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FLUORESCENT ANTIBODY TECHNIQUE IN CULTURED CELLS INFECTED WITH PORCINE ENTEROVIRUSES*

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The indirect method of the fluorescent antibody (FA) technique was applied to observe primary porcine kidney cultured cells infected with porcine enteroviruses (V 76 and Kr 69TC strains).

Halo-like specific fluorescence was present in one part of the cytoplasm adjoined to the nuclei of the infected cells and rather large fluorescent granules were also scattered there 5 hours after inoculation (HAI), the first observation time. In the following time, the fluorescent granules tended to become smaller and to aggregate densely in the cytoplasm surrounding the nuclei of the cells. Twenty to 25 HAI, a cytopathic effect (CPE) consisting of shrinking and rounding of the fluorescent cells became obvious. The cellular and nuclear membranes became indistinct and dust-like fluorescent granules filled the cytoplasm and the nuclei. The most suitable observation time in the diagnostic tests was confirmed to be from 15 to 20 HAI.

INTRODUCTION

LARSKI (1955), MAYR & SCHWÖBEL (1956) first used cell cultures to study the Teschen disease virus. Then the Teschen disease virus and other porcine enteroviruses were isolated from fecal and organ suspensions in many countries^{1,2,6,8,10,12,15,16,18,20,22}.

A considerable amount of research was done^{11,12,17,21} on the best time to reach the maximum infective titer in the cultured cells infected with porcine enteroviruses. From these results, it seems that a period of 12~78 hours must elapse to reach the maximum after inoculation. The stained cultured cells infected with porcine polioencephalomyelitis virus (T 80 strain) were observed by JENNINGS & KELLY (1960) who described a halo-formation around the nuclei and a condensation of the chromatin material next to the nuclear margin 8 HAI and the granulation and the acidophilic masses in the cytoplasm 16 HAI.

The FA technique was first used to study viral antigens in tissues by COONS

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et al. (1942). BUCKLEY (1957) observed the first fluorescence in the cytoplasm of monkey kidney cultured cells infected with poliomyelitis virus three HAI. The first appearance of the antigen of ECBO virus in cultured cells was observed by CHRISTOV et al. (1967) who described the halo-like fluorescence in the cytoplasm around nuclei 5 HAI. Rapid identification of human enterovirus isolates by indirect FA technique was described by NEUBERT & ROTHE (1969).

In the present study, the porcine kidney cultured cells infected with porcine enteroviruses were first observed by indirect FA technique time after time.

MATERIALS AND METHODS

Viruses

V 76 strain which belongs to the Konratice strain virus of the Teschen disease virus group, was isolated in Germany and supplied to the author by the Czechoslovakian Collection Center of Animal Pathogenic Microorganisms. It has been passaged 783 times in porcine kidney cell culture and the titer was $10^{9.7}$ tissue culture infectious dose (TCID)₅₀/0.1 ml. Kr 69TC strain which belongs to a porcine enterovirus group, was isolated from the lung of an apparently healthy pig in Moravia, Czechoslovakia and supplied to the author by Dr. D. VEŽŇKOVÁ of the Veterinary Research Institute. It has been passaged 35 times in porcine kidney cell culture and the titer was $10^{9.5}$ TCID₅₀/0.1 ml.

Antiserum production

Adult rabbits were inoculated subcutaneously with 5, 5, 5, 5 and 10 ml of the infectious cell culture fluid on the first, the third, the 5th, the 8th and the 38th days, respectively. On the 45th day, the rabbits were bled from the hearts. The defibrinated blood of the rabbits was centrifuged at 3,000 rpm for 20 minutes, then the supernatant serum was inactivated at 56°C for 30 minutes and stored at -20°C until used.

Cell culture

Primary monolayer cultures were prepared from the kidneys of the pigs younger than 4-weeks-old. Trypsin treatment was tried according to DULBECCO & VOGT (1954).

The cell suspension contained $8\sim 10\times 10^5$ cells/ml of growth medium. The growth medium consisted of Hank's balanced salt solution (BSS) with 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 20% inactivated calf serum, 200 U/ml of penicillin and 200 µg/ml of streptomycin. The medium was changed for maintenance medium 4 to 20 hours before inoculation with the viruses. The maintenance medium consisted of Earle's BSS with 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 200 U/ml of penicillin and 200 µg/ml of streptomycin.

Virus inoculation

The tubes containing confluent monolayers on pieces of slide glass were inoculated with 0.2 ml \times approximately 10^5 TCID₅₀/0.1 ml of the virus fluid. The inoculated tubes were incubated for absorption of the viruses onto cells at 37°C for one hour. Two ml of maintenance medium were then added into each tube and the tubes were further incubated.

The maintenance medium was also added to control tubes.

Fluorescent antibody technique

The slide glasses covered by the monolayer cells infected with V 76 or Kr 69TC strain were collected at 5-hour intervals from 5 to 25 HAI. They were washed in phosphate-buffered saline (PBS) solution (pH 7.1), dried at room temperature and fixed in acetone at room temperature for 5 to 10 minutes. The fixed cells, in Petri dishes, were stored at 4°C until stained.

The indirect method of the FA technique was used to stain the cells. At first, the cell layers were overlaid with unlabeled rabbit antisera which were diluted to 20 times in a physiological saline solution and absorbed once with porcine liver powder, in a moist chamber at room temperature for 30 minutes. They were washed three times with PBS solution for 5 minutes every time. After drying, the layers were secondarily overlaid with labeled anti-rabbit porcine globulin prepared in the Institute of Sera and Vaccines, Czechoslovakia, at room temperature for 30 minutes. They were then washed three times with PBS solution for 5 minutes every time and mounted with 10% PBS solution in glycerin.

The specificity of the fluorescent staining was checked in the noninfected cultured cells and in the infected cultured cells treated with normal rabbit serum and with rabbit antiserum of transmissible gastroenteritis virus for the first staining.

The samples prepared stated above were examined under a fluorescent microscope, type ML-2 made in USSR, equipped with a darkfield condenser.

RESULTS

Five HAI, halo-like fluorescence was observed in one part of the cytoplasm adjoined to the nuclei of the infected cultured cells (fig. 1). Diffused fluorescent granules were also present in the cytoplasm of some scattered cells. The size of the granules was various and rather large granules were often seen (fig. 2). In some cells, foggy fluorescence was observed. No fluorescence was localized in the nuclei at this time. In the 10-hour groups, findings were more varied. Fluorescent granules distributed more diffusely throughout the cytoplasm and dense fluorescence tended to be one-sided in the cytoplasm close to the nuclei (fig. 3). Feebly fluorescent granules were present in the nuclei of the cells showing dense fluorescent granules in the cytoplasm. Some of the cells were rounded. A nuclear fission was seen in one cell which showed one-sided fluorescent granules in the cytoplasm, infected with Kr 69TC strain.

Fifteen HAI, fluorescent granules tended to become rather small and distributed diffusely in the cytoplasm. Some fluorescent cells were shrunken and showed stippled fluorescent granules in the prolonged cytoplasm. Twenty HAI, CPE became obvious and typical. The nuclei of prolonged cells became ill-defined and the small fluorescent granules, dust-like fluorescence, showing rather feeble brilliance were present in the cells. Some foci of aggregated fluorescent cells appeared in the field of the monolayer cells. Some fluorescent granules were present in the nuclei also. Twenty-five HAI, the borders of both fluorescent cell cytoplasm and the nuclei tended to become more indistinct.

Fluorescent cells were obviously characterized by rounding and shrinking (fig. 4). Meanwhile, the noninfected controlled cultured cells which showed typical CPE by unidentified causes showed nonspecific fluorescence.

No obvious difference was observed in the fluorescent cells inoculated with V 76 and Kr 69TC strains during the experimental period.

DISCUSSION

The appearance of fluorescence was almost the same for both, in the cells infected with V 76 strain and in them infected with Kr 69TC strain. The first appearance, halo-like fluorescence 5 HAI, might be the same finding as the halo-like fluorescence found on ECBO virus reported by CHRISTOV et al. (1967).

Different strains of a porcine enterovirus or the same strain of the virus, but with different passage numbers in cell culture, were shown to have different rates of multiplication in porcine kidney cell culture (HANCOCK et al. 1959). But the first release of the multiplied viruses was recognized at a similar time after inoculation, 5 to 10 hours, as those of several reports^{11,12,21}. In the present experiment, many fluorescent granules were already seen in the cytoplasm of some scattered cells 5 HAI at the first observation time. It was sure that the viral antigens existed before 5 HAI also. The present results were similar to those reported by BUCKLEY (1957) who recognized the first viral antigens of poliomyelitis virus in monkey kidney cultured cells three HAI.

When fluorescent granules increased greatly after 10 HAI, dense fluorescence tended to locate in the periphery of the nuclei in the present experiment. Meanwhile, eosinophilic masses were first observed in the cytoplasm close to the nuclei of the cultured cells infected with porcine polioencephalomyelitis virus from 4 to 8 HAI by KASZA (1965). Granulation of eosinophilic masses in the cytoplasm increased and the masses became obvious 16 HAI (JENNINGS & KELLY, 1960). In the early stage of infection in the present experiment, fluorescent granules in the cytoplasm were rather large, then the granules became smaller. In proportion to the degeneration of the infected cells, fluorescent granules tended to be distributed diffusely throughout the cytoplasm and were seen in the nuclei also. Fluorescence was seen in both the cytoplasm and the nuclei of the cells showing typical CPE. Specific and nonspecific fluorescence might be mixed in the cells, because the noninfected cells which showed typical CPE by unidentified causes showed nonspecific fluorescence. So it was confirmed that the most suitable time to try the diagnostic tests was from 15 to 20 HAI. At that time, specific fluorescence was more apparent and nonspecific fluorescence was not.

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EXPLANATION OF PLATE

- Fig. 1 Halo-like fluorescence (HF) is seen in the cytoplasm adjoined to the nucleus (N) and some fluorescent granules in the under right part.
Five hours after V 76 strain inoculation × 540
- Fig. 2 Clear fluorescent granules are seen in the cytoplasm. Two to 6 fluorescent granules gather there.
Five hours after V 76 strain inoculation × 540
- Fig. 3 Fluorescent granules are dense in two parts of the cytoplasm adjoined to the nucleus (N).
Ten hours after V 76 strain inoculation × 540
- Fig. 4 Fluorescent granules become smaller and some are seen in the nuclei also. The gathered fluorescent cells are degenerated and shrunken.
Twenty-five hours after V 76 strain inoculation × 540

