruses of subgroup A (Chapter 3, Section A.2.f.). Chang cells have the added advantage of being continuously available and could be maintained longer than HEK cells in culture. These cells might be useful for the isolation of adenoviruses of all serotypes.

2. Culture Attempts Using All Available Specimens from Three Patients.

To evaluate the sensitivity of the Chang cell system for the isolation of established adenovirus serotypes, 107 specimens were tested in Chang cultures. These specimens were taken from 3 children who were known to excrete growing strains of adenovirus (patients no. 6, 8 and 25; see Chapter 2). All but 16 of the specimens were negative for adenoviruses by EM, and had not been tested in culture previously.

It was hoped that by testing consecutive specimens taken over a period of months from the same child, more information might be gained about the patterns of excretion of (a) established adenovirus

serotypes, and (b) adenoviruses related to the strains of patients no. 64, 68 and 69. In particular, it was important to determine (1) whether adenoviruses of this serotype might be present in specimens negative for adenoviruses by EM, and (2) whether these strains might show increased infectivity in cell culture before or after excretion in large enough numbers to be detected by EM.

Method. 39 specimens from patient no. 6, 31 specimens from patient no. 8, and 37 specimens from patient no. 25 were tested. The specimens from each child had been taken over 10 months, 9 months and 8 months respectively. All specimens were incubated in Chang cultures at 36.5°C in parallel, over at least 2 passages, as described in Chapter 1 Section 4. The cultures were observed on alternate days for CPE. Neutralisation tests were performed as described in Chapter 1. Enterovirus isolates were identified by Dr. Eleanor J. Bell (Enterovirus Reference Laboratory (Scotland)).

Results. See Table 7.3. 4 specimens which were originally designated 'NG' were tested (one from patient no. 6 (dated 12/2/76) and 3 from patient no. 25 (dated 19/7/76, 21/7/76 and 25/7/76). 2 specimens (from 21/7/76 and 25/7/76) caused CPE at the first passage but only the former caused mild CPE at the second passage. The cytopathic effects were similar to those caused by the specimens of patients no. 64, 68 and 69 but the agent(s) could not be identified. None of the remaining 103 specimens caused this CPE. Adenovirus strains of established serotypes were isolated from 30 specimens. Type 1 alone was isolated from patients no. 6 and 25 whereas patient no. 8 shed adenovirus types 5, 9, 14-16 and 31. Type 5 isolates were obtained from specimens taken over almost 7 months.

TABLE 7.3 Results of culture with 107 stool specimens from 3 children, using Chang Conjunctiva cells. No entry in the table indicates that no virus was detected

Patient No. 6			Patient No. 8			Patient No. 25		
Date of Stool	Virus by: EM‡	Culture	Date of Stool	Virus by: EM‡	Culture	Date of Stool	Virus by: EM‡	Culture
15/12/75			18/ 1/76			25/ 6/76		
16/12/75			20/ 1/76			29/ 6/76		
18/12/75			21/ 1/76	SRV		29/ 6/76		
12/ 2/76	Ad*		22/ 1/76			30/ 6/76		
13/ 2/76	SRV**		28/ 1/76	SRV	Ad 31	1/ 7/76		
13/ 2/76	SRV		15/ 3/76		Ad 5	2/ 7/76	Rota ¶	
16/ 2/76			16/ 3/76	Ad	Ad 5	3/ 7/76	Rota	
17/ 2/76			23/ 3/76		Ad 5	5/ 7/76		
4/ 3/76			31/ 3/76		Ad 5	12/ 7/76	Ast	
16/ 3/76			6/ 4/76	Ast	Ad 5	13/ 7/76	Ast	
22/ 3/76	Ast***		12/ 4/76	SRV	Ad 5	14/ 7/76	Ast	
24/ 3/76	SRV		21/ 4/76		Ad 5	15/ 7/76		
29/ 3/76			27/ 4/76			17/ 7/76		
16/ 4/76			4/ 5/76			19/ 7/76	Ad	
16/ 4/76			11/ 5/76		Ad 5	21/ 7/76	Ad	?[1]
18/ 4/76			12/ 5/76		Ad 5	25/ 7/76	Ad	
18/ 4/76			17/ 5/76	SRV		2/ 8/76		
20/ 4/76	SRV		24/ 5/76		Ad14-16	9/ 8/76		
27/ 4/76			31/ 5/76	Ad	Ad14-16	17/ 8/76		
4/ 5/76		Ad 1	9/ 6/76	Ad	Ad14-16	2/ 9/76		
7/ 5/76		P 1 §	21/ 6/76		Ad 5	16/ 9/76		
8/ 5/76	Ad	Ad 1	7/ 7/76			29/ 9/76		
10/ 5/76		Ad 1	15/ 7/76			11/10/76	SRV	
12/ 5/76	Ad	Ad 1	22/ 7/76			18/10/76		
12/ 5/76	Ad	Ad 1	26/ 7/76	SRV	Ad 5	8/11/76		?Ent†
13/ 5/76	Ad	Ad 1	2/ 8/76		Ad 9	22/11/76		Cox B5††
24/ 6/76	Ad		9/ 8/76	Ad		6/12/76	Ad	?Ent
14/ 7/76		Ad 1	18/ 8/76			13/12/76	Ad	Ad 1
23/ 7/76	Ad	Ad 1	25/ 8/76			21/12/76		
28/ 7/76			4/10/76		Ad 5	29/12/76		Ad 1
29/ 7/76			11/10/76			5/ 1/77		
18/ 8/76						10/ 1/77		
31/ 8/76						18/ 1/77		
31/ 8/76		Ad 1				24/ 1/77		
20/10/76						17/ 2/77		
21/10/76		Ad 1				24/ 2/77		
22/10/76		Ad 1				28/ 2/77		
25/10/76								
26/10/76								

* adenovirus ** small round virus *** astrovirus
§ poliovirus ¶ rotavirus
† enterovirus †† coxsackievirus
? isolate untyped [1] CPE over 2 passages only
‡ EM results of Professor C.R. Madeley

Comment. It was concluded that Chang Conjunctiva cells are suitable for the isolation of at least some established serotypes of adenovirus from stools. The prolonged excretion of type 5 by patient no. 8 further confirms that the commoner serotypes can be shed over several months (see also Table 2.10). It is remarkable that 4 different serotypes were isolated from this child over 9 months and that 3 different serotypes were shed within a 2 month period. Type 5 was shed before and after episodes of excretion of 2 other serotypes (type 9 and type 14-16). Therefore the presence of more than one adenovirus serotype in the alimentary tract at any one time may be a common phenomenon. This might be an important means of genetic recombination between wild strains, from which new serotypes might originate.

Sixty eight stool extracts were negative for adenoviruses by EM and also by culture in Chang cells. None of the specimens which were negative by EM yielded cytopathogenic agents similar to those from patients no. 64, 68 and 69. There is no evidence from this that such strains can be excreted over long periods or that they can be detected in stools other than those known to contain adenoviruses by EM. In this study, it was noted that Chang cells were not particularly sensitive to the toxic effects of stool extracts.

C. ATTEMPTS TO PASSAGE AND CHARACTERISE FURTHER NG AGENTS IN CELL CULTURE.

1. Introduction.

The adenovirus strains detected in Chang cells from NG specimens showed little or no cytopathogenicity for HEK cells. In the first passage HEK cell cultures inoculated with the specimens

of patients no. 62, 64, 68 and 69 there was an effect on the monolayer which did not develop again on passage. This was attributed to a toxic effect of the stool. These specimens were classed as 'NG' because the cytopathic effects in HEK cells were not typical of the commoner adenovirus serotypes, nor of the 'oncogenic' serotypes 12, 18 and 31 which were also responsible for some early cytopathic effects.

The term 'non-growing' (NG) adenovirus was no longer appropriate for the agents which could be passaged in Chang cells. A term was required which would emphasise the low degree of infectivity of these strains. The word 'fastidious' was chosen, at the risk of implying an intelligence on the part of the agents themselves. However, this term emphasises the more demanding (and ill-understood) requirements of these viruses in cell culture relative to the requirements of most established serotypes. The agents from patients no. 64, 68 and 69 are referred to hereafter as fastidious (F) adenoviruses.

It was important to determine whether the one serotype of F adenoviruses so far detected using Chang cells was present in most NG specimens.

2. Studies with Sixteen Further NG Specimens.

a. Simultaneous Culture at 33°C and 36.5°C. 2 of the 4 agents studied in Section A. failed to cause CPE after 3 passages in Chang cultures. It was necessary to determine the conditions under which F adenoviruses would best retain infectivity over several passages. In Chapter 3 Section C., one NG specimen caused CPE when incubated at 33°C but not 36.5°C (although the agent could not be typed). To determine whether de novo isolations of F adenoviruses would benefit

from incubation at 33°C, 16 NG specimens (not previously tested in Chang cultures) were incubated at both temperatures in parallel.

Method. The 16 NG specimens (from 12 children) were incubated in Chang cultures over at least 3 passages and observed on alternate days for CPE. Separate tests were carried out at 33°C and 36.5°C in parallel. The time in culture for each specimen during the first 3 passages (18-28 days) was approximately the same at each temperature.

Results. 11 specimens (from 8 children) caused CPE over more than one passage at 33°C, whereas only 5 specimens (from 5 children) caused CPE over more than one passage at 36.5°C (Table 7.4). CPE from all 11 specimens was observed over at least one more passage at the lower temperature. The cytopathic effects were of the kind caused by F adenoviruses from patients no. 64, 68 and 69. 14 of the stool extracts had been tested previously for the induction of adenovirus specific antigen production in KB cells (Table 4.11). The specimens which gave positive results by immunofluorescence all caused CPE in Chang cells incubated at 33°C, and, with one exception (patient no. 89 on 9/3/79), the specimens which were negative by immunofluorescence did not cause CPE (Table 7.4).

Comment. The close correspondence between the results of immunofluorescence and observations of CPE suggests that the effects on the cells were caused by replicating (or partially replicating) adenoviruses. Development of CPE over more passages at the lower temperature could be due to temperature sensitivity of the replicating agent(s) or of some stage of the replication process.

It was necessary to obtain further evidence that these cytopathogenic agents (like those from patients no. 64, 68 and 69) were

TABLE 7.4 Results of culture attempts on 16 stool extracts known to contain adenoviruses by EM. Incubations in HEK cell cultures were performed previous to incubations in Chang cell cultures. All incubations in Chang cells at 33°C and 36.5°C were performed in parallel at each passage.

Patient No.	Date of Stool	Cytopathic Effect										Immunofluorescence in KB Cells Inoculated with Stool Extract †
		HEK 36.5°C			Chang 36.5°C			Chang 33°C				
		P1*	P2	P3	P1	P2	P3	P1	P2	P3	P4	P5	
78	14/ 5/78	-	-	-	-	-	-	-	-	-	NT	NT	-
79	1/ 8/78	-	-	-	-	-	-	-	-	-	NT	NT	-
80	8/ 8/78	-	-	-	-	-	-	-	-	-	NT	NT	-
81	21/ 8/78	±	-	-	+	±	-	+	+	+	-	-	+
82	29/ 8/78	-	-	-	±	-	-	±	+	+	±	±	+
83	30/ 8/78	±	-	-	+	-	-	+	+	±	-	-	NT
83	31/ 8/78	±	-	-	+	±	-	+	+	±	-	-	+
84	21/10/78	±	-	-	+	±	-	+	+	±	-	-	+
85	18/ 9/78	-	-	-	-	-	-	-	-	-	NT	NT	-

TABLE 7.4 continued

Patient No.	Date of Stool	Cytopathic Effect											Immunofluorescence in KB Cells Inoculated with Stool Extract†
		HEK 36.5°C			Chang 36.5°C			Chang 33°C					
		P1*	P2	P3	P1	P2	P3	P1	P2	P3	P4	P5	
86	3/10/78	-	-	-	±	-	-	+	+	±	-	-	+
87	7/ 2/79	-	-	-	±	-	-	+	+	±	-	-	+
87	12/ 2/79	-	-	-	-	-	-	-	-	-	NT	NT	-
87	12/ 2/79	+	-	-	+	+	± §	+	+	+	±	-	NT
88	5/ 3/79	-	-	-	±	-	-	±	±	-	NT	NT	+
89	6/ 3/79	+	-	-	+	±	-	+	+	+	±	-	+
89	9/ 3/79	-	-	-	±	-	-	±	+	+	±	-	-

* passage number
\+ complete cytopathic effect (monolayer destroyed)
± incomplete CPE (slow progression or late onset)
\- no CPE
NT not tested
† results from Table 4.11
§ CPE did not develop again on passage at 36.5°C

adenoviruses, and to determine whether they were antigenically related to the F adenovirus strains studied previously.

b. Attempts to Characterise the Cytopathogenic agents. The agents detected after incubation at 33°C were tested for sensitivity to 5-BUdR in culture and for possible neutralisation by antiserum from rabbits 90 and 96. Selected second passage Chang culture fluids from cultures incubated at 33°C were tested by immunofluorescence for the ability to induce adenovirus-specific antigen production.

Part I. Sensitivity to 5-BUdR.

Method. The 11 first passage fluids from the NG specimens which caused CPE at 33°C (Section a. above) were re-passaged in the presence and in the absence of 5-BUdR. The method used was as described in Section A.3.a. Adenovirus type 1 ($10^{2.5}$ TCD_{50}/ml), type 8 ($10^{1.5}$ TCD_{50}/ml) and echovirus type 11 (obtained from Dr. E.J. Bell; $10^{2.5}$ TCD_{50}/ml) were tested for sensitivity to 5-BUdR in parallel with the unknown agents.

Results. The echovirus 11 and adenovirus control cultures without 5-BUdR showed CPE in at least 50 per cent of the cells by the seventh day. The cultures with 5-BUdR which received prototype adenovirus showed no CPE, whereas the cultures containing echovirus 11 were not protected. All the cultures without 5-BUdR which received first passage fluids from the NG specimens showed CPE. This affected at least 25 per cent of the cells by the seventh day. The cultures containing 5-BUdR did not develop CPE (Table 7.5).

Part II. Immunoflucrescence.

Method. 8 second passage fluids from Chang cultures incubated at

33°C were tested by immunofluorescence using KB cells, with fixation 3 days after inoculation. The tests were performed as described in Section A.3.c.

Results. 5 of the 8 fluids tested (each derived from NG specimens of a different child) caused intracellular adenovirus antigen production in KB cells (Table 7.5). The original specimens from which the 5 positive fluids were derived had also been found positive by the same test (Table 4.11). However, the specimens from which the 3 negative culture fluids were derived had also been found positive.

Part III. Neutralisation Tests Using Sera from Rabbits Inoculated with a NG Specimen.

Method. Test 1. 10 of the 11 first passage fluids from specimens which caused CPE at 33°C (Section a. above) were tested in duplicate for neutralisation by (a) pre-inoculation serum from rabbit 90 at 1:20 dilution, and (b) day 7 (post-inoculation) serum at 1:20. The tests were performed as described in Section A.3.d. (Part I) with virus controls in quadruplicate. The Chang cells were incubated at 33°C.

Test 2. The 10 corresponding second passage fluids from Chang cultures incubated at 33°C were tested in duplicate for neutralisation by (a) pre-inoculation serum from rabbit 96 at 1:20 dilution, and (b) day 7 (post-inoculation) serum at dilutions 1:80, 1:160, 1:320 and 1:640. The tests were performed as in Test 1 above, with virus controls in duplicate. The Chang cells were incubated at 33°C.

Results. Test 1. All 10 first passage fluids caused CPE in the virus controls, which affected at least 25 per cent of the cells by the seventh day. The 1:20 dilution of pre-immune serum from rabbit

90 did not protect the cells. In all but one case, the cultures inoculated with first passage fluids and day 7 serum (1:20) were protected (Table 7.5). The exception was the first passage fluid from the specimen of patient no. 82.

Test 2. Only 6 of the 10 second passage fluids tested caused CPE in at least 25 per cent of the cells in the virus controls by the seventh day. These fluids were derived from the specimens of patients no. 81, 82, 83, 87 and 89. The agents from 5 of these specimens were neutralised by post-inoculation serum at all dilutions (including 1:640). Pre-inoculation serum at 1:20 was not protective. The CPE from the specimen of patient no. 82 was not neutralised by rabbit 96 serum at any dilution tested (1:80 or higher).

Comment. As with prototype adenovirus strains, the cytopathic effects from the 11 NG specimens in Chang cell cultures were inhibited in the presence of 5-BUdR. Therefore it is likely that the cytopathic effects were caused by replicating or partially replicating agents. These results are in agreement with the results of immunofluorescence which suggested that the agents were adenoviruses. Only a proportion of the second passage fluids tested were positive by immunofluorescence although all the original specimens caused development of intracellular adenovirus antigens. It may be that the production of infectious virus was limited in some way and that both CPE and adenovirus antigen production at later passages were mainly due to carry-over of the semi-infectious virus from the original inoculum. This would explain the 'tailing off' of cytopathic effects seen.

Since 9 of the 10 agents tested (from 6 of 7 children) were neutralised by rabbit 90 serum at 1:20 dilution, all 9 were probably antigenically related to the strain of non-growing adenovirus from

TABLE 7.5 Results of cell culture and immunofluorescence tests on 16 NG specimens from 12 children.

Patient No.	Date of Stool	CPE Over >1 Passage in Chang Cells at 33°C §	Inhibition of CPE by 5-BUdR	Inhibition of CPE by R.90 Serum at 1:20**	Immuno-Fluorescence in KB Cells Inoculated with: S.E.†	Immuno-Fluorescence in KB Cells Inoculated with: P2*
78	14/ 5/78	-	NA	NA	-	NT
79	1/ 8/78	-	NA	NA	-	NT
80	8/ 8/78	-	NA	NA	-	NT
81	21/ 8/78	+	+	+	+	-
82	29/ 8/78	+	+	-	+	+
83	30/ 8/78	+	+	+	NT	NT
83	31/ 8/78	+	+	+	+	+
84	21/10/78	+	+	+	+	-
85	18/ 9/78	-	NA	NA	-	NT
86	3/10/78	+	+	+	+	+
87	7/ 2/79	+	+	+	+	+
87	12/ 2/79	-	NA	NA	-	NT
87	12/ 2/79	+	+	+	NT	NT
88	5/ 3/79	+	+	NT	+	-
89	6/ 3/79	+	+	+	+	+
89	9/ 3/79	+	+	+	-	NT

\+ positive result
\- negative result

NA not applicable
NT not tested

† results from Table 4.11
§ results from Table 7.4
* fluid from the second passage in Chang cells at 33°C
** day 7 (post-inoculation) serum

patient no. 25. Since the fastidious adenoviruses from 4 of the children were neutralised by rabbit 96 serum at 1:640 dilution, they were probably the same serotype as the F adenoviruses from patients no. 64, 68 and 69. The F adenoviruses from the specimen of patient no. 82 were not of the same serotype.

c. Investigation of Culture Conditions Under Which F Adenoviruses Might Replicate Best. 5 of the 16 NG specimens tested above did not cause CPE in Chang cells. One specimen was from a child (patient no. 87) who shed cytopathogenic agents (F adenoviruses) in another 2 specimens. One cannot assume that all 3 specimens contained the same serotype of adenovirus, but this seemed likely. Since there was some success in detecting F adenoviruses using Chang cells, CPE from the remaining specimens might be observed if slight variations in the culture conditions already used were tried. NG specimens which showed different degrees of cytopathogenicity were re-tested in rolling and stationary cultures of Chang cells at 33°C and 36.5°C. Tests at 33°C using maintenance medium without serum, or with adsorption periods, were also performed.

Methods. 6 NG specimens (from patients no. 80, 83, 85, 86 and 87) were used to inoculate cultures of Chang cells. Six different sets of culture conditions were used: (1) stationary incubation (33°C), (2) stationary incubation using Leibovitz' L15 medium with antibiotics, but with no serum (33°C), (3) stationary incubation (33°C) after adsorption at 33°C for 1 hour (carried out as described in Chapter 2), (4) incubation with rolling of the culture tubes (33°C), (5) incubation with rolling (36.5°C), and (6) stationary incubation (36.5°C). The test conditions were performed in duplicate in most

cases and cultures were inoculated with 0.1 ml amounts of extract. At degeneration, the cultures were frozen and thawed 4 times and the contents of each tube were added to each of 2 fresh cultures (0.1 ml per tube). Adsorption at 33°C, use of Chang cell maintenance medium without serum and rolling at 2 temperatures were done as in the first passage. The cultures were examined on alternate days for CPE.

Results. As before, the specimens tested of patients no. 80, 85 and 87 caused little or no CPE under any set of conditions (Table 7.6). The specimens from patients no. 83 and 86 caused CPE to some extent under all conditions at the first passage. At the second passage there was little evidence of CPE from these specimens, except in those cultures incubated stationary at 33°C with or without an adsorption period and with fetal calf serum in the maintenance medium. From the time taken for CPE to develop, there was little advantage in adsorption over straight inoculation.

Conclusion. One set of conditions under which the 16 NG specimens were originally tested in Chang cells (stationary incubation at 33°C) was best for the passage of CPE. Use of adsorption periods in subsequent isolation attempts was considered unnecessary and was avoided because of the increased risk of cross-contamination of cultures with different specimens.

3. Tests for the Presence of F Adenoviruses in Other Stool Extracts Known to Contain Adenoviruses by EM.

Since 9 of 16 NG specimens tested (from 6 of 12 children) in Section C.2.b. appeared to contain the same or a related serotype of F adenovirus, it was important to determine the frequency of

TABLE 7.6 Results of culture attempts with 6 NG specimens using Chang Conjunctiva cells, and with 6 different variations in method.

Patient No.	80		83		83		85		86		87	
Date of Stool	8/8/78		30/8/78		31/8/78		18/9/78		3/10/78		12/2/79	
Passage No.	1	2	1	2	1	2	1	2	1	2	1	2
Stat 33°C	-	-	$+_4$	$\pm$	$+_2$	$\pm$	-	-	$\pm$	$\pm$	-	-
Stat 33°C Adsorption	-	-	$+_4$	$\pm$	$+_4$	$+_6$	-	-	$\pm$	$\pm$	-*	-
Stat 33°C Serumless	-	-	$+_4$	-	$+_6$	-	-	-	$\pm$	-	-	-
Rolling 33°C	-	-	$+_4$	-	$+_4$	-	-	-	$\pm$	-	-	-
Rolling 37°C	-	-	$+_4$	-	$+_4$	-	-	-	-	-	-	-
Stat 37°C	-	-	$+_4$	-	$+_4$	-	-	-	$\pm$	-	-	-

- \- no cytopathic effect
- $\pm$ incomplete cytopathic effect (slow progression/late onset)
- + complete cytopathic effect (monolayer destroyed). Subscript refers to the number of days to reach completion
- stat stationary incubation
- * one plaque seen in one culture, which did not progress

occurrence of these adenoviruses in as many specimens as possible. Specimens in 2 categories were chosen for examination: (a) those known to contain adenoviruses by EM but negative in cultures of HEK cells (NG specimens), and (b) specimens which contained adenoviruses by EM but which were not previously tested in culture.

a. Culture at 33°C with Further NG specimens. 47 NG specimens not previously incubated at 33°C in Chang cells were tested. All cytopathic effects caused by the F adenovirus strains detected thus far had arisen during the first passage. Therefore it was considered sufficient to incubate the NG specimens over one passage for the detection of F adenoviruses. Any NG specimen which did not cause CPE after this treatment was considered culture negative, although in practice all but 6 specimens were re-passaged into fresh Chang cell cultures.

Method. 47 specimens (from 36 children) were incubated at 33°C in Chang cultures. Included in this group of specimens were 2 stool extracts from children in Tanzania, and one from a child in Malaysia. In most cases where CPE developed over 2 passages, the first passage fluids were tested for neutralisation by the day 7 (post-inoculation) serum (diluted 1:20) from rabbit 90 and/or rabbit 96.

Results. 13 specimens (from 13 children) caused CPE over more than one passage (Table 7.7). This was similar to the CPE caused by the specimens from patients no. 64, 68 and 69 tested previously. 12 agents from 12 first passage fluids tested were neutralised by a 1:20 dilution of serum from rabbit 90 and/or 96. Included in the 12 was an agent detected from the Malaysian child. The stools from Tanzania did not cause CPE.

TABLE 7.7 Further NG specimens which caused cytopathic effect over more than one passage in Chang Conjunctiva cells incubated at 33°C.

Patient	Date of Stool	Number of Passages		CPE Inhibited by Rabbit 90 and/or Rabbit 96 Serum (Post-inoculation; 1:20)
		Tried	In Which CPE Developed	
L	4/ 9/75	4	3	+
T	15/12/75	4	3	+
3	6/12/75	2	2	+
20	1/ 7/76	6	6	+
21	5/ 7/76	4	2	+
29	26/ 7/76	5	5	+
38	18/ 1/77	2	2	+
41	5/ 2/77	2	2	+
66	7/11/77	2	2	NT
68	21/11/77	5	4	+
71	19/ 1/78	2	2	+
76	23/ 3/78	5	4	+
Malaysian	received 4/ 4/78	2	2	+

- negative result
NT not tested
+ positive result

Culture fluids derived from the specimens of patients no. 21, 29 and 68 (from the first, second and third passage respectively) caused sufficient CPE to be tested further. The agents were neutralised by rabbit 96 serum at dilutions up to and including 1:1280. Of the 12 children who shed fastidious adenovirus strains neutralised by antiserum at 1:20, 4 children shed NG adenoviruses in other stools and these could not be passaged in Chang cell cultures.(Table 7.8). However, 2 stools from patient no. 68 (one tested here and one tested previously (Section A.)) both appeared to contain F adenoviruses.

Comment. Approximately 25 per cent of the NG specimens examined contained the same or a related serotype of fastidious adenovirus to the strain which had infected patient no. 25. The finding of a related agent in one stool from Malaysia probably indicates that these agents are widespread geographically.

Several of the specimens tested were from 1975 (21) and 1976 (8) and had been frozen more than once (for previous tests) and stored in small quantity without added protein. Therefore some specimens may have lost infectivity, and the true proportion of extracts containing related strains of F adenovirus may have been much higher than one quarter. It is notable that in 4 cases when more than one NG stool extract from the same child was tested, only one caused CPE which developed again on passage. This may have resulted from some difference in the history of storage of the specimens before or after preparation of the extracts, but this seems unlikely. Alternatively, the fastidious adenoviruses might have varying infectivity in culture depending on the stage of infection in the individual.

TABLE 7.8 Results of culture attempts with different NG specimens from the same child, using Chang Conjunctiva cells incubated at 33°C.

Patient	Date of Stool	Number of Passages: Tried	In Which CPE Developed
T	9/12/75	2	0
	15/12/75	4	3*
	17/12/75	2	0
3	2/12/75	2	0
	5/12/75	2	0
	6/12/75	2	2*
	8/12/75	2	0
20	26/ 6/76	1	0
	28/ 6/76	1	0
	1/ 7/76	6	6*
38	17/ 1/77	2	1
	18/ 1/77	2	2*
68	21/11/77	5	4*
	21/11/77	5 §	4

* CPE inhibited by post-inoculation serum from rabbit 90 and/or 96 diluted 1:20

§ results of Section A.2.b.

b. Neutralisation Tests to Screen Stool Specimens for the Presence of F Adenoviruses. All related strains of F adenovirus so far detected caused CPE at the first passage. Therefore it should be possible to screen stool extracts for the presence of strains related to that of patient no. 25 by mixing the original specimen with specific antiserum from rabbits 90 or 96. This might allow quick selection of specimens containing F adenoviruses without the need for further culture attempts (other than to confirm their presence). 29 stool extracts from 1979, known to contain adenoviruses by EM but not previously tested in culture, were examined for the presence of F adenoviruses. 6 specimens were from Malaysian children.

Method. Each of 29 specimens (from 25 children) were mixed (0.1 ml) in one well of a Linbro disposo tray with (a) 0.1 ml day 7 serum from rabbit 96 at 1:20 dilution, and (b) PBS (0.1 ml). The mixtures were left at room temperature for 1 hour, then added (0.2 ml) to fresh cultures of Chang cells and incubated at 33°C. The cultures were examined daily for CPE. To confirm the cytopathic effects separately from the neutralisation tests, 26 specimens were incubated over 2 passages in Chang cultures at 33°C as described in Chapter 1.

Results. 2 of the 29 tests were unreadable because of bacterial contamination of the original specimens. 6 specimens did not cause CPE. 4 specimens caused CPE in both the treatment and the control cultures, which affected at least 50 per cent of the cells by the eighth day. The appearance of the cytopathic effects suggested infection by either enteroviruses or adenoviruses of the commoner established serotypes. The other 17 specimens (from 15 children) caused CPE in the virus control cultures which affected at least

50 per cent of the cells by the seventh day. The corresponding test cultures (which received rabbit 96 serum) were completely protected. In the 2 cases where 2 extracts from the same child were tested, only one of the extracts caused CPE which was neutralised by rabbit 96 serum. 3 specimens (taken over 4 days) were tested from one child, and in each case the specimens caused CPE which was neutralised by rabbit 96 serum.

Separate tests for CPE revealed that 15 of the 17 specimens caused an effect on the monolayers over 2 passages similar to the effects caused by the F adenoviruses from patients no. 64, 68 and 69. All 17 specimens caused CPE by the fourth day after inoculation. The second passage fluids derived from 5 specimens (each from a different child) were examined by EM. All 5 fluids were found to contain adenovirus particles (greater than 5 particles per grid square). 3 of the 6 Malaysian stools (from different children) caused cytopathic effects which were neutralised by the rabbit antiserum.

Comment. More than 50 per cent of the total number of specimens examined (from 15 of 25 children) caused cytopathic effect characteristic of F adenoviruses. In each case the agent responsible was related to the strains of F adenovirus from patients no. 64, 68 and 69. It is not known why the number of adenoviruses of the same or a related serotype was high in this collection of specimens. Detection of these agents may have been improved over previous trials because the specimens were frozen and thawed once only.

Therefore adenoviruses related to the strain from patient no. 25 are probably common in stools containing virions in sufficient quantity to be detected by EM. Such strains were again detected in

stools from Malaysian children, indicating widespread occurrence. The detection of cytopathogenic strains in 3 stools of one patient reinforces the results from patients no. 87 and 89 (Table 7.4) that F adenoviruses can be shed over a number of days (at least 3-5 days). The 17 culture positive specimens were not previously tested in HEK cells and prolonged culture attempts were not done using Chang cells. Since the culture attempts were incomplete, it is perhaps not appropriate to describe these particular agents as fastidious. However, all 17 specimens appeared to contain adenoviruses related antigenically to the fastidious strains of one serotype from patients no. 21, 29, 64, 68, 69, 81, 83, 87 and 89.

D. ATTEMPTS TO EXPLAIN THE VARIABILITY OF DIFFERENT NG SPECIMENS IN CAUSING CYTOPATHIC EFFECTS IN CHANG CELLS.

1. Examination of Virus-Free Supernates and NG Specimens for the Presence of an Inhibitory Substance Specific for Fastidious Adenovirus.

There was no evidence from the work described in Chapter 5 for an inhibitor of adenovirus replication in NG specimens. However, there was still a possibility that NG specimens might contain an inhibitor to an uncommon serotype. Since adenoviruses of one previously unrecognised serotype could be detected in some NG specimens but not in others using Chang cells, the apparent lack of infectivity in the latter case might be due to the presence of an inhibitor specific for F adenoviruses. Therefore several NG specimens were tested for ability to inhibit the replication of one strain of F adenoviruses.

Method. 6 NG specimens which caused no CPE in Chang cells were selected (from patients M, 3, 20, 70, 78 and 85). Supernatant fluids taken after the ultracentrifugation of NG specimens (for EM examination) were also tested (from patients no. 38, 41, 43, 45 and 52). (For further details on the collection and storage of supernatant fluids, see Chapter 5 Sections 2.a. and 2.c.). Specimens corresponding to 4 of the 9 supernates were previously tested in Chang cultures and only one (from patient no. 41) caused CPE over more than one passage.

Extracts or supernatant fluids were mixed (0.1 ml + 0.1 ml) in duplicate wells of a Linbro disposo tray with fourth passage culture fluid derived from the specimen of patient no. 69 (see Section A.2.b). After 1 hour at room temperature, the mixtures and virus controls (with PBS in place of test extract or supernatant fluid) were added (0.2 ml) to fresh cultures of Chang cells. These were incubated at 33°C and observed on alternate days for CPE.

Results. The cultures which received virus from patient no. 69 showed CPE in at least 25 per cent of the cultures by the sixth day. None of the cultures which received both virus and stool extract, or virus and supernatant fluid, were protected.

Comment. There was no evidence for the presence of a substance in NG specimens which would inhibit the development of CPE caused by the F adenovirus strain from patient no. 69. None of the 6 stool extracts tested caused CPE when added previously to Chang cultures. Therefore it is unlikely that the apparent lack of infectivity of these viruses was due to the action of a free (unbound) inhibitor specific for the common serotype of F adenoviruses. However, a

type-specific inhibitor (possibly antibody) may have been present in these specimens, but firmly bound to the virus particles.

2. Treatment of Culture Fluids by Methods Which Might Improve Infectivity.

a. Introduction. The loss of CPE from some NG specimens with repeated passage in Chang cells and the inability of other NG specimens to induce CPE might be due to the failure of the viruses once produced to infect adjacent cells. Release of infectious virus from the cell, or entry to neighbouring cells after virus production, might require some condition or factor only present *in vivo*. For example, the F adenoviruses might require the presence of certain enteric enzymes for effective cell to cell spread.

b. Treatment of Culture Fluids with Chymotrypsin and Trypsin. To determine if chymotrypsin or trypsin might be required to maintain the infectivity of F adenoviruses, culture fluids which showed reduced infectivity on passage were treated separately with these enzymes.

Method. First passage cell fluids derived from single specimens from patients no. 12 and 25, and third passage fluids derived from single specimens from patients no. 87 and 89 (taken from cultures which showed CPE) were shown to cause little or no CPE on passage. They were treated with chymotrypsin and trypsin as follows:

Each fluid (0.1 ml) was mixed in duplicate wells of a Linbro disposo tray with (a) 0.1 ml α-chymotrypsin (Calbiochem; 200 µg./ml in PBS), (b) 0.1 ml trypsin (BDH Chemicals; 200 µg./ml in PBS), and (c) 0.1 ml PBS. Control mixtures containing chymotrypsin and PBS

or trypsin and PBS were also prepared in duplicate. The mixtures were incubated at 36°C for 1 hour and the contents of each well (0.2 ml) were added each to a fresh culture of Chang cells. The cultures were incubated at 33°C and observed on alternate days for CPE.

Results. The cultures which received chymotrypsin alone or trypsin alone were unaffected. The cultures which received culture fluids alone developed little or no CPE. The culture fluids treated with chymotrypsin or trypsin did not cause CPE.

Comment. The concentration of chymotrypsin and trypsin was chosen from a preliminary test to determine the highest concentrations of the enzymes that could be used without disrupting the Chang cell monolayers. Treatment of NG specimens has been described previously (Chapter 5 Section 3.c.) but in that case the enzyme was inactivated before addition to the cell cultures. In this case I hoped to establish whether treatment of culture fluids (failing in their ability to cause CPE) with chymotrypsin or trypsin, and incubation in culture in the presence of these enzymes, would improve the infectivity of F adenoviruses. However, there was no indication that these treatments were of benefit, although they might improve the infectivity of F adenovirus strains if carried out at each passage.

c. Sonication of Culture Fluids. Sonication of culture fluids to improve the release of virus was tried previously using HEK cells (Chapter 3 Section C.2.) but it did not allow development of CPE. However, it might improve the infectivity of fastidious strains from NG specimens (known to be cytopathogenic in Chang cell cultures), possibly by substituting for some other necessary condition required in vivo to facilitate virus release.

Method. The culture fluids used in Section b. above were tested, and also a first passage Chang cell culture fluid derived from the specimen from patient no. 71. For each specimen 0.1 ml of cell culture fluid was added to each of 2 cultures of Chang cells. A sample of the same culture fluid was sonicated for 60 sec in a Brensonic bath sonicator and 0.1 ml was added to each of 2 Chang cell cultures. All cultures were incubated at 33°C and observed on alternate days for CPE.

Results. All 5 culture fluids caused little or no CPE, whether or not they were sonicated.

Comment. Sonication failed to prevent the loss of infectivity shown by the agents in the 5 culture fluids tested. As stated in the previous section regarding enzyme treatment, sonication at each passage might have benefited the development of CPE with some fastidious strains, but this was not tested further.

E. INVESTIGATION OF ADENOVIRUSES IN INTESTINAL SEGMENT CULTURE FLUIDS.

1. Introduction.

In Chapter 3 Section B., some first and second passage fluids from intestinal segment cultures inoculated with 3 NG specimens were found to contain adenoviruses by EM (Table 3.6). Although adenovirus particles were detected in some day 14 fluids from the first passage, the fluids from days 12 and 16 did not cause CPE when added to cultures of HEK cells. The adenovirus particles were seen by EM after 6 complete changes of medium. This was unlikely to be just observation of input virus, and replication probably occurred. If the original specimens contained F adenoviruses, the culture

fluids would not be expected to cause CPE in HEK cultures. However, they might cause CPE in Chang cells incubated at 33°C.

2. Inoculation of Chang Cells with Intestinal Segment Culture Fluids.

a. First Passage Fluids.

Part I. The first passage fluids from intestinal segment cultures inoculated with the 3 NG specimens (from patients no. 5, 25 and 30) were used to inoculate Chang cell cultures.

Method. Each day 4, day 6, day 8, day 10, day 12 and day 16 culture fluid of the first passage (stored at -20°C) was added (0.1 ml) to a fresh culture of Chang cells and incubated at 33°C. Day 6, day 8 and day 10 fluids from control dishes without intestinal segments were tested in parallel with fluids from dishes with cultures. The Chang cultures were observed on alternate days for CPE.

Results. Cytopathogenic agents were detected in all fluids taken at day 16 from dishes inoculated with specimens from patients no. 5 and 25 (Table 7.9). One of the 6 day 16 fluids tested from cultures inoculated with the specimen of patient no. 30 caused CPE and this was the only positive fluid derived from the extract of this child. For patients no. 5 and 25, cytopathogenic agents were detected in the intestinal segment culture fluids taken at day 12 and day 8 respectively, and in fluids taken at later times. In all cases where CPE developed, it was similar to that caused by the specimens of patients no. 64, 68 and 69 (Plates 12, 13 and 14). The fluids from dishes without cultures taken at day 6, day 8 and day 10 did not cause CPE.

TABLE 7.9 Results of attempts to detect cytopathogenic agents in intestinal segment culture fluids using Chang Conjunctiva cells.
I. Tests using first passage organ culture fluids.

Patient No.	No. of Cultures† from Which Cytopathogenic Agents Were Detected:					
	Day 4	Day 6	Day 8	Day 10	Day 12	Day 16
5	0/6	0/6*	0/6*	0/6*	2/6	6/6**
25	0/6*	0/6*	4/6*	6/6*	6/6	6/6 §
30	0/6	0/6*	0/6*	0/6*	0/6	1/6 §
U.I.	0/2	0/2	0/2	0/2	0/2	0/2

† actual/possible

* fluids from 6 control dishes (no cultures) inoculated with stool extract were also tested in Chang cells and found to be culture negative

** 2 Chang cell culture fluids caused CPE again on passage. The CPE was inhibited by rabbit 90 serum (post-inoculation) diluted 1:20

§ all agents from Chang cultures showing CPE caused CPE again on passage, and were neutralised by rabbit 90 serum (post-inoculation) diluted 1:20

U.I. uninoculated control cultures

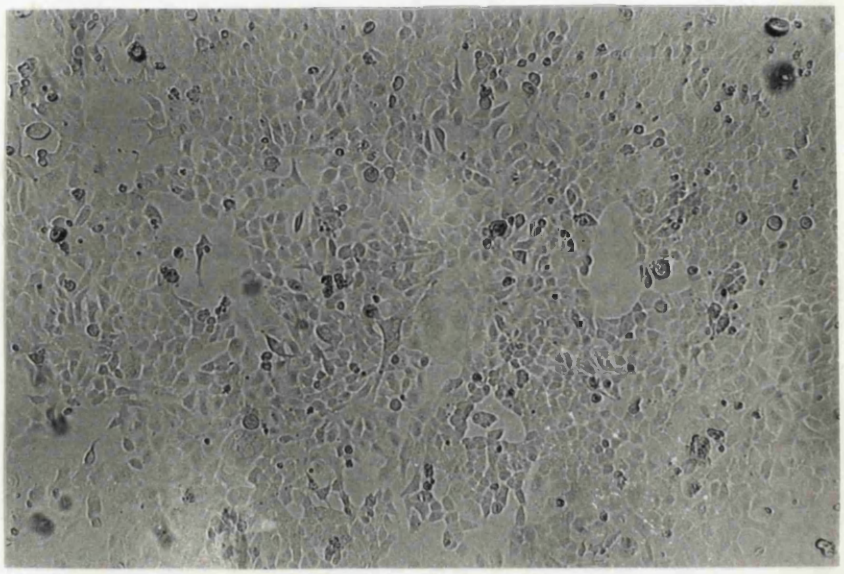

PLATE 12 Uninfected monolayer of Chang Conjunctiva cells.

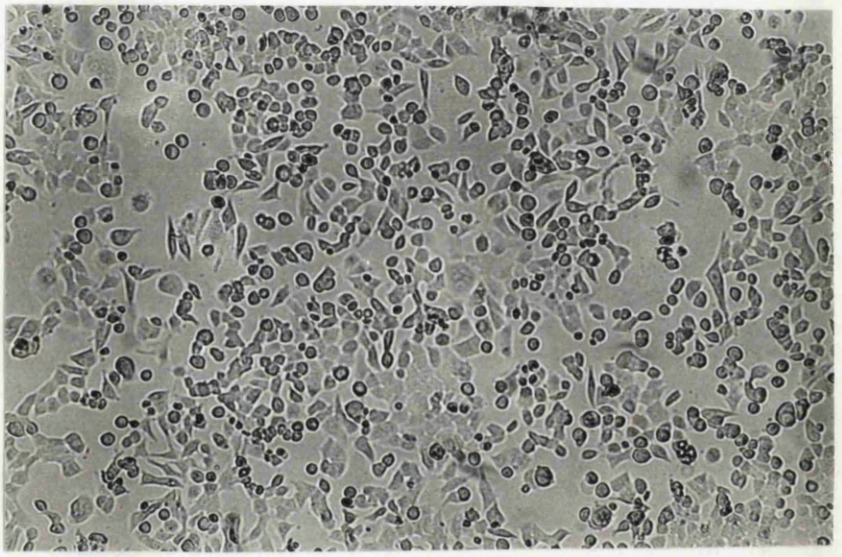

PLATE 13 Chang Conjunctiva cell monolayer inoculated with a day 16 intestinal segment culture fluid derived from a NG specimen from patient no. 25. The cytopathic effect is typical of those caused by all fastidious adenoviruses detected.

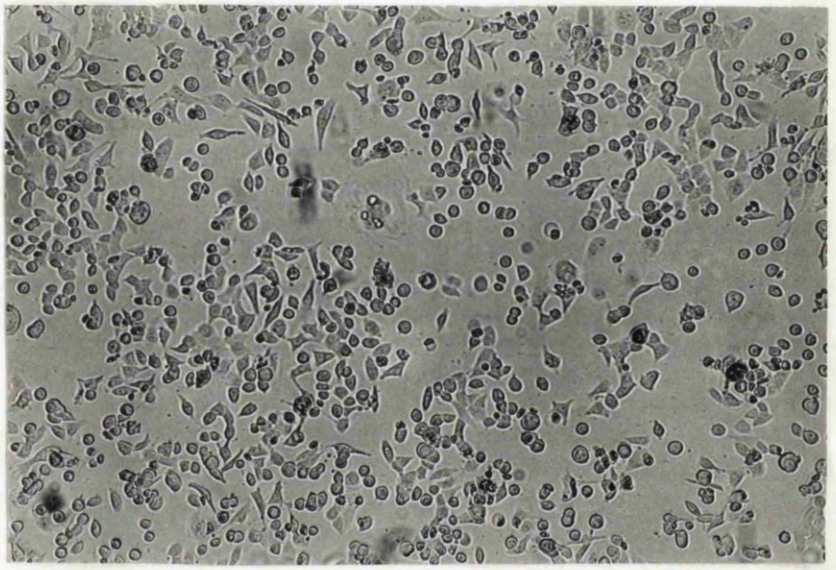

PLATE 14 Complete destruction of a Chang Conjunctiva cell monolayer inoculated as described in Plate 13.

Comment. The fluids from control dishes (uninoculated cultures or inoculated dishes with no cultures) did not cause CPE. However, CPE developed in Chang cultures inoculated with day 16 fluids taken from intestine cultures after 7 complete changes of medium. Therefore it was assumed that the cytopathic effects were caused by replicating agents. The results of EM using day 14 fluids derived from the specimens of patients no. 5 and 25 (Table 3.6) suggested that adenovirus replication occurred. The day 12 fluids which caused CPE in Chang cells (Table 7.9) were from the same cultures whose day 14 fluids contained adenoviruses. Therefore the agents which caused the cytopathic effects were probably adenoviruses.

Part II. Since the cytopathic effects seen were characteristic of the F adenoviruses previously studied, the cytopathogenic agents from the intestinal segment cultures were tested against antiserum which had neutralised most strains of F adenovirus.

Method. Chang culture fluids derived from the day 16 fluids which caused CPE were tested in duplicate for neutralisation by (a) pre-inoculation serum from rabbit 90 (diluted 1:20), and (b) day 7 (post-inoculation) serum from rabbit 90 (diluted 1:20). The tests were performed as in Section A.3.d. Part I., with virus controls in duplicate. The Chang cultures were incubated at 33°C.

Results. Cytopathic effects from 4 of 6 Chang cell fluids derived from the specimen of patient no. 5 did not develop again when the fluids were re-passaged. Cytopathic effects developed in the virus controls with all other fluids tested and affected at least 25 per cent of the cells by the seventh day. The cytopathogenic agents from patient no. 5 (in 2 day 16 culture fluids), patient no. 25

(in 6 day 16 culture fluids) and patient no. 30 (in one day 16 culture fluid) were neutralised by post-inoculation serum from rabbit 96 but not by the pre-inoculation serum (Table 7.9).

Comment. The agents present in the intestinal segment culture fluids were adenoviruses of the same or a related serotype as the fastidious strains from patients no. 64, 68 and 69.

Stool specimens from patient no. 25 (taken 2 days apart) had been used both for inoculation of the intestinal segment cultures (stool date 25/7/76) and for inoculation of rabbits 90 and 96 (stool date 27/7/76). Therefore it was expected that the cytopathogenic agent(s) from this patient would be the same serotype as the F strains from patients no. 64, 68 and 69 which were neutralised by the rabbit antisera at high dilution (1:160 or greater).

It is remarkable that the agent shed by patient no. 25 on 25/7/76 was more infectious in Chang cultures (incubated at 33°C) after one passage at 36.5°C in intestinal segment cultures than when the specimen was added direct to Chang cells and incubated at 36.5°C. (see Section B.2.). Incubation at 36.5°C in intestinal segment cultures may not be so detrimental to infectivity as incubation at 36.5°C in cell cultures. Moreover, one passage in intestinal segment cultures might possibly improve the infectivity of all F adenoviruses in cell culture. It is possible that the F strains would have multiplied more readily if the culture dishes had been incubated at 33°C.

b. Second Passage Fluids. The successful propagation of F adenoviruses would be further confirmed if the second passage intestinal segment culture fluids were also shown to cause CPE in Chang cell cultures.

Method. The second passage intestinal segment culture fluids from day 8, day 10 and day 14 were added (0.1 ml) to a fresh culture of Chang cells and incubated at 33°C. (The second passage intestinal segment cultures had been inoculated with (a) day 12 first passage culture fluids derived from the specimen from patient no. 25, and (b) day 16 first passage fluids derived from the specimens from patients no. 5, 25 and 30). The Chang cells were observed on alternate days for CPE.

Results. Only the day 14 second passage fluids derived from the specimens from patients no. 5 and 25 caused CPE (in 2 of 5 or 6 cases; see Table 7.10). The cytopathic effects were typical of those caused by F adenoviruses, but the agents could not be passaged further and were not identified by neutralisation.

Comment. The agents were detected in fluids taken from day 14 but not in fluids taken earlier than this. Since adenovirus particles were detected by EM in some day 12 fluids of the second passage (Table 3.6), it is probable that the cytopathic effects seen in Chang cultures inoculated with day 14 fluids were caused by F adenoviruses derived from the original specimens, and that these had multiplied in intestinal segments.

F. DISCUSSION.

The work described in this chapter indicates that although some stool specimens contain adenoviruses (detected by EM) which cannot be cultivated using HEK cells, a number of adenovirus strains are infectious or semi-infectious when incubated in Chang cell cultures at 33°C. Cytopathogenic strains from 9 children were neutralised by one rabbit antiserum (96) at a dilution of 1:640

TABLE 7.10 Results of attempts to detect cytopathogenic agents in intestinal segment culture fluids using Chang Conjunctiva cells.
II. Tests using second passage organ culture fluids.

Patient No.	Second Passage Cultures Inoculated with First Passage Fluids of Day:	No. of Cultures† from Which Cytopathogenic Agents Were Detected: Day 8	Day 10	Day 14
5	16	0/5	0/5	2/6*
25	12	0/6	0/6	2/6*
	16	0/5	0/5	2/5*
30	16	0/6	0/6	0/6
U.I.	16	0/2	0/2	0/2
	P 2**	0/4	0/4	0/4

† actual/possible

* the Chang cell culture fluids from cultures showing CPE did not cause CPE again on passage

** uninfected second passage (control) culture fluids

U.I. uninoculated cultures

or greater. This suggests that the 11 strains tested (from 9 children) and the strain used as immunogen (from patient no. 25) were the same serotype. 13 further strains (each from a different child) were neutralised by rabbit antiserum to the agent from patient no. 25 at low dilution (1:20) and were probably the same or a related serotype. Since 3 strains were not neutralised by standard antisera to adenovirus types 1 to 33, it is probable that the agents were either types 34 to 36, or new (as yet undescribed) serotypes. (The figures discussed here do not include the results of intestinal segment culture).

Only one agent from 25 agents tested was not neutralised by post-inoculation serum from rabbit 90 or 96, and was therefore a different (undetermined) serotype. Like other fastidious strains tested in parallel (Table 7.4) successful passage of this strain was achieved best on incubation of the Chang cell cultures at 33°C. In so far as a 33°C dependency has not been described previously for adenoviruses of established serotypes, this strain may also have been a new serotype.

Fastidious adenoviruses appear capable of infecting not only Chang cells but also cultures of fetal human intestinal segments. Growth in cultures of intestinal segments may have been a closer parallel to growth *in vivo* than cell culture, possibly because the intestinal segments possessed a (modified) intestinal epithelium.

Assuming that the large numbers of adenoviruses seen by EM in NG specimens are in some cases F adenoviruses, the partial success in growing these agents in culture does not appear to reflect their ability to multiply *in vivo*. Even if most viruses are non-viable or antibody-coated, replication must occur somewhere in the body

for such large numbers of particles to be produced. The frequency of detection of F adenoviruses in NG specimens, the large numbers of particles in these specimens and the finding of these agents in stools from Malaysia all suggest that F adenoviruses are successful and well adapted for growth in vivo.

One strain of F adenovirus (from patient no. 25) brought about the production of high levels of both group- and type-specific antibodies when injected into rabbits. This suggests that at least some antigenic determinants on these F adenovirus particles were free to act as immunogens and were not covered by human (gut-derived) antibody. Therefore, it is unlikely that association with antibody was the reason for the poor infectivity of this strain in cell culture.

A paradox exists: the viruses appear to survive or multiply best in culture at 33°C but are shed in large quantity from the alimentary tract, which suggests multiplication in vivo at around 37°C. The reason for improved passage at 33°C over 36.5°C in Chang cultures is not known. Antigenically related strains from different children showed the same temperature requirement. It is unlikely that all strains became temperature-sensitive mutants independently of each other and, in view of their antigenic relationship, they were probably all derived from a common ancestral strain. This again suggests successful spread and replication.

The adenoviruses are regarded primarily as etiologic agents which infect the respiratory tract or eyes (see Chanock, 1974), although some serotypes are isolated almost exclusively from stools (Rosen et al., 1962b). Like the rhinoviruses and coronaviruses (see Lennette and Schmidt 1979) adenoviruses may originally have been adapted to growth at cool respiratory surfaces at around 33°C. Those genetic variants which could survive and multiply successfully at

37°C would have had a selective advantage, since with opportunist invasion of the gut cells progeny particles could be shed in quantity in stools as well as in respiratory secretions. Strains of established serotypes also appear well adapted to other conditions which would be required for survival within the intestine, in that they are resistant to digestive enzymes and low pH.

Unlike strains of established serotypes, the adenoviruses which grow poorly or not at all *in vitro* (fastidious strains) may only be adapted to growth at cool respiratory surfaces (at around 33°C). If so, the large numbers of adenoviruses detected in stools by EM might be the result of downflow from a respiratory infection. The low infectivity of these viruses in stools might result from inactivation by extreme conditions within the intestine (for example, a higher temperature than 33°C, low pH, or the presence of enzymes to which only F strains were sensitive). Alternatively, the F adenoviruses detected in stools might result from defective replication of respiratory-adapted strains in the intestine. However, if F adenoviruses are respiratory agents adapted to growth at 33°C, they would be expected to be shed in respiratory secretions also and to have been detected in cell culture many years ago.

Rather than being respiratory-adapted strains, the F adenoviruses may be capable of infecting cells of the human intestine at around 37°C. If so, the progeny particles produced in large quantity *in vivo* might fail to replicate *in vitro* due to the absence of some condition or factor present within the intestine. The requirement for a lower temperature in cell culture in order to initiate CPE over more than one passage might result from instability of the virions at around 37°C. It is possible that any production of new virus at

each passage (even at 33°C) was insufficient to replace the loss of infectious (or semi-infectious) particles from the original inoculum by (a) dilution, and (b) temperature inactivation of virions with prolonged incubation, thus resulting in loss of CPE and fluorescence with passage (see Tables 7.4 and 7.5). However, if temperature inactivation of adenovirus particles occurred, this must have been severe, since 2 strains (from patients no. 82 and 89) caused CPE over at least 4 passages when incubated at 33°C and were almost undetected at 36.5°C.

If fastidious strains of adenoviruses multiply mainly in the intestine, they may have evolved a unique niche among the adenoviruses. Strains of common established serotypes appear adapted to growth within the intestine at 37°C and can be shed over several months. The period over which non-growing or F adenoviruses can be excreted remains to be determined. It is possible that these strains are shed only in large numbers over a relatively short period (days) and that their success as infectious agents depends on an ability to cause extensive infection of the intestine in a discrete episode. This being so, the virions might not normally be subject to temperatures of around 37°C for longer than 2 or 3 days, and in this case any temperature sensitivity of the virions might not be important. A short, severe episode of virus excretion would be compatible with suggestions that non-growing adenoviruses may be associated with diarrhoea (Flewett et al., 1975; Whitelaw et al., 1977; Richmond et al., 1979; Brandt et al., 1979). However, the association of these viruses with diarrhoea requires much further study, and at present their role in causing disease is still uncertain.

CHAPTER 8

CHAPTER 8 FINAL DISCUSSION.

1. The Work In Retrospect.

The work described was carried out to investigate the nature of adenoviruses seen in stools by EM which could not be isolated in cell cultures used routinely for detecting stool viruses (adenoviruses, enteroviruses and reoviruses). I hoped to find a reason for the 'non-growth' and, assuming that the particles were not completely non-viable, to find conditions under which these viruses might be grown in the laboratory.

In addition to conclusions about 'non-growing' adenoviruses (discussed below), I established the following points concerning the shedding of adenovirus strains of recognised serotypes in specimens positive for adenoviruses by EM taken from children in Glasgow:

1. Adenoviruses of established serotypes may be isolated from stools which are positive for adenoviruses by EM. 2 serotypes may be isolated from one stool.

2. Adenovirus types 1 and 2 are the most common established serotypes isolated from stools known to contain adenoviruses by EM.

3. Adenoviruses of one serotype may be shed by one child for several months. The same child may shed adenoviruses of more than one type - simultaneously or in succession.

4. Adenoviruses of subgroup A (types 12, 18, 31 and 12-31) may often be present in stools but may be undetected or unidentified in cultures used routinely. Chang Conjunctiva cells are particularly useful for the passage and identification of these serotypes.

In addition, it was confirmed that some stool extracts EM positive for adenoviruses do not cause CPE in cultures of HEK cells and human amnion cells. The non-growing adenoviruses can be shed in successive stools from one child. The maximum period detected over which these particles were shed by one child was 8 days. Children who excrete adenoviruses which cannot be cultured in HEK cells can shed common serotypes of adenovirus at other times. Moreover, one child in this study was found to excrete adenoviruses which could and could not be cultured from stools passed on the same day.

Much of the information gained about the non-growing adenoviruses in this study was the result of testing the relative likelihood of the 5 possibilities to explain the phenomenon of non-growth set out in Chapter 2 Section C.2.h. In brief, the possibilities were that (1) the non-growing adenoviruses are new (that is, previously unrecognised) serotypes requiring special culture conditions, (2) they are established human serotypes requiring special culture conditions, (3) they are defective, (4) they are viable but non-infectious due to the presence of an external inhibitor or agent, and (5) they are not of human origin.

These possibilities are not mutually exclusive and were not tested as distinct hypotheses. The viruses might have failed to grow for more than one of the reasons above. For example, they may have been of animal origin and also covered with antibody, or they may have been new serotypes but also defective.

Culture attempts with canine and bovine cells suggested that the non-growing adenoviruses were not from dogs or cattle. Bovine adenoviruses of subgroup 2 are best isolated from calf testis cells (Bürki et al., 1978). Had the non-growing adenoviruses belonged to bovine

subgroup 2, they might not have been detected in bovine embryo kidney cells. However, adenoviruses of bovine subgroup 2 do not share the same complement fixing antigen with the other adenoviruses of mammalian origin, whereas tests using antiserum against non-growing adenoviruses suggested that the latter did possess the common mammalian complement fixing antigen. The non-growing adenoviruses could have been of simian origin but this was unlikely since secondary rhesus monkey kidney cells inoculated with these viruses showed little or no CPE (Madeley et al., 1977). The possibility that they originated from mammals other than dogs, monkeys and cattle still exists.

There was no evidence for a free (unbound) inhibitor in the stools containing non-growing adenoviruses. Treatments to destroy an inhibitor or remove it from the virus particle did not lead to cytopathic effects. Preliminary attempts to use immune electron microscopy (IEM) for the detection of an IgA or an IgG coating on the particles failed. This would have involved mixing adenoviruses in NG specimens with anti-human IgA or anti-human IgG sera and observing for clumping of the particles. Apart from difficulties in interpreting the results obtained, the quantities of adenoviruses in G and NG stool extracts stored at -20^{o}C proved insufficient for this test. This was possibly a result of some instability of the particles on storage, but was not investigated further.

Adenoviruses which could not be grown from the stool of one child (patient no. 25) caused the production of both group-specific antibodies and type-specific (neutralising) antibodies when injected into rabbits. This suggests that (in one case, at least) some antigenic determinants were not covered with antibody, and were free to elicit an immune response.

Immunofluorescence using KB cells suggested that the adenoviruses in some NG specimens were at least partially infectious. The detection of cytopathogenic agents using Chang Conjunctiva cells alone, or intestinal segment cultures and Chang cells in series, also indicated that adenoviruses in some NG specimens are viable or partially viable. Three strains were not neutralised by standard antisera to serotypes 1 to 33 and this suggested that these were a previously unrecognised serotype. Agents from 20 children were detected and found to be related by neutralisation test (this excludes the results of organ culture in which strains from 3 other children were found to be related, and the results of screening stools in which strains from 15 other children were found to be related). Therefore it is probable that the adenoviruses in these specimens were of human origin and of a previously unrecognised serotype requiring special conditions for growth.

Different NG specimens from the same child showed different degrees of infectivity in some cases. It is unlikely that the quantity of viruses in itself limited the initiation of CPE, since large numbers of particles were present to be detected by EM. However, the relative proportion of infectious or semi-infectious particles may have varied between specimens.

2. The Work in Relation to Recent Results of Others.

There have been few reports of attempts to characterise adenoviruses in NG specimens. Richmond et al. (1979) briefly reported the detection of adenovirus-specific antigen production in HEK cells by indirect immunofluorescence, using stool specimens from an outbreak of gastroenteritis as inocula.

Gary et al. (1979) tested 13 stools containing adenoviruses

which could not be cultured, by a direct immunofluorescence test. Most first and second passage fluids from HEK cultures inoculated with these specimens induced adenovirus-specific antigen production in HEp 2 cells but the third passage fluids did not. This suggested that virus replication did not occur. The same fluids from HEK cultures were tested against antiserum to hexon antigen by counter-immunoelectrophoresis. Only the fluids from the first 2 passages contained detectable hexon antigen, which suggested that this was being diluted out and not produced in the serial passages. The results of counterimmunoelectrophoresis and immunofluorescence using anti-hexon serum suggested that these viruses shared group antigens with established human adenovirus serotypes, and the average buoyant density in sucrose of 5 strains (1.335 g./cm^3) was found to coincide with that of growing strains.

These workers found no evidence for the presence of an antibody coating on non-growing adenoviruses by IEM using partially purified virus and antiserum against human IgA, IgG and IgM. Rabbit antisera to 3 non-growing strains were prepared and tested for neutralising activity against the 35 established serotypes. No serum had a neutralising titre to any serotype greater than 1:8. By haemagglutination inhibition test against the 35 established serotypes, no serum had a titre beyond 1:16. Gary et al. concluded that adenoviruses which could not be cultured from stools were of human origin and were infectious, but incapable of effective replication in conventional cell cultures. They suggested that these adenoviruses should be considered a new distinct subgroup. Therefore, although Gary et al. did not succeed in passaging these agents, their conclusions exactly match my own.

Retter et al. (1979) tested 42 stool specimens (known to contain adenoviruses by EM) for the ability to induce intracellular adenovirus antigen formation in HAE-70 cells by indirect immunofluorescence test. (HAE-70 is a continuous cell line derived from human amnion). 32 of the specimens did not cause CPE in these cells, whereas the other 10 contained growing strains. All but one of the 42 specimens were positive by immunofluorescence. These workers recommended the use of this test for the detection of adenoviruses in general. It was noted that fluorescence in cultures inoculated with non-growing strains of adenovirus was confined to single cells. This is in agreement with the observations made using Glasgow stools (Chapter 4).

Acute and convalescent serum was obtained from one child with gastroenteritis at the time of shedding non-growing adenoviruses. Retter et al. found that the convalescent serum to a dilution of 1:512 suppressed the appearance of fluorescing cells in cultures inoculated with a stool specimen from this child. The acute-phase serum at dilutions higher than 1:16 did not have a neutralising effect. This is the first evidence to suggest that the presence of these non-growing strains in the alimentary tract may lead to a serum (neutralising) antibody response.

The almost complete correspondence between detection of adenoviruses by EM and by immunofluorescence does not agree with the results of the immunofluorescence tests reported in this thesis (Chapter 4). In this case, only 14 out of 20 G specimens and 15 out of 28 NG specimens were positive by immunofluorescence. The indirect immunofluorescence tests in both cases were similar, and both used antiserum to an established adenovirus serotype, prepared in guinea pigs. The HAE-70 cells used by Retter et al. may have been especially sensitive to non-growing adenoviruses, since superior results were obtained in

tests performed with fixation of the cells only 2 days after infection (compared with 3 days after infection for those tests described in Chapter 4). Retter et al. did not report the number of children from whom the specimens were taken. Specimens taken from a small number of patients may (by chance) have contained especially infectious or virulent strains of adenovirus (although still not capable of causing CPE). This would have enhanced the apparent ability of the immunofluorescence test to detect adenoviruses in stools.

DNA restriction site analysis (see Pettersson et al., 1973) has been performed by Dr. G. Wadell on serotypes of adenovirus belonging to all established subgroups (Dr. G. Wadell, personal communication). Dr. Wadell found that non-growing adenoviruses from 3 Scandinavian children had DNA restriction patterns which did not fit the patterns of the genomes from established serotypes. He agreed to examine specimens from Glasgow children. 7 specimens contained sufficient amounts of DNA for analysis using the restriction enzymes Bam I, Sma I and Eco RI. These specimens were from patients no. 12, 25, 29, 81, 83, 84 and 89 (see Table 8.1). All recognizates had the same DNA restriction site pattern to each other and to the Scandinavian recognizates but this pattern was different from those of established serotypes.

It is remarkable that the organisation of the genome of all 7 strains was apparently the same, when 3 different strains of one serotype (type 7) were found to have different DNA restriction maps (Wadell and Varsanyi, 1978). This is a strong indication that adenoviruses in at least some NG specimens have the same or a similar genome and are probably the same serotype. F adenoviruses were detected (using Chang cells) in specimens from 6 of the 7 patients listed above, and the strains were antigenically related (see Chapter 7;

also Table 8.1). Since more than one serotype may be isolated from one stool, it is not correct to conclude that the agents passaged in Chang cells (F adenoviruses) were the same agents as the recognizates analysed by Wadell. However, in view of the antigenic similarity of the 'isolates' and the genetic similarity of the recognizates, it is probable that both tests detected the same adenovirus.

Johansson et al. (1980) describe the development of an enzyme linked immunosorbent assay (ELISA) technique for the detection in stool extracts of (a) adenovirus group antigens, and (b) antigens peculiar to non-growing adenoviruses. The detection of non-growing adenoviruses was evaluated using 6 NG specimens from Glasgow (from patients T, 3, 12, 20, 25 and 29). The test for these viruses depended on the group-specific capture of adenovirus components in stools, followed by the reaction of previously unknown subgroup- or type-specific antigenic determinants on these components with indicator antibody. The antibodies against non-growing adenoviruses were prepared by inoculating rabbits with virus components derived from a Helsinki child with severe diarrhoea.

All 6 NG specimens gave a positive reaction in the test (Table 8.1) whereas 3 wild strains of established serotypes (types 14-16, 18 and 31) and prototype strains of all established serotypes were negative. Therefore, there was evidence that non-growing adenoviruses share previously unknown subgroup- or type-specific antigenic determinants. Since the specimens tested from 3 patients (numbers 3, 12 and 20) did not cause CPE over more than one passage in Chang cell cultures, it is probable that in many cases non-cytopathogenic and cytopathogenic (fastidious) strains are antigenically related.

TABLE 8.1 List of NG specimens tested by DNA restriction site mapping and/or ELISA* (G. Wadell, personal communication), and by cell culture.

Patient	Date of Stool	Tested by DNA Restriction Site Mapping	Tested by ELISA*	Tested by Culture Using Chang Cells (33°C)
T	15/12/75	No	Yes	Yes †
3	8/12/75	No	Yes	Yes §
12	26/ 4/76	Yes	Yes	Yes §
20	26/ 6/76	No	Yes	Yes §
25	25/ 7/76	Yes	Yes	Yes ¶
29	26/ 7/76	Yes	Yes	No **
81	21/ 8/78	Yes	No	Yes †
83	31/ 8/78	Yes	No	Yes †
84	21/10/78	Yes	No	Yes †
89	6/ 3/79	Yes	No	Yes †

* enzyme linked immunosorbent assay using antiserum from a rabbit inoculated with non-growing adenoviruses from a Helsinki child

** another NG specimen from the same day was tested (see Table 7.7) and found to cause CPE over 5 passages

§ no development of CPE over more than one passage

† CPE developed over more than one passage

¶ in this case, Chang cell cultures were incubated at 36.5°C. CPE developed at the first passage only. F adenovirus detected from this specimen after incubation in intestinal segment cultures (Table 7.9)

At the time of writing, Dr. R. Wigand is investigating the serotype(s) of some of the fastidious strains of adenovirus described in Chapter 7. He passaged serially agents from patients no. 29 and 68, and has confirmed the superiority of passage at 33°C over 37°C using Chang cells. These agents produced complement fixing antigen in Chang cells, reacting with a positive human serum pool. The F adenovirus strain from patient no. 29 was not neutralised by standard antisera to the 35 established serotypes and to candidate adenovirus type 36 (of subgroup D). Post-inoculation sera from rabbits 90 and 96 (at a dilution of 1:5) did not neutralise human adenovirus types 1 to 35 and candidate adenovirus type 36 (R. Wigand, personal communication).

In conclusion, there are many indications using different techniques that adenoviruses which do not grow in HEK cells are closely related antigenically, and constitute at least one new serotype.

3. Suggestions for Further Work.

Suggestions for further investigation of fastidious adenoviruses are discussed below under the headings, a. Identity, b. Replication Requirements, c. Etiologic Role, d. Prevalence and e. Mode of Transmission.

a. Identity. The degree of relatedness between strains of F adenovirus requires further examination. The relationship between 2 adenovirus strains of established serotypes is usually determined by reciprocal cross-neutralisation tests using various dilutions of antiserum to one strain against the isolate of the other strain at a standard infectivity (for example, 10^3 TCD_{50}/ ml). This procedure was not possible with F adenoviruses because the infectivity was so low that initiation of

CPE could not always be predicted. Therefore an alternative method to quantitate virus infectivity would have to be used in order to determine the relatedness of strains by neutralisation. Some F adenovirus strains caused adenovirus group antigen production in KB cells. With these strains, a fluorescent focus count might be used to quantify the degree of infectivity of different strains in different culture fluids. On adjustment of the virus concentration to achieve a standard infectivity in fluorescent focus units (FFU) per ml, standardised neutralisation tests might be performed using immunofluorescence. The possible superiority of Chang cells over KB cells for detecting adenoviruses by immunofluorescence was not tested in this study. A test for the most sensitive culture by immunofluorescence would be of advantage since this might allow a saving on the quantity of virus used.

The relationship between the adenoviruses in NG specimens which are detected in Chang cultures and those which do not cause CPE requires further investigation. This has been investigated to some extent using DNA restriction site mapping and ELISA (see Section 2.) but the number of specimens examined was small. The available evidence indicates that irrespective of cytopathogenicity in Chang cells, most adenoviruses which do not infect HEK cells productively are closely related strains of a previously undescribed serotype. F adenoviruses in different specimens from the same child can show differences in cytopathogenicity in Chang cells. This further suggests that the reason(s) for differences in infectivity may be other than differences in identity of the agents. However, one cannot be sure that F adenoviruses shed by one child in successive stools are always the same strain, although this seems likely. The relationship between strains will best be determined when the requirements for optimum replication are more fully understood (see Section b. below).

IEM might allow the identification of F strains without the need for cell culture. However, there are difficulties in interpretation with this technique (regarding what constitutes clumping of particles or antibody cover). When testing one recognizate against several different antisera, it would probably be difficult to achieve optimum proportions of virus and antiserum in every case. Moreover, antisera produced for use in neutralisation tests would be expected to have high levels of antibodies to group- and subgroup-specific antigenic determinants in addition to type-specific antibodies. Although the main group (hexon) antigen alpha does not appear to be exposed on the intact virion (Norrby et al., 1969), cross reactions between serotypes might be found unless group- and subgroup-specific antibodies were removed from the test antisera. Cross-reactions between serotypes by IEM have already been reported (Vassal and Ray, 1974).

Other methods of identifying F adenoviruses might include haemagglutination, analysis of structural polypeptides, or DNA sequence homology. Hierholzer and Gary (1979) reported that stool extracts containing non-growing adenoviruses agglutinate rat erythrocytes to moderately high titres. If confirmed, this might lead to the development of a haemagglutination-inhibition test specific for F adenoviruses. Adenoviruses of different subgroups have been shown to differ in the apparent molecular weight patterns of their internal structural polypeptides, by SDS polyacrylamide gel electrophoresis (Wadell, 1979). The members of 4 subgroups (A to D) could be distinguished, but type 4 did not fit any of the other 4 patterns. Adenoviruses which could not be cultured fell into a sixth, distinct group. This result requires to be confirmed by others.

Members of the different subgroups of human adenoviruses have also been distinguished on the basis of DNA sequence homology by liquid-phase molecular hybridisation (Green et al., 1979). This was claimed to be the most meaningful and definitive way to group adenoviruses.

Type 4 was considered to be a distinct subgroup (E) by this method also. It is possible that the adenoviruses which do not grow in HEK cells would fall into a sixth subgroup. However, in common with SDS-polyacrylamide gel electrophoresis of structural polypeptides and (to a lesser extent) haemagglutination, this technique requires the use of large quantities of purified adenovirus components. The study of adenoviruses from NG specimens by these techniques will be accomplished once more is known about the culture requirements and once strains can be found which multiply to high titre when passaged in cell cultures.

b. Requirements for Optimum Replication. As stated in Chapter 7, the decrease in apparent infectivity on passage (or absence of infectivity) may be due to temperature sensitivity of the virion rather than of some stage in the replicative cycle. Incubation of specimens (which cause CPE) at 37°C with inoculation of Chang cultures at intervals, and incubation of these cultures at 33°C, would indicate whether the infectivity of F adenoviruses decreases at 37°C more readily than that of established serotypes.

Success in detecting F adenoviruses with Chang cells (and Intestine 407 cells) may have been due to the use of Leibovitz' L15 medium rather than Eagle's medium for maintenance. There was some success in propagating F adenoviruses from 2 children in intestinal segment cultures using Leibovitz' medium. It is possible that the high concentration of one or more amino acids in the medium (L-arginine, L-histidine or L-threonine; see Leibovitz, 1963) promoted some replication of these agents. Arginine is an essential medium constituent for the multiplication of adenoviruses in cell cultures (Rouse, 1963), but the reason for inhibition of replication on arginine deprivation is unclear. Arginine may be required for the

formation of an essential protein which is synthesised late in the infectious cycle (Wigand and Kümel, 1978).

Fastidious adenoviruses may have a requirement for very high concentrations of arginine or other amino acids within the cell. If such a requirement exists, it might represent an adaptation to conditions at the usual site of replication of these viruses in the body. It is probable that F adenoviruses multiply within the intestinal epithelium and these cells may have higher concentrations of amino acids as a result of oligopeptide uptake and hydrolysis (see Mathews, 1974; Silk, 1974). A possible requirement for high concentrations of amino acids could be investigated by comparing the apparent infectivity over several passages of cytopathogenic strains in Leibovitz' L15 medium lacking one amino acid at a time. This might also be done using immunofluorescence to quantitate infectivity. Such a study might show that the use of L15 medium is more important for the detection of F adenoviruses than the use of Chang cells. However, Chang cells will continue to be useful because they last longer than HeLa, KB and secondary HEK cells in culture.

c. Etiologic Role. No attempt was made to relate excretion of F adenoviruses to signs of diarrhoea and/or vomiting, for 2 reasons: (1) clear clinical details for some patients were not available and in other cases the severity of 'loose stools' was unclear, and (2) the study was biased towards children with diarrhoea and/or vomiting (on admission or after admission to hospital). There were relatively few children described as 'well' who could be regarded as controls.

The study was based on stool specimens which contained sufficient quantities of adenoviruses to be detected by EM. Although strains of established adenovirus serotypes were isolated from some specimens, it is possible that all adenovirus particles seen were fastidious strains. (In addition, there may have been fastidious

strains in some specimens in quantities too low to be detected by EM). At present, neither cell culture using Chang cells nor EM is a reliable method of detecting all fastidious strains. Therefore a (potentially) sensitive immunological method such as ELISA (see Section 2.) or radioimmunoassay might be best for detecting these agents in stools and trying to relate their presence to signs of disease. For example, an ELISA might be set up to screen stools for the presence of antigenic determinants peculiar to the (apparently common) serotype of F adenoviruses. The test might be performed using specimens from children with and without diarrhoea when the stool was passed. This test would confine the investigation to one agent without considerations of cultivability and (to some extent) quantity of virus particles shed.

It is important to remember that the shedding of large quantities of adenovirus particles with diarrhoea may be the result of flushing of the intestine and does not necessarily imply a causative role for the virus. With a test such as ELISA or radioimmunoassay (type-specific) which lends itself to the study of large numbers of specimens, the finding that a significantly higher proportion of diarrhoea specimens contain F adenoviruses than normal stools would indicate an association between the viruses and illness. These sensitive techniques might also allow the demonstration of a temporal relationship between onset of illness and shedding of F adenoviruses in outbreaks of diarrhoea.

In theory, investigation of a causative role for F adenoviruses would best be done by experimental infection of humans. Oral administration of viruses to babies is unethical and such studies would have to rely on adult volunteers. However, it may be that most children from a certain age and most adults are immune to reinfection

by F adenoviruses after infection in infancy, in which case an etiological role for the viruses would not be found by this method.

d. Prevalence. The prevalence of F adenoviruses (of the common serotype) would best be determined by a method such as ELISA which detected subgroup- or type-specific antigenic determinants on adenovirus components in stools. (This test might indicate that F adenoviruses are also common in stools negative for adenoviruses by EM.) The prevalence of cytopathogenic strains could be determined using Chang cells in a screening-neutralisation test (see Chapter 7 Section C.3.b.).

The presence of neutralising antibody to the common serotype of F adenovirus in the sera of children could be tested using (a) a conventional neutralisation test with a cytopathogenic strain of a standard (constant) infectivity (in TCD_{50}/ml), or (b) a neutralisation test using immunofluorescence with a strain of standard infectivity (in FFU/ml). By testing sera from children of different ages, the proportion of individuals with previous experience of the agent and the common age of infection could be determined. (This assumes that children develop type-specific humoral immunity after infection by F adenoviruses, which may not be the case.)

e. Mode of Transmission. Transmission of F adenoviruses is likely to be from faecal material, in view of the large numbers of particles shed in stools. This might be by direct contact or droplet spread. However, transmission of these viruses might also occur from the respiratory tract or eyes.

The presence of F adenoviruses of the common serotype in respiratory or eye secretions might be tested by ELISA or radioimmunoassay. It is unlikely that CPE would be detected in Chang cells

inoculated with these secretions since the quantity of virus particles would be lower than in stools. However, if shedding from the respiratory tract or eyes is an important means of transmission, there might be a higher proportion of viable particles than in stools.

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