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Received January 21, 1966. P.S.E.B.M., 1966, v122.

Chromosome Studies of Human Cells Infected *in utero* and *in vitro* With Rubella Virus.* (31099)

T. H. CHANG,† P. S. MOORHEAD, J. G. BOUÉ,‡ S. A. PLOTKIN,§ AND J. M. HOSKINS‡‡
(Introduced by David Kritchevsky)
Wistar Institute of Anatomy and Biology, Philadelphia, Pa.

Published observations on the induction of chromosome breakage by a number of viruses such as measles, chickenpox and herpes simplex(15,1,2,7,8) as well as the recent occurrence of a major epidemic of rubella in the United States have stimulated interest in the possible effects of rubella on the chromosomes of human fetuses *in utero*. In fact, positive effects of rubella virus on chromosomes *in vivo* have been reported by Wiedemann(25) who found elevated breakage in 2 cases of Gregg's syndrome.

Further, it was demonstrated in our laboratory that infection of human fibroblasts *in vitro* resulted in an increased frequency of breakage, although neither major rearrangement nor evidence of non-disjunction was observed(4). On the basis of sex chromatin studies it has recently been suggested that a correlation may exist(20) between non-disjunction and epidemic rubella.

On the other hand, no aberrations were observed(13,3) in 2 chromosomal studies of leucocytes from abnormal newborn infants, known to be excreting rubella virus. Earlier, it was reported that no abnormalities were present in cells cultured from fetuses from rubella-infected mothers; however, since only "eight cells" were examined in one case(14) and "mitoses were few" in the other(23) further studies seemed warranted.

In the present study, fibroblast-like cell strains were derived from fetuses which had been aborted therapeutically because of confirmed diagnoses of maternal rubella. In all cases rubella had occurred in the first trimester of pregnancy, usually (60%) in the second month. Further, strains derived from

* Research supported in part by USPHS Training Grant TI-GM-142 from Nat. Inst. of General Med. Sciences, Contract PH 43-62-157 from Nat. Cancer Inst., Career Development Award K3-CA-18,372, Research Grant AI 01799 from National Inst. of Allergy & Infect. Dis. and Joseph P. Kennedy, Jr. Foundation.

† Present address: U. S. Dept. of Agriculture Metabolism and Radiation Research Laboratory, Fargo, N. D.
‡ Present address: Laboratoire de la S E S E P., Paris, France.
§ Joseph P. Kennedy, Jr. Foundation Scholar.
‡‡ Present address: Dept of Bacteriology, University of Sheffield, England.

TABLE I. Fetal Ages of Rubella Abortuses at Time of Initiation of Strains Used for Chromosome Studies.

Rubella abortus designation		Elapsed time (wk) from Last menstruation to rubella symptoms	Rubella symptoms to abortion	Approximate age of abortus (wk)
Series I	Series II			
R.A. 3		7	2	9
R.A. 4		11	2	13
R.A. 6		12	2	14
	R.A. 16	2	8	10
	R.A. 17	7	8	15
R.A. 20		6	3	9
R.A. 22		6	4	10
R.A. 25		7	3	10
	R.A. 26	7	4	11
	R.A. 27	9	3	12
	R.A. 28	8	3	11
R.A. 31		9	5	14
	R.A. 32	4	7	11
	R.A. 33	7	5	12
	R.A. 35	4	10	14

spontaneous and surgical abortions were infected *in vitro* in an effort to confirm our earlier observations of elevated breakage frequency.

Materials and methods. Rubella abortion cell strains. Pregnancy was terminated from one to 16 weeks (median of 4 weeks) after the onset of rubella. The ages of the various rubella abortuses contributing to the two series studied, (see below, Series I and II), as well as the time relationships regarding detection of virus in the mothers, are given in Table I.

Embryonic material was obtained by surgery and either whole embryo or fragmented tissues were used. In the case of fetuses obtained by curettage, only skin and muscle were used; in the case of intact fetuses, only individual organs were used. In all cases the material was washed 3 times in saline and explants were placed in organ culture according to the technique of Jensen *et al*(11). In this method the tissue rests on "teabag paper" supported on a metal grid and is bathed in modified Eagle's medium, incorporating double-strength amino acids and vitamins and 10% inactivated calf serum. Cells usually grow out from the fragments in 2 to 6 weeks and eventually give rise to a confluent culture. When the cultures (4 oz bottles) became confluent, they were trypsinized and subcultured 1:2 on a weekly schedule.

Fetal strains infected in vitro. Using the same culture procedures and methods described for the rubella abortion-derived strains, 27 diploid cell strains were established and propagated *in vitro* from embryos obtained by spontaneous and surgical abortions. In each case, no clinical indication of virus infection was noted.

All the strains were susceptible to rubella virus and a chronic infection was readily established in each. After a period of serial cultivation following infection, 4 strains from 3 fetuses were selected for examination of the chromosomes. The rubella virus used for infection was prepared by mixing 2 pools;‖ one was prepared from a permanent cell line of rabbit kidney origin (RK13) and the other from a human diploid cell strain (WI-38). The virus input dose was 4.8-5.3 $\times$ 10^4 $TCID_{50}$ per 4 oz culture bottle, each of which contained approximately 3 $\times$ 10^6 cells. All virus assays were performed according to the method of Parkman *et al*(17), using African green monkey kidney cells.

Chromosome preparations. Rubella abortion cell strains. The cytological studies of the strains from tissues of abortuses from rubella-infected mothers were performed in two parts. Preparations from Series I, comprising samples of 8 strains derived from 7 abortuses, were examined by one observer (J.G.B.) and preparations from Series II, of 9 similarly derived strains, were examined by another (T.H.C.). Except for the 4 samples from rubella abortuses R.A. 3, R.A. 4 and R.A. 6, all preparations for metaphase cell studies were made from suspensions of fixed cells using ignition-drying procedures following the usual pretreatments of colcemide and hypotonic trypsin solutions(21). The 4 exceptions mentioned were coverglass grown monolayers which were handled as such throughout treatments of 0.1 μg/ml colcemide and hypotonic serum, fixation and air-drying (12). No consistent differences could be

‖ One of these pools (RK13) was later discovered to have been contaminated with Mycoplasma (see *Discussion*).

attributed to either methodology or to observer.¶

In vitro *infected cell strains*. Four cell strains (5406 Lung, 5311 Pericardium, 5302 Skin, and 6211 Pharyngeal mucosa) from surgically or spontaneously aborted fetuses, were examined. Each of these was divided into "infected" and "control" substrains. Samples for chromosome studies were taken at various indicated periods during serial cultivation following the original exposure to rubella virus. Slide preparations were made by the same ignition-drying procedures described for the rubella abortus material.

Observations. All the data were obtained, using oil immersion optics, from metaphase cells first selected for their quality using low-power observation. Exact chromosome counts were made and all cells were examined for obvious karyotypic variation and for the presence of any rearrangements or breaks.

In Series I, approximately 50% of all cells examined were analyzed or karyotyped to the extent that the recognizable chromosome pairs and chromosome groups were accounted for individually. In Series II, 50 cells (50%) of each sample were subjected to the same karyotypic analysis. In the *in vitro* infection experiments from 20 to 50% of all metaphases examined were so analyzed.

General breakage frequencies referred to are expressed as the percentage of cells that had one or (rarely) more distinct achromatic gaps or breaks. Breaks in which a displacement of the fragment had occurred, often termed "true breaks," are tabulated together with the more common achromatic gaps. In presenting the data, both chromatid and chromosome types of lesions are considered together; thus, acentric fragments, "delayed isolocus breaks"(16), and unequivocal achromatic gaps of one or both chromatids are regarded as equivalent indicators of chromosomal damage and the criteria used are illustrated (Fig. 1).

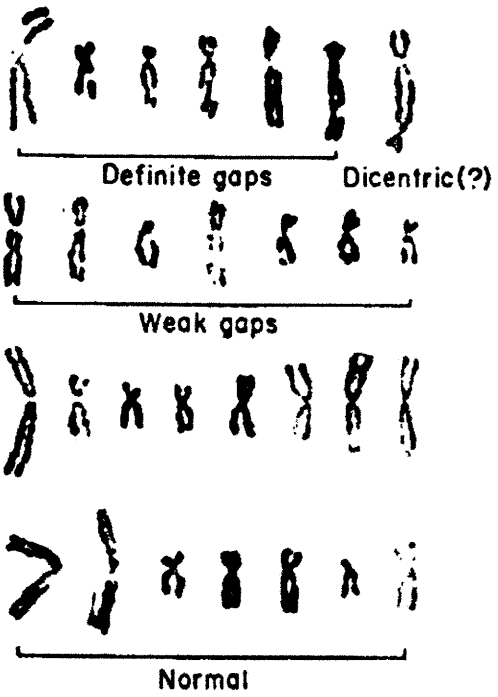

FIG. 1. Examples of achromatic gaps and breaks in metaphases from rubella abortus cell strains illustrating criteria used for scoring of frequency of breakage. Unequivocal or "Definite" gaps (upper row) were scored whereas "Weak" gaps (second row) were excluded as borderline instances. Fourth and fifth examples of upper row represent "true" breaks involving displacement of fragment.

Results. General observations. Rubella abortion cell strains. Morphologically, the cell strains resembled fibroblasts and appeared to be similar to the strains obtained from spontaneously aborted embryos. Out of the 40 rubella fetuses cultured, cell strains were derived from 34; in most cases they originated from pieces of skin and muscle obtained at curettage. Intact embryos, however, yielded cell strains from heart, lung, kidney, thymus, pituitary, eye, placenta, brain, pharyngeal mucosa and adrenal.

Rubella virus was isolated from the supernatant culture medium of cell strains derived from 18 fetuses; 16 fetuses yielded cell strains which were rubella-negative. Every cultured strain from 8 of the rubella-positive fetuses

¶ In order to determine whether criteria used by the two observers were the same, the same slide preparations from *Rubella abortus* 31.2, 8th passage, were examined. The results showed no significant difference in interpretations of unequivocal gaps and breaks. J. G. B. noted 14 breaks in 110 cells, equalling 12.7% (7.1% - 20.6%) and T.H.C. noted 8 breaks in 100 cells, equalling 8.0% (3.5% - 15.0%).

was positive for virus and 10 fetuses gave rise to both positive and negative strains. Virus-carrying cell strains were obtained from all types of organs cultured, which suggests a wide dissemination of infection within the fetus. Virus titers in the supernatant medium of positive strains varied between $10^{2.0}$ and $10^{4.0}$ $TCID_{50}$/ml. One hundred and twenty cell strains derived during these studies were subcultured weekly at a 1:2 ratio for 10-20 weeks before they ceased to divide; 48% of this number carried rubella virus throughout the entire period.

Fetal cell strains infected in vitro. Cells were subcultured as described and at 3-7 days postinfection culture fluid assays indicated, without exception, that rubella virus was present; however, the maximum titer was not reached until 2-3 passages after infection. Virus yields ranged from 2.0 to 5.3 $\times$ 10^4 $TCID_{50}$/ml, and did not differ significantly between different strains.

The active proliferation of cells during chronic infection was not affected, except that the lung strain 5406 could not be cultivated beyond the 10th passage. This early termination of growth was consistent with our earlier observations, *i.e.,* that cultures derived from human lung are usually inhibited by rubella, although the effect may not become apparent for a few passages (19,10).

At no time during chronic rubella infection was there any evidence of cell transformation in terms of unusual growth or morphologic changes, although the association between dividing cells and the virus extended to a maximum of 28 weeks. No ordinary cytopathic effects were noted in any strain during this period. All cell strains, infected or noninfected, as well as those from rubella abortus material, eventually ceased growing, as has previously been noted for such human fibroblastic strains (9,22).

Effects on the chromosomes. Neither obvious rearrangements nor non-disjunctional changes were prominent in any of the material studied. Among approximately 1400 cells from Series I and II of the rubella abortion strains, only 4 cells proved to have unquestionable changes in number. Two cells with dicentrics were observed among 600 cells of the *in vitro* infected material. Apart from these examples, chromosome counts gave no indication of a departure from euploidy. Since the proportions of hypodiploid and hyperdiploid counts were not excessive they are presumed to be due to artifacts of preparation. Thus, chromosome breakage provided the principal basis for comparisons.

TABLE II. Series I. Frequency of Chromatid and Chromosome Breakage in Cells Cultured from Seven Abortuses from Rubella-Infected Mothers.

Abortus & tissue of cell strain	Passage examined cytologically *in vitro*	Evidence of virus: *In vitro*—various passages	Evidence of virus: *In embryo*	Achr. gaps & true breaks	Total metaph. observed	Frequency of gaps & breaks (%)	Statistical limits at 95% confidence (%)
R.A. 6* Spleen & kidney	Primary	Neg.	Neg.	21	31	67.8†‡	51.4-84.3
R.A. 4* Skin	"	Neg.	Neg.	5	37	13.5	2.5-24.5
R.A. 3* Skin & muscle	"	"	"	4	45	8.9	1.3-16.4
R.A. 6* Skin	4th	"	"	2	48	4.2	0.4-14.4
R.A. 20 Skin & muscle	5th	"	"	6	57	10.5	4.0-21.6
R.A. 22.1 Heart	5th	"	"	1	102	6.9	2.9-13.9
Subtotal† of "negatives"	—	—	—	24	289	8.3†	5.4-12.1
R.A. 25A Skin & muscle	5th	Pos.	Pos.	22	118	18.6	11.6-25.8
R.A. 31.2 Skin	4th	"	"	36	124	29.0‡	21.0-37.0
Subtotal of "positives"	—	—	—	58	242	24.0‡	18.6-29.3

* These 4 preparations were air-dried coverglass monolayers; all others were from ignition-dried suspensions of fixed cells.
† Exceptional value from R.A. 6 (spleen and kidney) excluded from subtotal for "negatives."
‡ Values which differ significantly from virus-negative subtotal ($P < 0.05$).

Breakage in rubella abortion cell strains—Series I. An elevated frequency of general breakage was found in 3 of the 8 rubella abortion strains that made up Series I (Table II).

There appeared to be a partial correlation between the presence of virus and breakage since 2 of these 3 strains were positive for rubella. The R.A. 6 (Spleen and Kidney) sample from the original explant culture showed the highest break frequency of all, and although viral assay was not performed on media from the primary explant, no virus was found in either the embryo or in later subcultures of the strain.** The mother was definitely positive for rubella, as were all the cases considered in this work.

For purposes of comparison with the virus-positives the virus-negative strains may be regarded as a control group (Table II). Excluding the exceptionally high value of 67.8% for R.A. 6 (Spleen and Kidney), a frequency of 8.3% for the "negatives" is obtained. For the 2 rubella-positive strains a combined value of 24.0% is obtained which is significantly†† different from that for the rubella-negatives (Table II).

Breakage in rubella abortion cell strains—Series II. Breakage frequencies ranged from 3 to 14% for the 9 strains examined, and no individual value was elevated above any other to a significant degree (Table III). The combining of values from those strains which were rubella-negative failed to reveal any differences from those which were positive. The combined total for the 900 metaphases examined, without regard to virus, yielded a break frequency of 9.8% (7.9-11.9%).

Breakage in fetal cell strains infected in vitro. Of the 4 strains infected with rubella *in vitro*, strain *5406 Lung* provided the most extensive material, permitting a comparison between 300 cells from uninfected controls and 276 from the rubella-infected cultures (Table IV). Breakage frequencies obtained for each of the 3 different passage levels examined indicated no significant differences, and the combined data from the 3 passages also yielded no significant difference when infected and uninfected cultures were compared.

Of the 3 other strains studied, pharyngeal mucosa gave an elevated value for chromosome breakage which did differ significantly from its control material (Table IV). A tendency for a slightly greater breakage value for each infected culture over its control counterpart was noted and a comparison of all control with all infected values confirms

TABLE III. Series II. Frequency of Chromatid and Chromosome Breakage in Cells Cultured from Nine Abortuses from Rubella-Infected Mothers.

Abortus & tissue of cell strain	Passage examined cytologically *in vitro*	Evidence of virus: *In vitro*—various passages	Evidence of virus: *In embryo*	Achr. gaps	True breaks	Total metaph. observed	Frequency of gaps & breaks (%)	Statistical limits at 95% confidence (%)
R.A. 16.2 Skin	4th	Neg.	Pos.	2	1	100	3.0	0.6- 8.6
R.A. 27.6 Skin & muscle	4th	"	Pos.	5	2	100 D, Q	7.0	2.9-13.9
R.A. 35.10 Lung	4th	"	Neg.	9	5	100*	14.0	7.8-22.7
Subtotal				16	8	300	8.0	5.2-11.1
R.A. 17.1 Skin	5th	Pos.	Pos.	11	3	100	14.0	7.8-22.7
R.A. 26.6 "	4th	"	"	10	2	100	12.0	6.3-20.2
R.A. 28.4 "	4th	"	"	14	0	100	14.0	7.8-22.7
R.A. 31.2 "	8th	"	"	6	2	100	8.0	3.5-15.2
R.A. 32.8 Brain	4th	"	"	11	1	100	12.0	6.3-20.2
R.A. 33.3 Plac.	4th	"	"	4	0	100*	4.0	1.1-10.9
Subtotal				56	8	600	10.7	8.8-13.4
Combined totals				72	16	900	9.8	7.9-11.9

D = Cell with a dicentric chromosome. Q = Quasi-diploid cell. * Cell with 47±° or 48±° chromosomes.

**No virus could be recovered from 7 different organs, including spleen and kidney, nor from 22 different cell cultures.

†† The level of significance considered acceptable is that of $P < 0.05$, as determined from the tables of Stevens(24).

TABLE IV. Frequency of Chromatid and Chromosome Breakage in Cultured Fetal Cells Infected *in vitro* with Rubella.

Abortus & tissue of cell strain		Passage *in vitro* Infected	Passage *in vitro* Sampled	Rubella infection *in vitro*	Achr. gaps & true breaks	Total metaph. observed	Frequency gaps &/or breaks (%)	Statistical limits at 95% confidence (%)
5406	Lung	—	8th	Control	6	96	6.3	2.3 -13.1
		—	9th	"	5	100	5.0	1.6 -11.2
		—	10th	"	8	80	10.0	4.4 -18.8
Control subtotal					19	276	6.9	4.2 -10.6
5406	Lung	4th	8th	Positive	9	100	9.0	4.2 -16.3
			9th	"	7	100	7.0	2.8 -13.7
			10th	"	11	100	11.0	5.6 -18.9
Infect. subtotal					27	300	9.0	6.0 -12.8
5311	Pericardium	—	15th	Control	3	84	3.6	0.7 - 9.3
		4th	13th	Positive	6	100	6.0	2.2 -12.6
5302	Skin	—	14th	Control	3	70	4.3	0.8 -10.5
		4th	14th	Positive	11	100 D, D	11.0	5.6 -18.9
6211	Pharyng. mucosa	—	21st	Control	1	75	1.3	0.03- 7.3
		4th	21st	Positive	15	100	15.0*	8.0 -22.0
Combined total controls		—	—		26	505	5.2	3.4 - 7.5
Combined total infecteds		—	—		59	600	9.8*	7.6 -12.5

D = cell with a dicentric chromosome. * Significantly different from its control value ($P < 0.05$).

this trend: 5.2% *vs* 9.8% ($P < 0.05$, Table IV).

Discussion. In general, the effect of rubella infection on the chromosomes of human diploid fibroblasts was minimal. No evidence was obtained from these studies, which involved more than 2000 cells, that non-disjunction was significantly increased, even in the demonstrated presence of rubella virus. A small but demonstrable increase in achromatic gaps and breaks occurred in cultures derived from tissue which had become infected naturally *in utero*. Both series of strains from rubella abortuses showed this slight increase in breakage.

In Series I (Table II) among 8 samples observed, there were 3 instances of elevated breakage, 2 of which were positive for rubella virus. However, in the larger series of strains from rubella abortus material (Series II, Table III) 6 of the strains which were positive for rubella *in embryo* and *in vitro* revealed no striking increases in chromosome breakage. Only by pooling all the data from these 6 positive strains could a slight effect be imputed.

Possibly the production of rubella virus *in vitro* bears no relation to persisting chromosome damage stemming from an original exposure *in utero*. If this is true, break frequencies among the virus-negative strains would not, of course, constitute a proper control value for purposes of comparison. An acceptable control value might possibly be based upon cell strains from an extensive series of spontaneous abortion fetuses not exposed to rubella. However, according to one study(5) at least 22% of spontaneous abortion-derived cell cultures reveal major abnormalities of chromosome number. Fetal material, obtained surgically in the absence of known pathological factors, might be used to circumvent this objection. One available value for chromosome breakage frequency, from the normal lung strain WI-38, is 5.1% (3.4-7.2%) (Girardi, Weinstein and Moorhead, unpublished). This determination of break frequency agrees well with a value of 3.5% (2.5-5.0%) obtained independently by Weinstein (unpublished) Similarly, the overall value for break frequency obtained within the present study, in non-infected strains obtained from both spontaneously and surgically aborted material, was 5.2% (3.4-7.5%) (Table IV).

If one considers these 3 values (5.1%, 3.5% and 5.2%), each based upon 500-700 cells, as representative of the expected level

of spontaneous chromosome breakage *in vitro*, it is noted that the frequency of 9.8% from all strains of Series II (Table III), regardless of the presence of virus, is significantly greater than any control value. On this basis there is evidence in both series for slight, but significant, increases in chromosome breakage due to an original exposure to rubella infection *in utero*.

The results from the cell strains which were infected *in vitro* with rubella are subject to question, since, unfortunately, Mycoplasma organisms were present in one of the viral pools used. That the presence of Mycoplasma may induce chromosome damage(6,18) has been reported from two laboratories. With this reservation in mind, there was, as in the other two studies reported here, a slight but significant increase in breakage associated with rubella. In each of the intra-strain comparisons (Table IV) the rubella-infected material had a slightly greater frequency of chromosome breakage although only one of these, pharyngeal mucosa, was significantly increased above its control counterpart. Differences in response to rubella infection related to the tissue of origin of a strain may exist (19) and it may be of interest to note that pharyngeal mucosa was also the tissue source of the strain in which induced breakage had previously been demonstrated(4).

It must be emphasized that all these studies were limited to those cells which propagate most readily in culture, namely those which morphologically resemble fibroblasts.

With this limitation the findings show: (a) demonstrable, but small increases in chromosome breakage, (b) a partial correlation with the presence of virus, (c) lack of changes in chromosome number per cell and rarity of chromosome rearrangement. The findings, therefore, do not seem to encourage the viewpoint that cytologically visible chromosome damage or transmitted mitotic errors play a significant role in the embryopathy associated with rubella infection *in utero*.

Summary. A cytologic study of metaphase chromosomes was undertaken to determine if damage to the genetic apparatus of human cells may be induced by rubella virus and whether such damage produces the pathology seen in newborns from mothers infected early in pregnancy. Fibroblast cell strains were initiated *in vitro* from embryonic material obtained by therapeutic abortion of fetuses from rubella-infected mothers. Embryonic material from spontaneous and non-rubella therapeutic abortions yielded similar cell strains for infection *in vitro*. The results from both types of studies were as follows: (a) Virus shedding cell strains were obtained from all types of organs cultivated, which indicated wide dissemination of virus in the fetuses from rubella-infected mothers. (b) Chronic infection could readily be established by *in vitro* infection and no cytopathic effect was noted. (c) Although many strains continued to shed virus throughout months of *in vitro* cultivation, no changes in growth properties occurred with the exception that infected strains from lung tissue showed limited growth capacities. (d) From observations of over 2000 metaphases, neither obvious chromosomal rearrangements nor non-disjunctional changes were observed in strains infected naturally or experimentally. (e) Among 17 rubella abortion-derived cell strains there was a marked elevation in frequency of chromosome breakage in 3 instances (18%, 29%, 68%). (f) A correlation between virus shedding *in vitro* and increased chromosome breakage was noted although this was not complete. (g) The combined average frequency of chromosome breakage in one set of 7 rubella-abortion strains was 18%; in the other set of 9 abortus strains, 9.8%. (h) Similarly, a slight, but significant elevation in average chromosome break frequency (9.8%) was obtained in four cell strains which had been experimentally infected *in vitro* with rubella virus (uninfected cells: 5.2%).

Although effects upon the chromosomes were demonstrated, these were restricted to slight general elevations in frequency of breakage above normal values. Very high increases in breaks were sporadic. It appears more likely that the general fetal abnormalities associated with rubella infection *in vivo* result from possible effects of the virus upon growth rather than from effects upon the chromosomes. However, the studies were re-

stricted to fibroblasts cultured *in vitro;* and effects on other types of cells may have greater implications for this question.

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Received January 21, 1966. P.S.E.B M., 1966, v122.

Inhibition of Adenovirus Replication by 1-β-D-Arabinofuranosylcytosine.* (31100)

LAWRENCE A. FELDMAN AND FRED RAPP
Department of Virology and Epidemiology, Baylor University College of Medicine, Houston, Texas

Human adenovirus types 2, 5, 7, and 12 are unable to replicate in African green monkey (GMK) cell cultures(1-4). In these cells, the viruses are able to induce synthesis of adenovirus tumor (T) antigen but fail to induce synthesis of viral (V) capsid antigen (1). Co-infection with simian papovavirus 40 (SV40) potentiates replication of the adenoviruses(1-4) and results in induction of the adenovirus T and V antigens(1) as well as the synthesis of SV40 T and V antigens.

Previous studies in our laboratory indicated that 1-β-D-arabinofuranosylcytosine (cytosine arabinoside or ara-C) inhibited the replication of SV40(5,6) while allowing the synthesis of SV40 T antigen. Buthala(7) had suggested that ara-C does not inhibit replication of adenoviruses and when the antagonist was found to block the potentiation of adenovirus replication in GMK cells by SV40(8), it was concluded that SV40 T antigen was not responsible for the potentiation phenomenon. In order further to support such a conclusion, it was necessary to confirm that ara-C was not capable of inhibiting adenovirus replication. The results of quantitative studies designed to investigate the effect of ara-C on adenovirus replication in human cells are presented here.

Materials and methods. Cell cultures and viruses. Primary cultures of human embryonic kidney (HEK) cells were grown in one-oz bottles using Melnick-Hanks' (M-H) lac-

* This work was supported in part by grants CA-04600 from Nat. Cancer Inst. and AI 05382, and 5 T1 AI 74 from Nat. Inst. of Allergy & Infect. Dis., U.S.P.H.S.