THE EFFECT OF CRUDE PREPARATIONS OF VACCINIA ON
MITOSIS AND DNA SYNTHESIS OF KB CELLS

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PLATE 14
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The influence of vaccinia virus on mitosis and DNA synthesis of infected cell cultures has been studied by a number of investigators but their findings are contradictory. Carrère, Mandin, and Pourquier (3) have reported a slight increase of the incidence of mitosis in the KB cells at 48 hr after infection. Magee and coworkers (15, 16), on the basis of radioautographic and biochemical studies, have observed a temporary enhancement of total DNA synthesis of infected HeLa cells. These latter findings were not confirmed by other authors (7, 8). By contrast the results of Kitt and Dubbs (9–13) show plainly that infection of various kinds of cell cultures with the authors' strain of vaccinia virus causes a rapid reduction of mitosis and repression of the synthesis of cellular DNA. This lack of agreement is ascribed by the above mentioned authors to difficulties in interpretation caused by the fact that two opposing processes are taking place simultaneously in infected cells; i.e., a partial shutdown of host cell DNA synthesis and the initiation of viral DNA synthesis.

Recent experiments in our laboratory in the organ culture system indicate that our strain of vaccinia virus has a dual capacity well known from experiments on embryonated eggs (5) and in vivo: it causes a stimulation of the ectodermal cells as well as their destruction. The morphological observations and mitotic index determinations have suggested that the temporary increase of multiplication rate of cells after infection is not dependent upon the amount of infectious virus particles introduced into the medium. These findings have directly stimulated the quantitative studies in the cell culture system which are reported here. The influence of vaccinia virus on mitosis and DNA synthesis was examined at a wide range of input doses of virus. The use of very low amounts of virus to infect the cultures made it possible to observe the effects of vaccinia in course of the spread of infection.

Materials and Methods

Tissue Culture Methods.—All the experiments were carried out with the strain of KB cells. Growth medium consisted of 15% of Hank's saline with 0.5% lactalbumin hydrolysate, 10% pooled calf serum, and 75% of No. 199 medium. Cells were grown on cover glasses which lay loosely on the bottom of thin walled bottles (35 ml capacity) held vertically.
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Virus.—A calf lymph vaccinia virus was employed. The lesions were scraped into Hank's saline, ground, and centrifuged at 2000 rpm for 5 min. The supernatant when tested on chick fibroblasts monolayers had a titer of $2 \times 10^7$ PFU/ml. The virus stocks were kept frozen in sealed ampoules at $-20^\circ$C. Preparations of the virus were always sonicated before use.

Titration of Infective Virus.—The routine assays for vaccinia virus were made on chick fibroblasts cultures (CF) without agar overlay (14) using five test tubes for each dilution. The plaques scored at 48 hr were visible to the naked eye and the curve relating plaque numbers to virus dose was linear. The multiplicity of infection was then determined as the ratio of CF plaque-forming units (PFU) to the number of cells present at the time of infection.

General Experimental Procedure.—Large numbers of replicate cultures were made by putting 3.0 ml of the appropriate dilution of cells (200,000/ml) in growth medium in culture bottles. After incubation at 37°C for 24 hr the cells from three cultures at random were counted. Other cultures were drained of their medium and infected with three different multiplicities of virus: 20, 0.02, and 0.0002 PFU/cell. The infected cultures were incubated with occasional mild agitation for 60 min at 37°C. At the end of this period the cultures infected with vaccinia as well as control cultures were washed carefully with prewarmed phosphate-buffered saline (PBS) solution and prewarmed growth medium was added.

At indicated intervals the cultures were taken for (a) morphological studies; (b) estimation of mitotic indices; (c) estimation of proportions of infected cells; and (d) radioautography.

Morphological Methods and Estimation of Mitotic Indices.—The cultures were fixed in 25% acetic acid and stained with hematoxylin-eosin, Giemsa, or aceto-orcein. The proportion of cells in mitosis was estimated by examining 1500 to 2000 cells aceto-orcein stained on photographs taken at random ($\times 400$) from each preparation. All stages of mitosis were included.

Estimation of Proportion of Infected Cells.—The cells from infected cultures were washed three times with calcium- and magnesium-free PBS and then incubated at 37°C for 5 min with 0.02% trypsin solution. At the end of this period this solution was discarded and the growth medium was added. The resulting suspension was centrifuged at 800 to 1000 rpm and washed three times. The cell concentration was determined with the hemocytometer and appropriate dilutions made for the addition of infected cells to chick fibroblasts monolayer cultures. Depending on the input multiplicity of virus and the time after infection, 5 to 30 cultures were used for each cell dilution (1000 to 10 cells per ml). Cell suspensions were added directly to the CF cultures. The per cent of cells infected by the multiplicities of 0.02 and 0.0002 at the beginning of the experiment was deduced from the titer of the supernatant after an hour of incubation.

Radioautographic Experiments.—At various time intervals after infection, tritiated thymidine (Amersham, specific activity 3000 mc/mg) was added to the medium to give a concentration of 1.5 $\mu$g/ml. After 30 min exposure at 37°C to the radioactive precursor the cultures were fixed in Carnoy, washed with excess of 5% cold trichloroacetic acid (TCA) 1 min and with tap water (1/2 hr). Radioautography was carried out using AR10 Kodak stripping film by the method of Pelc (17) and an exposure time of 4 wk at 4°C in the atmosphere of CO$_2$. Cells labeled over nuclei were determined as percentage of the cells in random nonrepetitive oil-immersion fields. Artifactitious lines of silver grains caused no difficulty in interpretation since they occurred only over empty spaces on cover glasses. At least 1500 cells were counted for each cell preparation.

In these experiments in which prelabeled cells were used, the KB cells were grown for 24 hr in the medium with tritiated thymidine (1.5 $\mu$g/ml and 0.2 $\mu$g/ml). At this time the medium was removed, the cells were incubated in new growth medium which contained unlabeled thymidine ($10^{-5}$ M) and well washed before being infected with vaccinia at the multiplicity of 20 (CF assay).
RESULTS

Properties of the Experimental System.—It is known from studies of KB, Hela, and L cells infected with vaccinia that virus adsorption extends over a period of several hours (19).

The rate at which KB cells in monolayer cultures became infected when allowed to adsorb the virus (20, 0.02, and 0.0002 PFU/cell) during an hour was measured by scoring infectious centers and by titrating the supernatant as described in Materials and Methods. The data shown in Text-fig. 1 indicate the percentage of infected cells (virus-producing cells) depending on the amount of virus introduced into the cultures and the time after infection. The very wide range of virus doses used to infect the cultures gave the possibility of studying the effects of vaccinia virus on host cells, when all cells were infected, as well on partially infected cell populations.

Cellular Lesions in Cultures Exposed to Varying Amounts of Vaccinia Virus.—The time of appearance of cellular lesions was closely correlated with the input dose of virus, however their sequence and morphological characteristics were identical. The earliest noticeable change was swelling and rounding of most cells. Cytoplasmic degenerative alterations, which appeared at first, consisted of vacuolization, lipid droplets, and blebbing. Some cells were fused to form multinucleated syncytia. At the early stages of infection no nuclear or nucleolar alterations could be observed; at the final stages preceding cell desintegration, the nuclei became shrunken. At this time, in some of the cells remaining on
glass, nuclear changes consisted of margination of chromatin; in these altered nuclei, nucleoli were not recognizable. Following the greatest dose (20 PFU/cell) the first degenerative alteration (swelling and vacuolization) were found at 12 hr. Following smaller doses they appeared as late as at 36 hr (0.02 PFU/cell) and 60 hr (0.0002 PFU/cell) postinfection.

As revealed by detailed cytological studies of infected cultures at various time intervals, the infection was not followed by the occurrence of mitotic abnormalities and there was no accumulation of cells in any one stage of mitosis.

**Text-Fig. 2.** Mitotic index (---) and DNA synthesis (----) in KB cells as measured by the incorporation of tritiated thymidine. Cultures of KB cells were grown on cover slips and processed at the given time intervals, as described under Materials and Methods.

**Effect of Vaccinia Virus on Mitosis and DNA Synthesis of KB Cells.**—These experiments were designed to compare the temporal incidence of mitotic cells with the incidence of cells synthesizing cellular DNA. Vaccinia virus matures and aggregates in the cytoplasm of infected cells (2, 1, 4). It is therefore possible to study the problem with the aid of labeled thymidine.

Under the conditions of these experiments the KB cells (control cells) manifested a mitotic index which fluctuated about 5% and about one third of the cells showed labeled nuclei when subjected to a pulse of tritiated thymidine (Text-fig. 2). The data obtained from infected cultures are presented in Text-figs. 3, 4 and 5. At 2 hr postinfection the incidence of mitotic cells and cells with label over nuclei did not differ from control values. At 5 hr the infected cultures of all experimental groups (multiplicity 20, 0.02, and 0.0002) revealed a similar increase in the number of labeled nuclei as compared with untreated cultures and at 12 hr a markedly increased mitotic index (more than 140% of control cultures).
The initial elevation of mitotic rate for the first group of infected cultures (20 PFU/cell, Text-fig. 3) was quickly followed by a sharp decline. The time at which mitotic activity began to decline coincides with the time at which nearly 100% of the cells became infected (Text-fig. 1). At 48 hr no mitotic figures were observed. In contrast the fraction of cells synthesizing DNA increased during the time interval between 5 and 24 hr to reach a value of 40 to 50% which remained approximately constant in the course of the experiment (until the final destruction of cells at 72 hr). Thus the ratio of the per cent of cells labeled to the per cent in mitosis increased from 45:5 at 5 hr to 50:0 at 50 hr.

The infected cultures of the third group (Text-fig. 5) (0.0002 PFU/cell) revealed (until 72 hr postinfection) an increased portion of mitotic cells and cells undergoing DNA synthesis as compared with the control cultures. A slight decline of the fraction of cells synthesizing DNA was followed by a new increase at 60 hr which was parallel with the mitotic wave.

The data obtained for the second group of cultures (multiplicity 0.02) presented (Text-fig. 4) intermediate values between those for the first and those for the third group of cultures.

Effect of Vaccinia Virus on Host Cell DNA.—As already described, following the highest dose of virus the cultures quickly reveal (Text-fig. 3) a disparity
between the size of DNA-synthetic cell pool and mitotic cell pool. This disparity indicates that the cells labeled over nuclei represent individuals which are moving very slowly through the S stage. It was therefore of interest to examine the pattern of the labeling of these cells as compared with the untreated controls and with the cells infected with lower doses of virus.

In the control cells the label was confined exclusively to the nuclei and 90% of the cells were heavily labeled revealing some differences in grain count which can be expected in cultures growing asynchronously. At 2 and 5 hr after infection the pattern of labeling was similar in all groups of infected cultures and in control cultures. From 12 hr onwards after infection, the cells of cultures infected with the highest dose of virus showed a “light” pattern of labeling over nuclei (Fig. 1). In the second group of cultures (0.02 PFU/cell) the fraction of “lightly” labeled cells increased gradually (Fig. 2) to reach 70% at 72 hr. At this time only 20% of cells of the third group (0.0002 PFU/cell) showed a marked decline in the amount of radioactivity over nuclei.
One way to explain this gradually increasing reduction in the incorporation of tritiated thymidine into the nuclei is by supposing a different availability of DNA precursors within infected cells due to the degradation of DNA template. This possibility was tested by incubating KB cells with tritiated thymidine for 24 hr prior to their infection with vaccinia virus (20 PFU/cell). At 2, 5, 12, and 24 hr after introduction of the virus into the cultures the number of cells with label over nuclei and the pattern of labeling were determined. At the time of infection about 90% of the cells were found to have labeled nuclei, all showing heavy labeling. Throughout the 24 hr after infection the fraction of labeled nuclei as well as the pattern of labeling did not differ from control cultures. Since microscopical studies of cultures exposed at 12 hr to a pulse of tritiated thymidine reveal a great portion of KB cells labeled in the cytoplasm indicating viral DNA synthesis it may be deduced that the virus does not cause a marked breakdown of host cell DNA. Since the breakdown of nuclear DNA does not appear to contribute to the decrease in the rate of incorporation of tritiated thymidine other explanations should be taken into consideration. First it may be that the activities of an enzyme or enzymes involved in the utilization of nucleic acid precursors are different in infected and control cells. Another possibility is that there is an increased pool of intracellular nucleotides.

![Text-Fig. 5. Per cent of KB cells undergoing DNA synthesis (---) and mitosis (---) at different intervals after infection at multiplicity of 0.0002 PFU/cell. Vertical lines represent two standard deviations around the mean. All points up to 55 hr on the curve for labeled cells differ significantly from corresponding points on the curve for labeled control cells (Text-fig. 2). All points up to 60 hr on the curve for mitosis differ significantly from corresponding points on the curve for mitotic control cells.](image-url)
Control Experiments.—In these experiments vaccinia virus propagated several times on the membranes of embryonated eggs was used. The vaccinia preparation consisted of a supernatant obtained from a low speed centrifugation of a homogenate of egg membranes in which vaccinia had been growing for 72 hr. Results of experiments performed in the same conditions as in the case of the calf lymph virus did not differ significantly from those described above. In order to investigate the possible influence of normal tissue components, the controls included extracts obtained from noninfected CAM. In these experiments the incidence of mitosis did not differ from control cultures.

DISCUSSION

The study of the mitotic events during the infection of KB cells with vaccinia has demonstrated a sudden disappearance of mitosis under conditions when all cells are infected. Because infection was not followed by an accumulation of cells at any one stage of mitosis and because no abnormal mitoses were observed it seems clear that the virus does not act directly on the process of mitosis but prevents multiplication of cells by interfering with another stage in the growth cycle.

As revealed by our pulse-labeling experiments, the ratio of the per cent of cells labeled over nuclei to the per cent in mitosis increases with the time indicating that the synthesis of DNA of the infected cell is free to continue during the block of cell division. From further radioautographic analysis it becomes evident that with the increase of the amount of cell-associated virus particles the rate of incorporation of tritiated thymidine decreases. These results suggest that the virus interferes with the DNA synthesis of the host cell at the time when the bulk of vaccinia DNA has been synthesized. The above findings are in agreement with the results of experiments suggesting that a substantial proportion of the intact genome is required for the normal growth of vaccinia (18). The question remains open if the suppression of mitotic division is an independent event or results from the interference with the mechanism of cell DNA synthesis.

From the analysis of cellular activities, as expressed by the occurrence of mitosis and DNA synthesis under experimental conditions when only a fraction of the cells was infected, it becomes evident that infection with vaccinia results in a temporary increase of mitotic cell pool which is parallel with the increase of DNA-synthesizing cell pool. It is also worthy to note that the extent of the early stimulatory effect is not dependent upon the amount of the infectious virus particles introduced into the culture medium. This situation as well as the fact that the infectious virus particles inhibit mitosis gives reason to assume that the stimulation of mitosis is due to some additional action which is only coincident with the process of infection. A similar phenomenon of acceleration of growth rate of HeLa cells infected with low doses of virus was
observed by Gray et al. (6) for "natural" strain of herpes virus. On the basis of his experiments the author suggests that these are the uninfected cells which are stimulated to divide at a rate at least 8 times as rapid as the cells in the untreated cultures.

Our experiments currently in progress give evidence that vaccinia virus, in the course of the adaptive process to certain types of cells or tissues, undergoes cyclic changes which determine the acquisition or loss of ability to exert the above described stimulatory effect. Since the ability to stimulate mitosis is not correlated with the amount of infectious virus particles the possibility must be considered and explored that this ability may be due to the presence in extracts of some cells or tissues of cell-viral products or noninfectious virus particles deriving from the cell-virus interaction.

**SUMMARY**

A morphologic study has been made on KB cells infected with various doses of vaccinia as to DNA synthesis and mitosis. Determination of mitotic indices revealed that the mitotic cell pool depended on the proportion of infected cells and the time after infection. By cytologic examination neither mitotic lesions were found nor an accumulation of mitotic cells at any one stage of mitosis was demonstrated. Radioautographs of infected cultures have shown that the frequency of cells labeled over nuclei was significantly increased as compared with control cultures.

Following the greatest dose of virus (multiplicity of 20 PFU/cell) the ratio of cells synthesizing DNA to mitotic cells increased from 45:5 at 5 hr to 50:0 at 50 hr. Concomitant with the appearance of this disparity between the DNA-synthesizing cell pool and mitotic cell pool the nuclei of cells became "lightly" labeled.

Following the lowest dose of virus (multiplicity of 0.0002 PFU/cell) the increase of the fraction of mitotic cells was proportional to the increase of the fraction of cells which were labeled over nuclei.

**BIBLIOGRAPHY**

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EXPLANATION OF PLATE 14

Fig. 1. Culture infected at a multiplicity of 0.0002 PFU/cell and exposed to a pulse of tritiated thymidine (48 to 48 1/2 hr postinfection). Most nuclei are heavy labeled. Note cytoplasmic labeling indicating sites of viral DNA synthesis. X 700

Fig. 2. Sister culture infected at a multiplicity of 20 PFU/cell and exposed to a pulse of tritiated thymidine (48 to 48 1/2 hr postinfection). "Lightly" labeled nuclei. Cytoplasmic label in loci distant from nucleus. X 700
(Koziorowska and Wlodarski: Effect of vaccinia on KB cells)