Protoplasma 104, 75—80 (1980)

PROTOPLASMA

Animal-Specific Membrane Components Visualised on the Surface of Animal/Plant Heterokaryons

M. WARD [1], M. R. DAVEY [1], E. C. COCKING * [1], M. BALLS [2], R. H. CLOTHIER [2], and J. A. LUCY [3]

[1] Department of Botany and [2] Department of Human Morphology, University of Nottingham, and [3] Department of Biochemistry and Chemistry, Royal Free Hospital School of Medicine, University of London, U. K.

Received February 27, 1980
Accepted in revised form June 9, 1980

Summary

Immunofluorescence was used to demonstrate the presence and distribution of animal-specific membrane components within the plasma membranes of *Xenopus*/carrot heterokaryons. The inhibitor of cellulose synthesis, 2,6-dichlorobenzonitrile, was used to impair cell wall regeneration so that the plasma membranes of cultured heterokaryons would remain accessible to antibodies. *Xenopus*-specific surface components were observed in heterokaryons after 14 days of culture.

Keywords: Animal/plant heterokaryons; Carrot protoplasts; 2,6-dichlorobenzonitrile; Fusion; Immunofluorescence; *Xenopus* cells.

Animal/plant heterokaryons can be produced by fusing *Xenopus* cells with carrot protoplasts. These heterokaryons, like protoplasts, are able to regenerate cell walls under appropriate culture conditions (WARD *et al.* 1979). Intermixing of the animal and plant plasma membranes can be visualized by immunofluorescence shortly after the fusion process. However, this technique cannot be used to study the heterokaryon membrane over extended periods of culture as the cell walls synthesized by the heterokaryons are impervious to antibodies. At appropriate concentrations the inhibitor of cellulose formation, 2,6-dichlorobenzonitrile (DB), can prevent cell wall synthesis by protoplasts, thus averting cytokinesis whilst allowing nuclear division; multinucleate protoplasts are produced as a consequence (MEYER and HERTH 1978). We have employed this inhibitor so that the plasma membranes of cultured

* Correspondence and Reprints: Department of Botany, School of Biological Sciences, The University of Nottingham, University Park, Nottingham NG7 2RD, U.K.

0033-183X/80/0104/0075/$ 01.20

protoplasts and animal/plant heterokaryons would remain accessible to antibodies.

The intermixing of cell surface antigens following the fusion of two different mammalian cell types was studied by FRYE and EDIDIN (1970) using immunofluorescence. They concluded that diffusion was the most likely mechanism involved in the redistribution of molecules within the plasma membrane, and their observations have been used as a powerful argument in favour of the fluid mosaic model for the organization of biological membranes (SINGER and NICOLSON 1972). It was of interest to study whether animal and plant membrane components were able to intermix and, if so, how long the animal-specific antigens persisted on the surfaces of heterokaryons cultured under conditions suitable for maintenance of the plant protoplast partner. HARRIS *et al.* (1969) used the technique of haemadsorption to demonstrate hen-specific surface antigens on heterokaryons produced by the fusion of hen erythrocytes with mammalian cells. The loss of these membrane components during subsequent culture demonstrated the turnover occurring within the plasma membrane.

Protoplasts obtained from carrot (*Daucus carota*) cell suspension cultures and cultured cells of a line derived from the South African clawed toad (*Xenopus laevis laevis*) were fused by mixing in a glycine-NaOH buffer at pH 10.4 with 50 mM $CaCl_2$ for 15 minutes at 30 °C following a protease pretreatment (WARD *et al.* 1979). The products of fusion were washed in a salt solution containing 0.5 M mannitol (DAVEY *et al.* 1978) and resuspended in the same salt solution with 0.44 M sucrose replacing the mannitol. On centrifugation (90 × g, 5 minutes) the majority of protoplasts and heterokaryons rose to form a band at the surface, while the *Xenopus* cells formed a pellet at the bottom of the tube. The protoplasts and heterokaryons were removed and cultured in Medium 8 p (KAO and MICHAYLUK 1975) with 2 mg/l DB and with glucose at 100 g/l instead of 68.4 g/l. *Xenopus* cells are unable to survive in this medium. Approximately 10^6 carrot protoplasts and *Xenopus*/carrot heterokaryons were removed from the culture medium by centrifugation (90 × g, 5 minutes) and incubated on ice for 20 minutes with 4 drops of rabbit anti-*Xenopus* cell antiserum which had been diluted 1 : 5 v/v with salt solution containing 0.5 M mannitol. The cells were then washed four times by centrifugation and resuspension in the same salt solution, then incubated for 20 minutes with 4 drops of sheep anti-rabbit immunoglobulin antibodies conjugated with fluorescein isothiocyanate (Wellcome Reagents Ltd.) diluted 1 : 8 v/v with salt solution containing 0.5 M mannitol. After a further four washes, the cells were viewed with a Vickers M 41 Photoplan microscope with an incident light fluorescence unit. This method was specific for *Xenopus* cell surface components and no cross-reaction was observed with intact carrot protoplasts. An immunoprecipitate was produced by double immunodiffusion (OUCHTERLONY 1967), in agarose, of the rabbit anti-*Xenopus*

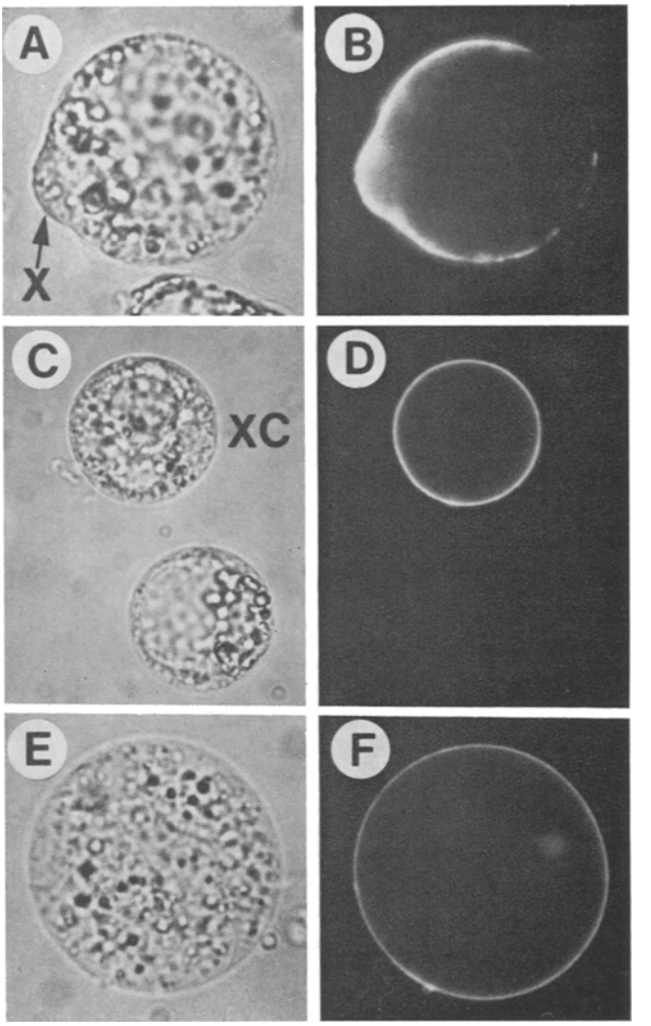

Fig. 1. Surface labelling of *Xenopus*/carrot heterokaryons by immunoflorescence. *A* Heterokaryon 0.5 hours after fusion; bright-field illumination. The *Xenopus* region appears as a small protrusion (*X*). ×1,000. *B* Same field as *A* but with ultra-violet illumination, showing polar pattern of fluorescence centred over the *Xenopus* region. ×1,000. *C* Heterokaryon (*XC*) 3.5 hours after fusion and an unfused carrot protoplast; bright-field illumination. ×625. *D* Same field as *C* but with ultra-violet illumination. The heterokaryon shows a complete ring pattern of fluorescence. ×625. *E* Heterokaryon cultured for 7 days in the presence of 2,6-dichlorobenzonitrile. It has remained spherical and has not undergone the change in shape normally associated with wall regeneration. Bright-field illumination. ×1,000. *F* Same field as in *E* but with ultra-violet illumination. The surface fluorescence indicates the continued presence of *Xenopus*-specific components within the plasma membrane. ×1,000

cell antiserum against *Xenopus* cell homogenates, but not against carrot protoplast homogenates. This confirmed that the immune serum would not react with the protoplasts.

Heterokaryons were of similar appearance to carrot protoplasts under bright-field illumination, but could be distinguished from them by their surface fluorescence when viewed with ultra-violet light. Heterokaryons could not be labelled if either rabbit anti-*Xenopus* cell antiserum absorbed with *Xenopus* cells, or if pre-immune rabbit serum were used. Sheep anti-rabbit immunoglobulin antibodies conjugated with fluorescein isothiocyanate did not stain the heterokaryons if used alone. Samples were taken from the same population of fusion products at various intervals after fusion and incubated on ice until all the samples could be stained simultaneously for immunofluorescence. Of the heterokaryons identified from samples taken 20 minutes after fusion, over 70% (99 of 134) showed a polar pattern of fluorescence centred over the *Xenopus* region (Figs. 1 *A* and *B*), while the remainder had a ring pattern of staining (Figs. 1 *C* and *D*). However, in samples taken 3 hours after fusion, 80% (74 of 93) of the heterokaryons identified showed the complete ring pattern of fluorescence and eventually all the heterokaryons showed this pattern. Incubation on ice after fusion inhibited the spread of *Xenopus*-specific antigens over the surface of the heterokaryons. After examination by immunofluorescence samples were fixed and heterokaryons identified by using carbol fuchsin (KELLER *et al.* 1973) which stains *Xenopus* and carrot nuclei differently. Fusion indices estimated by the two staining methods were in agreement. The number of heterokaryons that could be visualized by immunofluorescence diminished with time in culture, though some heterokaryons were found after culture for 14 days in medium containing DB.

The majority of carrot protoplasts cultured with DB for 14 days remained spherical, formed little or no cell wall detectable using the fluorescence brightener tinopal BOPT (CIBA-Geigy, U.K., Ltd.) (COCKING and EVANS 1975) and did not divide. Acridine orange staining (SCHWARTZ, LARSCH, and BARTELS 1977) revealed that the number of nuclei per protoplast increased during culture. As a further check for viability, protoplasts which had been cultured with the wall synthesis inhibitor for 14 days were washed free of the DB and further cultured in protoplast culture medium, whereupon the majority formed cell walls and many underwent division to produce cell colonies. The concentration of DB used did not prevent the proliferation of *Xenopus* cells cultured in 50% v/v MEM (Auto-Pow Minimum Essential Medium Eagle (modified), Flow Laboratories Ltd.) with 10% fetal calf serum (DAVEY *et al.* 1978).

These results show that animal and plant plasma membrane components are able to mix after heterokaryon formation. The time taken for the spread of *Xenopus*-specific antigens over the whole surface of a heterokaryon would be expected to depend on the relative sizes of the animal and plant com-

ponents and the number of *Xenopus* cells and carrot protoplasts which combined to form the heterokaryon. Some heterokaryons with a polar pattern of fluorescence may appear to have the ring pattern if viewed from above the *Xenopus* pole, but in any event the spread of *Xenopus* components appears to be complete within 3 hours for the majority of heterokaryons. Ultrastructural studies have shown that the underlying cytoplasmic components also intermix after polyethylene glycol-mediated fusion (DAVEY *et al.* 1978), though this takes much longer than the intermixing of membrane components. The formation of an apparently normal cell wall over the surface of heterokaryons (DAVEY *et al.* 1978, WARD *et al.* 1979) indicates that this structure can be laid down over a hybrid animal/plant plasma membrane. The disappearance of fluorescent heterokaryons from culture may be due to their degeneration or to the turnover of their plasma membrane leading to removal of the *Xenopus*-specific components. The development of a method for recognizing heterokaryons by bright-field illumination for use in conjunction with immunofluorescence would facilitate the study of cell membrane turnover. The use of DB to impair wall formation makes possible the examination of the cell membranes of heterokaryons by immunofluorescence after extended periods in culture, which would otherwise require the enzymatic removal of the newly-synthesized cell wall (Figs. 1 *E* and *F*). This latter alternative is unsuitable since heterokaryons and unfused protoplasts may be at different stages of wall formation and it may not be advisable to subject the plasma membrane to the effects of a crude mixture of enzymes prior to investigation by immunofluorescence.

Unlike many animal/animal or plant/plant heterokaryons, the *Xenopus*/carrot heterokaryons studied here have not been observed to undergo nuclear division or to divide. They have been cultured in plant protoplast medium and appear to behave like modified protoplasts. The immunofluorescence technique is currently being used to assess viability and the retention of animal-specific components in attempts to design more suitable media for the long-term maintenance of animal/plant heterokaryons.

Acknowledgements

This work was supported by a grant from the Medical Research Council. The authors thank the Cancer Research Campaign Laboratories (University of Nottingham) for producing the rabbit anti-*Xenopus* antiserum, and Mr. B. V. CASE for photographic assistance.

References

COCKING, E. C., EVANS, P. K., 1975: The techniques of plant cell culture and somatic cell hybridization. In: New techniques in biophysics and cell biology, Vol. 2, pp. 127—158 (PAIN, R. H., SMITH, B. J., eds.). London-New York: Wiley.

DAVEY, M. R., CLOTHIER, R. H., BALLS, M., COCKING, E. C., 1978: An ultrastructural study of the fusion of cultured amphibian cells with higher plant protoplasts. Protoplasma 96, 157—172.

FRYE, L. D., EDIDIN, M., 1970: The rapid intermixing of cell surface antigens after formation of mouse-human heterokaryons. J. Cell Sci. **7**, 319—335.

HARRIS, H., SIDEBOTTOM, E., GRACE, D. M., BRAMWELL, M. E., 1969: The expression of genetic information: a study with hybrid animal cells. J. Cell Sci. **4**, 499—525.

KAO, K. N., MICHAYLUK, M. R., 1975: Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at very low population density in liquid media. Planta **126**, 105—110.

KELLER, W. A., HARVEY, B. L., KAO, K. N., MILLER, R. A., GAMBORG, O. L., 1973: Determination of the frequency of interspecific protoplast fusion by differential staining. Colloq. Int. Cent. Natl. Rech. Sci. **212**, 455—463.

MEYER, Y., HERTH, W., 1978: Chemical inhibition of cell wall formation and cytokinesis, but not of nuclear division, in protoplasts of *Nicotiana tabacum* L. cultivated *in vitro*. Planta **142**, 253—262.

OUCHTERLONY, O., 1967: Immunodiffusion and immunoelectrophoresis. In: Handbook of experimental immunology, pp. 655—706 (WEIR, D. M., ed.). Oxford and Edinburgh: Blackwell Sci. Publ.

SCHWARTZ, D. S., LARSH, H. W., BARTELS, P. A., 1977: Enumerative fluorescent vital staining of live and dead pathogenic yeast cells. Stain Tech. **52**, 203—210.

SINGER, S. J., NICOLSON, G. L., 1972: The fluid mosaic model of the structure of cell membranes. Science, N. Y., **175**, 720—731.

WARD, M., DAVEY, M. R., MATHIAS, R. J., COCKING, E. C., CLOTHIER, R. H., BALLS, M., LUCY, J. A., 1979: Effects of pH, Ca^{2+}, temperature and protease pretreatment on interkingdom fusion. Somat. Cell Genet. **5**, 529—536.