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SHORT COMMUNICATION

LACK OF CELL PROLIFERATION IN THE FOCI OF VARIOLA VIRUS-INFECTED FL CELLS[1]

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Several viruses have been reported to produce so-called hyperplastic or proliferative foci on cultured cells infected with them (GRAY *et al.*, 1958; KITAMURA *et al.*, 1964; PADGETT *et al.*, 1962; SCHWERDT and SCHWERDT, 1962; SHEIN *et al.*, 1963; TEMIN and RUBIN, 1958; VERNA and EYLAR, 1962; VIEUCHANGE, *et al.*, 1958; VOGT and DULBBECCO, 1960; YOHN *et al.*, 1964). These foci consist largely of cells piled up on one another and look like microtumors. The formation of the foci by transformable oncogenic viruses such as Rous sarcoma virus (TEMIN and RUBIN, 1958) and several oncogenic DNA viruses (KITAMURA *et al.*, 1964; SHEIN *et al.*, 1963; VOGT and DULBBECCO, 1960) has been considered to be due to loss of contact inhibition of cultured cells. Shope fibroma virus also produces similar foci on infected culture cells (PADGETT *et al.*, 1962; VERNA and EYLAR, 1962). The number of foci is correlated with the amount of infectious virus inoculated onto the cells. Autoradiographic studies using ^{3}H-thymidine on focus formation of Shope fibroma virus on FL cells revealed that these foci consist exclusively of virus producing cells which could hardly multiply (KATO *et al.*, 1965). It was concluded that the formation of the foci of Shope fibroma virus was not due to proliferation of the infected cells, but to their aggregation. VIEUCHANGE *et al.* (1958) observed the formation of small proliferative buds on HeLa cells infected with small inocula of variola virus. A linear relationship was demonstrated between the number of foci formed and the relative concentration of variola virus present in the inocula (PIRSCH and PURLSON, 1962). KITAMURA (1968) and KITAMURA and YOTSUYANAGI (1968) studied the biological characteristics of hyperplastic foci of variola virus and showed that these foci consisted of viral antigen-positive cells. However, these authors did not give any evidence of proliferation of the infected cells to form the foci.

This communication reports autoradiographic studies with ^{3}H-nucleosides on the mechanism of formation of the so-called hyperplastic foci of variola virus-infected FL cells.

Variola virus (Harvey strain) was kindly provided by Dr. T. KITAMURA (National Institute of Health, Japan). FL cells were dispersed into Leighton tubes with cover slips on the bottom, at a concentration of 3×10^5 cells per tube. After 3 or 4 days, when the cells had formed monolayer sheets (1×10^6 cells per tube), the medium was replaced by

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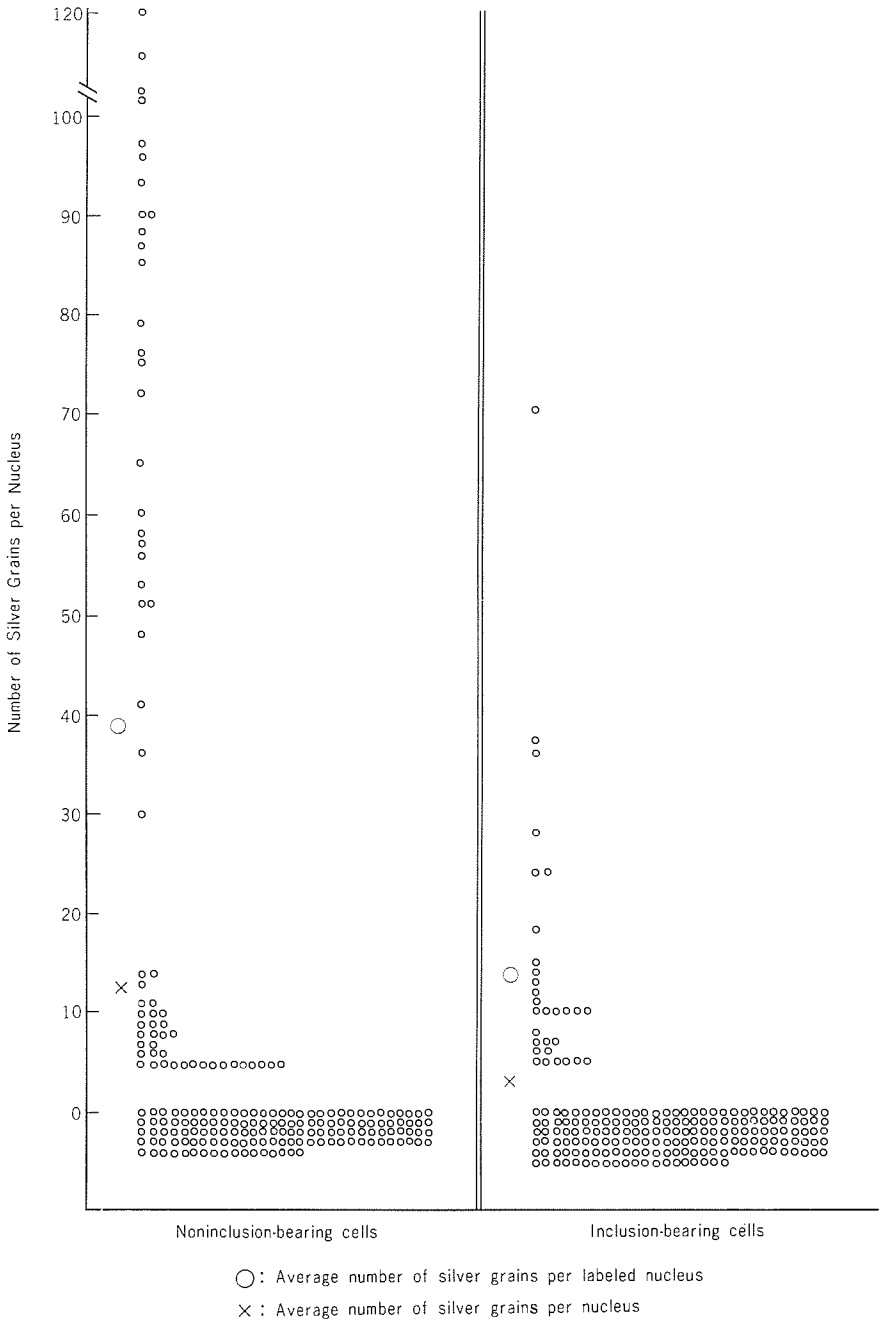


FIGURE 1 Effect of inclusion formation of variola virus upon nuclear DNA synthesis. (12 hours prparation of experiment)

one tenth ml of virus material (1×10^3 pock forming units/ml). Two hours later, medium consisting of Eagle's minimum essential medium (MEM) and 10 per cent calf serum with 100 IU penicillin and 0.10 mg streptomycin per ml was added. Six hours after infection, many spots consisting of a few cells showing inclusion formation were observed. Twelve hours after infection, infected focus consisting of more than 10 cells bearing inclusions could be seen. Twenty four hours after virus inoculation, the number of infected cells constituting each focus had increased up to scores. These cells appeared packed together in the focus and some cells were already piled up on one another. By seventy two hours after infection, the foci had reached diameters of up to 0.3–0.5 mm. Numbers of cells were packed and piled up in the focus (Fig. 2).

After these various periods, these infected cells were placed in a solution of ^{3}H-thymidine (10 μc/ml, specific activity 5 c/mM) for 1 hour. To demonstrate the viability of cells by RNA metabolism, some samples of these infected cells were placed in a solution of ^{3}H-uridine (5 μc/ml, specific activity 2.73 c/mM) with non-radioactive thymidine (2×10^{-5} M) for 1 hour. Then autoradiography using Sakura NR-M2 emulsion was carried out. The exposure time was 5 days.

Before autoradiography a part of each sample was submitted to the fluorescent antibody technique. From the beginning of focus formation, most of the cells constituting the focus showed specific fluorescence with fluoresceine-isothiocyanate coupled with anti-cowpox virus rabbit γ-globulin (Fig. 3).

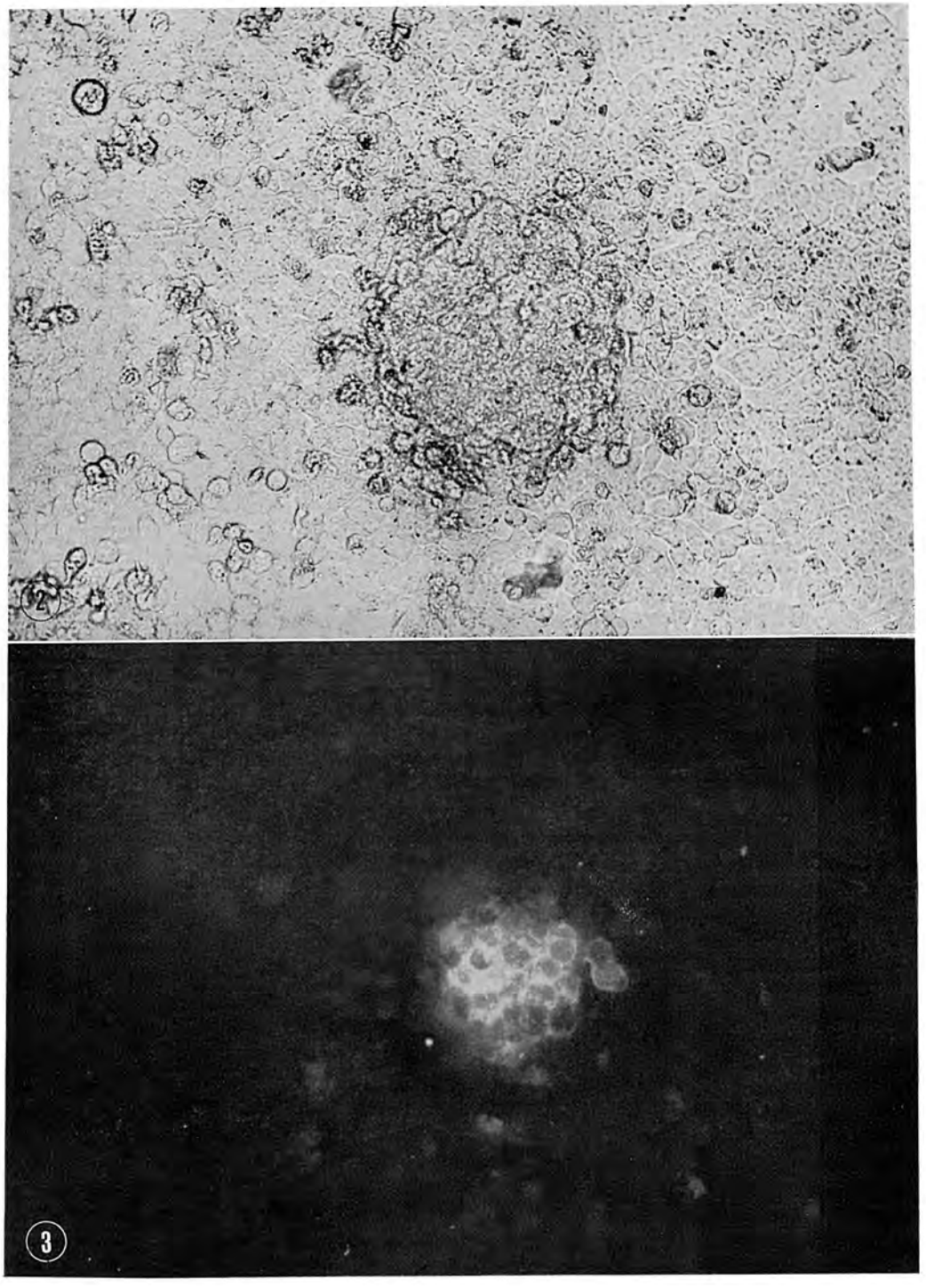
Autoradiography of ^{3}H-thymidine revealed that most of the cells constituting the focus had cytoplasmic inclusions showing viral DNA synthesis and did not show any remarkable nuclear DNA synthesis (Figs. 4, 5, 6). Mitotic figures were scarcely seen in the focus. For quantitative analysis of autoradiograms, the infected culture was kept in the solution of ^{3}H-thymidine (1 μc/ml). Then autoradiography was carried out. The exposure time was 3 days. Cells in the autoradiogram were divided into two groups: inclusion-bearing cells and noninclusion-bearing cells. Of 200 cells in each group which were counted at random, the numbers of silver grains per nucleus of these cells were counted and plotted as shown in Fig. 1. A distinct difference between the number of silver grains in the nuclei of inclusion-bearing cells and in the nuclei of noninclusion-bearing cells was found. DNA synthesis in the nuclei of inclusion-bearing cells was definitely suppressed. This result is in exact accordance with the results on other poxviruses (KATO *et al.*, 1964; KATO *et al.*, 1965). Autoradiogram of ^{3}H-uridine revealed that most of cells constituting the focus showed evident RNA metabolism at least until 72 hours after infection.

Our experiments showed that the foci formed in variola virus-infected cultures consist of inclusion-bearing cells which could hardly proliferate. It is concluded that the formation of the foci of variola virus is not due to proliferation of the infected cells. The infection seems to spread radially and these infected cells continue to metabolize for a considerable period, because of the mild cytopathogenicity of the virus. Thus the focus may be formed by the aggregation of infected cells which are passively pushed to the focus by the surrounding noninfected cells which are growing actively.

Samples were taken 3 days after virus inoculation.

FIGURE 2 A focus observed in an unstained FL monolayer infected with variola virus.

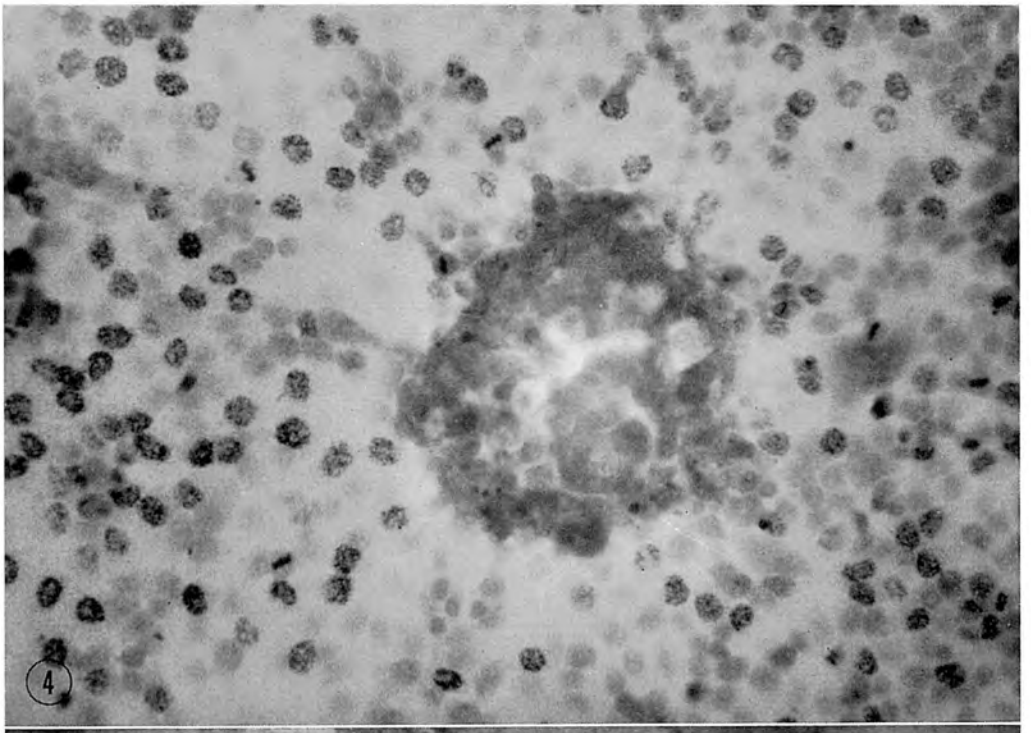
FIGURE 3 A focus showing viral antigen, stained with fluoresceine-isothiocyanate coupled with anti-cowpox virus rabbit γ-globulin.



Samples were taken 3 days after inoculation.

FIGURE 4 Autoradiogram of a focus. The infected culture was kept in ^{3}H-thymidine solution (10 μc/ml) for 1 hr. After the Feulgen reaction, autoradiography was carried out. None of the cells constituting the focus showed any remarkable nuclear DNA synthesis. Many cells surrounding the focus showed active nuclear DNA synthesis and mitotic figures.

FIGURE 5 Autoradiogram of the lower edge of the focus, stained with Giemsa solution. In the area adjacent to the focus many cells bearing inclusions showing active DNA synthesis were seen. Nuclear DNA synthesis of these inclusion-bearing cells was suppressed.



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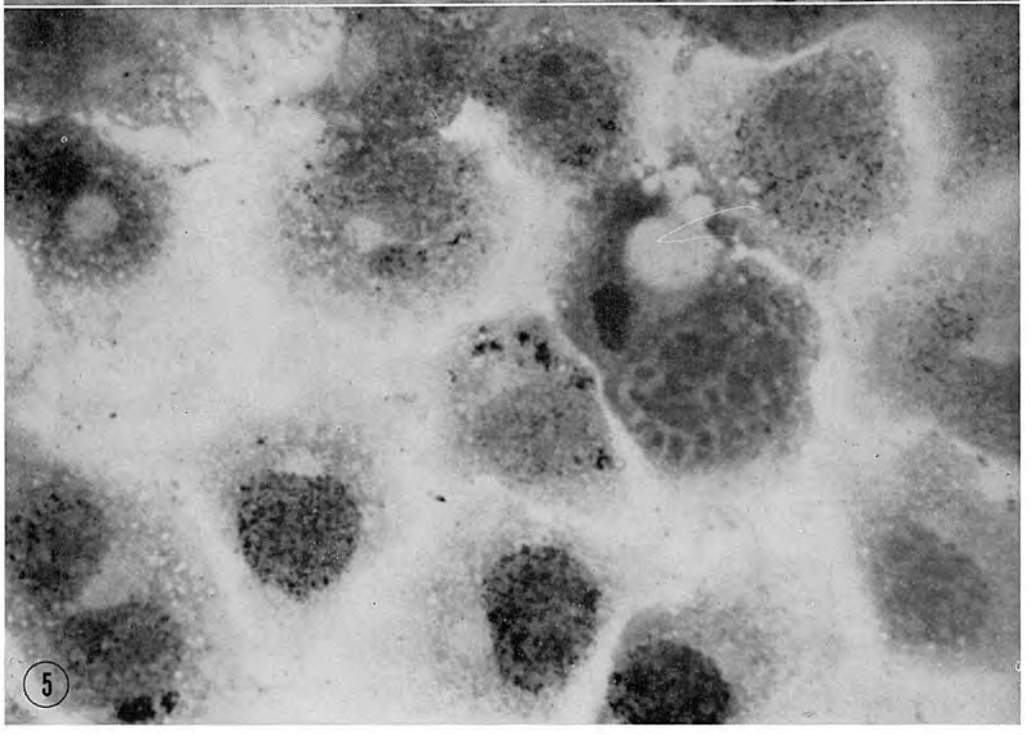

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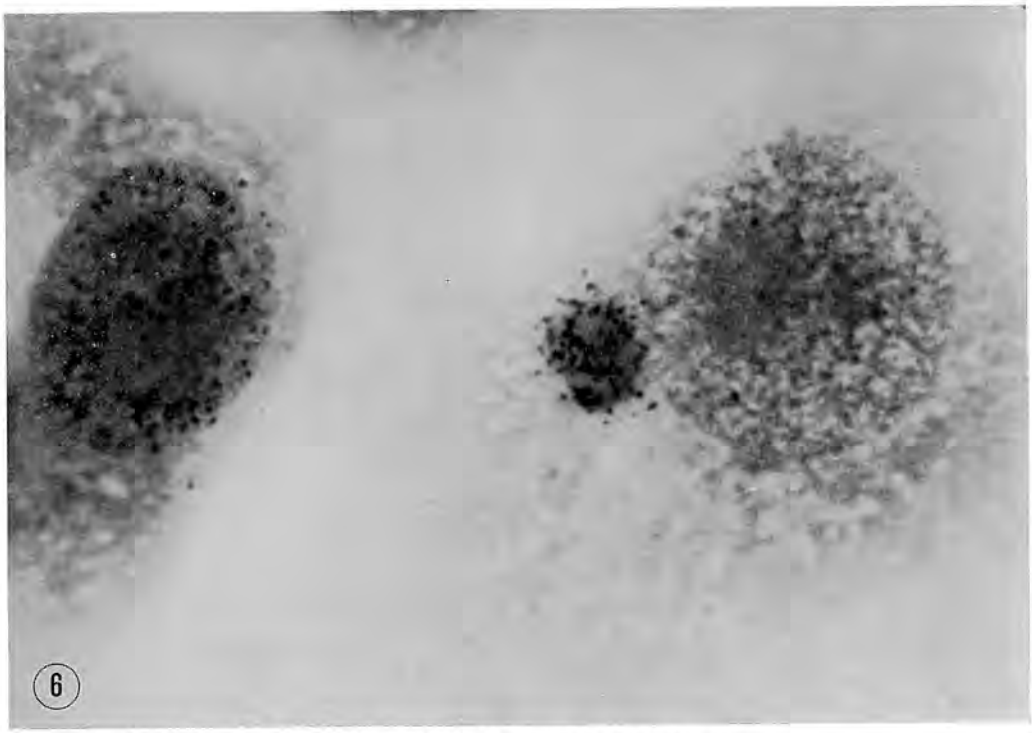

FIGURE 6 A cell bearing a typical variola " Guarnieri body " showing DNA synthesis, stained with Giemsa solution. Nuclear DNA synthesis of the cell is not as active as that of the left cell with no inclusion. (12 hours proparation of experiment)

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