Lipopolysaccharide-Free Conditions in Primary Astrocyte Cultures Allow Growth and Isolation of Microglial Cells

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Primary rat astrocyte cultures were used to isolate a macrophage population that does not adhere to the confluent glial cells. The cells multiplied vigorously in coculture with astrocytes during the 14 d culture period, provided that functionally active lipopolysaccharide (LPS) was either absent or present in very low concentrations. Based on morphological, immunocytochemical, and pharmacological data, it was concluded that the isolated cells were microglia, the resident macrophages of the brain. The findings characterized them as a distinct cell population that shares features both of peritoneal macrophages and of astroglial cells. Like peritoneal macrophages, the isolated cells were able to phagocytize as shown by their ingestion of latex beads and uptake of L-leucyl methylester. Furthermore, they were immunocytochemically stainable by a specific monoclonal antibody (ED 1) against a macrophage-specific antigen (Dijkstra et al., 1985). They also synthesized prostaglandin E₂ (PGE₂) and secreted interleukin 1 (IL-1) upon stimulation with LPS. Upon stimulation with the ionophore A23187, PGD₂, the predominant prostaglandin of the brain, was the major PG metabolite released by these cells. In contrast to peritoneal macrophages, microglial cells were able to multiply.

Proliferation of microglial cells in coculture with astrocytes was suppressed when 2 ng LPS/ml or higher concentrations were added to astroglial culture media. These astrocyte cultures, which contained approximately 1% microglia, were used to investigate the influence of LPS on prostaglandin and IL-1 secretion in order to compare astroglial and microglial features. Increasing LPS concentrations induced increased PGE₂ secretion, whereas PGD₂ secretion was essentially unaffected by LPS. The critical influence of LPS contaminations in most of the commercially available animal sera used for astrocyte cultures on cellular composition in general and on metabolism of hormones and growth factors in particular is discussed.

Microglial cells have been long known as a separate cell population of the brain (Rio-Hortega, 1932). Their features have recently been studied in great detail both morphologically (Mori and Leblond, 1969; Ling et al., 1973; Oehmichen, 1978) and cytochemically (Ling, 1981; Perry et al., 1985). They can be characterized as the primary scavenger cells during brain development and in the injured adult brain (Ling, 1981). It is widely accepted that microglia are specialized quiescent cells of the mononuclear phagocyte lineage (Werb and Chin, 1983) that retain their capacity to proliferate upon injury (Rio-Hortega, 1932; Oehmichen, 1983), a feature that distinguishes them from blood monocytes and macrophages. There is clearly a need to establish a pure culture of primary microglial cells suitable for investigation of their biochemistry and metabolism so as to better understand their potential role in normal and pathological brain states. Unfortunately, no antibodies against specific marker proteins of microglia are presently available.

Three attempts at culturing these cells have been reported (Ling et al., 1983; Giulian and Baker, 1986; Colton and Gilbert, 1987). The maximum enrichment in all cases was no more than 90–95%. Moreover, the first 2 procedures involved several complicated and time-consuming steps. Our report describes a simple one-step isolation procedure of pure microglial cells from primary astrocyte cultures that is based solely on maintaining the concentration of lipopolysaccharide (LPS) in nutrient media at a low level. The simplicity of the isolation procedure and the absolute purity of the cell population makes it extremely valuable for further studies on properties of brain-derived inflammatory cells. The purified microglial population has been characterized in this report by morphological, immunohistochemical, and biochemical data as a distinct cell population that shares several properties with peritoneal macrophages and several with astrocytes, the glial cells of the brain.

Materials and Methods

Cell cultures. Astroglial cells were prepared from cerebral hemispheres of newborn Wistar rats as described previously (Keller et al., 1985). In brief, after removal of the meninges, forebrains were minced and gently dissociated by trituration in Hank's balanced salt solution. Cells were collected by centrifugation at $200 \times g$ for 10 min, resuspended in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal calf serum (FCS), plated onto 35 mm Falcon culture dishes (5×10^5 cells/dish), and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO₂. Purity of the cells, as determined by glial fibrillary acidic protein (GFAP) immunohistochemical staining, was >90%. FCS batches from different suppliers were tested for functionally active LPS content by their ability to induce interleukin-1 (IL-1) production in cultured human blood monocytes (Northoff et al., 1986a, b). Media were prepared taking extreme care to avoid all potential sources of LPS contaminations, which may originate by way of preparation and storage

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of water used for nutrient media, reusable filtering devices, tubing, and glassware, as well as animal sera. Various concentrations of LPS, prepared from *Salmonella typhimurium* (Sebak, 8359 Aidenbach, FRG) were added as indicated in each experiment. Fourteen-day-old cultures grown in "low LPS" media were gently shaken; floating cells were then removed from astroglial cultures and reseeded (microglial cultures). Resting peritoneal macrophages were collected by lavage of adult rat peritoneum and maintained in DMEM as above. Astrocytes were also grown in defined media N1 (Bottenstein and Sato, 1979; Bottenstein et al., 1980) or in defined media as modified by Morrison and de Vellis (1981) with initial supplementation of serum for 3 d. Cell counts were determined in a Neubauer chamber after removal of cells from plates by trypsinization and subsequent trituration. Unless otherwise stated, all chemicals were from Sigma (8024 Deisenhofen, FRG).

³H-thymidine incorporation into cellular DNA. Isolated microglia or peritoneal macrophages (washed 3 times after 2 hr of attachment) were maintained for 24 hr in the absence or presence of 2 ng LPS/ml. Then, 0.5 μ Ci of ³H-thymidine (Amersham-Buchler, Braunschweig, FRG) was added to 2 ml of nutrient media for 6 hr. After removal of media and 3 washes, cells were fixed in ice-cold hypochloric acid (0.3 M) for 30 min at 4°C and washed again 3 times in acid. Then, 2 ml of 0.2 M NaOH was added, and solubilized material was counted in a Packard scintillation counter. Aliquots of NaOH-solubilized material were taken for protein determinations according to Lowry et al. (1951). Radioactivity was adjusted to protein content, and subsequently, ratios between results from LPS-deficient and LPS-containing samples were computed.

Immunocytochemistry. Cultures were fixed in ethanol-acetic acid (95:5) at -80°C for 12 min and incubated with specific antibodies (anti-GFAP and ED 1) as described previously (Gebicke-Haerter et al., 1984; Ignatius et al., 1986). Fluorescent second antibodies were from Cappel Laboratories (Cochranville, PA). Specimens were mounted in glycerol: 10 $\times$ PBS (10:1) with 0.2 M *n*-propylgallate as antifading agent (Valnes and Brandtzaeg, 1985) and viewed with a Zeiss fluorescence microscope under the appropriate wavelengths.

Cell incubations and radioimmunoassays. Cells were washed in DMEM/HEPES (20 mM), pH 7.4, and preincubated in the same media for 15 min at 37°C. After aspiration of media, they were incubated for 1 hr in the absence or presence of 10 ng LPS/ml or for 15 min with or without the calcium ionophore A23187 (1 μ M). Reactions were stopped by mixing supernatants with ice-cold phosphate-buffered gelatin (0.1%) solution containing 1 μ M indomethacin (Merck Sharp & Dohme, Rahway, NJ). Between 200 and 400 μ l of this solution was used for radioimmunoassays specific for PGD₂ and PGE₂. No cross-reactivity was detectable using authentic PGD₂ and PGE₂ with respective antisera.

Determination of LPS and IL-1. The functionally active LPS content of FCS (which is not identical with the total LPS content) from different suppliers was determined by the IL-1 production and assay system (IPAS) (Northoff et al., 1986a, b). In brief, sera were incubated with enriched human monocytes for 24 hr and levels of induced IL-1 measured by the C3H/HeJ mouse thymocyte costimulator assay (Northoff et al., 1980). IL-1 levels induced by sera under test were compared with those induced by a standard dilution curve of LPS (*S. typhi*, Difco, Detroit) in order to calculate the LPS equivalent (functionally active part of total serum-borne LPS). The C3H/HeJ assay, which was carried out in the presence of 50 μ g PHA-M (Serva, Heidelberg, FRG), was also used to determine IL-1 in media conditioned by isolated microglia or astrocytes. Media were either generated in the presence of indomethacin (1 μ M) or excessively dialyzed before testing to avoid inhibition by prostaglandins (PGs).

Results

Culturing of primary astrocytes and purification of a nonadherent population of microglia

LPS content of culture media determines cellular composition of primary astroglial cultures

In the first series of experiments, the influence of FCS on PG synthesis in rat astroglial cultures was determined. Astrocytes grown in the presence of 2 formulas of defined media (Bottenstein and Sato, 1979; Bottenstein et al., 1980; Morrison and de Vellis, 1981) showed no detectable PG synthesis. Optimum PG synthesis was obtained at 10% FCS and was significantly reduced at 5% FCS (approx. 25%). All further investigations were

therefore done in cultures supplemented with 10% FCS. With many sera randomly selected from different suppliers (Boehringer, Mannheim; Gibco, Karlsruhe; Sebak, Aidenbach, FRG), astrocyte cultures were obtained that showed the "typical" appearance described in the literature (Fig. 1a). The confluent monolayer of flat, polygonal cells is composed mainly of astroglial cells (see below), with occasional insertions of fibroblasts and macrophage (approx. 4 and 1%, respectively). A few sera, however, generated cultures containing substantial amounts of mononuclear cells. During the second week of culture, increasing numbers of these cells appeared on top of the astrocytic monolayer (Fig. 1b) and began to detach. Their phagocytotic activity could be shown by ingestion of latex beads (1.1 μ m diameter; Fig. 1c, arrowheads). Figure 1c further demonstrates the presence of additional phagocytes inserted in the astroglial cell layer. The LPS content of FCS used in these studies and the effect on cellular composition of astrocyte cultures is summarized in Table 1. It is evident that "phagocyte-accumulating" cultures resulted from use of sera that contained no detectable or very low amounts of functionally active LPS (up to 0.1 ng LPS equivalent/ml). Sera containing higher levels of LPS (only 3 of which are shown in Table 1, numbers 1, 10, and 22) resulted in the appearance of "typical" astrocyte cultures (Fig. 1a). Furthermore, addition of 2 ng LPS/ml or more to culture media supplemented with LPS-free FCS reliably eliminated the "phagocyte-accumulating" effect and led to the "typical" astroglial cultures shown in Figure 1a. In these cultures, occasional macrophages were identified by their pronounced cytotoxic activities, which resulted in the appearance of cell-free halos around their cell bodies (Fig. 1d).

LPS content of culture media affects protein content and cell number of primary astrocyte cultures

Fourteen-day-old astrocyte cultures grown with or without LPS were routinely checked for their protein content and cell numbers. Table 2 shows protein quantities and cell numbers typically found in 14-d-old astrocyte cultures maintained LPS-free or with 2 ng LPS/ml of culture media supplemented with LPS-free sera. LPS-free cultures contained 38 $\pm$ 4% more protein than cultures supplemented with 2 ng LPS/ml. Floating or weakly adherent cells were measured separately, and they represent another 9 $\pm$ 3% of protein to the value obtained with LPS-free cultures. Cell counts from LPS-free cultures were significantly higher than expected from the protein values. As shown in Table 2, 70 $\pm$ 14% more cells were found in LPS-free cultures, a feature probably resulting from a predominant proliferation of smaller cells. The dependence of protein content from LPS concentration is summarized in Table 3. The highest protein content was always found in LPS-free cultures. Low levels of LPS (about 1 ng/ml) caused a sharp drop in protein content that correlated with the transition from the "phagocyte-accumulating" type of culture to the "typical" astrocyte type of culture. At higher LPS concentrations, no significant further changes in protein content were observed.

Low LPS content of astroglial culture media allows purification of microglia after a 14 d culture period

Floating and loosely attached cells were harvested from "phagocyte-accumulating" (LPS-free) cultures at day 14 by gentle shaking or rinsing and were reseeded. Most of them rapidly attached to plastic (within several minutes to 2 hr) and assumed a typical macrophage morphology (Fig. 2, a-c). Addition of 2 ng LPS/

Figure 1. Morphological features of primary astroglial cultures maintained in the presence or absence of LPS. Cultures *a*, *c*, and *d* were grown for 2 weeks in culture media supplemented with LPS-free fetal calf serum and 2 ng LPS/ml. Culture shown in *b* was maintained under identical conditions, with LPS omitted. This resulted in accumulation of phase-bright phagocytes. Phagocytes atop (arrowheads) or inserted (arrows) in the astroglial cell layer are visualized by their ingestion of latex beads. Some inserted phagocytes show distinct cytolytic activities (*d*, center). Scale bar, 20 μ m.

Table 1. Influence of LPS content of sera on cellular composition of astrocyte cultures

	Sera numbers									
	1	4	9	10	13	18	21	22	23	24
LPS ^a	++	+-	-	++	-	+-	-	+	-	+-
Phagocyte accumulation	-	+	+	-	+	+	+	-	+	+

^a LPS-equivalent (IPAS): ++ >10 ng/ml; +- 1–10 ng/ml; +- = 0.1–1 ng/ml; - <0.01 ng/ml.

ml to these cultures did not seem to induce marked morphological changes in these cells compared with their morphology when maintained continuously under LPS-free conditions (Fig. 2, *a, b*), except that in the absence of LPS most cells are smaller in diameter (Fig. 2*b*). Essentially, all of these cells displayed phagocytotic activities (Fig. 2*c*). When cells were harvested too rigorously, occasional astrocytes or oligodendrocytes were recovered in microglial cultures (Fig. 2*d*). In this case, growing fibers of oligodendroglia often avoided microglial cell bodies or stopped elongation on encountering microglia (Fig. 2*d*, left). Astrocytes also maintained a certain distance from adjacent microglia when flattening out (Fig. 2*d*, right).

For comparative purpose, nonelicited peritoneal macrophages were grown in the same media with and without different concentrations of LPS. Only high LPS concentrations (1 μ g/ml) were able to induce discrete morphological changes (Fig. 2*e*). These were, however, distinct from those seen with isolated microglia (Fig. 2*a*) in that bipolar, elongated forms predominated over circular, flattened cell bodies. In the absence of LPS, most peritoneal macrophages retained their round, phase-bright morphology devoid of any cellular extensions (Fig. 2*f*).

Cells isolated from astroglial cultures belong to the mononuclear phagocyte cell lineage

Immunocytochemical studies with antibodies directed specifically against intracellular astrocytic (GFAP; Eng, 1980) and monocyte/macrophage (ED 1; Dijkstra et al., 1985) antigens were performed on astrocyte cultures maintained with and without LPS and on isolated microglia. Astroglia grown under LPS-deficient conditions were conspicuous, with ample numbers of ED 1+ cells that were inserted in the astrocytic cell layer (Fig. 3*a*, bottom). Loosely adhering or nonattached cells are not shown since they were removed by the labeling procedure. As described earlier (Keller et al., 1985), the majority of cells in "typical" cultures (contaminated by LPS-containing sera) are GFAP+ (Fig. 3*b*, middle). Here, cells labeled with ED 1 are rare and may become very large (>30 μ m diameter; see also Fig. 1*d*). Based on cell counts from randomly selected fields, approximately 1% of all cells in LPS-supplemented cultures and 15% in LPS-free cultures were ED 1+. Cells being removed from LPS-free cultures by the labeling procedure added another 9–

12% to the overall cell count, as mentioned above. Hence, LPS-free conditions generated 2 populations of ED 1+ cells, both of which actively proliferate. One population, inserted in the astroglial cell layer, was predominantly attached to plastic since, after trypsinization, cells remaining behind in the petri dish are more than 90% ED 1+. Thus, trypsinization was suitable for reliably enriching microglia and, simultaneously, reducing macrophage contamination in astrocyte cultures. The other cell population, which accumulated atop the confluent astroglial cells, was spherical and phase bright, with no extensions, and was gradually detaching.

Pinocytotic activities of cell types in primary astrocyte cultures were tested also using 1 mM of the lysosomotropic substance L-leucyl methyl ester (Thiele et al., 1983). LPS-deficient cultures revealed an almost exclusive uptake of this agent by ED 1+ cells, which was demonstrated by the formation of numerous intracellular vacuoles (Fig. 3*c*). LPS-containing cultures reacted in a very similar way (Fig. 3*d*). In both cases, astrocytic uptake of the ester remained remarkably reduced. Separated and reseeded microglial cells were also stained with ED 1 (Fig. 3*e*) and with anti-GFAP antibodies (not shown). All of them were ED 1+, which confirmed their identity as cells of the monocyte–macrophage lineage and the purity of the cultures.

LPS-free conditions, hence, result in astrocyte cultures that can be used to purify a population of mononuclear cells that is distinguished by their weak to nonexistent adherence to the glial cells. Conversely, astroglial cultures prove to be useful in the evaluation of LPS contaminations in culture media, respectively in supplementary sera.

Biochemical characterization of rat microglia compared with astrocytes and peritoneal macrophages

Synthesis of PGD₂ and PGE₂ in microglial, astroglial, and macrophage cultures

Synthesis of PGD₂ and PGE₂ in microglial and astroglial cultures was determined in the presence and absence of LPS. Astroglial cultures were maintained for 14 d in LPS-free or LPS-supplemented media (2 ng/ml), and isolated microglia were grown for 3 d under the same conditions. They were then washed 3 times in serum-free DMEM and incubated for 1 hr with or without challenging (1 μ g/ml) concentrations of LPS. The amounts of

Table 2. Protein content and cell numbers of 14-d-old astrocyte cultures

Protein		Cell numbers	
-LPS	+2 ng LPS/ml	-LPS	+2 ng LPS/ml
500 μ g/ml $\pm$ 45 (30)	362 μ g/ml $\pm$ 34 (78)	10.1 $\pm$ 0.8 $\times$ 10 ⁶ cells (12)	5.9 $\pm$ 0.6 $\times$ 10 ⁶ cells (12)
Ratio ^a 1.38 $\pm$ 0.04		Ratio ^a 1.70 $\pm$ 0.14	

Number of samples in brackets.

^a Fold increase over cultures treated with additional 2 ng LPS/ml.

Figure 2. Morphological features of microglia isolated from LPS-free astrocyte cultures. LPS (2 ng/ml) induced rapid flattening and enlargement of isolated microglial cell bodies (*a*), which was less pronounced under the continued absence of LPS (*b*). Latex bead phagocytosis by microglia in the absence of LPS is shown in *c*. Occasional oligodendrocytes and astrocytes in microglial cultures "avoid" microglia when growing fibers (*d*, left: arrows) or flattening out (*d*, right: arrows). For comparison, peritoneal macrophages were maintained for 24 hr in the absence (*f*) or presence of different concentrations of LPS. Cells cultured in the presence of 1 μ g LPS/ml are shown in *e*. Scale bar, 20 μ m.

Figure 3. Immunocytochemical labeling of 2-week-old rat astroglial cultures maintained in the absence or presence of 2 ng LPS/ml and of isolated microglial cells. Phase-contrast pictures are shown in *upper panels*, GFAP (astrocyte) labelings in the *middle*, and ED 1 (a macrophage marker) labelings in the *lower panels*. Under LPS-free conditions, numerous ED 1+ cells appeared in the astroglial cell layer (*a*, *lower panel*). Their percentage

PGD₂ and PGE₂ secreted during this period of time are shown in Figure 4. In the absence of LPS, microglial PGD₂ synthesis was intermediate between that for astroglial and peritoneal macrophage cultures, the levels for the latter being hardly detectable. Surprisingly, in the presence of LPS, astroglial PGD₂ synthesis

was reduced and a similar trend was observed in microglia, although not in peritoneal macrophages. PGE₂ release enhancement by LPS was greatest in peritoneal macrophages, followed by microglia. In order to evaluate the PG-synthesizing potencies of astroglial and microglial cells, the ionophore A23187 was

was markedly reduced in LPS-containing cultures (*b*, lower panel). Predominantly, ED 1+ cells showed pinocytosis of the lysosomotropic substance L-leucyl methyl ester, which resulted in the formation of vacuoles (*c*, no LPS; *d*, 2 ng LPS/ml). Ester uptake by astrocytes was significantly lower (*arrows*). ED 1 staining of isolated microglia is shown in *e*. They were GFAP- as can be seen in previous pictures. Scale bar, 20 μ m.

used for additionally stimulating PG synthesis. Astrocytes grown for 2 weeks with or without increasing concentrations of LPS (up to 10 μ g/ml) showed a slight but steady increase in their PGD₂ synthesis with LPS concentrations up to 100 ng/ml (Fig. 5). At higher LPS levels, the ionophore-stimulated PGD₂ syn-

thesis markedly declined. PGE₂ secretion increased sharply between the 0 and 1 ng LPS/ml levels and could be further enhanced by up to 1 μ g LPS/ml. Above that level, LPS repressed PGE₂ synthesis, too.

A comparison of the data collected from astroglia, microglia,

Figure 3. Continued.

and peritoneal macrophages that were stimulated with A23187 after culturing in media with or without 2 ng LPS/ml is given in Figure 6. Microglia produced the highest specific amounts of PGD_2 in the presence or absence of LPS, whereas peritoneal macrophages displayed the highest specific activity of PGE_2 synthesis both in the presence and absence of LPS. Astroglial and microglial PGE_2 synthesis was low in the absence of LPS and was significantly stimulated by LPS. In contrast, LPS markedly reduced ionophore-stimulated PGD_2 release (to about 50%) in peritoneal macrophages and, less strongly, in microglial cells.

IL-1 synthesis in astroglial and microglial cultures

The amount of IL-1 secreted into media of primary astrocyte and microglial cultures in the presence or absence of LPS was determined. Astrocytes were grown either with or without 10 ng LPS/ml for 14 d; microglia were harvested from LPS-deficient cultures and were allowed to attach for 8 hr or 3 d in the absence or presence of 2 ng LPS/ml. All of them were subsequently treated for 24 hr with or without challenging (1 $\mu\text{g}/\text{ml}$) concentrations of LPS (Table 4). During this time, indomethacin (1 μM) was present to inhibit PG synthesis. Secreted PGs can interfere with the IL-1 detection assay. Specific IL-1 synthesis was observed in LPS-treated microglia that were freshly isolated or treated with 2 ng LPS/ml for 3 d and in LPS-treated astrocyte cultures that were grown in LPS-deficient media throughout culture time ("phagocyte-accumulating" cultures). Isolated microglia maintained LPS-free did not produce significant amounts of IL-1. In addition, IL-1 could not be found in astrocyte cultures with low contaminations of microglia (10 ng LPS throughout), even when LPS was raised up to 1 $\mu\text{g}/\text{ml}$ during the last 24 hr of culture.

^3H -thymidine incorporation of isolated microglia in the presence or absence of LPS

Isolated microglia and freshly harvested peritoneal macrophages were maintained for 24 hr with or without 1 μg LPS/ml media. ^3H -thymidine incorporation into DNA of both cell populations is listed in Table 5. Peritoneal macrophages did not show any incorporation of radiolabel above background in the absence or presence of LPS. In the absence of LPS, however, microglia were found to incorporate significantly increased amounts of ^3H -thymidine. Addition of LPS suppressed microglial cell division. Furthermore, ^3H -thymidine incorporation into microglia maintained LPS-free also dropped to the background ratio of 1.0 between days 2 and 4 after their isolation (not shown).

Discussion

Studies on the PG-synthesizing capacities of astrocytes require serum-supplemented culture media. Astrocytes grown in defined media did not show any detectable PG synthesis in our hands. Most supplementary animal sera, however, contain significant amounts of LPS. In this report, convincing evidence is presented that final concentrations of LPS above 2 ng/ml culture media dramatically suppress microglial cell proliferation, whereas LPS-deficient cultures show a marked increase in mononuclear cell counts. The results presented here corroborate previous findings (Northoff et al., 1986a, b) that a considerable percentage of commercially available fetal calf sera is contaminated with LPS. When LPS is present in the critical range where microglial growth is affected most (<2 ng/ml), microglial percentages in astrocyte cultures are unpredictable, and, thus, their contribution in studies of astrocyte metabolism and biochemistry is

Table 3. Protein content of astrocyte cultures in relation to LPS concentration

LPS ($\mu\text{g}/\text{ml}$)	0	10^{-3}	10^{-2}	10^{-1}	1	10
Protein ($\mu\text{g}/\text{ml}$)	489 ± 43	367 ± 50^a	406 ± 70	380 ± 51	388 ± 46	371 ± 28

^a $p < 0.001$.

Figure 4. PGD₂ and PGE₂ synthesis in rat primary astrocyte, microglial, and peritoneal macrophage cultures stimulated with LPS. Astroglia were grown for 2 weeks, microglia and peritoneal macrophages for 3 d in the absence or presence of 2 ng LPS/ml. They were washed in serum-free culture medium and incubated serum-free for 1 hr in the absence or presence of 1 μ g LPS/ml. All experiments were done in triplicate. $n = 12$; *** $p < 0.001$.

difficult to evaluate. Whether astroglial proliferation is also influenced by LPS-deficient conditions cannot be resolved entirely by the present data. Once a certain level of LPS is reached, however, astroglial growth and development are apparently independent from further increases in LPS up to about 1 μ g/ml since neither cell counts nor protein levels at day 14 differed significantly over this concentration range of LPS (see also below). Additionally, basal and stimulated PGD₂ synthesis in astroglial cultures is minimally affected by increasing LPS concentrations up to 100 ng/ml. The steady increase in A23187-stimulated PGE₂ synthesis with LPS concentration (Fig. 5) is likely to originate both from microglia inserted in the astrocytic monolayer and from astrocytes. Although the sole contribution of microglia to PGE₂ secretion cannot be excluded, it seems to be highly unlikely. As seen in Figure 6, microglia produce approximately 70 ng PGE₂/mg protein compared with 14 ng PGE₂ in astrocyte

cultures at 2 ng LPS/ml media. Since microglial percentages in astrocyte cultures at LPS concentrations above 2 ng/ml remain essentially constant (approximately 1%), their specific PGE₂ production in coculture with astrocytes has to be at least an order of magnitude greater than that determined after their separation.

Astroglial IL-1 synthesis has been reported by others (Fontana et al., 1982). The present data do not support these findings. IL-1 was not detectable in astroglial cultures containing low amounts of microglial cells (in the presence of 10 ng LPS/ml throughout). However, when "phagocyte-accumulating" astrocyte cultures (no LPS throughout) were challenged for 24 hr with 1 μ g LPS, large amounts of IL-1 appeared in the culture media. This indicates that microglial cells, rather than astrocytes, are the source of IL-1 in astroglial cultures. IL-1 has been described as a potent mitogen for astrocytes (Giulian and Lachman, 1985).

Table 4. IL-1 activity^a in astrocyte and microglia culture media in the presence or absence of LPS

Astrocytes ^b	IL-1	Microglia ^b	IL-1
10 ng LPS throughout, then 24 hr no LPS ^c	—	8 hr no LPS, then 24 hr no LPS ^c	—
or 1 μ g LPS ^c	—	or 1 μ g LPS ^c	16
No LPS throughout, then 24 hr no LPS	—	3 d no LPS, then 24 hr no LPS	—
or 1 μ g LPS	64	or 1 μ g LPS	4

^a Expressed in titers (maximum dilution to stimulate significant ³H-thymidine incorporation in thymocyte assay).

^b Cell numbers in astrocyte cultures were $6.1 \times 10^6 \pm 0.5$ and $9.5 \times 10^6 \pm 0.8$ for "10 ng LPS throughout" and "no LPS throughout," respectively; cell numbers in microglial cultures were $0.6 \times 10^6 \pm 0.3$ cells.

^c LPS-free serum was used or LPS was added to 10% LPS-free serum plus indomethacin (1 μ M). All experiments were done at least twice in triplicate.

Figure 5. Ionophore A23187 stimulated PGD₂ and PGE₂ synthesis in rat primary astrocyte cultures. Astroglial cells were cultured for 2 weeks in the absence or presence of various concentrations of LPS as indicated, washed 3 times, and stimulated with 1 μ M ionophore for 15 min. $n = 12$; *** $p < 0.001$.

Figure 6. Ionophore A23187 stimulated PGD₂ and PGE₂ synthesis in astroglial, microglial, and peritoneal macrophage cultures in the absence or presence of 2 ng LPS/ml. Astrocytes were cultured for 2 weeks, microglia and peritoneal macrophages for 3 d in the absence or presence of 2 ng LPS/ml, washed 3 times, and stimulated with 1 μ M ionophore for 15 min. All experiments were done in triplicate. $n = 12$; *** $p < 0.001$.

However, since IL-1-producing microglia in LPS-supplemented astrocyte cultures are negligible and microglia in LPS-deficient (phagocyte-accumulating) cultures do not synthesize IL-1 (no IL-1 detectable in either case), IL-1 is probably not significant for proliferation of cultured astrocytes during the 2-week culture period. Therefore, astroglial proliferation is likely to be unaffected by the LPS concentrations routinely used, but rather depends on growth factors present in sera, e.g., epidermal growth factor (EGF) (Simpson et al., 1982).

LPS-free culture conditions have been shown to generate a floating microglial cell population in rat astroglial cultures ("phagocyte-accumulating" cultures). Presumably, this effect is strictly dependent on the presence of confluent astrocytes that prevent dividing microglia from attaching to a suitable substrate. This would imply that astrocytes seeded more densely at the beginning of culture and reaching confluency earlier would reinforce microglial accumulation. Accumulation atop the flat astrocytes may thus be due to some process of extrusion from the cell carpet. In this case, isolated microglia and microglia remaining behind in astrocyte cultures would belong to the same cell population. Observations made along with trypsinization would also argue for this hypothesis. Hence, astrocytes appear

to display cell-surface properties unfavorable for microglia, whereas plastic seems to represent a much better substrate. The finding that growing fibers or flattening membranes of occasional astrocytes in isolated microglial cultures avoid microglial cell bodies or stop elongation in the vicinity of microglia may be viewed along the same lines. This is in contrast to other observations on astrocytes being particularly good substrates for peripheral and central neurons (Hatten and Mason, 1986) and glial cells (unpublished observations). Another explanation for the "avoidance" reaction may reside in the cytolytic properties of microglia described above (Fig. 1*d*).

The signal(s) initiating microglial cell division in LPS-free astroglial cultures have as yet not been identified. Astroglia may secrete factors mitogenic for microglia, and LPS may antagonize this effect in the "standard" astroglial culture systems either by turning off factor production in astrocytes or directly inhibiting microglial cell division. In macrophages, LPS has been shown to increase secretion of IL-1 and PGE₂ (Werb, 1983) and specifically to repress the production of apolipoprotein E (Werb and Chin, 1983). In astroglial cultures, LPS evidently plays the crucial role in suppression of the phagocyte-accumulating effect. Independent from LPS, attachment of microglia to a suitable substrate may be an appropriate signal to turn off their cell division, an interpretation that can be deduced from the incorporation studies of radiolabeled thymidine after microglial isolation.

The origin and fate of microglia are still controversial. Bartlett's (1982) report on hemopoietic stem cells in adult mouse brain has been challenged by the findings of Hoogerbrugge et al. (1985). Others have shown that blood monocytes may give rise to "ameboid" cells of the brain (Ling et al., 1980), which may, after infiltration of a lesioned area, become resident microglia (Imamoto and Leblond, 1977). This cell population is reportedly quiescent (Mori and Leblond, 1969) and can be traced

Table 5. ³H-thymidine incorporation into microglia and peritoneal macrophages^a

Microglia	Macrophages (peritoneal)
5.27 $\pm$ 1.25 ^{b,c}	1.05 $\pm$ 0.11

^a Batches of sera were used that contained no detectable amounts of LPS (see Table 1) and cultures were grown with or without addition of 1 μ g LPS/ml for 24 hr.

^b Ratio of cpm/mg protein from -LPS/+LPS maintained cultures.

^c $p < 0.001$ in relation to "no effect" (ratio = 1.0).

as early as the fifth postnatal day in rat corpus callosum (Imamoto and Leblond, 1978). Ameboid microglia or microglial progenitor cells are believed to develop during embryogenesis or perinatally and to function, at this stage, as phagocytes of perishing cells. It is feasible that the special (LPS-free) conditions of astroglial cultures create an environment that particularly favors ameboid microglial or microglial progenitor cell proliferation. Whether blood monocytes would behave in the same way in this situation cannot be resolved. However, LPS has been described recently as inhibiting terminal maturation of blood monocytes into macrophages (Ganter et al., 1987).

The isolated cell fraction described in this report is characterized as a distinct cell population that shares important features with peritoneal macrophages and with astrocytes. In contrast to peritoneal macrophages, these cells divide in culture. After reseeding, their rate of attachment to plastic is much faster than that of peritoneal macrophages, and their ensuing morphological appearance is different. On the other hand, they do phagocytize latex particles and pinocytize L-leucyl methyl ester, as well as synthesize significant amounts of IL-1 upon stimulation with LPS. They also express an intracellular antigen confined exclusively to cells of the monocyte-macrophage lineage. Their PG-synthesizing capacities give them a position intermediate between astrocytes and peritoneal macrophages. Upon stimulation with LPS, reduction of PGD₂ release (Fig. 4) is more pronounced in astrocytes but not in macrophages, whereas PGE₂ is stimulated in microglia and macrophages but not in astrocytes. Stimulation of PG synthesis with the calcium ionophore A23187 in combination with LPS (Fig. 6) served as an additional means to gain information about the status of PG-synthesizing enzymes in the different culture systems. Specific PGD₂ production was most pronounced in microglia. Its reduction observed in the presence of LPS persisted upon stimulation with ionophore and appeared in peritoneal macrophages; in contrast, it was no longer seen in astrocytes. Hence, microglia may be able to switch their metabolic properties from a more macrophage-like pattern to a more astrocyte-like pattern depending on environmental conditions. The most prominent PG metabolite released by peritoneal macrophages upon A23187 stimulation was PGE₂. Although astrocytes, microglia, and peritoneal macrophages responded in a similar way to the presence of LPS by significantly elevating their PGE₂ levels, astrocytes were by far the least active, whereas microglial PGE₂ synthesis reached an intermediate level.

We have shown earlier that microglia (ED 1+ cells) in astrocyte cultures do not produce α_2 -macroglobulin, an acute phase protein made by rat hepatocytes and also synthesized by human macrophages (Ganter et al., 1987). α_2 -macroglobulin synthesis distinguishes the microglial cells from cocultured astrocytes (Gebicke-Haerter et al., 1987). Furthermore, it has recently been shown in our laboratory that NGF mRNA (mRNA^{NGF}), which was not detectable in freshly isolated microglia, was markedly induced upon a pulse of LPS. mRNA^{NGF} determined after 24 hr almost reached mRNA^{NGF} levels found in astroglial cultures maintained in the presence or absence of LPS (unpublished observations). In contrast, peritoneal macrophages are apparently unable to express substantial amounts of mRNA^{NGF}, as pointed out recently by Heumann et al. (1987). Finally, we have shown that ED 1+ cells in astroglial cultures, but not astrocytes, are immunoreactive for apolipoprotein E, an important secretion product of the macrophages (Ignatius et al., 1986). Under certain culture conditions (Pitas et al., 1987) and *in vivo* (Boyles

et al., 1985), however, astrocytes also showed apo-E immunoreactivity. Thus, microglia appear to be a distinct cell population in their failure to synthesize α_2 -macroglobulin but appear to be related to astroglia in their ability to express significant amounts of mRNA^{NGF} and to both macrophage and astroglia in their ability to secrete apo-E.

Taken together, the results presented here make it reasonable to conclude that the isolated cells originate from resident microglia of the brain. The purity of the cultured cells makes them extremely useful for studies on microglial biochemistry and metabolism, as well as for the production of specific antibodies against unique antigens of this cell population.

References

- Bartlett, P. F. (1982) Pluripotential hemopoietic stem cells in adult mouse brain. *Proc. Natl. Acad. Sci. USA* 79: 2722-2725.
- Bottenstein, J. E., and G. H. Sato (1979) Growth of a rat neuroblastoma cell line in serum-free supplemented medium. *Proc. Natl. Acad. Sci. USA* 76: 514-517.
- Bottenstein, J. E., S. D. Skaper, S. S. Varon, and G. H. Sato (1980) Selective survival of neurons from chick embryo sensory ganglionic dissociates utilizing serum-free supplemented medium. *Exp. Cell Res.* 125: 183-190.
- Boyles, J. K., R. E. Pitas, E. Wilson, R. W. Mahley, and J. M. Taylor (1985) Apolipoprotein E associated with astrocytic glia of the central nervous system and with nonmyelinating glia of the peripheral nervous system. *J. Clin. Invest.* 76: 1501-1513.
- Colton, C. A., and D. L. Gilbert (1987) Production of superoxide anions by a CNS macrophage, the microglia. *FEBS Lett.* 223: 284-288.
- Dijkstra, C. D., E. A. Doepp, P. Joling, and G. Kraal (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs. Distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED 1, ED 2, and ED 3. *Immunology* 54: 589-599.
- Eng, L. F. (1980) The glial fibrillary acidic (GFA) protein. In *Proteins of the Nervous System*, 2nd ed., R. A. Bradshaw and D. M. Schneider, eds., pp. 85-117, Raven, New York.
- Fontana, A., F. Kristensen, R. Dubs, D. Gerns, and E. Weber (1982) Production of prostaglandin E and interleukin-1 like factor by cultured astrocytes and C 6 glioma cells. *J. Immunol.* 129: 2413-2419.
- Ganter, U., J. Bauer, C. Schulz-Huotari, P. J. Gebicke-Haerter, H. Beeser, and W. Gerok (1987) Repression of α_2 macroglobulin in human mononuclear phagocytes by endotoxin. *Eng. J. Biochem.* 169: 13-17.
- Gebicke-Haerter, P. J., H.-H. Althaus, I. Rittner, and V. Neuhoff (1984) Bulk separation and long-term culture of oligodendrocytes from adult pig brain. I. Morphological studies. *J. Neurochem.* 42: 357-368.
- Gebicke-Haerter, P. J., J. Bauer, A. Brenner, and W. Gerok (1987) α_2 -Macroglobulin synthesis in an astrocyte subpopulation. *J. Neurochem.* 49: 1139-1145.
- Giulian, D., and T. J. Baker (1986) Characterization of ameboid microglia isolated from developing mammalian brain. *J. Neurosci.* 6: 2163-2178.
- Giulian, D., and L. B. Lachman (1985) Interleukin-1 stimulates astroglial proliferation after brain injury. *Science* 228: 497-499.
- Hatten, M. E., and C. A. Mason (1986) Neuron astroglia interactions in vitro and in vivo. *Trends Neurosci.* 9: 168-174.
- Heumann, R., D. Lindholm, C. Bandtlow, M. Meyer, M. J. Radeke, T. P. Misko, E. M. Shooter, and H. Thoenen (1987) Differential regulation of mRNA encoding nerve growth factor and its receptor in rat sciatic nerve during development, degeneration, and regeneration: Role of macrophages. *Proc. Natl. Acad. Sci. USA* 84: 8735-8739.
- Hoogerbrugge, P. M., G. Wagemaker, and D. W. van Bekkum (1985) Failure to demonstrate pluripotential hemopoietic stem cells in mouse brains. *Proc. Natl. Acad. Sci. USA* 82: 4268-4269.
- Ignatius, M. J., P. J. Gebicke-Haerter, J. H. P. Skene, J. W. Schilling, K. H. Weisgraber, R. W. Mahley, and E. M. Shooter (1986) Expression of apolipoprotein E during nerve degeneration and regeneration. *Proc. Natl. Acad. Sci. USA* 83: 1125-1129.
- Imamoto, K., and C. P. Leblond (1977) Presence of labelled monocytes, macrophages and microglia in a stab wound of the brain following an injection of bone marrow cells labelled with ³H-uridine into rats. *J. Comp. Neurol.* 174: 255-280.

- Imamoto, K., and C. P. Leblond (1978) Radioautographic investigation of gliogenesis in the corpus callosum of young rats. II. Origin of microglial cell. *J. Comp. Neurol.* 180: 134–164.
- Keller, M., R. Jackisch, A. Seregi, and G. Hertting (1985) Comparison of prostanoid forming capacity of neuronal and astroglial cells in primary cultures. *Neurochem. Int.* 7: 655–665.
- Ling, E. A. (1981) The origin and nature of microglia. In *Advances in Cellular Neurobiology*, Vol. 2, S. Fedoroff and L. Hertz, eds., pp. 33–82. Academic, New York.
- Ling, E. A., J. A. Paterson, A. Privat, S. Mori, and C. P. Leblond (1973) Investigation of glial cells in semithin sections. I. Identification of glial cells in the brain of young rats. *J. Comp. Neurol.* 149: 43–72.
- Ling, E. A., D. Penney, and C. P. Leblond (1980) Use of carbon labeling to demonstrate the role of blood monocytes as precursors of the “ameboid cells” present in the corpus callosum of postnatal rats. *J. Comp. Neurol.* 193: 631–657.
- Ling, E. A., C. Y. Tseng, F. C. T. Yoon, and W. C. Wong (1983) Isolation and culture of amoeboid microglial cells from the corpus callosum and cavum septum pellucidum in postnatal rats. *J. Anat.* 137: 223–233.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265–275.
- Mori, S., and C. P. Leblond (1969) Identification of microglia in light and electron microscopy. *J. Comp. Neurol.* 135: 57–80.
- Morrison, R. S., and J. de Vellis (1981) Growth of purified astrocytes in a chemically defined medium. *Proc. Natl. Acad. Sci. USA* 78: 7205–7209.
- Northoff, H., C. Carter, and J. J. Oppenheim (1980) Inhibition of concanavalin A-induced human lymphocyte mitogenic factor (interleukin-2) production by suppressor T-lymphocytes. *J. Immunol.* 125: 1823–1828.
- Northoff, H., D. Kabelitz, and C. Galanos (1986a) Interleukin 1 production for detection of bacterial polysaccharide in fetal calf sera and other solutions. *Immunol. Today* 7: 126–127.
- Northoff, H., D. Glück, A. Wölpl, B. Kubanek, and C. Galanos (1986b) Lipopolysaccharide-induced elaboration of interleukin-1 (IL-1) by human monocytes: Use for the detection of LPS in serum and influence of serum-LPS interactions. *Rev. Infect. Dis. (Suppl. 5)* 9: 599–602.
- Oehmichen, M. (1978) *Mononuclear Phagocytes in the Central Nervous System*, Springer-Verlag, New York.
- Oehmichen, M. (1983) Inflammatory cells in the central nervous system. *Progr. Neuropathol.* 5: 277–325.
- Perry, V. H., D. A. Hume, and S. Gordon (1985) Immunohistochemical localization of macrophages and microglia in the adult and developing mouse brain. *Neuroscience* 15: 313–326.
- Pitas, R. E., J. K. Boyles, S. H. Lee, D. Foss, and R. W. Mahley (1987) Astrocytes synthesize apolipoprotein E and metabolize apolipoprotein E-containing lipoproteins. *Biochem. Biophys. Acta* 917: 148–161.
- Rio-Hortega, P. del (1932) Microglia. In *Cytology and Cellular Pathology of the Nervous System*, Vol. 2, W. Penfield, ed., pp. 481–584, Hocker, New York.
- Simpson, D. L., R. Morrisson, J. de Vellis, and H. R. Herschman (1982) Epidermal growth factor binding and mitogenic activity on purified populations of cells from the central nervous system. *J. Neurosci. Res.* 8: 453–462.
- Thiele, D. L., M. Kurosaka, and P. E. Lipsky (1983) Phenotype of the accessory cell necessary for mitogen-stimulated T and B cell responses in human peripheral blood: Delineation by its sensitivity to the lysosomotropic agent, L-leucine methyl ester. *J. Immunol.* 131: 2282–2290.
- Valnes, K., and P. Brandtzaeg (1985) Retardation of immunofluorescence fading during microscopy. *J. Histochem. Cytochem.* 33: 755–761.
- Werb, Z. (1983) How the macrophage regulates its extracellular environment. *Am. J. Anat.* 166: 237–256.
- Werb, Z., and J. R. Chin (1983) Apoprotein E is synthesized and secreted by resident and thioglycollate-elicited macrophages but not by pyran copolymer- or bacillus Calmette-Guerin-activated macrophages. *J. Exp. Med.* 158: 1272–1293.