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FLUORESCENT ANTIBODY STUDY OF VACCINIA VIRUS PROPAGATION IN CHICK EMBRYO CHORIOALLANTOIC MEMBRANE

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Summary. — Chick embryo chorioallantoic membranes (CAM) were inoculated with vaccinia virus (Lister strain). The viral antigenic material was identified in the cytoplasm of CAM cells from 8 till 72 hours after inoculation (p.i.). A rapid diagnosis of variola by the fluorescent antibody (FA) technique on CAM was offered.

The localization of viral antigens in CAM infected with viruses of the variola-vaccinia group was studied by Kato *et al.* (1959). Experiments now reported were undertaken to study the dynamics of vaccinia virus development in CAM by the FA technique.

The Lister strain of vaccinia virus in its 5th CAM passage was inoculated onto CAM of Leghorn chick embryos. Three days p.i. membranes showing confluent viral lesions were used for virus titration and FA staining. At defined time intervals p.i., the membranes were collected, washed three times with buffered saline solution pH = 7.4 (BSS) and dried on filter paper. Imprints were prepared by gently rubbing microscope slides with the membrane. Whole membranes were quick-frozen in petroleum ether cooled to —78° C in a dry ice-acetone mixture and cut in a kryostat. Additional slides were made from abortive skin lesions of persons revaccinated with smallpox vaccine.

For direct FA staining, gamma-globulins from a rabbit immunized with vaccinia virus, conjugated with fluorescein isothiocyanate, were used. The indirect staining was performed with unconjugated rabbit anti-vaccinia gamma-globulin and labelled anti-rabbit goat gamma-globulin prepared as described by Serokowa *et al.* (1967). Appropriate controls of the specificity of immunofluorescent staining were included.

In frozen sections from CAM 2 and 4 hours p.i. with $10^{3.5}$ ID_{50} of virus, a small amount of antigenic material was identified in the cytoplasm of single leukocytes. No specific fluorescence was detected at that time in epithelial and connective tissue cells. Eight hours p.i. granules of viral antigens were found in the cytoplasm of single epithelial cells. After 12 and 16 hours the specific fluorescence was more intensive and involved many epithelial cells (Fig. 1). In some preparations finely granular fluorescent material was also observed in the endothelium of small subepithelial capillaries and in intracellular spaces. After 24 hours confluent finely granular fluorescence involved large fragments of the epithelium and groups of connective tissue cells (Fig. 2). The infected epithelial cells showed extensive regressive changes, manifested by cell enlargement and vacuolization of the cytoplasm. After 36 and 48 hours specific fluorescence was still more intensive

and after 72 hours both the whole epithelium and the majority of connective tissue cells were involved (Fig. 3). Irrespective of the degree of virus multiplication in connective tissue cells, the antigenic material was found in the cytoplasm of many leukocytes in the inflammatory exudate.

Specific fluorescence was observed in imprints from the CAM mainly in epithelial cells and single leukocytes (Fig. 4). The antigenic material was also localized extracellularly in the form of finely granular clusters (depending on the degree of virus multiplication). On the first day p.i. intracellular localization was predominant, while on the 2nd day and later the amount of extracellular antigenic material increased (Fig. 5). After infection with $10^{1.5}$ ID_{50} of virus, localization of the antigens in both imprints and sections was similar to that described above, but specific fluorescence was much less intensive, especially on the 1st day p.i.

In imprints from skin lesions after vaccination, the specific granular fluorescence was found in the cytoplasm of leukocytes and also extracellularly (Fig. 6).

After 2 hours' fixation in 4% buffered formalin pH 7.4, the infectivity of CAM was abolished, but the immunofluorescence pattern was similar to that described above. Prolonged fixation of CAM with formalin resulted in a marked decrease in the intensity of specific fluorescence, but the viral antigens were detected even after 24 hours' fixation.

The dynamics of virus multiplication in CAM. Two and 4 hours p.i. virus was detected only in undiluted membrane suspensions. Virus multiplication began 8 hours p.i.; then the logarithmic phase followed and lasted for 40 hours. Later virus multiplication was only slight, not exceeding 1 log unit. After infection with $10^{1.5}$ ID_{50} of virus, the growth curve was similar but the logarithmic phase began after 16 hours and the maximal infective titre was lower. These results did not differ from data reported by others (Anderson, 1954; Briody and Stannard, 1951).

When comparing immunofluorescence patterns of vaccinia virus multiplication in CAM described above with data reported by other authors (Gurvich and Roihel, 1965; Noyes and Watson, 1955; Sokolov and Parfanovich, 1964) who studied this process in tissue cultures, there is a marked similarity concerning both the time of appearance and the localization of viral antigens in the infected cells. The process of vaccinia virus multiplication occurs in the cytoplasm and the localization of the antigens is essentially cytoplasmic; nuclear localization remains disputable. Specific fluorescence in the cell nucleus was observed by many authors in tissue cultures in various periods after infection with variola and vaccinia viruses: in the log phase (Gurvich and Roihel, 1965), parallelly with the appearance of antigens in the cytoplasm (Sokolov and Parfanovich, 1964), and in the period of accumulation of viral antigens in the cell (Noyes and Watson, 1955). Nuclear localization was not detected in the present study. This is in accordance with findings reported by Loh and Rigss (1961). Kato *et al.* (1959) found only cytoplasmic localization of viral antigens in CAM sections on the second day p.i. with viruses of the variola-vaccinia group.

From the practical point of view the improved FA technique for rapid

diagnosis of variola in the material taken directly from skin lesions (Kirsh and Kissling, 1963; Murry, 1963) and in tissue cultures (Avakyan *et al.*, 1961; Kirillova *et al.*, 1961) is applicable also to CAM.

Infectivity of the tissue material for FA testing is eliminated by preliminary fixation which does not destroy virus antigenic properties. Buffered 4% formalin (pH 7.4) appeared to be the fixative of choice. A comparison of infectivity determinations and the results obtained by FA technique shows that the loss of infectivity occurs much earlier than the loss of antigenic activity.

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Explanation of Photomicrographs:

Fig. 1. Vaccinia virus antigens in the cytoplasm of CAM epithelial cells 12 hours p.i. × 300.
a — Single granules of antigenic material in the cytoplasm of CAM epithelial cell. × 500.
b — Single cells of CAM epithelium packed with large amounts of viral antigenic material. × 500.

Fig. 2. Intensive granular and globular fluorescence of viral antigenic material localized in all layers of CAM epithelium and many connective tissue cells. × 400.

Fig. 3. Vaccinia virus antigenic material in the cytoplasm of CAM cells, 3 days p.i. × 500.
a — Antigenic material in epithelial cells; *b* — globular fluorescence of viral antigenic material

in connective tissue cells; *c* — confluent and globular fluorescence of viral antigenic material in leukocytes of an inflammatory infiltration in CAM connective tissue.

Fig. 4. Confluent and globular fluorescence of viral antigenic material in the cytoplasm of many leukocytes and single epithelial cells of CAM 36 hours p.i. × 160.

Fig. 5. Intra- and extracellular localization of vaccinia virus antigens 72 hours p.i. in CAM. × 500.

Fig. 6. Localization of viral antigenic material in the cytoplasm of many leukocytes from a skin lesion after vaccination. × 800.

Figs 1—3 frozen sections, Figs 4—6 imprints; all stained by the direct FA method.