

ADENOVIRUS-INDUCED CHROMOSOME DAMAGE  
IN MAMMALIAN CELLS

THE EFFECTS OF INFECTION WITH ADENOVIRUSES  
ON THE CHROMOSOMES OF HUMAN CELLS AND SYRIAN  
HAMSTER CELLS

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SCOPE AND CONTENTS:

Seven adenoviruses, including oncogenic and non-oncogenic serotypes from human and simian hosts, were utilized to investigate their effects upon the chromosomes of human and Syrian hamster cells. Human cells support adenovirus multiplication while hamster cells do not support replication of infectious adenovirus.

The chromosome damage induced by adenoviruses in abortive infection of hamster cells was compared with respect to the effect of virus dose upon the incidence and the types of chromosome aberrations. The effect of different adenoviruses upon the amount and types of chromosome damage was also examined.

The effect of adenovirus infection upon DNA synthesis of human and hamster cells was examined, and the relevance of adenovirus-induced chromosome aberrations to the etiology of human cancers is discussed.

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## TABLE OF CONTENTS

	<u>Page</u>
SCOPE AND CONTENTS.....	ii
ACKNOWLEDGEMENTS.....	iii
LIST OF FIGURES.....	v
LIST OF TABLES.....	ix
INTRODUCTION.....	1
MATERIALS AND METHODS	
I    Tissue Culture Techniques.....	9
II   Virological Techniques.....	19
III  Radioautographic Techniques.....	24
IV   Cytologic Preparations.....	26
RESULTS	
I    Replication of Adenoviruses in Human and Hamster Cells.....	32
II   Effect of Adenoviruses on Human and Hamster Chromosomes.....	39
III  Effect of Inactivated Adenoviruses on Chromosomes of Hamster Cells.....	78
IV   Radioautography of Adenovirus 12 Effect of DNA Synthesis.....	82
DISCUSSION.....	100
SUMMARY.....	137
REFERENCES.....	139

LIST OF FIGURES

<u>Number</u>		<u>Page</u>
1	The categories of chromosome damage induced in Syrian hamster cells by adenoviruses.....	42
2	Adenovirus-infected BHK21 cells showing chromatid breaks, chromosome bridges and acentric fragments, and proximity of cells with normal metaphase morphology to those with virus-induced chromosome damage.	44
3-15	Chromosome aberrations induced in cultured BHK21 cells 27 hours after infection with Ad.12 or Ad.18.....	46
16-27	Chromosome aberrations induced in cultured BHK21 cells 27 hours after infection with Ad.7.....	48
28-41	Chromosome <u>aberrations</u> induced in cultured BHK21 cells 27 hours after infection with SV15.....	49
42-57	Chromosome aberrations induced in cultured BHK21 cells 27 hours after infection with Ad.2.....	51,52

LIST OF FIGURES (Continued.....)

<u>Number</u>		<u>Page</u>
58-61	Inclusion body formation in BHK21 cells 72 hours after infection with Ad.2.....	52
62-74	Chromosome aberrations induced in cultured BHK21 cells 27 hours after infection with Ad.4.....	53
75-86	Chromosome aberrations induced in cultured BHK21 cells 27 hours after infection with SA7.....	55
87	Incidence of chromosome aberrations with time in hamster cultures infected with Ad.12.....	62
88	Incidence of chromosome aberrations with time in hamster cultures infected with Ad.18.....	63
89	Incidence of chromosome aberrations with time in hamster cultures infected with Ad.7.....	65
90	Incidence of chromosome aberrations with time in hamster cultures infected with SV15.....	66
91	Incidence of chromosome aberrations with time in hamster cultures infected with Ad.2.....	67

LIST OF FIGURES (Continued.....)

<u>Number</u>	<u>Page</u>
92	68
Incidence of chromosome aberrations with time in hamster cultures infected with Ad.4.....	
93	69
Incidence of chromosome aberrations with time in hamster cultures infected with SA7.....	
94	71
Effect of time of sampling on the incidence of metaphase plates showing chromatid breaks, fragmentation, overcontraction or erosion of the chromosomes after infection of BHK21 cultures with adenoviruses.....	
95-106	74
Cultured human amnion cells 48 hours after infection with adenoviruses, showing inclusion body formation, chromatin condensation and micronuclei.....	
106a	88
Relative $G_2$ periods of cultured Syrian hamster cells, uninfected and infected with Ad.12.....	
107-110	92
Examples of metaphases from Syrian hamster cultures showing various amounts of radioautographic labelling (silver grains) localized over the chromosomes, indicating replication of DNA.....	

LIST OF FIGURES (Continued.....)

<u>Number</u>		<u>Page</u>
111-114	Examples of labelled metaphases from Syrian hamster cultures before and after removal of autoradiographic silver grains.....	93
115	Karyotype of a female cell from an uninfected Syrian hamster culture, before and after removal of silver grains.....	95
116	Karyotype of a male cell from an uninfected Syrian hamster culture, before and after removal of silver grains.....	96
117	Karyotype of a female cell from a Syrian hamster culture infected with Ad.12, before and after removal of silver grains.....	98
118	Karyotype of a male cell from an Ad.12-infected Syrian hamster culture, before and after removal of silver grains.....	99

LIST OF TABLES

<u>Number</u>		<u>Page</u>
I	Monolayer culture media.....	15
II	Source, passages, and titers of adeno- viruses.....	21
III	Replication of adenoviruses in human and hamster cells.....	37
IV	Inclusion body formation in human and hamster cultures infected with Ad.2.....	38
V	Effect of virus dose on the incidence of chromosome aberrations induced in hamster cells.....	56
VI	Effect of virus dose on degree of chromo- some damage per hamster cell.....	58
VII	Effect of virus dose on the type of chromosome damage induced in hamster cells.	60
VIII	Mitotic index in adenovirus-infected human cultures.....	75
IX	Incidence of chromosome aberrations in adenovirus-infected human cultures.....	76
X	Inclusion body formation in adenovirus- infected human cultures.....	77

LIST OF TABLES (Continued.....)

<u>Number</u>		<u>Page</u>
XI	Virus neutralization experiments.....	81
XII	Effect of Ad.12 infection on DNA synthesis of human and hamster cultures...	86
XIII	Effect of Ad.12 infection on chromosome replication of hamster cells.....	91
XIV	Ionizing radiation-produced chromosome breakage and exchange.....	113
XV	Adenovirus-induced chromosome breakage and exchange.....	115
XVI	Virus-induced DNA synthesis and enzymes of DNA synthesis.....	125
XVII	Chromosome aberrations and transformation by viruses.....	130

## INTRODUCTION

The important discovery, in 1911 (181), that a cancer in chickens was produced by a virus, gave rise to the viral theory of oncogenesis in addition to, and most often in opposition to, the somatic mutation theory attributed to Boveri (23). Since that time, the role of viruses in the etiology of cancers has received considerable attention, and many recent investigations increasingly have examined in great detail the interactions between the host cell genome and the infecting viral genome. At present, even though the precise role of viruses in oncogenesis is still unknown, an important fact has been established beyond all doubt within the last decade: viruses can induce chromosome aberrations.

The relevance of chromosome aberrations to the initiation and development of tumors is not all agreed upon at present (79,80,88,196), yet it may well be appreciated that, even while some tumors display apparently normal diploid karyotypes (81,134,170,214, 218), the majority of neoplasms show abnormal chromosome

complements (80,101,124) and, in fact, certain tumors continuously display a wide range of chromosome aberrations, illustrating a persistent karyotypic instability (200,203). Although many neoplasms may show a large proportion of tumor cells to have a similar or identical chromosome constitution, or at least common chromosome abnormalities (79,88,104,196), a chromosome aberration common to any one type of cancer has not convincingly been demonstrated, with the exception of the Philadelphia chromosome which is common to almost all cases of chronic myeloid leukemia (158).

The role of viruses or of chromosome aberrations as the primary cause of cancers is still controversial. The important fact remains, however, that viruses can cause chromosome aberrations, and in the study of such viral-induced aberrations may reside the means for better understanding of the mechanisms of oncogenesis.

The capacity to induce chromosome aberrations has been demonstrated for a wide variety of viruses in many *in vivo* systems, including polyoma virus (81), adenovirus type 12 (51,200,203), chickenpox virus (herpes zoster: varicella) (6), yellow fever virus (77), measles virus (rubeola) (6,155), mumps virus (6) and the leukemia viruses (2,105,107,214). Aberrations have also been induced in *in vitro* systems by each of

the above viruses (16,22,28,50,91,128,153,154) and in addition by simian vacuolating virus SV40 (213, 221), adenovirus type 18 (197), herpes simplex virus (21,91,176,198) and by several myxoviruses (7,22,28).

The types of chromosome aberrations induced by viruses range through a wide spectrum, from single chromatid breaks (6,37,75,128,156,197,203,216,221) to fragmentation (16,91,198,200,203) complete pulverization (7,21,22,28,77,153,203,198), to coiling anomalies (7,16,200,203) and even include anomalies of the mitotic apparatus (16,37). Rapp and Hsu observed, however, that widely different viruses induced dissimilar types of chromosome aberrations (176). Further comparisons reveal that different host cell systems infected by the same strain of virus can display strikingly different aberrations. Measles virus, for example, induces chromosome breakage and rearrangements in rabbit kidney cultures (135) and in a human cell line established from carcinoma of the larynx (50), yet induces pulverization of the chromosomes in human leukocytes (22,153). Herpes simplex virus induces pulverization in human lung cultures (198) but induces achromatic gaps and breaks in Chinese hamster cells (75,198), and breaks and fragmentation in *Mastomys* cells (91).

Most of the viruses with the potential to induce chromosome aberrations *in vitro* are oncogenic in suitable hosts, although there are several examples of apparently non-oncogenic viruses which also induce chromosome aberrations *in vitro* (16,21,75,198). It is very interesting that, to take the example of the Rous sarcoma viruses, the usually non-oncogenic Bryan strain does not induce chromosome aberrations (154), in contrast to the highly-oncogenic Schmidt-Ruppin strain which produces aberrations both *in vivo* (170) and *in vitro* (154). These two strains are very closely-related, as judged by their common complement-fixing antigen revealed by mammalian antibodies (93,217).

Comparative information on the chromosome aberrations induced by a group of genetically closely-related viruses might therefore yield valuable information that would contribute to an understanding of the mechanism(s) of viral oncogenesis. The adenoviruses comprise such a group of closely-related viruses, and include both oncogenic and apparently non-oncogenic strains from both human and non-human natural hosts. In addition, the chromosome-damaging capacity of one of the adenoviruses, the highly-oncogenic human adenovirus type 12 (67,97,222) has been investigated extensively during the past five years.

Although a single virus particle may be sufficient to obtain infection, many cellular responses to infection depend upon the dose of virus. For example, the frequency of transformation of cells *in vitro* depends upon the dose of SV40 virus (212), polyoma virus (130), adenoviruses types 12 or SA7 (30), and the supplemental dose of X-irradiation (201). A further influence of virus dose is on the incidence of tumours induced by adenovirus type 12 (223) and on the incidence and time of appearance of tumours induced by the "mammary tumour agent" (42). Viral dose also affects the yield of vaccinia virus (191), the rate of DNA synthesis (1), the rate of RNA synthesis (109) and the rate of protein synthesis (9). In addition, the fact that chromosome aberrations increase with increased dose of X-rays (13), ultraviolet light (32) or thymidine (225) has been well documented.

In view of the known dosage effects of physical and chemical agents and of viruses, it is surprising that the influence of virus dose on the induction of chromosome aberrations has been so little explored.

Infection of mammalian cells by adenoviruses *in vitro* may elicit two responses, a lytic response, or a non-lytic response. In a lytic response, such as

obtained with human adenovirus type 12 infection of human cells (17,84,128,171) or human adenovirus type 5 infection of human cells (145) or hamster cells (207), the results expressed include early cessation of mitosis (69,128), suppression of host cell DNA synthesis (66, 70,71), suppression of host cell RNA synthesis (71), clumping of chromatin and formation of micronuclei (128), alterations in the cellular fine structure (71), formation of mature infectious virus (17,84,128,171) and, eventually, cell death and disintegration (146).

A non-lytic response to infection with adenoviruses is illustrated by Syrian hamster cells infected with human adenovirus type 12. Morphologic and fine-structural alterations like those described above for Ad.12-infected human cells were not observed in hamster cells (71,128). No infectious virus was produced, as measured by immunologic techniques (84) or by virus assay (136), nor was virus structural antigen detected (128, 171). Hamster cells nevertheless were infected by adenovirus type 12, as demonstrated by the immunofluorescent-staining detection of virus-coded neoantigens, "T" antigens (17,84), in a high proportion of nuclei in infected cultures (128).

The capacity of adenovirus type 12 to induce chromosome aberrations during abortive infection of

Syrian hamster cells, the high oncogenic potential of adenovirus type 12 for a variety of mammalian species, the known influence of virus dose on several aspects of host cell: virus relationships, and the effects of adenovirus type 12 infection on host cell DNA synthesis and mitosis during a productive infection suggest the following questions:

Is the capacity to damage chromosomes a common property of adenoviruses, or is the chromosome-damaging potential restricted to the oncogenic adenoviruses?

Are chromosome aberrations virus-specific, i.e., do different types of adenoviruses induce different types and different amounts of chromosome damage?

Is the induction of chromosome aberrations a function of virus dose?

Is adenovirus infection capable of inducing chromosome aberrations during a lytic response (e.g., in human cells), prior to the virus-induced cessation of mitosis?

Does Ad.12 infection during a non-lytic response (e.g., in Syrian hamster cells) have effects on DNA synthesis and mitosis similar to those in a lytic response (e.g., in human cells)?

It was therefore decided to examine the chromosome aberrations, if any, induced in human and Syrian hamster

cells by several strains of adenoviruses, human and simian, including highly-oncogenic human adenoviruses types 12 (67,97) and 18 (67,96) and simian adenovirus SA7 (99), weakly-oncogenic human adenovirus type 7 (67), and the apparently non-oncogenic human adenoviruses types 2 and 4 (57) and simian adenovirus SV15. In addition, it was deemed of great interest to examine the effects of the known oncogen, human adenovirus type 12, on DNA synthesis and mitosis in a non-lytic system (Syrian hamster cells) as well as in a lytic system (a human cell line, AV3).

## MATERIALS AND METHODS

### I Tissue Culture Techniques

#### 1. Media

(a) CMRL 1066: This medium was prepared by weighing out the various ingredients according to the formula given by Parker (165).

(b) Eagle's Basal Medium (BME) (44): Originally, BME with Hank's salts was prepared from the dry ingredients according to the formula in Merchant *et al.* (142). It was later judged more convenient, less time-consuming and subject to less risk of error, to purchase the tissue culture medium as powder, and reconstitute it as needed. BME with Hank's salts was therefore purchased as "Powder Media" from Grand Island Biological Company, Grand Island, New York ("Gibco" cat. #G-12).

(c) Eagle's Minimum Essential Medium (MEM) (45): As with BME, MEM with Hank's salts initially was prepared from the various dry constituents according to a revised formula (142); later, "Powder Media" was purchased from "Gibco" (cat. #F-12).

(d) Medium 199 (147): For all experiments in which it was used, Medium 199 with Hank's salts was

purchased from "Gibco" as "Powder Media" (cat. #E-12).

(e) Preparation of Media: Routinely, 20 litres of media were prepared either by weighing out the various dry ingredients and dissolving them in the order required according to the standard formulae, or by reconstituting "Powder Media" from "Gibco" as directed. The liquid tissue culture media were then sterilized by filtration with 6 lb. pressure through a membrane filter, type MF, pore size  $0.22\mu$ , fitted with a microfibre glass disc prefilter. Filtration equipment and membrane filters were purchased from Millipore Filter Corporation, Bedford, Massachusetts. Filtered media were dispensed into sterile 32 oz. prescription bottles, 800 ml. per bottle, and stored at  $4^{\circ}\text{C}$  for not more than two months before use.

To prepare complete growth and maintenance medium, sterile fetal calf serum (FCS) and antibiotics were added aseptically, along with sufficient sterile bicarbonate solution to adjust the pH to 7.4. Media used for the various cells in this investigation are listed in Table I.

## 2. Reagents

(a) Antibiotics: Two antibiotics, penicillin and streptomycin, were used routinely in the tissue culture fluids. Penicillin G, sodium salt, U.S.P.,

1650 U/mg., and streptomycin sulphate, 740 µg./mg., were purchased from General Biochemicals, Chagrin Falls, Ohio. Penicillin and streptomycin were prepared as a single stock solution in phosphate-buffered saline (PBS), penicillin at  $2 \times 10^4$  U/ml., streptomycin at  $4 \times 10^3$  µg./ml. The stock solution was sterilized by filtration and stored at  $-20^\circ\text{C}$ . One part by volume of thawed antibiotic solution was added to 100 parts of medium, yielding final concentrations of penicillin at 200 U/ml. and streptomycin at 40 µg./ml.

(b) Bicarbonate Solution: This was prepared as a 7.5% solution of sodium bicarbonate in distilled water and was sterilized by autoclaving. Sufficient amounts of bicarbonate were added to media to adjust the pH to 7.4.

(c) Citrate Saline: This solution was used to prepare, and as a diluent for, stock solutions of trypsin. It comprised 1.0% potassium chloride together with 0.44% sodium citrate in distilled water, and was sterilized by filtration.

(d) Fetal Calf Serum (FCS): FCS was purchased from Grand Island Biological Company, Grand Island, New York, and was stored at  $-20^\circ\text{C}$ . Routinely, FCS was treated by heating to  $56^\circ\text{C}$  for 30 minutes in order to inactivate any non-specific virus-neutralizing components.

Heat-inactivated FCS was then added to chemically-defined media to a final concentration of from 1% to 5% for maintenance and from 5% to 10% for growth media (Table I).

(e) Phosphate-buffered Saline (PBS): This reagent was prepared as a 10 x stock solution by dissolving the following chemicals in 100 ml. distilled water:

- 8.0 g. Sodium Chloride
- 0.2 g. Potassium Chloride
- 1.15 g. Disodium Hydrogen Phosphate
- 0.2 g. Potassium Dihydrogen Phosphate
- 0.1 g. Calcium Chloride
- 0.1 g. Magnesium Chloride·6 H<sub>2</sub>O

The stock solution was sterilized by filtration, dispensed in 10 ml. amounts into tubes, and stored at 0°C. Working solution was prepared by diluting the stock with 9 volumes of distilled water.

(f) Trypsin Solution: Bacto trypsin 1:250 was purchased from Difco Laboratories, Detroit, Michigan, as lyophilized powder. It was reconstituted with sterile citrate saline and prepared as a 1.25% stock solution, dispensed into sterile tubes in 10 ml. aliquots, and stored at 0°C. Stock solution was thawed and diluted with sterile citrate saline to make a working solution of 0.125% trypsin.

### 3. Glassware

(a) Leighton Tubes: Leighton type tissue culture tubes were purchased from Bellco Glass, Inc., Vineland, New Jersey. The size used for cell cultures in this investigation was 16 x 85 mm., containing glass coverslips 10.5 x 35 mm., and fitted with silicone rubber stoppers.

(b) Prescription Bottles: "Saniglass" screw-cap prescription bottles (Brockway Glass Co., Inc.) in sizes from 3 oz. to 32 oz. were purchased from Brown's Bottles and Supplies, Montreal, Quebec. The bottles were used as received in their factory cartons, without additional cleaning; subsequent usages were preceded by standard cleaning procedures for tissue culture glassware.

### 4. Cell Cultures

The cell cultures employed in this investigation were grown as monolayers in glass culture vessels, in the media shown in Table I. The methods for transferring cell lines from one culture vessel to another are briefly outlined in a later section. The tissue culture terms used throughout this presentation are those adopted by the Tissue Culture Association (47).

#### (a) Hamster Cell Lines

(i) Embryo hamster (EH): Pregnant hamsters were obtained from High Oak Ranch, Ltd., Richmond Hill,

Ontario (H.O.R.F<sub>1</sub> random bred Syrian). Primary cultures of Syrian hamster embryo cells were initiated as described in a later section, and were subcultured through several passages.

(ii) Baby hamster kidney (BHK21): This established cell line, obtained from Dr. L. Weiss, Roswell Park Memorial Institute, Buffalo, New York, was serially subcultured over many months. Cytologic examinations revealed no gross karyotypic alterations throughout the period of experimentation.

(b) Human Cell Lines

(i) Human embryonic kidney (HEK): Primary cultures of HEK cells were purchased from Flow Labs., Inc., Rockville, Maryland, and were used as received for titration of human adenovirus preparations. In addition, HEK cells were subcultured and used both for passaging human adenoviruses and for growth of virus stocks.

(ii) Human amnion (AV3): This established cell line obtained from Dr. D.T. Karzon, The Children's Hospital, Buffalo, New York, was serially propagated for several months during the experimental studies, and was used to examine the effect of adenoviruses on human cells in culture.

TABLE I

Media for Growth and Maintenance of  
Monolayer Cell Cultures

<u>Cell Line</u>	<u>Growth Medium</u>	<u>Maintenance Medium</u>
Hamster		
EH	1066 + 10% FCS	1066 + 2% FCS
BHK21	MEM + 5% FCS	MEM + 5% FCS
Human		
HEK	BME + 10% FCS	199 + 1% FCS
AV3	MEM + 5% FCS	MEM + 5% FCS
HEp2	BME + 10% FCS	199 + 1% FCS
Simian		
BS-C-1	BME + 10% FCS	BME + 1% FCS
LLCMK <sub>2</sub>	BME + 10% FCS	BME + 1% FCS

(iii) Human epidermoid cells (HEp2): An established cell line derived from carcinoma of the larynx (49), HEp2 was obtained from Dr. A. Fjelde, Roswell Park Memorial Institute, Buffalo, New York. The cells were serially propagated throughout the experimental studies, and were employed chiefly to passage human adenovirus and to grow stocks of such viruses. In addition, HEp2 cells were used as control cultures in the adenovirus replication studies in hamster cells (BHK21).

(c) Monkey Cell Lines

(i) Cercopithecus monkey kidney (BS-C-1):

(ii) Rhesus monkey kidney (LLCMK<sub>2</sub>):

Both these established cell lines were serially propagated and were used for the growth of simian adenovirus stocks and for titration of such stocks.

5. Culture Methods

(a) Initiation of Embryo Hamster Cultures (EH):

Pregnant Syrian hamsters were obtained with known dates of conception. On the twelfth day of gestation, the parent hamster was sacrificed by dislocating the cervical vertebrae, and the uteri were removed aseptically.

Embryos were in turn removed aseptically from the uteri and were washed with sterile PBS. A median ventral incision made in each embryo permitted the entrails to

be removed quickly. The embryos were rinsed twice in sterile PBS and were then minced into pieces about 1 mm.<sup>3</sup> in PBS. Minced tissue was washed twice with PBS, the washings being discarded, and sterile trypsin solution was added to the minced tissue, about 30 ml. of trypsin for every 4-6 embryos. The mixture was incubated in a spinner flask at 37°C for five minutes, after which the supernatant was decanted from the pieces of tissue, and the trypsinization procedure was repeated four times with fresh trypsin each time. The first supernatant was discarded, but the next four were neutralized with 1 part FCS to 4 parts supernatant, and pooled. Trypsinized cells were subsequently centrifuged in a clinical centrifuge (International Clinical Centrifuge Model CL) for ten minutes at 800 r.p.m. The resulting supernatant fluid was poured off, and the pellet resuspended by trituration in a small volume of complete growth medium. Cell counts were performed on an aliquot of this cell suspension in a hemocytometer (142) and appropriate volumes of the suspension were dispensed into 32 oz. prescription bottles to yield  $4 \times 10^7$  cells per bottle. Fresh growth medium was added to each culture bottle, and twenty-four hours later the medium was replaced with fresh medium. The EH cells subsequently were

subcultured into a number of smaller prescription bottles appropriate to the experiment to be performed.

(b) Subculturing: Trypsinization of cells from the glass surface of culture vessels was the method routinely used in transplanting the cell lines from one culture vessel to another. Cultures were rinsed twice with PBS, and sterile trypsin solution, prewarmed to 37°C, was poured onto each monolayer of cells, and the cultures incubated at 37°C. The trypsin solution either was left on the cells for 10-20 minutes until the cells had detached from the glass, or was poured off after only 1-2 minutes, and the cultures allowed to incubate in the residual trypsin for 5 minutes, by the end of which time the cells were readily shaken from the glass surface. The former method was applicable to those cell lines which adhere tightly to the glass, and are detached only with difficulty (HEp2, BS-C-1, LLCMK<sub>2</sub>); this technique necessitates centrifuging the detached cells at 800 r.p.m. for 5 minutes, decanting the supernatant, and resuspending the cell pellet in fresh medium with FCS. The latter trypsinization method was suitable for those cells which are released readily from the glass surface (BHK21, EH, AV3), and involves merely resuspending in fresh medium the cells which have been shaken loose from the culture vessels.

Whichever of the above methods was used, an aliquot of the cell suspension was then counted in a hemocytometer according to the method given by Merchant *et al.* (142). Sufficient volumes of cell suspension were then added to culture vessels to yield the desired number of cells per bottle, and fresh growth medium was added.

The experiments in this investigation were designed to illustrate various aspects of host cell: virus relationships, and required different conditions of growth and sampling times. A comprehensive experimental design, applicable to each experiment, therefore cannot be given. For this reason, in each section in which experimental results are given, the appropriate experimental design will be described in more detail.

## II Virological Techniques

### 1. Viruses

Seed aliquots of the adenoviruses used in this investigation were obtained from the sources listed in Table II. The passage history of each virus in these laboratories, the cells in which virus stocks for the experiments were produced, and the titers of the virus stocks are also listed in Table II.

## 2. Virus Infection of Cell Cultures

A standardized technique for virus infection of monolayer cell cultures was adopted for use throughout the investigation. The cell cultures were washed twice with PBS or with medium without FCS. Virus was then added to the cultures so as to give a designated potential input multiplicity of infection per cell, in as little medium without FCS as just necessary to cover the cells. This virus was permitted to adsorb to the cells for three hours at 37°C, with intermittent rocking of the cultures to distribute the inoculum evenly over the cells. At the end of the adsorption time, residual unadsorbed virus was washed from the cells by rinsing the cultures three times with the appropriate medium containing FCS. Fresh medium was then added to the cultures and they were re-incubated at 37°C for times suitable to the experiment being performed.

## 3. Titration of Virus Preparations

Owing to the large numbers of titrations routinely to be performed, the tube titration method was employed. As a consequence of viral infection, cells may undergo characteristic morphologic changes, and may display cytopathic effect (CPE). The CPE induced in tubes of sensitive cells by different dilutions of adenovirus

TABLE II

## Source and Passages of Adenoviruses, and Titers of Stocks

Virus	Strain		Passage history of virus	Cells in which stock virus prepared	Titer of stock virus (TCD <sub>50</sub> /ml)
Ad.2	JSL	W. McD. Hammon <sup>a</sup>	1 x HEK	HEK	10 <sup>9.2</sup>
Ad.4	U-566	R. Huebner <sup>b</sup>	2 x HEK 3 x HEp2	HEp2	10 <sup>8.2</sup>
Ad.7	V-825	R. Huebner	1 x HEK	HEK	10 <sup>7.4</sup>
Ad.12	Huie	R. Huebner	4 x HeLa 1 x HEK	HEK	10 <sup>7.4</sup>
Ad.18	3-185	R. Huebner	2 x HEK 2 x HEp2	HEp2	10 <sup>7.0</sup>
SA7	030367	B.C. Casto <sup>c</sup>	1 x LLCMK <sub>2</sub>	LLCMK <sub>2</sub>	10 <sup>7.2</sup>
SV15	Pitt	W. McD. Hammon	1 x BS-C-1	BS-C-1	10 <sup>7.4</sup>

<sup>a</sup> University of Pittsburgh

<sup>b</sup> National Institutes of Health, Bethesda, Maryland

<sup>c</sup> Institute for Biomedical Research, Chicago, Illinois

stock was scored in the following manner: 16 mm. x 125 mm. culture tubes of confluent monolayers of HEK cells in the case of human adenoviruses and LLCMK<sub>2</sub> cells for titration of the simian adenoviruses were inoculated with 0.2 ml. of 10-fold dilutions of virus. The diluent for each virus was the medium (without FCS) appropriate for the cells. Each log<sub>10</sub> dilution of virus was inoculated into four tubes, the virus was adsorbed in the manner described previously and was left on the cultures, fresh medium being added to the tubes. The tubes were examined twice a week for the presence of, and the degree of, CPE shown by the cells. At the end of 21 days, the results scored as progressive CPE allowed the calculation of the original concentration of virus in the undiluted stock, according to the method of Reed and Muench (1977). The virus concentration, or infectivity, was expressed as 50 per cent tissue culture infectious dose (TCID<sub>50</sub>) per ml. of undiluted virus preparation.

#### 4. Passage of Virus and Growth of Virus Stocks

Seed aliquots of virus were titrated, and appropriate amounts of virus to yield potential multiplicities of infection of 2 to 3 TCID<sub>50</sub> per cell were inoculated into 16 oz. bottles containing confluent monolayers of the desired cell line. The inoculum

volume was adjusted to 5 ml. per bottle by addition of medium without FCS. Adsorption of the virus was according to the method previously described. Four to five days after virus inoculation, the cells were harvested as follows: the cells and medium of the 16 oz. bottles were rapidly frozen, and subsequently the contents were thawed and the cells scraped into the medium with a rubber policeman. The mixture was sonicated, and dispensed into 1 ml. ampoules, which were then stored frozen, to be used after an aliquot of the virus harvest had been titrated.

#### 5. Sonication of Virus Harvests

Intact cells and aggregated cell debris in the virus harvests were disrupted with ultrasonic vibration, by use of a Branson Sonifier Model S125 (Branson Instruments, Inc., Danbury, Connecticut). Harvest material was placed in a screw-cap vial in an ice bath and sonified aseptically, at power setting 3, for six seconds.

#### 6. Antisera

Virus neutralization experiments were performed with the following rabbit antisera: #3-122, to type 4 adenovirus and #3-136, to type 18 adenovirus, supplied by Dr. Wallace P. Rowe of the National Institutes of Health, Bethesda, Maryland; #18-9-24, to type 12 adeno-

virus, prepared in the laboratory of Dr. D.S. Yohn of Roswell Park Memorial Institute, Buffalo, New York; antisera to type 26 adenovirus, supplied through the National Reference Reagents Branch of the National Institutes of Allergy and Infectious Diseases, Bethesda, Maryland.

Undiluted antisera were mixed with equal volumes of undiluted stock virus and incubated at 37°C for one hour, with continuous gentle shaking of the incubation tubes. Control tubes containing stock virus diluted with an equal volume of medium without FCS received similar treatment.

### III Radioautographic Techniques

#### 1. Tritiated Thymidine

Thymidine-methyl- $H^3$  was purchased from New England Nuclear Corporation, Boston, Massachusetts, at a specific activity of 1.00 mC per 0.12 mg. (1.9 C/mM) in sterile water. Cell cultures were incubated in medium containing tritiated thymidine at a final concentration of 0.5  $\mu$ C/ml. for a time appropriate to the experiment being performed.

#### 2. Photographic Emulsion

Ilford Nuclear Research Emulsion K-5, in gel form, was purchased from Ilford Limited, Essex, London, England. The following technique for coating microscope

slides is essentially that method used by Kopriva and Leblond (115). The gel emulsion was melted in a water bath at 43°C for one hour, and then diluted with distilled water, also at 43°C, one volume of water to one volume of melted emulsion. The solution was mixed slowly with a glass rod, and residual air bubbles were carefully scooped from the surface with a porcelain spoon; the emulsion was then ready for coating the slides.

Before coating experimental slides containing tissue exposed to the isotope, the level of background fog in the emulsion was examined by coating test slides. Two slides were dipped vertically into the emulsion, back to back, for 1-2 seconds, lifted from the emulsion, and the excess allowed to drain onto the filter paper. The backs of the slides were wiped free of melted emulsion, and the slides placed in a rack for drying; a slide box with large openings cut into its bottom and lid, so that only the frame structure remained, was quite practical for this purpose. The drying rack was placed in a light-proof drying box equipped with a variable-speed electric air-blower. Emulsion-coated slides were dried in this apparatus in a gentle air stream at room temperature for 30 minutes. The test slides were then developed in Kodak D-19 developer for

two minutes, fixed in Kodak Rapid Fix for four minutes, and washed for 20 minutes in gently-running water, all at 19°C. The test slides were examined microscopically while still wet, and the level of background grains assessed. If suitably low background fog were present, then the experimental slides were coated with emulsion and dried in the same fashion as described above.

When the emulsion-coated experimental slides were dry, they were transferred to a light-proof slide box along with a dehydrating agent, Drierite, and the edges of the box were wrapped with tape. Exposure of the slides was at 4°C. In order to determine the best exposure time, slides were removed at several intervals and developed, fixed and washed as described above. After washing, the slides were dehydrated through an alcohol: butanol: xylol series, mounted with Permount, and examined microscopically.

#### IV Cytologic Preparations

##### 1. Reagents

(a) Colchicine solution: Two concentrations of colchicine powder (alkaloid U.S.P., Fisher Scientific Co., New York) were used, one solution at 0.01%, another at 0.001%, in distilled water. To every ml. of medium on the cultured cells, 0.02 ml. of the appropriate

colchicine solution was added, the former concentration for hamster cells, the latter concentration for human cultures. Final concentrations of colchicine were therefore 2  $\mu\text{g./ml}$  for hamster cells, and 0.2  $\mu\text{g./ml}$ . for human cells.

(b) Hypotonic solution: Sodium citrate was used to swell the cells prior to fixation; for cells cultured in bottles, 1.0% sodium citrate in distilled water was used, while cells grown on coverslips were treated with a solution of 0.8% concentration.

(c) Fixative: The harvested cells were fixed with acetic-alcohol solution, prepared by mixing one volume of glacial acetic acid with three volumes 95% ethanol. Subsequently, 45% acetic acid was used to rinse the acetic-alcohol from the fixed cells, prior to staining.

(d) Aceto-orcein stain: Staining solutions were prepared as 2% orcein by refluxing 2 grams of orcein powder (British Drug Houses Ltd., Poole, England) with 100 ml. of 45% acetic acid for 24 hours. The solution was cooled and filtered before use.

## 2. Techniques

Cultured cells were incubated in medium containing colchicine at the concentrations previously mentioned for 1 1/4 to 1 1/2 hours prior to sampling for chromosome

analysis.

Cells grown in prescription bottles were treated in the following manner at harvest: the cells were scraped from the glass surface with a rubber policeman, and the medium with suspended cells was centrifuged in 12 ml. conical tubes in a clinical centrifuge at 1000 r.p.m. for 5 minutes. The supernatant was decanted, 5 ml. of hypotonic solution at 37°C added, and the pellet suspended with a Pasteur pipette. The resuspended cells were incubated for 10 minutes in a water bath at 37°C, after which the cells were again centrifuged as before. The supernatant was carefully drawn off the pellet and 5 ml. of acetic-alcohol cautiously added in order not to disturb the pellet. After three minutes the first fixative was removed, and 5 ml. of 45% acetic acid carefully layered onto the pellet, again so as not to disrupt the pellet. After a further three minutes of fixation, the acetic acid was removed from the pellet, except for one or two drops, and 2-3 drops of 2% aceto-orcein added to the preparation. The cells were resuspended with a Pasteur pipette and allowed to stain for three minutes before beginning the squash procedure.

Alcohol-cleaned microscope slides (25 x 75 mm.) were used for the squash preparations, but coverglasses

(22 mm.<sup>2</sup>) were merely wiped clean with lens tissue to remove splinters of glass which might interfere with complete flattening of the cells. It was not found necessary that the coverslips be coated with silicone compounds, as most cells adhered to the slide and not to the coverglass, when the coverglass was removed later in the procedure.

One drop of the stained preparation of cells was placed on a slide, about one-third from the end. A coverglass was placed over the drop, and air pockets avoided by lowering the coverslip gently over the drop with one edge touching the slide initially. Filter paper was placed on the top of the coverslip and squashing was begun with the thumb directly over the centre of the coverslip. Gentle, even pressure was applied with the thumb, stain squeezed out from under the coverslip being absorbed by the filter paper. Stronger pressure was then applied with the thumb, both in the centre and at the corners of the coverslip. Caution was exercised, especially when the thumb was repositioned, to avoid sideways movement of the coverglass, because such movement would destroy the preparation.

The slides were examined microscopically several times during the squashing procedure to determine if further squashing were necessary. When the preparations had been squashed adequately, coverslips were removed by

a freezing procedure (35); slides were placed coverslip down on the flat surface of a block of dry ice for 3-4 minutes. For permanent preparations, the coverslips were removed quickly with a razor blade, by a single flipping movement, and the slides were immediately plunged into 95% ethanol for only a few seconds, then into each of a series of butanol: butanol/xylol; xylol for 5-10 seconds each. Finally, the preparations were mounted in Permount with a fresh coverglass. For radioautography, however, the coverslips were removed as above, but the slides were merely rinsed briefly in 10% acetic acid and permitted to air dry.

Cells grown on coverslips in Leighton-type tubes were necessarily treated by a different procedure than that above. A modification of the technique used by Fogh and Fogh (52) proved ideal for demonstrating excellent chromosome preparations on coverslips. Growth medium was removed from Leighton tube cultures and replaced with 0.8% sodium citrate. One minute later, an equal volume of distilled water was added dropwise to the hypotonic solution and the cells were incubated in the final solution for 10 minutes. An equal volume of acetic-alcohol was then added slowly and the coverslip was gently removed from the Leighton tube to be placed in a tube containing fresh acetic-alcohol. The coverslip

was air-dried, and subsequently stained with 2% aceto-orcein for 5 minutes. The stain was rinsed from the coverslip in 95% alcohol and the coverslip was dehydrated by passing for five seconds each through a butanol: butanol/xylol: xylol series. Finally, the coverslip was mounted in Permount cell side down on a microscope slide.

## RESULTS

### I Replication of Adenoviruses in Human and Hamster Cells

One of the principal aims of this investigation was to compare the effects of infection with adenoviruses types 2,4,7,12,18,SV15 and SA7 on chromosomes of cells which support a complete viral replication cycle with that on chromosomes of cells in which only an abortive infection is induced with adenoviruses. Detailed knowledge of the capacity for replication of the above adenoviruses in hamster cells and in human cells was therefore essential.

#### 1. Experimental Design

Leighton tubes were seeded with  $1.5 \times 10^5$  BHK21 or HEp2 cells per tube in their respective growth media (Table I). After 48 hours incubation at 37°C, confluent monolayers of each cell line had formed in the tubes. Cell counts were performed on several replicate cultures in order to estimate the number of cells per Leighton tube of each cell line. Each tube was then inoculated with an appropriate amount of one of the seven adenovirus stocks, types 2,4,7,12,18,SV15 or SA7, to yield a potential multiplicity of infection of 5 TCID<sub>50</sub> per cell. The virus adsorption was performed in the

fashion previously described, except that, at the end of the virus adsorption period, the cultures were rinsed three times with PBS containing 2% FCS, and 1.5 ml. of appropriate maintenance media was added to each tube.

Two tubes of each cell culture infected with each virus were collected at 0,6,18,24,40,48,72 and 96 hours after adsorption by swiftly freezing the Leighton tubes and their contents at  $-70^{\circ}\text{C}$ . Samples were subsequently thawed and the contents of duplicate samples were pooled and sonicated. Sonified preparations were titrated on HEK cells in the case of the human adenoviruses types 2,4,7,12 and 18, and on LLCMK<sub>2</sub> cells for the simian adenoviruses types SV15 and SA7. The resulting titers are expressed as TCID<sub>50</sub> per 0.2 ml., in Table III.

Control studies were performed on a parallel set of BHK21 cultures which had been killed by heating in a water bath at  $56^{\circ}\text{C}$  for 30 minutes. The heat-killed cultures were inoculated with adenoviruses in the same manner as the experimental cultures, and were sampled along with the experimental cultures at the same times after virus adsorption. The contents of adenovirus-infected heat-killed BHK21 cultures were titrated and compared with titrations obtained from experimental cultures. This comparison permitted detection of virus

replication in the experimental cultures as distinguished from residual cell-associated inoculum in the heat-killed cultures.

## 2. Adenovirus Replication in Human and Hamster Cells

During the 96-hour interval following virus adsorption, each adenovirus replicated in the human cell cultures to yield titers ranging from 3 to 5.5  $\log_{10}$  in excess of virus input (Table III). During the same interval, however, the cell-associated inoculum residual in BHK21 cultures was not significantly greater than that detected in heat-killed cultures, with the exception of Ad.2. This latter virus did appear to replicate in BHK21 cells to yield a 2.2  $\log_{10}$  increase in titer over the virus input (Table III).

## 3. Formation of Inclusion Bodies in Human and Hamster Cells

Previous studies have demonstrated the presence of inclusion bodies in nuclei of various human cell lines lytically-infected with adenoviruses (86). The inclusion body is known to be the site of replication of mature adenovirus (146), and in the electron microscope large numbers of mature virions are associated with the inclusion body (71,106,197) in human cells. It has also been shown that the inclusion body contains virus structural antigens (106), probably is the site of viral DNA replication (71,228) and has its components

involved in the formation of mature virions (71,106). In addition, titration of adenovirus stocks by the number of cells forming inclusion bodies indicates that titrations by this method may correspond very closely to titrations obtained by the tube titration method (197).

~~Formation of intranuclear~~ inclusion bodies in adenovirus-infected cells therefore is strongly indicative that a complete viral replication cycle has resulted in those cells.

It is of great interest, then, that in Ad.2-infected hamster cells, the rise in titer was accompanied by the appearance of intranuclear inclusion bodies (Figs. 58-61), similar to those observed in Ad.2-infected human cell cultures. However, only a small fraction, 0.6% of the interphase cells examined at 72 hours post-infection displayed inclusion bodies in BHK21 cultures exposed to 5 TCID<sub>50</sub> per cell, whereas in human cultures exposed to the same virus dose the incidence of inclusion bodies 72 hours after infection was over 30% (Table IV).

Although the data in Table III may also suggest a slight increase in titer for SV15-infected hamster cultures, aceto-orcein preparations failed to reveal any intranuclear inclusion bodies in over 4,000 cells

examined, whereas in human cultures infected with SV15 typical inclusion bodies were readily observed with the same staining technique (Fig. 98).

The above observations are interpreted to indicate that the adenoviruses tested in this study, with the exception of Ad.2, did not replicate in BHK21 cells during 96 hours post-infection. In the case of Ad.2, the small increase in titer in hamster cultures relative to that produced in human cultures, together with the low frequency of inclusion bodies, suggest that relatively very few cells supported virus replication of Ad.2 in the infected BHK21 cultures.

TABLE III

The Average TCD<sub>50</sub> of Adenovirus in BHK21 and HEp2 Cells at  
Three Intervals Post-infection

Virus	BHK21			Heat-Killed BHK21			HEp2		
	0 <sup>a</sup>	48 <sup>a</sup>	96 <sup>a</sup>	0	48	96	0	48	96
Ad.2	4.0 <sup>b</sup>	6.2	5.5	4.0	3.7	3.3	3.7	9.3	9.2
Ad.4	4.3	2.5	2.3	4.0	2.5	2.3	4.7	6.7	7.5
Ad.7	4.7	4.3	4.5	5.3	4.7	4.3	4.7	5.0	7.7
Ad.12	4.7	3.0	2.3	4.7	2.7	2.0	5.0	7.3	8.5
Ad.18	4.7	3.0	3.0	4.5	3.3	2.3	5.0	7.3	8.5
SV15	3.7	4.7	4.5	4.3	4.7	3.7	3.5 <sup>c</sup>	6.0 <sup>c</sup>	8.0 <sup>c</sup>
SA7	4.7	3.6	2.9	3.7	3.4	-	4.6 <sup>c</sup>	5.5 <sup>c</sup>	5.9 <sup>c</sup>

<sup>a</sup> Hours post-infection

<sup>b</sup> Log<sub>10</sub> TCD<sub>50</sub> titer per 0.2 ml. for HEK cells, coverage of 2 determinations.

<sup>c</sup> Data obtained on LLCMK<sub>2</sub> cell line

TABLE IV

Incidence (%) of Interphase Nuclei Showing Inclusion  
Bodies in Cultured BHK21 Cells and AV3 Cells  
Infected with Human Adenovirus Type 2 :  
*2000 cells examined*

Hours Post-Infection	Input Multiplicity (TCID <sub>50</sub> per cell)		
	1x	5x	25x
BHK21			
24	0	0	0.2
36	0	0.2	1.9
48	0	0.5	2.6
72	0	0.6	1.3
AV3			
24	0.5	1.6	
36			
48	3.0	9.3	
72	6.3	34.6	

## II Effect of Adenoviruses on Human and Hamster Chromosomes

Previous studies have shown that the highly-oncogenic adenovirus type 12 has potent chromosome-breaking capacities in both Chinese and Syrian hamster cells *in vitro* (128,198), and that Ad.12-induced hamster tumours display a wide array of chromosome aberrations and karyotypic abnormalities (200,203). In spite of the apparent lack of chromosome-damaging capacity of Ad.12 for human cells (128), it was nonetheless felt that other types of adenoviruses, perhaps even those non-oncogenic for hamsters, might induce chromosome aberrations in human cells, much as does the non-oncogenic measles virus (153).

Although the capacity of many viruses to induce chromosome aberrations in mammalian cells has been well documented (7,16,75,144,153,198,199,216) quantitative relationships between virus dose and the type and degree of chromosome damage have not been explored extensively. It becomes of great interest, then, to compare the chromosome-damaging capacities of a selection of adenoviruses, including types from human and simian hosts, and to examine the effects of virus dose on the severity and the type of chromosome damage induced in human and hamster cells.

### 1. Experimental Design

Human cells (AV3) and Syrian hamster cells (BHK21) were trypsinized from their respective cultures in growth phase and were planted into 4 oz. prescription bottles. BHK21 cells were seeded at 1.5, 1.0, .75, or  $.5 \times 10^6$  cells per bottle, while AV3 cells were seeded at 1.5 or  $.5 \times 10^6$  cells per bottle. Twelve hours later, cell counts were performed on replicate cultures; no significant increase in cell number per bottle had occurred over that initially inoculated into the bottles. Nonetheless, it could readily be observed in the inverted microscope that many cells were entering mitosis, and the cultures were considered then to begin growth phase again.

Appropriate dilutions of adenovirus stocks were prepared in medium without FCS to yield potential multiplicities of infection of 25, 5, and 1 TCID<sub>50</sub> per cell. Each dilution of virus was inoculated into twelve bottles of each cell line, and virus adsorption was permitted in the standard fashion, as described previously.

Subsequently, two bottles of each cell line inoculated with each virus dose were harvested at each of 6, 18, 27, 36, 48 and 72 hours post-infection. The cells were examined for chromosome aberrations by

arresting mitotic cells at metaphase through the use of colchicine, applied at a concentration of 2  $\mu\text{g}$  per ml. for the last 1 1/4 hours prior to harvesting. In addition, virus-infected cultures were sampled without colchicine pretreatment to determine whether other effects are induced by adenovirus infection, e.g., a colchicine-like effect such as that observed with herpes simplex virus (198). Harvested cultures were prepared according to hypotonic pretreatment and aceto-orcein squash techniques (p. 29).

## 2. Effect of Adenoviruses on Chromosomes of Hamster Cells

### (a) Types of Chromosome Aberrations

Each of the human and simian adenoviruses tested induced a wide array of chromosome aberrations in BHK21 cells. Chromosome damage was categorized as breakage, including chromatid breakage and fragmentation, and coiling anomalies, comprising overcontraction and erosion of the chromosomes (Fig. 1). Breakage phenomena ranged from single chromatid breaks through multiple breaks per chromosome to complete fragmentation or "pulverization"\* of the chromosome complement. The coiling anomalies usually affected the whole chromosome complement of the cell, so that all chromosomes in a

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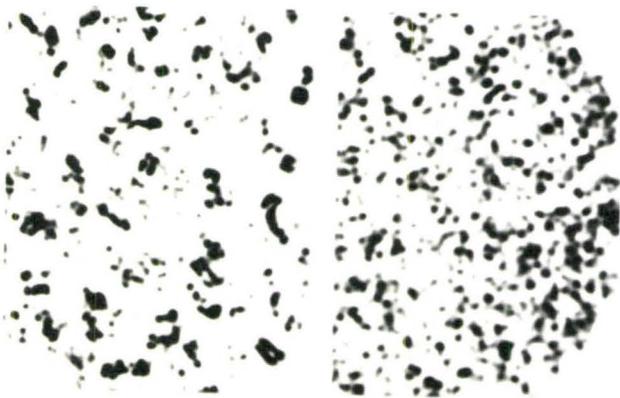
\* For reasons to be given later, chromosome "pulverization" is considered in the same category as "fragmentation".

Fig. 1      Portions of metaphase plates illustrating the categories of chromosome damage induced in Syrian hamster cells (BHK21) after infection with adenoviruses. The small arrows indicate chromatid breaks, the double arrow shows a possible isochromatid lesion, while the larger arrow demonstrates a chromatid gap; such chromatic gaps were not counted as chromosome damage, only the well-defined breaks being tabulated.

**Chromatid  
breaks**



**Fragmentation**

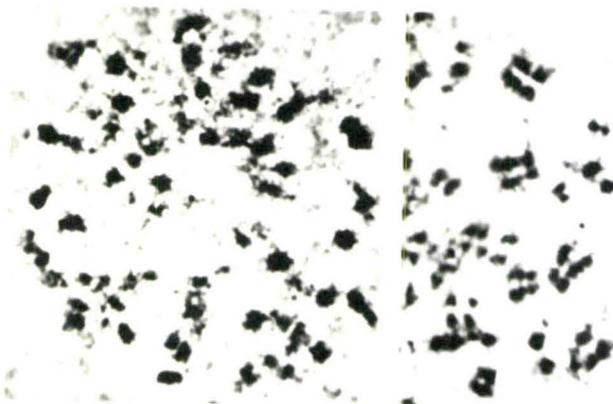


**Pulverization**

**Over-  
contraction**



**Erosion**

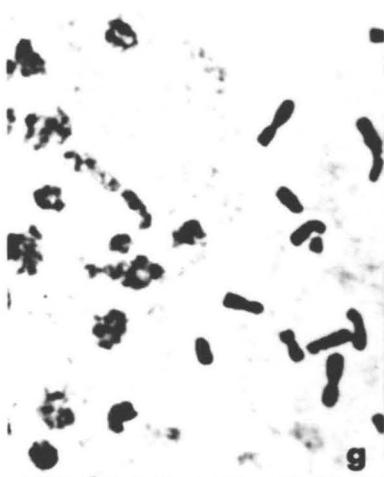
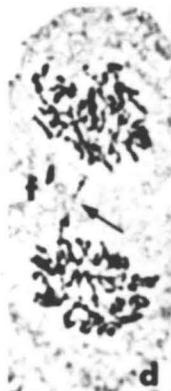
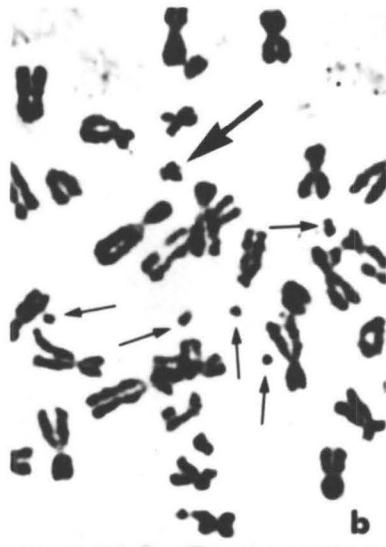
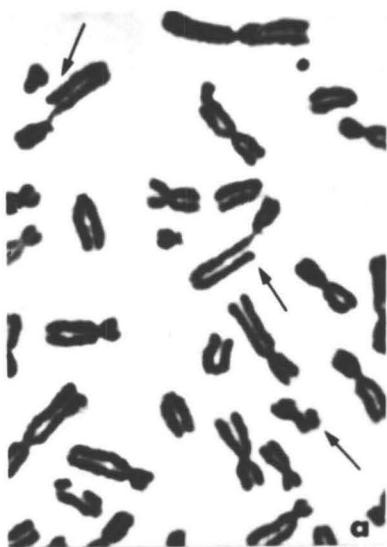


metaphase plate were overcontracted or eroded; occasionally, however, one or only a few chromosomes were affected.

That the observed aberrations were real phenomena, and not artefacts of fixation or of squash preparation is attested to by several observations. 1. It is unlikely that the aberrations categorized as breaks were merely achromatic, uncoiled chromatid segments, since dislocated chromatid segments (Fig. 2a), unattached fragments (Fig. 2b) and chromatid exchanges were also observed. 2. Anaphase and telophase figures with attached fragments, chromosome bridges, and acentric fragments were frequently seen in virus-infected cultures sampled without colchicine pretreatment (Figs. 2 c-f). 3. Overcontraction or erosion of the chromosomes were only rarely observed in uninfected cultures which had received the same preparatory treatments as the virus-infected cultures, yet were commonly seen in infected cultures, often in very high frequencies. 4. Cells showing chromosomes with normal colchicine-induced metaphase morphology and cells showing overcontracted or uncoiled chromosomes frequently lay side by side in the same preparation (Figs. 2 g,h).

Similarities were observed from virus to virus in the incidence or the type of chromosome aberrations

- Fig. 2a Part of metaphase plate from BHK21 culture infected with Ad.4, illustrating chromatid breaks with dislocated segments (arrows).  
X1800
- 2b Part of metaphase plate from BHK21 culture infected with Ad.18, demonstrating chromatid fragments (arrows) each noticeably smaller than the member (large arrow) of the smallest chromosome pair in the normal Syrian hamster complement. X1800
- Figs. 2c-f Mitotic figures from BHK21 cultures 27 hours after infection with Ad.12; no colchicine pretreatment. X1000
- c,d Anaphase figures showing chromosome bridges (arrows) and chromosome fragment (f).
- e,f Telophase figures showing chromosome fragment (f) not included in daughter nucleus.
- Figs. 2g,h Cultures of BHK21 cells infected with adenovirus showing proximity of apparently normal metaphase plates with cells showing chromosome erosion (g) or pulverization (h). X1500



induced in hamster cells. In order to make comparisons between the adenovirus-induced chromosome aberrations more clear, the adenoviruses are considered in groups, within which the types of aberrations are comparable. The groups consist of Ads.12 and 18, Ad.7 and SV15 and Ads.2,4 and SA7.

(i) Ad.12 and Ad.18: In BHK21 cultures infected with Ad.12 or Ad.18, the chromosome damage consisted almost exclusively of chromatid breaks. Metaphase plates from these cultures showed wide variation in the number of chromatid breaks, extending from a single break per complement (Fig. 3) to several breaks per metaphase plate (Fig. 4), through multiple breaks per chromosome (Figs. 5,6), even extending to fragmentation (Figs. 7,8) or complete "pulverization" (Fig. 9) of the entire chromosome complement. In addition, various translocation anomalies were observed in infected cultures, e.g. di- or tricentric chromosomes (Fig. 10), "double-minute" fragments (Fig. 10), extra-long chromosomes (Fig. 11) and single or multiple chromatid exchange figures (Figs. 12-15). The incidence of such rearrangements, however, was low, not exceeding 4.5% of the examined metaphases, even in cultures infected with the highest input multiplicity.

- Figs. 3-15 Chromosome aberrations induced in cultured Syrian hamster cells (BHK21) 27 hours after infection with Ad.12 or Ad.18 at input multiplicity of 25 TCID<sub>50</sub>/cell.
- Fig. 3 Part of metaphase plate which showed only a single chromatid break (arrow) in the complement (Ad.12). X2000
- Fig. 4 Part of metaphase plate showing several chromosomes each with a single chromatid break (arrows) (Ad.18). X2000
- Fig. 5 Multiple breaks per chromosome (Ad.12). X2000
- Fig. 6 Multiple breaks per chromosome (Ad.18). X1800
- Fig. 7 Metaphase plate showing fragmentation of most of the complement, only a few chromosomes remaining intact (Ad.12). X1500
- Fig. 8 Extensive fragmentation of the entire complement (Ad.18). X1800
- Fig. 9 "Pulverization" of the entire complement (Ad.12). X2000
- Fig. 10 Part of metaphase plate showing tricentric chromosome and several "double minute" fragments (arrows) (Ad.18). X2000
- Fig. 11 An extreme example of an aberrant, "extra-long", chromosome (Ad.12). X2500
- Figs. 12,13 Chromatid exchange figures (Ad.18). X3000
- Fig. 14 Multiple chromatid exchange figure (Ad.12). X3000
- Fig. 15 Extreme disarrangement of the chromosome complement (Ad.18). X1600



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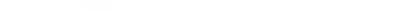
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(ii) Ad.7 and SV15: BHK21 cultures infected with Ad.7 or SV15 exhibited chromosome damage predominantly consisting of chromatid breaks. Similarly to the aberrations resulting from Ad.12 or Ad.18 infection, the chromatid breaks elicited by Ad.7 or SV15 showed wide variation in extent per metaphase plate, ranging from a single or a few breaks (Figs. 16,28,29), to fragmentation (Figs. 17,30) or "pulverization" (Figs. 18,31) of the chromosome complement. Diverse structural disarrangements were also observed, including dicentric (Figs. 21,22,32,33) and ring chromosomes (Figs. 23,34), and chromatid exchanges (Figs. 19,20,35,36), although only to a low frequency, a maximum of 6.7% for Ad.7-infected cultures, and 5.6% in the case of SV15.

In addition to chromatid breakage, however, significant amounts of coiling anomalies were also induced. Overcontraction (Figs. 24,37) or erosion (Figs. 25,26,38) of the chromosomes was frequently observed, and micronucleated cells (Figs. 27,39) were encountered in cultures exposed to 25 TCID<sub>50</sub> per cell. Often, metaphase plates were observed showing several types of anomalies concurrently (Figs. 40,41).

(iii) Ad.2, Ad.4 and SA7: Infection of BHK21 cultures with Ad.2, Ad.4 or SA7 elicited an array

- Figs. 16-27 Chromosome aberrations induced in cultured Syrian hamster cells (BHK21) 27 hours after infection with Ad.7 at input multiplicity of 25 TCID<sub>50</sub>/cell.
- Fig. 16 Several metaphase chromosomes each exhibiting a single chromatid break with dislocated segment. X2000
- Fig. 17 Fragmentation of the complement. X1800
- Fig. 18 "Pulverization" of the chromosomes. X1800
- Figs. 19,20 Chromatid exchange figures. X2500
- Figs. 21,22 Dicentric chromosomes. X1500
- Fig. 23 Part of metaphase showing two figures of 3 interlocking ring chromosomes (48 hours after infection). X1500
- Fig. 24 Metaphase plate showing overcontracted chromosomes. X1000
- Figs. 25,26 Parts of metaphase plates illustrating erosion of the chromosome complement. X1800
- Fig. 27 Interphase cell showing micronuclei (48 hours after infection). X1000



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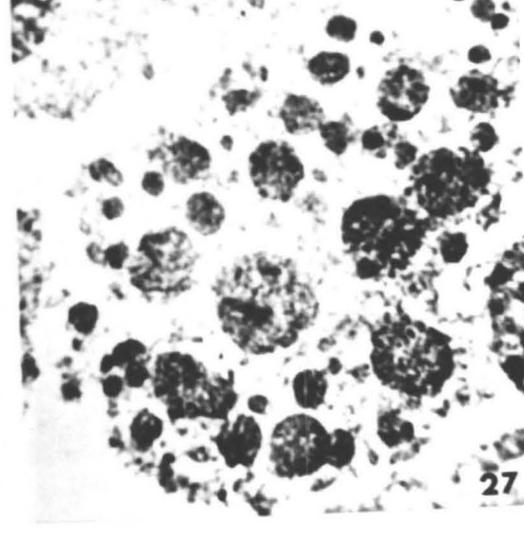
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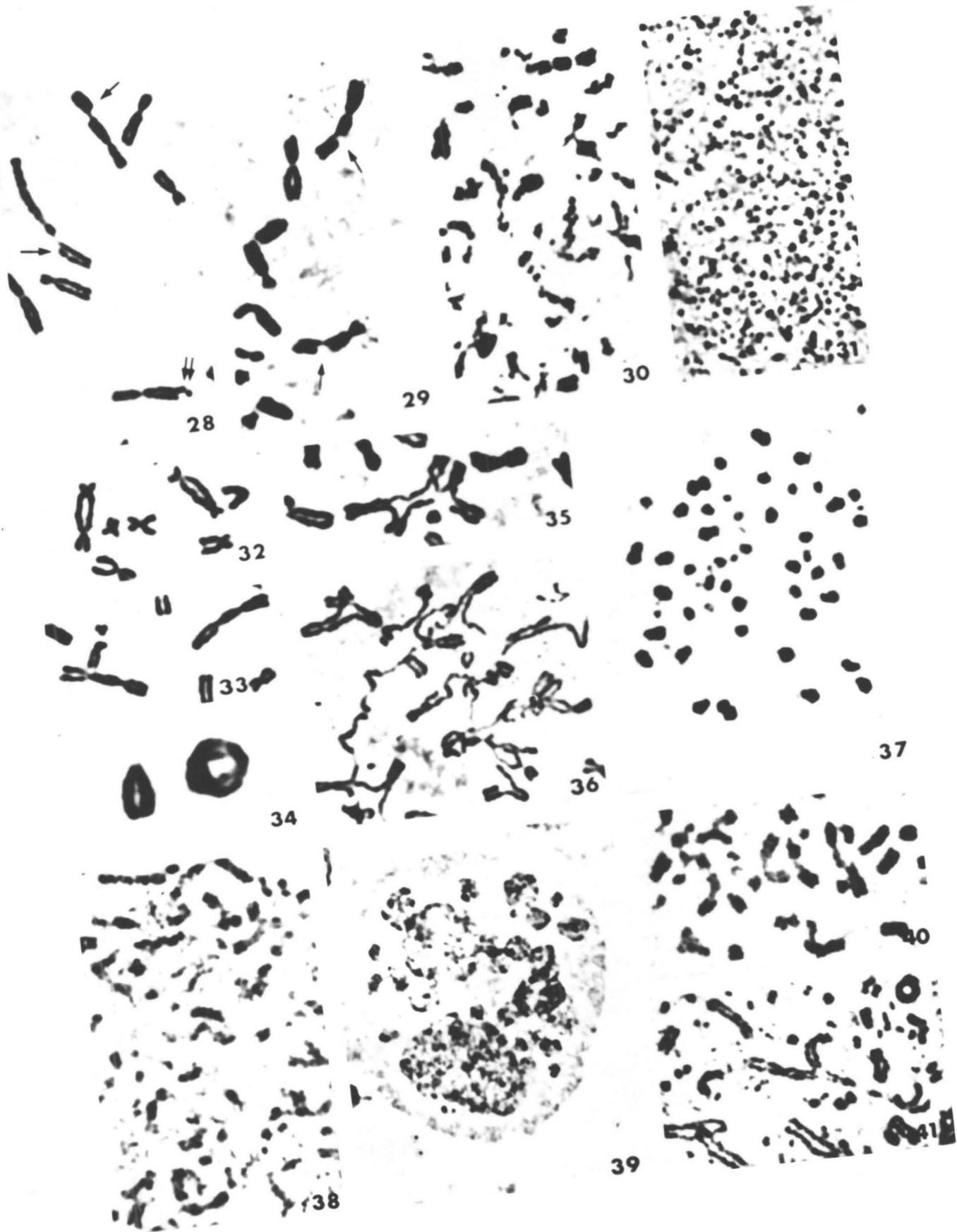


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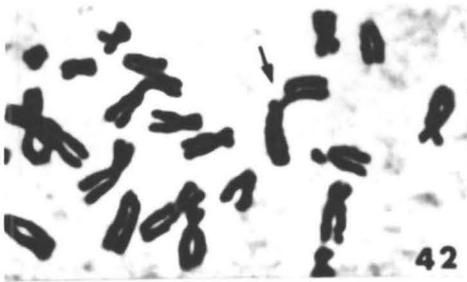
- Figs. 28-41 Chromosome aberrations induced in cultured Syrian hamster cells (BHK21) 27 hours after infection with SV15 at input multiplicity of 25 TCID<sub>50</sub>/cell.
- Fig. 28 Part of a metaphase plate showing chromatid breaks with dislocated chromatid segments (arrows). X1600
- Fig. 29 Part of a metaphase plate demonstrating chromosomes with chromatid breaks (arrows). X2000
- Fig. 30 Fragmentation of most of the chromosome complement. X1800
- Fig. 31 Part of a metaphase plate showing "pulverization" of the complement. X1800
- Figs. 32,33 Examples of part of metaphase plates, each showing two dicentric chromosomes. X1800
- Fig. 34 Ring chromosome, along with an apparently normal telocentric chromosome. X3500
- Figs. 35,36 Multiple exchange figures. X1800
- Fig. 37 Metaphase plate showing overcontracted chromosomes. X1000
- Fig. 38 Part of metaphase plate with chromosome erosion. X2000
- Fig. 39 Interphase cell showing micronuclei (48 hours after infection). X1000
- Fig. 40 Chromosomes showing overcontraction and erosion concurrently. X1800
- Fig. 41 Portion of metaphase plate with multiple chromosome aberrations (breaks, rearrangements, erosion) (48 hours after infection). X1600



of chromosome aberrations similar to those induced by Ad.7 or SV15, and in addition induced anomalies of the mitotic apparatus. Chromatid breakage was frequently displayed, ranging from a single break (Fig. 42) to many breaks (Figs. 43,62,76,77) per complement, through fragmentation of a single chromosome (Fig. 46) to fragmentation (Figs. 44,63,77) or "pulverization" (Figs. 45,64) of all chromosomes of a cell. Similarly to those cultures infected with Ad.12, Ad.18, Ad.7, or SV15, cultures infected with Ad.2, Ad.4 or SA7 displayed a variety of rearrangements of the chromosomes, encompassing dicentric (Figs. 47,49,65,78) or ring chromosomes (Figs. 66,79) "double-minutes" (Fig. 67) and chromatid exchanges (Figs. 48,49,68,69,80). The frequency of such structural disarrangements, however, was always low, occurring to a maximum of 5.6% of the metaphases examined.

Although significant amounts of chromatid breakage and fragmentation were observed, the major effect of Ad.2, Ad.4 or SA7 appeared to be overcontraction and erosion. Overcontraction usually involved the whole complement (Figs. 52,71,81) but varying amounts of erosion could be detected, involving from only a few chromosomes (Figs. 53,82) to the entire complement (Figs. 54,55,72, 83). Frequently, metaphase plates exhibited several types of aberrations concurrently (Figs. 50,57,74,86).

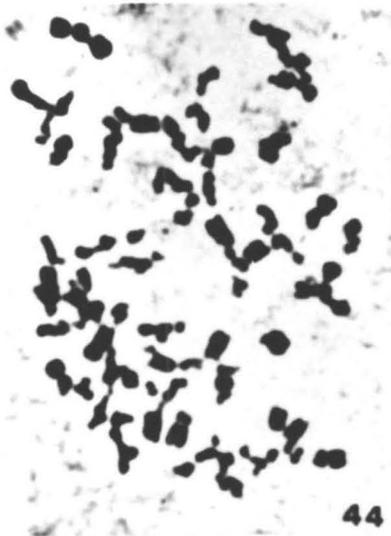
- Figs. 42-50 Chromosome aberrations induced in cultured Syrian hamster cells (BHK21) 27 hours after infection with Ad.2 at input multiplicity of 25 TCID<sub>50</sub>/cell.
- Fig. 42 Part of a metaphase plate showing a single chromatid break (arrow). X2000
- Fig. 43 Part of a metaphase plate with many chromatid breaks. X1800
- Fig. 44 Fragmentation of the chromosome complement. X1200
- Fig. 45 Pulverization of the entire chromosome complement. X2000
- Fig. 46 Fragmentation of a single chromosome in a metaphase plate (arrow). X1200
- Fig. 47 Part of metaphase plate showing dicentric chromosome. X2000
- Fig. 48 Chromatid exchange figure. X2000
- Fig. 49 Part of metaphase plate exhibiting a dicentric chromosome (arrow) and a chromatid exchange (double arrow). X2000
- Fig. 50 Part of metaphase plate showing multiple abnormal chromosomes. X1800
- Fig. 51 Metaphase plate from Ad.2-infected BHK21 culture showing apparently normal chromosome complement. X1200



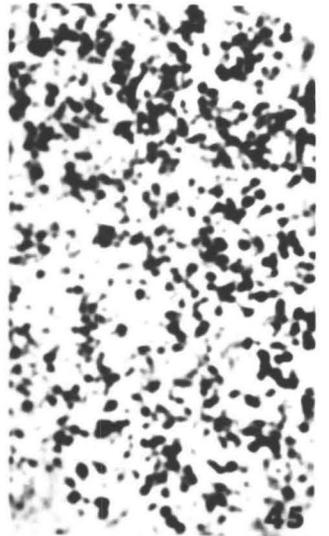
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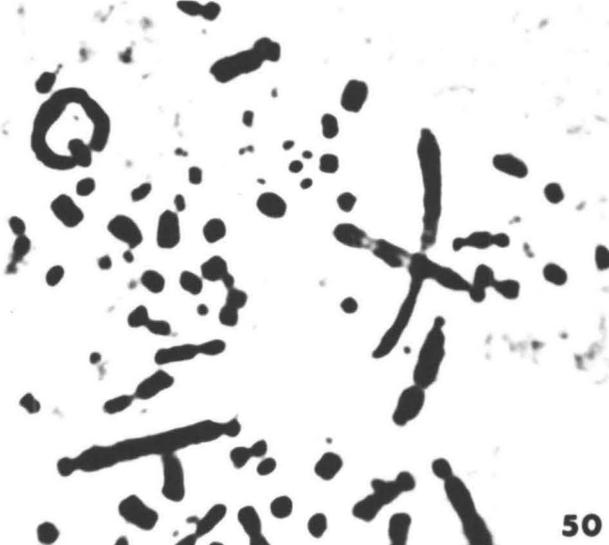
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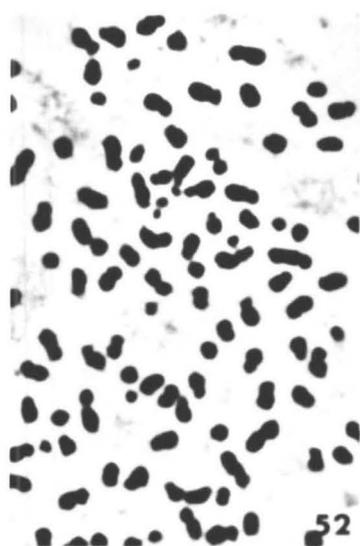


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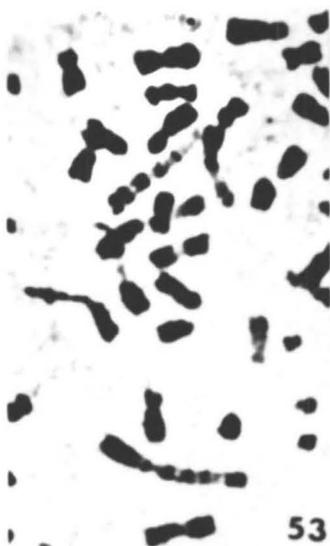


51

- Figs. 52-57 Chromosome aberrations induced in cultured Syrian hamster cells (BHK21) 27 hours after infection with Ad.2 at 25 TCID<sub>50</sub>/cell.
- Fig. 52 Overcontraction of all chromosomes in a polyploid cell. X1200
- Fig. 53 Part of metaphase plate showing erosion of part of chromosome complement. X1800
- Figs. 54,55 Metaphase plates showing erosion of all chromosomes. X1200
- Fig. 56 Micronuclei (48 hours after infection). X1000
- Fig. 57 Metaphase plate showing several chromosome aberrations concurrently (breakage, overcontraction, erosion). X1500
- Figs. 58-61 Examples of Ad.2-infected BHK21 cells showing intranuclear inclusion bodies (arrows) (27 hours after infection). X1200



52



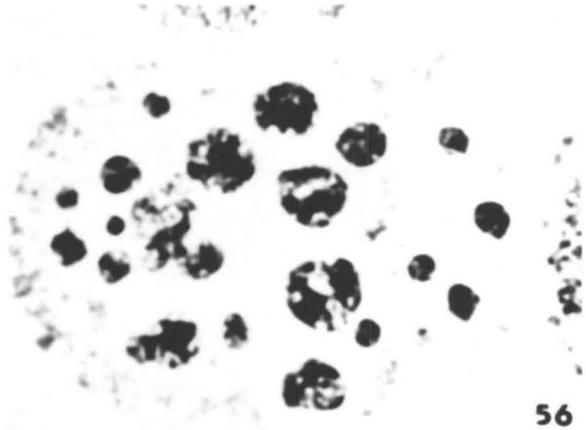
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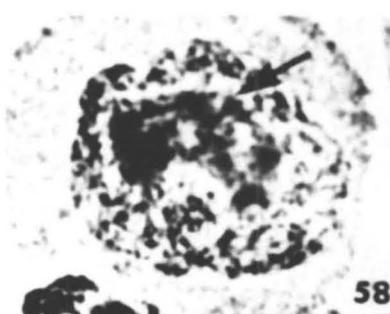
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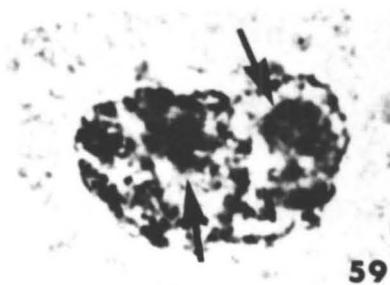
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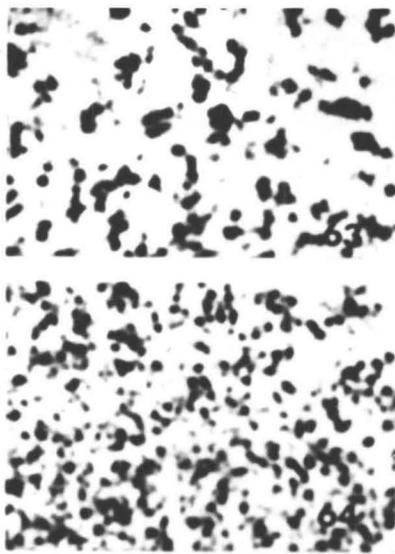


61

- Figs. 62-74 Chromosome aberrations induced in cultured Syrian hamster cells (BHK21) 27 hours after infection with Ad.4 at input multiplicity of 25 TCID<sub>50</sub>/cell.
- Fig. 62 Part of metaphase plate showing several chromosomes each with a single chromatid break (arrows). X2000
- Fig. 63 Part of fragmented metaphase plate. X1600
- Fig. 64 Part of metaphase plate showing "pulverization". X1600
- Fig. 65 Dicentric chromosome. X1800
- Fig. 66 Ring chromosome. X3500
- Fig. 67 Part of metaphase plate showing many "double minute" fragments (48 hours after infection). X1800
- Fig. 68 Multiple chromatid exchanges. X1000
- Fig. 69 Chromosome "stickiness". X1000
- Fig. 70 C-mitosis effect. No colchicine pretreatment. X300
- Fig. 71 Metaphase plate showing overcontraction of all chromosomes. No colchicine pretreatment. X800
- Fig. 72 Metaphase plate showing erosion of chromosome complement. X1000
- Fig. 73 Formation of micronuclei (48 hours after infection). X1200
- Fig. 74 Part of metaphase plate showing several chromosome aberrations concurrently (breaks, overcontraction, erosion). X1200



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63



65

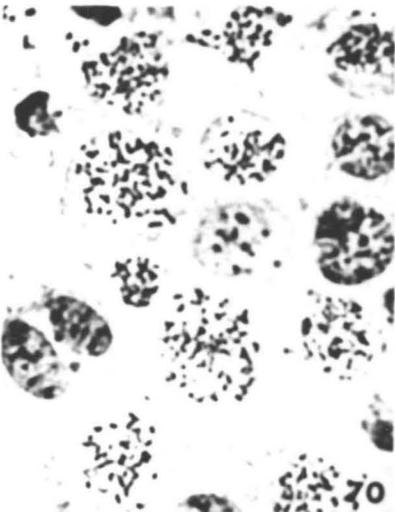
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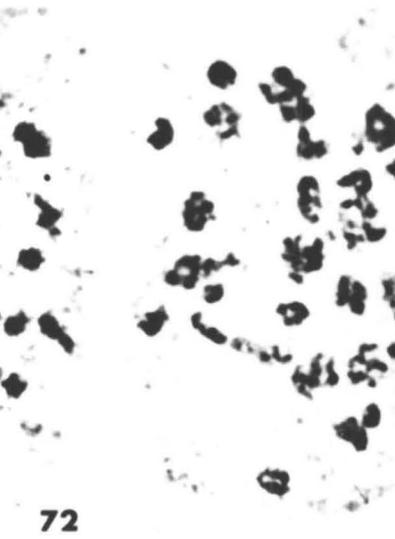
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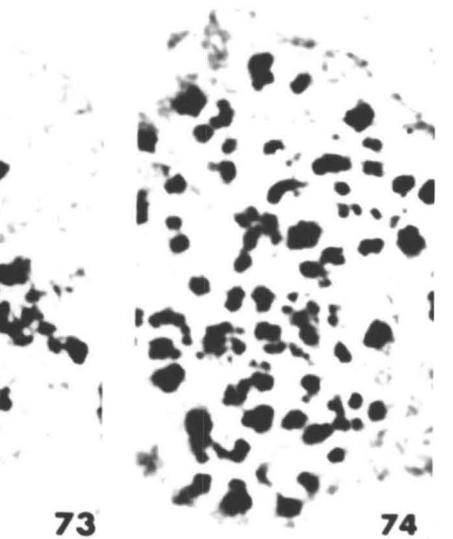
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74

Along with the chromosome aberrations, Ad.2, Ad.4 or SA7 infection of BHK21 cultures appeared to affect adversely the mitotic apparatus of the cells. Even without colchicine pretreatment, the contracted chromosomes at metaphase remained scattered throughout the cells for a prolonged time (Fig. 70), resulting in the accumulation of cells with the characteristic appearance of C-mitosis. In addition, a high frequency of micronucleated cells (Figs. 56, 73, 85), formed probably as a result of chromosomes entering interphase directly from "metaphase" (Figs. 73, 84), was observed in cultures infected with Ad.2 or Ad.4, and especially in cultures infected with SA7. This latter phenomenon again closely resembles that found in colchicine-treated cells.

(b) Influence of Virus Dose

Chromosome lesions induced in hamster cells (BHK21) after infection with adenoviruses types 2, 4, 7, 12, 18, SV15 or SA7 at 25, 5 or 1 TCID<sub>50</sub> per cell were tabulated by enumerating metaphase plates showing chromatid breaks, chromosome fragmentation, overcontraction and erosion of the chromosome complement (Table V). A high frequency of chromosome aberrations was found in each culture infected with adenovirus, and each adenovirus tested, even in cultures receiving

- Figs. 75-86 Chromosome aberrations induced in cultured Syrian hamster cells (BHK21) 27 hours after infection with SA7 at 25 TCID<sub>50</sub>/cell.
- Fig. 75 Part of a metaphase plate showing a single chromatid break. X1800
- Fig. 76 Part of a metaphase plate demonstrating multiple chromatid breaks. X1600
- Fig. 77 Part of a fragmented metaphase plate. X1800
- Fig. 78 Dicentric chromosome. X2000
- Fig. 79 Part of a metaphase plate containing two ring chromosomes. X2000
- Fig. 80 Chromatid exchange figure. X2500
- Fig. 81 Metaphase plate showing overcontraction of chromosome complement. X1000
- Fig. 82 Part of a metaphase plate showing erosion of several chromosome arms. X3500
- Fig. 83 Part of a metaphase plate showing erosion of most of the chromosomes. X1800
- Fig. 84 Metaphase plate showing erosion of chromosomes and illustrating possible method of formation of micronuclei. X1000
- Fig. 85 Interphase cell with many large micronuclei. X1000
- Fig. 86 Part of a metaphase plate with erosion of several chromosome arms (arrows) and showing overcontracted chromosomes and fragments. X1800



77

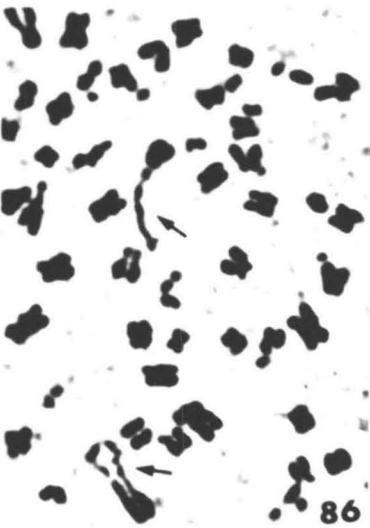
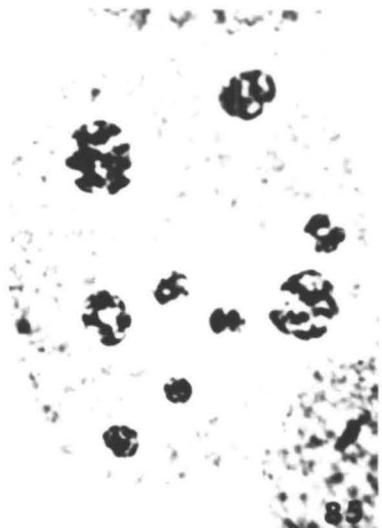
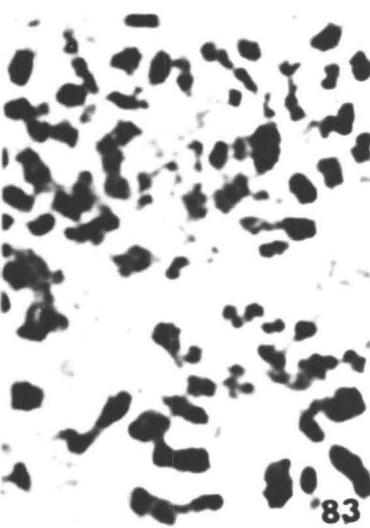
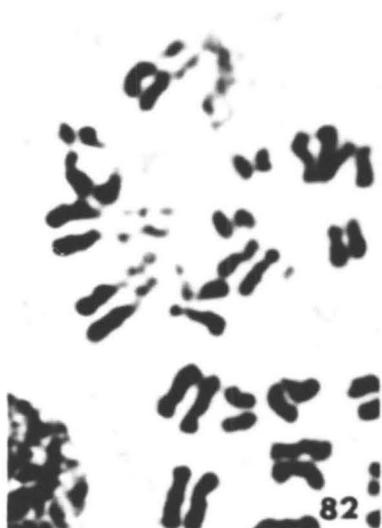
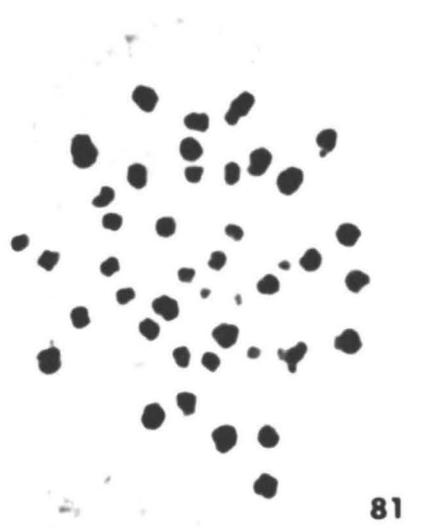


TABLE V

Effect of Adenovirus Dose on the Type of Chromosome Aberrations Induced  
in BHK21 cells 27 Hours Post-Infection

Type of Aberrations (% of Metaphases Examined) :150 metaphases examined per sample

Virus	Dose	Chromatid Breaks		Coiling Anomalies		Total Aberrations
		1-15	>15 (fragmentation)	Overcontraction	Erosion	
Ad.12	25x	38.2	17.3	0	0.9	56.4
	5x	18.2	1.8	0	0.9	20.9
	1x	9.6	0.8	0	0	10.4
	Control	4.0	0	0	0	4.0
Ad.18	25x	41.7	38.9	2.1	0.7	83.4
	5x	26.6	5.1	1.2	0	32.9
	1x	14.9	0.6	0.6	0	16.1
	Control	4.0	0	0.8	0	4.8
Ad.7	25x	16.7	8.7	8.0	5.3	38.7
	5x	9.6	1.6	0.8	1.6	13.6
	1x	7.2	0	0.8	0	8.0
	Control	4.0	0	0	0	4.0
SV15	25x	16.5	20.0	8.6	9.0	54.1
	5x	12.7	7.3	0	2.7	22.7
	1x	11.3	0.7	0	2.0	14.0
	Control	5.6	0	0	0	5.6
Ad.2	25x	24.0	8.0	12.0	8.8	52.8
	5x	12.0	0.7	3.3	2.0	18.0
	1x	8.0	0	4.0	0	12.0
	Control	4.0	0.7	0	0	4.7
Ad.4	25x	18.9	25.2	24.3	20.7	89.1
	5x	13.0	2.0	12.0	8.0	35.0
	1x	11.3	0	2.7	2.0	16.0
	Control	4.7	0	0.7	0	5.3
SA7	25x	10.5	5.2	43.3	19.0	78.0
	5x	14.3	4.0	14.2	5.3	37.8
	1x	8.0	0.8	4.0	3.2	16.0
	Control	6.0	0.7	0.7	0	7.4
*Control		4.0-6.0	0-0.7	0-0.7	0	4.0-7.4

\* Range of values from 7 control, uninfected experiments.

the low input multiplicity of 1 TCID<sub>50</sub> per cell, induced substantially more chromosome aberrations than could be detected in comparable uninfected cultures.

Table V further reveals that the total incidence of metaphase plates showing chromosome aberrations increased with increasing virus dose. This was evident whether the aberrations involved primarily chromatid breakage and fragmentation, for example, with Ad.12 or Ad.18, or also involved large amounts of overcontraction and erosion of the chromosome complements, as with Ad.4 or SA7.

Metaphase plates showing chromatid breaks were further grouped into four categories, according to the number of breaks per chromosome complement: one break; two to five breaks; six to fifteen breaks; and extreme fragmentation of the chromosomes (more than fifteen breaks per metaphase plate). Chromosome complements displayed progressively more chromatid breaks with increasing virus dose (Table VI); the category "fragmentation" makes this particularly evident for each virus examined, both in terms of absolute numbers of metaphases (Table VI) and in terms of the proportion of total aberrations constituted by "fragmentation" (Table VII). Not only chromosome breakage but also coiling anomalies (overcontraction and erosion) increased with greater virus input (Table VII). In cultures infected

TABLE VI

Influence of Virus Dose on Degree of Damage Per Metaphase Plate  
Showing Chromatid Breaks (% of Metaphases Examined)

Virus	Dose	1 Break	2-5 Breaks	6-15 Breaks	1-15 Breaks	>15 breaks (fragmentation)
Ad.12	25x	9.0	22.5	6.7	38.2	17.3
	5x	10.0	7.2	1.0	18.2	1.8
	1x	5.6	2.4	1.6	9.6	0.8
Ad.18	25x	6.0	9.7	26.0	41.7	38.9
	5x	5.6	17.0	4.0	26.6	5.1
	1x	8.2	6.7	0	14.9	0.6
Ad.7	25x	3.3	10.7	2.7	16.7	8.7
	5x	4.0	5.6	0	9.6	1.6
	1x	3.2	3.2	2.8	9.2	0
SV15	25x	3.8	10.3	2.4	16.5	20.0
	5x	4.7	6.7	1.3	12.7	7.3
	1x	4.0	6.0	1.3	11.3	0.7
Ad.2	25x	5.7	12.6	5.7	24.0	8.0
	5x	4.9	4.9	2.2	12.0	0.7
	1x	4.0	4.0	0	8.0	0
Ad.4	25x	2.7	11.7	4.5	18.9	25.2
	5x	3.7	7.3	2.0	13.0	2.0
	1x	6.3	5.0	0	11.3	0
SA7	25x	2.2	6.6	1.7	10.5	5.2
	5x	2.2	11.4	0.7	14.3	4.0
	1x	5.6	1.6	0.8	8.0	0.8

with Ad.7 or Ad.2, for example, erosion of the chromosomes was observed only in those cultures exposed to input multiplicities of 25 and 5. Similarly, in cultures infected with SV15, overcontraction of the chromosome complements occurred exclusively in those cultures receiving the highest viral dose, 25 TCID<sub>50</sub> per cell.

Interesting variations are revealed by Table VII in the proportions of the different categories of chromosome aberrations induced by the different types of adenoviruses. Ad.12 and Ad.18 induced almost exclusively chromatid breaks and fragmentation at each input multiplicity of infection. Although adenoviruses other than Ad.12 or Ad.18 also induced predominantly chromatid breaks and fragmentation, coiling anomalies were also elicited in varying amounts. The proportion of aberrations due to overcontraction and erosion was relatively minor at the low input multiplicities with Ad.7 and SV15, and increased with greater virus input to reach almost 35% of the total aberrations in cultures exposed to the highest virus dose. However, in BHK21 cultures infected with Ad.2, Ad.4, or SA7 the proportion of aberrations represented by coiling anomalies was as high even at low multiplicities as that induced by the highest multiplicity of SV15 and Ad.7. Coiling

TABLE VII

Effect of Adenovirus Dose on the Proportion of Types of Chromosome Aberrations Induced in BHK21 Cells 27 Hours Post-Infection

Virus	Dose	Type of Aberrations (% of total aberrations)		Total Breaks	Coiling Anomalies	
		1-15	>15 fragmentation		Overcontraction	Erosion
Ad.12	25x	67.7	30.7	98.4	0	1.6
	5x	87.1	8.6	95.7	0	4.3
	1x	92.3	7.7	100.0	0	0
Ad.18	25x	50.0	46.7	96.7	2.5	0.8
	5x	80.9	15.5	96.4	3.6	0
	1x	92.6	3.7	96.3	3.7	0
Ad.7	25x	43.2	22.4	65.6	20.7	13.7
	5x	70.7	11.7	82.4	5.9	11.7
	1x	90.0	0	90.0	10.0	0
SV15	25x	30.5	37.0	67.5	15.9	16.6
	5x	56.0	32.1	88.1	0	11.9
	1x	80.7	5.0	85.7	0	14.3
Ad.2	25x	45.5	15.1	60.6	22.7	16.7
	5x	66.7	3.9	70.6	18.3	11.1
	1x	66.7	0	66.7	33.3	0
Ad.4	25x	21.2	28.3	49.5	27.3	23.2
	5x	37.1	5.8	42.9	34.3	22.8
	1x	70.6	0	70.6	16.9	12.5
SA7	25x	13.4	6.7	20.1	55.5	24.4
	5x	37.8	10.6	48.4	37.6	14.0
	1x	50.0	5.0	55.0	25.0	20.0
*Control		100-86.5	0-9.4		0-9.4	0

\* Range of values from 7 control uninfected experiments.

anomalies then increased with greater virus input to become the preponderant types of chromosome aberrations with Ad.4 and particularly with SA7.

It is evident, therefore, that not only the total incidence of metaphase plates showing chromosome aberrations but also the types of aberrations and their proportions depended on the virus dose inoculated into BHK21 cultures. Moreover, the proportions of the different categories of chromosome damage induced by comparable input multiplicities depended upon the type of adenoviruses employed.

(c) Influence of Sampling Time

The observed incidence of chromosome aberrations induced by adenovirus infection of BHK21 cultures was markedly affected by the sampling time after infection (Figs. 87-93). With one exception (SA7), the viruses utilized in these studies induced maximum aberrations at 27 hours after infection; the maximum incidence of chromosome aberrations induced by SA7, however, occurred at 36 hours post-infection. These observations suggest that the majority of chromosome damage was observed in cells in their first and possibly second mitotic cycle after infection. The incidence of chromosome aberrations observed at subsequent sampling times, however, varied widely according to the strain of virus used.

Fig. 87      Effect of time of sampling on the frequency of metaphase plates showing chromosome aberrations after infection of BHK21 cultures with Ad.12 at 25, 5 and 1 TCID<sub>50</sub> per cell.

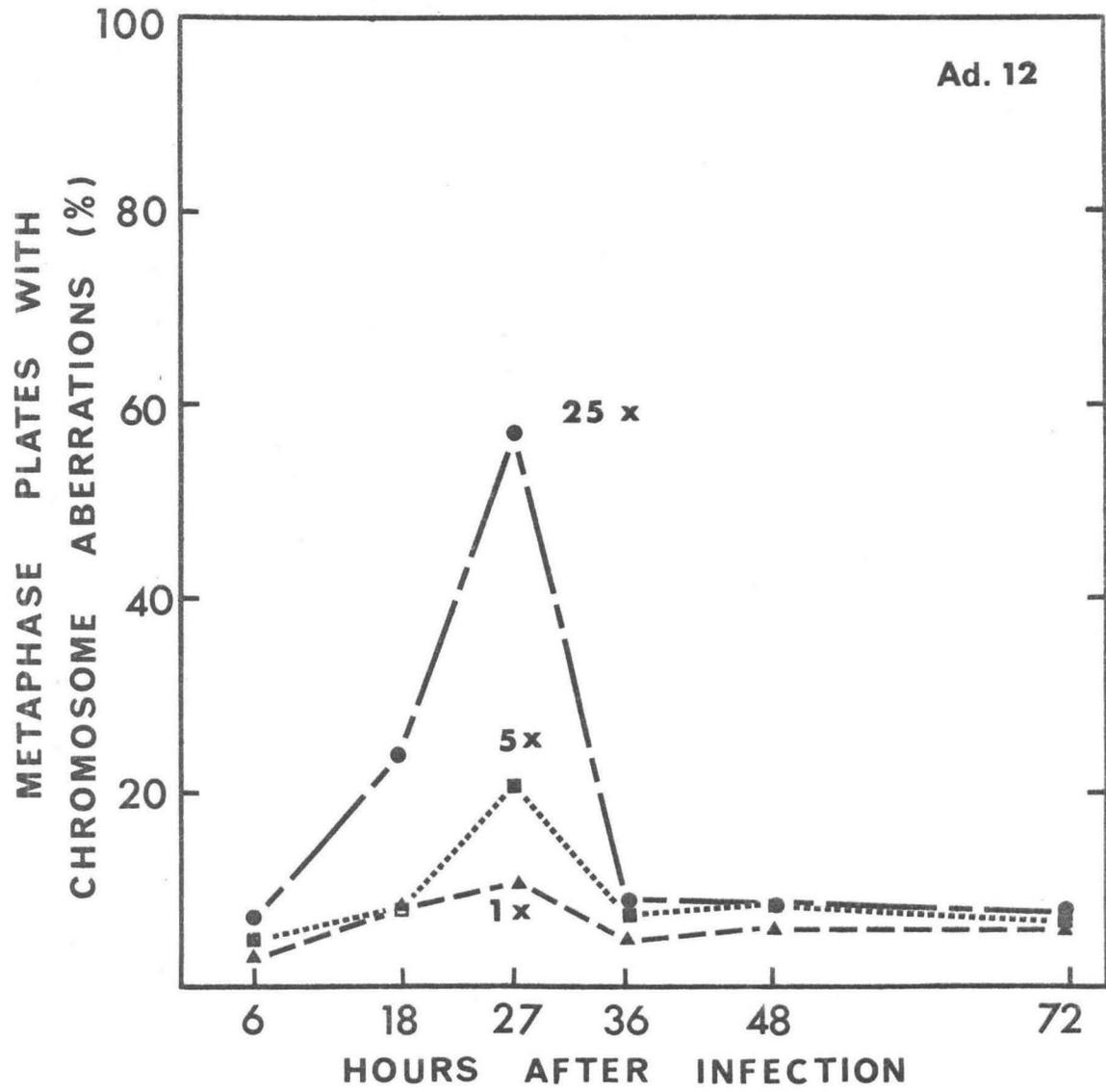
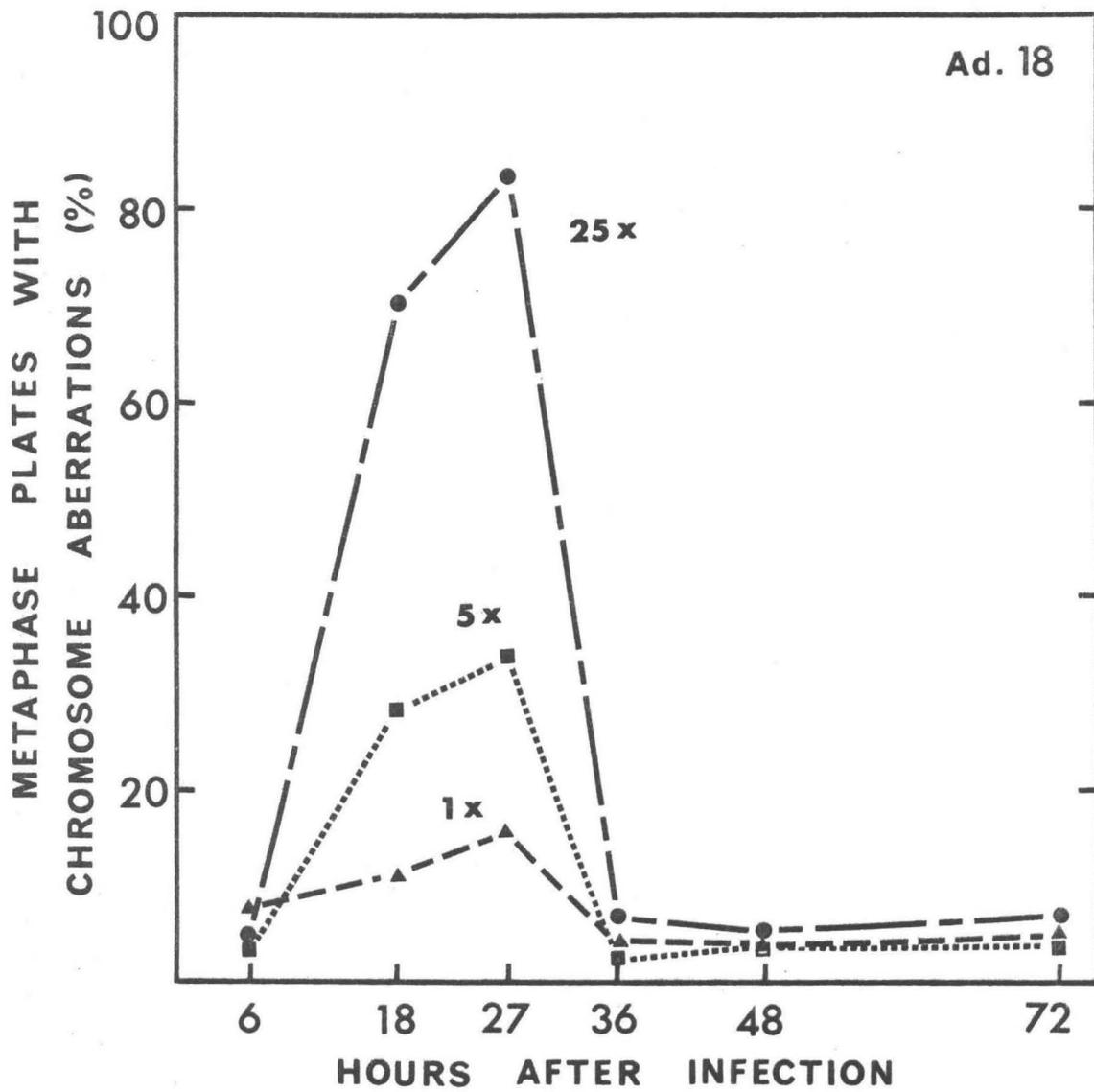


Fig. 88      Effect of time of sampling on the frequency of metaphase plates showing chromosome aberrations after infection of BHK21 cultures with Ad.18 at 25, 5 and 1 TCID<sub>50</sub> per cell.



A particularly interesting effect of the time of sampling is evident from cultures infected with Ad.12 or Ad.18 (Figs. 87,88). With each input multiplicity of virus, maximum levels of chromosome aberrations were detected at 27 hours post-infection, but by the next sampling time, the virus-induced aberrations had virtually disappeared. Indeed, in cultures exposed to the 25x input multiplicity the decrease from the maximum level of aberrations at 27 hours was especially precipitous.

The decrease from the maximum level of chromosome aberrations was not nearly so rapid in cultures infected with adenoviruses other than Ad.12 or Ad.18 (Figs. 89-93). Aberrations decreased to near control levels only slowly (Ad.7, SV15), or remained near the maximum levels for a prolonged time (Ad.2, Ad.4). The observed variations in the incidence of chromosome damage with time after infection in part depended upon the virus dose. Cultures infected with SV15 at 1 TCID<sub>50</sub> per cell, for example, showed that the incidence of chromosome aberrations reached control levels by 48 hours after infection while at 5x input multiplicity the aberrations decreased to control levels only by 72 hours. Moreover, in cultures exposed to 25 TCID<sub>50</sub> per cell the frequency of metaphases with chromosome aberrations was very high at 48 hours,

Fig. 89      Effect of time of sampling on the frequency  
of metaphase plates showing chromosome  
aberrations after infection of BHK21  
cultures with Ad.7 at 25, 5 and 1 TCID<sub>50</sub>  
per cell.

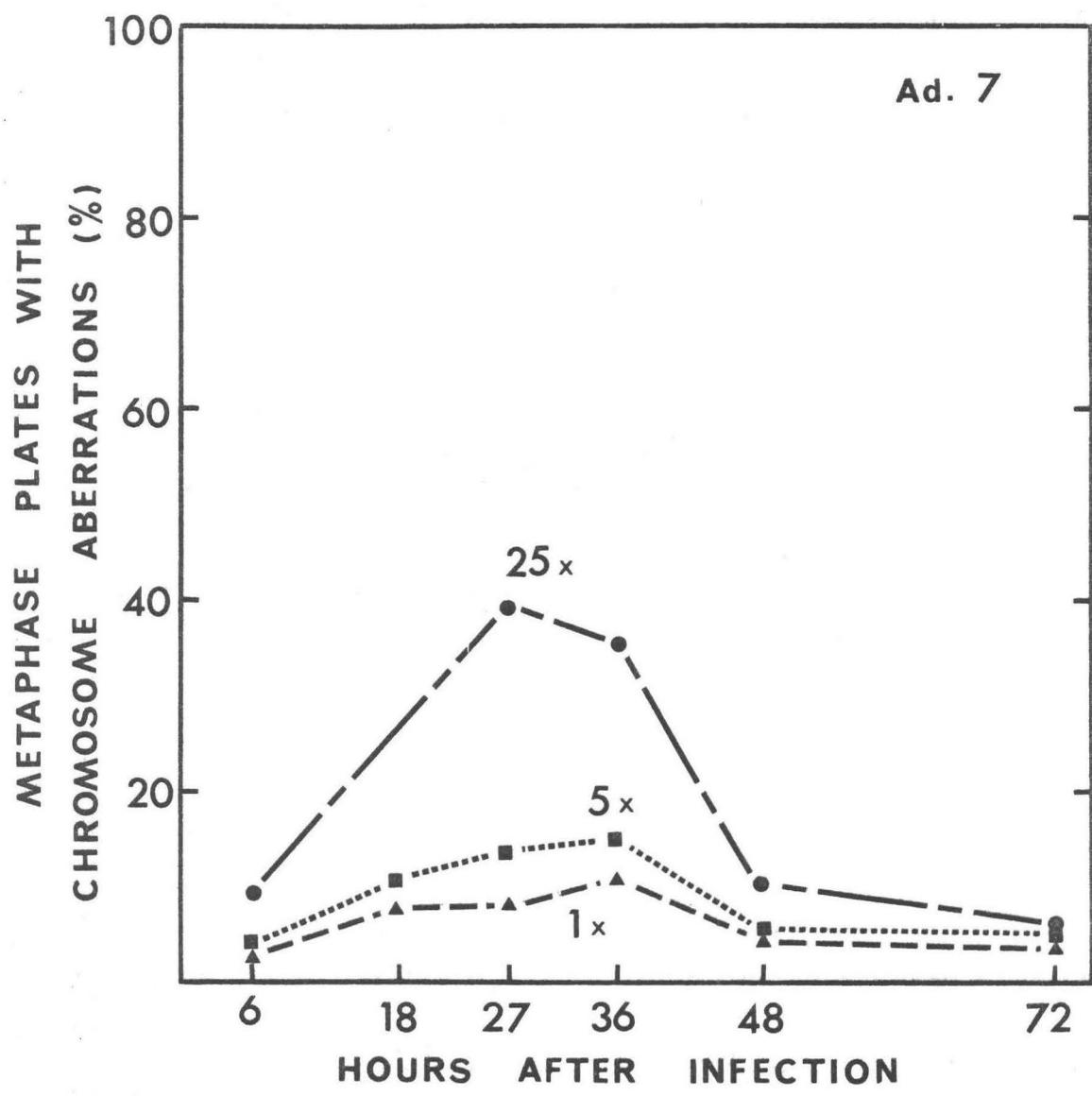


Fig. 90      Effect of time of sampling on the frequency of metaphase plates showing chromosome aberrations after infection of BHK21 cultures with SV15 at 25, 5 and 1 TCID<sub>50</sub> per cell.

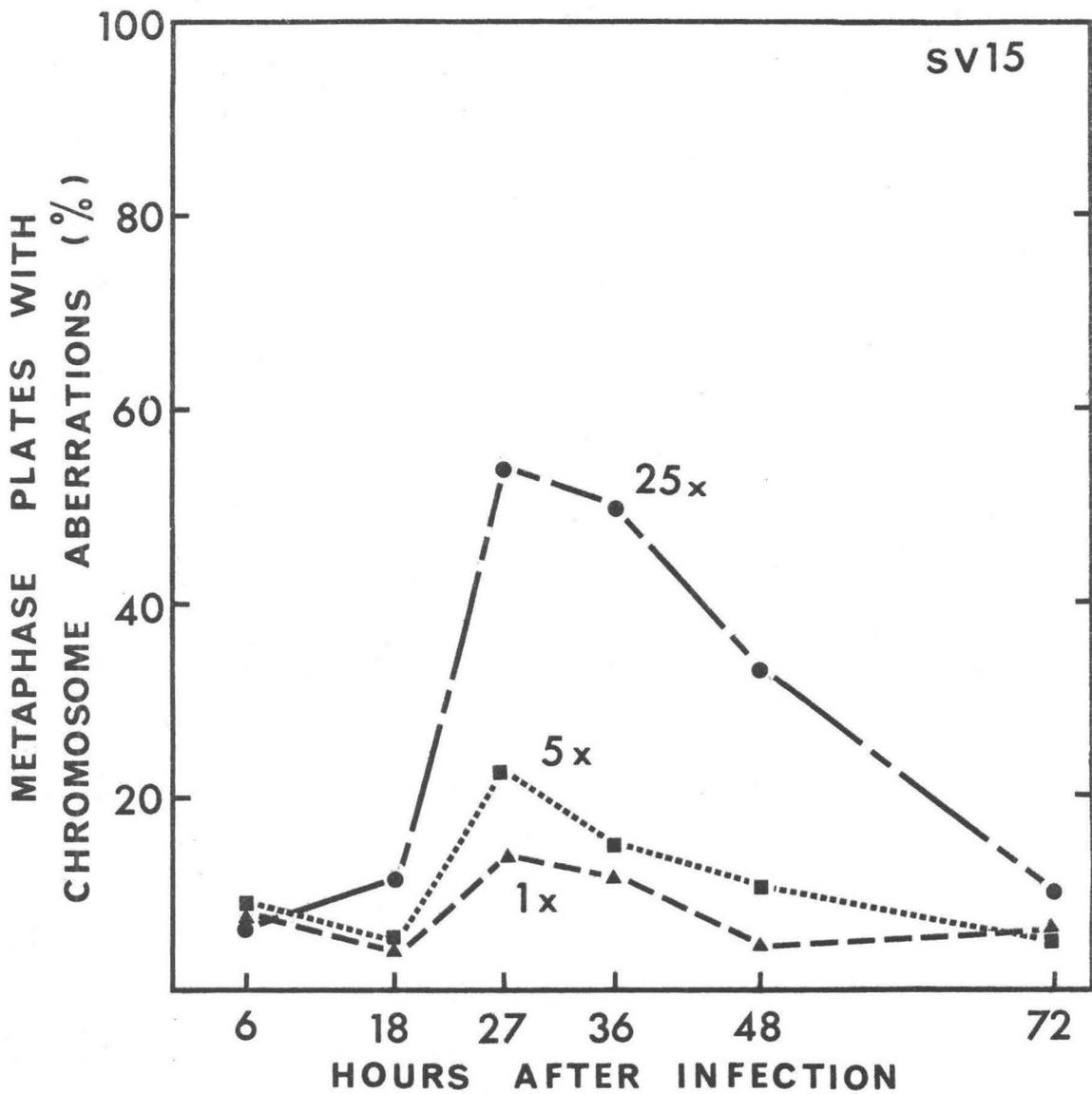


Fig. 91      Effect of time of sampling on the frequency  
of metaphase plates showing chromosome  
aberrations after infection of BHK21  
cultures with Ad.2 at 25, 5 and 1 TCID<sub>50</sub>  
per cell.

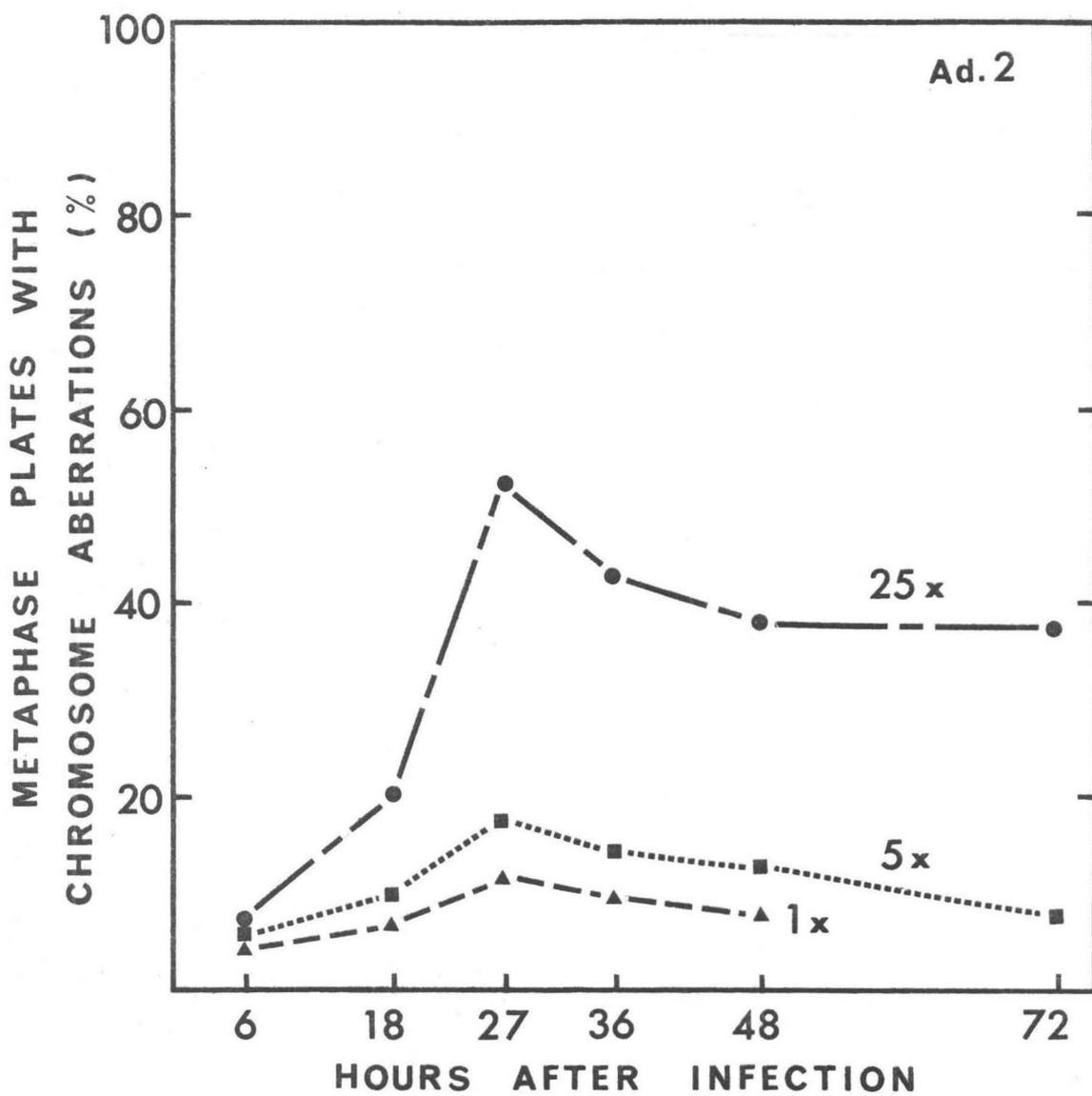


Fig. 92      Effect of time of sampling on the frequency  
of metaphase plates showing chromosome  
aberrations after infection of BHK21  
cultures with Ad.4 at 25, 5 and 1 TCID<sub>50</sub>  
per cell.

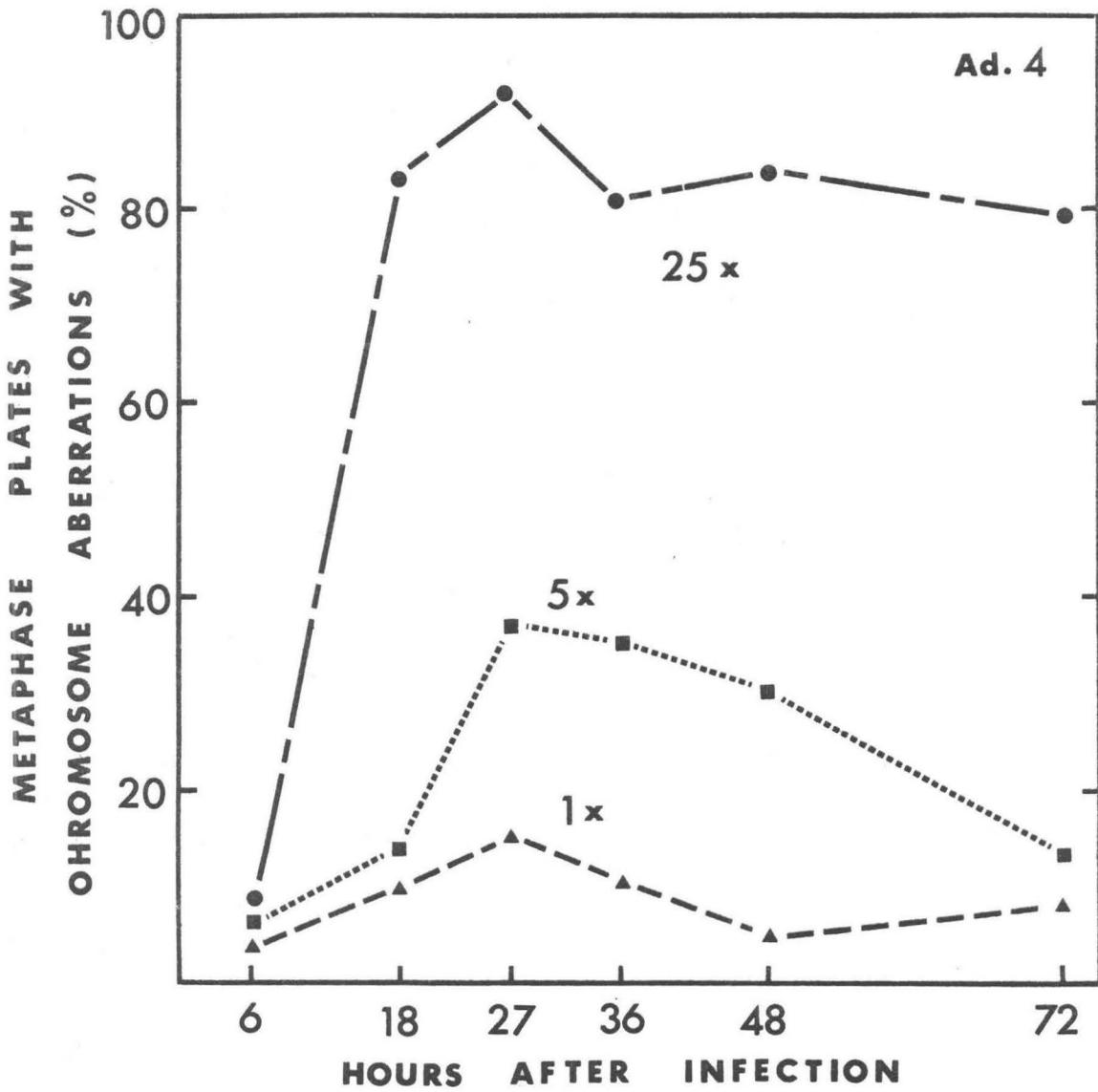
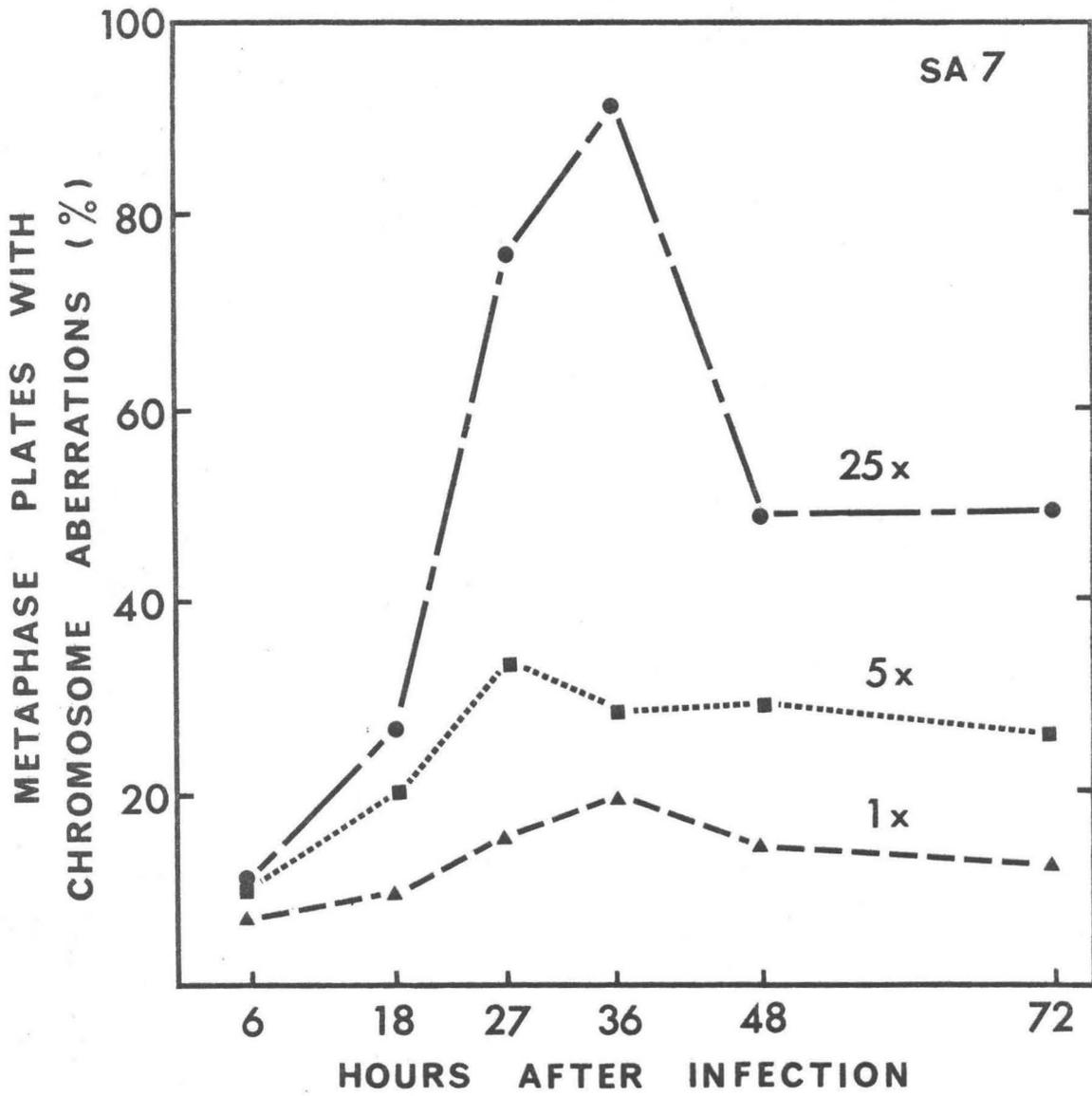


Fig. 93      Effect of time of sampling on the frequency of metaphase plates showing chromosome aberrations after infection of BHK21 cultures with SA7 at 25, 5 and 1 TCID<sub>50</sub> per cell.



and, although decreasing rapidly, was still substantially greater than that in uninfected cultures 72 hours after infection.

The level of chromosome aberrations in cultures infected with Ad.2 or Ad.4 (Figs. 91,92) similarly declined to near control levels by 48 hours for 1x input multiplicity, and by 72 hours for 5x input multiplicity. At 25 TCID<sub>50</sub> per cell, however, the incidence of aberrations remained near the maximum even to 72 hours after infection.

The relationship between the incidence of chromosome damage induced by the different adenoviruses and the time of sampling can be compared among cultures infected with comparable input multiplicities of 25 TCID<sub>50</sub> per cell (Fig. 94). The rapid decrease in the aberration level by 36 hours after infection with Ad.18 or Ad.12 (Figs. 94a,b) was due to the virtual disappearance by that time of the two categories of aberrations, chromatid breaks and fragmentation, which almost exclusively constituted the total aberrations. In cultures infected with other adenoviruses, the decrease in chromatid breaks to control levels usually occurred slightly later, by 48 hours after infection. The incidence of fragmented metaphase plates, however, declined only by 48 hours (Fig. 94c) or 72 hours (Fig. 94d), or remained high for

Fig. 94            Effect of time of sampling on the incidence  
                    of metaphase plates showing chromatid  
                    breaks, fragmentation, overcontraction or  
                    erosion of the chromosomes after infection  
                    of BHK21 cultures with adenoviruses.

94a            Ad.18

94b            Ad.12

94c            Ad.7

94d            SV15

94e            Ad.2

94f            Ad.4



a prolonged time (Figs. 94e,f). Similarly, the incidence of overcontraction and erosion decreased markedly by 48 hours (Fig. 94c) or 72 hours (Fig. 94d), or remained very high, at least to 72 hours (Figs. 94e,f). This latter phenomenon is characteristic of colchicine-treated cultures.

In summary, the data for the different types of adenoviruses suggest: firstly, that different proportions of the constituent types of chromosome damage were induced at the time of maximum aberration level; secondly, that the constituent types of chromosome aberrations decreased by different amounts after infection; thirdly, that there was a variation in the incidence of chromosome aberrations with time.

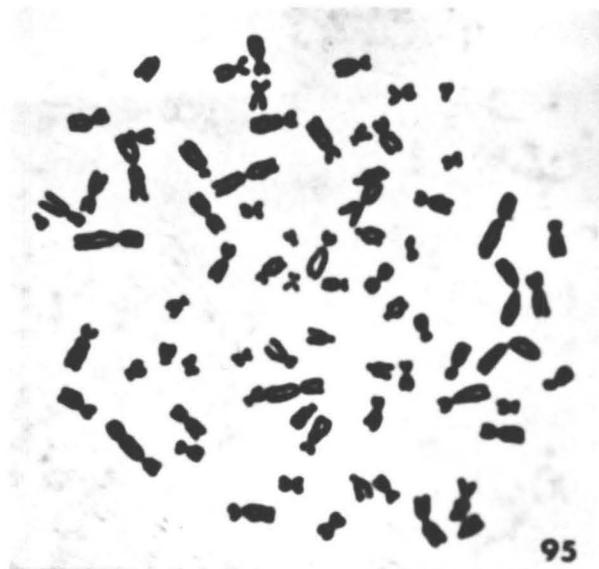
### 3. Effect of Adenoviruses on Chromosomes of Human Cells

Infection of the human amnion cell line AV3 with the adenoviruses used in this study resulted, with each virus, in progressive reduction in the mitotic index leading to eventual cessation of mitosis approximately three days after virus inoculation (Table VIII). Examination of metaphase plates (Fig. 95) prior to this virus-induced cessation revealed no detectable increase in the level of chromosome aberrations over that observed in uninfected cultures (Table IX). Unlike the wide array of chromosome aberrations found in adenovirus-infected

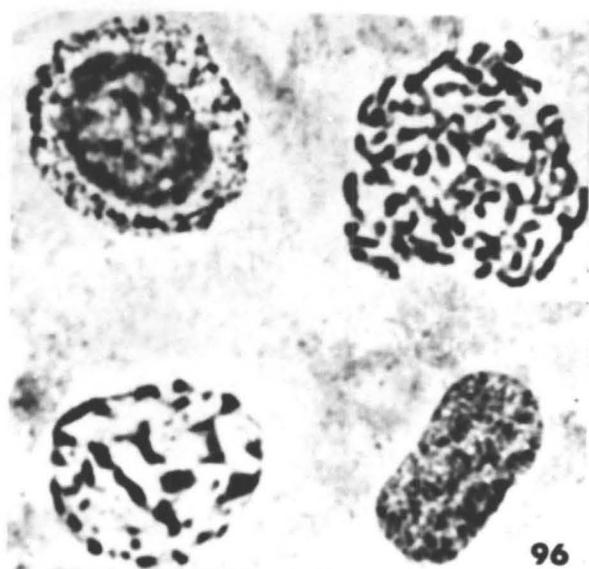
BHK21 cultures, the aberrations detected in adenovirus-infected AV3 cultures consisted principally of chromatid breaks, as did the aberrations in uninfected AV3 cultures. This observation, together with the progressive decline in mitotic index evidenced by infected cultures, makes it apparent that adenovirus-infected AV3 cells probably failed to enter mitosis.

AV3 cultures infected with adenovirus revealed many interphase nuclei containing intranuclear inclusion bodies (Figs. 97-102) and the incidence of the inclusion bodies increased with time (Table X). In addition, many interphase nuclei showed various degrees of chromatin condensation (Figs. 103-105) and formation of micronuclei (Fig. 106), especially in virus-infected cultures sampled 72 hours after infection. Frequently, normal interphase nuclei, chromosomes arrested at metaphase by colchicine, nuclei showing chromatin condensation, and nuclei containing inclusion bodies lay side by side (Fig. 96) in the same preparation; these phenomena were obviously not merely artefacts of treatment of the preparation.

- Figs. 95-106 Cultured human amnion cells (AV3) infected with adenoviruses at 5 TCD<sub>50</sub>/cell and sampled 48 hours later.
- Fig. 95 Metaphase plate from AV3 culture infected with Ad.2, exhibiting no viral-induced chromosome aberrations. X1500
- Fig. 96 Culture of AV3 cells infected with Ad.18; note the apparently normal interphase cell (lower right), an apparently normal metaphase (upper right), an interphase cell showing chromatin condensation (lower left), and a cell with a large intranuclear inclusion body (upper left). X800
- Figs. 97-102 Various examples of intranuclear inclusion bodies in adenovirus-infected AV3 cultures. X1200
- Fig. 97 Infected with Ad.7.
- Fig. 98 Infected with SV15.
- Fig. 99 Infected with Ad.12.
- Fig. 100 Infected with Ad.18.
- Fig. 101 Infected with Ad.4.
- Fig. 102 Infected with SA7.
- Figs. 103-105 Several examples of interphase nuclei showing chromatin condensation from adenovirus-infected AV3 cultures. X1200
- Fig. 103 Infected with Ad.18.
- Fig. 104 Infected with Ad.12.
- Fig. 105 Infected with Ad.4.
- Fig. 106 Interphase nucleus exhibiting micronuclei, from AV3 culture infected with Ad.18. X1200



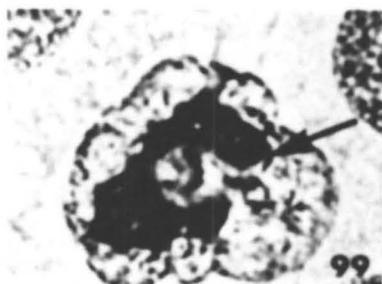
95



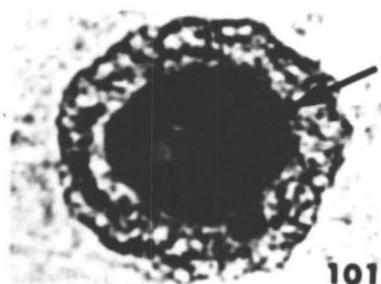
96



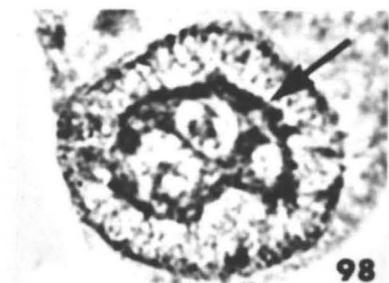
97



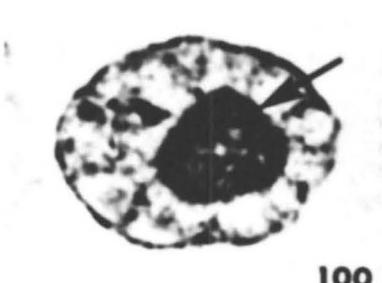
99



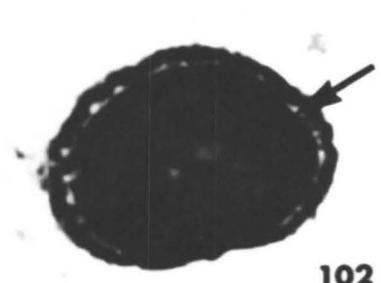
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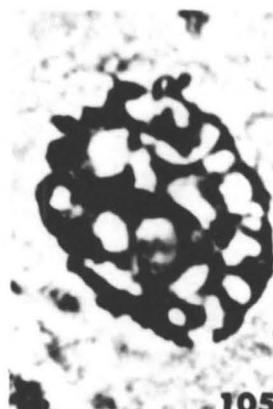
102



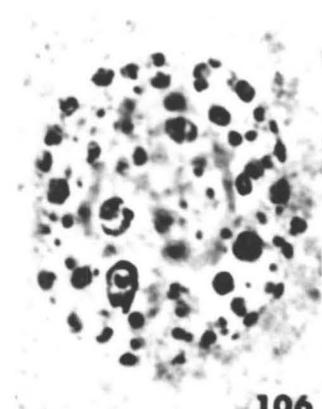
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105



106

TABLE VIII

Incidence of Mitoses (%)<sup>a</sup> in Cultured AV3 Cells Infected with Adenoviruses; Colchicine-treated, 0.2 µg./ml. for 1 1/4 Hours

Hours Post-Infection	Ad.2	Ad.4	Ad.7	Ad.12	Ad.18	SA7	SV15
Infected Cultures							
24	5.3	3.6	2.4	4.3	4.8	3.5	5.9
48	2.3	1.8	1.8	1.0	4.4	1.8	3.3
72	1.0	1.1	0.8	0	1.0	0.2	2.3
Uninfected Cultures							
24	5.2	4.5	6.2	4.5	5.2	4.5	5.2
48	4.2	3.5	5.8	3.5	4.2	3.5	4.2
72	6.1	3.7	4.8	3.7	6.1	3.7	6.1

<sup>a</sup>Based on examination of 2000 cells per sample

TABLE IX

Incidence (%)<sup>a</sup> of Metaphase Plates Showing Chromosome Aberrations  
in Cultured AV3 Cells: Input Multiplicity, 5 TCD<sub>50</sub>/Cell

Hours Post-Infection	Ad.2	Ad.4	Ad.7	Ad.12	Ad.18	SA7	SV15
Infected Cultures							
24	4.0	4.0	5.6	4.2	5.0	4.0	4.0
48	4.8	4.3	5.0	6.4	4.8	6.0	5.0
72	5.3	4.3	5.3	**	*	3.6	6.0
Uninfected Cultures							
24	5.0	4.8	6.4	4.8	5.0	4.8	5.0
48	4.0	7.0	5.6	7.0	4.0	7.0	4.0
72	5.6	4.0	6.4	4.0	5.6	4.0	5.6

\* Too few plates analysable.

\*\* No mitoses

<sup>a</sup>Based on examination of at least  
150 metaphases per sample

TABLE X

<sup>b</sup>  
Incidence (%) of Interphase Nuclei with Inclusion Bodies in  
Cultured AV3 Cells Infected with Adenoviruses

Hours Post-Infection	Ad.2	Ad.4	Ad.7	Ad.12	Ad.18	SA7	SV15
Infected Cultures							
24	1.6	0.6	1.6	0.7	1.0	1.4	2.7
48	9.3	4.4 ( 0.5) <sup>a</sup>	9.4	9.0	7.2	21.2	10.1
72	34.6 (6.4) <sup>a</sup>	15.6 (16.0) <sup>a</sup>	51.5 (3.5) <sup>a</sup>	32.4 (4.0) <sup>a</sup>	21.4 (8.4) <sup>a</sup>	62.0 (7.1) <sup>a</sup>	27.9 (6.0) <sup>a</sup>

<sup>a</sup> Incidence (%) of nuclei showing chromatin condensation or formation of micronulcei.

<sup>b</sup> 2000 cells examined per sample

### III Effect of Inactivated Adenoviruses on Chromosomes of Hamster Cells

The chromosome-damaging capacity of an adenovirus preparation may be not only due to the virus or some particular component of it, but also due to toxic or mutagenic products in the virus preparation, for example, residual components of disrupted cells or products formed in the course of viral replication. In an attempt to distinguish between the above possibilities, type-specific virus-neutralizing antisera were used to inactivate adenoviruses, and the chromosome-damaging capacities of such inactivated virus preparations were examined on BHK21 cells. Ad.18 and Ad.4 were chosen for these experiments, the former virus representing an adenovirus which has an effect on the chromosome complement simulating that of ionizing radiation, whereas the latter virus exerts a colchicine-like effect as well as radiomimetic effects.

#### 1. Experimental Design

Ad.18 and Ad.4 virus stocks were centrifuged at 100,000 x g for 90 minutes, thus sedimenting almost all of the virus. The supernatant fluid was removed from the pellet and retained, and the pelleted virus was resuspended in MEM without FCS. Appropriate amounts of the resuspended Ad.18 and Ad.4 stocks, preincubated with

either diluent (MEM without FCS) or antisera, were then added to BHK21 cultures in Leighton tubes to yield potential multiplicities of infection of 25 and 5 TCID<sub>50</sub> per cell. Virus adsorption was performed in the fashion previously described (p. 20), the major difference being that Leighton tubes were used in order to conserve antisera. In addition, tubes of BHK21 cells were inoculated with undiluted or 1:5 dilution of supernatant, corresponding to the fluid in 25x and 5x virus preparations, respectively. Cultures were incubated at 37°C and samples were taken 27 and 48 hours after inoculation. Coverslips with their adherent cells were treated for chromosome analysis (p. 30), and the chromosome aberrations were subsequently enumerated and tabulated (Table XI).

## 2. Virus Neutralization Experiments

That the chromosome aberrations induced in BHK21 cells in response to Ad.18 or Ad.4 infection were due to the virus is shown by the observations that only type-specific antibody effectively diminished the induction of chromosome aberrations, whereas antisera to an unrelated adenovirus did not (Table XI). Adenovirus 18 type-specific antisera completely abolished the effects of Ad.18 at both input multiplicities, while adenovirus 12 antibody moderated to a large extent the chromosome-damaging activity of Ad.18; this is a reflection of the known close

antigenic relatedness of Ad.12 and Ad.18 (117). Adenovirus type 4 antisera completely abolished not only the chromosome-damaging capacity but also the mitotic-arresting activity of Ad.4 at 5x input multiplicity, and reduced the activity of 25 TCID<sub>50</sub> dose of Ad.4 per cell by more than 60%. Antibody to adenovirus type 12, however, did not affect the ability of Ad.4 to induce chromosome aberrations and mitotic arrest.

Furthermore, it is demonstrated that no chromosome-damaging or toxic components were present in the virus stocks, other than viruses, since virus stock supernatants did not induce significant amounts of chromosome aberrations, neither from Ad.4 stocks nor from Ad.18 stocks. The chromosome aberrations induced by undiluted supernatant from Ad.18 preparations corresponding to 25 TCID<sub>50</sub> per cell, was less than the level of aberrations induced by preparations containing virus at 5x input multiplicity, and can be accounted for by a small amount of virus, less than 10%, remaining in the supernatant.

TABLE XI

Neutralization by Specific Antiserum of the Capacity of  
Adenovirus Types 18 and 4 to Induce Chromosome Aberrations  
in BHK21 Cells

Materials added to BHK21 Cells	Percent of metaphase plates with chromosome aberrations <sup>d</sup>			
	27 hrs. <sup>a</sup>		48 hrs. <sup>a</sup>	
	25 <sup>b</sup>	5 <sup>b</sup>	25	5
Ad.18 plus diluent	65.6	26.8	29.6	14.7
Ad.18 plus type 18 antibody	6.5	7.6	5.6	6.2
Ad.18 plus type 12 antibody	12.4	9.2	9.7	8.7
Ad.18 plus type 26 antibody	71.5	45.6	29.0	17.6
Virus-stock supernatant fluid <sup>c</sup>	17.5	8.0	9.9	7.4
Uninfected control	6.9	7.2	5.6	6.8
Ad.4 plus diluent	41.6	16.6	43.0	15.0
Ad.4 plus type 4 antibody	15.2	7.7	16.8	8.0
Ad.4 plus type 12 antibody	46.0	18.0	48.6	16.0
Virus-stock supernatant fluid <sup>c</sup>	8.3	6.4	6.4	6.4
Uninfected control	6.2	6.7	6.8	7.0

<sup>a</sup> Hours post-infection

<sup>b</sup> Potential multiplicity of infection.

<sup>c</sup> Fluid obtained from virus stocks centrifuged at 100,000 x g for 90 minutes.

<sup>d</sup> Based on examination of at least 150 metaphases per sample

#### IV Radioautography of Adenovirus 12 Effect on DNA Synthesis

A previous radioautographic investigation revealed that Ad.12 infection of human cells results in early suppression of DNA synthesis (71). This effect occurs in lytically-infected host cells, but the effects of Ad.12 during non-lytic infection were not compared. The question arose, whether early suppression of DNA synthesis was also a feature of Ad.12 infection of Syrian hamster cells, which are only abortively infected with Ad.12. The technique of incorporation of tritium-labelled thymidine coupled with radioautography was therefore used to compare the effects of Ad.12 infection on DNA replication in an established human cell line, AV3, and in a hamster cell line, EH.

Previous investigations have also established that Ad.12 infection of hamster cells induced large amounts of chromosome damage (128,203). Integration of the viral genome is thought to occur in Ad.12-induced hamster tumor cells (84,106,128), and the viral-induced chromosome breakage has been suggested as providing the opportunity for such integration to occur (203). If this were true, then detection of abnormal patterns of DNA replication, perhaps associated with the sites of

chromosome breakage, might be an effective means of demonstrating the importance of viral-induced chromosome breakage in oncogenesis. Deterioration of the orderly replication of the chromosome has, in fact, been suggested as a factor in the initiation of cancer (224). Therefore, metaphase plates of hamster cells were examined by radioautography to determine both the normal pattern of DNA replication in the chromosomes of uninfected cells, and whether the pattern was disturbed in the Ad.12-infected cells.

#### 1. Experimental Design

Primary cultures of Syrian hamster embryo cells (EH), initiated as described previously, were subcultured through two passages and were then planted into 3 oz. prescription bottles at  $5 \times 10^5$  cells per bottle. Serially subcultured human amnion cells (AV3) were also seeded into 3 oz. bottles at  $5 \times 10^5$  cells per bottle. Twenty-four hours after planting, the monolayer cultures sparsely covered the glass bottom of the culture bottles. The AV3 and the EH cultures were rinsed twice with their appropriate culture medium without FCS, and each culture was then inoculated either with 1.8 ml. of medium without FCS or with 1.5 ml. of medium lacking FCS plus 0.3 ml. of human adenovirus type 12. Those cultures receiving Ad.12 were therefore exposed to an input multiplicity of

4°C, and developed in Kodak D-19.

2. Effect of Ad.12 on DNA Synthesis of Human and Hamster Cells

Microscopic examination of the slides from cultures infected with Ad.12 and from uninfected cultures showed cells with silver grains localized over the nucleus. In addition, both infected and uninfected EH cultures showed mitoses, which had been arrested at metaphase by colchicine, with label localized over the chromosomes (Figs. 107-110).

It is known that thymidine is a specific precursor of DNA (56); according to the technique of radioautography employed, localization of label (silver grains) therefore closely represents the site of incorporation of tritiated thymidine into newly-synthesized DNA (29).

Examination of the proportion of labelled interphase nuclei and of the proportion of cells in mitosis from cultures without colchicine pretreatment revealed several interesting effects due to adenovirus infection (Table XII). AV3 cultures infected with Ad.12 showed decreased proportion of cells synthesizing DNA. However, by 48 hours after infection, the proportion of cells incorporating tritiated thymidine was much greater than in uninfected cultures. In addition, there was an inhibitory effect on AV3 cell mitosis, leading to cessation of mitosis by 48 hours post-infection. These

TABLE XII

Thymidine-H<sup>3</sup> Uptake by Cultured Embryo Hamster Cells and  
AV3 Cells, 24 Hours After Exposure to Ad.12

Hours post-infection prior to incubation in H <sup>3</sup> -TdR for 1 hour	<u>Uninfected Cultures</u>		<u>Ad.12-Infected Cultures</u>		
	% cells labelled <sup>a</sup>	mitotic coefficient <sup>b</sup>	% cells labelled <sup>a</sup>	mitotic coefficient <sup>b</sup>	Inclusion <sup>a</sup> bodies (%)
	<u>EH Cells</u>				
12	26.0	0.5%	30.0	0.6%	0
24	17.5	0.4%	37.7	1.0%	0
36	15.0	0.9%	23.3	1.1%	0
48	23.0	1.0%	28.0	0.6%	0
	<u>AV3 Cells</u>				
12	27.0	2.7%	22.2	1.7%	0
24	35.3	2.0%	23.0	0.1%	5.6%
36	26.3	2.1%	25.3	0.1%	28%
48	17.7	1.4%	41.0	0	79%

<sup>a</sup> 1000 cells examined

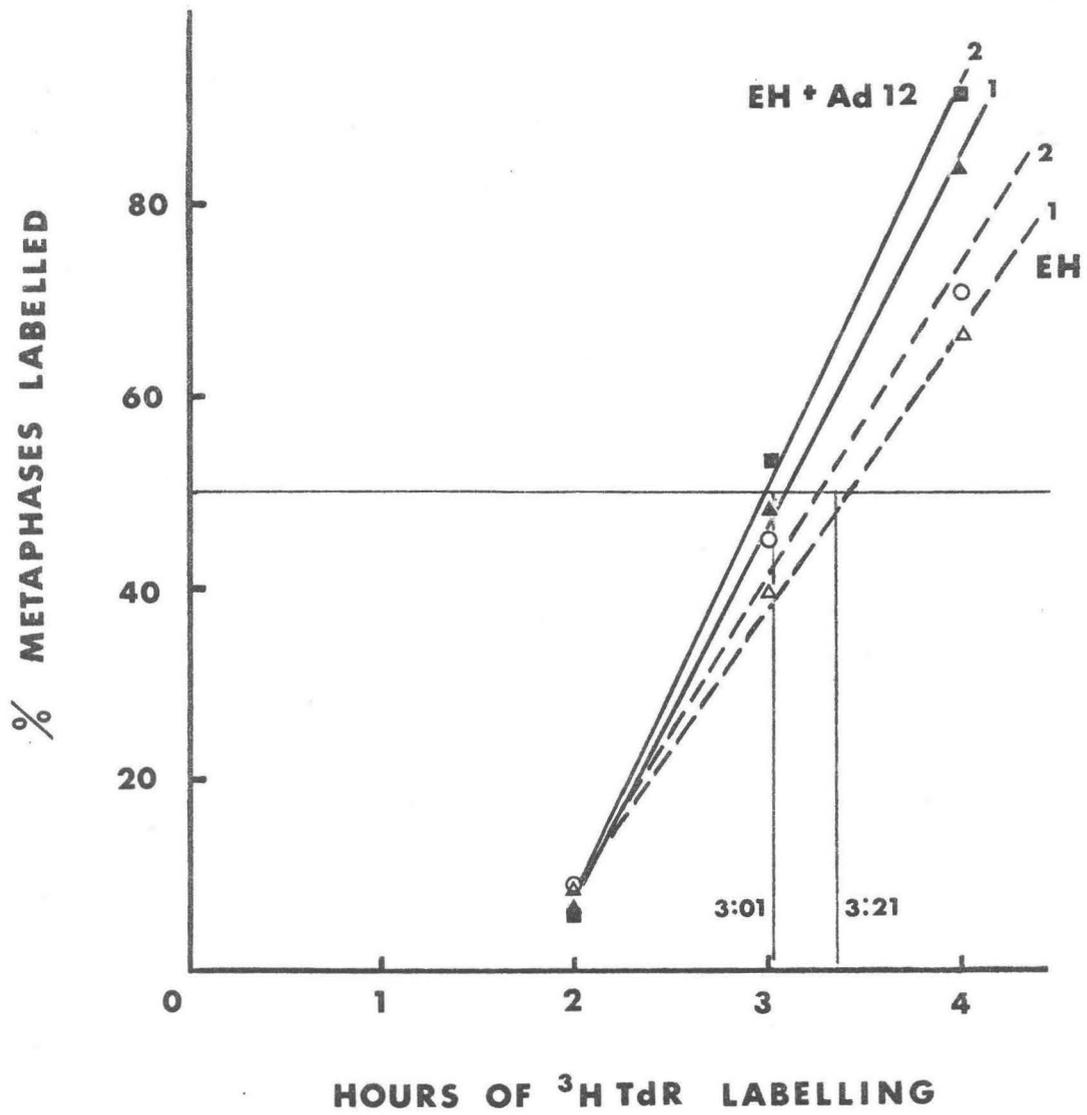
<sup>b</sup> 2000 cells examined

observations are in accord with the early cessation of mitosis (128) and suppression of host cell DNA synthesis (71) found in other investigations on Ad.12-infected AV3 cells.

EH cultures infected with Ad.12 showed increased proportion of cells incorporating tritiated thymidine, and in addition may have shown stimulated mitosis, in contrast to the effects observed in Ad.12-infected human cultures. Moreover, analysis of the mitoses from infected and uninfected EH cultures revealed that the average  $G_2$  period, as measured by the time of exposure to isotope by which 50% of the metaphases become labelled (169, 190), was slightly shorter in Ad.12-infected cultures than in uninfected cultures, in each of two experiments (Fig. 106a).

While uninfected AV3 cells showed readily detectable amounts of silver grains localized over their nuclei, Ad.12-infected cells exposed to the same isotope-containing medium were labelled with fewer grains. This, together with the background scatter of silver grains, made detection of DNA-synthesizing infected cells more difficult than detection of uninfected cells which incorporated isotope. This resulted in the anomalous observation that a higher proportion of infected cells showed inclusion bodies

Fig. 106a      Relative  $G_2$  periods of uninfected Syrian hamster cultures (EH) and cultures 24 hours after infection with Ad.12 (EH + Ad.12). Results from two experiments, expressed as per cent of metaphases labelled at various times after exposure to tritiated thymidine ( $^3\text{H-TdR}$ ). The time of 50% labelling represents the average  $G_2$  period.



than showed incorporation of tritiated thymidine.

The above results must therefore be considered only as the results of a preliminary investigation. Examination of the effects of Ad.12 infection on DNA biosynthesis has proven to be an investigation which necessitates a variety of new and more sensitive techniques for the detection of, and capable of distinguishing between, viral and host cell DNA synthesis. Accordingly, a far more detailed study would be necessary to elucidate the effects of adenovirus infection on DNA synthesis. Such an investigation lies beyond the scope of the present studies.

### 3. Effect of Ad.12 on Chromosome Replication in Hamster Cells

While the radioautographic technique is useful in determining that part of the cell cycle during which DNA synthesis occurs, it is also a technique which is sensitive enough to yield useful information concerning the chronology of chromosome replication. It has been well documented that asynchrony of duplication exists both between different chromosomes of the same complement (60,89,188,208) and between different parts of the same chromosome (31,33,59,188). It became of interest, then, to determine whether any differences existed between the chromosome replication pattern in uninfected EH cultures and those infected with Ad.12.

Examination of the labelled mitoses from EH cultures revealed variable amounts of silver grains associated with the metaphase chromosomes (Table XIII); some cells had heavy labelling over almost all of the chromosome complement, some displayed localized regions of heavy label on most chromosomes (Fig. 107), others showed well-defined areas of lighter label (Fig. 108) and still others had very small amounts of well-localized label (Figs. 109-110).

In order to determine the replication pattern, those metaphases with a relative absence of label from most chromosomes were analysed. These cells presumably must have been exposed to tritiated thymidine only towards the end of their DNA synthesis period. Suitably well-spread late-labelled metaphases were therefore photographed in transmitted light with oil-immersion optics, and the co-ordinates of the adjustable microscope stage were recorded for each cell in order to permit relocation of each metaphase plate in the future. The silver grains were bleached from the slides with Kodak Farmer's Reducer (1 minute at 23°C), and suitable mitoses were relocated and rephotographed using phase contrast optics (Figs. 111-114). Each of these latter photographs was used chiefly to aid in karyotyping the radioautograms of each cell.

Figs. 107-110 Examples of metaphases from Syrian hamster cultures showing various amounts of radioautographic labelling (silver grains) localized over the chromosomes, indicating DNA replication. X2000

Fig. 107 A metaphase plate showing very heavy labelling over most of the chromosomes; exposed to  $^3\text{H-TdR}$  for 4 hours before sampling.

Fig. 108 Example of a metaphase plate illustrating localized areas of heavy labelling associated with most of the chromosomes; 3 hours exposure to  $^3\text{H-TdR}$ .

Fig. 109 A metaphase plate demonstrating well-defined areas of moderate labelling over many of the chromosomes; exposed to  $^3\text{H-TdR}$  for 3 hours.

Fig. 110 An example of a metaphase plate showing extremely sparse labelling restricted to only a few chromosomes; 2 hours exposure to  $^3\text{H-TdR}$ .

TABLE XIII

Uptake of Tritiated-thymidine into Chromosomes of  
Cultured EH Cells, 24 Hours After Exposure to Ad.12

Hours of Incubation with H <sup>3</sup> -TdR	Metaphases Examined	Labelled <sup>d</sup> Metaphases (%)		Type of Labelling (% of Total Labelled Metaphases)		
		<u>1</u>	<u>2</u>	Intermediate <sup>a</sup>	Late <sup>b</sup>	Very Late <sup>c</sup>
Uninfected EH						
2	200	8.5	8.5	0	9	91
3	150	39.3	45.0	0	38	62
4	150	66.0	70.0	45	40	15
Ad.12-Infected EH						
2	200	6.5	6.5	0	0	100
3	200	48.0	53.6	0	55	45
4	200	83.5	92.0	50	44	6

<sup>a</sup> Heavy label over most of chromosome complement.

<sup>b</sup> Label in distinct segments over most chromosomes.

<sup>c</sup> Label in distinct segments over only few chromosomes.

<sup>d</sup> Results of two experiments.

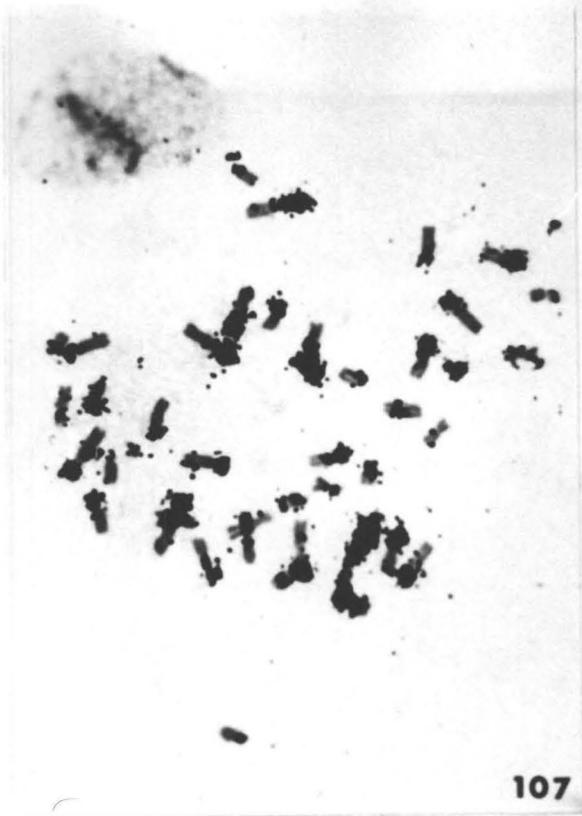
Figs. 107-110 Examples of metaphases from Syrian hamster cultures showing various amounts of radioautographic labelling (silver grains) localized over the chromosomes, indicating DNA replication. X2000

Fig. 107 A metaphase plate showing very heavy labelling over most of the chromosomes; exposed to  $^3\text{H-TdR}$  for 4 hours before sampling.

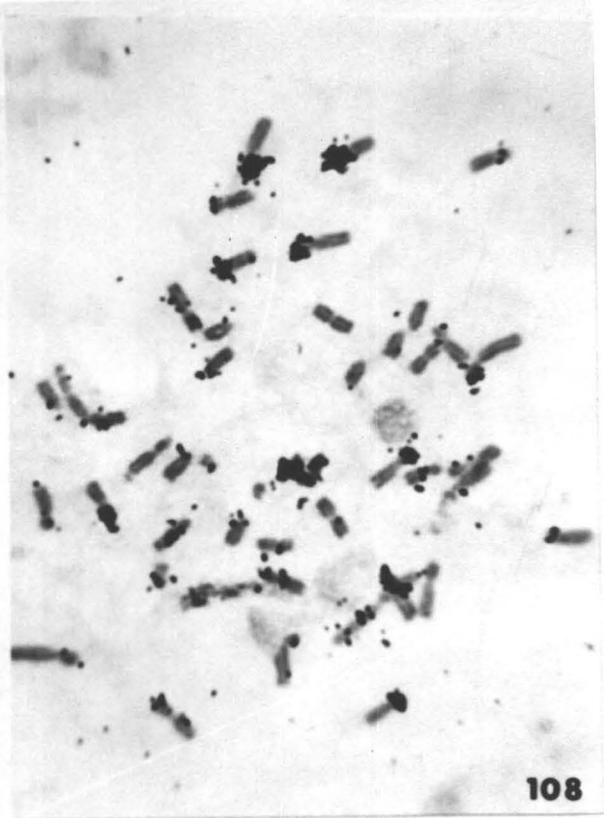
Fig. 108 Example of a metaphase plate illustrating localized areas of heavy labelling associated with most of the chromosomes; 3 hours exposure to  $^3\text{H-TdR}$ .

Fig. 109 A metaphase plate demonstrating well-defined areas of moderate labelling over many of the chromosomes; exposed to  $^3\text{H-TdR}$  for 3 hours.

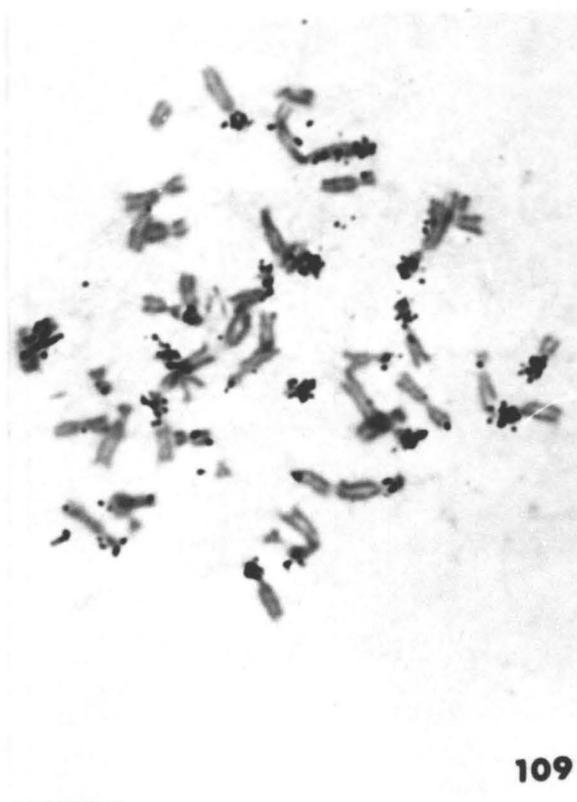
Fig. 110 An example of a metaphase plate showing extremely sparse labelling restricted to only a few chromosomes; 2 hours exposure to  $^3\text{H-TdR}$ .



107



108



109



110

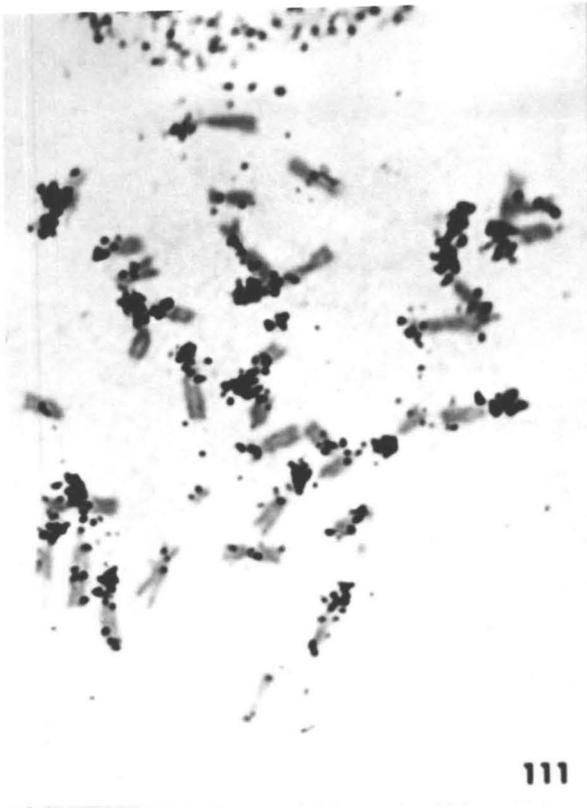
Figs. 111-114 Examples of labelled metaphases from Syrian hamster cultures before and after removal of radioautographic silver grains. X2000

Fig. 111 Metaphase, photographed in transmitted light, illustrating localization of silver grains over chromosomes; 3 hours exposure to  $^3\text{H-TdR}$ .

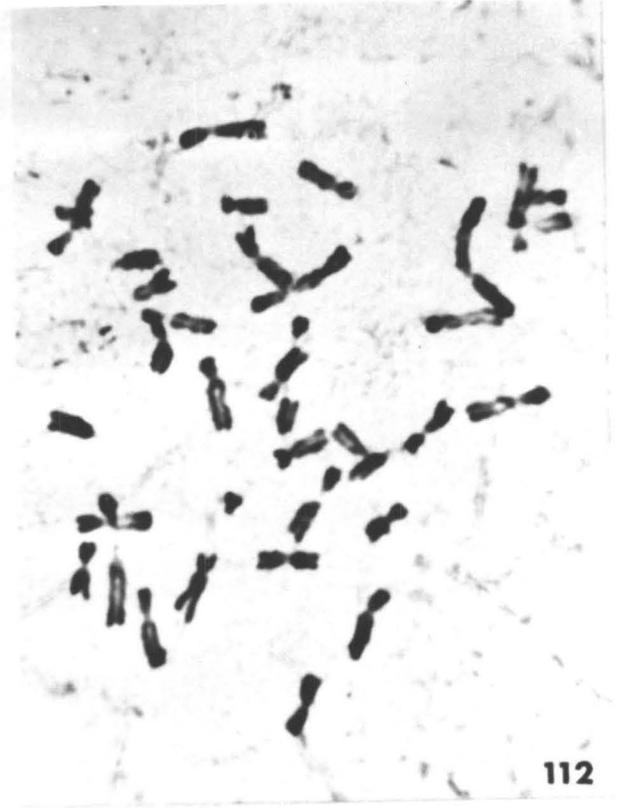
Fig. 112 The same metaphase as in Fig. 111, with the silver grains removed, and photographed with phase contrast optics.

Fig. 113 An example of a metaphase showing localized, light labelling, photographed in transmitted light.

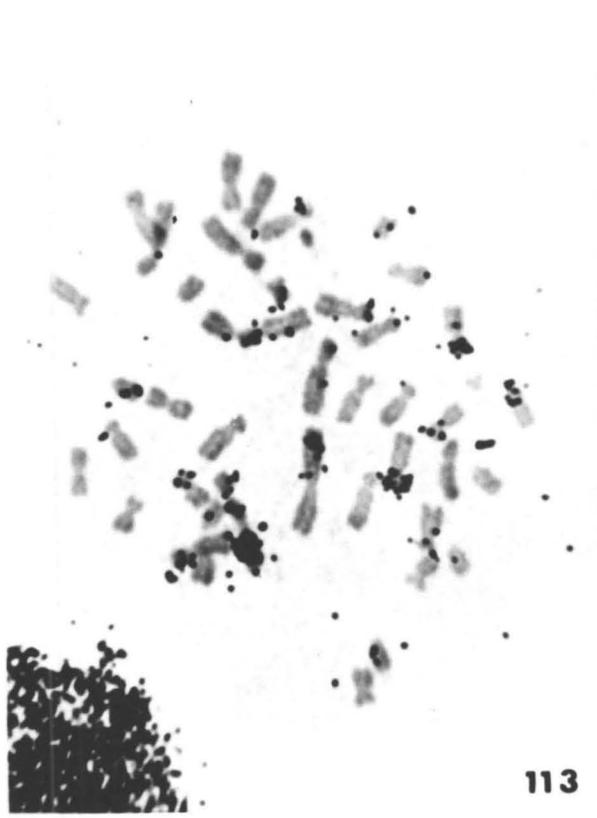
Fig. 114 The same metaphase as in Fig. 113, relocated and photographed with phase contrast optics, after removal of the silver grains.



111



112



113



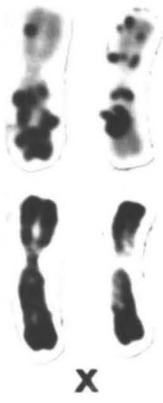
114

Examination of the karyotypes prepared from the radioautograms revealed a distinctive labelling pattern for both female and male EH cells. In uninfected female cells (Fig. 115) one entire X-chromosome and the long arm of the other X-chromosome were late-replicating. In addition, the short arms of most of the submedian metacentric chromosomes also showed late-labelling patterns. Male EH cells from uninfected cultures (Fig. 116) displayed the long arm only of the single X-chromosome to be late-replicating. Although, like the female EH cell, the short arms of most of the submedian metacentric autosomes also were late-replicating, frequently the whole of one medium-sized submedian metacentric chromosome in male cells displayed late-replication. Although this chromosome was not of the size found for the suspected Y-chromosome by Galton and Holt (59), polymorphism of the Y-chromosome is known to occur in the Syrian hamster (122); therefore, this chromosome is considered the putative Y-chromosome by virtue of its late-labelling pattern (59,60,89,92,169, 188,192).

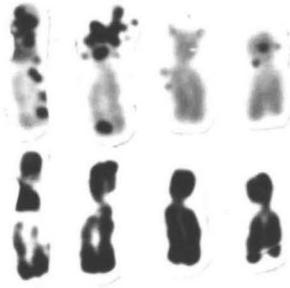
The radioautograms from cultures infected with Ad.12 presented much the same replication pattern as that above (Figs. 117-118); one and one-half X-chromosomes in female cells, one-half X-chromosome and the whole of one medium-sized submetacentric in male cells, and, in

Fig. 115

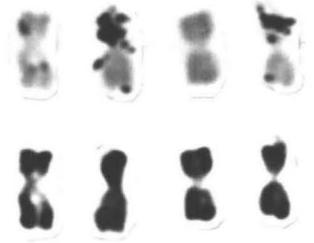
Karyotype of a female cell from an uninfected Syrian hamster culture (EH), before and after removal of silver grains. This cell displays the typical late-replicating pattern of DNA in female hamster cells; note the labelling over one entire X-chromosome and the long arm of the other X-chromosome, and over the short arms of most of the submedian metacentric autosomes. Exposed for 3 hours to  $^3\text{H-TdR}$ . X4000



X



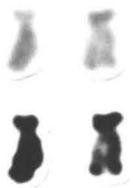
A



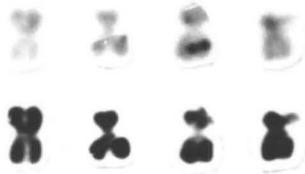
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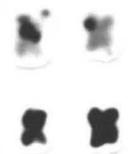
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D



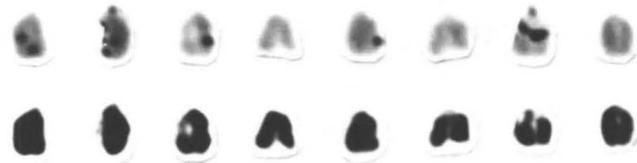
E



G



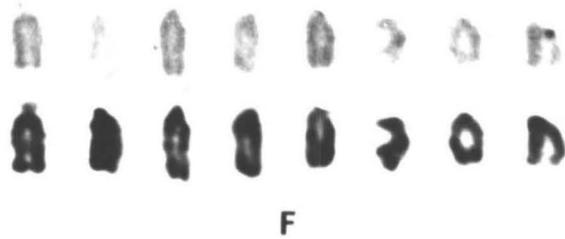
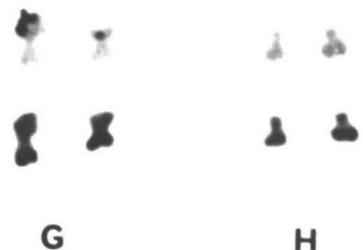
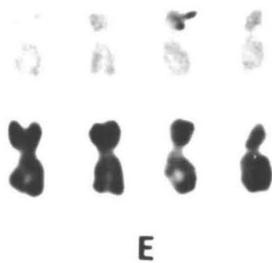
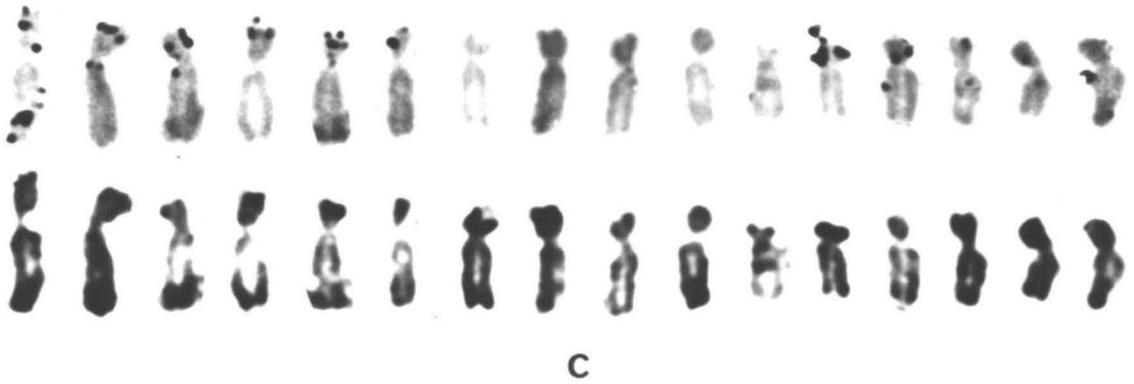
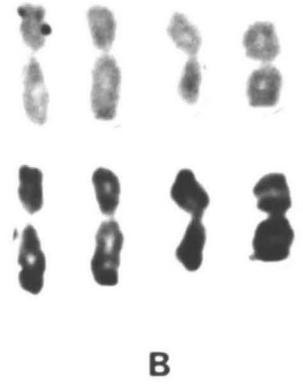
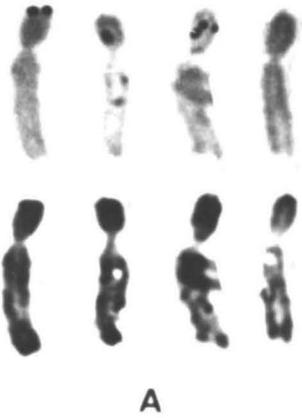
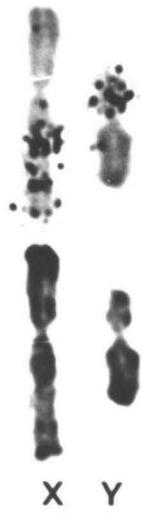
H



F

Fig. 116

Karyotype of a male cell from an uninfected Syrian hamster culture (EH), before and after removal of silver grains. This cell displays a very late DNA replication pattern; the long arm only of the single X-chromosome is late-replicating, along with the short arms of many submedian metacentric autosomes. Exposed for 3 hours to  $^3\text{H-TdR}$ . X4000



both male and in female cells, the short arms of most of the submedian autosomes were late-replicating.

The observed late-labelling patterns correspond closely to those previously described for the Syrian hamster (59) and the Chinese hamster (89). Moreover, no difference could be detected between the patterns from EH cultures infected with Ad.12 and those from uninfected cultures. Ad.12-infected cultures displayed up to 33.3% of the metaphases examined with chromosome aberrations, consisting of chromatid gaps and breaks, and including 7.0% showing fragmented chromosomes, whereas control uninfected cultures displayed only 6.0% total aberrations. Yet, even in the presence of chromatid gaps or breaks (Figs. 117-118), there was no detectable difference in the late-replication pattern of Ad.12-infected cells from that in uninfected cultures.

Fig. 117

Karyotype of a female cell from a Syrian hamster culture (EH) 24 hours after infection with Ad.12, before and after removal of silver grains. The late-replicating pattern remains as in Fig. 115. Note the isochromatid break in the long arm of the entire late-replicating X-chromosome (arrow). Exposed for 3 hours to  $^3\text{H-TdR}$ . X4000

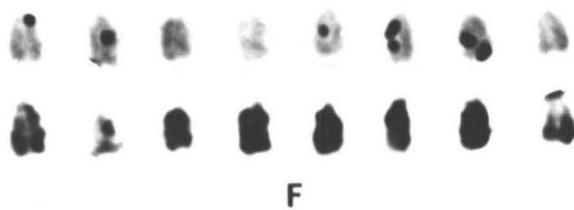
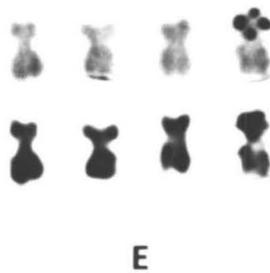
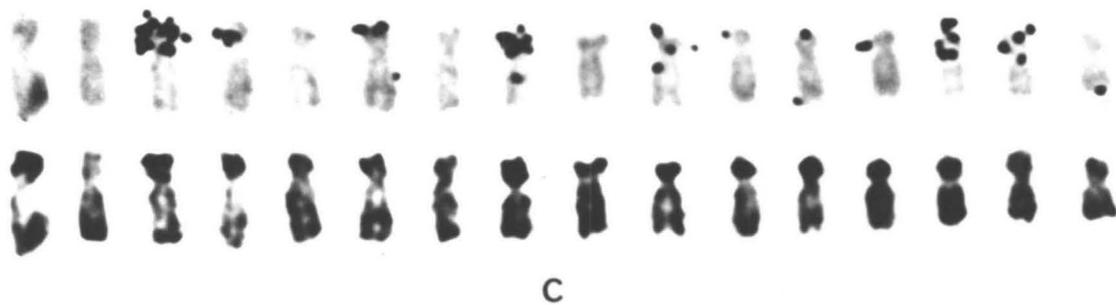
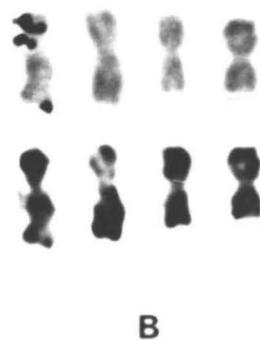
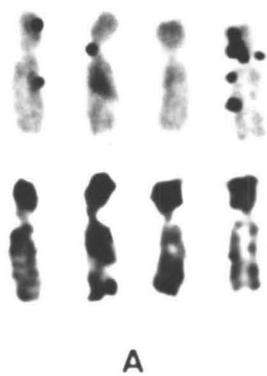
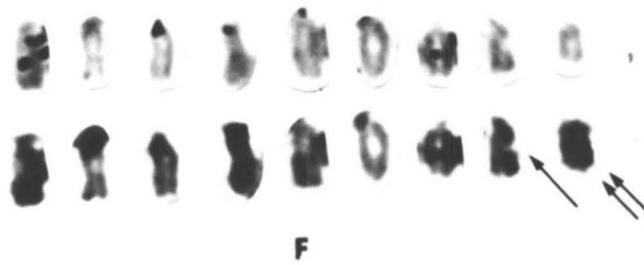
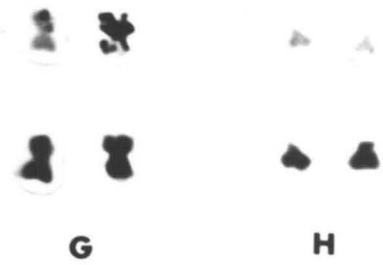
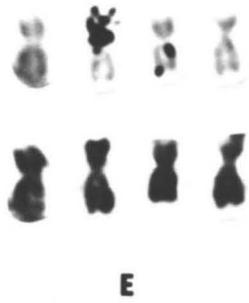
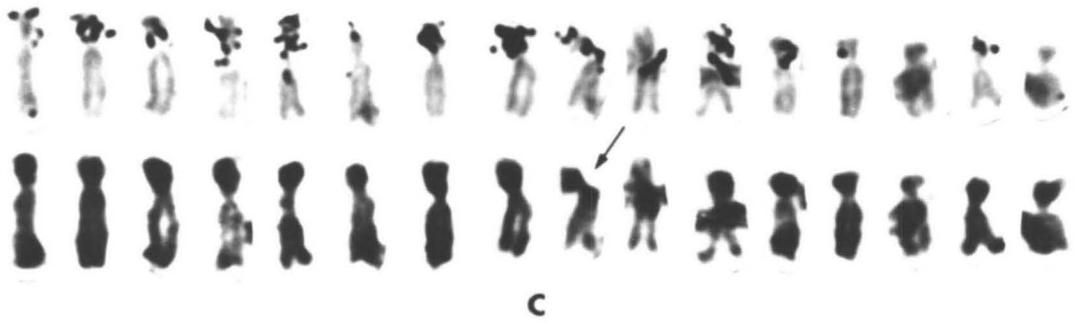
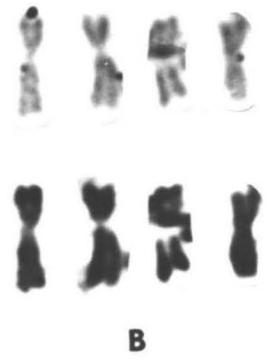
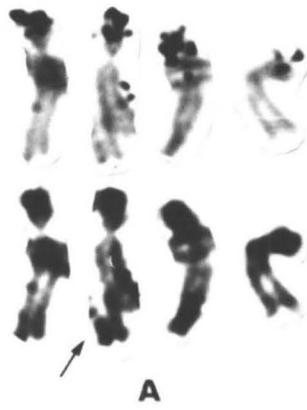


Fig. 118

Karyotype of a male cell from a Syrian hamster culture (EH) 24 hours after infection with Ad.12, before and after removal of silver grains. The late-replicating pattern remains essentially as that in Fig. 116. Note the presence of several chromatid breaks, some with dislocated chromatid segments (arrows) and an extra telocentric chromosome (double arrow). Exposed for 3 hours to  $^3\text{H-TdR}$ . X4000



## DISCUSSION

Human cells and Syrian hamster cells, grown *in vitro*, were used to examine some effects of infection with each of seven adenoviruses, human types 2,4,7,12 and 18, and simian types SV15 and SA7. The capacity of the cells to support adenovirus replication was investigated along with the ability of the above adenoviruses to induce chromosome aberrations in each cell line. Major differences were shown by the two systems in response to adenovirus infection, and it is these differences which deserve detailed discussion.

### Adenoviruses Induce Chromosome Aberrations in a Non-Lytic Infection

The cells used in the present study represent examples of a lytic response (human cells) and a non-lytic response (hamster cells) to adenovirus infection.

Human cells supported a complete virus replication cycle (lytic infection) and in addition showed inhibition and eventual cessation of mitosis, formation of intranuclear inclusion bodies, and morphologic changes of the interphase nuclei including chromatid condensation and formation of micronuclei. These responses to adenovirus

infection are similar to those previously described for adenovirus type 12 infection of human cells (71,128). In addition, virus replication experiments demonstrated that each adenovirus tested forms mature infectious virus in human cells.

In contrast to the results obtained with human cells, infection of Syrian hamster cells with adenoviruses did not result in inhibition of mitosis, nor in morphologic changes in nuclei, such as clumping of chromatin or formation of intranuclear inclusion bodies. Neither was infectious virus produced, Syrian hamster cells being only abortively infected with each of the adenoviruses tested with the exception of Ad.2. Even in this latter case, only a small proportion of hamster cells supported the replication of Ad.2, as judged by the occurrence of very few cells showing inclusion bodies.

Examination of metaphase plates revealed another striking difference between the two virus: cell systems. Abundant chromosome aberrations were induced in a non-lytic infection (hamster cells infected with adenovirus), whereas no adenovirus-induced chromosome aberrations could be detected in a lytic infection (human cells infected with adenovirus). The absence of detectable chromosome aberrations in human cells lytically infected with adenoviruses does not necessarily mean that virus infection

had no effect on the chromosomes of human cells, but merely that there was a lack of infected cells entering into mitosis. Therefore chromosome aberrations, even if present in the majority of cells, would remain undetected. That there was, in fact, some effect on the chromosome material of infected human cells is evident from the chromatin clumping and formation of micronuclei.

#### Chromosome Aberrations Depend on the Virus Dose

Infection of a hamster cell line, BHK21, with human adenoviruses types 2,4,7,12 or 18, or with simian adenoviruses types SV15 or SA7, resulted in a wide array of chromosome aberrations, including chromatid breakage, fragmentation, overcontraction, and erosion. In addition, anomalies of the mitotic apparatus were encountered in the infected cell cultures. The incidence of metaphase plates showing chromosome aberrations and the incidence of breaks per metaphase plate are related to the input multiplicity of each virus tested. To a lesser degree, the virus dose also influences the type of chromosome damage induced.

The relationship between virus dose and the incidence of chromosome aberrations was readily demonstrable due to the particular properties of the test system

employed: 1. The BHK21 cell line does not support multiplication of the viruses tested, with the exception of Ad.2 to a limited extent. Thus, quantitative differences observed between virus doses are a function of input multiplicity. 2. Infection of BHK21 cells with adenoviruses does not inhibit entry into mitosis, unlike adenovirus infection of human cells. Thus, anomalies of the chromosomes or of the mitotic apparatus are detectable at metaphases following infection. 3. The BHK21 cell line has a relatively stable chromosome complement (202). Thus, even a relatively small increase in the incidence of chromosome aberrations can be quantitatively evaluated.

The capacity to induce chromatid breakage is a common feature of the adenoviruses examined in this study. Although there appears to be a variation in the incidence of breakage induced by the different viruses, it is questionable whether these differences are real or only due to variable proportions of coiling anomalies induced by the different types of adenoviruses. Since chromosome breaks are difficult to detect in metaphase plates with overcontracted or eroded chromosomes, the incidence of chromatid breakage probably was underestimated in BHK21 cultures infected with adenoviruses other than Ad.12 or Ad.18.

Although each of the adenoviruses induces a wide array of chromosome anomalies, the proportions of the different chromosome and mitotic aberrations depend not only on the viral dose but also on the type of virus. If the types of chromosome and mitotic anomalies induced by comparable input multiplicities of virus are compared, strain-specific differences become evident. The adenoviruses examined can be grouped into two categories according to the spectrum of chromosome aberrations elicited in hamster cells: 1. viruses which induce, almost exclusively, chromatid breaks (Ad.12 and Ad.18); 2. viruses which induce chromatid breakage, and in addition, coiling anomalies and adverse effects on the mitotic apparatus (Ad.7, SV15, Ad.2, Ad.4 and SA7). These mitotic anomalies are particularly evident in BHK21 cultures infected with Ad.2, Ad.4 or SA7, leading to a characteristic colchicine-like C-mitosis (61,123), arrest at metaphase and formation of micronuclei (41,194).

#### The Incidence of Chromosome Aberrations Changes with Sampling Time

Previous investigations have indicated that the incidence of virus-induced chromosome lesions decreased with time after infection; the decreases to near control levels occurred relatively quickly (Ad.18 (37), measles (6)) or only after many days post-infection (Ad.12 (199),

herpes simplex (75)). Widely differing cell and virus systems were used in those studies, whereas in the present investigation the same test system was used throughout. Under the latter experimental conditions, virus-specific differences become evident. The chromosome aberrations induced by Ad.12 or Ad.18 had virtually disappeared from cultures sampled only 36 hours after infection, whereas the aberrations induced by the other adenoviruses could remain at very high levels even 72 hours after infection, indeed, near the maximum level in the case of Ad.4. These differences reflect variation in the proportion of the categories of aberrations induced by each type of adenovirus.

Ad.12 and Ad.18, which induced almost exclusively chromatid breakage, seem to affect only the first one or two mitotic cycles after infection. Presumably, cells with chromatid breaks either may reconstitute their damage relatively quickly, as with radiation-induced damage (119), or may be selected against in subsequent cell divisions. It is well-established that radiation-induced chromosome aberrations tend to be lost at mitosis (46,100,183) and that the majority of cells carrying such aberrations are rapidly eliminated from the cell population (46,100). At subsequent sampling times, therefore, an increasing proportion of cells in the sample presents an apparently

normal chromosome complement (46).

Selection against cells with chromatid breaks and abnormal chromosomes may therefore explain the rapid decreases in cells showing such aberrations induced by Ad.12 or Ad.18. It is also probable that the observed chromosome damage induced by the other adenoviruses, human types 2,4,7 and simian types SV15, SA7, is similarly restricted to the first and possibly second cell divisions post-infection. However, the inhibitory effect on the mitotic apparatus induced by these latter viruses, particularly Ad.2, 4 and SA7, results in a prolonged "metaphase", delaying completion of cell division, and therefore resulting in chromosome aberrations lingering to relatively late times after infection.

#### Adenovirus-Induced Chromosome Damage Involves a Viral Component

Virus neutralization experiments with type-specific antisera demonstrate that chromosome aberrations induced by the preparations of adenoviruses used in this investigation are due to the presence of virus and are not due to toxic or mutagenic products in the supernatant of the stock virus preparations. The possibility exists, however, that the chromosome damage is a response to some particular component(s) of proteins of the infecting virions, rather than due to the viral genome or a product of it.

Several virus-induced effects are known to be associated with the viral structural antigens, e.g., the early cytopathic effect shown by lytically-infected cell cultures is a response to specific components of the capsomeres (166). Moreover, the capsid proteins of infecting virions may be responsible for selectively inhibiting host cell DNA synthesis (65,125), the biosynthesis of host DNA being effectively suppressed several hours before viral capsid proteins are produced in adenovirus-infected cells.

Evidence against the above possibility includes the observations that a relatively great amount of purified viral structural (fibre) antigen is required to block host cell DNA synthesis (125), and that inhibition of DNA synthesis apparently does not occur in hamster cells infected with adenoviruses. In addition, the adenovirus-induced chromosome aberrations only appear quite late following infection, unlike other virus-induced damage to the chromosomes, as, e.g., the pulverization effect elicited by myxoviruses, which characteristically has a very rapid onset after infection (7).

The observation that virus-induced blockage of host cell macromolecular synthesis may be due to accumulation of newly-synthesized viral structural antigens (12,65) suggests that chromosome aberrations

may also be due to accumulation of newly-synthesized viral proteins. However, this possibility is obviated by the fact that, in hamster cells, adenovirus structural antigens are not produced (84,128,171), whereas abundant chromosome aberrations are still elicited. Also, the capacity of adenovirus to induce chromosome damage declines with ultraviolet light inactivation of the virus (197,228,229), apparently showing single-hit kinetics (228,229), while attachment, penetration, and uncoating of adenovirus remain unaffected by UV treatment (118).

The above data are interpreted to indicate that a viral component sensitive to UV is therefore not required for intracellular uncoating of adenovirus, but a viral component sensitive to UV is necessary for induction of chromosome aberrations. Thus, the production of chromosome aberrations is likely due to, or in some manner under the control of, the infecting viral DNA.

#### Chromosome Aberrations Induced by Adenoviruses and by Other Agents

The chromosome aberrations induced in hamster cells by adenoviruses types 2,4,7,12,18,SV15 and SA7 comprise a wide range of anomalies, including chromatid breakage, fragmentation, coiling anomalies and anomalies of the mitotic apparatus. On a morphological basis, the adeno-

virus-induced aberrations are comparable to those produced by a great variety of agents. Adenovirus-induced chromatid breakage, e.g., closely resembles that produced by other viruses (6,75,151,198), certain chemicals (38, 92), chemical carcinogens (92), inhibitors of DNA synthesis (111) and ionizing radiations (119). Coiling anomalies induced by adenoviruses are similar to those produced by viruses (151,198), and chemicals (111,151), while anomalies of mitosis resemble those induced by viruses (16), mitotic inhibitors (18,61) and ionizing radiation (119).

The morphology of the extreme fragmentation produced in hamster cells by Ad.12 and Ad.18 at high input multiplicities resembles that produced by large doses of ionizing radiation. However, high doses of adenoviruses types 2,4,7,SV15 and SA7 produce an extreme fragmentation which resembles the pulverization phenomenon, described by Nichols *et al.* (153) and observed to be produced by a variety of viruses (7,21,28,77,152,153,198) and by the application of colcemid (108).

The extensive fragmentation of the chromosome complement induced by adenoviruses should not be confused with the pulverization phenomenon, for there are several differences between the two aberrations. 1. Pulverization is characterized by rapid onset following virus infection

(7), whereas the adenovirus-induced phenomenon appears only late after infection. 2. Pulverization is almost exclusively observed in multinucleated cells (108), but the adenovirus-induced aberration is found in mononucleate cells and is not confined to detectably polyploid cells. 3. Polyploid cells showing all chromosomes pulverized are rare, almost always at least one set of chromosomes being intact (108), whereas most often the adenovirus-induced fragmentation involves all the chromosomes, very rarely showing intact chromosomes together with a large amount of "pulverized" chromosomes.

Recent data obtained from radioautography of the pulverized chromosomes (108,152) suggests that pulverization occurs in those chromosomes which were in the period of DNA synthesis. Pulverization, then, as the term currently is applied in the literature, apparently depends upon the presence, in the same cytoplasm, of nuclei asynchronous with respect to their DNA synthesis (108,152). It has been suggested that the major role of viruses which produce pulverization may be merely the induction of syncytium formation (108). Adenoviruses, however, do not induce syncytia in infected cultures.

Altogether, the data indicate that the extreme fragmentation seen in adenovirus-infected hamster cultures, while it superficially resembles pulverization induced by

the myxoviruses, is not the same phenomenon. More probably, the "pulverization-like" effect shown by some cells in the present investigation represents extreme chromosome breakage, simulating an X-ray effect, coupled with uncoiling of the chromosomes. Indeed, cells are found in adenovirus-infected cultures which might represent stages of a progression, from chromosomes showing chromatid breaks together with moderate uncoiling (Fig. 57), more extensive breakage with more severe uncoiling (Fig. 30), through fragmentation of the chromosomes with drastic uncoiling (Fig. 8) to a "full-blown pulverization" (Fig. 9).

The chromosome aberrations produced in this study by adenoviruses types 2,4,7,12,18,SV15 and SA7 may be considered to be of three major categories; breaks (including fragmentation), coiling anomalies (including the "pulverization-like" effect) and mitotic abnormalities. Just as ionizing radiation induces visible chromosome aberrations in addition to a great number of point mutations which are not visible in microscopic examination (119), it has been suggested that virus-induced chromosome aberrations may also be indicators of point mutations (151). Recent reports indicate that animal viruses are, indeed, capable of producing point mutations (10,25). It is therefore considered that, of the categories of

chromosome damage induced by adenoviruses, the single break is the most important type of chromosome aberration with regard to its potential mutagenic significance (151).  
Coefficient of Aberration Production by Viruses and by Ionizing Radiation

Since the morphology of the chromatid breaks produced by a wide variety of agents is very similar, it becomes of interest to compare the incidence of adenovirus-induced chromosome breakage with that elicited by other agents. Among the agents frequently employed for inducing and studying the kinetics of chromatid breaks are ionizing radiations.

The coefficients of production most often encountered in the literature for ionizing radiation-produced aberrations (Table XIV) are  $3-4 \times 10^{-3}$  deletions per cell per rad (R) (13,14,46,48,119) and for exchanges (primarily dicentrics and rings),  $4-6 \times 10^{-6}$  per cell per  $R^2$  (14,187). A recent report (46) suggests that this latter relationship may be modified according to the experimental time of sampling. Rather than varying with the square of the radiation dose, the production of exchanges may only be a linear function of the dose. Ionizing radiations therefore produce almost equal proportions of chromatid breaks leading to deletions, and exchanges, approximately  $4 \times 10^{-3}$  of each per cell per R.

TABLE XIV

Relationship Between Chromosome Breakage and Exchange Produced by Ionizing Radiation

Type of Radiation Used	Dose (Rad)	Breaks and Exchanges (per cell)	% of Aberrations Due to Exchanges	Coefficient of Aberration Production (Aber./cell/R)	Reference
Chronic γ		0.18	50	$10 \times 10^{-3}$	48
Therapeutic γ	2000 -6000		21-45 (Aver. 36)		4
X-rays	750 500	3 1	29 15	$4 \times 10^{-3}$ $2 \times 10^{-3}$	226
γ	300	0.6-0.8	45-48	$2-3 \times 10^{-3}$	116
X-rays	300-30		43		46
X-rays	54	0.4	41	$7 \times 10^{-3}$	111
γ and neutron fission	47 12		40 16	$6 \times 10^{-3}$ $2 \times 10^{-3}$	14
Diagnostic X-rays	50 5	0.08 0.02	(Aver. 27)	$1.4 \times 10^{-3}$ $4 \times 10^{-3}$	187

In comparison, the coefficient of production of chromatid breaks by the adenoviruses types 2,4,7,12,18, SV15 and SA7 may be calculated on the basis of chromatid breaks per cell per infectious unit, the term "infectious unit" corresponding to an input multiplicity of 1 TCID<sub>50</sub>\*. On this basis, the adenovirus preparations induce from 2 to 4 x 10<sup>-1</sup> breaks per cell per infectious unit (Table XV). This indicates that one infectious unit may induce more than 100 times as many visible chromatid breaks as 1 rad of ionizing radiation. To express this another way, exposure of cells to 1 infectious unit per cell yields an incidence of chromatid breaks comparable to that obtained by 50-200 R; 5 infectious units per cell gives a result similar to that obtained by 200-1000 R, and 25 infectious units per cell elicits severe chromosome damage comparable to that resulting from 1000-4000 R of ionizing radiation.

Another difference observed between the chromosome damage induced by viruses and by ionizing radiation resides in the amount of rejoining of the induced chromatid breaks. The proportion of adenovirus-induced chromosome aberrations due to exchanges is especially low at the higher viral doses, indicating that rejoining of viral-induced chromatid breaks is a rare event (Table XV).

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\* An infectious unit of adenovirus may comprise 10-100 physical particles (168).

TABLE XV

Relationship Between Chromosome Breakage and Exchange  
Produced in Hamster Cells by Adenoviruses

Virus	Dose (TCID <sub>50</sub> /cell)	Breaks and Exchanges Per cell	% of Aberrations Due to Exchanges	Coefficient of Aberration Production (Aber./cell/ infect. unit)
Ad.18	25	10.0	0.7	.40
Ad.12	25	5.0	1.5	.20
Ad.7	25	1.8*	3.3	.07
SV15	25	1.4*	3.5	.05
Ad.2	25	2.5*	2.3	.10
Ad.4	25	1.5*	0.9	.06
SA7	25	1.0*	1.6	.04
Controls	(Average)	0.07	21.0	

\* Many breaks were probably not counted, due to their being obscured by coiling anomalies.

However, exchanges make up a relatively large proportion of the damage induced by radiation at all doses, from exposure of but a few R (187) to well over 6000 R (4) (Table XIV). A high frequency of exchanges is therefore characteristic of all doses of ionizing radiation.

A high frequency of rejoining of breaks is also characteristic of the aberrations induced by many chemicals, including antibiotics, alkylating agents, nitrosocompounds, in fact, most chemical mutagens (111). However, the chromatid aberrations induced by chemical mutagens and by ionizing radiations differ in that breaks induced by X-rays, e.g., are randomly distributed both between and within chromosomes, whereas the breaks induced by chemicals follow a non-random distribution, frequently being localized to heterochromatic areas (88,111,193).

Since the radiomimetic effects of nitrogen mustards were first analysed (38), the chromosome-breaking effects of a wide spectrum of chemicals, as exemplified by alkylating agents, nitrosocompounds, antibiotics, and precursors or inhibitors of DNA (111), have been extensively investigated. Only certain inhibitors of DNA synthesis, however, such as cytosine arabinoside (araC), fluorodeoxyuridine (FUdR) and deoxyadenosine

(AdR), produce chromosome aberrations similar to those produced by adenoviruses (111,151). These inhibitors of DNA synthesis induce chromatid breaks and fragmentation, with only a small proportion of exchanges, suggesting that, for these chemicals, rejoining of the induced breaks is a rare event (111), just as for virus-induced breaks (151,155).

The observed similarity between the virus-induced chromosome aberrations and the lesions induced by chemicals which interfere with DNA synthesis, as mentioned above, lead one to wonder whether the virus-induced aberrations might also be due to a similar inhibitory effect of DNA biosynthesis.

Radioautographic examination of Ad.12-infected human and hamster cell cultures revealed different effects on DNA synthesis in the two systems. Ad.12 infection of human cells resulted in early suppression of DNA synthesis, as measured by the decreased number of cells incorporating tritiated thymidine relative to uninfected cultures. Similar observations (66,70,71, 113,189) have been attributed to viral-induced suppression of host cell DNA synthesis (66,70,71). Later increased DNA synthesis in infected human cultures is almost certainly attributable to synthesis of viral DNA (66, 69,71,175,207), the host cell DNA and RNA synthesis having

been almost completely inhibited (71) and viral DNA synthesis occurring in the intranuclear inclusion body (71,228).

In contrast to the results obtained with human cells, infection of Syrian hamster cells with Ad.12 resulted in increased numbers of cells incorporating tritiated thymidine, a slight increase in mitosis, and slightly decreased  $G_2$  period. The latter observations suggest that Ad.12 infection of hamster cultures might have resulted in decreased total cell cycle, since the length of the  $G_2$  period relates more or less directly to the length of the cell cycle (27,190).

The observed stimulation of DNA synthesis in hamster cells induced by Ad.12 infection agrees with the results of several investigators (121,175,207). The objection, that the observed increase in the number of cells incorporating tritiated thymidine may merely have been due to addition of fresh medium (127), is obviated by the fact that both infected and uninfected cultures received the same treatment regarding media changes, the only difference being the presence of Ad.12 in media added to cultures to be infected.

Increased DNA synthesis in virus-infected hamster cultures not only may be due to stimulation of host cell DNA synthesis (64,79), but also may be due to synthesis

of viral DNA even in the absence of formation of mature virus, as suggested for Ad.5 or Ad.12 infection of hamster cells (207) and as shown for SV40 virus (64,79), and adenovirus in green monkey kidney cells (175). Moreover, it has been shown that synthesis of mature infectious virus is not necessary for induction of DNA synthesis in adenovirus-infected cells (175). At present, however, whether the stimulation of DNA synthesis in Ad.12-infected hamster cells is due to viral or to host cell DNA synthesis remains to be elucidated.

If, indeed, viral DNA synthesis occurs in adenovirus type 12-infected hamster cells, it may well proceed at replication sites associated with the host cell chromosomes. Recent reports suggest that Ad.12, e.g., is detected in close association with the chromosomes of human cells (156,228) and hamster cells (229), but was not associated with the sites of chromosome breakage in these cells (156,228,229). However, these investigations did not examine the possibility of replication of viral DNA, even if only a small amount in the abortively-infected cells, in association with the host cell chromosomes or with the sites of virus-induced breakage.

In the present investigation, however, just as Rapp and Hsu (176) detected no replication sites of

herpes simplex virus associated with the chromosomes of Chinese hamster cells, so also did this preliminary investigation not detect significant amounts of DNA synthesis associated with the chromosome breaks in Ad.12-infected Syrian hamster cells. Although incorporation of tritiated thymidine was readily detected in chromosomes at metaphase, the late-replication patterns of Ad.12-infected cells were not different from those in uninfected cultures, even in the presence of multiple chromatid gaps and breaks.

The similar pattern of chromosome replication in Ad.12-infected and uninfected EH cultures, as demonstrated by radioautography of the chromosomes, indicated that the chronology of DNA replication has been maintained in Ad.12-infected cells, and that the presence of chromosome aberrations does not alter this pattern.

Deterioration of the orderly sequence of chromosome replication has been suggested as a factor in the initiation of cancer (224). Some human tumor cells have been investigated, however, and in those cancer cells with minimal deviation from the normal diploid karyotype, no difference in the chronology of replication was observed between normal and cancer cells (62,224). Clones of Chinese hamster cells with karyotypes widely

divergent from the normal displayed different patterns of chromosome replication (227) but no differences were detected for those chromosomes which were recognizable as, ~~and not~~ present in excess of, their counterparts in normal diploid cells.

It would be interesting, then, to examine the pattern of replication of the chromosomes of Ad.12-transformed cell lines, as well as Ad.12-induced tumor cells, in which Ad.12 infection results in the presence of "T" antigen (84,128,171). Such cells are suspected to carry persistently at least part of the viral genome, and its continued presence may upset the chromosome replication pattern of the host cell. Abnormal chronology of replication might then be responsible for the continuing karyotypic instability of Ad.12-induced tumor cells (203).

#### Possible Mechanisms of Adenovirus-Induced Chromosome Aberrations

A wide variety of non-viral agents capable of producing chromosome aberrations can be placed into one of two categories. The first comprises those agents which directly attack the DNA of the chromosomes, as exemplified by ionizing radiations (119), cross-linkage of DNA molecules (102) and enzymatic digestion of DNA (3a). The second category comprises agents which apparently prevent normal DNA biosynthesis, for example, incorporation

of nucleotide analogues into DNA (3,90,111,225), faulty ATP and protein synthesis needed for repair mechanisms (220) and outright inhibition of DNA biosynthesis (111).

In the present investigation the possibility that the adenovirus-induced chromosome aberrations are caused by a direct viral "attack" on the host chromosomes can be ruled out by the observations that, although viral DNA is likely involved in induction of aberrations (228,229), no direct association of infecting viral DNA with sites of chromosome breakage was found (156,228,229), nor was there detectable DNA synthesis specifically associated with sites of chromosome breakage (176). Moreover, it has been shown that the adenovirus-induced chromosome aberrations are very similar to those produced by certain inhibitors of DNA synthesis. The mechanism of action of the chromosome-damaging adenoviruses might then be considered to be similar to that of some of the agents which adversely affect normal DNA synthesis.

Kihlman recently has reviewed (112) the possible mechanisms of induction of chromosome aberrations by inhibitors of DNA synthesis. These chemically-induced aberrations have been postulated as resulting from interruption of DNA synthesis in late S period (209). Later data (11) suggest that interruption of a later

DNA synthesis, during the  $G_2$  period, needed to complete chromosome replication, resulted in chromosome aberrations. Still more recent observations led to the hypothesis that chromosome aberrations were due to suppression of a special type of DNA synthesis, occurring in small amounts, which normally is undertaken subsequent to S period and which is necessary for the structural integrity of the chromosomes at metaphase (112).

Evidence is rapidly accumulating to indicate that a small amount of DNA synthesis does occur as late as prophase of meiosis and possible mitosis (112,150,180). This DNA synthesis may be involved in genetic recombination (72) and may involve certain common steps with repair processes (87). Enzymatic repair mechanisms, which excise damaged nucleotides from DNA and resynthesize the damaged DNA strand, have been intensively studied in microorganisms (5,24) and recently have been demonstrated in mammalian cells, operating after heavy doses of X-rays (162) or of ultraviolet light (85). Therefore, inhibition of repair of normally-occurring lesions in chromosomes may also be responsible for chemically-induced chromosome aberrations (112). The question then arises, might the mechanism of virus-induced chromosome breakage also be the result of inhibition of a special DNA synthesis?

Preliminary observations in the present report

suggest that Ad.12 infection of cells which then show chromosome aberrations does not inhibit, but instead may stimulate, DNA synthesis. Nonetheless, large amounts of chromatid breakage and chromosome fragmentation are induced in these cells. Stimulation of cellular DNA synthesis has been observed by several other viruses both in lytic and non-lytic infections (Table XVI). In addition, enhanced synthesis of several enzymes involved in DNA biosynthesis has been demonstrated for a wide variety of viruses in lytic and non-lytic infections.

While a detailed analysis of enzyme action is beyond the scope of this discussion, it is still interesting to speculate on the involvement of viruses and virus-induced enzymes in the production of chromosome breakage in mammalian cells. Viral-directed enzymatic breakdown of DNA has been suggested as a mechanism of virus-induced chromosome breakage (151,228,229). In addition, enzymatic degradation of DNA is the basis of a recent hypothesis to explain chemically-induced chromosome breakage (3,150). The proposed mechanism involves reversal of action of DNA polymerase, due to an upset in the deoxyribonucleotide pool, caused by inhibitors of various steps in DNA biosynthesis. Viral-induced enzymes involved in DNA synthesis might also result in imbalance of the DNA precursor pool, subsequently resulting in breakdown of DNA by DNA polymerase.

TABLE XVI

Stimulation of DNA Synthesis and Induction of Enzymes Involved  
in DNA Synthesis by Virus Infection of Mammalian Cells

Virus	Cell Type	Type of Infection	DNA <sup>a</sup> Synthesis	Enzyme Induction <sup>a</sup>			Ref.
				TdRk <sup>b</sup>	DNAP <sup>c</sup>	dCMDd <sup>d</sup>	
Polyoma	Mouse	nonlytic	+	+	+	+	43
SV40	Green monkey	lytic	+				78
	Mouse line (3T3)	nonlytic	+				64
	Green monkey line (BS-C-1)	lytic	+				64
Ad.2	Human (HEK)	lytic	+	+	+	0	121
	Rhesus monkey	lytic	0	+	0	+	120
	Green monkey	nonlytic	+				175
Ad.5	Human (HeLa)	lytic	+				66
	(KB)	lytic	+				207
	(HEK)	lytic		+			206
	Hamster	lytic	+	+			206,207
Ad.12	Human (KB)	lytic	+				207
	(HEK)	lytic		+			206
	(HEK)	lytic	+	+			121
	Hamster	nonlytic	+	+			206,207
SV15	Green monkey line (CV-1)	lytic	+	+	+	0	114
Rous Sarcoma virus	Human		+				127
Pseudo-rabies	Rabbit	lytic	+				15
Vaccinia	Human (KB)	lytic		+	+		70
	(HeLa)	lytic			+		131

<sup>a</sup> +, stimulation; 0, no stimulation

<sup>b</sup> thymidine kinase

<sup>c</sup> DNA polymerase

<sup>d</sup> deoxycytidylate deaminase

Thus, two possible mechanisms to explain virus induction of chromosome aberrations are suggested. The first, based on the observations that adenovirus-induced aberrations are similar to those induced by inhibitors of DNA synthesis, involves inhibition of a special DNA synthesis which is required in small amounts either for establishing normal metaphase morphology of the chromosomes or for repair of normally-occurring chromosome lesions. The second mechanism, involving enzymatic breakdown of DNA by DNA polymerase, is based on the observed induction, by viruses, of a variety of enzymes involved in control of DNA synthesis.

It is clear that, since both oncogenic and non-oncogenic viruses induce similar chromosome aberrations, induction of chromosome aberrations by itself cannot be involved in viral oncogenesis. It is generally accepted that at least part of the viral genome is integrated into the host cell (8,57,58,63,97,171,205). Indeed, maintenance of the neoplastic state may require persistence of part of the viral genome (203). At the same time, it is becoming clear that integration of the viral genome necessitates host cell DNA replication (210,212). It may well be, in fact, that a factor contributing to the high oncogenic potency of some viruses may be their ability to induce the synthesis of cellular DNA (64); the observed

chromosome aberrations may therefore only be secondary phenomena, a result of the induction of various enzymes involved in DNA synthesis.

The effects of virus infection on the enzymes involved in DNA synthesis represents a promising area of investigation for elucidation of virus-induced chromosome damage. At present, the absence of data demonstrating definitively either suppression of a special DNA synthesis (repair mechanism?) or enzymatic breakdown of small quantities of cellular DNA does not permit exclusion of either of the above mechanisms for viral induction of chromosome aberrations. Nonetheless, the foregoing observations suggest that the second postulated mechanism, that involving enzymatic breakdown of DNA by DNA polymerase, may be the more likely.

#### Viruses as Mutagens

A multitude of agents are capable of acting as mutagens and of producing chromosome damage in mammalian cells. Among these are ultraviolet light (32), ionizing radiations (119,104), antimetabolites (3,90,112,150,193, 209,225), mitotic poisons (18,61) and chemical carcinogens (79,196). In addition, such treatments as exposure to low temperature (76) and to halucinogens (34) can induce chromosome aberrations in cultured mammalian cells.

The search to discover causative agents of human cancers continues. For an agent to be effectively

involved in the etiology of cancer, it must satisfy the following criteria: it must be active in relatively small amounts, certainly below toxic levels; it must be ubiquitous since cancer has a world-wide distribution; it must affect a large proportion of the population, about one person in six now being killed by neoplastic cells (182) . Some mutagenic chemicals may satisfy the first of these criteria, but only rarely are large segments of the population exposed to such chemicals, exposure largely being an occupational hazard (98). Small doses of X-rays, such as used in diagnostic procedures, have been shown to elicit detectable amounts of chromosome breakage and disarrangements (187). However, the proportion of population exposed even to such small amounts of ionizing radiation remains small (215). Even accidental exposure to large amounts of radiation affects only small segments of the population involved in particular occupations (215) or receiving specialized treatments (48,148,215).

Viruses, however, can satisfy each of the above criteria for agents responsible for human neoplasia; a single virus particle is sufficient to obtain infection (26), viruses are ubiquitous (86) and viruses affect large segments of the human population (86). In addition, it has been well established that viruses can cause cancers

in animals and induce chromosome aberrations in human and other mammalian cells in both *in vivo* and *in vitro* systems, and can cause transformation of human and other mammalian cells *in vitro* (Table XVII). It is natural, then, that investigations increasingly have turned to viruses as possible causative agents of human neoplasia.

The viral etiology of spontaneous tumors in animals is supported by the data in a recent review (103), which shows that tumors experimentally induced in a variety of animals by viruses more closely approach the relatively high proportion of diploidy retained by spontaneously-occurring tumors than do tumors experimentally induced by either chemical or physical agents. An additional study reveals that animal tumors induced by a highly-oncogenic virus, Rous sarcoma virus, also retain a high degree of diploidy (134). Similar data for comparison of experimentally induced cancers with spontaneously-occurring neoplasms are obviously unavailable for man. The etiology of human cancers thus cannot be deduced merely from the observations that the majority of human cancers are overwhelmingly aneuploid (79,132,224).

The possible viral etiology of human neoplasms must be explored by direct search for evidence of association of viruses with such neoplasms. Recent studies show, for

TABLE XVII

## The Capacity of Oncogenic and Non-oncogenic Viruses to Induce Chromosome Aberrations in, or to Transform, Mammalian Cells

DNA Viruses	Oncogenicity		Chromosome Aberrations		In vitro cell Transformation	
	Ref.	Species	In vivo	In vitro	Ref.	Species
<b>Papovaviruses</b>						
SV40	68	hamster		213 human A 221 human AB	20	rabbit, mouse, porcine
					36	hamster AB
					40	bovine
					144	human AB
SV40/Ad.7 hybrid	95	hamster				
SV40/Ad.12 hybrid	186	hamster				
Polyoma	74	rabbit, rat, guinea pig	81 mouse tumor A		39	bovine
	137	hamster			129	hamster A
	173	<i>Nastomys</i>			216	hamster B
					219	rat
Human papilloma (Wart virus)	141	human			150	human
Shope papilloma	163	rabbit	138 rabbit tumor A			
Bovine papilloma	161	horse			19	bovine
<b>Adenoviruses</b>						
Human type 2		unproved		37a hamster ABCFP	53	rat
" 3	94	hamster			55	rat
" 4		unproved		37,37a hamster ABCFP		
" 7	67	hamster		37a hamster ABCFP		
" 12	67,96	hamster	51 mouse tumor AB 200,203	37a hamster ABF 91 <i>Nastomys</i> AB	30,136	hamster rat
			hamster tumor ABCFP	128 hamster BF 151,156 human B 199 Chinese hamster AB	54	
" 14	58	hamster				
" 16	58	hamster				
" 18	67,96	hamster		37,37a hamster ABF		
" 21	58	hamster		197 human AB		
" 31	167	hamster				
Simian: SV1	96	hamster				
SV20	99	hamster			174,179	hamster
SV30	143	hamster				
SV33,34,37,38	99	hamster				
SA7	99	hamster, rat		37a hamster ABCFP	30,179	hamster
Avian: CHLO	184	hamster				
Canine: ICH	185	hamster				
<b>Herpes viruses</b>						
Herpes simplex		unproved		21 simian 7,198 Chinese hamster B		
				91 <i>Nastomys</i> BF		
Herpes zoster		unproved	6 human B	16 human CF		
<b>Toxoviruses*</b>						
YFVA	157	monkey				
Kojima virus contagiosus	141	human				
<b>RNA Viruses</b>						
Reovirus type 3	110	mouse	110 mouse tumor A			
<b>Arbovirus</b>						
Yellow fever		unproved	77 human BP			
<b>Nyxoviruses</b>						
Rubeola (measles)		unproved	6 human B 155 human B	22,153 human P 50 human AB		
Rubella (German measles)		unproved		135 rabbit B 22 human AB		
Mumps		unproved	6 human B	28 human P		
Parainfluenza 1 (Sendai)		unproved		7 human P		
NDV (Newcastle's disease)		unproved		28 human P		
<b>Leukemia viruses</b>						
Bovine sarcoma	67,172	hamster	2 Chinese hamster A	154 human AB	195	bovine, human
	93	guinea pig	107 rat A		205	rat
	149	monkey	170 chick A			
	204	Amphibian, reptile				
Marino	178	mouse	105,178 mouse A			
<b>Miscellaneous</b>						
Acute Infectious Hepatitis		unproved	139 human AB			

example, that a large proportion of children with leukemia, 79%, demonstrate the presence of myxovirus-like particles in blood cells in the electron microscope, whereas only a small proportion, 10%, of non-leukemic children show such particles (140). Another study reports that a high percentage of Americans with cancer of the post-nasal space or with Burkitt's lymphoma have antibodies against a virus found in the Jiyoye cell line of Burkitt lymphoma cells (160).

With the advent of recent techniques for immunofluorescent detection of viral-induced neoantigens ("T" antigens), several recent investigations have attempted to discern the possible role of viruses in human neoplasia by using such viral "footprints" for detection of previous virus infections in cancer patients. Lewis *et al.* (126) found that sera from some cancer patients demonstrated antibodies against adenovirus type 12-induced "T" antigen present in Ad.12-transformed hamster cells. Malmgren *et al.* (133) have demonstrated only a slightly increased incidence of antibodies to Ad.12 "T" antigen in cancer patients compared with that in non-cancer patients. However, relatively high frequencies of serum antibodies to Ad.12 "T" antigen were detected in patients with specific cancers, i.e., breast tumors and cancers of the colon. These results must not, however, be interpreted to mean that such tumors are caused by Ad.12, but only

that association between tumors and adenovirus infection must be more thoroughly investigated.

In view of the increasing number of reports concerning antibodies to adenovirus-induced neoantigen in humans, it is important then to re-examine the role of adenoviruses in human disease. Adenoviruses may cause three types of disease in humans, epidemic keratoconjunctivitis, acute respiratory disease (ARD) and pharyngitis and pharyngoconjunctival fever (86). The first is commonly due to adenovirus type 8, an apparently non-oncogenic serotype. The relationship between ARD and adenovirus was first demonstrated by Hilleman and Werner (83), who isolated adenovirus type 4 from an epidemic of ARD among newly-inducted troops. Since then, it has been established that most outbreaks of ARD are due to adenoviruses types 4 and 7. However, outbreaks of Ad.4 or Ad.7 are rare in civilian populations, therefore suggesting that the epidemic nature of ARD in military recruits principally occurs because most patients have not previously been exposed to types 4 and 7 adenoviruses. A different situation pertains to adenovirus-caused pharyngitis and pharyngoconjunctival fever. The most frequent adenoviral cause of these childhood febrile respiratory illnesses is Ad.3 (94). Moreover, Ad.3

enjoys a world-wide distribution, surveys showing that 40-50% of children and 50-70% of adults demonstrate neutralizing antibodies to this serotype (94).

In view of ~~the~~ wide distribution of adenoviruses in the human population, it may be of great interest to examine the degree of relatedness shown by the adenoviruses. It is known, e.g., that Ad.4 demonstrates cross-reaction of viral-induced neoantigens (T antigen) with adenoviruses types 3,7,11,14,16 and 21 (94). All of the above serotypes, except types 4 and 11, have already demonstrated their oncogenic potentials in various mammalian species (Table XVI). Under the proper conditions it may yet be shown that types 4 and 11 also display oncogenic capabilities.

Since the oncogenicity of many adenoviruses has been demonstrated, the use of vaccines against adenoviruses has been largely curtailed (164). However, a large number of people may already have been exposed to potential oncogens in the form of bivalent and trivalent vaccines, involving the known oncogens (in non-humans) Ad.3 and Ad.7 (73,82). More importantly, however, an even larger proportion of the population has been exposed naturally to adenoviruses types 3,4 and 7 (94). Moreover, Ad.3 infects at high incidence a large segment of the population at an early age. Since adenoviruses normally are oncogenic only in young mammals, and if adenoviruses prove to be

oncogenic in man, then Ad.3 may well be a prime suspect (94).

The search for a viral agent of neoplasia in man must likely concern itself with viruses infecting man as their natural host (211). The human adenoviruses represent a group of widely-distributed viruses, affecting large segments of the population, and including serotypes which are oncogenic in non-humans. Each of the adenoviruses tested in this investigation induced large amounts of chromosome aberrations in abortively-infected cells from a host in which some adenoviruses have been shown to be oncogenic. Adenovirus infection of human cells, however, usually results in a lytic response (86). Such a response to viral infection obviously precludes development of the neoplastic state. The present investigation demonstrates that human cells lytically-infected with adenoviruses display no chromosome aberrations prior to viral-induced cessation of mitosis.

Several recent studies indicate that chromosome aberrations are, in fact, induced in human cells *in vitro* by adenoviruses (156,197,229). Aberrations induced by Ad.12 were produced in cells which did not replicate virus, i.e. abortively-infected cells. In addition, inactivation of the replicatory abilities of adenovirus by ultraviolet light does not result in equivalent inactivation of other

virus-induced responses, such as induction of "T" antigen (197,228) and chromosome damaging capacity (197,228,229). An abortive infection may therefore be obtained with defective particles in a cell which normally supports virus multiplication; this system may then yield chromosome aberrations.

While the importance of chromosome aberrations in the initiation and development of tumors is still controversial, it has been suggested that chromatid breakage may be the most important chromosome aberration induced by viruses, with regard to possible relevance to neoplasia. It is of great interest, then, that each adenovirus tested, including oncogenic and non-oncogenic types, induced large amounts of chromatid breakage in hamster cells, which do not support adenovirus replication. It is entirely possible that each adenovirus type will also be shown capable of inducing chromosome aberrations in abortively-infected human cells. Abortive infection may result from the presence of defective particles in a population of virus particles, as recently shown for SV40 virus (214), yet such defective particles may still display potent tumorigenic capacities (214).

The present study thus demonstrates that abortive infection with adenoviruses can result in vast amounts of chromosome damage. Moreover, it has been pointed out that

defective adenoviruses can retain their chromosome-breaking activities, and can then induce chromosome aberrations in abortively-infected human cells. Further investigations therefore must actively pursue the relevance of defective adenovirus particles to chromosome aberrations in human cells and to the possible viral etiology of human neoplasms, for the natural exposure of man to adenoviruses is potentially a factor of great importance in his exposure to possible oncogenic agents.

## SUMMARY

The capacity of human adenoviruses types 2,4,7, 12 and 18 and simian adenoviruses SV15 and SA7 to induce chromosome aberrations in cultured Syrian hamster cells (BHK21) and human amnion cells (AV3) was examined at input multiplicities of 25, 5 and 1 TCID<sub>50</sub> per cell.

The human cells are lytically-infected with each adenovirus tested, supporting a complete virus replication cycle. Prior to the viral-induced cessation of mitosis, adenovirus-infected AV3 cultures show no detectable increase in the incidence of chromosome aberrations. In contrast, hamster cells are only abortively-infected with each adenovirus tested, not supporting multiplication of infectious virus, with the exception of Ad.2 to a limited extent. Each adenovirus induces a wide variety of chromosome aberrations in hamster cells, including chromatid breaks and fragmentation, overcontraction and erosion of the chromosomes, and mitotic anomalies.

The incidence of chromosome aberrations, the intensity of damage per cell, and the type of chromosome aberrations depend upon the input multiplicity of each virus tested. In addition, the types of chromosome

aberrations depend upon the type of adenovirus employed. The time of sampling also markedly influences the incidence of chromosome aberrations seen in adenovirus-infected hamster cultures.

The chromosome damage induced in hamster cells by adenovirus infection is due to the infecting virus particles, yet adenovirus-induced chromosome aberrations most closely resemble those produced by certain chemical inhibitors of DNA biosynthesis. Preliminary evidence is presented, however, that adenovirus infection does not inhibit, indeed, may stimulate, DNA synthesis in abortive infection of hamster cells, and in relation to this, a possible mechanism for adenovirus-induced chromosome aberrations is discussed.

The capacity to induce large amounts of chromatid breakage during abortive infection is a common feature of the adenoviruses tested, including the highly-oncogenic types and those apparently non-oncogenic serotypes. Chromosome aberrations induced by adenoviruses during abortive infection of mammalian cells is potentially of great importance in relation to the possible oncogenicity of human adenoviruses for man.

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