

# Cytology of rheumatoid synovial cells in culture

## I. Composition and sequence of cell populations in cultures of rheumatoid synovial fluid

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There are relatively few reports of the culture of synovial fluid cells *in vitro* in the study of rheumatoid arthritis (RA). This is perhaps surprising in view of the obvious advantages of this material in the study of a chronic process, offering, as it does, the opportunity of several examinations from the same donor.

Lackington (1959), cultured synovial fluids from ten RA joints and six from osteoarthrotic joints. He noted the appearance of large vacuolated round cells, some of which developed multiple nuclei within the first week of culture. Giant cells were present in both types of joint fluid but were much greater in size and number in the cultures from RA fluids. Within the first 2 weeks both types of culture gave rise to networks of cells resembling fibroblasts.

Castor and Dorstewitz (1966) compared the cultures obtained from four RA synovial fluids and five RA synovial membranes (one patient was donor of both) with cell strains derived from thirteen normal synovial membranes. These authors used trypsin to dissociate the membrane cells. They observed no reliable morphological differences between normal and rheumatoid cell strains all of which grew on glass as stellate or spindle-shaped fibroblast-like cells. They noted that cultures from joint fluid were in every way similar to those from synovial tissue and believed them to have arisen from desquamated synovial lining cells. In the discussion of their results, they observed that changes similar to those described by Lackington (1959) had been seen in their RA material but that they had similar findings in material from healthy persons. However, these authors were primarily concerned with cell strains which were studied in their fourth to eighth trypsin subculture; *i.e.* fibroblast-like cells. There were, however, some differences between fibroblasts from RA and other sources, notably in their growth rate, hyaluronate synthesis, and response

to cortisone. Later, ultrastructural investigation of synovial fibroblasts (Wynne-Roberts and Castor, 1972) failed to reveal morphological features which would distinguish those from rheumatoid and from non-rheumatoid sources.

Palmer (1971) cultured synovial fluids from 25 RA joints and from six joints affected with osteoarthritis. In contrast to the previous authors, he found that the RA synovial fluids gave rise only to epithelioid macrophages and polykaryocytes and that these remained as such despite periods of culture of up to 7 weeks. By contrast, cells from osteoarthrotic fluids developed similarly but, in addition, multipolar fibroblast-like cells appeared, and if the cultures were maintained long enough a fibroblastic monolayer developed and the other cell types were overgrown.

Mackay, Alexander, and Neill (1971) briefly reported the culture of synovial fluids from 47 RA patients, twelve patients with other inflammatory articular disease, and two with primary generalized osteoarthritis. They commented on the fact that the majority of the cells present in such cultures were similar to those seen in cultures of peripheral blood from healthy donors. The purpose of the present communication, and that of Panayi, Mackay, Neill, McCormick, Marmion and Duthie (1974) which follows, is to record the functional and ultrastructural characteristics of the cells seen in such cultures.

### Material and methods

Synovial fluids from patients with classical or definite rheumatoid arthritis, or from those with other forms of inflammatory joint disease and osteoarthritis, were set up in culture as soon as possible after aspiration. An equal volume of synovial fluid and cell culture medium were mixed and 1 ml. was inoculated into a Leighton tube

containing a 35 × 8 mm. coverslip and incubated at 37°C. in an atmosphere of 5 per cent. CO<sub>2</sub>: 95 per cent. air. The cell culture medium consisted of Eagle's Minimal Essential Medium (Burroughs Wellcome), supplemented with 10 per cent. newborn calf serum and 10 per cent. horse serum (Flow Laboratories) and containing 100 i.u./ml. penicillin and 100 µg./ml. streptomycin. All sera were inactivated by heating at 56°C. for 30 min. Medium changes of the cultures were carried out at twice-weekly intervals. At intervals some cultures were terminated; the coverslip was washed twice with phosphate buffered saline (PBS), fixed for 10 minutes in methanol, stained with Giemsa, dehydrated, and mounted in DePeX\*.

The phagocytic activity of these cultures was shown by the rapid uptake of inert particles (Zymoan, Koch-Light Laboratories). Zymoan was suspended in PBS at a concentration of 0.1 µg./ml. and 0.1 ml. added to each Leighton tube and held for 1 hr at 37°C. Alternatively, starch granules prepared from *Aramanthus cruentus* were prepared at a concentration of 5 mg./ml. and 0.1 ml. added to each tube. The cultures were then washed with PBS to remove excess Zymoan or starch particles, fixed, stained by the periodic acid-Schiff (PAS) method for 10 min., washed for 60 min. in running tap water, and then counterstained with Giemsa. They were then dehydrated through acetone, acetone-xylo, and xylo before mounting in DPX.

The response of the cells to a vital dye was examined by adding to each tube 1 ml. of 0.1 per cent. solution of neutral red in Eagle's medium. The cultures were then incubated overnight at 37°C., washed in Eagle's medium and examined by phase-contrast microscopy. Acid phosphatase was demonstrated by the Gomori technique. Cell cultures for electron microscopy were grown on araldite surfaces, as described by Smith, Gray and Mackay (1969).

## Results

Five types of cell adhered to the glass or araldite surfaces in cultures of synovial fluid. The proportion of cell types varied from one culture to another and the distribution of the principal types in relation to the source of the cultures is analysed in the accompanying paper (Panayi and others, 1974). The cell types are now described.

### (a) Macrophages

These were present in all cultures if any cells were viable. The cells were large and round with abundant often foamy cytoplasm, and under phase contrast exhibited a typical ruffled membrane with many vesicles and refractile inclusions. They were occasionally binucleate and, very occasionally, up to ten nuclei were present within one cytoplasmic membrane. They were actively phagocytic as judged by their rapid uptake of starch or Zymoan particles and, typically, they also concentrated neutral red into granules of varying size. Within the first few days of culture, many smaller cells with similar characteristics were present and these were classified as monocytes. The latter showed little acid phosphatase

staining, whereas this was evident in the majority of the macrophages present. These appearances at the level of the light microscope were confirmed by the ultrastructural studies. The cytoplasm was rather dense with many ribosomes and cisternae of rough endoplasmic reticulum were also often present. A well-developed Golgi apparatus was a prominent feature; associated with this there were numerous membrane bound lysosomes in the perinuclear area. The nuclear chromatin tended to be marginal in distribution and the cell surface showed microvilli (Fig. 1). With longer incubation the proportion of cells showing phagolysosomes, lacunae, and dense lysosomal bodies appeared to increase. Even by the third day occasional multinucleate cells with similar ultrastructure were present.

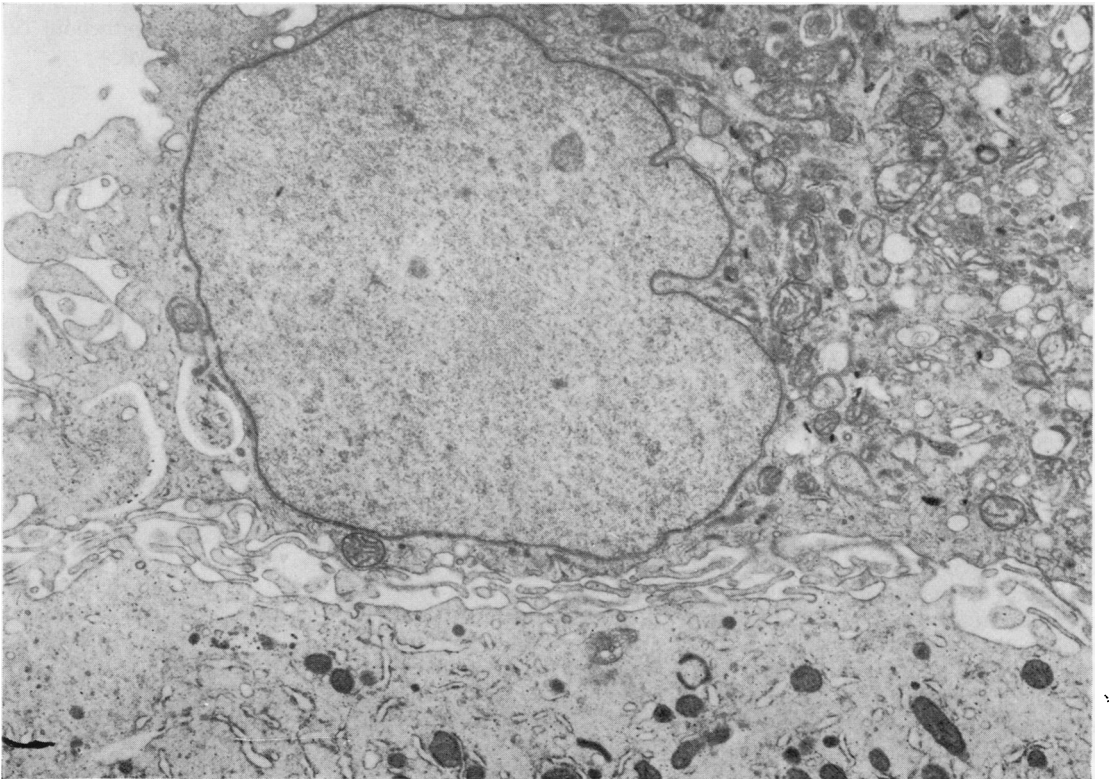
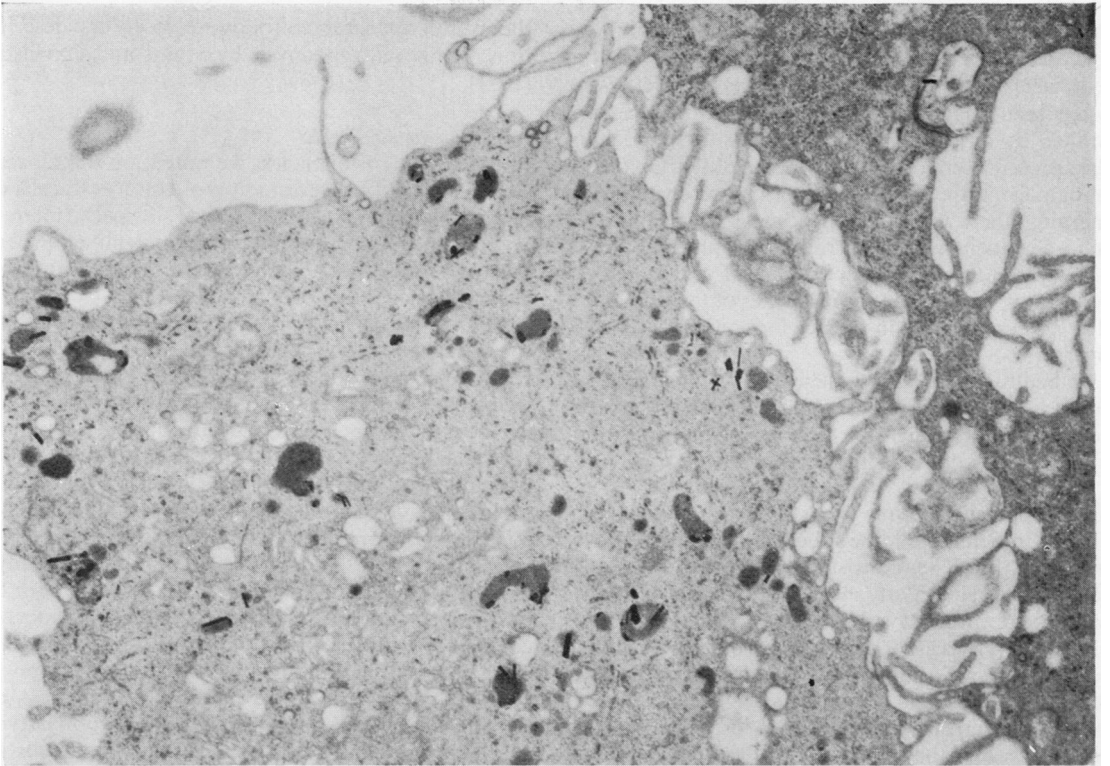
After 7 to 10 days' incubation some cultures remained as ones composed largely of collections of discrete macrophages with the properties just described, whereas other cultures, particularly those in which polykaryocytosis was to develop, contained other discrete mononuclear cells with some of the characteristics of epithelioid cells. The latter failed to take up starch or yeast particles and had large pale nuclei with a single prominent nucleolus. Ultrastructurally, they possessed filopodia which interdigitated extensively with those of neighbouring cells (Fig. 2). The mitochondria were numerous, usually elongated, and sometimes tortuous. They showed lysosomal dense bodies but phagolysosomes were rarely seen.

### (b) Polykaryocyte cultures

These developed after a variable period of incubation and the polykaryocytes had several to over fifty nuclei. Nuclear size was distinctly smaller than in macrophages and there appeared to be a tendency for the size of nuclei to diminish with increasing numbers of nuclei in a single polykaryocyte. Polykaryocytosis occurred at scattered foci throughout a culture, so that, on stained preparations, polykaryocytes could be distinguished macroscopically as large 'colonies' on the culture vessel. Microscopically, these collections of polykaryocytes were seen to be surrounded by mononuclear macrophages. The polykaryocytes that developed later also failed to show evidence of phagocytic ability for yeast or Zymoan particles. When first examined, at 7 days, they showed many features in common with the 'epithelioid' cells described above, but with further incubation, at 10

FIG. 1 *Junction of macrophage with epithelioid cell (upper right); numerous interdigitating villi at interface of cells. × 10,000*

FIG. 2 *Junction of an epithelioid cell (upper part of figure) with macrophage (lower cell), showing interdigitation of filopodia. × 10,000*



days or later, many of the cytoplasmic contents were lost and the cytoplasm was electron lucent and rather featureless. Large lacunae and cytoplasmic clefts were often seen together with formless grey areas not limited by membranes (Fig. 3, opposite). Mitochondria present were often elongated and with evidence of damage in the form of loss of cristae or indistinct margins; there were few lysosomes. There appeared to be a sequential change from macrophage to epithelioid cell to polykaryocyte marked by a loss of lysosomes, a change in the character of the mitochondria and an increase in surface filopodia (Figs 4 to 6, overleaf).

Subcultures of macrophages and polykaryocytes could not be established despite repeated efforts; the cells did not appear to be multiplying and could not be detached from the culture surface with trypsin.

#### (c) *Fibroblastic cultures*

These, however, could be subcultured many times by trypsinization. They consisted of large spindle-shaped cells arranged in orderly sheets with whorled patterns; widespread multi-layering never occurred. Fibroblasts often overgrew macrophage or polykaryocyte cultures after variable intervals of time. Wynne-Roberts and Castor (1972) have given an excellent detailed description of the ultrastructure of such fibroblasts and they need not be described in detail here.

#### (d) *Strap cells*

There are no previous descriptions of the EM appearances of such cells. They were seen in ten synovial cultures in the present series and closely resembled those described by Stanfield and Stephens (1963), who reported that their appearance in the light microscope was that of stellate and short cells arranged in patterns resembling 'angel' fish. Those in the present series tended also to reflect the findings of these authors in the formation of peculiar tissue-like aggregates. Ultrastructurally, there were accumulations of lysosomal dense bodies in the longitudinal parts of the cell each side of the nucleus; they resembled the type A cell of the synovial membrane described by Barland, Novikoff and Hamerman (1962) (Figs 7 to 9, overleaf).

#### (e) *Reticular cells*

These were present in varying proportions in nearly half of all the cultures examined. They have abundant cytoplasm, and are stellate or elongated, often with branching processes. They took up neutral red into granules that were more widely distributed than in fibroblasts but showed no tendency to concentrate dye in the perinuclear region as did the macrophages. A striking feature in the cells we studied was the presence of sacs lined with a single layer of ribosomes and filled with finely granular osmiophilic material;

the overall appearances followed closely the description of this cell type given by Stuart and Davidson (1971).

#### (f) *'Transformed' cells*

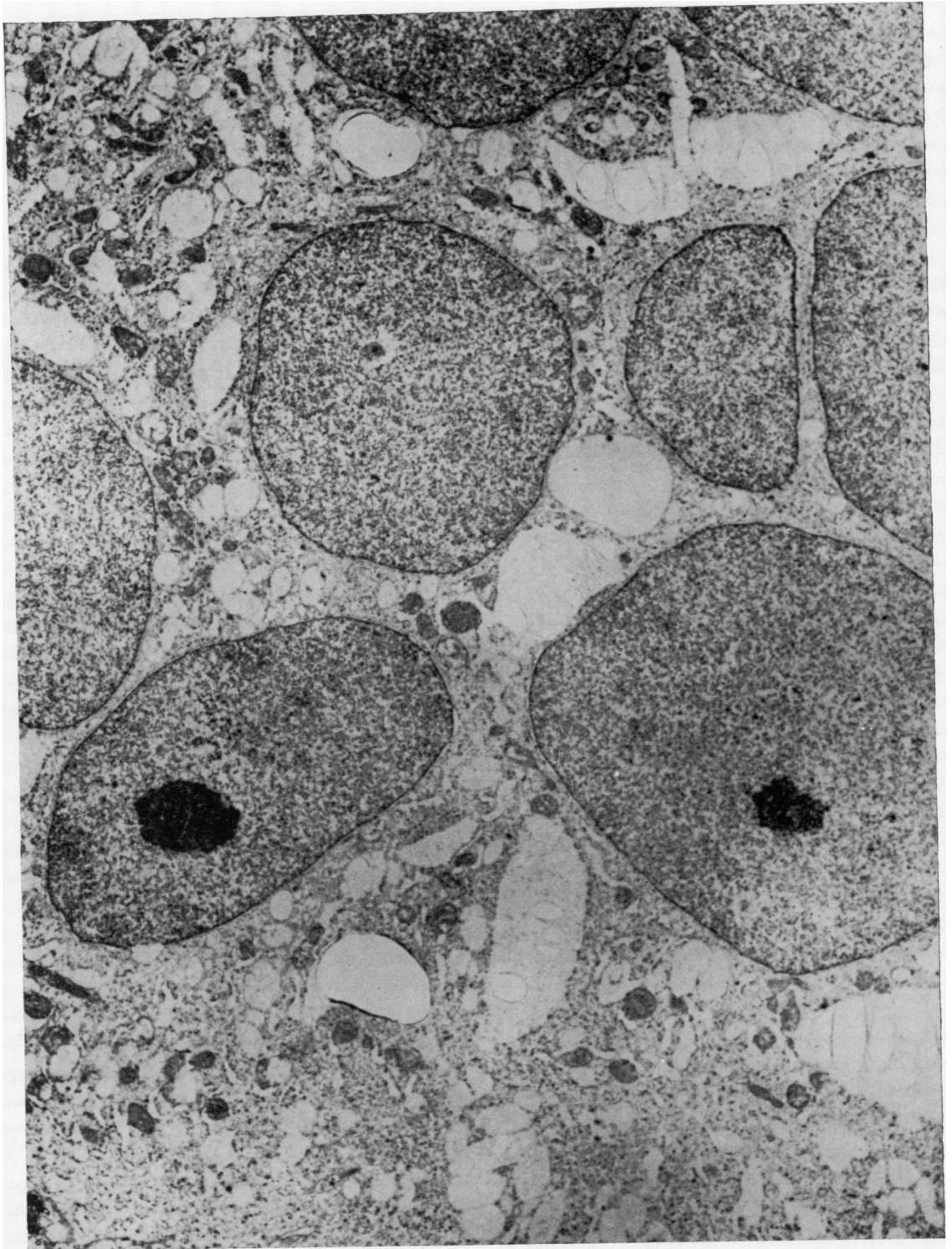
In the course of this work, we noted, on three occasions, apparent transformation of cultures to cells of an epithelial type. These were clearly of special interest and this phenomenon is described in detail elsewhere (Mackay, Marmion, Neill, Norval, Robinson, and Tait, 1974).

The preceding description has been restricted to the cells seen on the glass or araldite surfaces. Cells which do not adhere include erythrocytes and, frequently, large numbers of polymorphonuclear leucocytes. These are lost early from the system and the latter are certainly no longer apparent after the first change of medium at 3 days. On the other hand, preliminary time-lapse cinematography studies have confirmed the presence of large numbers of lymphocytes in the cultures during the whole period of study; see also Stanfield, Stephens, and Hill (1966). In particular, there was some evidence that lymphocytes could enter and leave polykaryocyte formations.

In general these studies indicated that the polykaryocytes probably arise mainly by a process of fusion. Ultrastructural examination of polykaryocytes suggests that reticular cells and strap cells at least may also become involved in such fusions, in addition to the macrophages and epithelioid cells that we have considered in detail above.

### Discussion

Many of the previous publications on cell cultures from patients with rheumatoid arthritis have been designed to detect persistent differences between fibroblastic cell strains from RA synovia and those from normal or other non-rheumatoid joints. Such studies have usually involved repeated subcultures of cells with trypsinization; thus discriminating against the carryover of cells of the phagocytic series. However, Bartfeld (1965) and Palmer (1968, a, b; 1970) clearly described macrophage and polykaryocytes similar to those observed in the present study, despite the fact that both authors used trypsin to dissociate the synovial membrane cells. Thus trypsin *per se* does not prevent the establishment of phagocytic cells in primary cultures; the failure of these cells to persist in trypsinized subculture is presumably related to the tenacity with which they adhere to glass and to their failure to multiply. Stanfield and Stephens (1963), and Stanfield and others (1966) used a variant of the explant method, without trypsin, to initiate their synovial membrane culture and studied them by cytological and time-lapse cinematography techniques. Our cytological findings agree closely with theirs; they found no morphological differences be-



**FIG. 3** *Central area of a polykaryocyte after 14 days in culture, showing lacunae and cytoplasmic clefts and some mitochondria but few phagolysosomes.  $\times 6,000$*

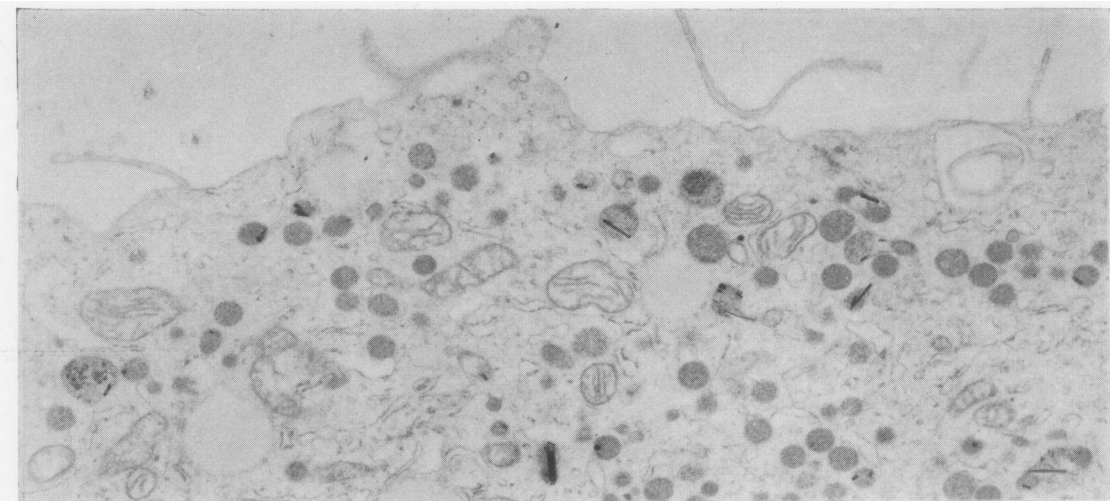


FIG. 4 *Margin of macrophage showing numerous lysosomes. 7 days in culture. × 10,000*

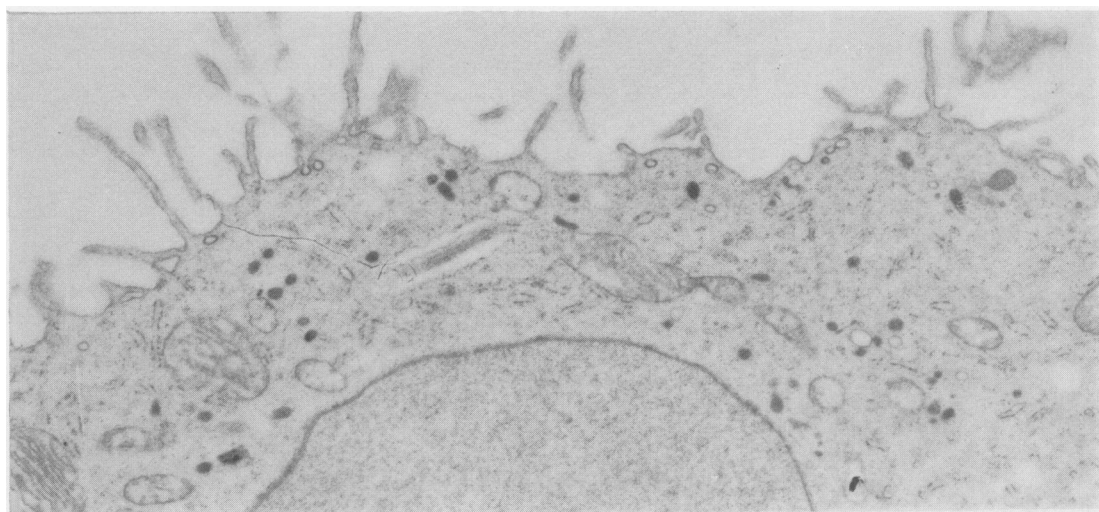


FIG. 5 *Similar cell to that in Fig. 4, but changing to epithelioid type. Note pinocytotic vesicles at base of numerous filopodia, large mitochondria, and small lysosomal dense bodies. × 10,000*

tween tissue cells cultured from RA and non-RA synovia. Highton and Palmer (1971) briefly described the EM appearances of the macrophage and multinucleate cells from rheumatoid synovia. They commented that the giant cells resembled those seen in cell cultures infected with certain syncytia-forming viruses but neither they nor we have seen inclusions or other virus components in such cells.

We have, in our present observations, been particularly impressed with the change of cell populations in the cultures with time. Initially there are lymphocytes and macrophages (with some other cells) and in sequence there appear epithelioid and giant cells and later still fibroblastic overgrowth; an interpretation

and working hypothesis is required. The sequence of events is remarkably similar to the (postulated) development of monocyte → macrophage → epithelioid cell → giant cell described by Sutton and Weiss (1966) in cultures of the buffy coat cells from chickens. Similar sequential changes also occur 'spontaneously' in cultures of monocytes from human peripheral blood and are, presumably, a reflection of the non-immune aspects of macrophage activation. The changes seen in our cultures have occurred much more quickly than those observed by Sutton and Weiss and those seen in human peripheral monocytes 'in vitro'. The fact too that there were differences between synovial fluids from different patients with

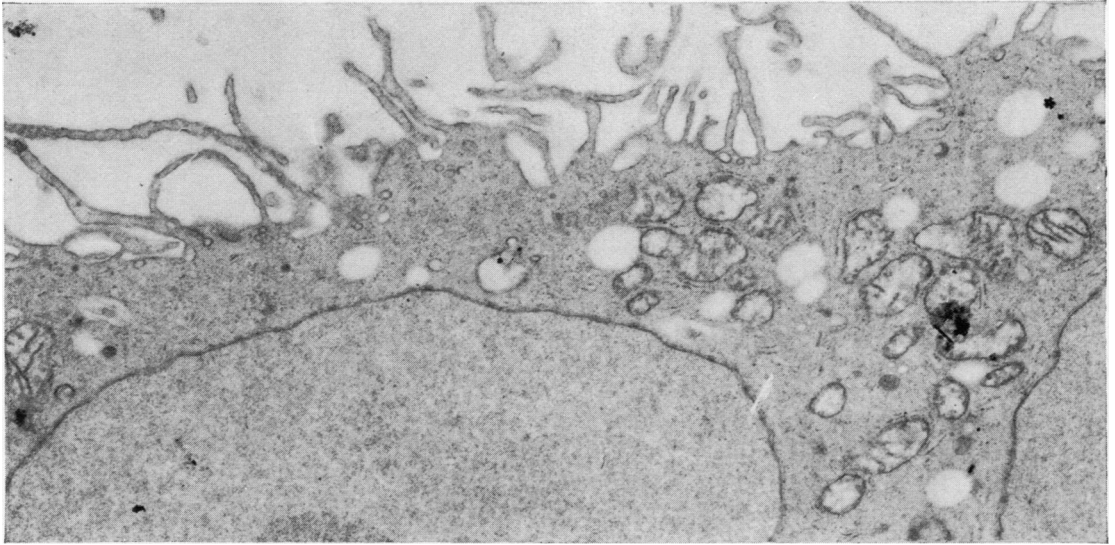


FIG. 6 Edge of polykaryocyte; numerous filopodia, large mitochondria, and virtual absence of lysosomes. 14 days in culture.  $\times 10,000$

RA and with other conditions suggests that the changes imply more than simply a series of different stages of development or de-differentiation *in vitro*. One interpretation of these findings is that, at the time of collection, the fluid contains many cells that are at different stages of the monocyte  $\rightarrow$  macrophage  $\rightarrow$  epithelioid cell evolution; the early appearance of polykaryocytes could be accounted for in this way. In the whole animal, this multicellular reaction, a kind of granuloma, arises in response to

extraneous, discrete antigens, or cell-associated antigens, or indigestible particles; Papadimitrou and Spector (1971) have, for example, described a similar sequence of cellular change in response to BCG, inactivated staphylococci, or colloidal gold. There was no morphological evidence of extraneous particles in our material.

If our findings are regarded as evidence of a granulomatous response within a mixed population of synovial fluid cells in culture, it follows that this

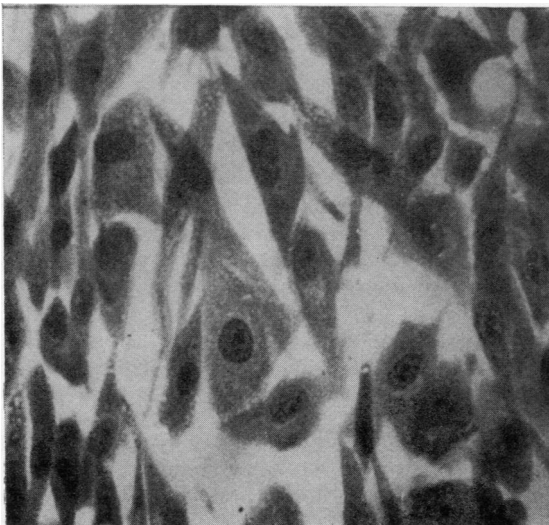


FIG. 7 'Strap' cells stained by Giemsa arranged in tissue-like aggregate or as 'angel' fish.  $\times 400$



FIG. 8 Phase-contrast preparation of 'strap' cells, again showing pavement or tissue-like arrangement.  $\times 450$

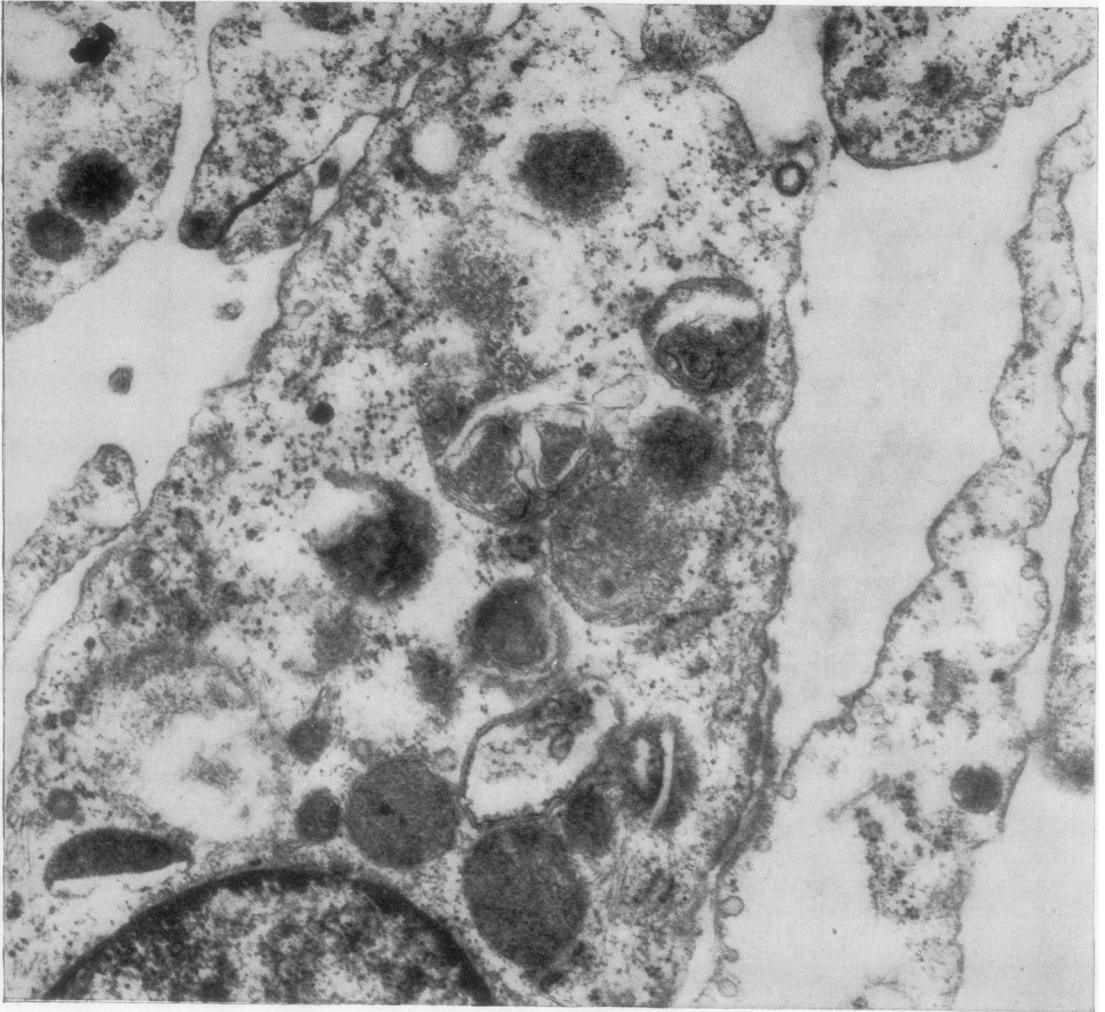


FIG. 9 Thin section of 'strap' cell, showing distribution of lysosomes, some phagosomes, and in an adjacent cell, numerous pinocytotic vesicles lining cell membrane.  $\times 26,000$

implies the presence of an extraneous antigen or 'target' cell with foreign antigens.

In the presence of an antigen and sensitized lymphocytes, activation of macrophages and subsequent polykaryocyte formation—the cellular immune response (CMI)—takes place much more quickly (Galindo, 1972). It is therefore of substantial interest that, if a CMI is involved, it should take place sooner (and to a greater extent as judged by the number of polykaryocytes formed) in cultures from the rheumatoid synovium than in those from traumatized or degenerative joints.

The central questions remain: what is the cell, or cellular antigen(s), against which the CMI is directed in the RA joint and what are the conditions governing its acceleration and completion *in vitro*? If this line

of thought is extended, it becomes clear that the hypothetical target cell will most probably not be available for examination as it will be eliminated during the rapid development of the CMI during the first few days in culture. The problem then becomes one of inhibiting the CMI in order to detect unusual cells that might be 'target' cells. Recent observations suggest that the use of autologous synovial fluid rather than calf and horse serum in the cultures appears to damp down the rate of development of the CMI in culture. The reasons for this protective effect are probably complex and are uncertain at present; it has however provided some confirmation of the views advanced above; the findings will be described in detail in a later paper of this series.



## Summary

Cell cultures have been established from the synovial fluids of patients with rheumatoid arthritis and from those with various arthroses. The cultures were fed with media containing inactivated calf and horse serum but trypsin was not used to disaggregate or passage cells.

Monocytes, macrophages, epithelioid cells, and polykaryocytes were observed in the cultures and, as a late event, fibroblasts were sometimes dominant.

There appeared to be a sequential change from macrophage to polykaryocyte that appeared earlier and was more intense in cultures from RA joints than in cultures from other sources.

The observations suggested that a cell-mediated immune reaction was being observed *in vitro*, but the antigen or target cell against which the reaction was directed was not identified; perhaps because of the efficiency of the reaction.

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