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Nucleocytoplasmic Interaction in Poxvirus-infected Cells I. Relationship between Inclusion Formation and DNA Metabolism of the Cells.

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SUMMARY

The development and the role of the inclusions of cowpox virus were investigated from the standpoint of cytoplasmic and nuclear DNA synthesis by quantitative autoradiography using ^{3}H-thymidine. Cytoplasmic DNA synthesis was first detectable 2 hours after virus infection. The development of the "B" type inclusion from a small compact to a large diffuse body was confirmed from inclusion curves. DNA synthesis was detectable in all "B" type inclusions except those in the terminal stage with a diffuse form. The most active DNA synthesis was found in "B" type inclusions with diameters of 5 μ - 10 μ. The activity of DNA synthesis of the inclusions was nearly the same as that of the nuclei of normal cells and noninclusion bearing cells. The rate of synthesis of DNA per unit area of "B" type inclusion was far higher than that of the normal nuclei. The most active DNA synthesis per unit area was found in "B" type inclusions with diameters of less than 2 μ.

The relationship between DNA synthesis in the nucleus and that in the "B" type inclusions was studied. Regardless of the size or the degree of DNA synthesis of the "B" type inclusions, inclusion bearing cells all showed remarkable suppression of nuclear DNA synthesis. There was no evidence that virus infection caused an increase in nuclear DNA synthesis.

INTRODUCTION

Several independent studies (Kato et al., 1960a, 1960b; Magee et al., 1960; Cairns, 1960) have been reported on cytoplasmic DNA synthesis in poxvirus infection using autoradiographic techniques with ^{3}H-thymidine. Cytoplasmic DNA synthesis was found exclusively in the "B" type inclusions of poxvirus. Furthermore, it was concluded that 1) host nuclear DNA is not transferred to cytoplasmic DNA, 2) the host nucleus is not the site of viral DNA synthesis, 3) cytoplasmic DNA synthesis occurs independently of host nuclear DNA synthesis, and 4) host nuclear DNA synthesis is suppressed after inclusion formation (Kato et al., 1960a, 1960b; Kato et al., 1963a). As to the last item, biochemical and autoradiographic evidences were also presented using primary culture cells (Kit et al. 1963).

No quantitative studies have been made on viral infected cells by grain counting of autoradiograms. This paper reports quantitative studies on the significance of inclusion formation with respect to cytoplasmic and nuclear DNA synthesis.

Parts of this work have already been reported in preliminary forms on several

occasions (Kato et al., 1962b).

MATERIALS AND METHODS

1. *General experimental procedure*

FL cells, at a density of 3×10^5 cells per ml, suspended in LE medium (Earle's balanced salt solution containing 0.5 per cent lactalbumin hydrolyzate) with 10 per cent bovine serum, penicillin (100 units per ml) and streptomycin (100 μg per ml), were put into Leighton tubes containing coverslips. The cells were allowed to become attached and grow for 2 days at 37°C. Then 0.5 ml of cowpox virus (LB red strain) was added to the Leighton tubes and the tubes were reincubated for 30 min to permit virus absorption. Then the virus solution was replaced by ordinary medium. Coverslips were taken out from the tubes at two hours intervals, as shown in Figs. 1 and 3. One hour prior to removing the coverslip, the medium was replaced by medium containing ^{3}H-thymidine. Two series of experiments were made at input multiplicities of 12 and 5 respectively.

2. *Autoradiography*

Autoradiography was carried out by the dipping film technique, as described previously (Kato et al., 1963a). To each Leighton tube was added 1.5 ml of ^{3}H-thymidine solution (concentration 1 μc/ml, specific activity 2.52 c/mM, purchased from the Radiochemical Center, Amersham, England). The degree of incorporation of ^{3}H-thymidine into the nuclei depends greatly upon the temperature (Kato et al., 1963b). Incubation of the cells in ^{3}H-thymidine solution, previously adjusted to 37°C, was carried out in a water bath at 37°C. After incubation for an hour in the presence of the isotope, the cultures were chilled to 4°C in an ice bath. Then the coverslips were taken out. After being rinsed with Hanks' solution, the cells were fixed by a few minutes immersion in methanol. The dipping emulsion used was Kodak NTB 2 nuclear track emulsion. Exposures were made in the presence of silica gel. The exposure time was 48 hours at 6°C in a cold room. After development and fixation, the autoradiograms were stained with Giemsa solution. The percentage of labeled cells was estimated from a count of at least 1,000 cells. Grain counts for each group of experiments were performed on at least 50 labeled subjects. The background fog was nearly negligible, being 0.25 - 0.30 grains per 100 μ^2.

RESULTS

1. *Relationship between labeled cells and inclusion bearing cells*

(*A*) *Experiments at a multiplicity of 12 (Expt. 1)*

(*a*) *Inclusion curves*

The inclusion curve is shown in Fig. 1. The percentage of "B" bearing cells increased steadily to 96 per cent at 14 hours. "A" type inclusions appeared about 6 hours later. The "B" type inclusions were classified into four groups according to their diameters: diameter of less than 2μ, diameter of 2 μ~5 μ, diameter of 5 μ~10 μ and large diffuse forms. The numbers of each group of inclusions in 500 cells in each preparation are shown in Fig. 2. The "B" type inclusions were first detectable 2 hours after infection. The first peak of the curve of "B" inclusions with diameters of below 2 μ was found 6 hours after infection. From morphological evidence, most cells seem to start cytoplasmic DNA synthesis 2 to 6 hours after infection. The appearance of a second and lower peak in the curve of "B"

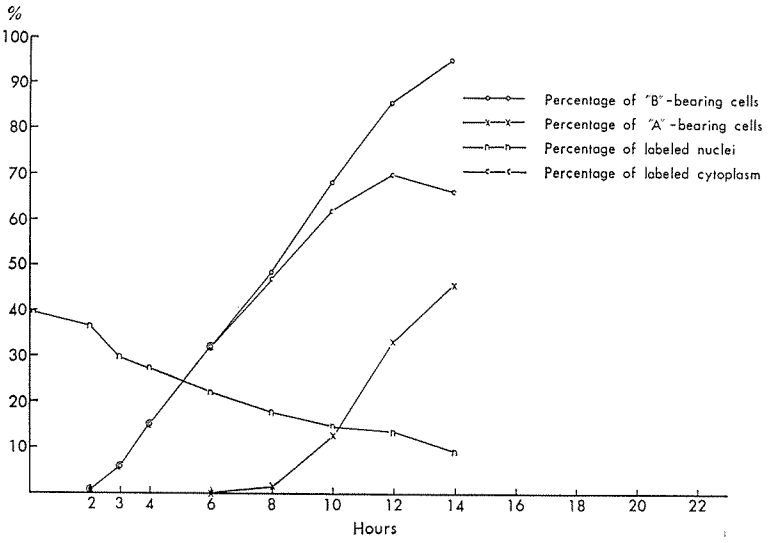

Fig. 1. Relationship between Labeled Cells and Inclusion-bearing Cells in Cowpox Virus-FL Cell System (Experiment I)

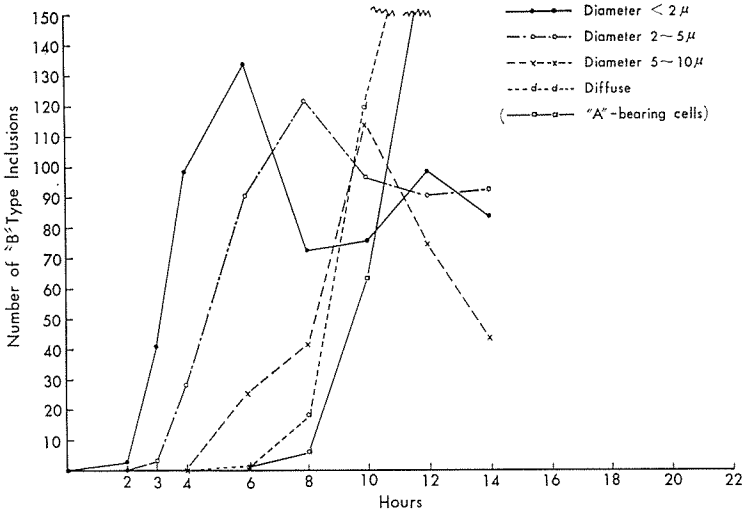

Fig. 2. "B" Type Inclusion Curves in Cowpox Virus-FL Cell System (Experiment I)

inclusions with a diameter of below 2 μ at 12 hours must indicate the occurrence of a second cycle of infection. As the period of infection progresses, degenerative changes generally become more predominant. This is probably due to the cytopathic effects of substances derived from the infected cells. With a multiplicity of 12, by 6 hours after infection many cells already showed cytopathic effects

such as cell rounding and aggregation of cells which made morphological studies difficult.

(b) Curves of labeled cells

As shown in Fig. 1, the percentage of labeled nuclei decreased gradually. On the contrary, the percentage of labeled cytoplasm increased. The rate of

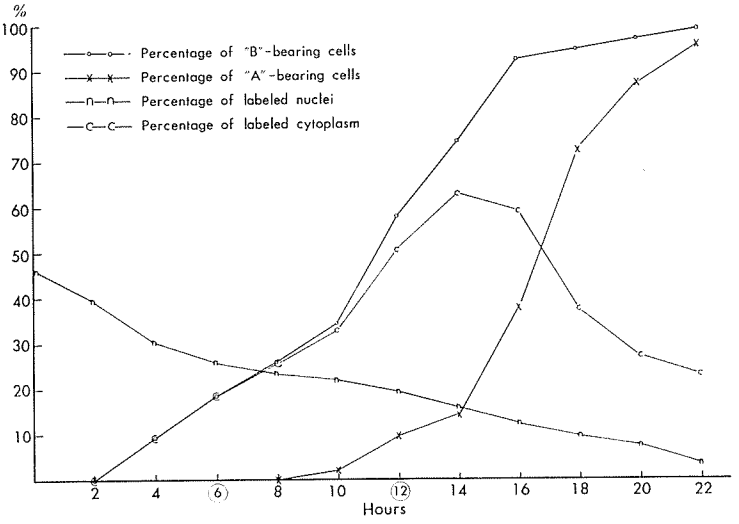

Fig. 3. Relationship between Labeled Cells and Inclusion-bearing Cells in Cowpox Virus-FL Cell System (Experiment 2)
(⑥ and ⑫ show that these preparations were chosen for grain counting)

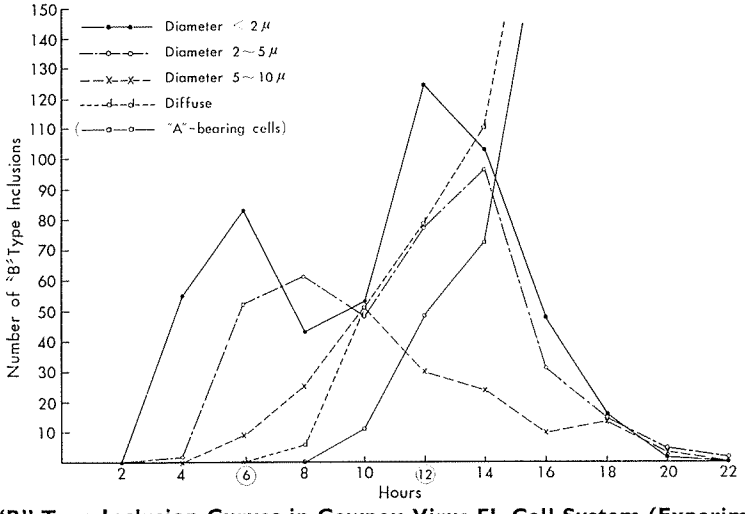

Fig. 4. "B" Type Inclusion Curves in Cowpox Virus-FL Cell System (Experiment 2)
(⑥ and ⑫ show that these preparations were chosen for grain counting)

increase in the percentage of labeled cytoplasm was identical with that of "B" bearing cells for about 10 hours, and then gradually decreased.

(*B*) *Experiments at a multiplicity of 5 (Expt. 2)*

Because of the severe cytopathic effects of a high multiplicity, experiments similar to the above were carried out at the lower multiplicity of 5. With this multiplicity, most cells retained their normal appearance at least in the early stage of infection. As shown in Figs. 3 and 4, the changes of the curves were generally slower than those in the previous experiment. However, the general tendency of the curves and the times of appearance of the highest peak of the number of inclusions in each group were nearly the same as in Expt. 1.

2. *Relationship between the development of "B" type inclusions and their ability to synthesize DNA*

Two samples from Experiment 2 were chosen for this study: the preparation taken at 6 hours (6 h preparation) and at 12 hours (12 h preparation). The silver grains per "B" type inclusion were measured quantitatively on these. The "B" type inclusions were classified into 4 groups on the basis of their diameters: less than 2 μ, 2 μ~5 μ, 5 μ~10 μ and larger diffuse. Fifty inclusions were chosen at random from each group. Silver grains appearing on the "B" inclusions were counted and results are plotted in Figs. 5 and 7. In the 6 h preparation (Fig. 5),

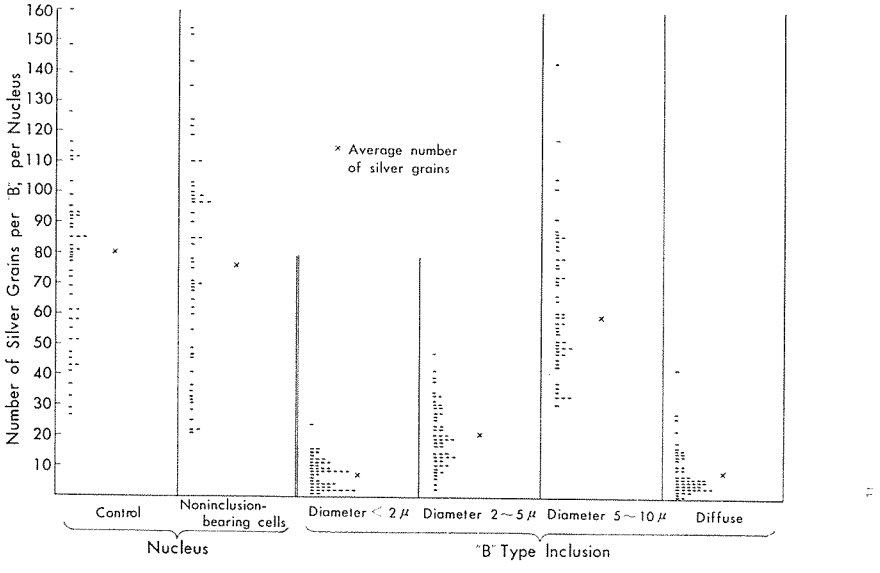

Fig. 5. Relationship between Development of "B" Type Inclusions and Their Ability to Synthesize DNA (6 Hour Preparation of Experiment 2)

incorporation of ^{3}H-thymidine into "B" inclusions occurred at all 4 stages. The

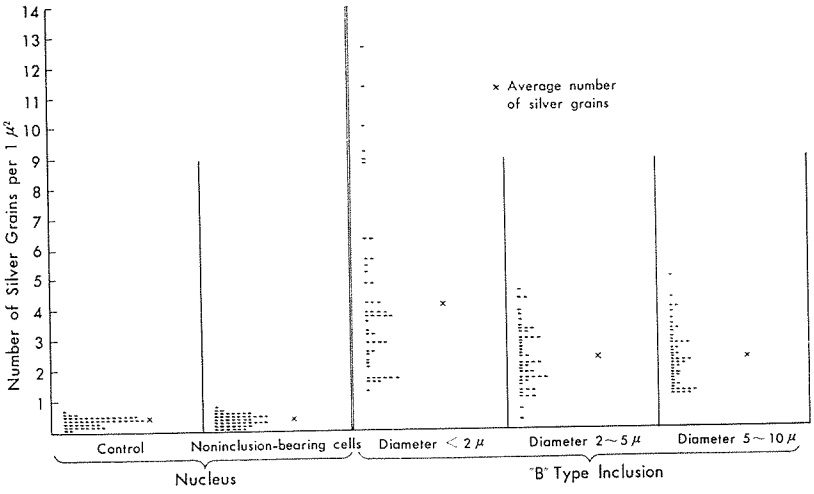

Fig. 6. Relationship between Development of "B" Type Inclusions and Their Ability to Synthesize DNA (6 Hour Preparation of Experiment 2)

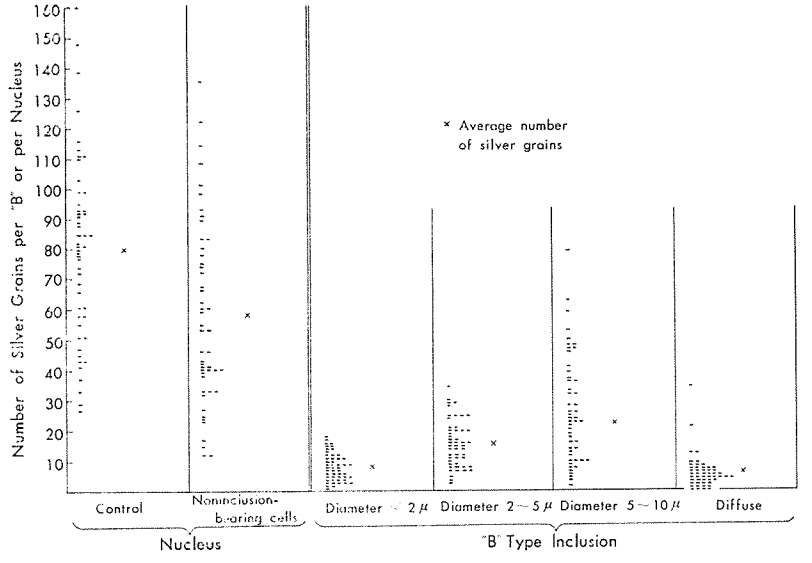

Fig. 7. Relationship between Development of "B" Type Inclusions and Their Ability to Synthesize DNA (12 Hour Preparation of Experiment 2)

most intensive incorporation, however, was found in well developed "B" inclusions with diameters of 5 μ~10 μ. The degree of incorporation decreases at the final stage of development of "B" inclusions, the large diffuse form. This must explain the dissociation between the "B" bearing cell curve and the curve of labeled

cytoplasm in Figs. 1 and 3. A comparison of the right 4 colums of Fig. 5, which show the number of silver grains per "B" inclusion, with the left two colums, which show the number of silver grains per nucleus shows that the distribution and the average number of silver grains per "B" inclusion with diameter of 5 μ~10 μ seems to be nearly identical with that per nucleus of normal or noninclusion bearing cell. This suggests that the efficiency of cytoplasmic DNA synthesis of poxvirus is as high as that of nuclear DNA synthesis of normal cells or noninclusion bearing cells.

The ability to synthesize DNA per unit area was studied. As shown in Fig. 6, the rate of synthesis of DNA in "B" inclusions per unit area was far higher than that in the nuclei of normal cells or noninclusion bearing cells. The highest activity was found in "B" inclusions with diameters of below 2 μ.

A similar study was made on the 12 h preparation. As shown in Figs. 7 and

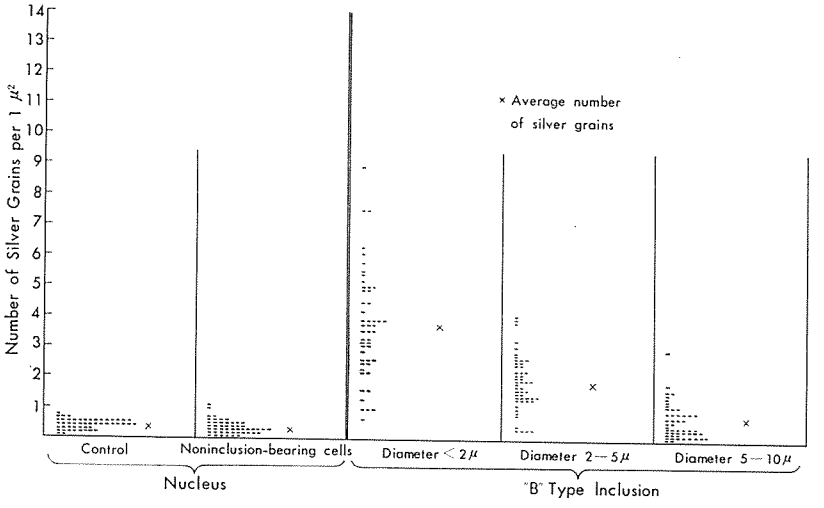

Fig. 8. Relationship between Development of "B" Type Inclusions and Their Ability to Synthesize DNA (12 Hour Preparation of Experiment 2)

8, the general tendency of the results was nearly the same as in the 6 h preparation. However, the activity of DNA synthesis both in "B" inclusions and in the nucleus of noninclusion bearing cells was lower than that in the 6 h preparation. As mentioned previously, degenerative changes increased at the later stage of infection. This may reduce cellular metabolism. As a matter of fact, "B" inclusions generally have a rough and diffuse structure.

This shows that the intensity of DNA synthesis in "B" inclusions varies not only with their size, but also with factors which might be influenced by the metabolic condition of the cells.

3. Effects of "B" inclusion formation upon nuclear DNA synthesis

As shown in Figs. 1 and 3, a gradual decrease in the percentage of labeled nuclei with time was noticed. Cells were distinguished into two groups, i.e. inclusion bearing cells and noninclusion bearing cells. The results obtained in Experiment 1 are shown in Fig. 9 and correspond to those in Fig. 1. The percentage of

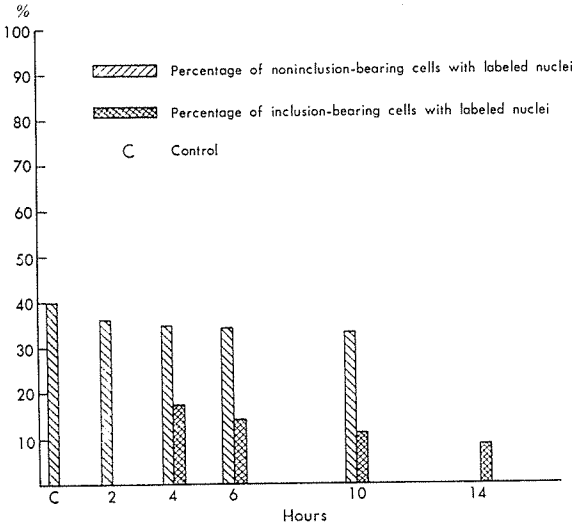

Fig. 9. Effect of "B" Formation upon Nuclear DNA Synthesis (Experiment 1)

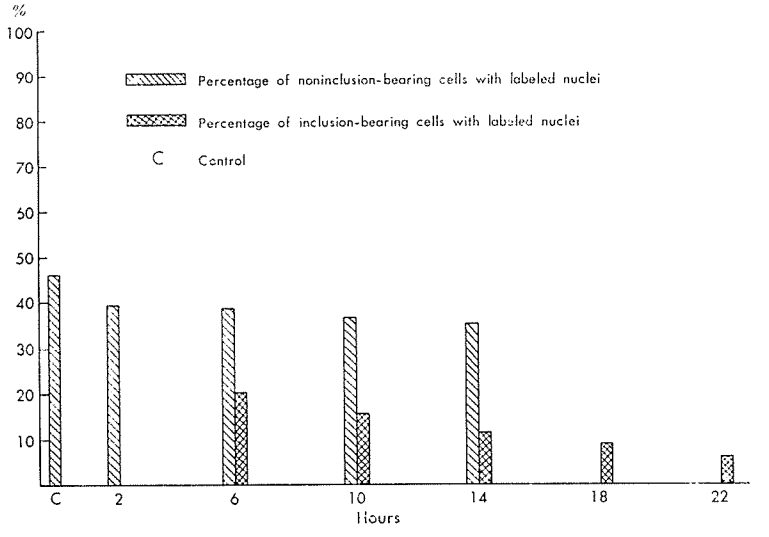

Fig. 10. Effect of "B" Formation upon Nuclear DNA Synthesis (Experiment 2)

noninclusion bearing cells with labeled nuclei was scarcely affected by time. Many noninclusion bearing cells seen in the preparation taken 10 hours after infection, could be regarded as infected cells, because most of the cells showed "B"

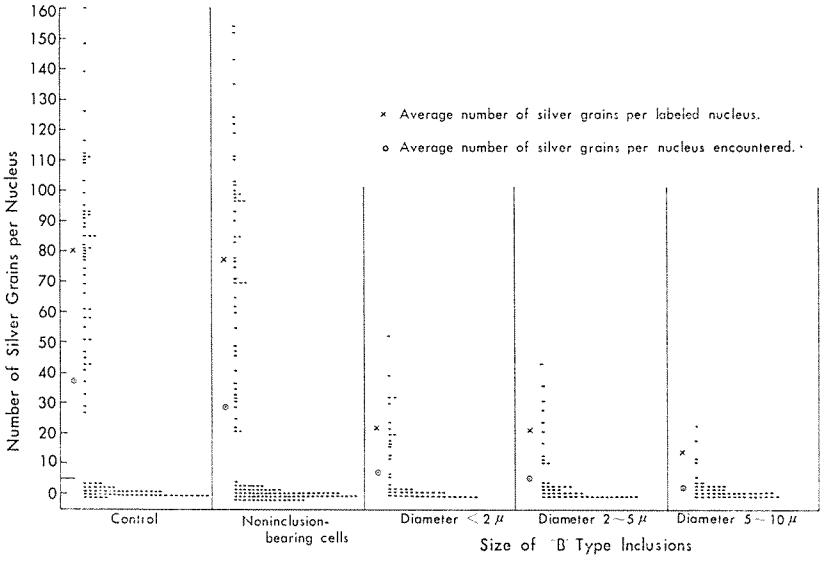

Fig. 11. Effect of "B" Formation upon Nuclear DNA Synthesis (6 Hour Preparation of Experiment 2)

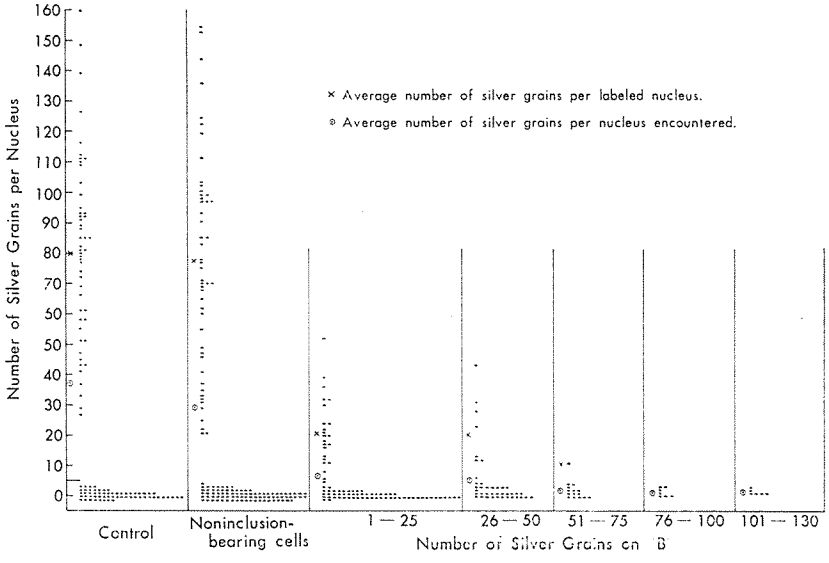

Fig. 12. Effect of "B" Formation upon Nuclear DNA Synthesis (6 Hour Preparation of Experiment 2)

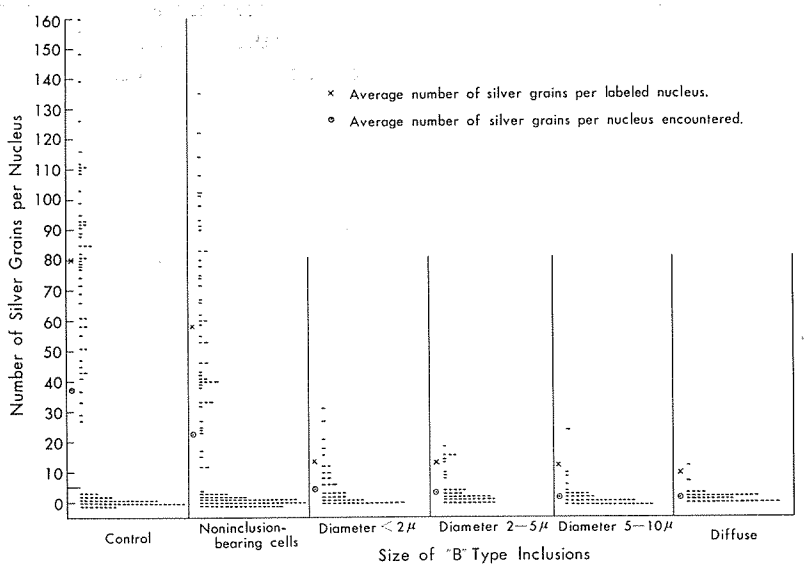

Fig. 13. Effect of "B" Formation upon Nuclear DNA Synthesis (12 Hour Preparation of Experiment 2)

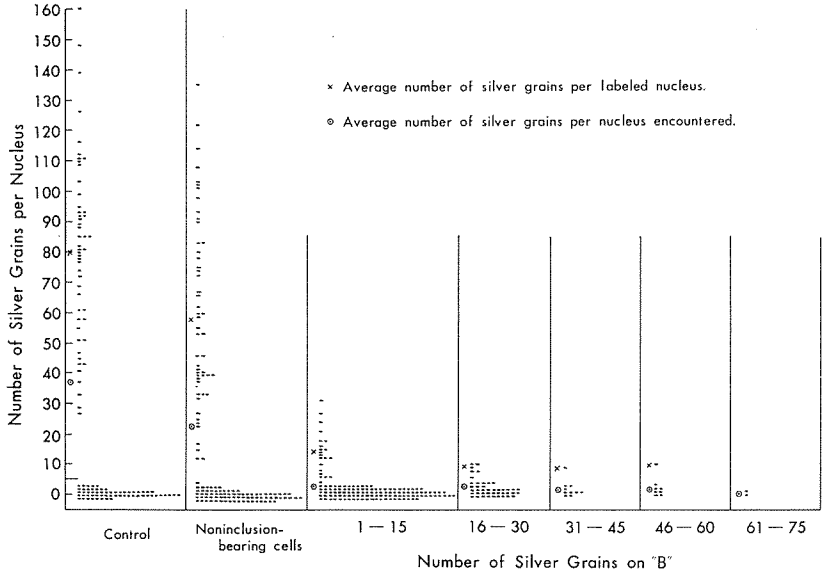

Fig. 14. Effect of "B" Formation upon Nuclear DNA Synthesis (12 Hour Preparation of Experiment 2)

inclusion formation 2 to 4 hours later. Similar studies were carried out with the preparation of Experiment 2. The result shown in Fig. 10 which corresponds

to Fig. 3 is nearly identical with the result of Experiment 1.

The study was extended to the relationship between the number of silver grains in the nuclei and the "B" type inclusions in the preparations of Experiment 2. Cells were divided into five groups: noninclusion bearing cells, cells bearing "B" inclusions with diameters of below 2 μ, cells bearing "B" inclusions with diameter of 2 μ~5 μ, cells bearing "B" inclusions with diameters of 5 μ~10 μ and cells bearing diffuse "B" inclusions. The numbers of silver grains per nucleus of noninclusion bearing cells and inclusion bearing cells were studied. The numbers of silver grains found in the 6 h preparation are plotted in Figs. 11 and 12 and those obtained in the 12 h preparation, in Figs. 13 and 14. A definite difference between the number of silver grains in the nuclei of noninclusion bearing cells and in the nuclei of inclusion bearing cells was found. Regardless of the size of the "B" inclusions (Figs. 11 and 13) and of the number of silver grains on the "B" inclusions (Figs. 12 and 14), DNA synthesis in the nuclei of inclusion bearing cells was definitely suppressed. The number of silver grains in the nuclei of noninclusion bearing cells was usually slightly less than in the nuclei of control noninfected cells. In the 6 hour preparation, this difference was slight while in the 12 hour preparation it was great. This difference between the two preparations seems to be due to the general degenerative tendency of most cells at the later stage of infection.

DISCUSSION

1. *The significance of the size and characteristics of "B" type inclusions with respect to DNA synthesis*

The first report of the development of inclusions of poxvirus was made by Bland and Robinow (1939) using so-called vaccinia virus and a cultured rabbit corneal cell system. The significance and characteristics of the "B" type inclusions of poxvirus have been studied extensively (Kato et al., 1962). It is now clear that the "B" type inclusion develops from a small compact to a large diffuse form. The results reported here confirmed quantitatively that the size and characteristics of the "B" type inclusions indicate not only the stage of infection at the cellular level, but also their DNA synthesizing activity.

2. *Relationship between "B" inclusion formation and nuclear DNA synthesis*

Poxvirus is known to show proliferative effects upon cells. As a matter of fact, most poxviruses produce pock which is formed as a result of cell infiltration and cell proliferation. Furthermore, several viruses of this group produce tumors such as Shope fibroma or Yaba monkey tumor. The present experiments were carried out to test whether virus infection causes an increase or decrease in nuclear DNA synthesis. The results show that at no stage of infection of cowpox virus, is there any evidence that cells with or without inclusions have increased nuclear DNA synthesis. On the contrary, the nuclei of all the cells bearing "B" type

inclusions showed definite suppression of DNA synthesis. The degree of suppression of nuclear DNA synthesis was irrespective of the size and characteristics of the "B" type inclusions. Therefore it is certain that immediately cytoplasmic DNA synthesis begins, nuclear DNA synthesis is suppressed.

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