VIROLOGY

Rapid Method for demonstrating Intracellular Pleuropneumonia-like Organisms in a Strain of Hamster Kidney Cells (*BHK* 21 *C*13)

THERE is as yet no ideal method for demonstrating pleuropneumonia-like organisms (PPLO) in tissue culture. Isolation of an infecting strain, particularly if this is intracellular, is frequently technically difficult and is always time-consuming. Cytological examination of monolayer cultures for evidence of PPLO infection is rapid, but staining artefacts may give rise to difficulty in interpretation. Indirect evidence of infection by transmission to susceptible 'indicator cultures'[1] is only valid if these can be maintained free from accidental contamination by PPLO.

We became acutely aware of these problems when PPLO were isolated from a strain of hamster kidney cells (*BHK* 21 *C*13)[2] in use in this laboratory. Two factors led us to believe that infection was intracellular. PPLO could rarely be isolated from the supernatant medium from cell cultures grown in the absence of antibiotics, but could readily be isolated when the cells were mechanically disrupted in a Griffiths tube and the cell debris plated on a suitable medium (Difco PPLO agar with 20 per cent inactivated horse serum and 5 per cent fresh yeast extract) which was then incubated anaerobically at 37° C for 5 days. In addition, PPLO could still be isolated from disrupted cells even after continuous cell culture for 3 weeks in medium containing kanamycin sulphate ('Kannasyn') in a concentration of 500 μg/ml., although the organism, when tested *in vitro*, was sensitive to a dose of 25 μg/ml.

Since PPLO are known to contain deoxyribonucleic acid (DNA), a modification of the fluorescent Feulgen staining technique[3] was developed which demonstrated the presence of abnormal foci of extranuclear DNA in infected cells. Subsequent investigations showed that these contained PPLO antigens.

Monolayer cultures of *BHK* 21 *C*13 cells infected with PPLO were grown in coverslips until a semi-confluent monolayer was obtained. Cultures of *BHK* 21 *C*13 cells from which PPLO had never been isolated served as a control. These preparations were washed twice in phosphate buffered saline (*p*H 7·2) and fixed for 10 min in formaldehyde saline (4 per cent) at room temperature. Preparations were then processed as follows. (Unless specifically stated otherwise, all procedures were carried out at room temperature.)

(1) Washed twice in distilled water (5 min each). (2) Immersed in 1·0 N hydrochloric acid at 60° C for 10 min to hydrolyse cell ribonucleic acid (RNA). (3) Hydrolysis stopped by plunging preparation into ice-cold water. (4) Washed twice in distilled water (5 min each). (5) Stained for 20 min in acriflavine–Schiff solution (acriflavine hydrochloride, 250 mg; potassium metabisulphite, 500 mg; 0·1 N hydrochloric acid, 50 ml.). (6) Washed twice in distilled water (5 min each). (7) Brought to 70 per cent ethyl alcohol by successive immersion in 20, 40 and 70 per cent ethanol. (8) Rinsed twice for 10 min in acid alcohol (0·1 N hydrochloric acid, 30 ml.; absolute ethanol, 70 ml.). (9) Rehydrated by immersion successively in 40 per cent and 20 per cent ethanol; mounted in buffered glycerol (glycerol, 90 ml.; phosphate buffered saline, *p*H 7·2, 10 ml.) and sealed with paraffin wax.

The preparations were then examined microscopically in short wave-length light (approximately 400 mμ: light source '*HBO* 200' mercury vapour lamp: Schott exciter filters *BG* 38/2·5, *BG* 12/4, Schott barrier filters 53/44).

In infected and in control preparations, cell nuclei fluoresced orange against a background of very pale yellow cytoplasmic fluorescence. Orange fluorescent foci were observed in the perinuclear region of 15–80 per cent of cells in infected preparations. The number of foci varied from one to one hundred per cell. The majority of cells had more than ten foci. In control preparations of uninfected cells, fewer than 3 per cent showed fluorescent foci. No cell had more than one focus. In both infected and control cells foci were still seen after treatment with ribonuclease (recrystallized five times; 100 μg for 8 h) but were not seen in cells which had been treated with deoxyribonuclease (recrystallized once; 100 μg/ml. for 8 h).

A comparison was made between cultural methods and fluorescent Feulgen staining as a means of detecting PPLO in cultures of *BHK* 21 *C*13 cells. Culture and cytological examination were carried out independently. The results are shown in Table 1.

Table 1. COMPARISON BETWEEN CULTURE AND FLUORESCENT FEULGEN STAINING AS A MEANS OF DETECTING PLEUROPNEUMONIA-LIKE ORGANISMS (PPLO) IN STRAINS OF HAMSTER KIDNEY CELLS (*BHK* 21 *C*13)

No. of specimens from which PPLO were isolated	Percentage of cells showing fluorescent foci	No. of specimens from which PPLO were not isolated	Percentage of cells showing fluorescent foci
22	15–84 (average, 30)	10	0·5–1·9 (average 1·0)

The agreement between the results of culture and of fluorescent Feulgen staining is good, and it is suggested that fluorescent Feulgen staining is a reliable, quick method for detecting intracellular PPLO.

The likelihood that the majority of fluorescent Feulgen-positive foci in infected cells might represent sites of PPLO multiplication was increased when it was found that they fluoresced specifically when infected cell preparations were exposed to rabbit prepared antiserum against *Mycoplasma hominis* followed by sheep anti-rabbit serum conjugated with fluorescein isothiocyanate. Control uninfected preparations when examined by this technique showed no specific fluorescence. It was concluded that the fluorescent Feulgen-positive foci observed in a small preparation of these cells are probably micronuclei.

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AGRICULTURE

Absence of Bitterness in Navel Oranges from Rooted Cuttings

MOST Washington Navel oranges yield a juice which eventually becomes unacceptably bitter. The bitterness, which develops slowly on standing or rapidly on heating, is due to limonoid compounds, principally limonin, present in the fragments of albedo and segment walls incorporated into the juice during extraction. As the fruit ripens, the bitterness becomes less evident, and the rate at which bitterness decreases during ripening is affected markedly by the rootstock on which the tree is grown. Thus, Marsh[1] in California and Kefford and Chandler[2] in Australia showed that some rootstocks (for example, trifoliate orange) promoted early disappearance of bitterness, while others (for example, rough lemon) encouraged high levels of bitterness at and beyond normal harvesting maturity. In the latter work, organoleptic tests were supported by the yields of crude limonin extracted from the peel of the oranges used in preparation of the juice.

When an unidentified Navel cutting was used as a rootstock for a Washington Navel scion the fruit showed