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Dynamic features of cells expressing macrophage properties in tissue cultures of dissociated cerebral cortex from the rat

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Summary. Two previously identified forms of macrophage were investigated in primary cultures of cerebral cortical cells. Dynamic features were revealed through time-lapse video recording and aspects of macrophage function were assessed. The two cell forms were shown to be different pre-mitotic stages of a single cell type. The cell cycle for these cells involved an initial large, flat, quiescent cell which retracted to yield a slightly rounded form with numerous processes. This latter form lost processes and developed profuse filopodia as it became very rounded just prior to division; both resulting daughter cells then regained the initial large flat appearance. These cells possessed several properties of macrophages, including phagocytosis, nucleoside diphosphatase enzyme, and CR3 receptors. These properties were transient, expressed just before and after mitosis, but subsequently down-regulated in the flat daughter cells. Because of this feature, it was difficult to determine the exact size of this cell population; however, the observed rate of proliferation suggests it may be substantial. It is suggested that these cells correspond to non-microglial macrophages of brain tissue and, because of their significant down-regulation, they may be difficult to detect. This may be important in studies of brain accessory immune cells in tissue culture.

Key words: Brain, vertebrate – Macrophages – Tissue culture – Mitosis – Microglia – Astrocytes – Rat (Sprague-Dawley)

While it is becoming increasingly clear that the brain does not possess the "privileged" immunological status previously attributed to it, the mechanisms of immune function in this tissue remain largely unresolved (Farrar et al. 1987); in particular, the identity of cells serving in an accessory role has been an issue of some interest (Wong et al. 1985; Fontana et al. 1987). Accessory cells facilitate or contribute to the immune response in several ways, including the secretion of cytokines and antigen processing and presentation for lymphocyte activation. Thus, these cells constitute a vital link for immune function. Although several other cells types can serve in an accessory capacity, macrophages form the major component of accessory cell function in most tissues; however, whether this is also the case in the central nervous system is unclear.

In an effort to develop a model system in which to investigate functional properties of brain macrophages, this laboratory has utilized a primary culture system of dissociated cerebral cortical cells. Three different forms of macrophages were initially identified in these cortical cultures based on morphological features, Fc receptor immunohistochemistry, and esterase histochemical staining (Jordan and Thomas 1987a). While all 3 forms originally were thought to be related to microglial cells, ramified microglia were subsequently identified definitively and shown to be distinct (Glenn et al. 1989a). More recent studies have resulted in the determination that a derivative of ramified microglia, reactive microglia (Glenn et al. 1986), is the predominant species of active phagocytic macrophage in these cultures (Jordan et al. 1990). One of the 3 originally identified macrophage forms was, in fact, amoeboid microglia, the developmental precursor for ramified cells. Thus, all 3 types of microglia have been established, and this class of brain macrophages is currently being investigated (Thomas 1990; Glenn et al. 1990). However, the nature and identity of the other 2 macrophage forms initially detected has remained undetermined. These cells were distinguished not only by macrophage-related staining properties, but also on the basis of relatively selective aspects of their morphology (Jordan and Thomas 1987a).

While tissue culture provides an amenable preparation to investigate individual cells or cell populations, it also necessitates the identification of cell types and a correlation in vivo as a prerequisite to investigating cellular function. This is particularly relevant for macrophages since they display heterogeneity of morphology

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and functional state, and since brain tissue contains several different macrophage populations (reviewed in Jordan and Thomas 1988). The purpose of the present study was to characterize the 2 previously detected, unidentified macrophage forms in cerebral cortical cultures, and to attempt to correlate these cells with known or established cell types in vivo. Portions of this work have been presented in 2 abstracts (Thomas and Jordan 1989; Thomas et al. 1990).

Materials and methods

Tissue culture

Primary tissue cultures of dissociated cerebral cortical cells from fetal Sprague-Dawley rats were prepared and maintained as previously described (Thomas 1985; Jordan and Thomas 1987a). Cultures displayed mature morphology after 2–3 weeks in vitro, with all major classes of brain cells represented.

Microscopy and video recording

Cultures which were stained histochemically following fixation were observed and photographed using a Zeiss Standard 16 microscope, equipped with phase-contrast optics and epifluorescence. Living cultures were observed using an inverted microscope equipped for phase-contrast fluorescence photography and time-lapse video monitoring. Time-lapse recording was performed according to described procedures (Thomas 1990); during extended periods of video recording, cultures were perfused continuously with fresh growth medium using a Dvorak-Stotler controlled environment culture chamber (Nicholson Precision Instruments, Gaithersburg, Md., USA).

Cell-labelling techniques

Histochemical staining for thiamine pyrophosphatase (TPP) followed the original protocol of Novikoff and Goldfischer (1961), with modifications described previously (Glenn et al. 1989a). Functional labelling of macrophages via phagocytic activity was assessed using 0.9 μm fluorescent latex beads (Polysciences, Warrington, Pa., USA) by a method previously described by this laboratory (Jordan et al. 1990). Immunohistochemical staining was performed using an indirect fluorescence technique as described (Jordan and Thomas 1987b). Labelling of active macrophages was achieved utilizing a monoclonal antibody against CR3 receptors, MAC-1 (prepared in this laboratory from M1/70 hybridomas; Thomas 1990).

Results

The 2 macrophage forms previously identified in this culture system (Jordan and Thomas 1987a) were observed consistently and distinguished readily by their morphology. The first had an overall rounded or oval cell body (approximately 50–100 μm) and, while sometimes exhibiting processes or pseudopodia as well, was particularly marked by profuse, long, spiny filopodia (Fig. 1A). This cell-type often occurred as fused or joined pairs. The second form was more flattened in appearance, quite large in size (80–200 μm), and possessed numerous stout processes, sometimes pseudopodia, and few or no filopodia (Fig. 1B). These 2 forms correspond to those previously suggested as reactive and ramified microglia, respectively (Jordan and Thomas 1987a). The surface membrane of both forms was characteristically rough and occasionally displayed a bubbly or ruffled appearance; the cytoplasm became increasingly granular with culture age. While these 2 subpopulations of cells were intrinsic to the confluent monolayer and could be observed throughout cultures, they were clearly more frequent near the proliferative edge of the monolayer. In most instances, they resided in a small recess or hole in the layer of cells, with only their processes or filopodia contacting surrounding cells.

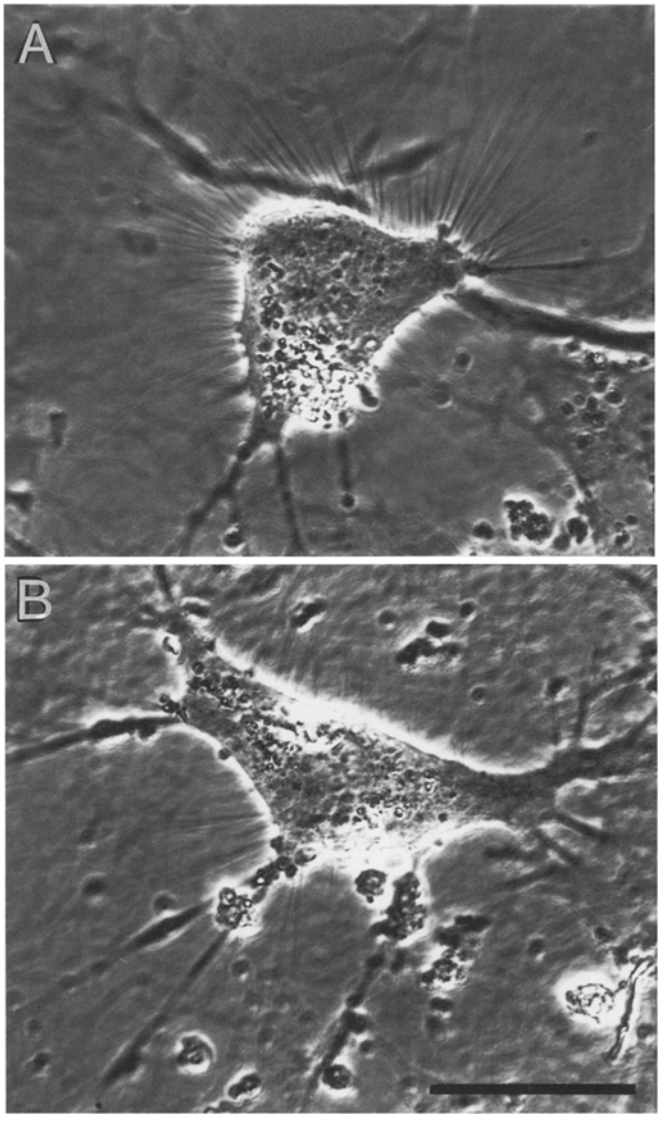

Fig. 1. **A** Individual macrophage displaying rounded cell body, numerous spiny filopodia, and grainy cytoplasm. **B** Another macrophage form with flatter cell body, stout processes, and fewer filopodia. *Bar*: 50 μm

Initially it was noted that when the same cell in an individual culture was observed at successive times, the cell seemed to disappear or alter its appearance. Therefore, time-lapse video recording was implemented to monitor or track single cells. When the filopodia-bearing

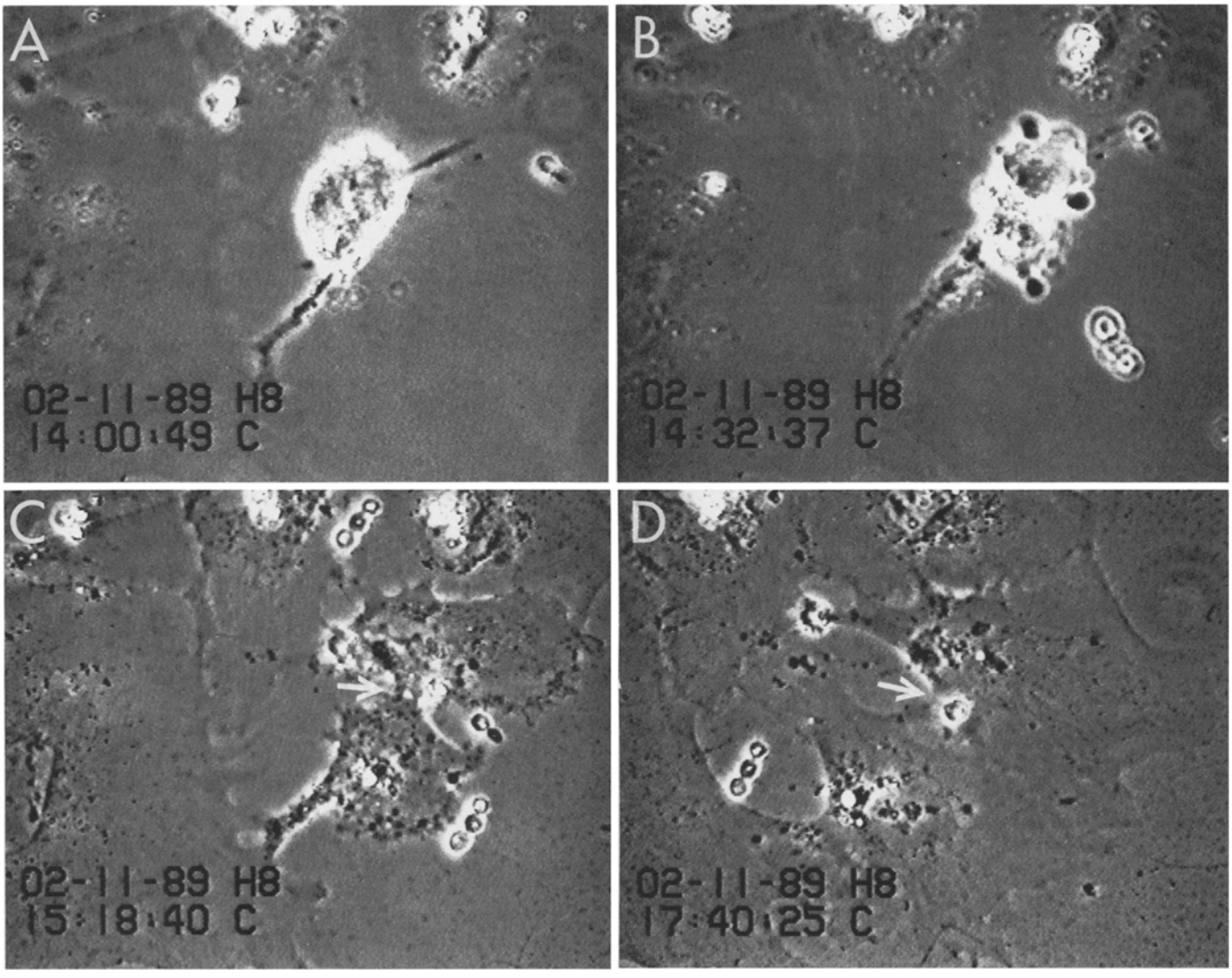

Fig. 2A–D. Time-lapse video sequence (covering total of 219 min 36 sec, time is at lower left in each frame) of a filopodia-bearing macrophage undergoing mitosis. Note retention of cytoplasmic connection between daughter cells (*arrows* in **C** and **D**) as they flatten and approach confluency. ×630

cells were recorded, it was revealed that they always initiated mitosis within 1 h (Fig. 2); this cell form corresponded to a pre-mitotic configuration. The resultant daughter cells then spread greatly as they flattened and approached confluence. Very often the flat daughter cells appeared to retain a cytoplasmic bridge or connection for an extended period (Fig. 2C, D). While both macrophage forms were thought initially to be distinct cell types, the presence of certain forms which appeared intermediate to these 2 suggested that they might be developmental or functional stages of the same cell population. This idea was confirmed by the video recording; over time the large process-bearing cells were shown to retract processes and develop more filopodia. Thus, the process-bearing cell form gave rise to the filopodia-laden form which then underwent division.

The observation of flat cells in the culture layer converting into the process-bearing form provided evidence for completion of the cell cycle. Typically, a flattened cell located near the proliferative edge of the culture monolayer would begin to round-up, though leaving some processes extended; as rounding continued, the cell lost processes, gained filopodia, and then underwent mitosis (Fig. 3). The daughter cells flattened and merged to become confluent. A complete cycle of cell division, beginning and ending with cells flattened or confluent, ranged from 2–4 h. During and immediately following division, a very vigorous "bubbling" of the cell membrane characteristically occurred, with cells sometimes extending processes and ruffles in every direction which were quickly retracted (see Figs. 2B, 3D). When cells became flattened, this activity of the surface membrane subsided. However, a residual level of surface activity consistently remained, characterized by small processes or finger-like projections extended up into the culture medium and then retracted (Fig. 4). Such activity appeared to transpire continuously and rapidly in the flattened cells. Thus, the macrophage forms appear to be related and correspond to a single cell-type engaged in significant proliferative activity. They may represent a

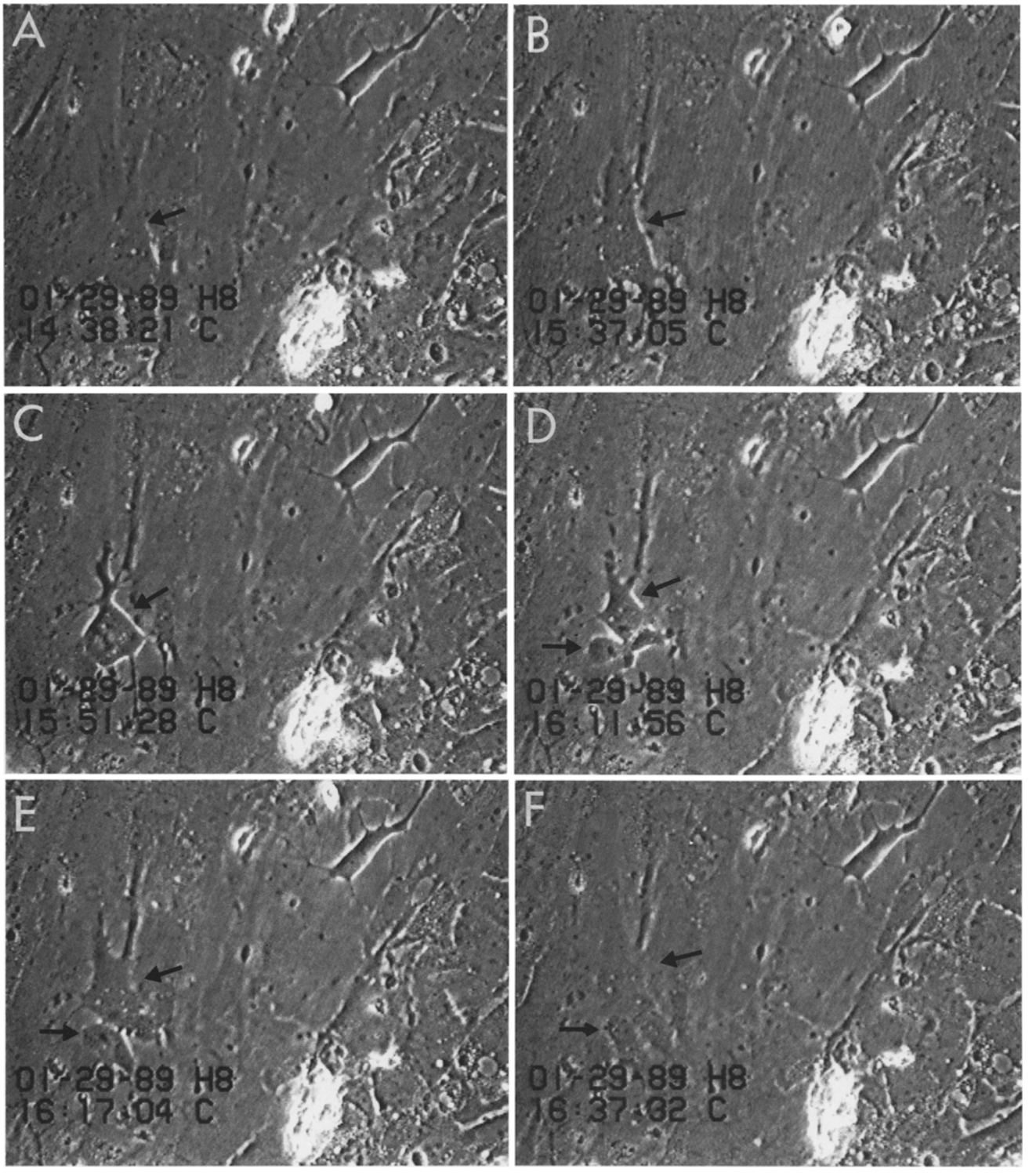

Fig. 3A–F. Six sequential frames from time-lapse recording showing flat cell (*arrows* at *lower left*, neuron-like cell at *upper right*) near proliferative edge of culture background layer converting into process-bearing form and dividing, with daughter cells again fusing with monolayer (total time covered is 119 min 11 s). ×400

major component of the culture population, in view of the finger-like processes exhibited by many cells.

These cell forms were originally identified based on the possession of macrophage properties, and in the course of subsequent studies of microglial cells, were noticed to be labelled frequently by macrophage-specific markers. To investigate this aspect further, phagocytic activity was assessed by incubation of cultures with fluorescently-labelled latex beads. The cell forms investigated here were found to be labelled, indicating phagocytosis. In addition, many flat cells at the margin of the monolayer accumulated beads (Fig. 5). Ingested beads

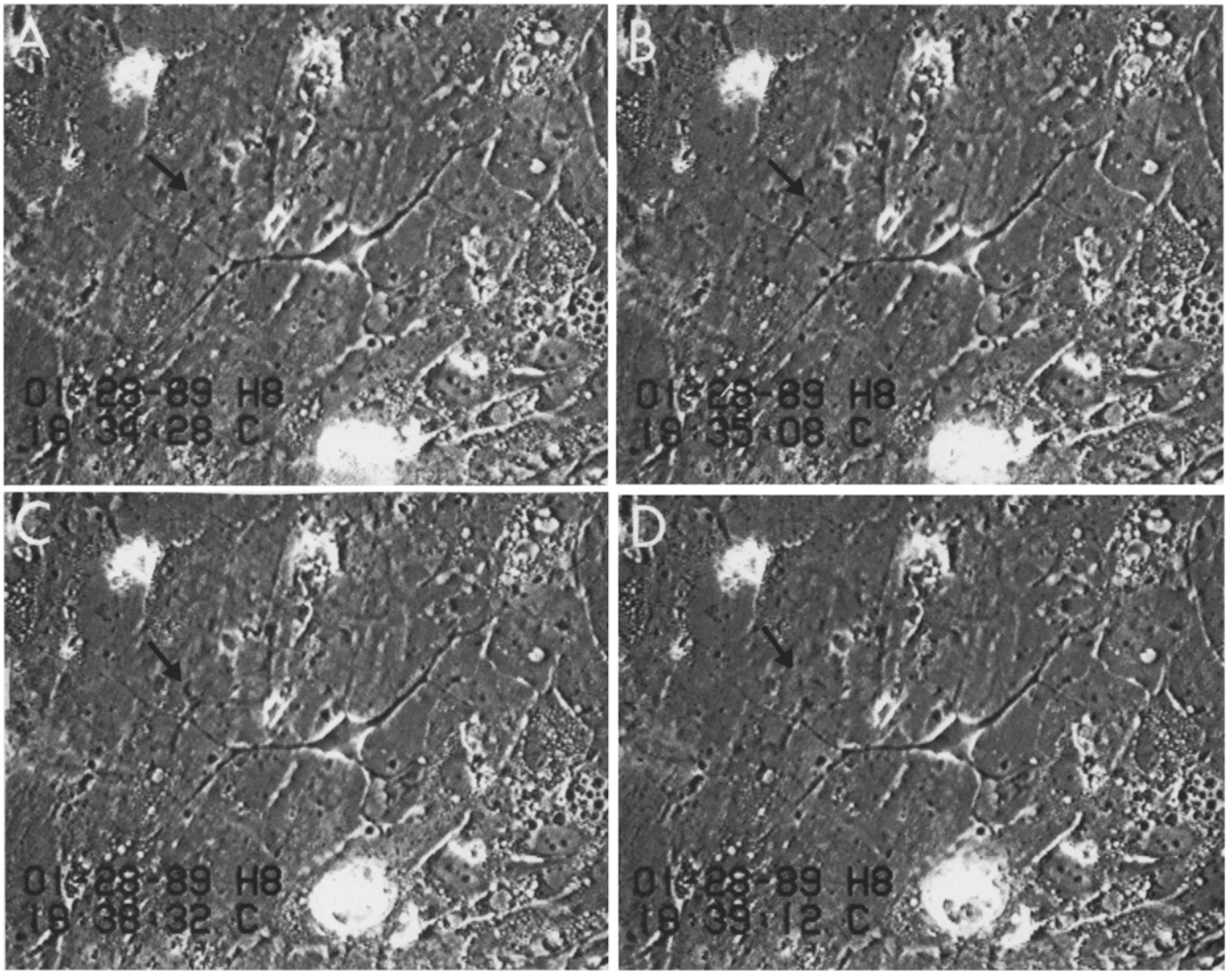

Fig. 4A–D. Four frames of time-lapse sequence depicting flattened cell in monolayer extending and retracting small processes (*arrows*). Total time equals 4 min 44 s. This activity was constant at low level across monolayer and was especially vigorous immediately after flattening following mitosis. Characteristic neuron is present in center of field. ×400

were distributed mainly around the nucleus and rarely in processes or pseudopodia, regardless of the amount of uptake. In studies employing TPP histochemistry for nucleoside diphosphatase degradative enzyme, the same cell forms as well as additional flat cells near the culture edge were stained. Individual flat cells near the margin were intensely stained at random, while surrounding and sometimes confluent cells were essentially unstained (Fig. 6). The dark brown to black TPP reaction product was distributed over the cell membrane and throughout the cytoplasm, but was less intense in the cell nucleus. The staining within the cytoplasm outlined or highlighted profuse vacuoles and reticular structures.

The pattern of labelling or spatial distribution of labelled cells was the same for each of these 2 activities (phagocytosis and TPP) as well as other macrophage markers. The filopodia- and process-bearing forms were labelled and occurred more frequently near the culture edge, and other flat cells in this edge region were also labelled. However, there was little or no staining of cells more central to the monolayer. Even in cultures from the same plating at widely different ages, where the size or area of the monolayer was significantly different, these cell forms were stained and preferentially located near the edge region. This suggests either that these cells remain near the proliferative margin as the monolayer increases in size, or that they decrease the expression of macrophage properties as they merge with the confluent monolayer. Several observations supported the latter possibility: (1) the number of stained cells in older cultures was not sufficient to correlate with the observed rate of proliferation; (2) while stained filopodia- and process-bearing cells were occasionally observed in the central region of the monolayer and these cells also exhibited mitosis, no stained or labelled flat cells were present in this region; and (3) when individual daughter cells, exhibiting the typical extension and retraction of finger-like processes were subsequently assessed after staining, they did not exhibit any label. Finally, a decrease in expression of macrophage properties was confirmed by

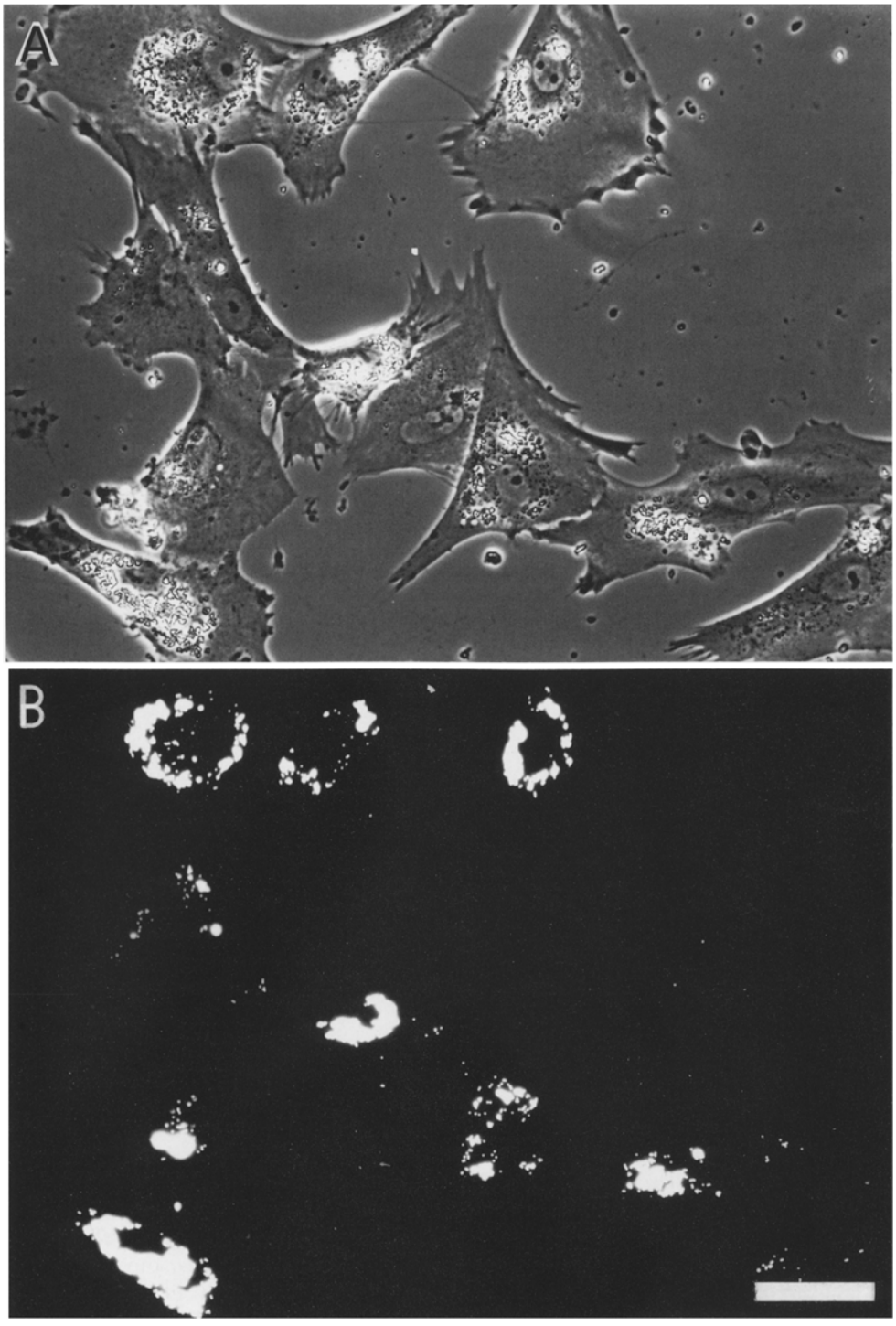

Fig. 5. A Phase-contrast image of flattened macrophages near growing edge of culture which have phagocytized 0.9 μm fluorescent latex beads. **B** Corresponding fluorescence view showing ingested beads distributed mainly in perinuclear region, regardless of degree of uptake. *Bar*: 50 μm

recording individual filopodial cells. After division, and after the daughter cells had existed for some time, these flat cells were shown not to ingest beads nor to stain for TPP (or MAC-1).

To demonstrate both identity and activity in the same cell, dual labelling was performed with MAC-1 immunohistochemistry and the latex beads. Once again, the same cell forms were identified and dual labelling could be observed in individual flat cells (Fig. 7). The MAC-1 immunofluorescence revealed cells in their entirety and was associated with the plasmalemma as well as cytoplasmic vesicles. The intensity of staining varied greatly between individual flat cells in a manner consistent with decreased expression of the receptor antigen. In MAC-1-stained cells, fluorescent beads were arranged in the usual perinuclear fashion. While all MAC-1-positive cells contained beads, some labelled cells exhibited very faint or no MAC-1 staining. This supports independent regulation of these 2 properties, with expression of the antigen being down-regulated at a faster rate than phagocytic activity.

While flattened cells within the monolayer had signifi-

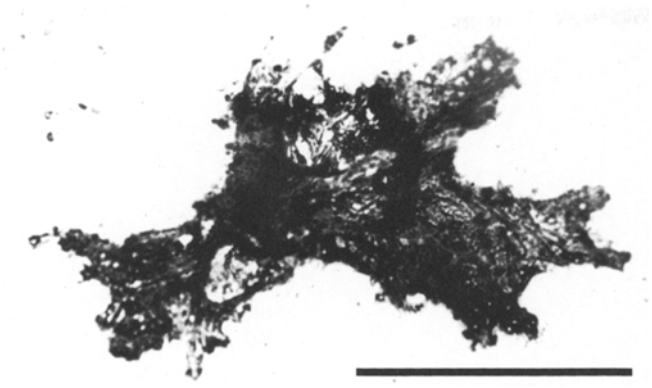

Fig. 6. Single flat macrophage just outside culture margin stained via TPP histochemistry. Staining was most intense at, or near, culture edge, with essentially no staining of flat cells within monolayer. *Bar*: 100 μm

cantly reduced membrane activity, they did constantly extend small processes into the culture medium as described earlier. The video microscopy also resulted in direct visualization of particle uptake by these cells. At least some of the small processes had a phagocytic function, as they were observed to capture floating particulate matter that they contacted and bring it into the cell (Fig. 8). Process extension, capture, and retraction usually occurred over a time of approximately 15–20 min, with most of this time elapsing between capture and retraction. If no particles were captured, extension and retraction of a given process would occur in under 5 min. Thus, the flat cells appear to retain a marginal or low level of phagocytic activity, not directly detectable by the bead ingestion technique utilized here. This apparently corroborates the suggestion mentioned above that phagocytosis is down-regulated at a slower rate than some other macrophage properties.

In considering the potential identity of the cell population investigated here, based on previously reported properties of astrocyte growth and proliferation in culture (Woodhams et al. 1981; Fedoroff et al. 1990) and studies indicating an involvement of this cell type in immune function (see Discussion), it is tempting to propose them as astroglia. To confirm this, an attempt was made to label these cells via glial fibrillary acidic protein (GFAP) immunohistochemistry. While GFAP is a selective marker for astrocytes, it appears to label protoplasmic astrocytes in culture less intensely than the fibrous astrocytes. Therefore, the identification of protoplasmic astroglia can sometimes be tenuous. It is our assessment that the cells presently described did not exhibit specific GFAP staining. These cells either appear not to be astrocytes or are an astrocyte subpopulation with little or no GFAP (see Fedoroff et al. 1990). Additionally, these cells did not label with the oligodendrocyte marker galactocerebroside, nor with the endothelial cell marker factor-VIII; however, they did consistently stain for the filament protein vimentin. In other studies not directly reported here, these cells were also seen to label with the macrophage-specific monoclonal antibodies OX-42 and ED1, as well as for interleukin-1.

Discussion

Two cell forms expressing macrophage properties in tissue cultures of rat cerebral cortex were investigated. These 2 forms were found to be different pre-mitotic stages of a single cell type which was actively engaged in proliferation. The expression of macrophage properties, including phagocytosis, TPP enzyme, MAC-1 staining, and other markers not directly considered here, was associated with cell division. These properties were subsequently lost or significantly down-regulated in the resultant daughter cells, with a reduced level of phagocytic activity being the only residual property consistently noticed. Thus, the present characterization revealed several features of the cell forms. Overall, these forms belong to a proliferative cell population transiently expressing macrophage properties, and this population may comprise a significant cell number in these cultures.

Initially, the underlying rationale in studying brain macrophages was that they might function as accessory cells in the immune response, as is the case for macrophages in many other tissues. However, several different cell types have recently been proposed as accessory cells in brain tissue and this has developed into a controversial area. There is much work supporting astroglial cells as a major component of accessory immune function in this tissue (Hirsch et al. 1983; Fontana et al. 1984; Fierz et al. 1985; Levi-Strauss and Mallat 1987; Massa and ter Meulen 1987; Massa et al. 1987); however, it has also been suggested that oligodendroglia in addition to astrocytes function in this capacity (Wong et al. 1984; Suzumura et al. 1986). Other studies indicate astrocytes and endothelial cells as accessory cells (Hickey et al. 1985; Traugott and Raine 1985; Traugott et al. 1985; Frank et al. 1986; Traugott 1987). Finally, still other, numerous studies suggest that microglial cells are the major and possibly sole component of accessory function (Hauser et al. 1983; Lampson and Hickey 1986; Matsumoto and Fijiwara 1986; Matsumoto et al. 1986; Woodroofe et al. 1986; Hayes et al. 1987; Hickey and Kimura 1987; Akiyama et al. 1988). Thus, the exact identity of immune accessory cells within the brain is currently a rather unclear and controversial issue; however, the participation of at least some macrophages is supported by the proposed involvement of the microglia, presumed intrinsic brain macrophages (Jordan and Thomas 1988).

Brain macrophages represent an understudied cell population both in vivo and in vitro. These macrophages are a heterogeneous group containing several different types (see Jordan and Thomas 1988) with microglia being the largest component. The microglia are rather distinct from the other macrophage types in their definitively lower level of intrinsic expression of macrophage properties. These other macrophage types, or non-microglial macrophages, include pericytes, meningeal macrophages, epiplexus cells, and supraependymal cells. Collectively, these nonmicroglial macrophages exhibit similar properties. While we have identified and characterized microglia in this cortical culture system (Jordan and Thomas 1987a; Glenn et al. 1989a; Jordan et al.

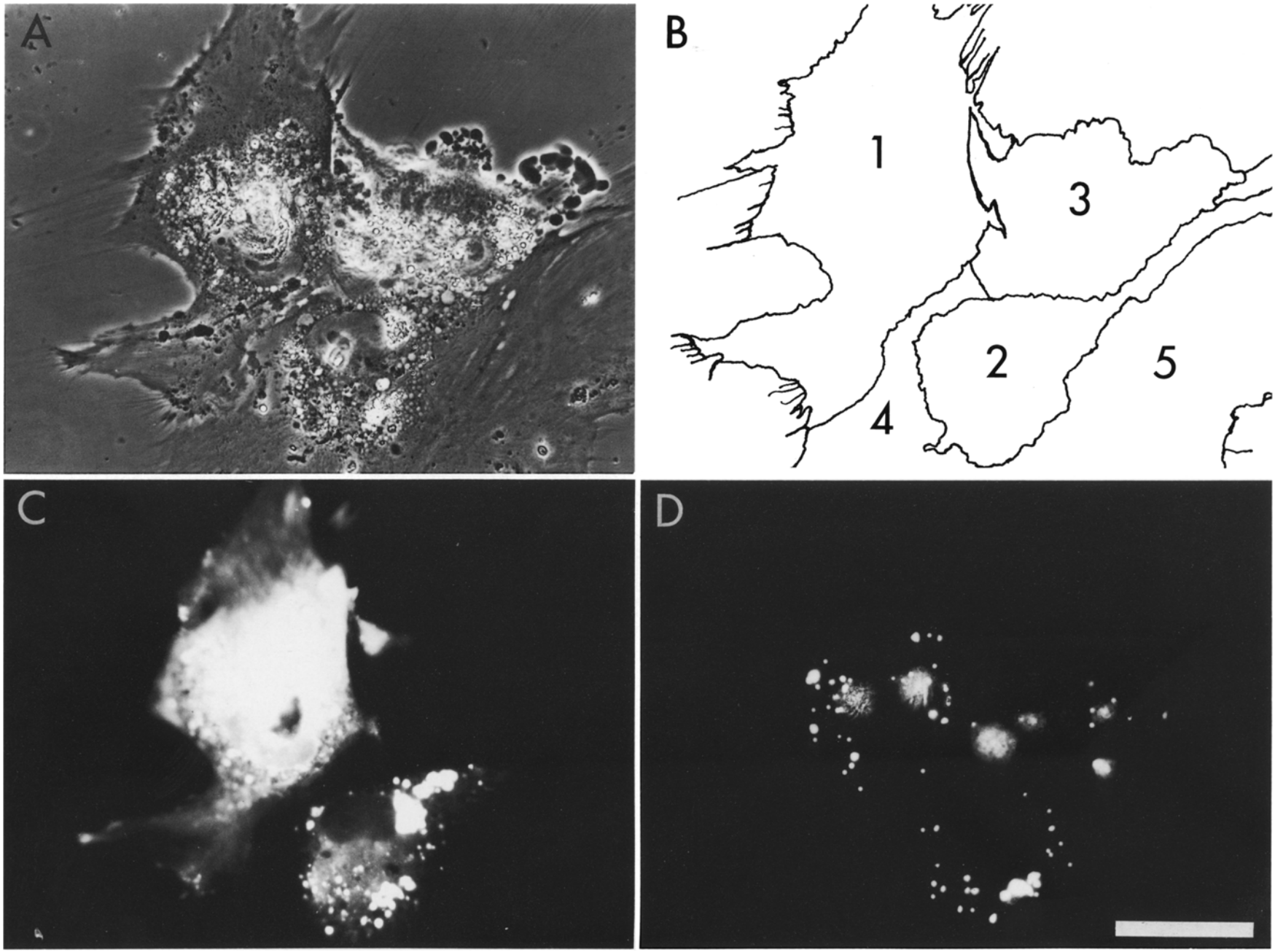

Fig. 7 A–D. Field of flattened macrophages dually stained for CR3 receptor with MAC-1 antibody, and for phagocytic capability with fluorescently-labelled beads. **A** Phase-contrast view of cells; note bubbling of membrane in cell no. 3 and filopodia extended throughout. **B** Line drawing indicating margins of 5 cells present in field. **C** MAC-1 immunofluorescence, demonstrating wide variability of labelling in different cells; note vesicular labelling of cell no. 2 and lower left of cell no. 1. **D** Epifluorescence pattern of latex beads ingested by 3 cells (nos. 1–3) in field. Expression of phagocytosis was typically more constant across culture than that of CR3 receptor. *Bar*: 50 μm

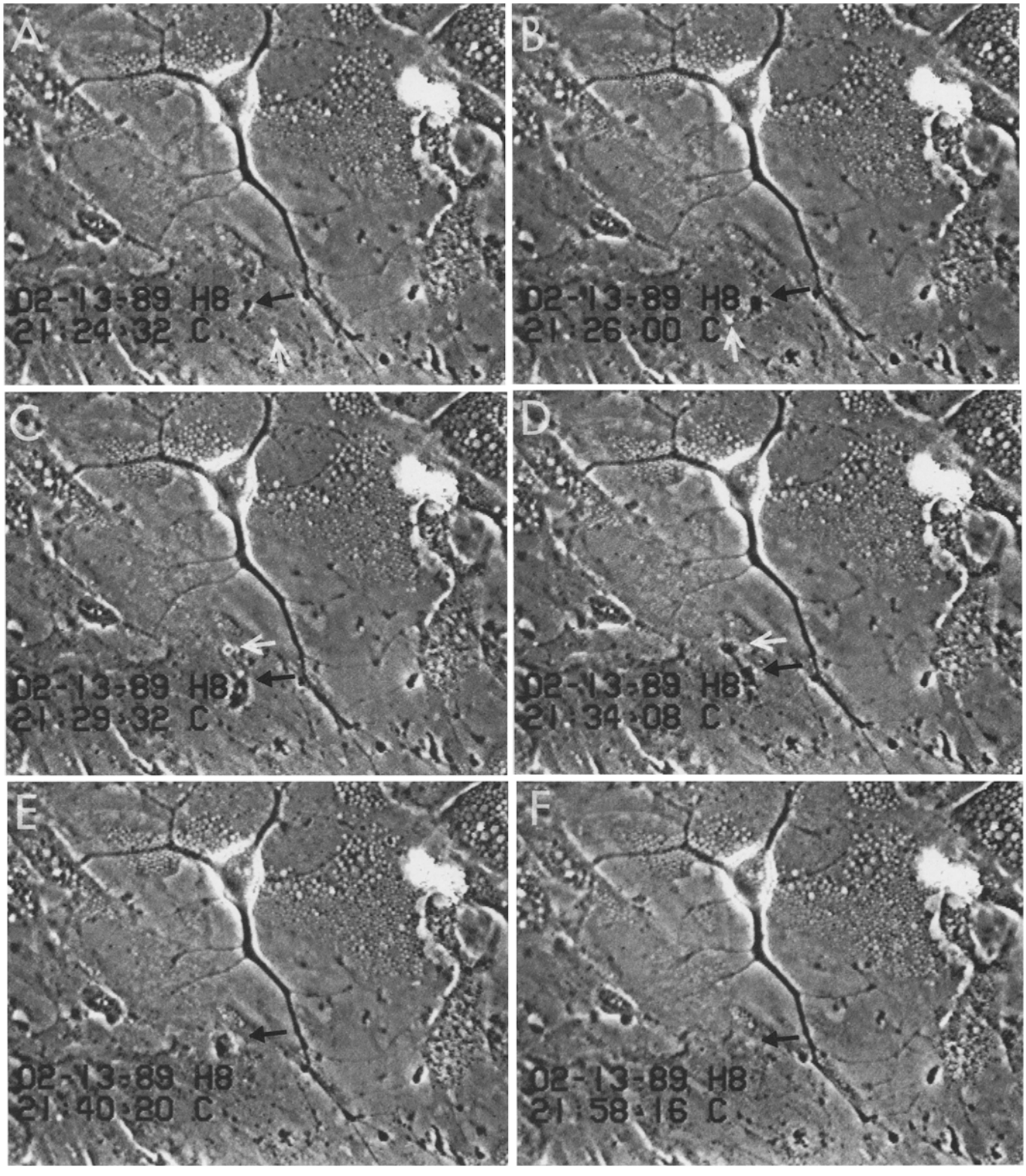

Fig. 8A–F. Time-lapse video sequence of flattened macrophage in monolayer extending process up into medium, capturing some particulate matter, and drawing both process and particle back into cell (total time is 33 min 44 s). Field is marked by presence of a large neuron. **A**, **B** Single, short process is extended (*black arrows*). **C** Contact is made with particle (*white arrows*). **D** Process tip bearing particle moves down and slightly to left; particle changes in appearance from light to dark as it comes into plane of focus. **E**, **F** Process is retracted with particle being ingested. ×630

1990; Thomas 1990), we have not previously been able to identify non-microglial macrophages. It seems possible that the present cells may correspond to a collective representation of the non-microglial macrophages. This is certainly supported by the properties described here and is consistent with the lack of GFAP staining and presence of vimentin, although it must also be cautioned that some astrocytes are vimentin-positive (Federoff et al. 1990). Also, the down-regulation of macrophage function would explain the previous inability to detect them.

If the present cells are indeed non-microglial macrophages, then they define another class or entity in culture – one which previously has not been considered extensively and which may have significant bearing on other findings. It has been suggested, or is even clear in some cases, that some of the immunological properties attributed to other cells (see above) are in fact a result of mis-identification or confusion with microglia (Akiyama et al. 1988); this appears particularly germane for astrocytes in culture (Hetier et al. 1988). Based on the present findings, not only microglia but also these potential non-microglial macrophages must be taken into account in efforts to identify accessory cells. Potential non-microglial macrophages may warrant consideration in studies of astrocytes as immunocompetent cells in tissue cultures; this is especially true in instances where a small percentage of the protoplasmic astrocytes (type 1) appear to display immune function (DuBois et al. 1985; Sasaki et al. 1989).

While the cells proposed as non-microglial macrophages possessed all of the properties of macrophages investigated, it is interesting that these properties were transient, being significantly down-regulated after mitosis. This makes it difficult to determine the full population and may contribute to confusion in their identification. However, it may also reveal some aspects of immune function within the brain. The non-microglial macrophages are normally located on the periphery or margins of the parenchyma of the brain in situ and, in contrast to conditions in vitro, consistently express properties of active macrophages, while microglial cells are located within the parenchyma and are consistently down-regulated. The non-microglial macrophages, which certainly should be present in these cultures since they are present in the tissue at the time it is obtained, would be exposed in vitro to cells of the parenchyma which they normally don't contact in situ. Thus, these cells may be behaving like microglia with respect to macrophage properties by down-regulating. The suggestion is that the parenchymal cells have a repressive influence on macrophage function.

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