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Studies on the Inclusion Bodies of Ectromelia Virus using the Fluorescent Antibody Technique

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SUMMARY

Ehrlich ascites tumor cells infected with ectromelia virus ("G" and "H" strains) were studied with the fluorescent antibody technique (indirect method).

All fluorescent spots were found to correspond to the sites of "B" type inclusion. Neither "A" type inclusions of either virus strains, nor nuclei showed any distinct fluorescence. Thus the "B" type inclusion was considered to be the main site for virus multiplication. Fluorocarbon (Daifron S3) purified elementary bodies were also studied with the fluorescent antibody technique and Feulgen reaction.

The purified material had only weak fluorescence. The Feulgen reaction of the same material was very weak. From the results it is concluded that "B" type inclusions may contain more maked DNA and viral protein than "A" type inclusions.

INTRODUCTION

As already reported, two kinds of inclusion bodies named "A" and "B" were found in ectromelia virus (Kato *et al.*, 1955). Recently two strains of ectromelia virus have been isolated, named "G" and "H" strains, which can produce two different "A" type inclusions (Hagiwara, 1958).

The "A" type inclusion of the "G" strain has many elementary bodies and that of "H" strain none. "B" type inclusions of ectromelia virus could be stained with antimyxoma fluorescent antibody, so that this type of inclusion consists of the viral antigen common to these pox group viruses.

On the other hand, "A" type inclusion bodies in both strains did not stained with myxoma fluorescent antibody (Takahashi *et al.*, 1959). This presented the problem of why even "A" type inclusion of the "G" strain, containing elementary bodies, could not be stained.

A possible explanation is that in the first place, non-stainability might depend upon the specificity of the antibody, because the antibody used was not homologous. Secondly "A" type inclusion might be covered with some stiff membrane which prevents conjugated antibody from entering. Finally, elementary bodies in the inclusion might be too small or so few that they could not be detected by fluorescent antibody technique.

The present study was performed to solve these problems.

MATERIALS AND METHODS

1. *Virus*

"G" and "H" strains of ectromelia virus isolated by Hagiwara were used.

1) *Crude virus material:* The strains were inoculated into the abdominal cavity of mice. The livers were removed from the moribund mice, and a ten percent suspension was made with a Waring blender at 45,000 rpm for 30 seconds.

2) *Purified virus materials* Daifron S3) (Trichlorotrifluoroethane) was used for purification of the virus material. One volume of Daifron was mixed with two volumes of crude virus material and blended twice each for 20 seconds at 45,000 rpm in a Waring blender.

The resulting gelatinous mass was centrifuged at 3,000 rpm for 10 minutes. The aqueous supernate (DF1) constituting the Daifron treated virus antigen was collected and further treated with Daifron in the same way as described above.

The supernate (DF2) was harvested and centrifuged in a Spinco L for 30 min. at 15,000 rpm. The pellets attached to the bottom of the tubes were resuspended in one-fourth of the original volume of phosphate buffer saline, which was used as the concentrated purified virus material.

2. *Smear preparation*

1) *Infected Ehrlich tumor cells*

Ehrlich tumor cells were mixed with about the same volume of crude virus material and the mixture was injected into the peritoneal cavities of mice. At various intervals, cells were harvested from mice and smear preparations were made.

2) *Purified virus material*

0.05 ml of the respective virus material, crude, DF1, DF2 and concentrated materials, were dropped onto slide glasses to cover about the same area and dried in the air for 30 min.

3. *Section preparation*

The infected Ehrlich tumor cells described above were harvested and centrifuged at 1,500 rpm for 15 min. The packed cells were then placed in the cryostat and section preparations were made.

4. *Fluorescent antibody technique*

The indirect method of Weller and Coons (1954) was employed. Hamsters were chosen as the immunizing animals with ectromelia virus, since their susceptibility to this virus had been observed in our laboratory. 0.5 ml of the suspension of CAM infected with ectromelia virus was inoculated intraperitoneally into hamsters three times at intervals of one week and blood was collected 10 days after the last injection. The titer of this immune serum was 1:40 with the antigen of a liver emulsion infected with ectromelia virus in the complement fixing reaction. The globulin fraction of normal hamster serum was injected subcutaneously into rabbits with Freund's adjuvant twice at an interval of one month and blood was taken 1 week later.

The precipitation titer of this immune serum was 1:1,000 by the antiserum dilution method, and the globulin fraction was conjugated with fluorescein isothiocyanate following Marshall's method (1958) in the same way as described in our previous report. However, as a slight cross reaction was detected between antihamster rabbit serum and normal mouse serum, both immune sera were repeatedly absorbed with acetone treated mouse liver powder.

Stain specificity was established in the following way. 1) Uninfected Ehrlich tumor cells showed no fluorescence. 2) When immune hamster serum was replaced by normal hamster serum, no fluorescence appeared in the infected cells. 3) Direct application of the conjugated antibody to the infected cells failed to show any fluorescence.

RESULTS

1) *Smear preparation of Ehrlich tumor cells infected with ectromelia virus*

Infected tumor cells at an early stage of infection were examined by the fluorescent antibody technique. As shown in Fig. 1, compact form of brilliant fluorescence was seen in the cytoplasm of most cells, and on restaining with Giemsa solution it was found that the fluorescent area corresponded precisely to "B" type inclusion meaning that this inclusion had much viral antigen.

In the cells at a later stage of infection, about 20 hours after infection, diffuse fluorescence was visible around the dark area beside the nucleus, representing bright "B" type inclusion around the dark "A" type inclusions. This was like the case with myxoma fluorescent antibody. Thus the possibility that the nonstainability of "A" type inclusions might be due to heterologous antibody was disproved.

2) *Preparation of sections of infected Ehrlich tumor cells*

Preparation of sections of infected cells bearing numerous "A" type inclusions were examined. The average diameter of the Ehrlich tumor cell is about 20 μ and as the thickness of the sections was less than 10 μ, sections of "A" type inclusions must be present in some of cells.

However no distinct fluorescence was seen in any of the "A" type inclusions, so that there was no appreciable difference between "A" type inclusions of smear preparations and those of section preparations.

The possibility that the membrane enclosing "A" type inclusions might be impermeable to the conjugated antibody was thus disproved.

3) *Purified virus material*

Fluorocarbon treated virus material was examined to see if purified virus material become visible with fluorescent antibody, when concentrated. In case of the crude virus material, a few of irregular fluorescent spots were detected in the pale blue autofluorescence of liver cell components. These were suspected to be debris of "B" type inclusions. In the DF1 preparation, rather homogeneous and weak fluorescence spread over the field, containing some masses of fluorescence. The DF2 preparation, however, showed a more homogeneous faint fluorescence and fewer irregular masses. This might be caused by breakdown of "B" type inclusions during the vigorous blending. Concentrated purified virus material had some fluorescence, so that concentration or aggregation may be essential for fluorescence of the purified virus material.

DISCUSSION

1) *Inclusion bodies of ectromelia virus*

Attempts to visualize the antigen of ectromelia virus with its specific antibody coupled with fluorescein isocyanate in infected cells were unsuccessful by direct method. This was probably due to difficulty in gaining hyperimmune serum in rabbits and to the inferiority of isocyanate as a reagent for conjugation. The indirect method mediated by hamsters was therefore attempted. Almost all fluor-

escent spots corresponded to the sites of "B" type inclusions. Even in "G" strain neither nuclei nor "A" type inclusions showed any distinct fluorescence. These results agreed with those of previous experiments using fluorescent antimyxoma rabbit serum, implying that the "B" type inclusions of the ectromelia virus contain much common viral antigen.

2) *Daifron purified virus material*

As already mentioned, "A" type inclusion of "G" strain contain many elementary bodies. Since no remarkable fluorescence was noticed in either of them in smear and section preparations raised the problem of whether the elementary bodies were stainable with fluorescent antibody.

Our results showed that purified virus material has very weak fluorescence, and it is most likely that the remarkably strong fluorescence of "B" type inclusions is due to the condensed and naked antigenic protein mass. The elementary bodies in "A" type inclusions are too diffuse to be detected with fluorescent antibody. This seems similar to the results of Feulgen reaction. The Feulgen reaction of purified virus particles is very weak. The facts obtained explain the very weak and sometimes almost negligible Feulgen reaction of the "A" type inclusion in "G" strain infections.

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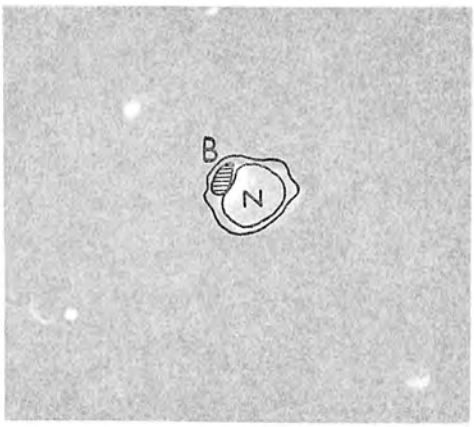

Fig. 1. Brilliant fluorescence was visible in the cytoplasm of Ehrlich tumor cell at an early stage of infection.

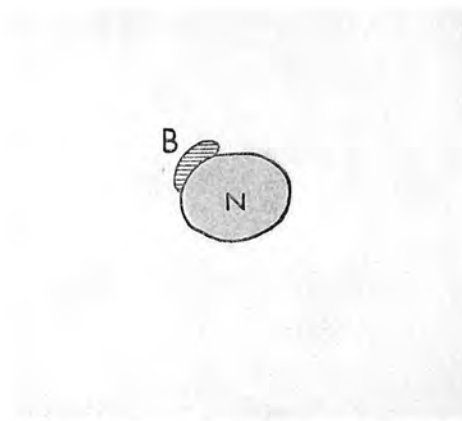

Fig. 2. The same cell as in Fig. 1, restained with Giemsa solution. The fluorescence proved to be located in a "B" type inclusion.

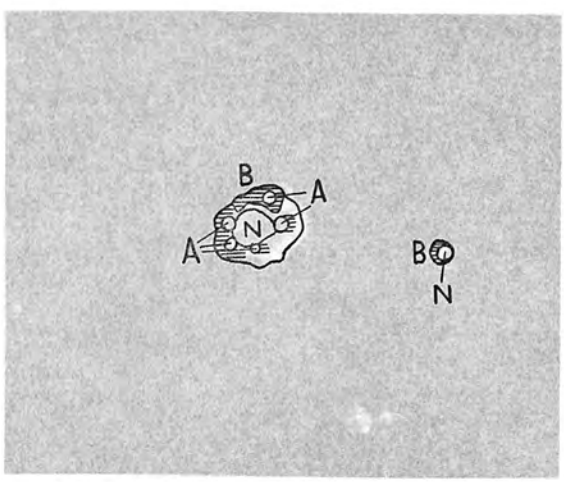

Fig. 3. Ehrlich tumor cell at a later stage of ectromelia infection. Diffuse fluorescence was visible around round dark areas.

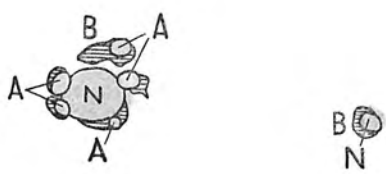

Fig. 4. The same cell as in Fig. 4, restained with Giemsa solution. It is evident that the round dark areas were "A" type inclusions and that the brilliant fluorescence spreading in cytoplasm correspond to "B" type inclusions.

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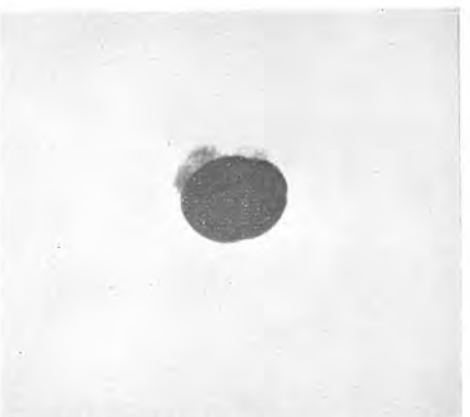

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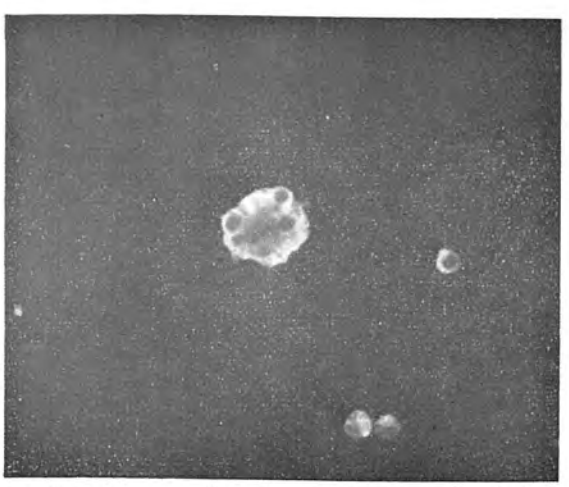

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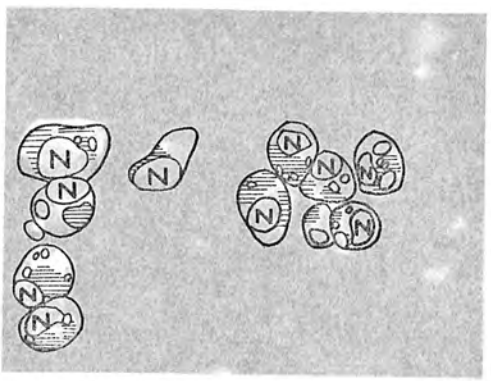


Fig. 5. Ehrlich tumor cells at a later stage of ectromelia infection, stained with antimyxoma fluorescent antibody. Just as in Fig. 3, brilliant fluorescence was conspicuous in the cytoplasm, while round areas remained unstained.

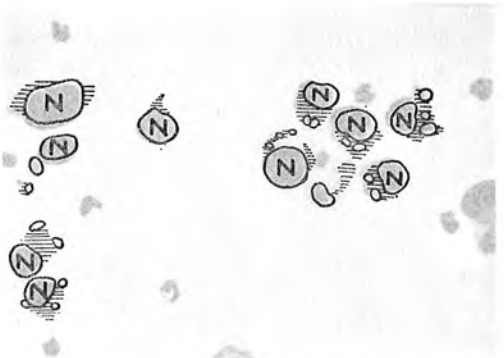


Fig. 6. The same cells as in Fig. 6, restained with Giemsa solution.

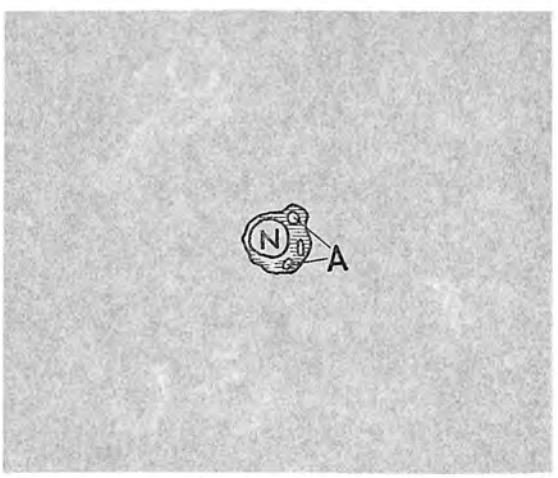
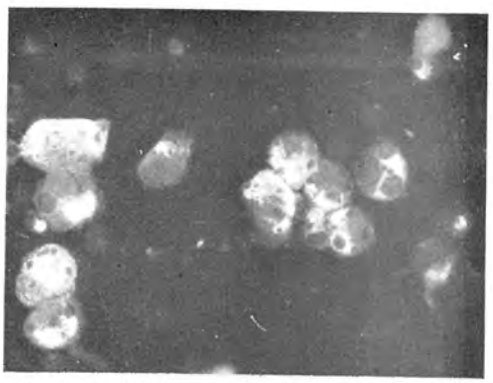


Fig. 7. Section preparation of infected Ehrlich tumor cells. The distinct fluorescence was not detected in "A" type inclusions.

Fig. 8. The same field as in Fig. 8, restained with Giemsa solution.



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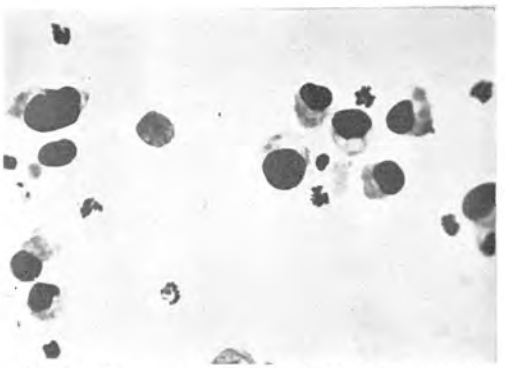

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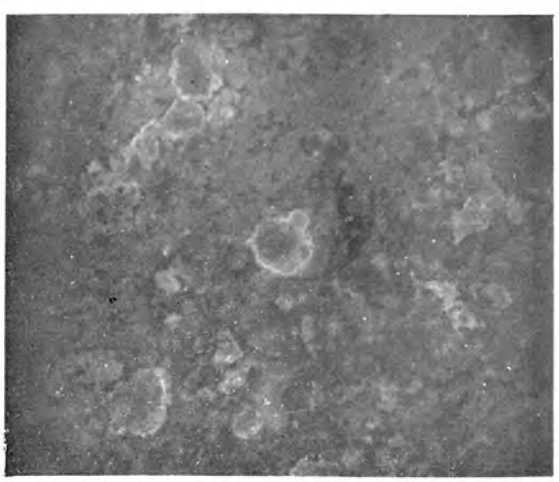

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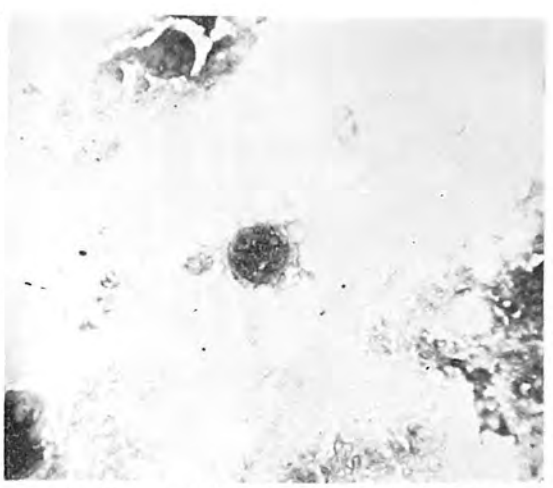

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Flg. 9. Crude virus material. A few irregular spots of fluorescence were detected in the pale blue autofluorescence of the liver cell components.

Fig. 10. DF1 (once Daifron treated) preparation. Rather homogeneous and weak fluorescence spreads, but still has some masses of fluorescence.

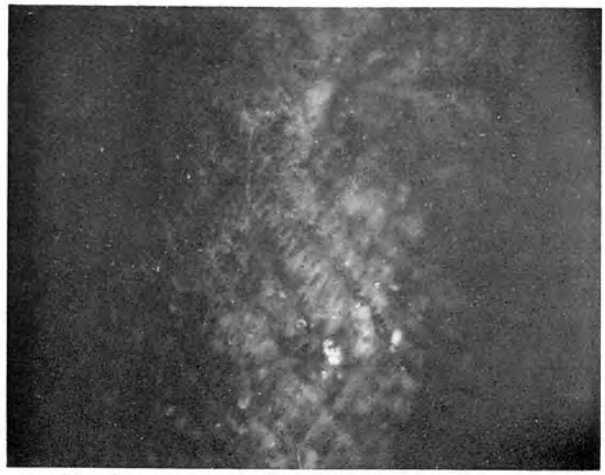


Fig. 11. Concentrated purified virus material had some fluorescence. The streaks seem to be caused by the evaporation process.

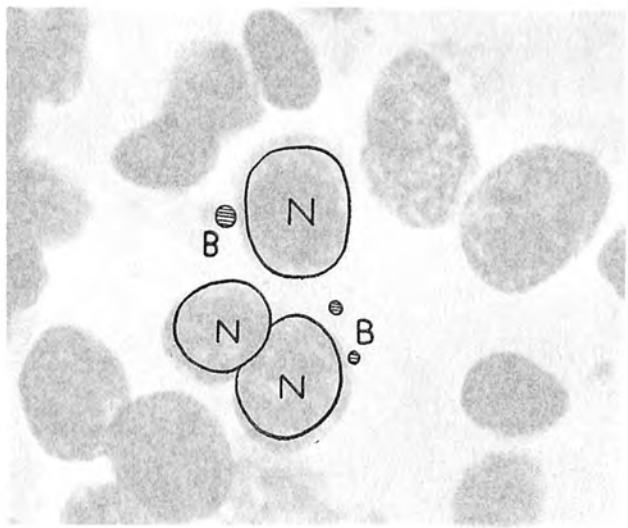


Fig. 12. Ehrlich tumor cells infected with ectromelia virus. Feulgen reaction. "B" type inclusions were Feulgen positive.

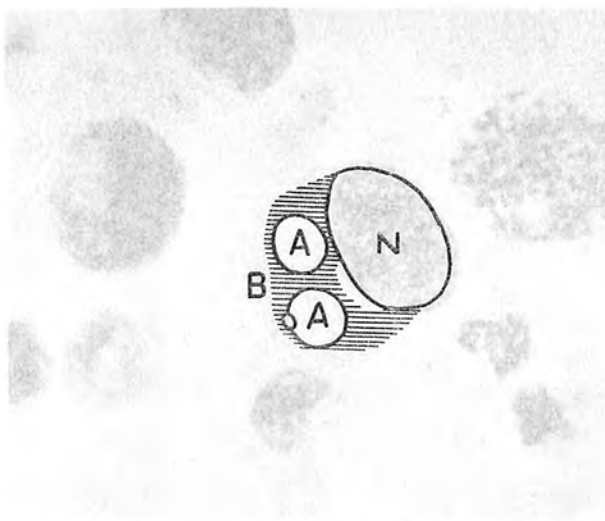
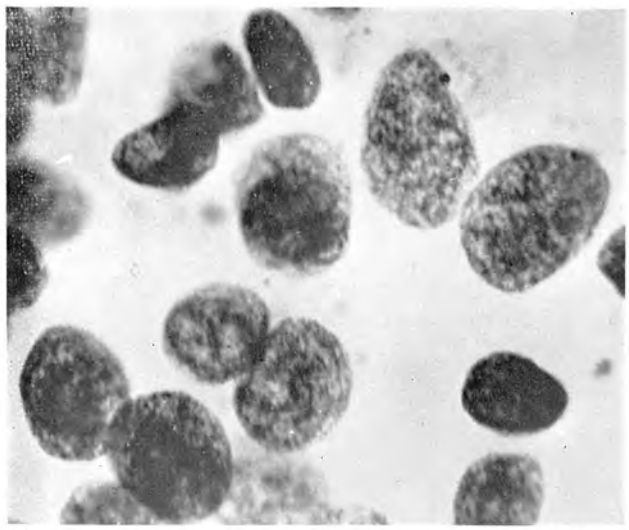


Fig. 13. Ehrlich tumor cells infected with ectromelia virus. Feulgen reaction. "B" type inclusions showed a positive reaction, while "A" type inclusions gave almost a negative reaction.



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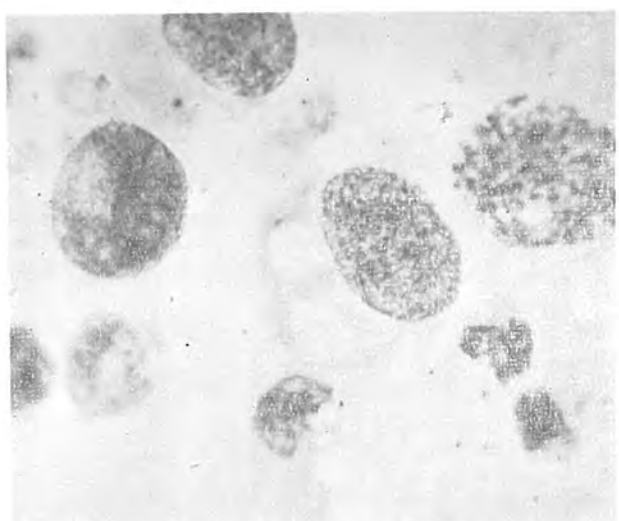

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