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Inhibition of Mitosis and Macromolecular Synthesis in Rat Embryo Cells by Kilham Rat Virus

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The effects of Kilham rat virus multiplication were studied in cultured rat embryo cells to examine the mechanisms by which virus infection might be related to developmental defects in rats and hamsters. The virus was found to inhibit mitosis and deoxyribonucleic acid (DNA) synthesis within 2 to 10 hr after infection. However, total ribonucleic acid synthesis was relatively unaffected until about 20 hr after infection, and total protein synthesis did not decline significantly until loss of viable cells was apparent in the cultures. No effect on chromosomes was detected. The effect of Kilham rat virus on DNA synthesis appears to be due to inhibition of macromolecular synthesis rather than to an inhibition of uptake of precursors into cells. The effect of the virus on mitosis may be an addition to the effect on DNA synthesis, since mitosis is inhibited even in cultures in which cells are able to divide at the time of infection and which have presumably completed DNA synthesis.

Kilham rat virus (KRV) is a minute, single-stranded deoxyribonucleic acid (DNA) virus (10, 11, 17, 19, 24) that can induce developmental defects in rats and hamsters (11-14). KRV and other members of the parvovirus group—including H-1 virus, minute virus of mice, and feline panleucopenia viruses—have been shown to induce a characteristic cerebellar hypoplasia (15) in neonatal animals. The cerebellar lesions induced by these viruses in several species are identical to the lesions induced by cytosine arabinoside (4) and similar to cerebellar lesions induced by low doses of X rays (1). KRV and H-1 have a preferential effect on fetal and neonatal tissues in vivo and at sites of tissue regeneration in adult animals (3, 14, 18, 23). A detailed study of the cerebellar lesions induced by KRV led Margolis and Kilham (13-15) to propose that the virus had a preferential effect on actively dividing cells. Subsequently, we (21) found that the efficiency of infection by KRV in cultures of rat embryo cells is related to the synthetic activity of the cells and, further, that the replication of KRV is dependent upon at least one cell function that may involve DNA synthesis (22). The present study examines the effects of KRV on cellular synthetic activity, mitosis, and chromosomes; the results are discussed in relation to the mechanisms of KRV-induced developmental defects.

MATERIALS AND METHODS

Virus and cell cultures. The original stock of KRV strain 171 was obtained from Lawrence Kilham, Dartmouth Medical School, and pools were prepared in Fischer or Sprague-Dawley strain rat embryo cells. The stock was tested as described previously and found to be free of other known rodent viruses (20). The origin and preparation of virus-free cell cultures used in these studies have been described elsewhere (20, 21).

Examination of chromosomes and determination of mitotic index. To test for an affect of KRV on chromosomes or mitosis, the cells were treated with colchicine after infection. The growth medium contained 10% calf serum plus Eagle's basal medium made up in Earle's balanced salt solution. A final concentration of 10^{-6} M colchicine was used in the medium for cells that were to be exposed to the drug for longer than 10 hr. Cells treated for intervals shorter than 10 hr were exposed to a 10^{-7} M concentration. At the end of the experiment (usually 24 hr), the cells to be examined for chromosome defects were treated with trypsin and scraped from the plates into a solution of 0.7% sodium citrate. Cells to be examined for mitosis were grown on glass cover slips—which, after collection, were placed in the sodium citrate solution for 10 min at room temperature. All cells were subsequently fixed for 20 min in a solution of three parts absolute methanol and one part glacial acetic acid. The cells for chromosome analysis were air-dried and stained with acetoorcein, whereas the cells to be examined for mitosis were stained with hematoxylin.

Radiochemicals and assays for DNA, ribonucleic acid, and protein synthesis. Tritiated thymidine ([^{3}H]dThd), specific activity 2 Ci/mmole; tritiated uridine, specific activity 2 Ci/mmole; and tritiated adenine, specific activity 5 Ci/mmole, were obtained from New England Nuclear Corp. L-[^{14}C]leucine,

specific activity 20 mCi/mmole, was purchased from Mallinckrodt Nuclear Corp.

For assays of incorporation of labeled precursors, the cells were grown in 60-mm plastic petri dishes (Falcon Plastic, Div. of B-D Laboratories, Inc.) and were exposed at various intervals to the appropriate precursor, usually at a concentration of 1 μCi/ml, for 1 hr. The medium was then removed, and the cells were scraped into 1 ml of cold phosphate-buffered saline and quick-frozen. The amount of the respective precursor incorporated into trichloroacetic acid-insoluble precipitate was determined by the method of Regan and Chu (16). Autoradiography of cells labeled with [^{3}H]dThd was performed as described elsewhere (21).

Measurement of [^{3}H]adenine incorporation. At intervals after infection, the cells were exposed for 1 hr to medium containing 1 μCi of [^{3}H]adenine per ml. At the end of incorporation, the cells were scraped from the plates and frozen. The samples were treated with 0.2 N perchloric acid after addition of 10^{-4} M adenine as a carrier. The resulting precipitates containing DNA and ribonucleic acid (RNA) were washed twice with 0.2 N perchloric acid. A sample of each precipitate was then treated with 0.5 N KOH for 16 hr at 37 C; afterward, the samples were chilled and treated with 0.2 N perchloric acid and their precipitates were washed twice with 0.2 N perchloric acid. The precipitates containing DNA and the supernatant fluid containing RNA were plated on Whatman filter paper discs (3 mm by 2.3 cm), and radioactivity was determined by scintillation spectrometry.

Labeling of cell DNA. Secondary cultures of rat embryo cells were planted in Eagle's basal medium containing 5% fetal bovine serum and 10 μCi of [^{3}H]dThd per ml. After 20 hr of growth, the medium was changed to unlabeled culture fluid and the cells were held an additional 18 hr after infection.

RESULTS

Effect of KRV on mitosis. Experiments were designed to determine the effect of KRV on the ability of rat embryo cells to divide after infection. We have reported previously that secondary, rat embryo cells enter a stationary phase at about 10^6 to 2×10^6 cells (per 60-mm petri dish) but can be stimulated to renew metabolic activity and cell division by the addition of fresh medium containing serum (21). Thus, in these experiments, secondary cells grown on cover slips were infected with KRV and given fresh medium. At 0.5, 2.5, 11, and 22 hr after infection, colchicine was added to the medium so that the cells would accumulate as they entered mitosis. The cover slip cultures were harvested at 24 hr after infection, and the proportions of mitotic cells were compared with those in similar cultures not exposed to the virus (Fig. 1). Cultures infected by KRV did not undergo any appreciable mitosis for the duration of the experiment. Although the efficiency of infection (number of cells synthesizing virus within the first replication cycle) was not determined for this experiment, the maximum efficiency, as seen with the fluorescent-antibody technique, usually did not exceed 30%.

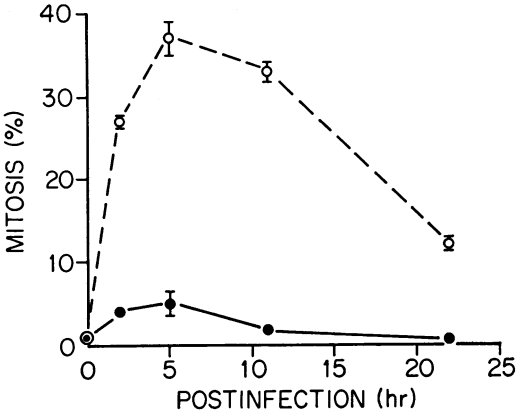

FIG. 1. *Effect of Kilham rat virus (KRV) on mitosis in rat embryo cell cultures. Secondary, rat embryo cells grown on glass cover slips were exposed to KRV (about 10 plaque-forming units/cell) or normal cell lysate for 2 hr at 37 C with occasional shaking and then given fresh medium. At various intervals (0.5, 2.5, 11, and 22 hr) thereafter, colchicine was added to give a final concentration of 10^{-6} M for cells to be treated for over 10 hr or 10^{-7} M for cells exposed for less than 10 hr. Cultures were harvested at 24 hr after infection; cells were fixed and stained, as described in Materials and Methods, before the mitotic cells were counted.* ○, *Control cells;* ●, *virus-infected cells.*

Thus, it would appear that mitosis is inhibited even in cells which do not immediately synthesize virus and could, therefore, be an indirect effect of the virus or the process of infection. However, as we have shown elsewhere (21), the efficiency of infection by KRV is dependent upon cell metabolic activity. Thus, cells can be infected, but virus synthesis is not completed until the cells provide the function(s) required for virus replication. It is not yet possible to determine if mitosis is inhibited only in infected cells, but the multiplicity of infection was sufficient to expose all cells to infectious virus. The addition of colchicine at 0.5 and 2 hr after infection should have resulted in the accumulation of mitotic cells, but did not—possibly because many cells overcame colchicine inhibition when the drug was given at the early intervals. The decline in percentage of mitosis after 5 hr is presumably the result of fewer cells entering mitosis and to the colchicine-mitotic cells leaving mitosis.

Although KRV induces cytopathic effects in cell cultures and inhibits mitosis, it is possible that the entering virus could affect chromosomes if cells were in mitosis at the time of infection. If

virus multiplication was abortive, such cells might then emerge to produce altered growth patterns. Therefore, in another experiment, the rat embryo cells were infected at 24 hr after plating, a time when a number of cells were dividing or in adjacent periods of the cell cycle. Three hours after infection, colchicine was added and the cells were incubated for an additional 3 hr before fixation. At least five fields, including up to 2,000 mitotic cells, were examined, but no appreciable chromosomal damage was seen. Occasional chromosome breaks appeared with the same frequency as in control cells.

Effect of KRV on DNA synthesis in rat embryo cells. The effect of KRV on the rate of total DNA synthesis was determined by measuring the incorporation of [^{3}H]dThd into trichloroacetic acid-precipitable material at various intervals after infection. The results (Fig. 2) show that DNA synthesis in uninfected cultures increased within 5 hr after infection but that incorporation of [^{3}H]dThd declined in cultures infected with the virus. The inhibition was usually apparent by 5 hr but not significant until the rate of synthesis increased in control cells. The amount of soluble precursor, released after the cells were disrupted by sonic treatment and treated with trichloroacetic acid, was also measured (Fig. 2 insert). These results suggest that the infected cells are able to maintain an intracellular soluble pool of the precursor but lose the ability to synthesize DNA. The inhibitory effect of KRV was prevented by incubating the virus with KRV antiserum prior to infection but was not reversed by incubation with normal serum.

KRV also inhibits the initiation of DNA synthesis in a cell line of normal rat kidney established by Duc-Nguyen et al. (2). Actively growing cultures of these cells showed a marked decrease in rate of total DNA synthesis when infected with KRV (Fig. 3). When the incorporation of [^{3}H]dThd was measured autoradiographically in

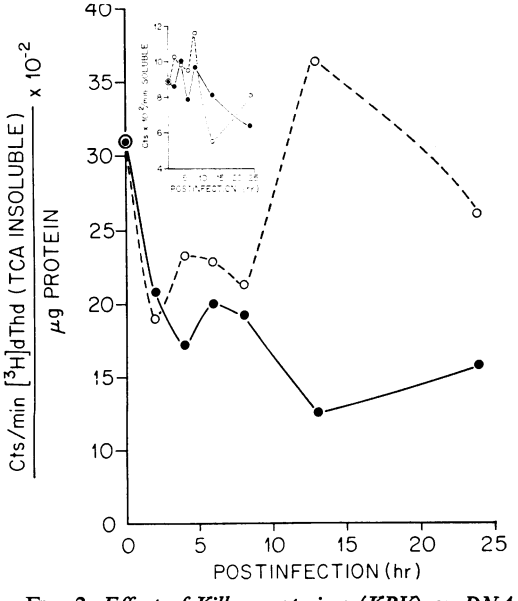

FIG. 2. *Effect of Kilham rat virus (KRV) on DNA synthesis in rat embryo cell cultures. Secondary, rat embryo cultures were infected with KRV (about 10 plaque-forming units/cell) or treated with normal cell lysate for 2 hr at 37 C and then given fresh medium. At the indicated intervals (2, 4, 6, 8, 14, 24 hr), two plates per group were given medium (Eagle's basal medium with Hanks balanced salt solution) containing 1 μCi of [^{3}H]dThd per ml for 1 hr. The medium was aspirated, and the cells were scraped into 2 ml of cold phosphate-buffered saline and quick-frozen. Incorporation of [^{3}H]dThd was measured by the method of Regan and Chu (16). Insert presents counts of soluble intracellular [^{3}H]dThd as a function of time after infection. ○, Control cells; ●, virus-infected cells.*

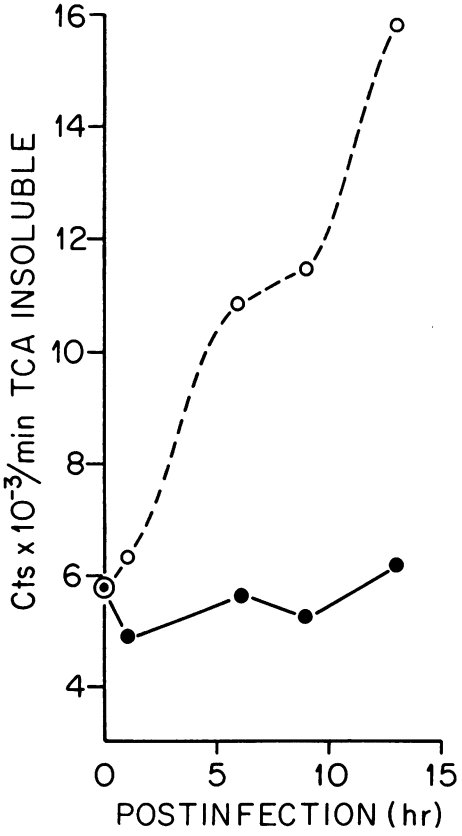

FIG. 3. *Effect of Kilham rat virus (KRV) on DNA synthesis in a normal rat kidney cell line (2). Kidney cells were infected with KRV and at intervals (2, 6, 8, 14 hr) thereafter were given medium containing [^{3}H]dThd (1 μCi/ml) for 1 hr. Incorporation of the precursor was measured as described previously (21). ○, Control cells; ●, virus-infected cells.*

secondary rat embryo cells, a proportion of the cells were able to initiate DNA synthesis after infection (Fig. 4). Thus, it is possible that some of the labeled cells were those which had initiated viral DNA synthesis, but there was no significant difference between the density of labeling in control and infected cells. It is possible also that the cells initiating DNA synthesis between 10 to 14 hr were uninfected or abortively infected. No effect of KRV on the rate of total DNA synthesis was seen in mouse embryo cells which do not support replication of the virus, suggesting that the effects in rat cells may be related to some replicative function of KRV.

Effect of KRV on cellular DNA. To determine if KRV had a degradative effect on existing cellular DNA, cells were labeled in medium containing [^{3}H]dThd. After 24 hr, the labeled medium was removed and the cells were held an additional 24 hr in unlabeled medium to deplete any cellular pools of [^{3}H]dThd. The cells were then infected with KRV and, at intervals, plates were harvested and the cells were assayed for trichloroacetic acid-insoluble and -soluble [^{3}H]dThd. As shown in Table 1, soluble [^{3}H]dThd was detected in both infected and control cells at 2 and 5 hr after infection. However, the essentially negative results at the 14- and 24-hr periods suggest that there was not extensive degradation of cellular DNA. Possibly, any cellular DNA degraded at the 14- and 24-hr periods could be masked by reutilization of the labeled material for viral DNA synthesis. However, as shown in the previous experiments, total DNA synthesis was quite depressed at these intervals. It is likely that the soluble [^{3}H]dThd seen in the early intervals after infection represents a nonspecific release of label from cells destroyed by the process of infection or treatment with infected cell lysate. In this case, it should be possible for the degradation products to be reutilized, but the effect does not seem virus specific.

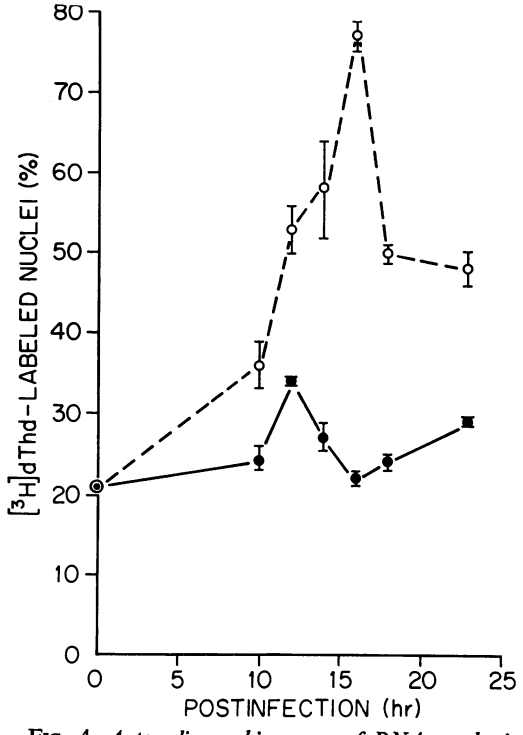

FIG. 4. *Autoradiographic assay of DNA synthesis in cells infected by Kilham rat virus (KRV). Secondary cultures of rat embryo cells were plated on glass cover slips. After 24 hr of growth, the cells were infected with KRV or normal cell lysate. At various intervals (10, 12, 14, 16, 18, 22 hr), cultures were labeled with [^{3}H]dThd (1 μCi/ml) for 1 hr. At the end of the exposure period, the cells were washed three times with phosphate-buffered saline, and the cover slips were fixed in acetone. Radioautograms were prepared as described previously (21). ○, Control cells; ●, virus-infected cells.*

Effect of KRV on RNA and the protein synthesis. Total synthesis of RNA or protein was assayed by measuring the incorporation of [^{3}H]uridine or [^{14}C]leucine, respectively. As shown in Fig. 5, KRV had relatively little effect on total RNA or protein synthesis compared to its effect on DNA synthesis. In another experiment, an effect on total RNA synthesis was seen by 24 hr in the virus-infected cells, but total protein synthesis remained similar to that in control cells until about 36 hr, when a cytopathic effect was evident.

The specificity of the effect of KRV on DNA synthesis was confirmed by exposing cells to [^{3}H]adenine and then determining the amount of labeled material incorporated into DNA and RNA. As in the previous experiments, infected or control cells were pulse-labeled at intervals after

TABLE 1. *Effect of Kilham rat virus infection on prelabeled cell deoxyribonucleic acid*

Time after infection (hr)	Group	Counts per min (μg of protein)	
		Trichloroacetic acid-insoluble [^{3}H]dThd	Soluble [^{3}H]dThd[a]
2	Infected	370	189
	Control	519	55
5	Infected	473	62
	Control	426	125
14	Infected	459	26
	Control	591	—
24	Infected	596	—
	Control	533	—

[a] Dashes indicate that counts per minute were below background level.

virus infection. As shown in Table 2, incorporation of the precursor into DNA was relatively unaffected, thus confirming that the effect of KRV is an inhibition of DNA synthesis and not an effect on the ability of the cells to take up the precursor. The quantitative differences in inhibition of DNA synthesis (Fig. 5 and Table 2) probably represent only variation between experiments. The percentage of control value is arbitrary because, if control cells are dividing more rapidly, the effect of the virus becomes apparent at an earlier time than in an experiment where normal cell activity is delayed.

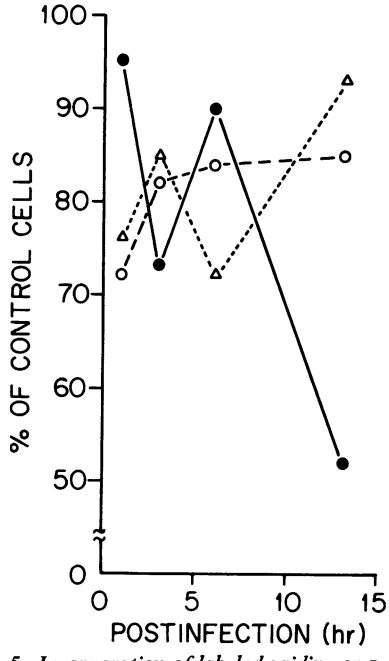

FIG. 5. *Incorporation of labeled uridine or L-leucine by Kilham rat virus (KRV)-infected cells. Secondary cultures of rat embryo cells were infected with KRV, and, at the indicated intervals (1, 2, 5, 6, 13 hr), the cultures were exposed to [^{3}H]uridine (1 μCi/ml), [^{3}H]dThd (1 μCi/ml), or L-[^{14}C]leucine (1 μCi/ml) for 1 hr at 37 C. At the end of the exposure, the cells were washed, scraped into 1 ml of cold phosphate-buffered saline, and quick-frozen. The amount of label incorporated was determined as described (21). Rate of incorporation by infected cells is expressed as per cent of label incorporated by control cells at the same intervals.* ●, [^{3}H]dThd; ○, [^{3}H]uridine; △, L-[^{14}C]leucine.

DISCUSSION

These results show that Kilham rat virus markedly inhibits DNA synthesis and mitosis in rat embryo cells. Several features of this effect are of interest. (i) The inhibition is quite rapid, since some cells are unable to divide within 2 hr after infection. (ii) Although the cells in these experiments were infected with a relatively high multiplicity of virus (about 10 plaque-forming units/cell), the efficiency of infection, determined from the number of cells synthesizing viral protein within 14 hr after infection, generally did not exceed 30%. The low efficiency of first-cycle infection is probably related to the metabolic state of the cells at the time of infection (21). Thus, it appears that mitosis and DNA synthesis may be inhibited even in cells probably infected with virus but not immediately able to initiate virus synthesis. (iii) The inhibition seems to be specific for DNA synthesis, as well as mitosis, since the KRV-infected cells synthesize RNA and protein at about 80 to 90% of the rate of uninfected cells. (iv) It appears that KRV inhibits some step in the synthesis of DNA, but not the formation of an intracellular pool of DNA precursors, so no effect of the virus on cell membranes is indicated. Fong et al. (4) have reported that H-1 virus infection caused a drop in the rate of incorporation of [^{3}H]dThd and a decline in growth by cells of two different lines. Further, they have reported a decline of RNA synthesis in cells infected with H-1 (5). However, the rate of RNA synthesis did not decrease until 16 to 24 hr after infection and could represent an indirect effect of the inhibition of DNA synthesis.

TABLE 2. *Incorporation of [^{3}H]adenine into ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) after Kilham rat virus infection*

Time after infection (hr)	Group	Counts per min per sample					
		RNA + DNA[a]	Per cent of control	RNA	Per cent of control	DNA	Per cent of control
7	Infected	940	56	187	81	407	36
	Control	1,665		230		1,134	
14	Infected	929	79	238	103	376	67
	Control	1,178		230		561	

[a] Initial trichloroacetic acid precipitate, taken before hydrolysis.

The mechanism by which KRV produces inhibitory effects on rat embryo cells has not been determined. It is possible that the viral nucleic acid, or a product of the virus, compete for a cell function required for DNA synthesis and mitosis, or inhibit the cell function(s) directly. For example, Salzman (20) has found DNA polymerase activity associated with purified KRV, which might have an as yet undetermined effect on cell functions.

KRV requires at least one radiosensitive cellular function for virus synthesis (22) and is therefore unlike some other DNA viruses that replicate in cells which have received similar doses of radiation (7–9, 21). Presumptive evidence (21) suggests that the cell function required by KRV involves DNA synthesis. This presents an apparent paradox, in that a cell function, presumably involving DNA synthesis, is required by KRV, but the virus in turn inhibits cell DNA synthesis. It is possible that the virus-required cellular function appears at a time prior to cell DNA synthesis; for example, an enzyme used for cell DNA synthesis could be involved. However, the inhibition of mitosis suggests that the infected cell would be unable to progress through the normal cell cycle. Cells in which mitosis is apparently blocked appear to be able to synthesize virus in subsequent replication cycles, since cells stained with fluorescent antibody continue to appear and the cytopathic effect progresses with time. Thus, the block in mitosis is either temporary or does not prevent the appearance of the virus-required function. It has not been possible to determine if KRV replication is abortive in a proportion of the cells. Cells in which virus infection was abortive would presumably be able to overcome the block. The true relationship between the virus-required cell function and the inhibition of cellular functions can be resolved by defining the cellular function(s) required by KRV.

The results reported here are of value in understanding the mechanism of developmental defects induced in animals by KRV. From the evidence presented (15, 21, 22), it appears that the virus preferentially infects actively dividing cells. Therefore, whereas a variety of tissues may be infected by KRV, the infection is productive only in cells capable of providing the required function, and apparently such cells are involved in the synthesis of DNA. Upon infection, KRV appears to permanently inhibit DNA synthesis and mitosis in cells that replicate the virus and to produce no obvious damage to chromosomes. Thus, it seems reasonable that the developmental defects induced by KRV could result from a loss of generative cells required for normal development and not from virus-induced chromosome damage.

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