Rheumatoid synovial cells from intact joints

Morphology, growth, and polykaryocytosis

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SUMMARY   Synovial cell lines were isolated by instillation of trypsin or chymotrypsin into intact knee joints of patients with persistent rheumatoid effusions resistant to conventional therapy. Morphology and growth in the primary phase were compared with rheumatoid cells isolated from excised synovium and nonrheumatoid synovial cells obtained from intact joints of cadavers or amputated limbs. Cell populations from all sources included varying proportions of marcophage-like and fibroblast-like cells, with only 1–3 % multinucleated cells. In medium supplemented with calf serum alone, rheumatoid cells from intact joints showed negligible changes in morphology. However, in the presence of nonrheumatoid, autologous rheumatoid or homologous rheumatoid serum a rapid increase occurred in size of the macrophage-like cells and numbers of polykaryocytes, including some giant syncytial cells. These effects were directly proportional to serum concentration and were identical in fresh or heat-inactivated serum. In most of these rheumatoid cell lines no multiplication occurred, regardless of serum type or concentration. In rheumatoid synovial cells from excised synovium, human serum induced both polykaryocytosis and rapid growth of fibroblasts. Non-rheumatoid synovial cells grew rapidly but few polykaryocytes developed, mostly with less than 6 nuclei.

Evidence of viral infection in rheumatoid synovial cells was sought by electron microscopy after stimulation of polykaryocytosis by human serum. In one of the cultures many cells were found with intranuclear particles possessing characteristics of the adenovirus group.

Some years ago it was shown that large numbers of cells could be separated from synovial intima without dissection, simply by instillation of trypsin solution into intact joints (Fraser and Catt, 1961). Apart from one patient with a persistent knee joint effusion (J. R. E. Fraser, 1964, unpublished), the technique was restricted in this laboratory to cadaver joints and amputated limbs. However, Ford and Oh (1965) applied it to a virological study of rheumatoid synovial cells. In this and subsequent studies no deleterious effects were observed in the patients (D. K. Ford, personal communication, 1975). In considering a possible alternative to synovectomy in the treatment of rheumatoid joint effusions, it was decided to explore the therapeutic potential of the technique in patients with effusions resistant to local steroid and conventional systemic therapy. The

clinical results of treatment with intra-articular proteolytic enzymes will be considered separately. This paper is primarily concerned with the cytological and cultural characteristics of the synovial cells isolated from intact rheumatoid joints.

Material and methods

ISOLATION OF RHEUMATOID SYNOVIAL CELLS
From intact joints
The procedure has so far been used only on knee joints. A sterile 13 or 16 g catheter is inserted beneath the patella on the medial side. The synovial fluid is aspirated with a syringe and the joint rinsed thoroughly with physiological saline to remove fibrin and free cells. Approximately 40 ml of saline containing 125 000 units trypsin (Tryptar, Armour Pharmaceutical Co. Ltd.) or 10 000 units of α-chymotrypsin (Chymar, Armour Pharmaceutical Co. Ltd.) are introduced and left *in situ* for 10–15 minutes. The cell suspension is withdrawn, centrifuged, and the pellet resuspended in culture medium

(Eagle's basal medium with 20% fetal calf serum). The procedure is usually repeated, with an intervening saline rinse. The cells adhere readily to glass or polystyrene culture flasks within 24 hours. As shown later, the behaviour of the cells depends on the type and concentration of serum in the culture medium.

From excised synovial tissue
Synovial tissue is transported to the laboratory in Eagle's medium. The synovial surface is dissected from underlying tissue, then scraped gently with a sterile scalpel blade into Hanks's balanced salt solution (Ca/Mg free). Trypsin is added (final concentration 0·25%), and the cell suspension incubated at 37°C for 20–30 minutes, then centrifuged and mixed in culture medium as above.

ISOLATION OF NONRHEUMATOID SYNOVIAL CELLS
These were obtained from intact cadaver joints using the procedure of Fraser and Catt (1961). Methods of maintaining these cells in culture have been described previously (Fraser and McCall, 1965).

CULTURE MEDIA
Eagle's basal medium (EBM), animal sera (fetal calf, newborn calf, adult horse), trypsin, and salt solutions (Hanks's balanced salt solution=HBSS; Dulbecco-Vogt phosphate buffered saline=PBS) were obtained from Commonwealth Serum Laboratories, Parkville, Victoria.

Nonrheumatoid serum was obtained aseptically from several fasting donors. Rheumatoid serum was prepared from a patient who had received no drugs other than gold in the previous 3 months.

LIGHT MICROSCOPY
Living cultures were examined by phase contrast illumination (Olympus PBM 6). Coverslip cultures were stained with Mayer's haematoxylin-eosin.

ELECTRON MICROSCOPY
In some isolations a portion of the trypsin-saline cell suspension direct from the joint was immediately centrifuged and the pellet fixed in 4% glutaraldehyde, for 4 hours at 4°C. Cell cultures were rinsed with warm PBS or HBSS and prepared for electron microscopy as described previously (Le Marshall *et al.*, 1977).

PHAGOCYTIC ACTIVITY
This was examined by incubating cultures overnight at 37°C with washed polyvinyl chloride (Geon, B. F. Goodrich—C.S.R. Chemicals Pty. Ltd., Glen Iris, Victoria) in culture medium.

Results

ULTRASTRUCTURE OF RHEUMATOID SYNOVIAL CELLS FROM INTACT JOINTS BEFORE CULTURE
Light and electron microscopy of joint aspirates showed mainly neutrophils, lymphocytes, erythrocytes, and fibrin. Trypsin-saline cell suspensions contained some cells of these kinds, but the dominant cell was a mononuclear cell with macrophage-like features (Fig. 1), similar to the type A synoviocyte in rheumatoid arthritis (Barland *et al.*, 1962). Borders were irregular, with many filopodia which were often long and branched. The cytoplasm contained a variety of vacuoles, primary lysosomes, and cytolysosomes filled with debris. Other features were cytoplasmic filaments, prominent Golgi zones, free ribosomes but little endoplasmic reticulum, mitochondria (sometimes swollen with disrupted cristae), and occasional myelin figures and lipid droplets. Between the cells were granular debris, fibrin, and a few collagen fibres.

BEHAVIOUR OF SYNOVIAL CELLS IN CULTURE
Within 24 hours synovial cells from all sources attached firmly to the culture surface and assumed various shapes by flattening and extending cytoplasmic projections (Fig. 2). At this stage some cells were elongated, with 2 or more projections, although few could be identified unequivocally as 'fibroblasts'. Others remained more or less circular in outline. Cytoplasmic detail in the smaller cells was often difficult to assess by phase contrast, but stained preparations showed the presence of 1–3% multinuclear cells (polykaryocytes = polykaryons).

Growth and morphology later in culture were related to the origin of the cultures and the type and concentration of serum in the culture medium.

(i) Nonrheumatoid synovial cells
Typically there was a 'lag' phase of 2–10 days in which cell numbers did not change appreciably in any growth medium. In this period some cells assumed the elongated forms of 'fibroblasts'; others appeared stellate or 'tulip-shaped'. The remainder showed morphological characteristics of macrophages. These were either small and irregular with 2 or more projections or circular cells with a relatively smooth margin. The fibroblasts later multiplied and formed confluent monolayers which could be subcultured repeatedly. In medium containing human serum, some of the macrophages enlarged considerably and a few polykaryocytes appeared. The latter usually contained less than 6 nuclei. Few of the macrophages could be dislodged by trypsin and only small numbers could be found in subcultivated cells.
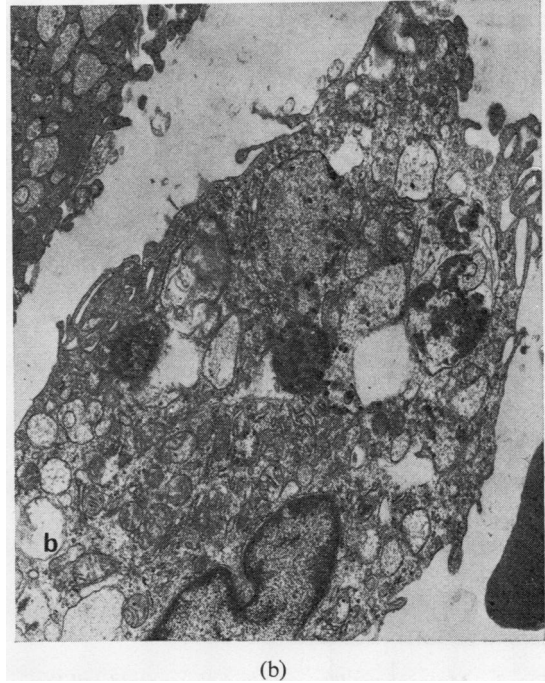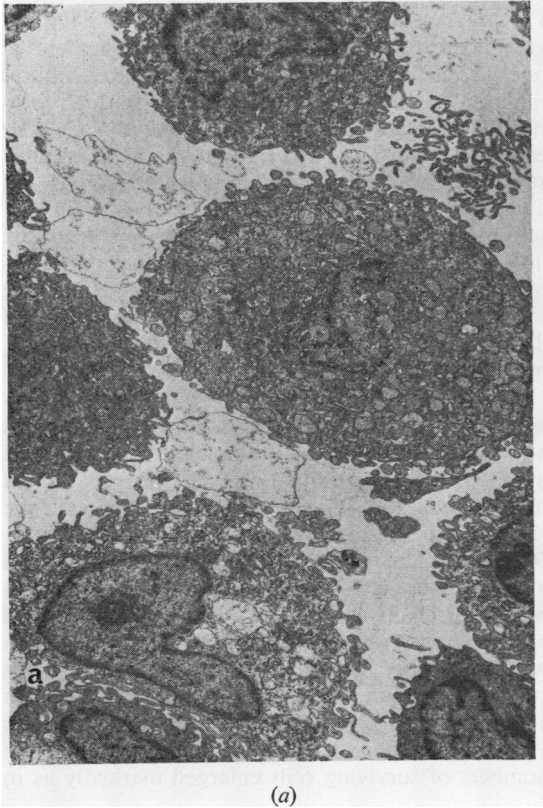
(a)

(b)

Fig. 1 *Electron micrographs of cells washed from an intact rheumatoid knee joint (Case C.S.) after trypsin instillation. (a) Group of macrophage-like cells characterized by rounded outline with numerous filopodia, many vacuoles and mitochondria, and prominent Golgi zones. × 4300. (b) Section of a macrophage-like cell showing features typical of type A synovial lining cells. × 8750.*

Fig. 2 *Synovial cells in culture 24 hours after isolation from an intact rheumatoid knee joint (Case A.V.), showing heterogeneous morphology of the cells. Phase contrast × 240.*

(ii) Rheumatoid synovial cells from excised tissue
Growth and culture behaviour were similar to non-rheumatoid synovial cells except that polykaryocytes usually became more numerous and some syncytial cells with many nuclei appeared (Fig. 3).

(iii) Rheumatoid synovial cells from intact joints
Cell growth was rarely sufficient to permit successful subculture irrespective of composition of the growth medium, although late multiplication of fibroblast-like cells occasionally occurred in widely separated patches. With human serum in the culture medium, the macrophage-like cells typically enlarged to several times their original diameter and after 3–7 days cultures often consisted predominantly of round or polygonal strongly phagocytic cells up to 80 μm in diameter. Many cells developed dense clusters of organelles around the nucleus, which was often partially or totally obscured. Some macrophages appeared to be attached to the substratum by filamentous processes radiating from the entire

periphery. Large round cells were often seen with a single long process extending from the central cytoplasm rather than from the margin (Fig. 4).

Fig. 3   *Macrophage-like cells in a rheumatoid cell line isolated from excised synovium (Case M.C.), after 21 days in medium containing human and fetal calf serum (20% each). A large polykaryocyte is surrounded by smaller macrophage-like cells with dense central clusters of organelles. Phase contrast ×240.*

Polykaryocytes became common after 3–7 days and giant syncytial cells containing large numbers of nuclei could often be found (Fig. 5). In later cultures multiple small vacuoles appeared in a high proportion of the enlarged macrophages. Cellular enlargement and polykaryocytosis were equally striking in nonarthritic serum and homologous and autologous rheumatoid serum. In contrast, fetal calf serum (FCS) or newborn calf serum alone produced little increase in cell size or numbers of polykaryocytes even after months of culture. In adult horse serum macrophages began to enlarge after several days in culture, and some polykaryocytes formed, most with less than 6 nuclei.

The effects of human and bovine sera on polykaryocytosis were compared by counting polykaryons and mononuclear cells in multiple randomly selected microscope fields, using living cultures or stained preparations. Fig. 6 shows the typical reaction of the rheumatoid cell lines isolated from intact joints. In 50% FCS the polykaryon index (PI) remained approximately constant throughout the 13 days in both heat-inactivated (56°C, 30 min) and unheated serum. In contrast, changes in 50% human serum were even more rapid than those seen previously in cultures grown in mixed FCS and human serum. In 10% human serum, cell numbers declined and the PI increased only slightly, although considerable numbers of surviving cells enlarged markedly as in
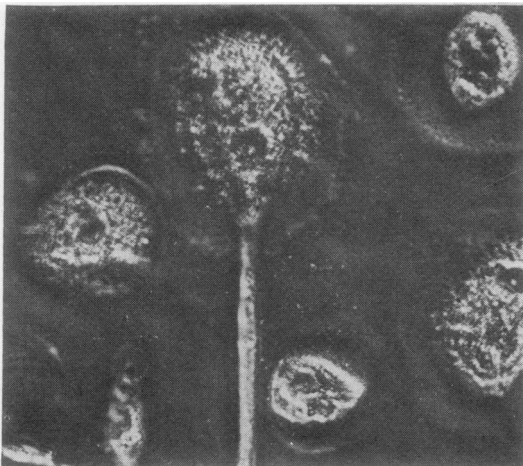

Fig. 4   *Macrophage-like cells in a synovial cell line isolated from an intact knee joint and cultivated in medium containing nonrheumatoid human serum. Note the cytoplasmic process extending from the nuclear region. Phase contrast ×300.*
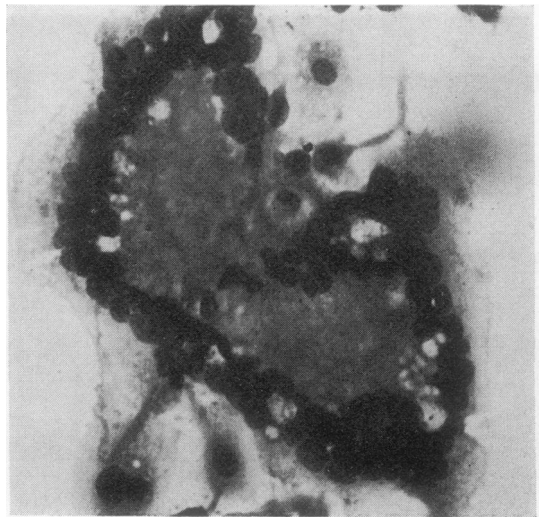

Fig. 5   *A giant syncytial cell in a rheumatoid synovial cell line isolated from an intact knee joint (Case M.S.), after 11 days in 50% heat-inactivated nonrheumatoid serum. Cells fixed in methanol, stained with Mayer's haematoxylin-eosin ×330.*
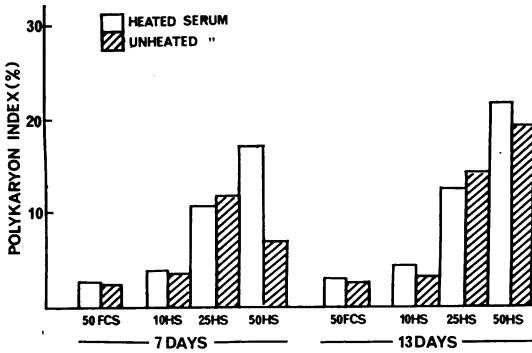
Fig. 6 *Effect of 50% fetal calf serum (FCS) and varying concentrations of nonrheumatoid human serum (HS) on the polykaryon index (percentage of the counted nuclei found in polykaryons) of a rheumatoid synovial cell line isolated from an intact knee joint (Case M.G.).*

the higher serum concentration. In 25% human serum changes in cell size and numbers of polykaryocytes were intermediate between 10% and 50%. The effects of human serum were similar whether heated or unheated.

Fig. 7 shows the distribution of polykaryons in one of the rheumatoid cell lines from an intact joint, grown in 50% FCS or human serum. In the former nearly all polykaryons contained 2 or 3 nuclei through the 11 days of observation. However, in 50% human serum the proportion of cells with 2 or 3 nuclei declined as cells with much greater numbers of nuclei appeared.
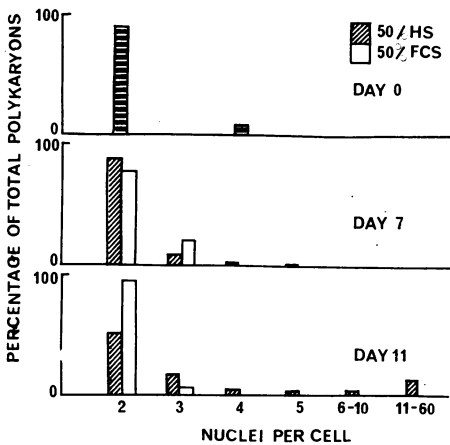

Fig. 7 *Distribution of numbers of nuclei per polykaryon in a rheumatoid cell line isolated from an intact knee joint (Case M.S.). The effect of 50% FCS is compared with 50% nonrheumatoid HS. Vertical bars show numbers of cells with designated number of nuclei, as a percentage of total polykaryons counted. Bars at day 0 show polykaryons before serum treatment.*

Fig. 8 shows the pattern of polykaryon formation in four nonarthritic synovial cultures in the primary phase. Polykaryons were often obscured by rapid overgrowth of fibroblasts, e.g. in 50% human serum, cell lines 1N and 8N could not be counted accurately after 5 and 9 days respectively. Some polykaryons formed but were proportionately fewer than in rheumatoid cultures and rarely contained more than 2 or 3 nuclei.

ELECTRON MICROSCOPY OF CULTURED RHEUMA-
TOID CELLS FROM INTACT JOINTS (FIG. 9)
The cells were much larger than the cells originally washed from the joint. Although there were many polykaryons in the cells scraped from the culture surface, few could be recognized as such in electron micrograph sections, probably due to thinness of the sections or orientation of cells. The cytoplasm was pleomorphic and showed the same features found in the macrophages before culture.

Evidence of viral infection has been sought in a small number of these cell lines and virus-like particles were found in one of the cultures. These consisted of intranuclear structures of constant size (approximately 80 nm in diameter) occurring in many cells. Some were closely grouped in clumps, others were scattered singly through the nucleus. Occasionally a dense core was seen and less commonly a halo surrounded the particle. The outer margin was irregular, suggesting either 'spikes' or a many-faceted surface. The identity of the structures is uncertain but their size, shape, and location are consistent with virions of the adenovirus group. The nuclei and the surrounding cytoplasm showed no other unique features. The particles were not found in the cytoplasm or seen to bud from the nuclear or plasma membrane.
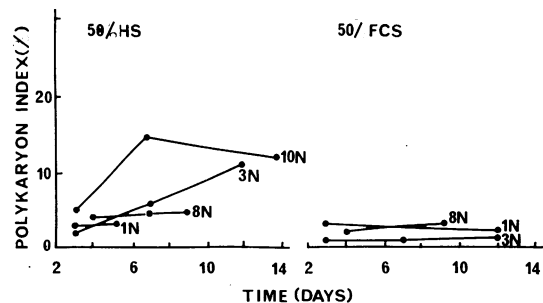

Fig. 8 *Change of polykaryon index (see Fig. 6) of 4 nonrheumatoid synovial cell lines in 50% nonrheumatoid HS and 50% FCS. N = cell line.*

Discussion

Synovial cells in primary cultures were morphologically heterogeneous regardless of the source. After spreading on culture surfaces, cells with macrophage-like or fibroblast-like features could be recognized. The relative proportions of these cell-types varied considerably from one isolation to another, but rheumatoid synovial cells from intact joints were invariably distinguished by high proportions of macrophage-like cells, which is consistent with the preponderance of type A synoviocytes in the surface of rheumatoid synovium (Ghadially and Roy, 1969). These cultures might also include inflammatory macrophages of haematogenous origin.

When rheumatoid synovial cells were first isolated from an intact joint in 1964, specific differences were not observed in the primary culture and the subsequent life history of the cell line was typical of the many nonrheumatoid synovial cell lines grown in this laboratory, and of rheumatoid cells obtained by trypsinizing cellular material scraped from the surface of synovial tissue excised during surgery. Thus, although polykaryocytosis has long been recognized as a feature of human rheumatoid synovial cells in culture (Lackington, 1959; Stanfield and Stephens, 1963; Bartfeld, 1965), the morphological peculiarities and apparently suppressed growth of the rheumatoid cell lines from intact joints examined in the present study were somewhat unexpected.

Palmer (1971) examined the influence of rheumatoid serum on synovial cells. Giant syncytia were found to occur in rheumatoid synovial cells from trypsin-dispersed tissue when cultured in heat-inactivated rheumatoid serum, but did not develop in synovial cells from osteoarthrosis. Palmer also noted suppressed growth of fibroblasts in rheumatoid synovial cultures, apparently related to the number of macrophages present.

In the present study rheumatoid synovial cells from intact joints were compared with rheumatoid
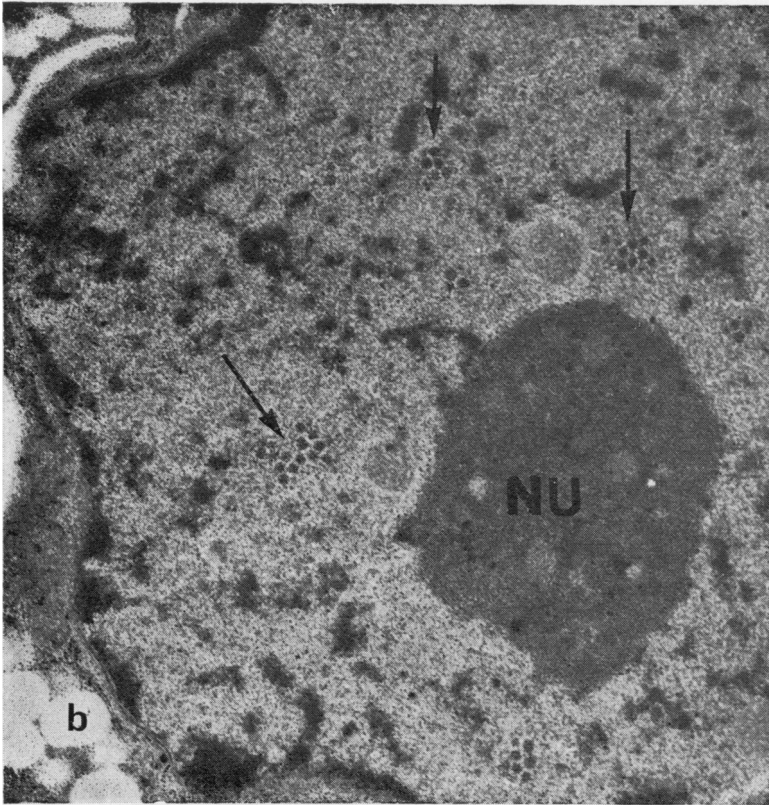
(a)

(b)

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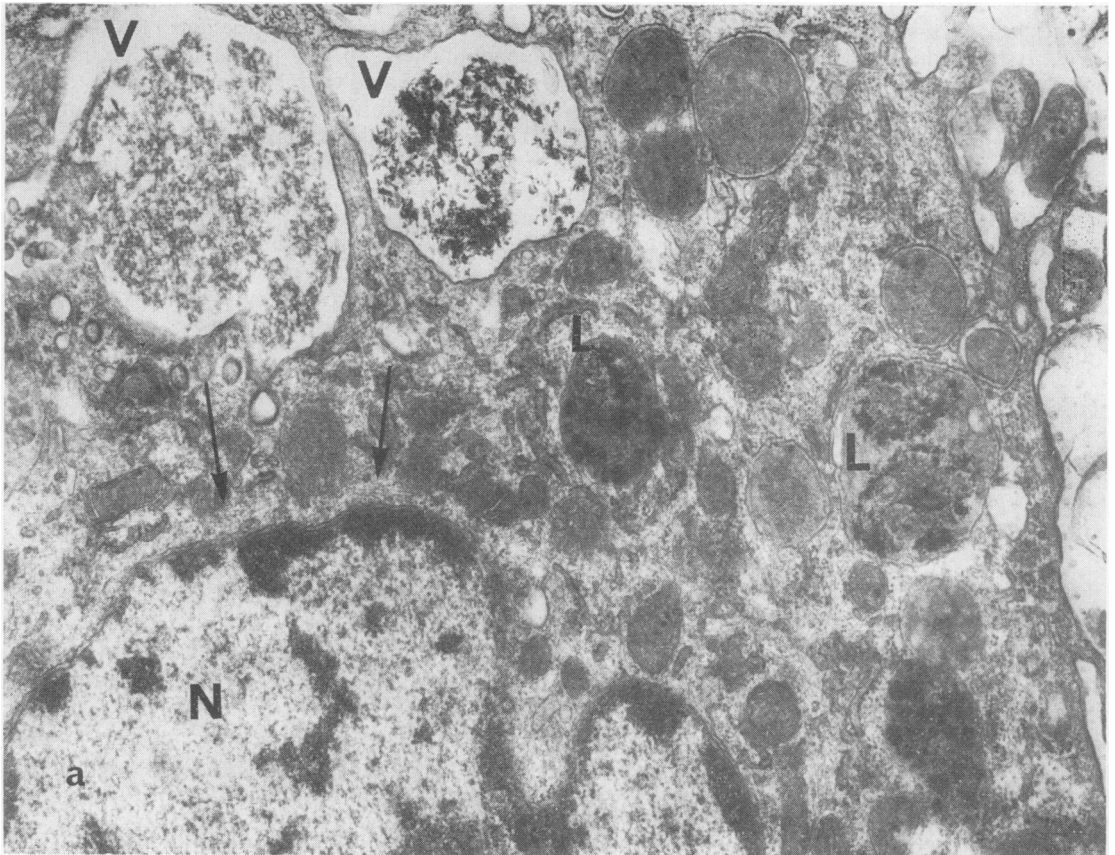
Fig. 9 *Electron micrographs of cells isolated from an intact joint of a rheumatoid patient (Case J.B.) and cultured for 31 days in 50% nonrheumatoid human serum. (a) Cytoplasmic features of a typical enlarged macrophage-like cell. Large vacuoles (V) containing debris apparently phagocytosed from the cell environs, and several lysosomes (L) can be seen. The nucleus (N) is surrounded by a narrow zone (arrows) apparently containing microtubules. × 32 300. (b) Portion of a nucleus showing clusters of virus-like particles (arrows) with diameter of 80 nm in the vicinity of the nucleolus (NU). × 20 000. (c) A higher power section including virus-like particles (arrows), showing dense cores in some, and a granular surface suggesting 'spikes' or multifaceted structure. × 27 500.*

cells from excised synovium and nonrheumatoid cells from intact joints of cadavers or amputated limbs. Responses were examined in a range of serum types including rheumatoid and nonrheumatoid sera and serum from animals. After 24 hours in culture, only 1–3% of the synovial cells were multinucleate. No clear-cut difference could be established between the proportion of polykaryocytes in rheumatoid and nonrheumatoid synovial cells, although such a difference was found by Kinsella *et al.* (1970) in trypsin-dispersed synovial cells before culture. Polykaryocytes occurred as both macrophage-like and fibroblast-like cells but the former predominated greatly. Occasionally, larger polykaryocytes with 8–12 nuclei were detected in the first 24 hours of culture, possibly representing the giant cells identified in rheumatoid synovial tissue (Grimley and Sokoloff, 1966).

In fetal calf or newborn calf serum rheumatoid synovial cells from intact joints remained virtually unaltered in appearance or numbers, sometimes for months in culture. Nonrheumatoid synovial cells or rheumatoid cells from excised tissues cultured in similar media were usually overgrown with fibroblast-like cells within 6–30 days. When human
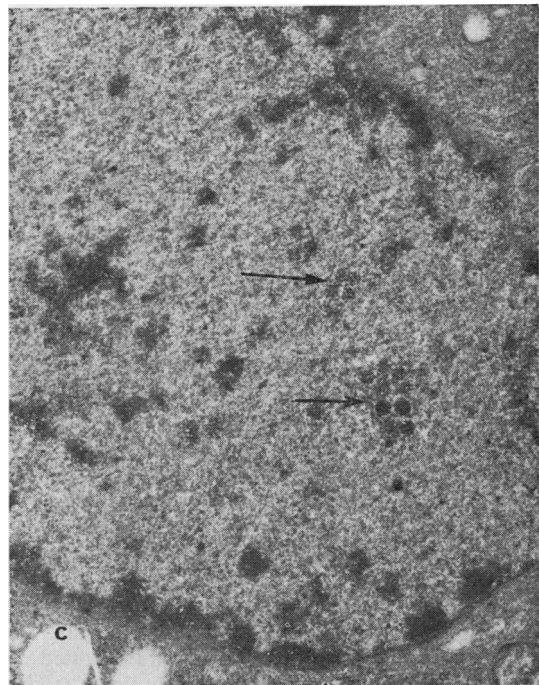
serum was included in the medium, macrophages in the rheumatoid cultures enlarged rapidly and large polykaryocytes appeared. Polykaryocytes became particularly numerous in the rheumatoid cultures from intact joints and the process was accelerated when human serum alone was used to supplement the medium, but growth of fibroblasts usually remained suppressed. The rate of the morphological changes in these rheumatoid synovial cells was directly proportional to serum concentration. Similar changes could be produced when rheumatoid synovial cells cultured for long periods in fetal calf serum were changed to human serum. The effects of human serum were not altered by mild heating. Non-rheumatoid serum and rheumatoid serum appeared to be equally active in inducing polykaryocytosis. Interactions between rheumatoid synovial cells and serum were not species specific since some polykaryocytosis also occurred in medium containing adult horse serum. Polykaryocytosis also occurred in some nonrheumatoid cell lines, although the changes were comparatively slow and it was unusual to find cells with more than 6 nuclei.

These findings support other evidence (Palmer, 1971; Mackay *et al.*, 1974) that rheumatoid synovial cells are especially prone to polykaryocytosis *in vitro*; although negligible data are available on comparable *in vitro* responses of synovial cells from connective tissue diseases related to rheumatoid arthritis. Since blood macrophages from rheumatoid subjects also form numerous polykaryocytes *in vitro* (Panayi *et al.*, 1974), it is not clear whether the responses of rheumatoid synovial cells to human serum are associated with type A synoviocytes alone or also with infiltrating macrophages from the blood stream.

Although polykaryocytes of limited size can develop by amitotic division (Roizman and Schlue-derberg, 1962), the larger syncytia seen in rheumatoid synovial cells in the present study are more likely to form by fusion between cells. This is supported by observations of nuclei in cytoplasmic bridges between rheumatoid synovial macrophages (Palmer, 1971) and in cytoplasm linking fusing blood macrophages (Sutton and Weiss, 1966). Further investigation would be required to elucidate the nature of the factor in serum responsible for induction of cell fusion.

The apparent relationship between poor growth of rheumatoid synovial cells and the presence of macrophage-like cells suggests that the former inhibit mitosis in neighbouring cells (Palmer, 1971). In a later study Palmer (1975) was unable to show direct inhibition of fibroblast growth by rheumatoid cultures. However, soluble inhibitors secreted by macrophages have been found in animal studies (Nelson, 1973; Calderon *et al.*, 1974). Work is underway in this laboratory to investigate the effect of rheumatoid macrophages on cell growth and the reason for the difference in growth behaviour found between rheumatoid synovial cells from intact joints and excised tissue.

Viruses have been much sought in rheumatoid synovium and cultured synovial cells. The large syncytia seen in the rheumatoid cultures might reflect activation of a syncytium-inducing virus (Roizman, 1962). A variety of unidentified structures, which might be viruses, have been found in rheumatoid synovium by electron microscopy (Highton *et al.*, 1966; Györkey *et al.*, 1972; Serre *et al.*, 1972; Schumacher, 1975). However, no relationship has yet been established between viruses or other organisms and causation of rheumatoid arthritis. In the present study intranuclear inclusions were found in synovial cells from one patient with rheumatoid arthritis, after extended culture in human serum. The structure of the particles strongly suggested virions of the adenovirus group. However, since these viruses are extremely common, their occurrence may have been purely by chance and investigation of the significance of this initial finding is continuing.

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