

Synovial and peritoneal macrophages in organoid culture

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Summary. Cultivation of macrophages and their progenitors has been very useful for elucidation of function, behaviour and morphology of these cells. The purpose of this contribution is to describe a new in vitro system (organoid, high density or micromass culture) which proved to be convenient for cultivation of macrophages derived from human synovial fluid and tissue and mouse peritoneal fluid. Using this method, highly differentiated and functionally active macrophages of marked purity and long maintenance (up to 2 weeks) could be obtained even after previous cultivation and subcultivation in monolayer culture. The macrophages were identified by electron microscopy and immunomorphology using HLA-DR-DP, CD-68 (markers for human macrophages), anti-human-polymorphonuclear leukocyte-gelatinase and F4/80 (a mouse macrophage surface marker). The significance of this method as a research tool in the study of cartilage degradation by macrophages in co-cultures is stressed.

Key words: Macrophages, Rheumatoid synovial fluid, Rheumatoid synovial tissue, Organoid culture

Introduction

Considerable information about macrophages has been accumulated within the last years, especially due to marked technical advances of cell culture methods (Johnson et al., 1977; Hammerstrom, 1979; Zuckerman et al., 1979). Since the first chick macrophages were obtained in monolayers (Baker, 1933) numerous methods have been devised to study macrophages in vitro (Parker 1938; Jacoby, 1944). Most of these methods, which are based on monolayer culture techniques, suffered from the following disadvantages:

1. difficulties in obtaining pure macrophage populations.
2. restricted maintenance of the cultures.
3. low yield of differentiated macrophages.

Such disadvantages are conspicuous, especially when dealing with synovial fluid or synovial tissue. These synovial materials contain different cell populations which are predominantly composed of fibroblast-like B-cells, macrophage-like A-cells, PMN-granulocytes, monocytes and lymphocytes.

The heterogeneity of the cell populations varies rapidly due to elimination of PMN granulocytes within a few days (Jacoby, 1965; D'Onofrio and Paradisi, 1983) and later due to overgrowth of fibroblastic cells.

Looking for improved in vitro methods, an organoid or high density culture system seemed to be most suitable for the cultivation of macrophages. It was herewith possible to obtain pure populations of differentiated macrophages which could be maintained for up to 2 weeks in culture. This method has become very important for the study of cellular differentiation. Cells from different organs and tissues (cartilage, bone, liver, lungs, bowel, and neoplasms) has been successfully cultivated in organoid culture (see «Discussion»).

Since human synovial material was not available whenever needed, mouse peritoneal fluid, as the best known mammalian source for obtaining macrophages, was also employed in our experiments. Mouse peritoneal macrophages can be considered as a useful alternative to synovial macrophages, for instance, for co-cultivation with cartilage in experiments designed for studying matrix breakdown (Shakibaei and Mohamed-Ali, 1994), since macrophages of different species and origin share important functional characteristics despite their structural and functional heterogeneity (Furth et al., 1972; Auger and Ross, 1992).

Materials and methods

Synovial cell cultures

Synovial fluid and synovial tissue from patients with rheumatoid arthritis were prepared as previously described (Mohamed-Ali et al., 1991; Mohamed-Ali, 1992). Briefly, the synovial cells were initially grown for

3-4 days as monolayer cultures. After subcultivation once or twice (according to the cell density), the monolayer cultures were digested by trypsin-EDTA and centrifuged at 180 g for 10 min. The cell pellet was resuspended with a small amount of growth medium, consisting of Dulbecco's modified medium supplemented with 15% foetal calf serum (Gibco-BRL), penicillin, streptomycin, amphotericin B, L-glutamin and vitamin C (Biochrom KG, Berlin, Germany). Finally, 10- μ l drops (cell density about 3×10^6 /ml) of the sediment suspension were pipetted onto a sartorius filter (pore size 0.2 μ m), placed on a V4A stainless steel bridge at the medium/air interphase, and the cells were then grown at high density in organoid culture at 37 °C in a humidified atmosphere with 5% CO₂ and 95% air for 7-14 days. The medium was changed daily.

Mouse peritoneal macrophage cultures

Macrophages of peritoneal fluid from NMRI mice were isolated and cultivated according to the method of Stuart et al. (1978). Briefly, the peritoneal cell suspension was centrifuged at 200 g for 10 min and the pellet subsequently resuspended in RPMI 1640 (Biochrom KG, Berlin, Germany)/FCS medium. The cell density was adjusted to 1×10^6 /ml in RPMI 1640 + 15% FCS and the cell suspension was then cultivated either as monolayer culture for 1 day or as organoid culture for 4-5 days.

Immunofluorescence:

A) Organoid cultures of both synovial and mouse macrophages were dipped into O.C.T. embedding compound (Bayer, München, Germany) and snap-frozen in liquid nitrogen. 8 μ m-thick sections were cut for indirect immunolabelling according to Barrach and Angermann (1977):

1) The sections of synovial cell cultures were incubated with 1:20 diluted primary antibodies (purchased from Serva Feinbiochemica, Heidelberg, Germany) against surface antigens of human monocytes and macrophages (Anti-HLA-DR/DP) and with antibodies against cytoplasmic antigens which are associated with lysosomal granules in human monocytes and macrophages (CD 68) for 2 h at room temperature and with 1:10 diluted Anti-Human-Fibroblast and Anti-Human-PMNL-gelatinases (prepared by Dr. Kolkenbrock/Institute of Biochemistry, Berlin, Germany) for 1 h at room temperature.

2) The sections of mouse macrophage cultures were incubated with the specific cell surface marker F4/80 (kindly provided by Prof. Gordon, Oxford, England) for 2 h at room temperature.

After rinsing all the samples for 3 x 5 min in PBS, the sections of synovial cell cultures were incubated with 1:30 diluted second antibodies RAM-FITC: conjugated Rabbit-Anti-Mouse and SAR-FITC: Swine-Anti-Rabbit immunoglobulins (Dakopatts, Denmark) for 1 h at room

temperature and the sections of mouse peritoneal macrophage cultures were incubated with GARa-FITC: conjugated Goat-Anti-Rat gammaglobulins (Dianova, Hamburg, Germany) for 1 h at room temperature.

Finally, the sections were rinsed for 3 x 5 min in PBS, air-dried, coverslipped with glycerin and photographed using a Universal-Microscope Axioplan (Zeiss).

B) Monolayer cultures of both synovial and mouse macrophages (grown on glass covers placed in Costar-Multiwell-Plates) were snap-frozen in liquid nitrogen and the monolayer cultures of synovial cells were further treated as in the case of synovial organoid cultures (see above).

The monolayer cultures of mouse macrophages (cultivated for 17 hours) were prepared for immunofluorescence staining according to Coons et al. (1941). After incubation with the primary antibodies F4/80, Anti-Human-Fibroblast-gelatinases and Anti-Human-PMNL-gelatinases for 60 min in a humidified chamber at room temperature and subsequent treatment with the second antibodies GARa-FITC and SAR-FITC (diluted 1:50 with PBS) for 60 min at room temperature, the cultures were counterstained with Evans' blue (Merck, Darmstadt, Germany), air dried, coverslipped with fluoromount mountant (BDH, England) and photographed using an Axiophot 100 light microscope (Zeiss).

Electron microscopy

The cultures of human synovial cells and mouse peritoneal macrophages were fixed in a mixture of 2% glutaraldehyde with 1% tannic acid in 0.1M phosphate buffer, pH 7.4, and postfixed in a 1% OsO₄ solution in phosphate buffer. After rinsing and dehydration in graded ethanol, the cultures were embedded in Epon, cut with an ultracut (Reichert) and the sections contrasted with 2% uranyl acetate/lead citrate. The specimens were examined with an EM 10 transmission electron microscope (Zeiss).

Immunoelectron microscopy

a) Immunolabelling of mouse peritoneal macrophages with the cell surface marker F4/80 (pre-embedding technique) for TEM:

Peritoneal macrophages were incubated with the primary antibody F4/80 (diluted 1:10 in PBS/BSA) for 5 min immediately after isolation and subsequently treated with the second antibody GARa 10 nm (diluted 1:30 in PBS/BSA) for 5 min. These procedures were carried out at 4 °C. Finally, the cells were fixed in 1% glutaraldehyde and contrasted with 2% OsO₄. These procedures were carried out at room temperature. After rinsing and dehydration in graded ethanol the cells were embedded in Epon, cut with an Ultracut (Reichert) and the sections contrasted with 2% uranyl acetate/lead

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citrate. All specimens were then examined with an EM 10 electron microscope (Zeiss).

b) Immunolabelling of mouse peritoneal macrophages with the cell surface marker F4/80 for SEM:

For this purpose the macrophages were cultivated on thermanox covers (Thermanox Plano, Marburg, Germany) for 17 hours. The cultures were fixed in 1% glutaraldehyde and subsequently processed as discussed above (see: a). This was followed by incubation with the second antibody GARa (labelled with 10 nm gold particles) and silver-enhancement (IntenSE, Amersham, Braunschweig, Germany) for 20 min. Finally, the cultures were postfixed with 1% OsO₄, dehydrated in graded ethanol, treated with HMDS: Hexamethyldisilazan (Sigma, München) and air-dried. The specimens were metallized with gold in a cathode vapouriser installation (Technics, Hummer V) and examined with Cambridge Stereoscan SEM (250 MK2) of 20 KV.

Results

I. Synovial cell cultures

The morphological features of synovial cells in monolayer culture are not a matter to be discussed here. This has been extensively described elsewhere (Mohamed-Ali et al., 1991). Briefly, after 2-3 days in monolayer culture synovial cells from both synovial fluid and tissue were still heterogeneous, mainly consisting of fibroblast-like cells (B-cells), macrophage-like cells (A-cells), monocytes and a number of special cells (polycaryocytes, dendritic cells and stellate cells). After 4-7 days the remaining cell populations were mostly fibroblast-like and macrophage-like cells.

The macrophages in organoid culture were large cells with abundant cytoplasm lodging numerous organelles. The cell surface was very irregular, bearing processes of varying length and width with interposed invaginations and blunt protrusions. The cell nucleus presented an oval to round profile, containing, in addition to euchromatin and heterochromatin, a pronounced nucleolus (Figs. 1B, 2A).

The cytoplasm appeared enriched with lysosome-like structures which could be assigned (due to morphological features) to primary and secondary lysosomes. The primary lysosomes were usually small in size and membrane-bordered, containing homogeneous electron-dense material.

In addition, straight elongated (up to 1000 nm) very narrow structures showing a content of 20-40 nm width with terminal extensions were seen. They predominantly presented curved profiles and in cases of appropriate sections through these cup-shaped structures they seemed circular. These structures are also ascribed to primary lysosomes (Ghadially, 1988). The secondary lysosomes appeared to be bigger and also membrane-bordered, mostly containing homogeneous electron-dense material. However, certain areas with differently

granulated material, membrane fragments and occasionally vacuoles could be demonstrated. In addition, vacuoles (30-120 nm) predominantly of an empty appearance, which probably represented endosomes, were present. Numerous coated and uncoated vesicles adjacent to well pronounced Golgi-complexes as well as high activity of endocytosis were also observed. The rough endoplasmic reticulum was markedly developed and numerous straight elongated mitochondria of the Crista-type were dispersed among the other organelles (Fig. 2B).

The few monocytes in the organoid culture were smaller than macrophages and showed a curved or lobed nucleus. The cytoplasm contained a smaller number of organelles than in macrophages, causing a luminous appearance of the cells. The cell surface was smooth, exhibiting only a few processes and a slightly ruffled membrane, thus distinguishing them from macrophages (Fig. 1A).

Immunomorphology of synovial macrophages in monolayer and organoid cultures:

Using the antibodies against cell surface antigen (HLA-DR-DP) and lysosomal-associated antigen (CD-68) of human macrophages as well as anti-human-PMNL-gelatinase, a varied proportion of cells in monolayer culture could be stained (not shown). In organoid culture (Fig. 3) almost all cells exhibited positive immunostaining with the three antibodies mentioned above, while there was no evidence for immunostaining with anti-human-fibroblast-gelatinase.

II. Mouse peritoneal macrophage cultures

The morphological features of mouse peritoneal macrophages, when cultivated in monolayer culture (Fig. 4B), have been thoroughly discussed in several previous works (North and Mackaness, 1963; Cohn et al., 1966; Carr, 1967, 1973) and need not be repeated here. The electron microscopic appearance of mouse peritoneal macrophages in organoid culture corresponded to the description of the synovial cells (see above) and did not differ from that of monolayer cultures. The cells were large, showing a highly irregular surface due to numerous prominent processes of various length and shape. The abundant cytoplasm contained numerous lysosomal dense bodies (primary and secondary lysosomes), vesicles and vacuoles (Fig. 5A). The cell density of the cultures (mostly 2-3 layers), however, was not pronounced to such an extent as in the synovial cell cultures.

Immunomorphology of mouse macrophages in monolayer culture:

Immunofluorescence assay (IFA): Immunomorphological investigations of macrophages cultivated for 24 hours in monolayer culture yielded a positive

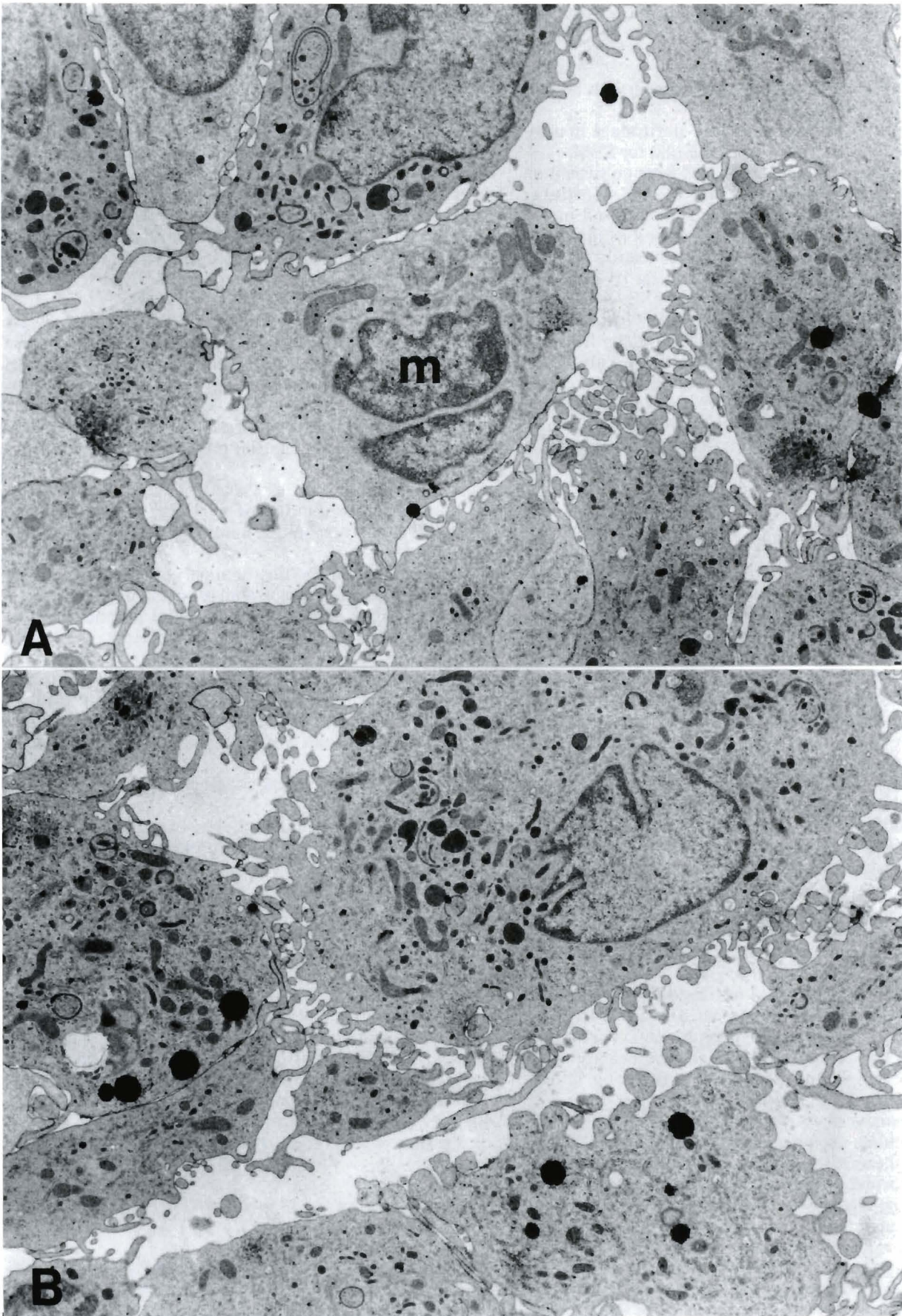


Fig. 1. Synovial cells in organoid culture. **A.** Culture period: 7 days. Monocyte-like cells (m) with a few processes and cell organelles, and indented nucleus. These cells are surrounded by macrophages with a very irregular surface and numerous cell organelles. x 8,000. **B.** culture period: 12 days. Macrophages showing numerous processes, i.e. irregular surface, and cell organelles. x 8,000

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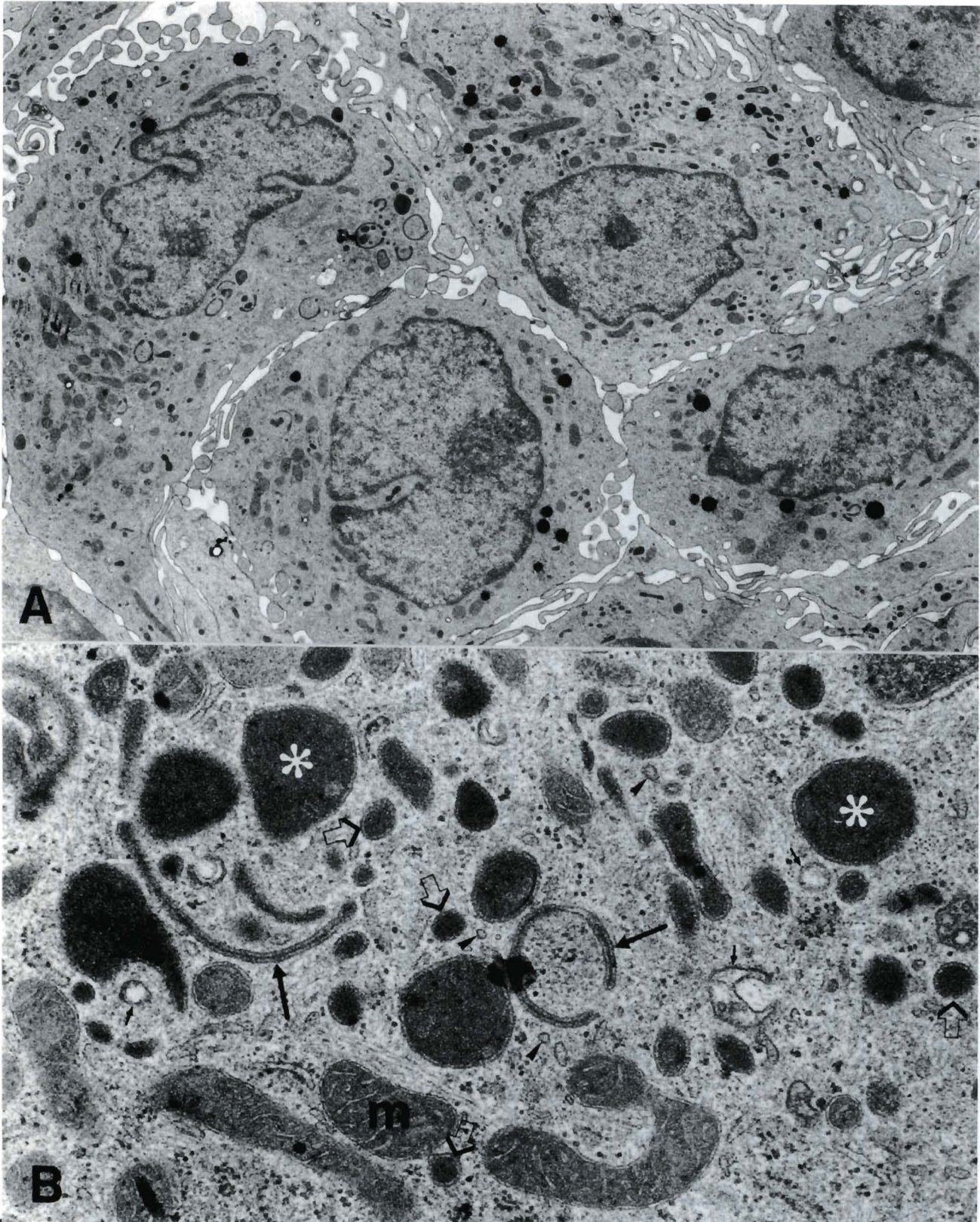


Fig. 2. **A.** Macrophages from synovial tissue in a 7-day-old organoid culture after previous cultivation in monolayer culture for 2 passages (= 7 days). Numerous processes and cell organelles. $\times 8,000$. **B.** Section of fig. 1B. Part of a macrophage in organoid culture from synovial cells showing numerous cell organelles, such as mitochondria (m), lysosome-like inclusions, probably primary (arrows and open arrows) and secondary (*) lysosomes, coated vesicles (arrowhead) and endosomes (small arrows). $\times 45,000$

reaction (Figs. 4-6) with the specific monoclonal antibody (F4/80) against mouse macrophage surface antigen which was diffusely distributed over the entire surface of the cells. This was clearly demonstrated by

TEM (Fig. 5B). In SEM macrophages could be identified due to numerous processes of varying length and width causing a marked irregularity of the cell surface (Fig. 6). The cell surface markers were clearly

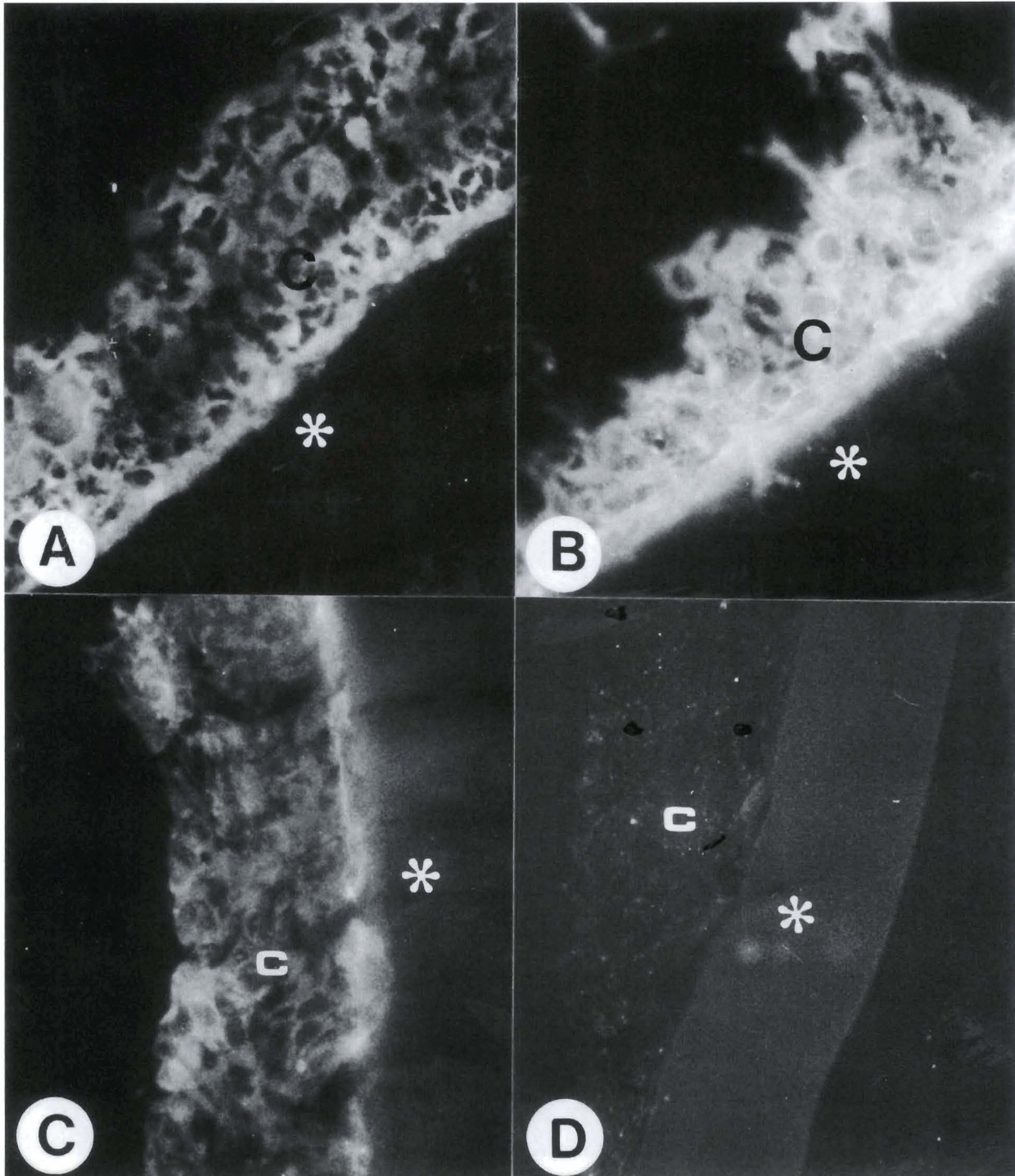


Fig. 3. Synovial cells in a 7-day-old organoid culture. *: filter, c: densely packed macrophage cells. Immunofluorescence microscopic demonstration of HLA-DR-DP (A), CD-68 (B), PMNL-gelatinase (C) and fibroblast-gelatinase (D). x 160

demonstrated with gold-labelled second antibody and silver-enhancement (Fig. 6).

Immunomorphology of mouse macrophages in organoid culture:

Immunofluorescence assay (IFA): The immunoreactivity of the cells cultivated in organoid culture for 4 days was strongly positive with the specific antibody (F4/80) against macrophage surface antigen (not shown) and with anti-human-PMNL-gelatinase (Fig. 4E), while there was no reaction with anti-human-fibroblast-gelatinase (data not shown). The latter represents a polyclonal antibody raised in rabbit against human fibroblast 72 KDa-gelatinase (gelatinase A) and strongly crossreacts with mouse fibroblast-gelatinase as it has been demonstrated by immunoblot (Dr. Hecker-Kia/Institute of Biochemistry, Berlin).

Discussion

The need to look for more advanced methods to cultivate macrophages arose from the inadequacy of the hitherto used *in vitro* methods which lacked the ability to improve the yield, purity and maintenance of macrophages in culture. For this purpose the organoid high density culture was used in our experiments. This culture method has been proved to be useful for triggering and stabilizing cell differentiation processes and development of organo-typical cell pattern in various tissues, such as cartilage (Merker et al., 1981; Zimmermann et al., 1990; Schröter-Kermani et al., 1991; Ghaida and Merker, 1992), bone (Zimmermann, 1992a), liver and haematopoietic system (Elkasaby et al., 1991), lungs (Zimmermann, 1987), nervous system (Zimmermann, 1992b), intestinal epithelium (Zimmermann, 1991) and neoplasms (Köpf-Maier and Zimmermann, 1991). When several cell-types are co-cultivated in organoid culture, e.g. prechondroblasts with mesenchymal cells and epithelial cells with connective tissue cells, sorting out procedures take place, i.e. prechondroblasts develop cartilage islands within mesenchymal tissue and epithelial cells from glandular-like structures within connective tissue. Hence, the organoid culture technique represents a convenient method for cell differentiation procedures. Using this method, it was indeed possible to obtain pure macrophage populations which were highly differentiated. They exhibited specific cell surface antigens and produced gelatinases of PMNL-type.

Transmission electron micrographs demonstrated all

features of activated macrophages, such as irregular cell surface, pronounced endocytosis, primary and secondary lysosomes, large Golgi apparatus, rough endoplasmic reticulum and abundant cytoplasm. The occurrence of secondary lysosomes (phagolysosomes) might be due to ingestion of serum proteins from the culture medium and phagocytosis of cell debris.

A further advantage of the organoid culture technique is the high purity of macrophage populations gained by this method. The other cell-types, such as fibroblast-like cells and PMN-granulocytes of the synovial cells and PMN-granulocytes of mouse peritoneal fluid obviously could not survive in the organoid culture and hence disappeared rapidly. Consequently, the organoid culture seems to be more convenient for differentiation and maintenance of macrophages than monolayer culture, which merely makes a restricted maintainability of the cells possible. Finally, the organoid culture technique enables not only cells derived from the primary cultures, but also cells which were initially cultivated in monolayer culture and passaged (once or twice) to grow and differentiate. This peculiarity is of special advantage when dealing with synovial cells, as has been observed in our laboratory while studying the effects of rheumatoid synovial cells on chondrogenesis and cartilage matrix breakdown in organoid co-cultures using mouse embryonal chondrogenic tissue (Mohamed-Ali, 1992; Mohamed-Ali et al., 1993).

This favourable way of cultivating macrophages might be useful for studying the pathological mechanisms of rheumatoid arthritis, since synovial macrophages play a pivotal role in the breakdown of cartilage matrix causing articular cartilage destruction in this disease. Upon stimulation by various agents, macrophages produce and secrete large amounts of matrix metalloproteinases (MMPs) as well as miscellaneous factors which affect other cells to produce considerable amounts of degrading enzymes (Vaes, 1981).

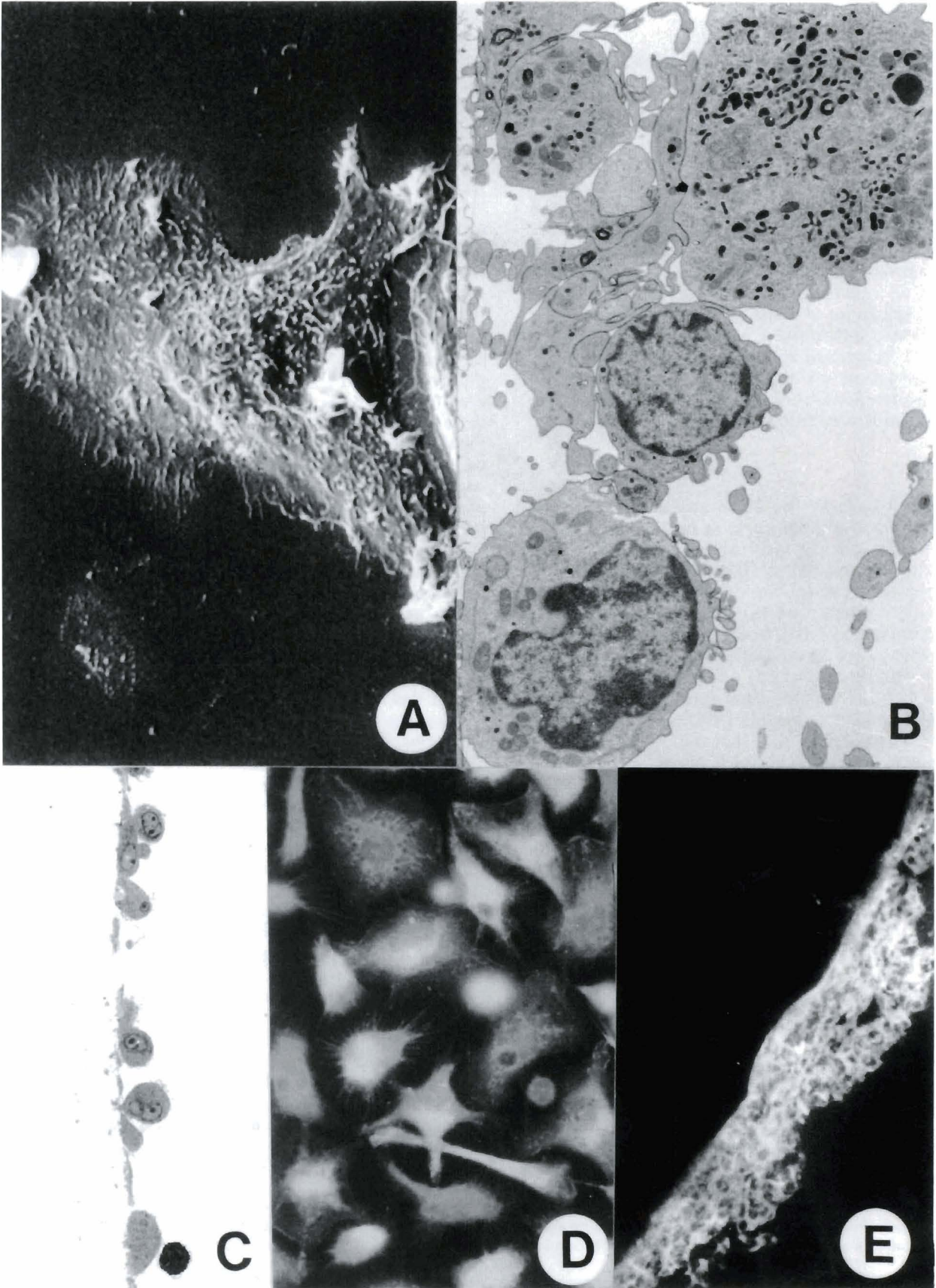
Investigation of both cellular and enzymatic mechanisms of cartilage degradation in rheumatoid arthritis which our laboratory in particular deals with, however, presupposes the availability of highly differentiated pure macrophage populations of long maintenance.

Due to high synthesis efficiency, macrophages are, additionally, involved in numerous physiological and pathological (inflammatory and immunoinflammatory) processes (Nathan, 1987) which accentuate, much more, their pure obtainability for *in vitro* studies.

Fig. 4 A-D. Monolayer culture of mouse peritoneal macrophages (culture period: 24 hours). **A.** Scanning electron microscopic picture of a macrophage with numerous processes. x 3,500. **B.** Transmission electron microscopic picture of a cross-sectioned monolayer culture. x 5,000. **C.** Like B. Light microscopic picture of a semi-thick section. x 180. **D.** Fluorescence microscopic demonstration of F4/80 (a surface marker). x 240

Fig. 4E. Fluorescence microscopic demonstration of PMNL-gelatinase in a cross-section of an organoid culture of mouse peritoneal macrophages (culture period: 4 days). x 160

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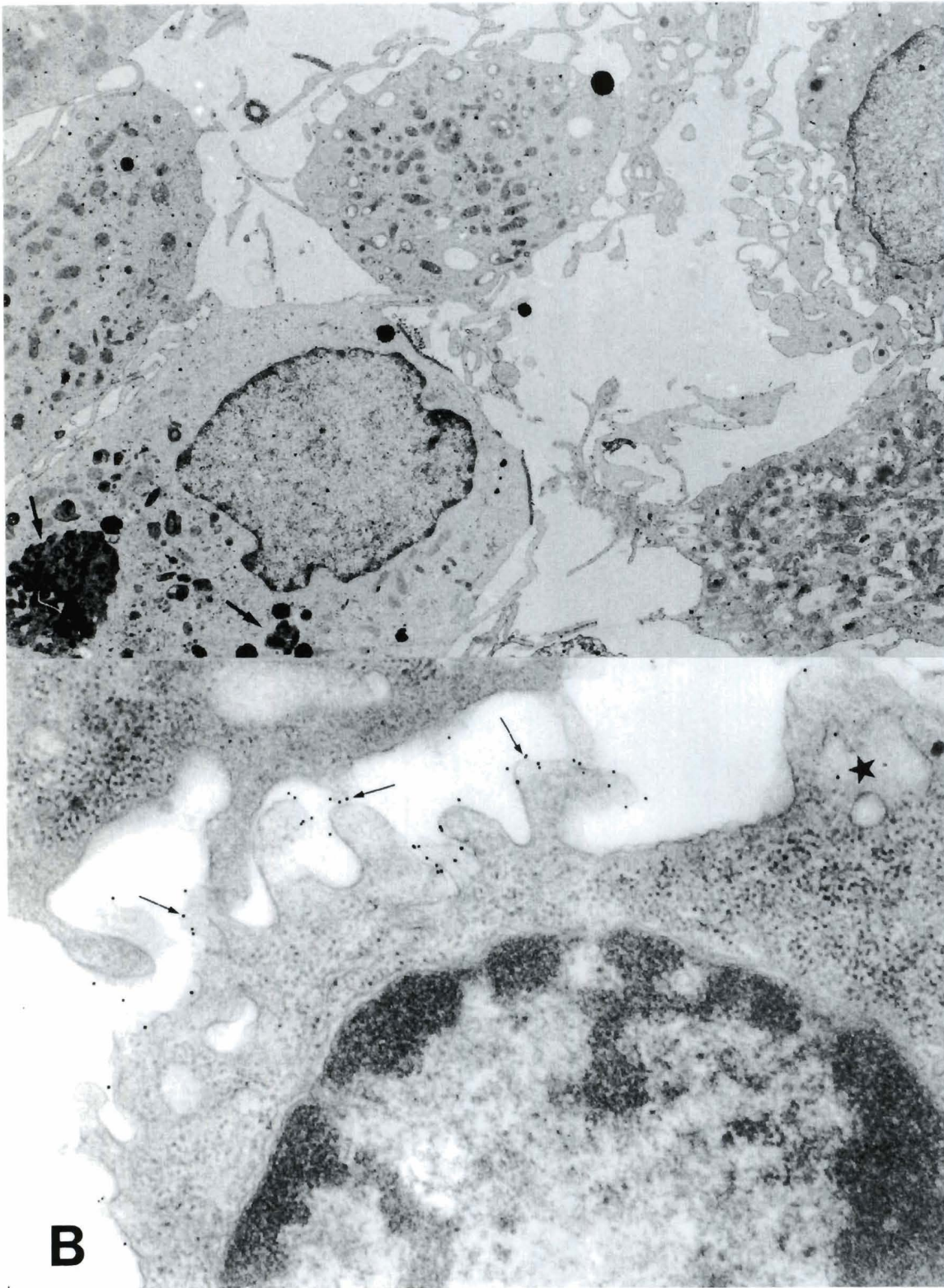


Fig. 5. A. 4-day-old organoid culture of mouse peritoneal macrophages. Typical morphology: numerous processes at the surface and many cell organelles, e.g. secondary lysosomes (arrow). x 6,500. **B.** Mouse peritoneal macrophages immediately after withdrawal from peritoneum. Immunoelectron microscopic demonstration of F4/80 at the surface by gold-labelled antibodies (arrow). Gold granules within the endosomes (*). x 53,000

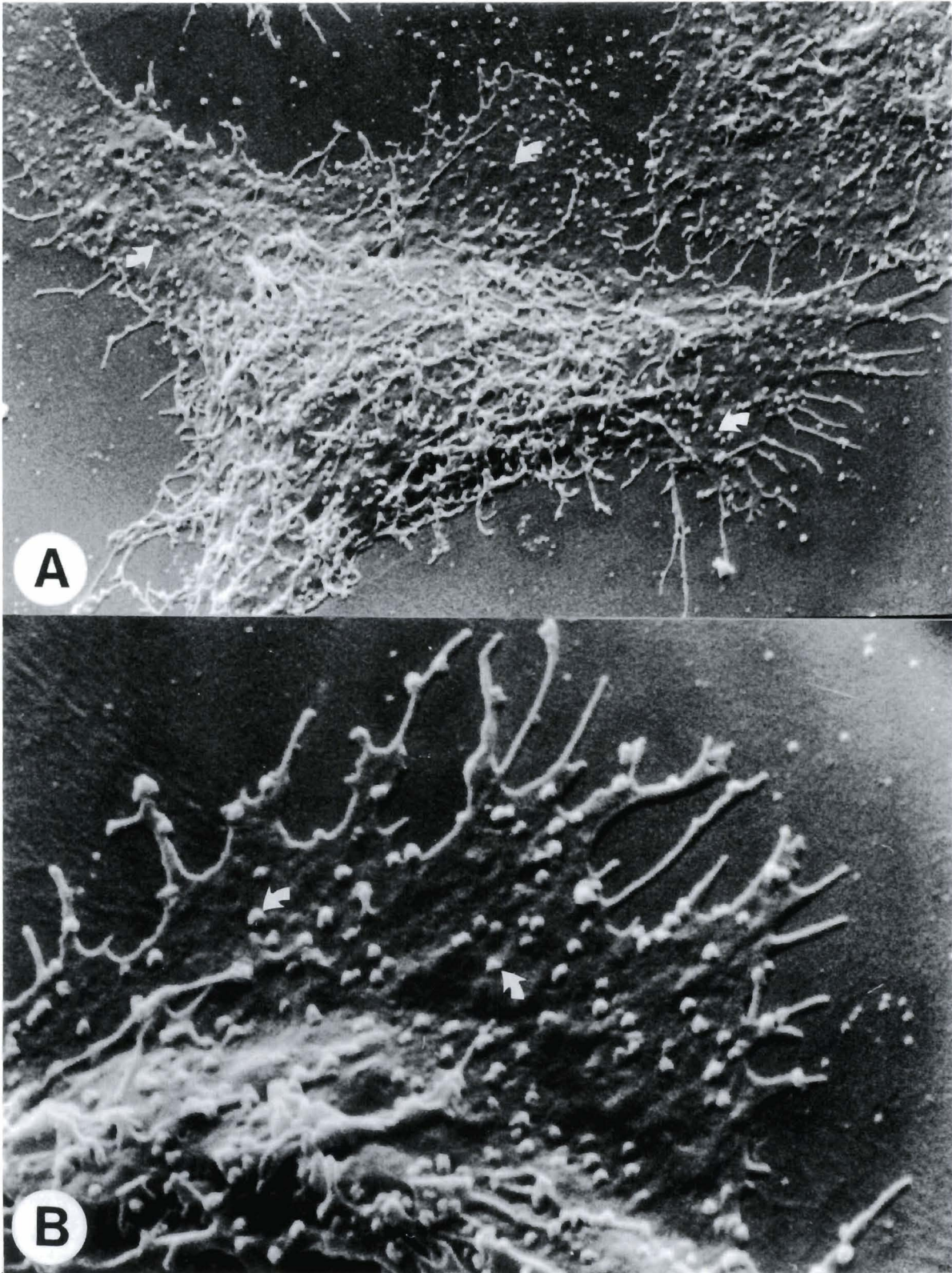


Fig. 6. Mouse peritoneal macrophages of a 24-hour-old monolayer culture. Demonstration of F4/80 by gold-labelled antibodies and silver enhancement at the surface (arrow). A. x 5,500; B. x 15,000

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