The Wistar Institute of Anatomy and Biology,
Philadelphia, Pennsylvania, U.S.A.

Behaviour of Rubella Virus in Human Diploid Cell Strains*

I. Growth of Virus

By

J. M. Hoskins** and **S. A. Plotkin**

With 2 Figures

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Introduction

The relation between rubella virus infection and congenital malformation in man is now well known, infection during the first three or four months of pregnancy giving rise to a high proportion of infants with congenital defects. Damage induced by the virus characteristically involves the brain, heart, eyes and ears (11, 6, 20, 24), although other organs and tissues can be affected. The virus multiplies in both affected and unaffected areas, and may be recovered from widespread sites throughout the fetus and newborn infant weeks or months after known maternal infection during early pregnancy (1, 13, 15, 3).

It seemed possible that a study of the behavior of rubella virus in human diploid cell strains in *vitro* might help in understanding the effect of the virus on the developing organs during human infection. Preliminary work already reported from this laboratory has shown that when human diploid cell strains are infected and propagated in tissue culture their response depends on the organs from which they were derived (2, 17, 9). The purpose of the present communications is to extend these observations, paying particular attention to the long-term aspects of rubella virus infection.

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** Holder of USPHS International Postdoctoral Research Fellowship. Present address: Swiss Serum and Vaccine Institute, Bern, Switzerland.

Matorials and Methods

Human Fetuses

Two groups of human fetuses. 8—20 weeks of age, were used for the initiation of diploid cell strains. The first group consisted of normal embryos obtained by hysterotomy and flown from Scandinavia, bathed in physiologic saline at 0° C. The second group represented spontaneous abortions. obtained from the g-ynecologic service of the Philadelphia General Hospital. Intact fetuses of both groups were kept at 0—4° C from the time of abortion until used, 12 to 96 hours later.

Human Diploid Cell Strains (HDCS)

Except for WI 38, the HDCS were initiated from surgically and spontaneously aborted fetuses by means of an organ culture technique (12) described in the following communication (8).

The WI 38 strain (7) was obtained from Dr. *Leonard Hayflick*. This strain, originally derived from fetal lung. had been reconstituted from a frozen cell stoclr and was used only from the 13th passage onwards. Cell strains initiated in our own laboratory could be used at lower passage levels.

Established HDCS — both WI 38 and those derived in our own laboratory — were grown in 110 ml "milk dilution" bottles and usually subcultured at seven-day intervals. The medium mas removed from confluent monolayers and the latter overlaid with trypsin solution, pre-warmed to 37° C. After 30 seconds' agitation, the trypsin mas aspirated and the cultures allowed to stand at room temperature until the monolayer had detached completely. The cells were then vigorously pipetted in about 10 ml of growth medium, and tlie entire yield divided between two bottles, each having the same surface area as tlie original bottle. Additional medium was tlien added to make a total volume of 25 ml and tlie bottles were incubated at 36—37° C.

Growth and maintenance media. The HDCS growth medium consisted of double-strength Eagle's minimum essential medium (MEM) in Earle's saline, 10% heated (56° C for 30 minutes) calf serum, and sodium bicarbonate at a concentration of 0.055%. Penicillin and streptomycin were also incorporated, at 100 I.U. and 100 μg/ml, respectively. These antibiotics were substituted in certain experiments by chlortetracycline, used at 50 μg/ml. The maintenance medium for HDCS was identical in composition to the growth medium unless otherwise specified.

Green monkey kidney cultures were obtained commercially and maintained in a medium consisting of single-strength Eagle's MEM in Earle's saline, and 2% calf serum (heated). In addition, the bicarbonate concentration was raised to 0.22%.

Trypsin. Trypsin (Difco, 1:250) was prepared as a 0.20% solution in the double-strength Eagle's MEM — Earle's saline — bicarbonate medium used as a basis for the HDCS growth medium. Antibiotics were also incorporated.

Inoculation of HDCS. Confluent diploid cell cultures. usually at less than the 6th passage level, were inoculated with virus in order to establish chronic infections. Two procedures were employed, and gave similar results: (a) addition of 1 ml of virus in fresh medium to eacli culture. and (b) addition of 0.2 ml of rirus to drained cultures, followed by adsorption for 1 hour at 36° C. Fresh growth medium was then added to the culture. In both cases, inoculated bottles were incubated for 3—7 days before being subcultured in the usual may.

Viruses

1. Rubella Virus

Four strains of rubella virus mere used in these studies: West Point (19). R. W. (25), RA-27-3 (18). and a strain — designated H 600 — irolated in our own laboratory from the aborted fetus of a woman who had developed rubella. Pools of the four strains were prepared by growing the viruses in RK13 cells (14), strain WI 38 cells. BHK 21 cells (23), and primary African green monkey *(Cerco ithecus aethiops)* kidney cells, respectively. In each case, the pool consisted of lightly centrifuged tissue culture fluid, harvested at the peak of virus production and stored frozen in aliquots at approximately —50° C.

2. ECHO Virus Type 11

ECHO virus type 11, used for assay of rubella virus by interference. was the Gregory strain originally obtained from the American Type Culture Collection. Pools were prepared by growing the virus in African green monkey kidney cultures and harvesting them when about 75% of the cell sheet was destroyed. Cell debris was separated by light centrifugation. and the supernatant fluid diridetl into aliquots and stored frozen at approximately —15° C.

Assay of Rubella Virus

Assays of rubella virus were usually done by an interference technique, employing ECHO rirus type 11 in primary tube cultures of African green monkey kidney (16). Serial tenfold dilutions were prepared in phosphate-buffered saline (4). and 0.2 ml volumes inoculated into each of four tubes per dilution. After incubating at 36° C for 7 days, the cultures were challenged with 1.0×10^4 (± 0.5 log$_{10}$) $TCID_{50}$ of ECHO virus type 11. This was done by replacing tlie maintenance medium in each tube with an equal volume of fresh medium containing the requisite dose of ECHO virus. After a further 3 days' incubation at 36° C, the tubes were examined for the presence of ECHO virus-induced cytopathic changes: absence of any such changes was interpreted as being due to inhibition by rubella virus. Titers were calculated by the method of *Reed* and *Muench* (22).

Results

Susceptibility of HDCS to Rubella Virus

Cell strains derived from 29 fetuses were examined. The following tissues and organs yielded viable cell strains: skin, lung, pharyngeal mucosa, pericardium, brain, pituitary, kidney and thymus.

All diploid cell strains examined were susceptible to the three strains of rubella virus tested (West Point, R.W. and H600), and a chronic infection could be readily established in them without any special manipulations. Thus, in a series of experiments, fully confluent cultures at different passage levels were inoculated with virus doses ranging from 2.5×104 to 2.5×10^5 $TCID_{50}$, and the cultures incubated for 3—7 days. They were then subcultured and the medium saved for assay. Without exception, the cells became infected and virus could be recovered from the medium. The titer of supernatant virus did not, however, reach a maximum until after 2 or 3 cell passages had taken place. Thereafter,

virus production was determined by assay of culture fluid at every weekly passage, and was continuous at a low but approximately constant level for the entire life-span of the infected cell strain. These findings are summarized in Table 1, which shows that there were no pronounced differences in virus yield between any of the cell strains examined. Some importance, however, based upon the very large number of assays, may be attached to the slightly elevated average yields of lung, pharyngeal mucosa and pericardial cell strains. Virus production by skin, lung and pharyngeal mucosa cells was not affected by their in *vitro* age (passage history) at the time of infection (Table 2). The three strains of rubella virus used also showed no significant differences in yield when they were tested in several cell strains derived from skin, lung and pharyngeal mucosa.

Table 1. Yield of Rubella Virus during Chronic Infection of HDCS

HDCS	Number of strains	Mean titer and standard deviation	Number of assays
Sh*	29	3.2±0.20**	164
LU	17	3.7±0.81	62
PM	5	3.4±0.75	81
THYRI	4	3.2±0.64	6
PC	2	3.4±0.88	34
PIT	2	3.1±0.64	4
BR	2	3.0±0.54	3
KID	2	3.2±0.42	4

* SK = skin; LU = lung; PM = pharyngeal mucosa; PC = pericardium; BR = brain; KID = kidney; PIT = pituitary; TIITBI = thymus.
** $Log_{10}TCID_{50}$/ml of culture medium.

Table 2. Production of Rubella Virus by HDCS Infected at Different *in vitro* Ages

HDCS	Passage level at infection	Average virus yield	Number of assays
SK* (144)**	1	2.7***	9
	15	3.0	5
PM (6212)	4	3.0	12
	21	3.2	10
LU (6210)	7	3.6	7
	18	3.6	5

* For key to abbreviations, see Table 1.
** Cell strain designation.
*** $Log_{10}TCID_{50}$/ml of culture medium.

Absence of Cytopnthic Effect

Virus multiplication took place in the absence of ordinary cytopathic effects in any cell strain, while coverslip cultures treated with May-Grünwald-Giemsa stain also failed to reveal any differences between infected and control material.

A number of individual chronically infected skin, pharyngeal mucosa and pericardiurn cultures were kept without subculturing in standard growth medium, which was changed and assayed at weekly intervals. The same low-grade virus production found in regularly subcultured cells persisted in the stationary cell populations for periods of up to seven

months (Table 3). In a number of cases, virus production continued in the latter cells long after active replication of the cell strain from which they were derived had ceased. The cells did not appear to be affected by their role as a virus factory.

Table 3. Indefinite Rubella Production by Individual HDCS Cultures

Interval niter infection (Days)	Cell strain				
	5602* SK**	6201 SK	5311 PC	1219 PM	1220 PM
0— 15	3.9***		2.2	4.0	4.0
16— 30	3.2	3.2	2.7		
31— 45	2.2	3.4	2.9		
46— 60	2.4	2.2	2.2		
61— 75	2.0	3.2	2.7		
76— 90	2.3	1.5	2.0	2.4	
91—105	2.7	2.4	2.2		
106—120	(99)†	2.2	2.7		3.0
121—135		(111)	(120)		
136—150				3.2	3.4
151—165					4.0
166—180				3.2	3.2
181—195				3.4	(169)
196—210				3.2	
				(208)	
Total observation period (Days)	99	111	120	208	169
Mean titer	2.9†	2.4	2.5	3.1	3.5
Number of assays	13	13	14	11	7

* Numbers refer to cell strain designation.
** For key to abbreviations, see Table 1.
*** $Log_{10}TCID_{50}$/ml of culture medium.
† Figures in parentheses represent day of last assay.

Rubella Virus Growth *Curves in HDCS*

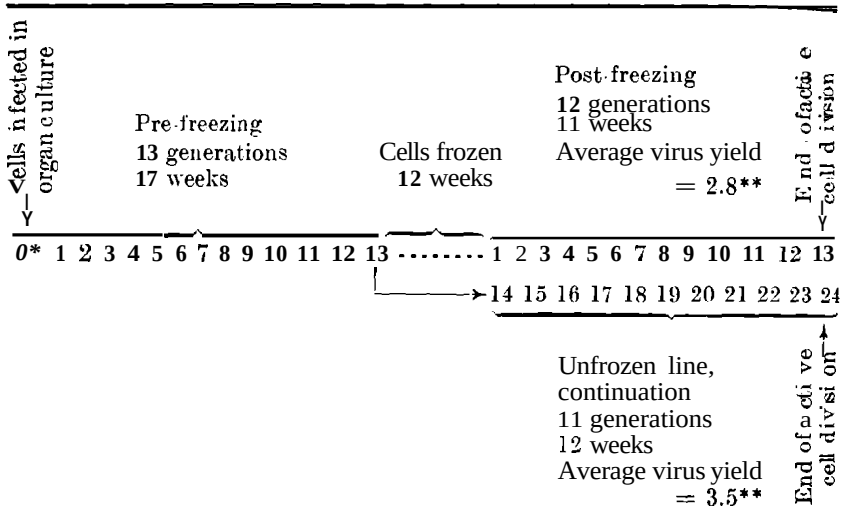
Since slight differences in production of rubella virus by HDCS of different tissue origins were suggested by the chronic infection experiments (e.g., see Table 1), it seemed profitable to examine virus growth curves in these strains.

Skin and lung cell strains were selected for comparison, since they seemed to give the biggest differences in virus yield and could be grown readily.

It was necessary to establish first the sensitivity of rubella virus to various procedures attendant upon disrupting infected cells and subsequent storage of the product. The effects of freezing and thawing and of ultrasonic vibration were investigated.

strain. Freezing was done by removing the cells with trypsin, and suspending them in a freezing medium at 1.5×10^6 cells/ml. The freezing medium consisted of single-strength Eagle's MEM, containing 10% glycerin and 10% heated (56° C for 30 minutes) calf serum; sodium hicarbonate was included at a concentration of 0.044%, and penicillin and streptomycin at 100 IU/ml and 100 µg/ml respectively. The suspension was then chilled overnight at 4° C, and the temperature finally lowered st about 1° C/minute until it reached approximately −78.5" C, when the frozen material was stored in a chest of solid carbon dioxide. After

Table 5. Freezing and Resuscitation of a Chronically Infected Pharyngeal Mucosa Cell Strain



* Serial figures represent cell generation number.
** $Log_{10}TCID_{50}$/ml of culture medium.

storing in the frozen state for 12 weeks, a 1 ml ampoule of the cells was thawed rapidly by swirling in a 37°C water bath and diluted to 10 ml with growth medium. The resulting suspension was transferred to a 5 cm Petri dish and incubated in a 5% carbon dioxide atmosphere at 36° C for 24 hours, when the growth medium was replaced with fresh medium. A confluent monolayer was established after a week, when the cells were subcultured in the usual way. Rubella virus was detected in the medium after the first post-freezing subcultivation and continued to be present until active cell growth ceased spontaneously after 12 generations (11 weeks). These findings are illustrated in Table 5. There was no significant difference in survival of the frozen and unfrozen chronically

infected cells. Moreover, the average virus yield from the post-freezing cell line did not differ significantly from that produced by the same unfrozen cells at a comparable passage level.

Discussion

Our observations amply confirm the ability of rubella virus to produce a low-grade chronic infection in all human diploid cell strains tested.

Our observations on the growth curve of rubella virus in HDCS show that approximately one-fourth to one-third of the cells produce virus at any given time, and that infected cells carry an average of only one complete virus particle at a time. Since loss of virus due to thermal inactivation at 37° C is high (0.3 $\log_{10}$ per hour), it appears that at peak production each infected cell must average a completed virus every hour.

Recently, *Rawls* and *Melnick* (21) have reported studies of rubella-carrying fibroblasts cultured from naturally infected fetuses. Their results are similar to ours in that their cell strains also produced rubella virus over long periods of time without evident cytopathic effect. In their naturally infected strains, the percentage of cells bearing virus was close to 100%.

Summary

Observations upon the growth of four strains of rubella virus in human diploid cell strains (HDCS) are reported. Sixtythree cell strains, derived from 29 fetuses by means of an organ culture technique, were studied. All HDCS tested were susceptible to rubella virus, and a chronic infection couid be established readily in them. The virus multiplied and was continuously produced at a low level for a maximum of 27 weeks while cell subcultivation proceeded normally. Stationary cell populations produced virus for at least 30 weeks. Experiments to define the growth curves of rubella virus were performed in skin and lung cell strains. Strains of lung cell origin yielded greater amounts of virus than skin cell strains. Both growth curve and chronic infection experiments showed that a maximum of approximately 30% of the cells were infected at any given time. Pharyngeal mucosa cells were stored for three months in the frozen state while chronically infected with rubella. Their life history and virus yield after resuscitation did not differ from unfrozen cells.

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Authors' address: Dr. ***J. M. Hoskins,*** Swiss Serum and Vaccine Institute, Bern, Switzerland.

The Wistar Institute of Anatomy and Biology,
Philadelphia, Pennsylvania. U.S.A.

Behaviour of Rubella Virus in Human Diploid Cell Strains

II. Studies of Infected Cells*

By

J. M. Hoskins** and S. A. Plotkin

With 2 Figures

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Introduction

Rubella virus infection of human fibroblast cells *in vitro* results in chronic infection with long term production of virus (6, 10, 11, 18). Infected cells appear normal, but on subsequent passage various effects are noted, which are the subject of the present communication.

Materials and Methods

Most of the methods used in this study have been described in the previous article (10). We add here only a description of our method fur developing cell strains from human fetuses.

According to this procedure (13), a stainless steel supporting grid stands in a 5 cm Petri dish containing 10 ml of growth medium. A disc of coarse. mesh, "tea-bag" paper rests upon the steel grid in such a way that it remains permanently at the air-culture medium interface. Two to four fragments of fetal tissue, about 1–2 mm3 in size, are placed upon the paper disc. and the entire apparatus is incubated at 36° C in a humid atmosphere of 5% carbon dioxide in air. Cells migrate from the tissue fragments and fall through the mesh onto the floor of the dish where they attach and start to grow. After 2–3 weeks, a more-or-less uniform cell monolayer was produced in most cases and consisted completely or mainly of fibroblastic elements,

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** Holder of USPHS International Postdoctoral Research Fellowship. Present address: Swiss Serum and Vaccine Institute, Bern, Switzerland.

depending upon the tissue of origin. Occasionally, tissues required 4–6 weeks before a confluent monolayer was produced. In all cultures, the growth medium was replaced with 10 ml of fresh medium at meekly intervals to maintain the nutritional factors and to offset evaporation.

Confluent monolayers were trypsinized as described below, and the entire cell population transferred in 10 ml of growth medium to a fresh 5 cm Petri dish. This transfer is arbitrarily defined as ***cell passage** number 1.* After 3–4 days' incubation at 36° C, a confluent monolayer was usually obtained. This was trypsinized and the cells transferred completely to a single 120 ml "milk dilution" bottle, representing ***cell passage** number 2.* Thereafter, passages mere usually made at weekly intervals, the contents of a single 120 ml bottle being divided between two fresh bottles, giving a 1:2 increase ratio. Since virtually no increase in human diploid cell populations occurs once a given cell monolayer becomes confluent (9), each passage made after the first bottle stage – i.e., passage number 2 – is reached represents an addition of exactly ***one generation*** to the cell strain. The life-span of a cell strain from passage 2 onwards may thus be measured accurately in terms of cell generations, thereby enabling valid comparisons of individual cell strain life-spans to be made. The method does not of course allow similar accuracy before the passage 2 stage is reached.

The particular advantage of this technique for the present studies is that it enables cultures to be established from exceedingly small amounts of tissue, an obvious advantage when working with organs derived from imniature fetuses. One should also note an inherent disadvantage of the procedure; namely, that it is highly selective, yielding, after a maximum of one or two passages, a cell population consisting only of fibroblastic elements. Nonetheless, it will be apparent that fibroblasts derived from different tissues may behave differently in a reproducible fashion.

Results

Growth of Human Diploid Cell Strains (HDCS)

Cell strains derived from 29 fetuses were examined. Twentyone of these originated from surgical abortions, while 7 came from spontaneously aborted fetuses. One cell strain was of uncertain origin. At the start of these studies, most importance was attached to HDCS derived from the surgically aborted fetuses since these could be presumed to be normal. In fact, no differences in any of the parameters studied could be found between the two groups of fetuses, and no distinction will henceforth be made between them.

We arbitrarily selected for study only those cell strains which gave a good initial culture and appeared to be growing well at the second Passage. The following tissues and organs yielded viable cell strains: skin, lung, pharyngeal mucosa, pericardium, brain, pituitary, kidney and thymus. The mean survival times of these strains are given in Table 1, which shows that considerable differences were found between strains obtained from different tissues and organs. In general, cell strains derived from skin, lung and pharyngeal rnucosa grew readily and possessed life

spans up to a maximum of 45 generations. On the other hand, strains originating from pericardium, brain, kidney, pituitary and thymus grew much less readily; not only were fewer strains able to be initiated, but those that were initiated had a shorter life-span. Considerable variation in life-span was found among the cell strains derived from any given organ.

Long-term Effect of Rubella Virus upon HDCS

Although chronically infected cell strains showed no obvious differences at first from uninfected control cultures, differences did become apparent in certain strains with the passage of time. This was manifested as a selective inhibition of mitosis, so that active multiplication of infected cell strains ceased before that of the controls. No cytopathic effect

Table 1
Life-span of Uninfected Human Diploid Cell Strains (HDCS)

HDCJ	Number of strains	Mean survival time (generations)
Skin (SK)	29	17 (6—36)*
Lung (LU)	17	21 (10—31)
Pharyngeal mucosa (PM)	5	33 (11—45)
Pericardium (PC)	2	16 (15—16)
Brain (BR)	2	11 (10—12)
Kidney (KID)	2	10 (10—10)
Pituitary (PIT)	2	6 (6— 6)
Thymus (THYM)	4	6 (4— 7)

* Figures in parentheses give minimum and maximum observed survival times.

accompanied this mitotic inhibition, and the infected cells appeared indistinguishable from their uninfected counterparts. On the basis of this criterion, human diploid cell strains can be classified into three groups, illustrated schematically in Table 2.

The first group (A) is made up of those cell strains whose life-span, while chronically infected with rubella, did *not* differ from that of uninfected control cells. Included in this group are strains derived from thymus, pericardium, brain, kidney and pituitary.

The second group (B) consists of cell strains whose growth was inhibited by rubella virus to a moderate degree; it includes pharyngeal mucosa and skin strains, which, when infected, had life-spans about 60 to 70% those of controls. Pharyngeal mucosa cell strains were uniformly inhibited by the virus, although the effect was not always obvious until several months after infection. The results with skin strains were equivocal. Slightly less than half (41%) of 29 strains examined were clearly inhibited by rubella, although, like strains derived from pharyngeal

mucosa, the inhibition occasionally became apparent only after several weeks or months of infection. The remaining fraction of skin cell strains (59%) showed no inhibition of mitosis.

The third group (C) in the classification consists only of lung cell strains, which were all regularly inhibited by rubella virus to a marked degree: namely, to between 25% and 50% of control values. However, the pattern of response with lung cells, apparently uniquely, was dependent upon the *in vitro* age of the strains at the time of infection. Thus, older lung strains, infected after 14 or more passages in culture, were inhibited

Table 2. Survival Time of HDCS Following Infection with Rubella Virus

Group	Organ from which strain derived		Number of strains	Mean survival time (generations)	
				Control	Infected
A	THYM*		4	3 (2— 3)**	3 (2— 3)
	PC		2	11 (11—11)	11 (10—12)
	BR		2	7 (4— 9)	6 (4— 7)
	KID		2	4 (4— 4)	4 (3— 4)
	PIT		2	2 (2— 2)	3 (2— 3)
B	PM		5	30 (6—41)	19 (5—27)
	SK		29***	14 (3—35)	10 (3—20)
C	LU†	Inf. <11	10	17 (9—28)	8 (4—20)
		Inf. >13	7	6 (4— 9)	1.6 (0— 3)

* For key to abbreviations, see Table 1.
** Figures in parentheses represent minimum and maximum observed survival times.
*** Inhibition shown by 12/29 (= 41%) strains.
Inhibition not shown by 17/29 (= 59%) strains.
† Lung strains are grouped according to whether they were infected at less than the 11th or greater than the 13th *in vitro* passages.

fivt times more rapidly than those infected at an early *in vitro* age. No similar correlation was found with strains derived from other tissues and organs. Fig. 1 shows the response of 17 individual lung strains to rubella infection. When cultures were infected at less than the 11th *in vitro* passage they had a mean survival time of 8 cell generations, while cultures infected at greater than the 13th passage had a mean survival time of only 1.6 cell generations.

The relation between *in vitro* age and accelerated reaction time following infection is indicated clearly by the data relating to four specified lung cell strains, shown in Table 3. An extended amount of data was available for the WI 38 cell strain, also of human fetal lung origin. These data were collected from eight separate experiments, done over a period

This appearance of pericardial cells remained unchanged during their entire life-span, extending up to a maximum of 16 generations. However, when these strains were chronically infected with either the West Point or H600 strains of rubella virus there was a marked decrease in cytoplasmic granularity about 4–6 generations after infection, which was correlated with a decrease in enzyme activity as judged by specific staining. After a further 4—5 passages, observable granule- disappeared altogether in unstained living cells, while stained cells revealed a small amount of acid phosphatase activity. These findings are shown diagrammatically in Table 6. Cultures stained for acid phosphatase are shown in Fig. 2. There was no correlation between the observed phenomenon and virus production by the cells, which continued unrestricted for their entire life-span.

Absence of Neoplastic Changes

At no time was there any evidence of cell transformation during chronic infection of human diploid cells with rubella virus, although the association between actively dividing cells and virus extended up to a maximum of 27 generations (27 weeks). We also examined two pharyngeal mucosa cell strains, infected 21 and 23 generations earlier, respectively, with the West Point rubella strain, for their ability to produce neoplastic lesions when inoculated into the checli pouch of 6—12-week-old hamsters. Up to 2.9×10^6 cells, in the logarithmic phase of growth, were inoculated into groups of animals. Nodules developed in all animals, and were grossly indistinguishable from those in controls inoculated with uninfected cells. All nodules regressed completely within 10—14 days. Histologic examination of the lesions revealed no eridencc of neoplasia.

Discussion

In human cells infected with rubella virus no cytopathic effect was observed at any time, but there was inhibition of cell growth. Inhibition occurred at any time from a few days to several months after infection had been initiated, depending upon the kind of cell stmin. HDCS could therefore be grouped according to whether they responded quickly, slowly, or not at all. In the present study, many cell strains were examined over extended periods, and it appears that certain of the conclusions reported earlier (6, 18) should be modified. Thus, strains derived from pharyngeal mucosa and skin, which were thought to remain unaffected by rubella virus, did tend to be inhibited before the homologous, uninfected controls. The inhibition was not observed, however, until after the cells had been passaged for several weeks, or even months, and it is thus possible that all diploid cell strains might show inhibition if they could be grown for

sufficiently long period. It may therefore be pertinent that the kinds of HDCS which were not inhibited (Group A of the classification) were those strains which had a shorter average life-span than those included in Groups B and C. Thus, the view held earlier that strains of IIDCS give an all-or-nonc response to rubella infection with respect to inhibition of mitosis appears not to be completely true. Rather, the action of the virus is graded, with cell strains from different tissues having different average responses.

Strains of lung origin were most sensitive to inhibition by rubella virus (6,11, 18), responding in general more quickly than others. However, no absolute distinction could be made between HDCS of lung and other tissue origins, since there was considerable overlapping in the time of response. One of our most interesting observations was that the response of lung cell strains was strictly related to the *in vitro* age of the strain at the time of infection. Thus, cells infected at greater than the 13th *in vitro* passage responded 5 times more rapidly than those infected at less than the 11th passage. It is possible that the accelerated response of older lung cell strains may reflect an incapacity of the mitotic mechanism of older cells to tolerate virus infection. It is known, for example, that late passage WI 38 strain lung cells react more readily to SV_{40} virus than cells of younger *in vitro* age (14). *In* the latter case, however, the cell- react by becoming transformed, and the mechanism of response may be different.

Our observations on chronically infected pericardial cell strains are of interest, since they indicate a relationship between rubella infection and acid phosphatase activity. In pericardial cells chronic rubella infection caused the gradual lysis of lysosomal particles, and loss or redistribution of acid phosphatase. The amount and distribution of acid phosphatase, together with that of other lysosomal enzymes, is known to be altered in variety of cytopathologic conditions, including infection by some viruses (1, 2, 4, 16). The significance of the rubella-pericardial cell system is uncertain, however, since in the examples just referred to a cytopathic effect follows infection. Although rubella virus can induce a cytopathic effectin a number of cells of human and non-human origin, e.g., human amnion (20), RK13 (17), RK1 (12), BHK-21 (19), continuous lines of African green monkey kidney cells (8) and rabbit cornea cells (15), it does not do so in HDCS. We are at present carrying out quantitative studies in order to establish the mechanism of the changes detectable histochemically.

In earlier communications (5,18), it was observed that 43% of mitoses examined in skin and pharyngeal mucosa cell strains had achromatic gaps and breaks. Recently, in association with the present study, an

examination of more than 1,100 cells obtained from four kinds of HDCS (skin, lung, pharyngeal mucosa and pericardium) derived from 3 fetuses, was performed (7). As before, virus infection was associated with minor chromosomal lesions in chronically infected strains. However, although in every instance the lesions were more frequent than in control cells, in three cases the difference was not significant when tested at the 95% level of probability. In only one of the four strains (derived from pharyngeal mucosa) was chromosomal damage in the infected cell line significantly raised above the control level. When the data for all the cell strains examined were combined, the virus effect upon the chromosomes was still statistically significant.

These results are quite consistent, both qualitatively and quantitatively, with our findings relating to HDCS derived from human fetuses aborted after maternal rubella infection (7). However, in both groups, individual cell strains were found which responded more dramatically than usual to rubella infection.

Although rubella virus was able to remain in a state of active association with HDCS for long periods — up to 27 weeks in actively dividing cells, and at least 30 weeks in stationary cell populations — no evidence of cell transformation was found. At all times, the chronically infected cells were morphologically indistinguishable from the controls. There was also no evidence of neoplastic change when cells which had been infected up to 23 generations earlier were inoculated into the cheek pouch of hamsters.

Summary

Rubella virus infection of human diploid cells did not result in an ordinary cytopathic effect, but several kinds of human diploid cell strains (HDCS) responded to infection by ceasing to multiply. Inhibition occurred quickly (most lung strains), or after many weeks (skin and pharyngeal mucosa strains). HDCS could be classified according to their response. The response of lung cell strains was strictly related to the *in vitro* age of the strains at the time of infection. Older strains stopped multiplying several times more quickly than younger ones. The relationship was not found for HDCS of other organ and tissue origins. Pericardial cell strains possessed large amounts of acid phosphatase-containing granules in a small proportion of the cells. During chronic rubella infection, histochemical activity of the enzyme decreased. The chromosomes of chronically infected skin, lung, pharyngeal mucosa and pericardial strains, infected up to 17 cell generations earlier, showed a mild but definite increase in achromatic gaps and breaks. No evidence of cell transformation or induction of neoplastic changes was found in HDCS which had been actively dividing for up to 27 weeks while infected with rubella.

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Authors' address: Dr. **J. *31. Hoskins,*** Swiss Serum and Vaccine Institute, Bern, Switzerland.

Department of Microbiology, Oregon State University, Corvallis, Oregon, U.S.A.

Incorporation of ^{14}C-Labeled Carbohydrates, Pyrimidines, Lipid Components and Amino Acids into Lee Influenza Virus and Chorioallantoic Tissue in Culture*

By

Patricia **M.** Buckley, S. **McGregor,** and K. S. Pilchcr

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Introduction

In cultures of chicken chorioallantoic tissue the susceptible entodermal and to a lesser extent the ectodermal cells are known to contain all the materials essential for the formation of new influenza virus. The only required component besides a balanced saline medium is glucose, needed to maintain cellular respiration. Nevertheless, labeled compounds in such a medium may be taken up and incorporated in developing virus. A method has been described for the isolation of labeled virus with high purity **(1).** This report presents the results of measuring the relative contributions of thirty 1%-labeled compounds to Lee influenza virus replicating in tissue culture. The virus was isolated by the purification method previously outlined.

Materials and Methods

Tissue culture and virus purification methods. The tissue culture method and virus inocula used are described separately **(1).** In each experiment, a labeled compound was present in the medium at a concentration of **8 mM** unless otherwise indicated, and unlabeled glucose was also present at a concentration of **4 mM.** A group of **10** culture tubes was prepared for each test, yielding about **25** ml of culture fluid for virus purification and **20** fragments of chorioallantoic tissue. For each compound **2** identical experiments comparatively measured incorporation of ^{14}C into noninfected and infected tissues. The procedures were similar to those used for virus incorporation studies except that less label was used. Radioactivity in media for these experiments

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