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Isolation and culture of amoeboid microglial cells from the corpus callosum and cavum septum pellucidum in postnatal rats

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INTRODUCTION

On the basis of ultrastructural (Stensaas & Reichert, 1971; Booz & Felsing, 1973; Penney, 1978; Ferrer & Sarmiento, 1980), scanning electron microscopic (Tseng, Ling & Wong, 1983), cytochemical (Ling, 1977; Ling, Kaur & Wong, 1982; Ferrer & Sarmiento, 1980) and immunofluorescence (Valentino & Jones, 1981) findings, it has been unequivocally established that amoeboid microglial cells in the perinatal rat brain are active macrophages. Together with the meningeal macrophages, supra-ependymal cells and epiplexus cells, they probably constitute the mononuclear phagocyte system of the central nervous system (Oehmichen, 1978).

Because monocytes and mononuclear phagocytes, such as alveolar and peritoneal macrophages, are known to have the property of adhering to glass surfaces (Parakkal, Pinto & Hanifin, 1974; Leake, Wright & Myrvik, 1975; Leake & Wright, 1979) and because our earlier study postulated that amoeboid microglial cells are monocyte-derived macrophages (Ling, 1981), the present study was undertaken in an attempt to culture the amoeboid microglial cells on glass coverslips in order to find out if they too show the same characteristic. The fact that the amoeboid microglial cells in the perinatal rat are aggregated at specific sites in the brain, viz. loosely structured supraventricular corpus callosum and cavum septum pellucidum (Ling, 1976; Tseng *et al.* 1983) meant that here were ideal sources of cells for culture because these areas could be removed readily from the brain. It was hypothesized that if indeed amoeboid microglial cells were endowed with the property of adhering to glass surfaces, they could then be separated from glioblasts and neuronal elements with the culture method. The availability of amoeboid microglial cells in culture will facilitate further experimental investigations which should subsequently lead to the characterisation of the cell type *in vitro*. This information will not only help in the better understanding of the cells which are unique to the perinatal brain but also in elucidating the controversial problem regarding the origin and nature of microglial cells (Fujita & Kitamura, 1976; Fujita, 1980; Oehmichen, 1978, 1980; Ling, 1981).

MATERIALS AND METHODS

One to five days old albino rats were used in this study. Under ether anaesthesia, the animals were perfused through the left ventricle with normal saline solution for about 10 minutes. This was to ensure that blood was flushed out completely from the circulation in order to prevent contamination by circulating monocytes as a

source of macrophages in the culture. After a good perfusion, the brain was whiteish and the liver was pale. Immediately after perfusion, the brain was exposed and a coronal slice about 1 mm in thickness was dissected out at the level of the optic chiasma and transferred to a petri dish containing Tyrode's solution at pH 7·6. A small block of tissue (approximately 1·0 mm × 1·5 mm × 1·5 mm), which contained the part of the corpus callosum immediately above the lateral ventricle, was dissected out; in addition, the septal area containing the cavum septum pellucidum was also removed. The tissue blocks were first transferred to a small plastic vial containing culture medium. The culture medium was prepared from 9·0 ml of single strength Earle's medium (Gibco), 1·0 ml of fetal calf serum (Gibco) (Flow), and 0·5 ml of penicillin–streptomycin (penicillin, 2500 i.u.; streptomycin, 2500 mcg). The tissue block was gently aspirated with a Pasteur pipette for about 1 minute. The dissociated tissue/cells in suspension were then transferred into a culture chamber designed by one of the present authors (Voon, 1980). Briefly, each culture chamber consisted of a glass microscope slide which measured 76 × 30 mm, with a thickness from 1·0 to 1·2 mm, and had a central circular aperture which was 15 mm in diameter. The chamber was bounded above and below by two microscope glass no. 1 coverslips which measured 32 × 32 mm each. Each chamber had a capacity of 0·3 ml. A chamber was first set up by adhering the lower coverslip to the underside of the microscope slide with a small amount of the culture medium. The dissociated tissue/cells suspension was transferred into the chamber and allowed to settle onto the coverslip. This lower coverslip provided the substrate for the cells to settle and to spread upon. The top coverslip was then slipped onto the upper surface of the microscope slide. The chambers were subsequently placed in trays in a humidified incubator at 37 °C, with changes of fresh medium at 24 hourly intervals. For the observation of the cultured cells, each culture chamber was viewed under a Nikon inverted microscope (Diaphot-TMD). Photomicroscopy was carried out with a single lens reflex camera (Nikon FE) attached to the inverted microscope (FX 135 Panatomic-X film (Kodak) was used). After phase contrast photomicroscopy, the lower coverslips were removed from the chamber for fixation. For routine light microscopy, the coverslips were stained with Leishman's stain.

For scanning electron microscopy, a round coverslip smaller (12 mm in diameter) than the aperture of the slide was placed on the lower coverslip, which therefore served as the substrate for the cultured cells. After 24 hours, this round coverslip, with the adherent cells, was removed from the chamber and washed twice with the culture medium before fixing in 4 % glutaraldehyde in 0·1 M cacodylate buffer for about 1 hour. Post-fixation was in 1 % osmium tetroxide for 20 minutes at 4 °C. The coverslip, with the adherent cells, was then dehydrated and critical point dried in liquid CO_2. It was then mounted on a specimen stub, sputter coated with gold for 3 minutes and viewed in a Philips 505 scanning electron microscope.

Test of phagocytosis by the cultured cells was done as follows: one drop of colloidal carbon solution (Günther Wagner Pelikan India ink; batch no. C11-1431A described as containing about 100 mg carbon per millilitre) diluted to one twentieth of its original concentration with Hank's balanced salt solution (HBSS) was added to the culture chamber before sealing the top coverslip. After 24 hours in culture, the lower coverslip was removed and stained in Leishman's stain. Alternatively, a drop of latex solution (Polysciences latex beads 0·05 μm in diameter) was mixed with the culture medium. Coverslips from this method were processed for scanning electron microscopy.

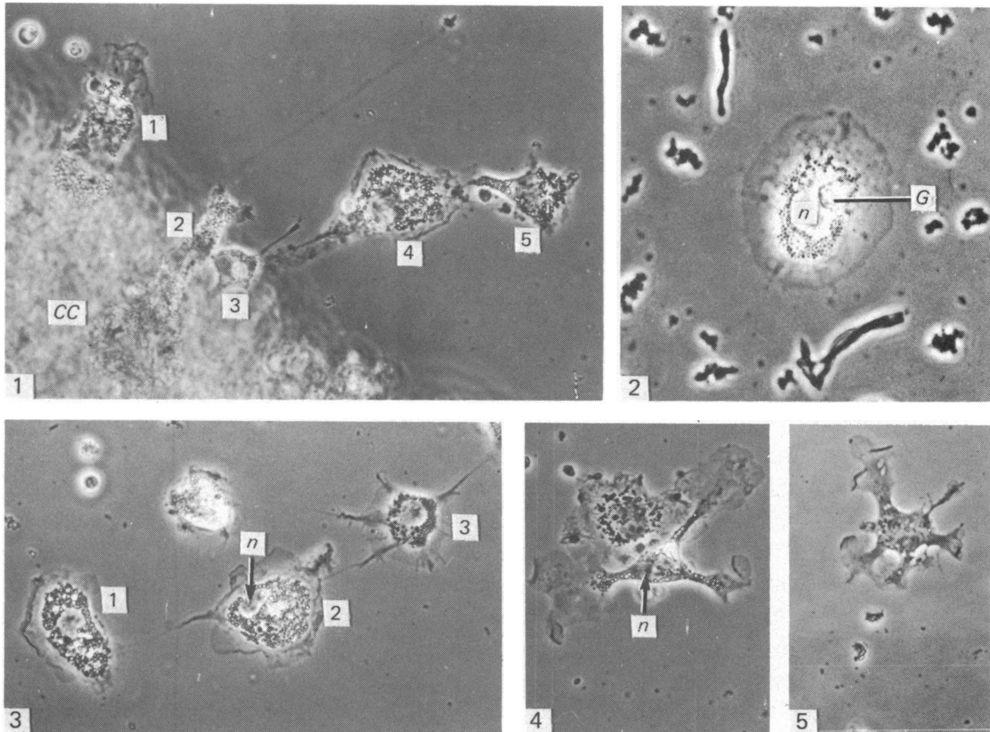


Fig. 1. Phase contrast micrograph showing five cells (numbered) migrating from a piece of corpus callosum (*CC*) in a day 1 culture. ×360.

Fig. 2. Phase contrast micrograph of a large round and flattened cell observed in a day 1 culture. The reniform nucleus (*n*) is evident. The pale zone at the nuclear indentation is likely to be the Golgi apparatus (*G*). Surrounding the nucleus are abundant dense granules. The peripheral cytoplasm appears homogeneous and is well spread. ×360.

Fig. 3. Phase contrast micrograph of three cells (numbered) in a day 1 culture. The two cells on the right project spinous processes. There is an accumulation of large numbers of granules in the perinuclear zone. *n*, nucleus. ×360.

Figs. 4–5. Phase contrast micrograph of diverse forms of cells in a day 4 culture. The cells send out pseudopodial processes with terminal expansions. Their cytoplasm is vacuolated. *n*, nucleus. ×360.

For the demonstration of endogenous peroxidase, the lower coverslips with the adherent cells were rinsed with phosphate-buffered saline before fixation in a diluted mixed aldehyde solution consisting of 1% paraformaldehyde and 1·25% glutaraldehyde in 0·1 M phosphate buffer at pH 7·4 for 4 hours. They were kept in 0·1 M phosphate buffer containing 10% sucrose overnight followed by incubation in a medium using 3, 3′, 5, 5′ tetramethyl benzidine (TMB) as a substrate according to the method of Mesulam (1978). The incubated coverslips were counterstained with neutral red.

For the demonstration of non-specific esterase, the coverslips with the adherent cells were fixed in 10% neutral formalin for 2 days at 4 °C. They were incubated in a medium containing alpha-naphthyl butyrate (Sigma, MO.) according to the method of Li, Lam & Yam (1973). Incubation times ranged from 1–2 hours and were at room temperature. After incubation, the coverslips were rinsed in distilled water and counterstained with 1% methyl green. They were dehydrated rapidly through a graded series of alcohol and mounted in Dammar xylol.

OBSERVATIONS

In day 1 (18–24 hours) cultures, examined using phase contrast microscopy, variable numbers of cells were observed migrating from the brain tissue (Fig. 1). The majority of the cells which had emigrated were large and round with smooth contours (Fig. 2). The nucleus was reniform and at the nuclear indentation was a pale zone which corresponded to the Golgi apparatus. Abundant phase-dense cytoplasmic granules and organelles were aggregated at the perinuclear zone (Fig. 2). The peripheral cytoplasm formed a homogeneous zone. Some cells in day 1 cultures displayed fine, spinous processes (Fig. 3).

After 4 days in culture, most of the cells sent out broad cytoplasmic expansions or pseudopodial processes (Figs. 4, 5). The cells were thus irregular and branched. With time, fibroblasts and other neuroglial elements, including astrocytes and oligodendrocytes, appeared to migrate from the tissue pieces.

In coverslips stained with Leishman's stain, the nucleus showed coarse chromatin clumps (Fig. 6). The copious cytoplasm was highly vacuolated except at the paranuclear zone (Fig. 6). Almost all the cells observed in day 1 cultures showed carbon particles ingested from the ambient medium (Fig. 7).

All the cells adherent to the coverslips observed in day 1 cultures were stained strongly positive for non-specific esterase. Thus, they were readily recognisable by this staining method. Sometimes, five to eight of them were observed in a field (Fig. 8). Their round nucleus, which was stained with methyl green, contained a prominent nucleolus (Fig. 9). It was also common to see the cultured cells containing two or more nuclei. The perinuclear zone was conspicuously stained and had a diffuse red reaction. The cytoplasm at the periphery was vacuolated and was unstained for the enzyme. For comparison, an amoeboid microglial cell taken from Araldite-embedded corpus callosum which was incubated for non-specific esterase activity described in a previous study (Ling *et al.* 1982) is presented in Figure 10. Note its similarities with the cultured cell in Figure 9, although, in the latter, the peripheral cytoplasm was often well spread and appeared vacuolated.

In day 4 and day 5 cultures, most of the cells sent out broad or fine vacuolated

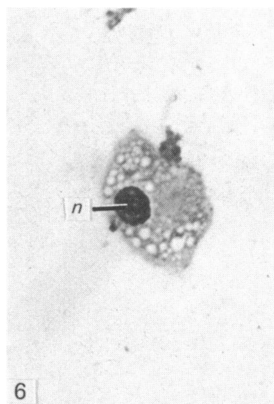
Fig. 6. A cell from a day 1 culture stained with Leishman's stain. The round, eccentric nucleus (*n*) displays conspicuous chromatin clumps. The copious cytoplasm is vacuolated, except at the paranuclear zone. × 420.

Fig. 7. A large vacuolated cell laden with carbon particles in its cytoplasm. Day 1 culture with carbon particles added to the medium. Lightly counterstained in Leishman's stain. *n*, nucleus. × 690.

Figs. 8–9. A group of 6 cells (numbered 1–6) as seen in a day 1 culture stained for non-specific esterase (Fig. 8). A representative esterase-positive cell is shown in Fig. 9. The round nucleus (arrow), which is greenish in the original preparation, carries a prominent nucleolus. The cytoplasm is strongly stained with diffuse red in the original preparation, and contains two large clumps (*Pg*) which are probably phagosomes. At the periphery, the cytoplasm is vacuolated. Counterstaining is with methyl green. Fig. 8, × 190; Fig. 9, × 690.

Fig. 10. Two amoeboid microglial cells (n_1, n_2) among the immature glioblasts (*GB*) in the corpus callosum of a 5 days old rat. The Araldite-embedded tissue had been incubated for non-specific esterase reported in an earlier study (Ling, Kaur & Wong, 1982). Note its similarity in morphological features with the cell in Fig. 9. The vacuolated cytoplasm dislays dark granular reaction products for non-specific esterase. 1 μm section counterstained with methylene blue. × 1080.

Fig. 11. A non-specific esterase-positive cell from a day 5 culture. The nucleus (*n*) is oval. The flattened cell body sends out long vacuolated pseudopodial processes (*p*). Compare the cells seen with phase contrast microscopy in Figs. 4, 5. × 690.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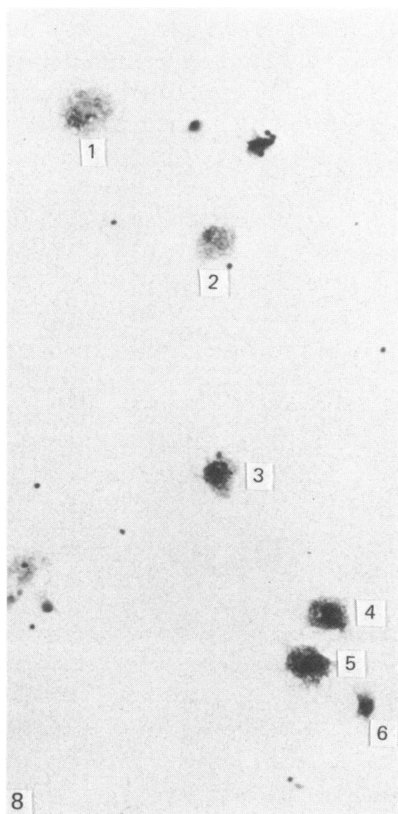
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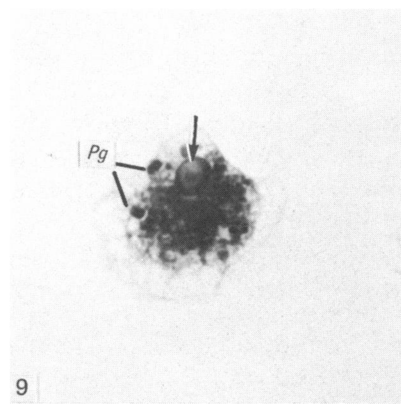
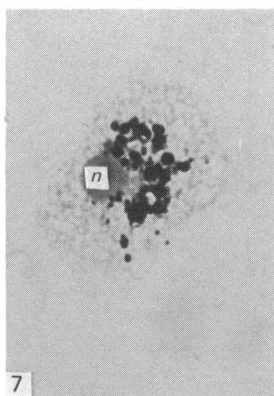

Pg
9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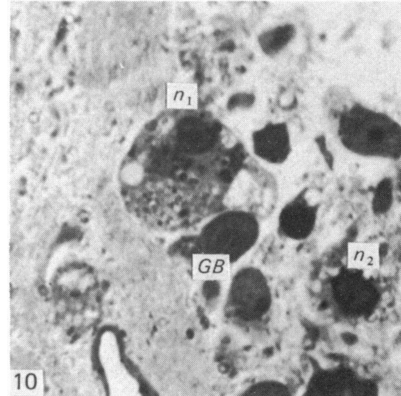
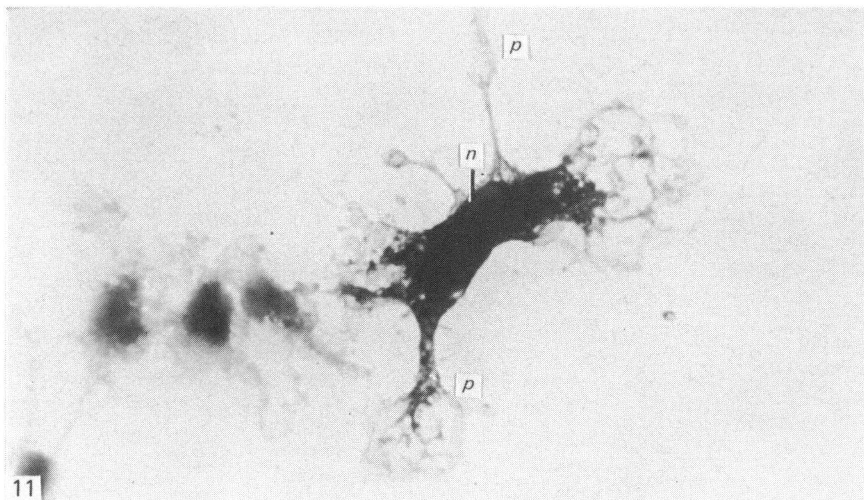
7


n_1
GB
n_2
10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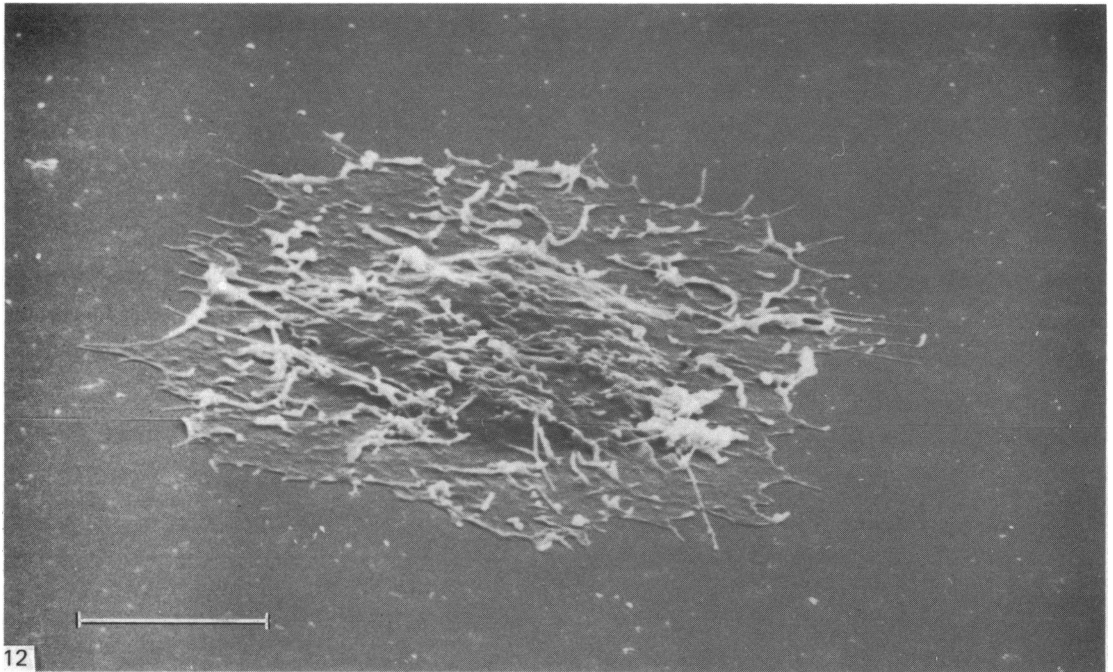
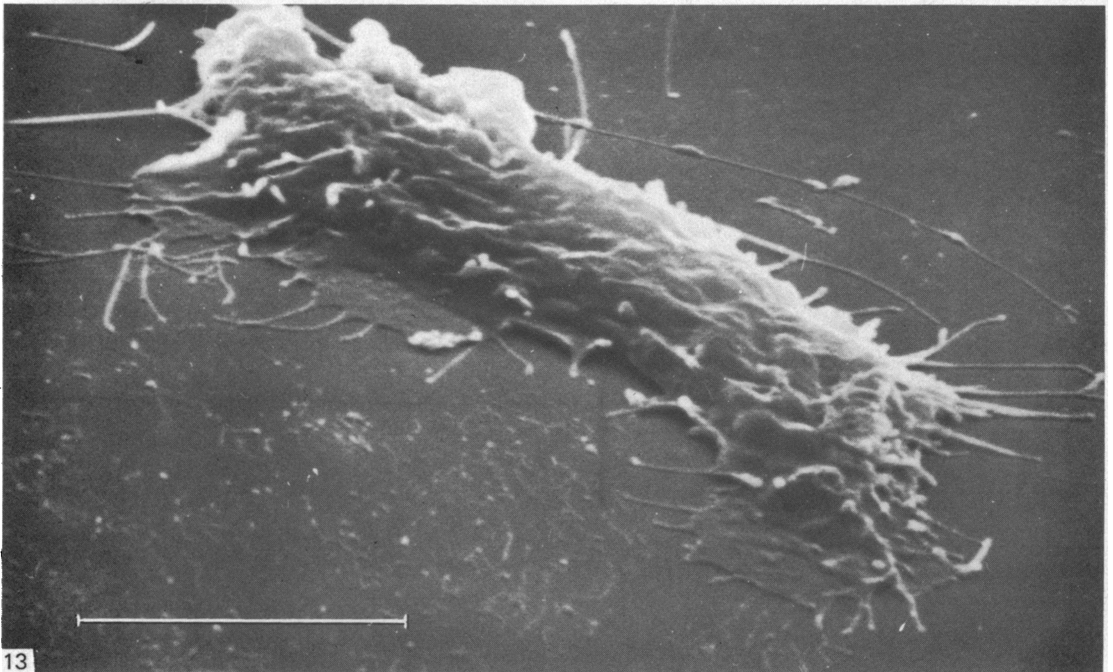
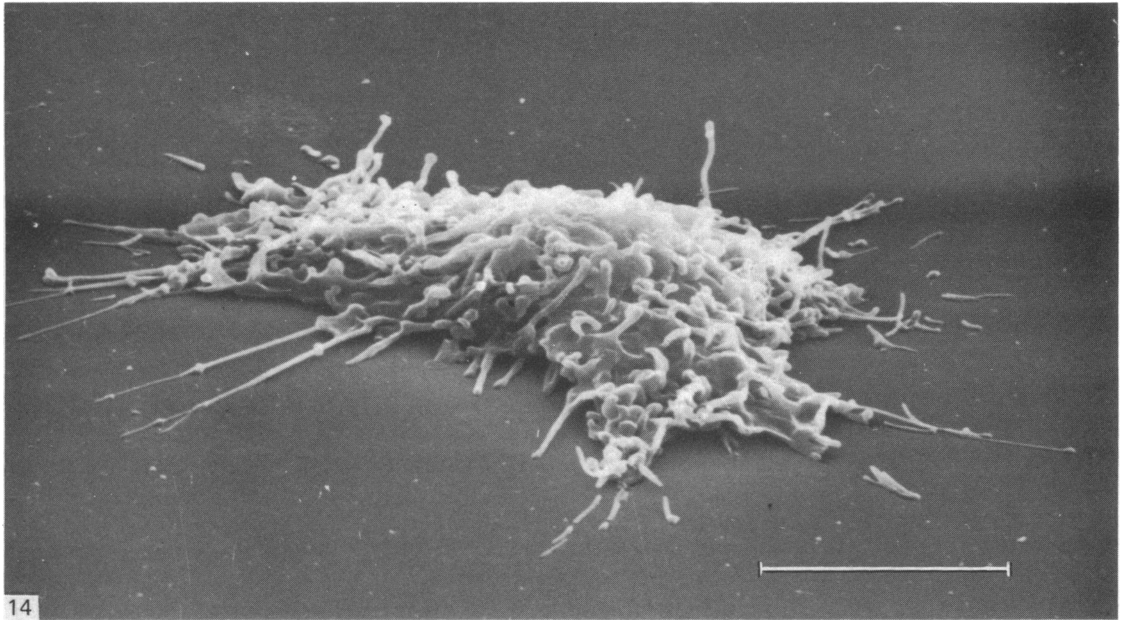
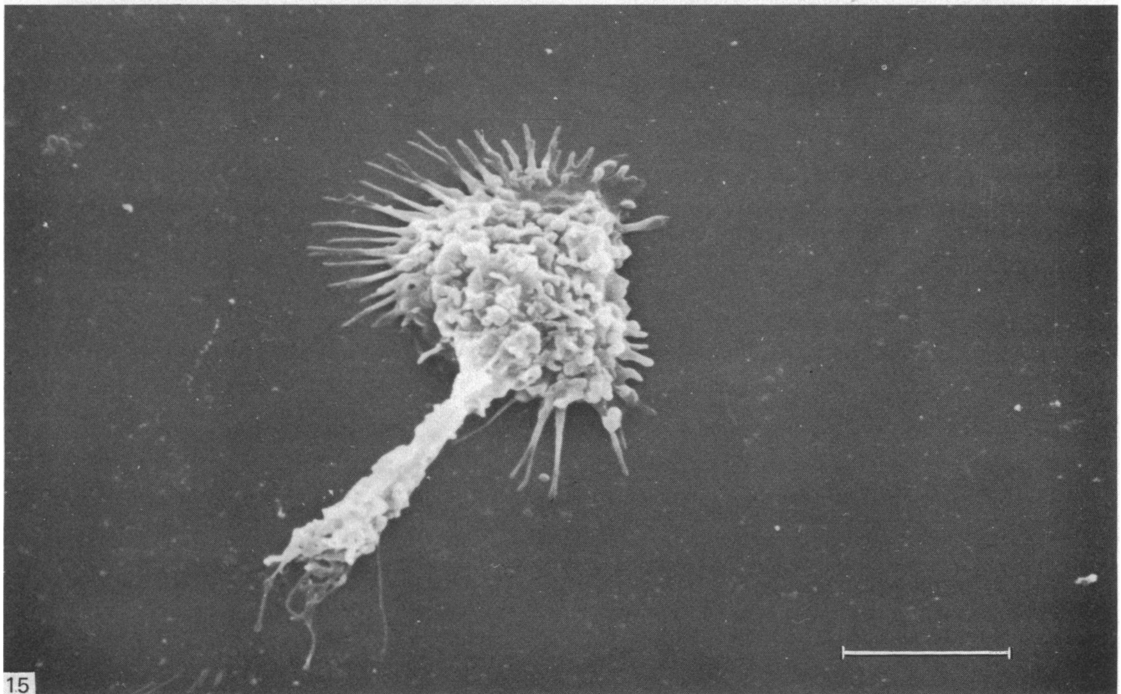




Figs. 12–15. Scanning electron micrographs of the various forms of cells adherent to the coverslip in day 1 cultures. The cell in Fig. 12 is well spread; the elevation in the centre is probably the nucleus. Fig. 13 shows an elongated cell with membrane ruffles. Fig. 14 is a cell endowed with filopodial processes. The cell depicted in Fig. 15 displays filopodia, blebs and a long pseudopodia-like process. Scale, 10 μm.





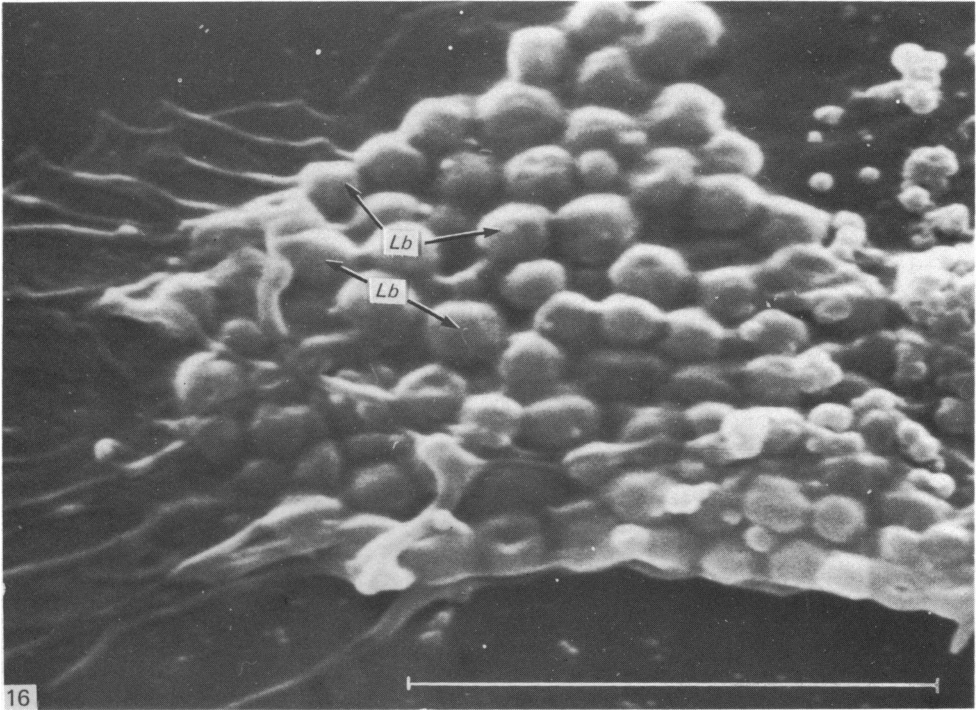


Fig. 16. A cultured cell, which is well spread on the left, has ingested numerous latex beads (*Lb*). Scale, 10 μm.

pseuodopodia with terminal expansions (Figs. 4, 5, 11). The cell body was strongly reactive for non-specific esterase (Fig. 11).

All the cells adherent to the coverslips in the culture were peroxidase-negative.

At no time did the culture show any contamination by blood elements, as indicated by the absence of red blood cells, lymphocytes and polymorphonuclear leucocytes in the culture chamber after the cultures were set up.

Scanning electron microscopic study in day 1 cultures showed diverse morphological features in cells adherent to the surface of the coverslips. They ranged from round flattened forms (Fig. 12) to elongated ones (Fig. 13). In some, the cell surface appeared relatively smooth with membrane rufflings (Fig. 13) while others showed long filopodial processes (Fig. 14) or blebs (Fig. 15). The cell depicted in Figure 15 also sent out a long cytoplasmic process. Most of the cells observed in day 1 cultures showed uptake of latex beads (Fig. 16).

DISCUSSION

The culture of postnatal corpus callosum and cavum septum pellucidum in the present study yielded a variable number of pleomorphic cells morphologically similar to the alveolar or peritoneal macrophages (Leake *et al.* 1975; Knyszynski, Leibovich, Skutelsky & Danon, 1978; Nabarra, Cavelier, Dy & Dimitriu, 1978; Leake & Wright, 1979; Tseng & Ling, unpublished work) and bone marrow mononuclear phagocytes (Buhles, 1979). In scanning electron microscopy, their surface features resembled closely the amoeboid microglial cells in the *in vivo* study (Tseng *et al.* 1983) and the supra-ependymal cells (Bleier, 1975; Walsh, Brawer & Lin, 1978; Sturrock, 1979;

Albrecht & Bleier, 1979; Bleier & Albrecht, 1980; Bleier, Siggelkow & Albrecht, 1982; Cohen, Albrecht & Bleier, 1982). These data, taken together with their property of adherence to glass coverslips as well as their avid ingestion of carbon particles and latex beads introduced in the culture medium, strongly suggest that they are mononuclear phagocytes in the brain.

In order to rule out the possibility of circulating monocytes contaminating the culture and giving rise to the observed phagocytes, the animals were first perfused with normal saline to flush out the blood cells from the circulation. The absence of granulocytes, lymphocytes and red blood cells in culture indicated the success of the perfusion. It is therefore definite that the observed phagocytes were indeed indigenous to the neural tissues. In this connection, the most likely source of the cells would be the amoeboid microglial cells which have been shown overtly to be active macrophages (Ling, 1977; Valentino & Jones, 1981; Ling *et al.* 1982; Leong, Shieh, Ling & Wong, 1983). This is further substantiated by the facts that the cultured cells, in particular in the day 1 cultures, were intensely stained for non-specific esterase, and that, in the *in vivo* study in the postnatal corpus callosum and cavum septum pellucidum, the only cell type that was stained for this enzyme was the amoeboid microglia (Ling *et al.* 1982).

Because the adherent meninges had been removed from the brain before culture and because only the supraventricular corpus callosum and cavum septum pellucidum were dissected out for culture it is highly unlikely that the culture was contaminated with meningeal macrophages or intraventricular macrophages which are structurally similar to amoeboid microglia (Ling, 1979, 1981; Bleier & Albrecht, 1980).

Based on light microscopic, cytochemical and scanning electron microscopic observations, it is therefore concluded that the glass-adherent phagocytes observed in the present study represent amoeboid microglial cells which have migrated from the brain tissue and which have remained viable as demonstrated by their uptake of carbon particles as well as latex beads from the culture medium.

SUMMARY

In phase contrast and scanning electron microscopy, diverse structural forms of cells tenaciously adherent to glass coverslips were observed in the culture of the corpus callosum and cavum septum pellucidum from postnatal rats. In day 1 culture, many of the cultured cells were round, with well spread peripheral cytoplasm which appeared homogeneous. Cell organelles aggregated mainly around the reniform or round nucleus. Some cells showed spinous projections. In day 3–5 culture, the cells became irregular, sending out long branching pseudopodial processes; often they displayed a vacuolated cytoplasm. The cultured cells were highly phagocytic, as shown by their uptake of colloidal carbon particles and latex beads, in light microscopy and scanning electron microscopy, respectively. Cytochemical studies have shown that the cells were peroxidase-negative but were strongly positive for non-specific esterase, similar to the amoeboid microglial cells in the postnatal corpus callosum. On the basis of their structural features, both in phase contrast and scanning electron microscopy, experimental as well as cytochemical properties, it is concluded that the cells in the present culture are in fact amoeboid microglial cells which are active macrophages in the developing corpus callosum.

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