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FOCUS ASSAY FOR VARICELLA-ZOSTER VIRUS IN HUMAN EMBRYO CELLS STAINED WITH IMMUNOPEROXIDASE METHOD

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Summary. — Rapid titration of varicella-zoster virus (VZV) in human embryonic fibroblasts (HEF) based on staining of virus-infected cells by indirect immunoperoxidase technique (IPA) is described. Cell monolayers were grown in wells of plastic plates (two different diameters). Foci of virus-infected cells as revealed by IPA could be counted either 48 hr post-infection, if cell-associated virus (VZV infected cells) was used as inoculum, or 72 hr p. i. if cell-free virus was used. A linear relationship was observed between virus dilution and number of foci. The first virus was detected 12 hr p. i., the highest titre at 36 hr, when cytopathic effect (CPE) involved about 50% of the monolayer.

Key words: *varicella-zoster virus*; *focus assay*; *immunoperoxidase staining*

Introduction

During cultivation of varicella-zoster virus (VZV) which tends to remain cell-associated, little if any infectious virus is released into culture medium (Shiraki and Takahashi, 1982). Cell-free virus can be prepared by ultrasonic disruption of infected cells as first described about twenty years ago (Caunt, 1963; Gold, 1965; Brunell, 1967). Quantification studies of the viral material prepared by this way are commonly preformed by plaque assay under solid or semisolid overlay. Development of VZV quantification was influenced by the cell-associated nature of the virus. Determination of the number of VZV-infected cells by usual plaque assay was introduced by Rapp and Benyesh-Melnick (1963). Later on, the method has been used for establishment of infectivity of the virus liberated from infected cells by sonic treatment (cell-free virus). The titration results can be obtained in such case after incubation period of 6—7 days (Brunell, 1967; Caunt, 1969).

In this report we describe a more rapid titration method, in which foci of infected cells are stained by indirect immunoperoxidase method (IPA). By the described method the results of titration are known on the third day. The infectivity is expressed in focus-forming units (FFU). The optimal conditions of the method have been determined

Materials and Methods

Tissue cultures. Primary cultures of human embryonic fibroblast cells (HEF) between 5th and 20th passage were used. HEF cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% inactivated calf serum (ICS) and antibiotics. For the IPA staining cells were grown in 24-well tissue culture plates (TCP) and 96-well TCP (A/S Nunc, Denmark) for two days.

Virus. The VZV strain used was isolated from vesicular fluid of a 14-year-old boy suffering from chickenpox. The virus was serially passaged in HEF roller cultures in Leibovitz L-15 medium supplemented with glutamine (Flow Laboratories, U.K.) and 2% ICS.

Cell-associated virus (infected cells) was prepared by infection of HEF flask cultures with VZV-infected cells at a ratio of one infected to five uninfected cells. After 48 hr incubation, the infected monolayer was harvested by trypsinization and resuspended in small amount of MEM containing 2% ICS (1 ml MEM per one 150 cm^2 tissue culture flask).

Preparation of cell-free virus. Cell-free virus was prepared from VZV-infected cells as follows: when about 70% of HEF cells showed CPE, the medium was aspirated, the cells have been removed from the flask surface with glass beads into a small amount of fresh medium. The cells were pelleted by centrifugation at 1000 rev/min for 20 min and suspended in Hank's balanced salt solution and then subjected to sonic treatment at 60 cs/sec for 60 sec. Residues of the disrupted cells were removed by centrifugation at 2000 rev/min for 15 min at 4 °C and the supernatant (cell-free virus) has been used in further experiments.

Focus assay. Adequate volumes of the appropriately diluted virus material (200 µl per well on 24-well TCP and 30 µl per well on 96-well TCP) were added to confluent monolayers. After adsorption, the viral inocula have been aspirated and MEM containing 2% ICS has been added to the monolayer. Pilot experiments showed in accordance with data of Asano and Takahashi (1978), as well as Walz-Cicconi and Weller (1984) that semisolid overlay was not superior to the fluid medium. After the appropriate incubation period in a 5% CO_2 atmosphere the medium has been removed, the cells have been washed with phosphate-buffered saline (PBS) pH 7.2 and fixed with 0.07% glutaraldehyde in PBS at 4 °C for 1 min. After fixation, the cells have been washed two times with 0.05% Tween 20 in PBS and pooled human anti-VZV serum diluted 1 : 100 (diluent solution: 0.05% Tween 20 in PBS containing 1% bovine serum albumine and 0.02% sodium azide) has been allowed to react with the antigen at 37 °C for 30 min. The pool serum constituted a mixture of sera obtained from persons suffering from chickenpox which previously had been shown highly anti-VZV positive in ELISA. After washing with 0.05% Tween, peroxidase-conjugated antibody to human IgG (SEVAC, Praha) diluted 1 : 500 was added for 30 min (incubated at 37 °C). After further washing with 0.05% Tween and with 0.05 mol/l Tris-HCl (pH 7.6), the peroxidase reaction was accomplished according to the method of Graham and Karnovsky (1966), using 0.3 mg/ml 3-3'-diaminobenzidine tetrahydrochloride (Serva, Heidelberg) in 0.05 mol/l Tris-HCl pH 7.6 containing 0.01% H_2O_2. Then the monolayers were washed with PBS followed by washing with distilled water and drying at room temperature. The number of foci was counted under ×12.5 magnification and the virus infectivity has been expressed as FFU/ml.

Results

The optimal incubation and adsorption periods

Confluent HEF monolayers have been infected with appropriately diluted cell-free virus or with cell-associated virus. At different intervals after infection the cells were fixed and stained with the IPA. The optimal incubation period was different for cell-free virus and for cell-associated virus (Fig. 1). In the case of cell-free virus, optimal incubation period was shown to be 72 hr, while for the titration of infected cells (cell-associated virus) it was 48 hr. At later intervals the numbers of foci did not increase, but they became larger and confluent.

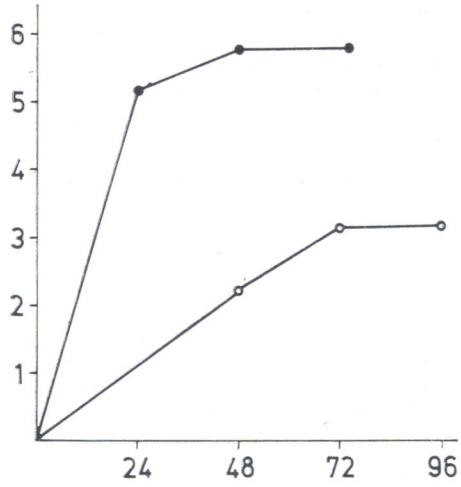


Fig. 1.
Optimal incubation period for the focus assay
HEF monolayers were infected with cell-free virus and/or infected cells (cell-associated virus); every 24 hr cells have been fixed and stained by IPA. Titres of cell-free virus (○——○) and infected cells (●——●) were plotted at given intervals. Abscissa: hr p.i.; ordinate: log FFU/ml.

After addition of cell-free virus the cells were incubated at 37 °C for various adsorption periods; then they were fixed and stained by the IPA (Table 1). The length of adsorption period influenced the levels of the VZV infectivity. A 90 min adsorption was shown optimal. All further experiments have been conducted under these conditions.

Quantitative assay

For quantitative evaluation of the assay, the relationship between virus dilutions and numbers of foci per well was engaged. Twofold dilutions of cell-free virus have been inoculated into HEF monolayers. The plates were fixed and stained after incubation period of 72 hr (Fig. 2). A linear relationship between virus dilutions and numbers of foci was observed in 24-well as well as in 96-well plates. These results wittness about the reliability of the method described (Fig. 3).

Growth kinetics of VZ virus

HEF monolayers prepared in 150 cm^2 tissue culture flasks have been inoculated with VZV-infected cells passaged in roller tubes. When about 70% CPE has developed, the cells were harvested by trypsinization and used immediately as inocula to infect other flask monolayers at a ratio of one inoculum cell to five uninfected cells. The inoculum volume of 5 ml MEM covered the cell monolayer during adsorption for 20 min at room temperature. Afterwards, MEM containing 2% ICS was added. At 12 hr intervals after infection duplicate sets of infected monolayers were used to prepare cell-free virus samples. Freshly prepared cell-free virus samples have been assayed on 24-well TCP. The HEF flask cultures infected by the same procedure were used for the study of the VZV infectivity expressed

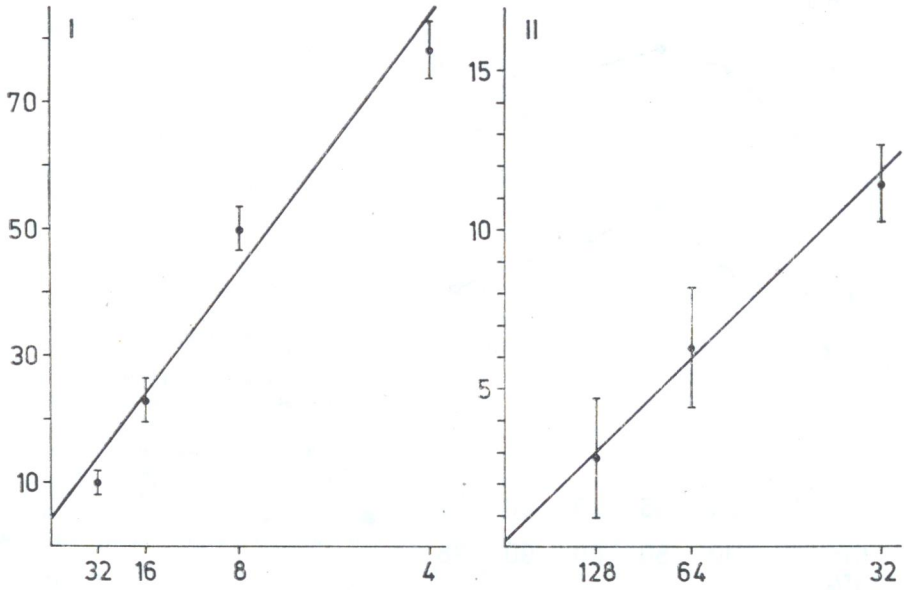
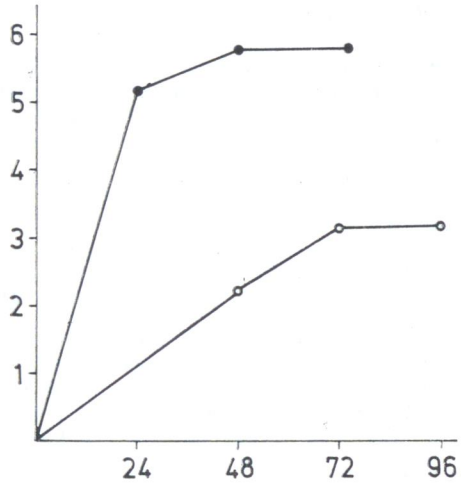


Fig. 2.
Relationship between VZV dilutions and numbers of foci
I — 24-well TCP; II — 96-well TCP
Numbers of foci were calculated from two parallel, independent experiments with quadriduplicate wells for each dilution (mean ± standard deviation)
Abscissae: virus dilution reciprocals; ordinates: no. of foci per well (16 mm or 8 mm in diameter, respectively).

Table 1. Adsorption time and VZV titre obtained in human embryonic cell monolayers grown in 24-well and 96-well tissue culture plates

Adsorption time (min)	VZV titre	
	24-well TCP	96-well TCP
30	$0.67 \times 10^4 \pm 0.65$	$0.75 \times 10^3 \pm 0.95$
60	$1.40 \times 10^4 \pm 0.58$	$2.66 \times 10^3 \pm 0.44$
90	$2.60 \times 10^4 \pm 0.22$	$4.70 \times 10^3 \pm 0.78$
120	$2.65 \times 10^4 \pm 0.27$	$4.87 \times 10^3 \pm 0.64$

Mean FFU/ml (± standard deviation) from two independent, parallel experiments in which quadriduplicate monolayer cultures were used for each virus dilution. Cell-free virus for the 24-well TCP assay and for the 96-well TCP assay was prepared separately.
The difference in titre between 60 min and 90 min adsorption time was $P > 0.005$ in both types of TCP.



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advantage is the convenience of handling plastic microplates as compared with HEF monolayers in tissue culture flasks (Gerna and Chambers, 1976).

Good reproducibility of VZV infectious titre determinations (whether cell-associated or cell-free virus) makes this modification more suitable and versatile to perform large number of estimations, the other known methods being more laborous and exacting. It is interesting that despite of several studies employing the IPA method for the study of antibody response to the infection by VZV (Gerna *et al.*, 1977; Haikin *et al.*, 1979), the IPA method has not been introduced for VZV infectivity measurements.

We employed HEF cells grown in 24-well TCP and in standard 96-well TCP. Both systems were shown convenient for this purpose. In 96-well TCP less cells and reagents are required, but not more than 15—20 foci per well can be reliably distinguished. Taking into account the volume of the inoculum, more accurate assays can be achieved in 24-well TCP, where it is possible to discriminate between 80-100 FFU per well. This was the reason for using 24-well TCP to determine VZV growth kinetics. The results were in agreement with the observations of Grose and Brunell (1978), who investigated the propagation of VZV at 36 °C in human melanoma cells. Follow-up of infectious VZV accumulation in HEF cells infected with different virus materials, points out at the importance of an appropriate harvesting time for obtaining of cell-free VZV of standard infectivity, needed e. g. for immunological studies.

In our experiments, the incubation period was clearly different when cell-free virus or cell-associated virus was used as inoculum. This finding is in agreement with the previous studies suggesting a more rapid spread of the virus in cell cultures, when cell-associated virus (infected cells) was used as inoculum (Takahashi, 1983). In conclusion, the advantage of the IPA technique is not only in the faster and easier obtaining of the results but also in the fact, that plates with stained foci can be kept without changes for a long time. This may be of special importance when the biologic variants of VZV with different growth potential are studied. The IPA method in conjunction with microassay using the HEF cultures seems also suitable for rapid detection of VZV in isolation experiments from clinical materials (non-published data), as it was described in the case of cytomegalovirus (Swenson and Kaplan, 1985).

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Explanation to Figure (Plate LVI):

Fig. 3. VZV foci stained with IPA in a 24-well TCP. Twofold dilutions of cell-free virus were inoculated into HEF monolayers and after 72 hr incubation the foci have been stained by IPA. II — Detail of the part I.