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Cytology of rheumatoid synovial cells in culture

III. Significance of isolates of epithelial cell lines

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Castor, Prince, and Dorstewitz (1961) and Neupert and Sommer (1973) have reported the emergence of continuous cell lines in cell cultures from rheumatoid synovia. Hsu, Pomerat, and Moorhead (1957) noted heteroploid transformation in an epithelial cell derived from a culture of knee joint synovium; the patient was stated to be free from malignant disease, but it was not recorded whether the joint was rheumatoid, arthritic, or healthy. Chessin and Hirschhorn (1961) referred to a permanent cell line which had been established from a normal human synovial tissue.

Ford and Smiley (1973) found aggregations of small round cells in relation to fibroblasts in a synovial culture from a rheumatoid knee; these cells grew in suspension, were not adherent to glass, and synthesized IgG and IgM. They clearly differed from the cells described above and were considered to be B-immunocytes. It might be expected that the ability to grow continuously in culture would be a reflection of the presence of Epstein-Barr virus genome in the cells; however, herpes-like virions were not seen in the cells.

The other cell transformations, as distinct from that described by Ford and Smiley (1973), were glass-adherent cells showing many of the characteristics of the continuous cell lines in common use. During the course of work described in the previous two papers in this series, we have experienced three episodes of such apparent 'spontaneous transformations' in cell cultures from patients with rheumatoid arthritis.

In an investigation of the possible role of viruses in rheumatoid arthritis these observations were initially interesting for evident reasons, namely, the known ability of some viruses to transform cell strains of finite life into lines with indefinite growth potential. However, we were very aware of the results of Gartler (1968), who showed that the majority of continuous cell lines described in the literature as being from different human sources probably had a common origin in the HeLa cell. The most reasonable explanation is laboratory contamination. Investigation along the lines used by Gartler (1968) has shown that the 'epithelial transformation' we have observed in synovial cultures probably represents contamination with Chang 'liver cells'.

Nevertheless, we record our observations for two reasons. First, though these cells were clearly of the Chang (HeLa) type, they differed from the Chang cells we had in our laboratory and may have acquired additional characteristics by virtue of accidental co-cultivation with rheumatoid cells. Secondly, we draw attention again to the dangers of inadvertent contamination with cells, this time in the particular context of cell culture applied to rheumatology.

Materials and methods

Synovial fluid cultures were prepared as previously described (Mackay, Panayi, Neill, Robinson, Smith, Marmion, and Duthie, 1974).

Chang 'liver' cells were obtained from Flow Laboratories (Irvine, Scotland). These cells and the synovial membrane cultures, were prepared or passed by trypsinization; the same basic media were used for all cell cultures but calf serum, rather than mixtures of horse and calf sera, was used for the Chang cells. The general procedures for handling cells followed the methods of Paul (1970).

Chromosome preparations were made by a technique similar to that of McDougall (1970). Species identification by cytotoxicity tests (Greene, Coriell, and Charney, 1964), or by the indirect immunofluorescence reaction, was done with a horse globulin fraction containing antibody to human lymphocytes (a reagent kindly provided by Dr. Keith James, Department of Surgery, University of Edinburgh). Cells were further identified at species level by determination of lactic acid dehydrogenase isoenzyme patterns; the race of origin was checked by electrophoretic

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examination for the fast variant of the glucose-6-phosphate dehydrogenase isoenzyme. This isoenzyme is characteristic of many Negro populations and is present in HeLa cells, a line derived from a cervical cancer in a Negress, and is also present in many continuous cell lines, presumably originating from contaminating HeLa cells (Gartler, 1968). Two techniques for isoenzyme assay were used: first the method of de Oca, Macy, and Shannon (1969), but without a vertical starch gel apparatus our results were equivocal, and then the method of Ellis and Alperin (1972) which gave reproducible patterns.

Cells were tested for ability to grow in semisolid agar by the method of MacPherson and Montagnier (1964). For examination in the electron microscope, cells were grown on araldite by the method of Smith, Gray, and MacKay (1969), fixed with 3·5% glutaraldehyde in 0·1 mol/l. sodium cacodylate buffer pH 7·2, and postfixed in 1% osmic acid in the same buffer. They were sectioned with an LKB/ultratome and stained with 1% ethanolic uranyl acetate and Reynolds's lead citrate. Immunofluorescence was by the indirect technique with fluorescein tagged antiglobulin and human sera with antibody or rabbit antisera to various virus antigens. For fluorochrome examination, cells were fixed in Carnoy's fluid and stained with 1/10,000 dilution of acridine orange.

Mycoplasmas were cultured by placing cells in 'sloppy' mycoplasma medium under microaerophilic conditions, subculturing on solid media and identifying the species of isolates by disc inhibition with known antisera.

For estimation of reverse transcriptase activity, cell extracts were prepared after disruption (Coffin and Temin, 1971) and assayed by the method of Temin and Mizutani (1970).

Results

Apparent epithelial transformations were seen on three occasions in a laboratory in the Department of Bacteriology. The first change occurred in a rheumatoid synovial membrane culture (Ref. no. 710025) after 5 serial subcultures; a total of 100 days *in vitro*. When first noticed in a culture held in a Roux flask, large areas of cells of epithelial morphology were seen in association with an intervening monolayer of fibroblasts of the type normally present in synovial membrane cultures prepared in this fashion. When this flask was subcultured an obviously enhanced growth rate was evident. In the two subsequent instances the epithelial 'transformation' was observed in primary synovial fluid cultures as distinct rounded foci, just visible to the naked eye, which enlarged in diameter and in the centres of which dense piling up of cells occurred as incubation was continued. The appearance is illustrated in Fig. 1. These changes were seen after 35 days (Ref. no. 720151) and 16 days (Ref. no. 720301) *in vitro*. When flasks containing these colonies were subcultured they gave rise to even monolayers of epithelial cells resembling those in culture Ref. no. 710025.

Serological characterization by means of cytotoxicity and immunofluorescence tests confirmed that all three cells were of human origin and isoenzyme analysis of lactic acid dehydrogenase confirmed these findings (Table I). At that stage there was some

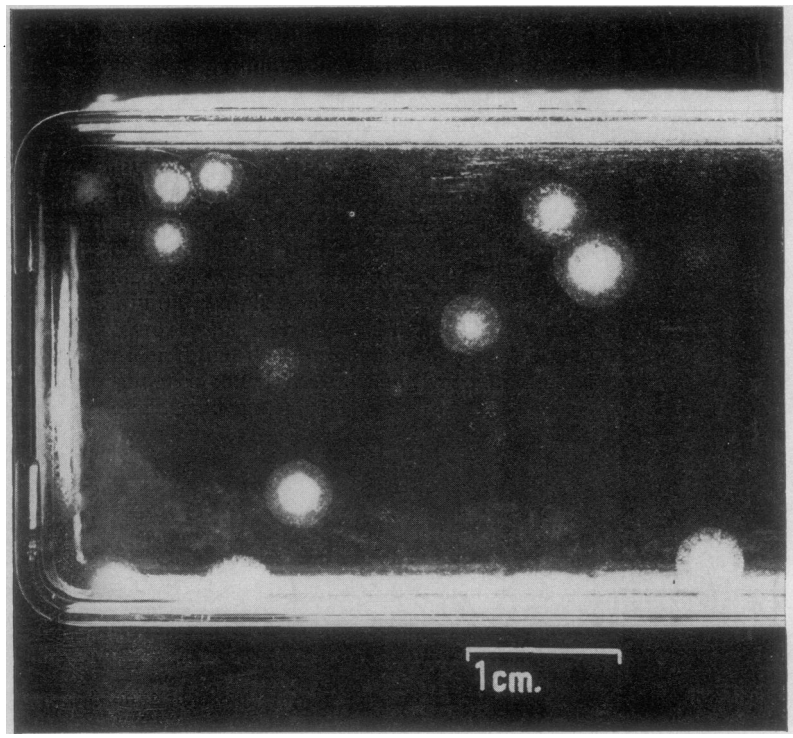

FIG. 1 *Primary synovial fluid culture at 45 days* in vitro, *Ref. no. 720151, showing distinct rounded foci of 'epithelial cells' with dense central areas of thickening*

Table I *Characterization of epithelial cells isolated from rheumatoid synovial cultures by serological and isoenzyme pattern*

Cell isolate or control strain	*Test with antihuman lymphocyte globulin*		*Isoenzyme analysis*	
	Cytotoxicity tests	*Immunofluorescence*	*LDH type*	*G6PD type*
710025	+ve	+ve	Human	A
720151	+ve	+ve	Human	A
720301	+ve	+ve	Human	A
Chang liver cells	+ve	+ve	Human	A
WI-38	+ve	+ve	Human	B
BHK fibroblasts	−ve	−ve	Hamster	Hamster
RK 13	−ve	−ve	Rabbit	Rabbit
710025 RBCs	ND	ND	ND	B
720151 RBCs	ND	ND	ND	B
720301 RBCs	ND	ND	ND	B

ND ≡ Not done.

difficulty in obtaining reproducible glucose-6-phosphate dehydrogenase zymograms and, pending the results of these, certain other features of the cell lines from rheumatoid synovia were examined. These features ultimately included an analysis of the karyotypes of the cells, determination of their ability to form colonies in soft agar, examination of the fine structure of the cells in the electron microscope before and after attempts to induce virus replication by treatment of the cells with bromodeoxyuridine (BUDR) and iododeoxyuridine (IUDR) with dimethylsulphoxide (DMSO) and to assist synthesis of viral proteins by treatment of the cells with actinomycin D.

The problem of the origin of the epithelial cells was finally resolved by determination of their glucose-6-phosphate dehydrogenase isoenzyme patterns on electrophoresis in cellulose acetate strips. A comparison was made between the mobility of G6PD extracted, on the one hand, from the cultured epithelial cells no. 710025, 720151, and 720301, and, on the other hand, from extracts from the erythrocytes of the patients from whom the cells had (ostensibly) come, together with rheumatoid synovial fluid fibroblasts cultured *in vitro*, homogenates of rheumatoid synovial membrane, human embryonic cells, WI-38 human lung fibroblasts, and Chang liver cells. The latter were included because they were the only continuous line cells of human origin present in the laboratory at the time when the apparent 'transformations' had taken place. Some of the results obtained are shown in Table I. It will be seen that the isoenzyme in the 'transformed' cells was in each instance of the fast moving A variant, and quite different from that found in the erythrocytes of the donor. On the other hand, the isoenzyme was the same as that found in the Chang cells. It was concluded that the contamination of the 710025 culture had probably been from the Chang cells and that the other synovial cultures had probably been contaminated with this line. It is of interest in this context that cells 710025, 720151, 720301 were all shown to be contaminated with *Mycoplasma orale*, serotype I; a species known to be present in the Chang cells carried in the department and absent from synovial cultures that had not yielded a 'transformed' epithelial cell.

FURTHER COMPARISONS OF THE EPITHELIAL CELL ISOLATES WITH CHANG CELLS

Although the G6PD isoenzyme analysis eventually clearly showed that cell line 710025 was a contaminant and identified its possible subclones (720151 and 720301), other investigations revealed certain differences between it and the Chang cells.

Karyotype analysis

The frequency distribution of chromosome numbers in cells of 710025 and cells of the Chang cell line is given in Table II. It will be seen that the mean number and standard deviation suggest that the karyotypes of the two populations of cells are different, but both are markedly aneuploid. In addition to these numerical differences, a small submetacentric satellite chromosome was present in 116 out of 200 (58%) metaphase plates of 710025 cells, but was not seen in any of 200 Chang cells (Fig. 2). A similar chromosome was present in 720301 preparations.

GROWTH OF CELLS IN SEMISOLID AGAR

Cell lines 710025, 720151, and Chang were seeded at a density of 10^2, 10^3, and 10^5 cells/Petri dish in semisolid agar and incubated in 5% carbon dioxide in air. Colonies had developed after 7 days' incubation of the plates inoculated with 10^3 cells of 710025 and 720151, and these eventually reached a size of 0·2–0·5 mm diameter by 14 days. There were no colonies in the plates inoculated with 10^5 Chang cells after 14 days' incubation. The colonies in 10^3/710025

Table II *Frequency distribution of chromosome numbers in 100 cells of epithelial isolate 710025 from a patient with rheumatoid arthritis and in 100 cells of the Chang liver continuous cell line*

Cell	*Number of cells with chromosome number of*																*Mean*	*SD*
	<63	*63*	*64*	*65*	*66*	*67*	*68*	*69*	*70*	*71*	*72*	*73*	*74*	*75*	*76*	*>76*		
710025	—	—	1	—	1	4	4	3	13	8	14	31	9	8	2	2	71·84	±2·38
Chang liver	4	7	7	17	25	12	13	3	2	2	2	—	—	—	—	6	66·00	±2·89

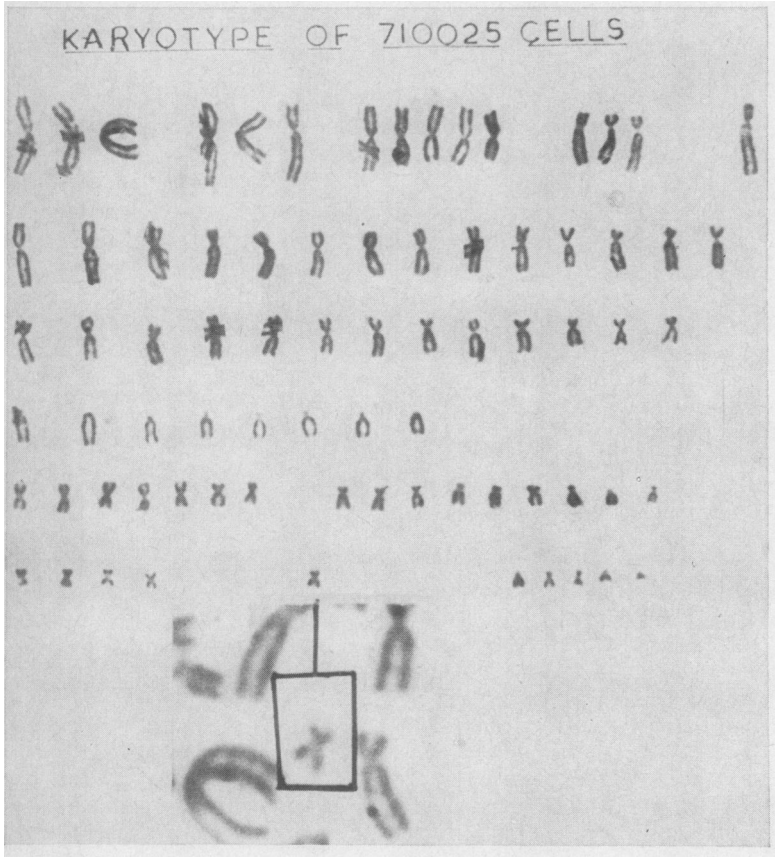

FIG. 2 *Karyotype of 710025 cells illustrating small submetacentric satellite chromosome*

were picked and subcultured in a small Falcon flask and then replated in agar where they again formed colonies.

ULTRASTRUCTURAL STUDIES

Examination of thin sections of all three cell lines showed that approximately 30% of the cells in the culture had surface 'blebs'—that is, distortions of the cytoplasmic membrane with underlying collections of beaded strands of material (Figs 3a and 3b). The beaded appearance may have been produced by the section of tubules, short lengths of which were present in most areas. The outer margins of these structures were electron dense and they varied in diameter from 20 to 25 nm. Collections of this material were also seen within the cell causing severe distortion of the normal cell architecture with displacement of ribosomes, endoplasmic reticulum, and other organelles of the cell (Fig. 3a). These collections were not bounded by membrane and were variable in size. In addition to this material, in a small proportion of the cells there occurred areas of fibrillary material with a more definite outline (Fig. 3a). This sometimes occurred in cells without blebs. The strands were longer and narrower (approximately 15 nm) and arranged in a concentric fashion. Neither type of inclusion was seen in preparations of Chang cells. Because the structures in the cytoplasmic

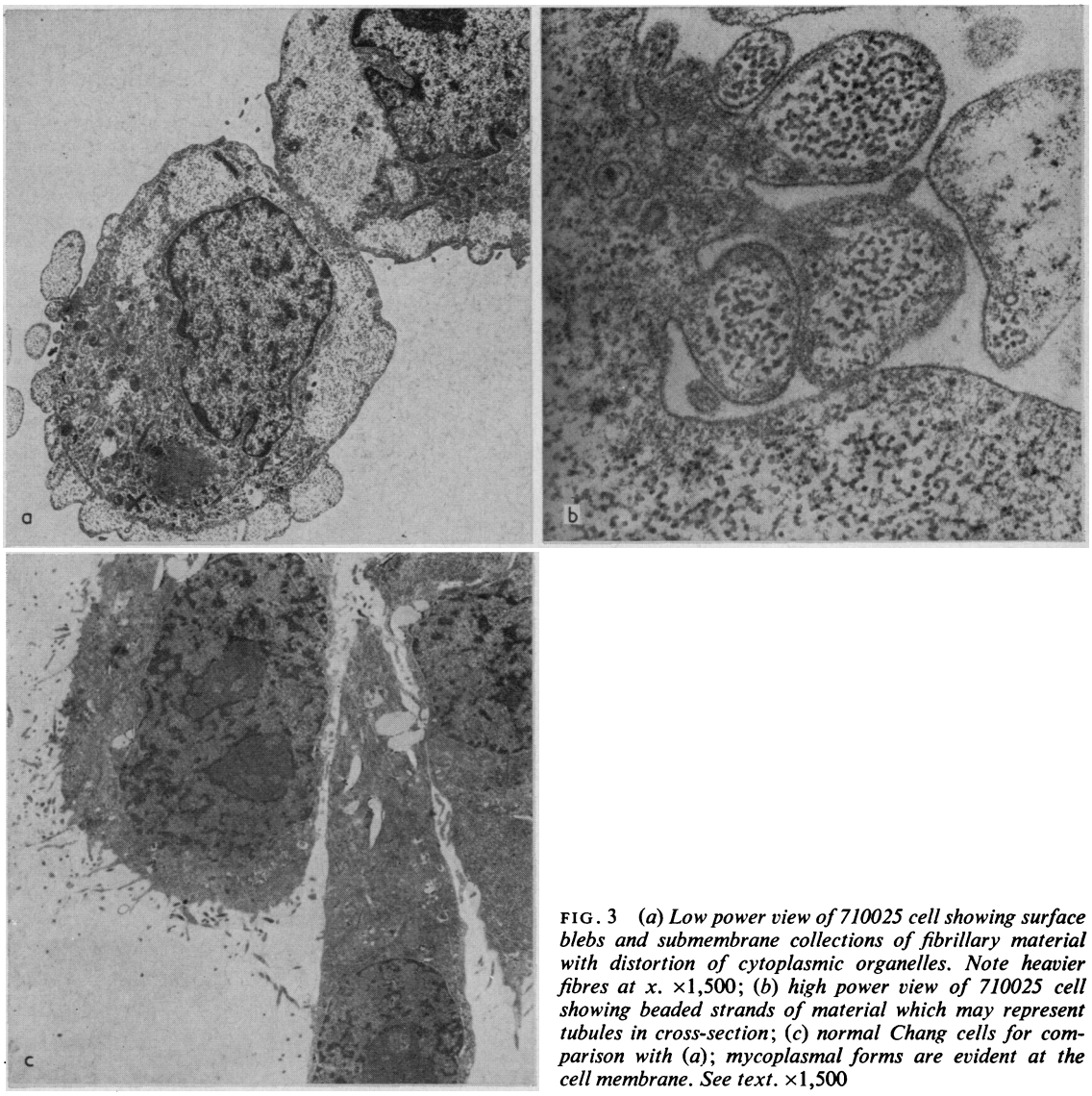

FIG. 3 (*a*) *Low power view of 710025 cell showing surface blebs and submembrane collections of fibrillary material with distortion of cytoplasmic organelles. Note heavier fibres at x.* ×1,500; (*b*) *high power view of 710025 cell showing beaded strands of material which may represent tubules in cross-section*; (*c*) *normal Chang cells for comparison with* (*a*); *mycoplasmal forms are evident at the cell membrane. See text.* ×1,500

blebs resembled the nucleocapsids of certain viruses, notably those of a myxovirus or a paramyxovirus, attempts were made to 'induce' the cells to produce vegetative virus. Treatment with either BUDR or IUDR apparently increased the number of cells with blebs.

Cells were treated with actinomycin D (Sigma) to see if bleb formation and filaments were dependent on a transcription of DNA and also if suppression of cellular mRNA would increase the amount of material. The number of blebs was assessed in preparations stained with acridine orange and by electron microscopy.

Treatment with actinomycin D increased the number of blebs seen in acridine orange preparations and also increased the amount of red cytoplasmic fluorescence. The nucleic acid present was identified as RNA by the use of RNA'ase and DNA'ase treatment.

The treatment with nucleic acid base analogues and with actinomycin D did not lead to the detection, by thin section or negative contrast electron microscopy, of structures resembling complete virus virions, nor did co-cultivation or cell fusion experiments (with lysolecithin as a fusing agent) with these cells and rabbit kidney cells (RK_{13}), human fibroblasts (WI-38), and human glioma transformed by Rous sarcoma virus (118 MG-EH cells) yield virus as judged by cytopathic changes.

In attempts to detect the presence of noncytopathic

or incomplete virions, ^{3}H-uridine was added (5 μCi/ml for 24 hours) to the culture medium of 710025 or 720301 cells previously treated with BUDR (20 μg/ml for 2–3 days). The cells were washed with PBS, harvested, and lysed with distilled water and the cytoplasmic contents fractionated on caesium chloride and sucrose gradients in combination with caesium chloride in the ultracentrifuge (Compans and Choppin, 1967; Yeh and Iwasaki, 1972). Fractions were collected and any fraction forming a peak was examined by negative contrast staining; no structures resembling virions or nucleocapsids were seen.

Incubation at 37°C for 20 mins with 0·1% trypsin removed some labelled material from the cells, but this again had no viral structure either before or after treatment with ether. In addition, lysis of the cells followed by assays for RNA polymerase after sucrose density gradient centrifugation failed to reveal any activity in the appropriate fraction for a paramyxo-like virus (Caliguiri and Tamm, 1970; Compans and Caliguiri, 1973).

There was evidence of reverse transcriptase activity in Chang cells and 710025 and 720301 cells: 720151 were lost due to contamination and therefore were not available for this purpose. The activity was completely dependent on the presence of RNA as shown by the sensitivity of the reaction to pre-incubation with RNA'ase. There were no apparent differences in activity between the three cell lines.

Discussion

Cross-contamination of cells in culture has been described from time to time, but the extent of the hazard is probably less appreciated than the more easily detected contaminations with bacteria, mycoplasmas, or viruses.

In none of the published work on cell lines from human synovial sources is there any evidence of the species of origin of the cells apart from chromosome studies. Chessin and Hirschhorn (1961) observed aneuploidy in the synovial cell line they used. They pointed out that such an appearance could be produced by breakage at the centromeres as was suggested by the high number of telocentric chromosomes. If the karyograms were constructed by re-assembly of the separated chromosome arms then somewhere near the normal human diploid number of 46 could be arrived at. Neupert and Sommer (1973) stressed that their cells diverged in karyotype from other human permanent cell lines. They considered that breakage at the centromeric region may occur during preparation of the chromosome spreads. In a similar fashion to Chessin and Hirschhorn, they constructed karyograms by re-assembly to reach the human diploid karyotype. Neupert and Sommer discarded the simpler explanation that cellular contamination 'specially by L cells' had taken place because continuously propagated cell lines of animal or human origin were not being cultured in their laboratory during this period.

We have been unable to trace the original description of the McCoy cell whose derivation is listed in the Flow Laboratories* catalogue as human synovial, but in correspondence with Dr. Kathleen Cartwright of Flow Laboratories it was pointed out that these cells were still available provided that the customer is fully aware that they are no longer of human origin but have been contaminated with a mouse cell strain. There are no McCoy cells available which are of completely human origin.

The human synovial cell line described by Hsu and others (1957) was lost as a result of yeast contamination and is therefore not available for further analysis. Castor and others' (1961) strain was destroyed in a laboratory accident (C. W. Castor, personal communication, 1973).

Intraspecies contamination may be much more difficult to detect. It has been shown, on the basis of isoenzyme studies, that many of the 'transformed' cell lines of human origin reported between 1952 and 1957 may in fact represent contamination with HeLa cells. 24 human heteroploid cell lines were shown to possess the A variant G6PD isoenzyme found only in some Negroes. Furthermore, the phosphoglucomutase (PGM) phenotypes of 20 of the commonly used cell lines (including Chang liver), were the same as that of the HeLa cell (Gartler, 1968).

Chang liver cells were probably the source of the contamination of the rheumatoid synovial culture yielding the epithelial cell line 710025. After this experience, special precautions were taken to avoid contamination of primary rheumatoid materials, including separate cell culture hoods solely for primary cultures. Despite these precautions, contamination occurred on two further occasions and it is reasonable to suppose either that the source was the Chang cells or the modified line represented by 710025.

It is probable that if the G6PD isoenzyme analyses of the epithelial isolates had worked efficiently at the outset we would have accepted at once the obvious interpretation of cellular contamination and not have examined them further. However, all three rheumatoid cell lines were shown to have other characteristics which were not present in the Chang cells, the probable source of the contamination. It must be admitted that we have no means of ruling out the possibility that the other two rheumatoid lines (720151, 720301) may represent contamination from 710025 cells, and the presence of the marker chromosome in both 710025 and 720301 preparations would suggest this.

The published reports of the isolation of epithelial

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cell lines from synovia have sometimes referred to the possibility of cell contamination but have excluded it on the grounds of quantitative karyotypic differences or because no continuous cell lines were being handled in the laboratory. Our experience suggests that the epidemiology of these contaminations is obscure and that all apparent cell transformation should be examined for isoenzyme patterns before it is concluded that transformation of donor's cells has in fact taken place.

Apart from some differences in karyotype and ability to grow in semisolid agar, the most striking feature distinguishing the 710025 epithelial cell line from its presumed parent, the Chang cell, is the presence of blebs and the collections of fibrillary material under the cell membrane and within the cell.

The acquisition of these properties is of interest from several points of view. First, they bear some resemblance to the nucleocapsids of paramyxovirus and certain other RNA viruses. Secondly, they recall the virus-like structures seen in vascular endothelium of the kidney, muscle, skin, and other organs in systemic lupus erythematosis (SLE) (Norton, 1969; Györkey, Min, Sincovics, and Györkey, 1969; Kawano, Miller, and Kimmelstiel, 1969; Schumacher, 1970; Hashimoto and Chandler, 1972). The actual content of the inclusions in 710025 cells is very similar to that seen in the SLE material. The cytoplasmic inclusions are, however, more numerous. Surface blebs are not a feature of SLE tissue in section, but a recent report (Silverman, Chandler, Nieland, and Hashimoto, 1972) mentioned apparent buds of cell membrane containing inclusions which were noted more frequently in SLE cells maintained in culture. The similarity of the tubular inclusions in cultured SLE cells to those in our material is strikingly illustrated in fig. 9 of Hashimoto and Chandler's (1972) paper.

The occurrence of reverse transcriptase activity was not apparently related to the presence of the 'blebs' in 720301 and 710025 cells, but it is nevertheless of interest in view of the recent demonstration by Bykovsky, Miller, Yershov, Ilyin, and Zhdanov (1973) of similar activity in human cancer cell lines. In our case, however, we were unable to find morphological evidence of B-type particles in the cells.

Preliminary investigations suggest that 710025 cells have a surface antigen that differs from those on Chang cells and is stained in membrane immunofluorescence tests with sera from certain patients with rheumatoid arthritis (J. N. McCormick, and others, unpublished data). Both 710025 and Chang cells react with a variety of animal sera containing antibody to paramyxoviruses and other viruses. Adsorption experiments suggest that the antibodies concerned are not directed to viruses but may be part of the T-agglutinin-agglutinogen systems (J. N. McCormick, and others, unpublished data).

There is no explanation at present of the nature of the change produced in Chang cells to give the 710025 cell. This is constantly present on subculture, and is clearly hereditable. A defective viral genome acquired from co-cultivation with the rheumatoid synovial cells is the simplest explanation. It is also possible that actual cell fusion may have occurred since polykaryocytosis is a prominent feature of such synovial cultures.

Summary

On three occasions during the course of culturing cells from synovial membranes and fluids of patients with rheumatoid arthritis the appearance of apparently 'transformed' epithelial cells was observed. When these isolates were characterized by isoenzyme analysis they were found to resemble HeLa cells rather than the cells of the patients from whom the cultures were supposedly derived. Thus, they probably arose as a result of laboratory contamination and attention is drawn to this hazard. The only human cell line in the laboratory at the time the incidents occurred was the Chang liver cell. When the karyological and ultrastructural characteristics of the rheumatoid and Chang cells were examined they were found to differ. In particular, the rheumatoid-associated cells showed accumulations of beaded strands in the cytoplasm and in bleb-like projections of the cytoplasmic membrane. The possibility must be considered that these differences may have arisen as a result of co-cultivation of Chang with rheumatoid cells, albeit accidental.

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