Cytology of rheumatoid synovial cells in culture

IV. Further investigations of cell lines cocultivated with rheumatoid synovial cells

MARY NORVAL, ALEXIA GRAHAM, AND B. P. MARMION

From the Department of Bacteriology, Edinburgh University Medical School, Teviot Place, Edinburgh EH9 9AG

Norval, M., Graham, A., and Marmion, B. P. (1976). *Annals of the Rheumatic Diseases*, 35, 297–305. **Cytology of rheumatoid synovial cells in culture. IV. Further investigations of cell lines cocultivated with rheumatoid synovial cells.** A previous report described a cell isolate presumed to have arisen by accidental cocultivation (contamination) of the Chang 'liver' cell line and rheumatoid synovial cells. This cell isolate had the same glucose-6-phosphate dehydrogenase isoenzyme as the Chang cell and also some shared antigens. It clearly differed in its karyotype, its ability to grow in semisolid agar, and in the possession of bleb-like projections of the cytoplasmic membrane filled with collections of beaded or granular material. In addition, it had a novel antigen(s) not present in the Chang cell.

As these properties might have been acquired from the synovial cells and because the bleb structures resembled those seen in some cell lines transformed by leucovirus the cell isolate has been further studied. Cytochemical methods at the light and electron microscope level showed that the granular material was polysaccharide in nature, probably glycogen. No evidence was found of the presence of a virus or a viral genome using a variety of techniques including attempted induction followed by ³H-uridine labelling of the cultures, and assay of the supernatant fluid from the culture for viral RNA-dependent DNA polymerase. In addition, cell extracts were not found to contain viral RNA-dependent DNA polymerase or RNA-dependent RNA polymerase. No rubella virus or leucovirus interspecies antigens were detected on the cell membranes.

Mackay and others (1974) and Mackay, Marmion, and McCormick (1975) reported the isolation of an epithelial cell line, 710025, which had apparently arisen from a culture of rheumatoid synovial cells and gave reasons for concluding that it was probably the result of a laboratory contamination of the synovial culture with Chang 'liver' cells. However, the cell line from this unintentional cocultivation had features that differed from the presumed parent, the Chang cells. First, there was a difference in karyotype, in particular the presence of an additional satellite chromosome in the 710025 cells. Second, the 710025 cells were able to form small colonies in semisolid agar whereas the Chang cells did not. Third, sera from a proportion of patients with rheumatoid or nonrheumatoid arthritis reacted by

membrane immunofluorescence with Chang and 710025 or equivalent cells but absorption analysis indicated that the latter had additional, unique, antigens to those shared with Chang cells; this point has been substantiated by antigenic analysis with antisera prepared in rabbits rendered tolerant to Chang cells (J. N. McCormick, personal communication). Finally, ultrastructural examination of the cells showed bleb-like projections of the cytoplasmic membrane—not seen in the Chang cells—and in some of the cells there were extensive collections of beaded or pseudofilamentous structures, either deep in the cytoplasm or in the blebs, or in both places.

Two additional cell lines, 720151 and 720301, with essentially similar characteristics were isolated

shortly afterwards, presumably again from contamination, this time with 710025 cells. Since this period, with added precautions against contamination, some 150 synovial membranes or exudates from rheumatoid or nonrheumatoid patients have been examined in culture without isolation of any 'transformed' cell lines. Efforts to reproduce the phenomenon by deliberate cocultivation of freshly taken synovial exudates and Chang cells were unsuccessful (Mackay and others, 1975); in many instances the Chang cells were eliminated by macrophages in the cultures and the survivors did not show the blebs or fibrillary material.

The epithelial cell isolates, typified by 720301 cells, required further investigation for a number of reasons. The cytoplasmic fibrillary material superficially resembled paramyxovirus nucleocapsids, or, alternatively, glycogen, and it was important to establish its nature. The bizarre morphology of 720301, and the related cell lines, resembled similar cytology in human pleural mesotheliomas (Davis, 1974), and that in a variety of tumour or transformed cells resulting from infection with avian or murine leucoviruses. Thus, for example, surface blebs and accumulations of glycogen, together with distinctive, additional, marker chromosomes, have been observed in a line of hamster cells (HT-1) transformed by murine sarcoma virus (Karpas and others, 1971); virus particles and murine leukaemia group antigen were not detected in those cells.

This paper reports the results of investigations designed to define the nature of the beaded or pseudofilamentous structures in the cocultivated cells, and attempts to detect the genome or gene products of rubella, paramyxovirus or leucovirus.

Materials and methods

CULTURE METHODS

Cell lines were grown in Eagle's complete medium (1959 modification) containing 5% calf serum normally in Roux bottles or on cover-slips in tubes at a temperature of 37°. They were passed by trypsinization.

CYTOCHEMISTRY

For light microscopy, 720301 and Chang cells were removed from Roux flasks using glass beads, pelleted in Difco agar (cooled to 48°) and fixed in Bouin's fixative (Gurr, 1957). Paraffin sections were prepared, some being treated with 1% diastase (BDH) for 1 hour at 37°. The sections were stained with Schiff (Gurr, 1957) and Best's Carmine (Gurr, 1957). Cells grown on cover-slips were also stained similarly.

For electron microscopy, 720301 and Chang cells were removed from Roux flasks with glass beads. They were stained specifically for polysaccharides according to the methods of De Bruijn (1973) and Schaff, Barry, and Grimley (1973). The blocks were sectioned on an LKB Ultratome 11 and the sections viewed on a Hitachi

HU11A electron microscope. Some preparations were stained by the ruthenium red method (Vorbrodt and Kroprowski, 1969) to detect differences in surface carbohydrate 'coat'.

Chang and 720301 cells were also examined for content of carbohydrate by growth for 24 hours in Eagle's medium containing either no glucose or increasing concentrations of 1 mmol/l, 5 mmol/l, and 10 mmol/l glucose. They were then harvested, solubilized with 30% potassium hydroxide, and the polysaccharide concentration of the cell-free extracts determined colorimetrically by the anthrone-sulphuric acid method of Shetlar (1952).

ATTEMPTS TO INDUCE CELLS TO PRODUCE VIRUS

(a) In various regimens 720301 cells were cultured in medium containing BUDR 20 µg/ml for 3 days; or IUDR 20 µg/ml for 3 days (Lowry and others, 1971); or IUDR 20 µg/ml for 3 days followed by DMSO (2%) for 4 days (Stewart and others, 1972); or in medium without arginine for 4 days (Kaplan, Wilbert, and Black, 1972). Cells were removed with glass beads, fixed in Karnovsky's fixative, embedded in Araldite, and the sections examined in the electron microscope. In addition, washed cells were lysed by freezing and thawing and the lysates used to inoculate BHK-12, RK₁₃ and Vero cells growing on cover-slips. Cover-slips were stained with Giemsa after 4 and 11 days' incubation.

(b) 720301 and Chang cells of various passage levels, the former ranging from 7 to 28 passages, were incubated in the presence of BUDR 20 µg/ml for 3 days; or IUDR 20 µg/ml for 4 days; or IUDR 20 µg/ml for 3 days followed by DMSO 2% for 3 days; or puromycin 10 µg/ml for 1 day (Aaronson and Dunn, 1974); or testosterone acetate 5 µg/ml for 3 days (Holder, Robey, and Vande Woude, 1974); or mitomycin C 1 µg/ml for 18 hours in the dark (Weiss and others, 1971). The attempted induction was followed by ³H-uridine labelling as outlined by Norval and Marmion (1976). For one experiment, after treatment with testosterone, 720301 cells were incubated in medium containing 2.5 µCi/ml (methyl-³H) thymidine and 0.4 µCi (2-¹⁴C) uridine (Amersham).

DNA POLYMERASE ACTIVITY

(a) Preparation of cells and polymerase assay

720301 and Chang cells were washed in medium and prepared as outlined by Norval, Ogilvie, and Marmion (1975).

(b) Preparation of culture supernatants and polymerase assay

20 ml of culture supernatant from confluent 720301 and Chang cultures, either before or after attempted induction with BUDR and testosterone acetate were prepared according to the method of Lieber and others (1973), and the sedimented pellet resuspended in 0.1 ml polymerase buffer as used in (a). To this was added double quantities of the assay constituents and incubation carried out at 37° for 2 hours. Samples of 50 µl were spotted on filter paper discs at 30-minute intervals and acid-precipitation and counting performed as above.

MEMBRANE ANTIGENS

720301 and Chang cells were examined for interspecies antigens associated with mammalian leucoviruses by membrane immunofluorescence following the method of Yoshiki and others (1974). The antisera used were rabbit antifeline leukaemia virus, HC1-guanidine disrupted (kindly provided by Dr. O. Jarrett), goat antifeline leukaemia virus p27 and p12, and goat anti-Mason Pfizer monkey virus p27 and p10-12 (kindly provided by the National Cancer Institute). The cells were also examined for rubella antigens using the method described by Hart and Marmion (1976).

Results

PSEUDOFILAMENTOUS CELLULAR INCLUSIONS
Cytochemical techniques were used in attempts to identify the material within the bleb-like structures and to distinguish glycogen granules and nucleocapsids of incomplete viral structures containing nucleic acid. Most of the experiments with Chang and 720301 cells were done with cultures infected with *Mycoplasma orale* 1 (Mackay and others, 1974). However, a subline of 720301 cells which had been

freed of mycoplasmas by 'pulse' treatment with sodium aurothiomalate and *M. orale* 1 antiserum and complement was used in ultrastructural studies.

Paraffin sections of pellets of 720301 cells, stained for polysaccharide, showed Schiff-positive material occurring in large particulate areas in the cytoplasm. There were also masses of this material outside the cells. On the other hand, Chang cells only occasionally showed traces of Schiff-positive material and small amounts extracellularly. Best's carmine staining of paraffin sections gave less striking results but the majority of 720301 cells seemed to have some glycogen, whereas very few Chang cells contained glycogen.

On the other hand cover-slip cultures of 270301 cells in monolayer showed much less Schiff-positive material in the form of discrete inclusions. Thus the difference in polysaccharide content with Chang and 720301 cells was less marked in cover-slips than in the paraffin sections, even so there were more Schiff-positive 720301 cells than Chang cells.

The results of the tests at the light microscope level were confirmed by ultrastructural examination.

FIG. 1 Ultrathin section of 720301 cell stained with $K_3Fe(CN)_6$ to show (a) glycogen granules in the cytoplasm; (b) absence of glycogen granules after pretreatment with diastase. $\times 440$

The results with the technique of De Bruijn (1973) with $K_3Fe(CN)_6$ are illustrated in Fig. 1a and 1b. Approximately 70% of the 720301 cells showed large stained areas in the cytoplasm which were removed after incubation with diastase, but not if the enzyme had been inactivated first by boiling. The same result was obtained after the 720301 cells had been freed of Mycoplasma contamination. The stained areas in the Chang cells were much smaller and were found in only about 40% of the cells. Finally, chemical analysis of cell lysate showed consistently higher levels of glycogen in 720301 cells compared with Chang cells, although in each instance higher levels of storage were attained if the concentrations of glucose in the culture medium was increased (Table). Ruthenium red staining did not show a difference in cell 'coat' between Chang and 720301 cells.

Table Analysis of glycogen content (estimated as glucose in hydrolysed cell lysate) of 720301 and Chang cells grown in Eagle's medium with varying content of glucose

Cell	Glucose content of medium (mmol/l)	μ g glycogen/mg protein
720301 pass 19	—	68
	1	72
	5	107
	10	150
Chang pass 284	—	20
	1	19
	5	45
	10	50

INVESTIGATION FOR INFECTIVE VIRUS OR VIRAL GENE PRODUCTS

(a) Infection with cytoplasmic DNA viruses and RNA viruses, other than leucoviruses

As previously reported (Mackay and others, 1974) paramyxovirus (measles, mumps, and parainfluenza) antigens were not detected in cell lysates of 720301 by complement fixation reactions with known antisera. Rubella antigen was not detected by membrane or other immunofluorescence with rabbit antisera that reacted satisfactorily with LLC-MK₂ cells chronologically infected with rubella virus (Hart and Marmion, 1976). In addition immunization of rabbits with cell suspensions did not stimulate viral antibodies.

Lysates of 720301 and Chang cells were also assayed for RNA-dependent RNA polymerase using the method of Scholtissk and Rott (1969). Results were negative but activity was detected in the corresponding lysates from LLC-MK₂ cells chronically

infected with rubella and PK₁₅ and MDBK cells chronically infected with Newcastle Disease virus (kindly provided by Mr. E. Gowans).

(b) Infection with leucoviruses

In view of the striking blebs and glycogen accumulation and the observations that cells with leucovirus genomes sometimes exhibit such morphological changes, extensive attempts have been made to induce the 720301 cells to form complete virions or gene products such as RNA-dependent DNA polymerase or to show interspecies antigens characteristic of the mammalian leucoviruses.

INDUCTION EXPERIMENTS

Many methods have been described in recent years whereby viruses have been induced from an integrated state to form complete virions. In particular, activation of RNA-tumour viruses in various situations including cell lines from human sources has been reported (Stewart and others, 1972; Holder and others, 1974; Bykovsky and others, 1973). RNA tumour viruses thus produced have a density of 1.16–1.18 g/cm³ (Robinson, 1967) on sucrose gradient equilibrium centrifugation, while Mycoplasmas have a density around 1.22 g/cm³ (Todaro, Aaronson, and Rands, 1971).

Attempts were made to induce the 720301 cells by several methods, including BUDR, IUDR, IUDR followed by DMSO, puromycin, and testosterone acetate. In the first instance ultrathin sections were examined in the electron microscope. The presence of the inducers had a general harmful effect on various cell structures except where DMSO was used in addition to IUDR. Puromycin caused an inhibitory effect with cessation of growth and rounding of cells. Testosterone had an even more toxic effect with much of the cell sheet detaching from the glass over the 3-day incubation period. No virus particles were seen and the bleb-like areas did not alter in shape and frequency. The Chang cells were noticeably less affected with all treatments including testosterone.

In addition, washed cells after induction were lysed by freezing and thawing, and the effect of the lysates on BHK-21, RK₁₃ and Vero cells growing on cover-slips found. There was no indication of virus infection as demonstrable by Giemsa staining.

As an alternative method for the detection of RNA virus virions, the cultures were labelled with tritiated uridine and the labelled constituents of the concentrated culture supernate separated by sucrose gradient equilibrium centrifugation. As a positive control for these experiments feline leukaemia virus (FeLV-B) grown in feline embryo cells was labelled with 10 μ Ci/ml of ³H-uridine. This had a density of 1.16–1.17 g/cm³ after sucrose gradient density centrifugation as outlined in Methods.

FIG. 2 Acid-insoluble cpm in sucrose density gradient fractions from 720301 culture supernatant, labelled with ^3H -uridine as in Methods

A double-labelling experiment after testosterone induction using ^3H -thymidine and ^{14}C -uridine showed that the peak at density around 1.21 contained both DNA and RNA (Fig. 4). *Mycoplasma orale* 1 was cultured on cell-free medium from an equivalent fraction containing this material. Thus it seems that the 720301 cells are not producing an RNA virus even after induction, and that the incorporation of labelled uridine and thymidine into cellular material is into Mycoplasmas.

When 720301 cells were treated similarly, there was a small peak of radioactivity around density 1.22, but no evidence of any incorporation of ^3H -uridine at density 1.16–1.18 (Fig. 2). This peak was enlarged by the presence of any of the inducers listed in the Methods but particularly by treatment with testosterone acetate (Fig. 3). On the other hand, the Chang cells showed no incorporation at this density even after induction with testosterone (Fig. 3).

FIG. 3 Acid-insoluble cpm in sucrose density gradient fractions from 720301 (—) and Chang (....) culture supernatant, induced with testosterone before labelling with ^3H -uridine

FIG. 4 Acid-insoluble cpm in sucrose density gradient fractions from 720301 culture supernatant, induced with testosterone before double labelling with ^3H -thymidine (—) and ^{14}C -uridine (....)

ASSAY FOR RNA-DEPENDENT DNA POLYMERASE AS AN INDICATOR OF LEUCOVIRUSES

Enzyme extracts were prepared from lysed cells and tested for the presence of endogenous RNase-sensitive DNA polymerase. Fig. 5 shows a typical result using 10 μ l of Chang and 720301 preparations. The enzyme activities may be expressed as an increase in acid-precipitable counts/min over the first 30 minutes of the incubation per μ g protein. For 720301 cells the result is 6.1; for Chang cells 7.6.

FIG. 5 Endogenous DNA polymerase activity of 720301 and Chang cells. Assay conditions as in Methods, using 10 μ l crude enzyme preparation

The product of the reaction was completely sensitive to DNase in both cases as shown by the reduction in acid-insoluble counts to background level after treatment with DNase at the end of the

FIG. 6 Effect of preincubation with RNase (●) and post-incubation with DNase on endogenous DNA polymerase activity of 720301 cells. Assay conditions as in Methods using 10 μ l crude enzyme preparation and 10 μ l after preincubation with RNase. DNase was added (arrow)

incubation period (Fig. 6; 720301 cells). The dependence of the polymerase activity on the presence of endogenous RNA is shown by pre-incubation of the enzyme preparation with RNase (Fig. 6).

Various methods have been described, such as by Norval and others (1975) to distinguish the RNA-dependent DNA polymerase characteristic of leucoviruses from other cellular polymerases, in particular the DNA polymerase primed by RNA but directed by DNA, found in normal stimulated human lymphocytes (Bobrow and others, 1972). Thus, the sensitivity of the enzyme from the Chang and 720301 cells to actinomycin D was ascertained. The result (Fig. 7) for 720301, and the Chang enzyme was the same. The antibiotic inhibited the reaction by over 90% even for the first sample taken 5 minutes after the start of the incubation period. Secondly, the ability of the polymerase to utilize the synthetic templates poly-d(A-T) and poly-A·d(T)₁₀ was determined. It was found that poly-d(A-T) stimulated the activity over 15-fold while poly-A·d(T)₁₀ had no effect.

FIG. 7 Effect of actinomycin D on endogenous DNA polymerase activity of 720301 cells. Actinomycin D (50 μ g/ml) dissolved in polymerase buffer was added to the assay mixture (●) or polymerase buffer alone (○).

Attempts were also made to analyse the product of the endogenous reaction in caesium sulphate and glycerol gradients. All the radioactivity was at the appropriate density for DNA, that is around 1.41 on Cs₂SO₄ equilibrium density centrifugation and near the top of the glycerol gradient. There was never any evidence of the formation of a labelled RNA-DNA hybrid which should band to a density of around 1.66 g/cm³ on Cs₂SO₄ (Sarngadharan and others, 1972), and have sedimentation coefficient of 70S on a glycerol gradient (Gulati, Axel, and Spiegelman, 1972).

The results therefore do not indicate the presence of a viral RNA-dependent DNA polymerase in the cell lysates. As a positive control in these experiments, use was made of the cell line 118MG-EH containing the Rous sarcoma virus genome with expression of reverse transcriptase activity (Norval and others 1975).

Also assay of the culture supernatants of Chang and 720301 cells did not indicate the presence of a viral reverse transcriptase. There was a very slight increase in acid-precipitable counts over the 2-hour incubation period in the case of the 720301 cells. This was not increased after induction with BUDR but did increase slightly after treatment with testosterone. The raised activity is probably due to the release of DNA polymerase by cell lysis or death and is certainly not sufficiently high to indicate budding of RNA tumour virus into the culture medium as shown by Lieber and others (1973), even in the presence of poly-A·d (T)₁₀ in the assay mixture.

LEUCOVIRUS ANTIGENS

Although mature leucovirus particles may never be formed by an infected cell line, there may still be expression of some viral antigens in the cell, such as the interspecies antigen associated with the major core protein p27 of the virus. These interspecies antigens were thus looked for in 720301 and Chang cells by immunofluorescence (Yoshiki and others, 1974) using antiserum prepared against HCl-guanidine disrupted feline leukaemia virus. In addition, antisera prepared against p27 and p12 proteins of feline leukaemia virus and Mason-Pfizer monkey virus were tried. There was no staining of either cell line with any of the antisera.

TRANSFECTION AND XC TEST

Two final experiments were tried in an attempt to show leucovirus information within the cells. It has been reported that DNA extracted from a human rhabdomyosarcoma cell line caused transformation of human fetal fibroblasts (Karpas and Tuckerman, 1974). Nuclear DNA was therefore extracted from the 720301 and Chang cells and used to 'infect' fetal skin fibroblasts which were split at intervals until they began to die out after about 8 months. There was no change in cell shape, growth, or other evidence of transformation.

Lastly an XC test was carried out. It has been shown that, when cells containing murine leukaemia virus genomes were overlaid with XC cells, syncytia and plaques developed in the cell sheet (Rowe, Pugh, and Hartley, 1970). No effect of this nature was observed in the case of the 720301 or Chang cell lines.

Discussion

The cell lines described in this paper were assumed to have arisen by accidental cocultivation of the Chang cell line with synovial fluid cells. As viruses have been proposed as infective agents in rheumatoid arthritis it was therefore of interest to discover if the unusual characteristics of these cells could be attributed to a viral infection of some kind—either productive and noncytopathic, or defective in some way, or integrated.

One of the most striking features of these cells was the bleb-like projections of the cytoplasmic membrane with associated accumulations of granular material in the cytoplasm. Originally it was thought that this material might be nucleocapsid in origin at occurs during chronic infection of cells with News castle disease virus (Mackay and others, 1974), but subsequently the results of various staining methods at the light and electron microscope level showed that the material was polysaccharide, probably glycogen. This was interesting as glycogen can accumulate in cells infected with either Mycoplasmas or leucoviruses. To resolve this difficulty, the cell line 720301 was cleared of Mycoplasma contamination by pulse treatment with sodium aurothiomalate and *M. orale* 1 antiserum plus complement. No Mycoplasmas were obtained on subsequent subculture and immunofluorescence using goat anti-*M. orale* serum was negative. However after this treatment the cells were shown to contain glycogen accumulations by electron microscopy.

No virus particles have been seen budding from the cell membrane or associated with the cell, and there was no evidence of a productive viral infection of any kind. Paramyxovirus antigens were not detected in cell lysates nor rubella antigens at the cell surface. It is known that leucoviruses can exist in an integrated state within the host cell's DNA, and may express some of their viral genes by the synthesis of RNA-dependent DNA polymerase or viral-coded antigens. Some may be induced by a variety of agents to complete the replicative cycle and to bud from the cell membrane into the culture supernatant. Several different inducers were therefore tried and the effect of these found by electron microscopy, by treatment of other cell lines with cell lysates, and by labelling with ³H-uridine and thymidine followed by sucrose gradient centrifugation. With the latter method, the results were complicated by the presence of Mycoplasmas which seemed to be released from the cell membrane after induction. However, they could be separated from leucoviruses as Mycoplasmas have a higher density and could be labelled with both radioactive uridine and thymidine. No peak of radioactivity with density appropriate for a leucovirus was found. The number

of Mycoplasmas in the culture supernatant appeared to be increased by treatment with inducers, or perhaps more are liberated from the cell by membrane effects, particularly with testosterone. The Chang cell line seemed to be much more stable than the 720301 cells in all respects, and despite containing *Mycoplasma orale* 1, did not release it in detectable quantities into the medium, even after induction. This may also be a reflection of relative population numbers.

The culture supernatant was examined for the presence of RNA-dependent DNA polymerase but none was found, even after induction with BUDR or testosterone acetate. The endogenous DNA polymerase within the cells was shown to have the characteristics of a DNA-directed and not RNA-directed enzyme. In addition no leucovirus inter-species antigen was detected on the cell membrane. Both a transfection experiment and an XC test were not successful.

With regard to RNA viruses other than leucoviruses, no RNA-dependent RNA polymerase was found in cell lysates, and tests for rubella specific antigens on the cell surface were negative.

Thus by all the techniques mentioned above, no evidence was obtained that there was a viral genome, in particular a leucovirus, present in the 'cocultivated' cell lines. The accumulation of glycogen granules remains to be explained, although it is still possible that such a genome is there but is not expressed as mature virus particles, or even as new viral-coded membrane antigens or polymerase enzymes necessary for viral replication.

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