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SHORT COMMUNICATION

INTERACTIONS OF ECTROMELIA VIRUS WITH MACROPHAGES AND ASCITES TUMOR CELLS FROM NORMAL AND IMMUNE MICE

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The behavior of macrophages and lymphocytes in response to antigen drew attention to the possible role of these cells in the immunity. However, until recently the role of these cells in viral infections has attracted less attention. Our previous works revealed that Ehrlich ascites tumor cells, Yoshida ascites tumor cells as well as peritoneal macrophages of mice were susceptible to ectromelia virus and produced " B " type inclusions of poxvirus.[1,2] Chick peritoneal macrophages infected with fowlpox virus[3] and rabbit peritoneal macrophages infected with myxoma virus also produced " B " type inclusions.[4] The " B " type inclusions are the sites of viral DNA synthesis and of viral antigen in these cells, and have been considered to be evidence for cytological viral multiplication.[5~8] Growth of poxvirus in macrophages from normal[9] and immune animals[10] has been reported using infectivity titration. ROBERTS[11] using fluorescent antibody staining described the susceptibility of macrophages to ectromelia virus, from normal and virus immune mice.

This communication presents direct cellular evidence for viral DNA synthesis in both Ehrlich ascites tumor cells and macrophages from normal and immune mice, and refers briefly to the susceptibility of lymphocytes to ectromelia virus.

Virus strain: Ectromelia virus Hampstead strain carrying the "A"v^+ marker (the "A" type inclusions contain elementary bodies) and cowpox virus LB red strain carrying the "A"v^- marker[12] were used. These viruses were passed through FL cells. Stock virus was prepared on FL cells. Aliquots of a single preparation of the virus, stored at $-25°C$, were used throughout. Virus titers of ectromelia virus and cowpox virus were 1.7×10^7 plaque forming units (pfu) on L cells per ml and 6×10^5 pfu on chick fibroblasts per ml respectively. Mice: Four week-old ddO mice which were supplied from the Laboratory Animal Center, Osaka University, were used. Macrophage cultures: Cultures were prepared by a method modified from those of FURNESS[13] and SEAMER[9] Mice were inoculated intraperitoneally with 2 ml of a 2 per cent suspension of starch in normal saline. On the third day each mouse was bled to death, and the peritoneal cavity was washed out with 5 ml of Parker's medium 199 containing 10 IU heparin per ml. The cells were then separated by centrifugation (1,000 rpm for 10 min), and resuspended in 199 medium containing 20 per cent inactivated calf serum, to give a final concentration of 5.5×10^6 cells per ml and 1 ml amounts were dispensed to Leighton tubes. These were closed with metal caps

and incubated at 37°C in an atmosphere of 5 per cent CO_2. Two hours later these were thoroughly washed with a jet of medium to remove all loose cells[14]. An almost pure culture of macrophages remained. After another twelve hours, the medium was replaced with 1 ml of fresh medium and henceforth the tubes were closed with rubber stoppers. Inclusion staining: Giemsa staining, which is the best to demonstrate the "B" and "A" type inclusions caused by poxvirus, was used. Furthermore, Giemas staining of inclusion-bearing cells pretreated with 1 N HCl at 60°C for 5 min is most suitable for demonstrating any kinds of "B" type inclusions[1,15]. The pretreatment with HCl removes cytoplasmic RNA which takes on a blue tinge from methylene blue. Only DNA-containing material, which takes on the reddish purple tinge of azur II, is clearly recognizable with Giemsa staining. Fluorescent antibody staining: Anticowpox virus rabbit γ-globulin coupled with fluorescein-isothiocyanate was used, since the fluorescent antibody could react with both the cells infected with cowpox virus, and those infected with ectromelia virus. Autoradiography: Dipping autoradiography with a Kodak NTB2 nuclear emulsion was carried out. Duration of exposure was 6 days. Giemsa stain was used for poststaining.

1. *Ability of cowpox virus to make mice immune against ectromelia virus*

The utility of immunization with vaccinia virus for controlling an outbreak of ectromelia has been reported.[16] Because of the close antigenic resemblance between ectromelia virus and cowpox virus,[17,18] Jenner's vaccination with cowpox virus was attempted on mice. Thirty mice were inoculated intraperitoneally with 0.25 ml of cowpox virus having 6×10^5 pfu per ml. Groups of five of these mice were then inoculated intraperitoneally with 0.25 ml of ectromelia virus having 1.7×10^7 pfu per ml

TABLE 1 *Vaccination of mice with cowpox virus against ectromelia virus*

(a)

Number of mice inoculated with CPV	Days of inoculation of EV after CPV inoculation	Number of mice inoculated with EV	Number of mice survived	Mortality of mice
5	1	5	0	100%
5	2	5	0	100%
5	3	5	0	100%
5	5	5	5	0%
5	7	5	5	0%
5	10	5	5	0%

CPV: cowpox virus EV: ectromelia virus

(b)

Number of mice inoculated with CPV	Doses of CPV inoculated per mouse*	Number of mice inoculated with EV	Number of mice survived	Mortality of mice
5	6×10^4 pfu/ml	5	5	0%
5	6×10^3 pfu/ml	5	5	0%
5	6×10^2 pfu/ml	5	0	100%
5	6×10^1 pfu/ml	5	1	80%
5	6×10^0 pfu/ml	5	0	100%

CPV: cowpox virus EV: ectromelia virus Days of inoculation of EV after CPV inoculation: 7 days
* Inoculum size: 0.25 ml

on 1, 2, 3, 5, 7 or 10 days after the cowpox virus inoculation. The mice were observed at least 20 days after ectormelia virus inoculation. As shown in Table 1 (a), for 3 days after cowpox virus inoculation, mice rarely escape from ectromelia death. However none of the mice which had been inoculated with cowpox virus more than 5 days before ectromelia virus inoculation showed any sign of infection and death.

Various doses of cowpox virus were inoculated intraperitoneally to 25 mice as shown in Table 1 (b). Seven days later all these mice were inoculated intraperitoneally with 0.25 ml of ectromelia virus having 1.7×10^7 pfu per ml. The mice were observed at least 20 days after ectromelia virus inoculation. As shown in the Table, a single inoculation of 0.25 ml of cowpox virus having 6×10^3 pfu per ml is enough to make the mice immune to ectromelia virus.

2. *In vivo observations of viral DNA synthesis in the Ehrlich ascites tumor cells and macrophages from normal and immune mice*

Three series of Ehrlich ascites tumor cells were prepared. 1) Ehrlich ascites tumor cells were injected into mice which had been immunized with 0.25 ml of cowpox virus (6×10^5 pfu per ml) twice and 0.25 ml of ectromelia virus (1.7×10^7 pfu per ml) once at 7 to 10 days intervals. Nine days later tumor cells were taken out (Group I cells). 2) Ehrlich ascites tumor cells were injected into normal mice intraperitoneally. One and seven days later, 0.25 ml of cowpox virus (6×10^5 pfu per ml) were injected intraperitoneally. After another seven days, the mice were inoculated with 0.25 ml of ectromelia virus (1.7×10^7 pfu per ml). Six days later the tumor cells were taken out (Group II cells). 3) Group III cells consisted of Ehrlich ascites tumor cells from nonimmunized mice.

Cells from all three groups were washed by centrifugation with resuspension three times in Hanks' solution. The number of tumor cells in each group was adjusted to 3.6×10^6 and these cells were resuspended in 1 ml of ectromelia virus suspension having 1.7×10^7 pfu. The cell and virus mixtures of each group were injected intraperitoneally into normal and hyperimmunized mice. The ascites cells of mice were taken out after 24 and 48 hours. One hour before taking out the sample, 1 ml of ^{3}H-thymidine solution (50 μc per ml) was injected intraperitoneally. Four smear preparations were made and observed by ordinary Giemsa staining, Giemsa staining with HCl pretreatment, fluorescent antibody staining and autoradiography.

Cells from all three groups, when injected into normal mice produced typical " B " and "A" type inclusions of ectromelia virus as described before (Fig. 2, 3, 4). Macrophages also produced these two types of inclusions (Fig. 5, 6). In order to identify the macrophages, carbon particles were injected intrapperitoneally before or after virus inoculation. Most of the macrophages showing phagocytosis of carbon particles also showed " B " inclusion formation simultaneously (Fig. 6). Generally, the number of carbon particles taken up was small when the carbon was given after virus inoculation. This may explain the decreased phagocytic activity of the infected cells. Most of the small lymphocytes which do not take up any carbon particles seem not to be susceptible to the virus. However a certain number of the cells showed a small inclusion in the thin cytoplasmic rim (Fig. 2, 6). The low susceptibility of small lymphocytes to the virus might be correlated with the low activity of RNA metabolism of the cells. Polymorphonuclear leucocytes which lack both DNA and RNA metabolism, show no signs of virus multiplication. The viral susceptibility of lymphocytes, especially those from immune animals, requires further investigation.

Fluorescent antibody staining with anti-cowpox virus rabbit γ-globulin coupled to fluorescein-isothiocyanate showed specific fluorescent areas in the cytoplasm. Irregular fluorescent areas generally correspond to " B " type inclusions, while the ring form of fluores-

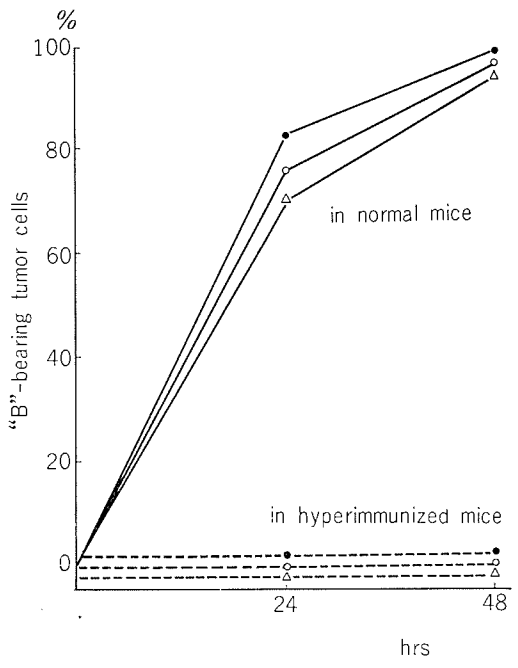


FIGURE 1 Comparison of the percentages of " B " type inclusion-bearing Ehrlich ascites tumor cells among the experimental groups.
△: Group I ○: Group II ●: Group III

scence corresponds to "A" type inclusions (Fig. 7).

Autoradiographs revealed that most of the " B " type inclusions labeled (Fig. 3, 4, 5), with the intensity of labeling in the Ehrlich ascites tumor cells being far stronger than that in the peritoneal macrophages. This may be a reflection of the difference in metabolic activity between these cells. Generally, nuclear DNA synthesis in inclusion-bearing cells was suppressed.

The percentages of inclusion-bearing tumor cells were compared among these groups of cells. As shown in Fig. 1, no intrinsic difference could be seen among the three groups after injection into normal mice. The infected cells which were transferred into hyperimmunized mice showed no sign of inclusion formation.

3. *In vitro observations of viral DNA synthesis in peritoneal macrophages from normal and immune mice*

In the *in vivo* experiment, the viral susceptivility to macrophages taken from immune mice is uncertain, because the immunized macrophages become mixed with nonimmunized macrophages after their injection into the peritoneal cavity of nonimmunized mice. Therefore an *in vitro* experiment was carried out.

Peritoneal macrophages from normal and immune mice were cultured as described above. Twenty six hours later these cells were challenged with 0.5 ml of ectromelia virus having 1.7×10^7 pfu per ml. One hour after adsorption of the virus, the virus suspension was replaced with medium 199 containing 20 per cent calf serum. Twenty hours after infection the cover slips were taken out. One hour prior to taking out the sample, the culture medium was replaced with 1.5 ml of ^{3}H-thymidine solution (1 μc per ml). As a control, uninfected macrophages were labeled in the same way. Then, autoradiography was carried out on them. Of the infected groups, almost one hundred per cent of the macrophages from either normal or immune mice produced " B " type inclusions and these inclusions were definitely labeled (Figs. 8 and 9).

The present experiments have shown that intraperitoneal immunization of mice with ectromelia virus does not confer any definite cellular immunity upon either peritoneal macrophages or upon Ehrlich ascites tumor cells grown in mice.

Summary

1. A single inoculation of cowpox virus into mice is enough to make the mice immune to ectromelia virus. 2. Ehrlich ascites tumor cells and peritoneal macrophages taken from normal and immune mice showed a similar degree of susceptibility to ectromelia virus, as indicated by inclusion formation and by cytopasmic viral DNA synthesis.

References

1. KATO, S. (1955). Studies on the inclusion bodies of ectromelia virus propagated in the ascites tumor cells. *Virus* **5**, 111–119.
2. HAGIWARA, K. (1956). Studies on the growth cycle of ectromelia virus propagated in Ehrlich ascites tumor cells. *Virus* **6**, 23–41.
3. KATO, S., HAGIWARA, K., BABA, E., SATO, Y. and KAMAHORA, J. (1955). Studies on the new inclusion bodies of fowlpox virus. *Virus* **5**, 318–325.
4. KATO, S. and CUTTING, W.C. (1959). A study of the inclusion bodies of rabbit myxoma and fibroma virus and a consideration of the relationship between all pox virus inclusion bodies. *The Stanford Medical Bulletin* **17**, 34–45.
5. KATO, S., KAMEYAMA, S. and KAMAHORA, J. (1960). Autoradiography with tritium-labeled thymidine of poxvirus and human amnion cell system in tissue culture. *Biken's J.* **3**, 135–137.
6. KAMEYAMA, S., TAKAHASHI, M., TOYOSHIMA, K., KATO, S. and KAMAHORA, J. (1959). Studies on the inclusion bodies of ectromelia virus using the fluorescent antibody technique. *Bicken's J.* **2**, 341–344.
7. KATO, S., TAKAHASHI, M., KAMEYAMA, S. and KAMAHORA, J. (1959). A study on the morphological and cyto-immunological relationship between the inclusions of variola, cowpox, rabbitpox, vaccinia (variola origin) and vaccinia IHD and a consideration of the term " Guarnieri body ". *Biken's J.* **2**, 353–363.
8. KATO, S. (1964). Diagnosis of smallpox and cowpox by fluorescent antibody technique. *Sōgō Rinshō* (*Clinic all-round*) **13**, 1239–1247.
9. SEAMER, J. (1965). Mouse macrophages as host cells for murine viruses. *Arch. Ges. Virusforsch.* **17**, 654–663.
10. MARAL, R. (1957). Etude du developpement du virus de la myxomatoses en cultures de tissus. *Ann. Inst. Pasteur* **92**, 742–751.
11. ROBERTS, J. A. (1964). Growth of virulent and attenuated ectromelia virus in cultured macrophages from normal and ectromelia-immune mice. *J. Immunol.* **92**, 837–842.
12. KATO, S., HARA, J., OGAWA, M., MIYAMOTO, H. and KAMAHORA, J. (1963). Inclusion markers of cowpox virus and alastrim virus. *Biken J.* **6**, 233–235.
13. FURNESS, G. (1958). Interaction between Salmonella typhimurium and phagocytic cells in cell culture. *J. Infect. Dis.* **103**, 272–277.
14. MACKANESS, G. B. (1962). Cellular resistance to infection. *J. Exptl. Med.* 116. 381–406.
15. KATO, S. (1964). Cytological observation techniques of virus-infected cells. *Soshikibaiyo* (*Tissue culture*) 442–448, Asakura.
16. PEARSON, A. E. G. (1957). Attempted control of ectromelia in a mouse-breeding colony. *Nature* 180, 1300.
17. DOWNIE, A. W., and MCCARTHY, K. (1950). The viruses of variola, vaccinia, cowpox and ectromelia, Neutralization tests on the chorioallantois with unabsorbed and absorbed immune sera. *Brit. J. Exptl. Pathol.* **31**, 789–796.
18. GISPEN, R. (1955). Analysis of pox-virus antigens by means of double diffusion. *J. Immunol.* **74**, 134–141.

FIGURE 2 Nonimmunized Ehrlich ascites tumor cells infected with ectromelia virus (Group III, 48 hour preparation). Giemsa staining with pretreatment of 1 N HCl at 60°C for 5 min. Three Ehrlich tumor cells produce large diffuse " B " type inclusions and one small lymphocyte also shows perinuclear " B " inclusion formation.

FIGURE 3 Autoradiogram of the nonimmunized Ehrlich ascites tumor cells infected with ectromelia virus (Group III, 24 hour preparation). Labeled " B " type inclusions are shown. The nuclei of the " B "-bearing cells are not labeled.

FIGURE 4 Autoradiogram of the immunized Ehrlich ascites tumor cells infected with ectromelia virus (Group II, 24 hour preparation). Labeled " B " type inclusions are shown. The nuclei of the " B "-bearing cells are not labeled.

FIGURE 5 Autoradiogram of peritoneal macrophages infected with ectromelia virus (Group I, 24 hour preparation). Labeled " B " type inclusions are shown. The nuclei are free from any silver grains.

ETC : Ehrlich ascites tumor cell
SLC : small lymphocyte
MP : macrophage
PM : polymorphonuclear leucocyte
" B " : " B " type inclusion
" A " : "A" type inclusion

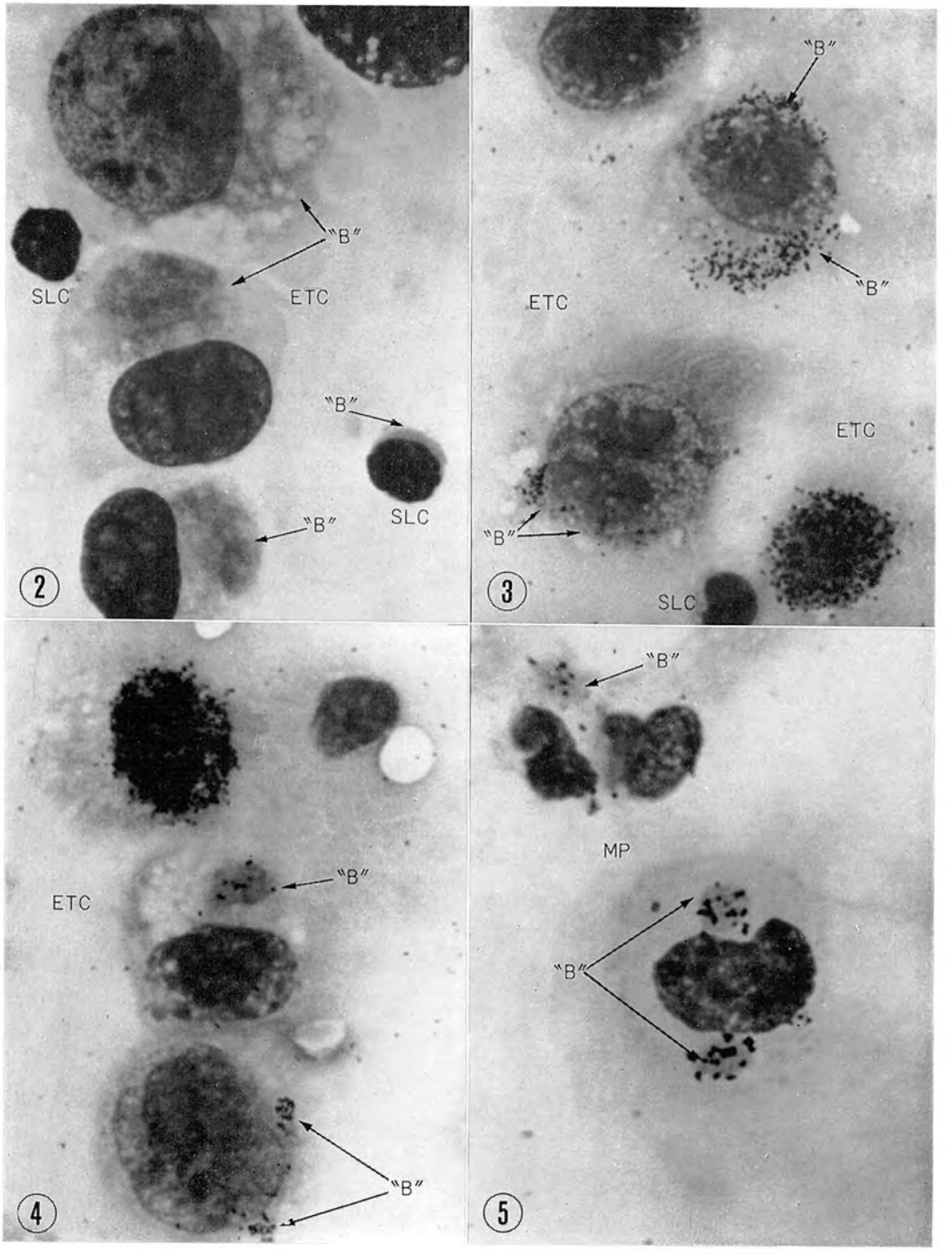
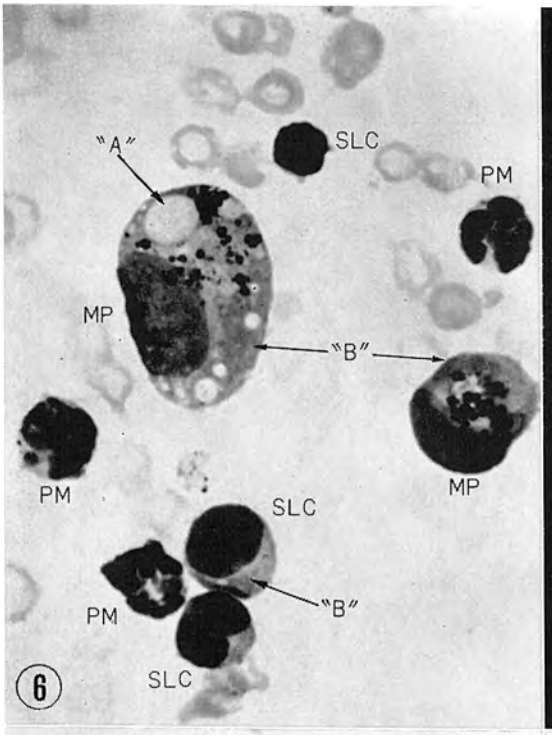

"B"
SLC
ETC
"B"
"B"
SLC
2
"B"
ETC
"B"
ETC
"B"
SLC
3
"B"
ETC
"B"
"B"
4
"B"
MP
"B"
5

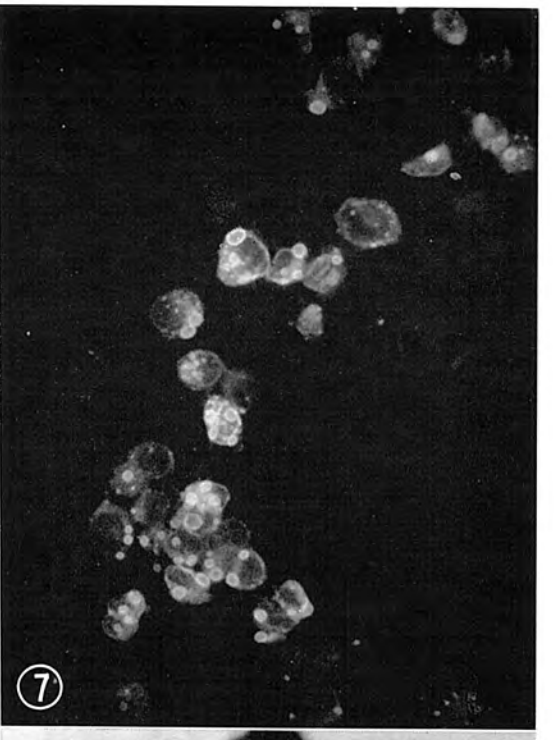
FIGURE 6 Two macrophages infected with ectromelia virus, showing phagocytosis of carbon particles and simultaneously showing inclusion formation. Giemsa staining. One small lymphocyte free from any carbon particles showing " B " inclusion formation.

FIGURE 7 Nonimmunized Ehrlich ascites tumor cells infected with ectromelia virus, stained with fluorescent anti-cowpox virus rabbit γ-globulin (Group III, 24 hour prepaation). Many distinct fluorescent areas are seen.

FIGURE 8 Autoradiogram of the cultured nonimmunized peritoneal macrophages infected with ectromelia virus. All macrophages have labeled " B " type inclusions.

FIGURE 9 Autoradiogram of the cultured immunized peritoneal macrophages infected with ectromelia virus. All macrophages have labeled " B " type inclusions.


"A"
SLC
PM
MP
"B"
PM
MP
SLC
"B"
PM
SLC
6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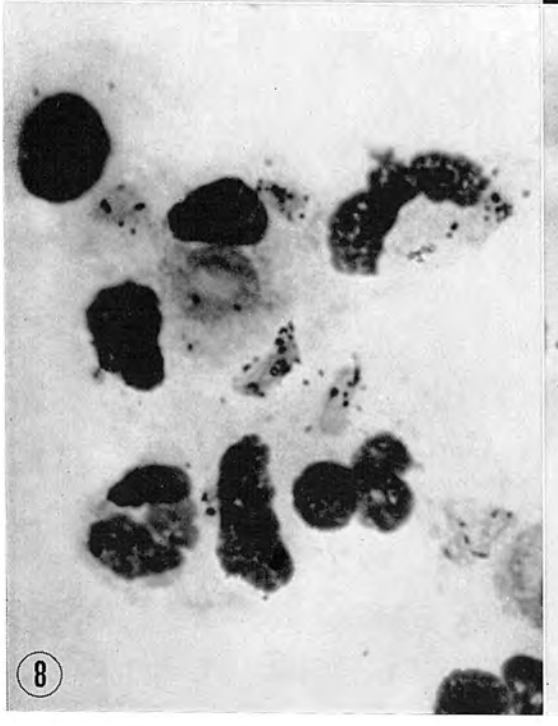
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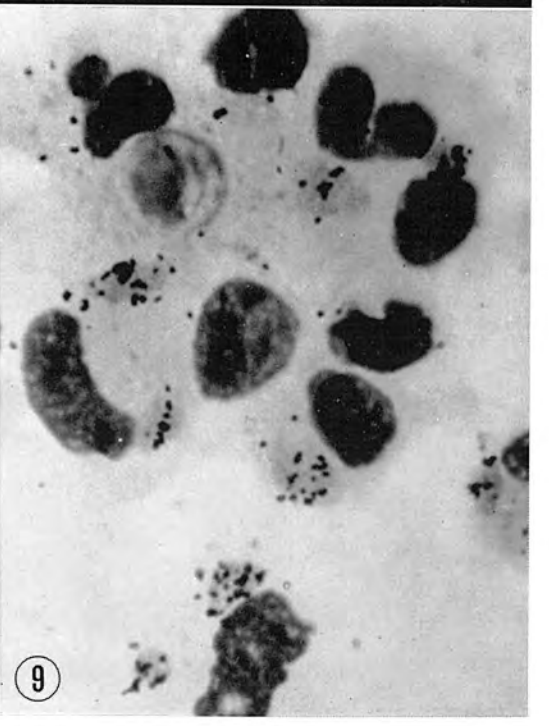
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