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Microglial progenitors with a high proliferative potential in the embryonic and adult mouse brain

(hemopoietic stem cells/macrophages/colony-stimulating factors)

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ABSTRACT **Single cell suspensions, prepared from brain stem, cerebellum, and forebrain parenchyma of embryonic and adult mice, were plated on monolayers of an astroglial cell line derived from a spontaneously immortalized mouse cerebellar culture, the D19 clone. A few of the brain cells adhering to the D19 monolayers were immunoreactive to the Mac-1 antibody, which labels all cells of the monocytic and granulocytic lineages. The Mac-1-positive cells proliferated vigorously and later most of them acquired the F4/80 epitope specific for macrophages and microglia cells. Studies in clonal conditions allowed development of large colonies of about 2×10^5 cells that expressed typical microglia markers. Bone marrow Mac-1-positive cells cocultured on D19 monolayers were also induced to proliferate, whereas peritoneal macrophages were not. D19 astrocytes express macrophage colony-stimulating factor (CSF-1) activity at a high level, and their conditioned media induced the proliferation of brain and bone marrow Mac-1-positive cells. A specific anti-CSF-1 antiserum completely blocked bone marrow macrophage progenitor proliferation and significantly reduced the multiplication of microglial precursors induced by the D19-conditioned medium. These data indicate that the embryonic and adult mouse brain parenchyma contains potential progenitors for microglial cells.**

Microglia, first described by del Rio Hortega (1), form a distinct population of glial cells in the central nervous system (CNS). Recent reports strongly suggest that microglia are specialized cells of the mononuclear phagocyte lineage. Indeed, it has been shown that cells morphologically similar to those originally described as microglial in the brain parenchyma of developing and adult mice are labeled by monocyte- and macrophage-specific markers. Microglia express FcR, type 3 complement receptor, and a macrophage-specific membrane glycoprotein of unknown function, recognized by the 2.4G2, Mac-1, and F4/80 monoclonal antibodies (2), respectively. Thus, it appears that brain microglia, like the other resident macrophages, could be the progeny of hemopoietic bone marrow progenitors. However, it has not been established whether the progenitors are present in the CNS before the formation of the blood–brain and meningeal barriers or if they can penetrate the brain during the adult life of normal mice to replace the decaying microglial cells. It is also known that cells that multiply *in vivo* after a brain injury (3) or *in vitro* in cultures of cerebral hemispheres from perinatal rat (4) express microglial markers; however, these studies did not indicate the actual number of cell doublings, and they have not determined whether the multiplying cells are microglial cells or their progenitors.

In this study, we have investigated the presence and the proliferative capacity of microglial progenitors in different regions of the developing and adult mouse brain parenchyma. To this end, we have used an experimental system whereby brain cells were cultured on monolayers of the D19 astroglial cell clone, derived from an immortalized mouse cerebellar culture (5, 6), which expresses the astrocyte marker glial fibrillary acidic protein and is morphologically comparable to the velate protoplasmic astrocytes of the cerebellar cortex. We report here that the brain parenchyma of the embryonic and adult mouse contains cells that proliferate very actively in response to a mitogenic signal(s) released by the D19 astroglial clone. These cells each give rise to colonies comprising about 2×10^5 cells that exhibit the typical Mac-1, FcR, F4/80, microglial-macrophage phenotype. When cultured under the same conditions, a subset of bone marrow cells exhibited a proliferative capacity and a phenotype similar to that of brain microglial cells. These results indicate that *embryonic* and *adult* mouse brain parenchyma contains microglial progenitors comparable to the bone marrow macrophage progenitors.

MATERIALS AND METHODS

Cell Cultures. Cell suspensions were obtained essentially as described (6). Embryonic (16–18 days) or adult (1 month or older) C57BL/6J mice were used after extensive heart perfusions with Hanks' solution. Cerebellum, forebrain, and brainstem were carefully freed from meninges, dissected out, extensively rinsed, minced, and incubated in a solution of 0.25% trypsin (GIBCO, no. 043-05090) diluted in Versene 1:5000 (GIBCO, no. 043-05040) for 20 min at 37°C. After removal of trypsin, tissues were mechanically dissociated with a pipet in 5 ml of complete medium [BME (GIBCO, no. 073-1300)/10% fetal bovine serum] and centrifuged at 200 × *g* for 10 min. The cell pellet was resuspended in complete medium and dissociated. Under these conditions >95% of the cells counted with a hemocytometer were single. Cell viability, estimated by trypan blue exclusion, was around 80%. The D19 astroglial cells were seeded at a concentration of 5×10^3 per ml in 24-well Falcon plates containing 1.5-cm^2 glass coverslips in a volume of 1 ml of complete medium. After 7–10 days, D19 cells were confluent and stopped multiplying. Brain cell suspensions were plated onto confluent D19 monolayers at various densities in complete medium (as indicated in *Results*). Bone marrow cells were obtained from adult femurs cleaned from surrounding tissues. The epiphyses were removed, a needle was inserted into one end of the bone cavity, and the bone marrow was flushed out. Monocellular suspensions were obtained by repeated pipetting. Peritoneal cells were obtained from adult mice by washing of the peritoneal cavity with glucose-containing phosphate-buffered saline (PBS).

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Abbreviations: CSF, colony-stimulating factor; CNS, central nervous system; CFU-GM, colony-forming unit, granulocyte/macrophage.

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All cultures were maintained in complete medium at 37°C in an atmosphere of 95% air/5% CO_2. Medium was changed 1 week later and every week thereafter.

Immunocytochemistry. Cocultures were double labeled with F4/80 hybridoma culture supernatant (gift of S. Gordon, University of Oxford) and Mac-1 (Boehringer Mannheim, no. 1118 129) or 2.4G2 (NEI-032, New England Nuclear) monoclonal antibodies. After successive incubations with undiluted F4/80 supernatant and fluorescein isothiocyanate-labeled goat anti-rat IgG antibody (Southern Biotechnology Associates, Birmingham, AL, no. 3030-02), cocultures were fixed with a 4% solution of paraformaldehyde in PBS, incubated with Mac-1 (1:100) or 2.4G2 (1:1000), followed by incubation with a rhodamine isothiocyanate-labeled goat anti-rat IgG antibody (Cappel Laboratories, no. 15049). In some experiments, F4/80 was replaced by 2.4G2. All incubations were carried out at 4°C for 30 min.

Cell Counting. Cells derived from a single progenitor were counted as Mac-1^+ cells 4–5 weeks after the beginning of the coculture. Cells in suspension, collected from each well, and plated onto glass coverslips, were all Mac-1^+. The number of Mac-1^+ cells in the corresponding monolayers was added to the number of cells in suspension.

Effect of D19-Conditioned Medium (D19 CM) and of Anti-Colony-Stimulating Factor 1 (CSF-1) Antiserum. To prepare D19 CM, culture medium of confluent D19 monolayers was replaced by fresh complete medium (BME/10% fetal bovine serum), collected 4 days later, and Millipore-filtered. Embryonic day 16 brainstem cells (10^4 single cells) were plated per cm^2 on glass coverslips in 24-well plates with BME/10% fetal bovine serum, with D19 CM (diluted 1:5), or with D19 CM (1:5) incubated overnight with an anti-CSF-1 antiserum (gift of E. R. Stanley, Albert Einstein College of Medicine) at a final dilution of 1:100, 1:300, or 1:1000. One week later and every week thereafter, all wells (control and experimentals) were changed with BME/10% fetal bovine serum.

Northern Blot Analysis. Poly(A)$^+$-enriched RNAs were purified from total RNAs of the D19 cell line according to published procedures (7, 8). Northern blot analyses were performed as described (9).

RESULTS

Embryonic Brain Parenchyma Contains Microglial Progenitors. The markers used in this study were essentially two monoclonal antibodies, Mac-1 and F4/80. Mac-1 labels a subset of colony-forming unit, granulocyte/macrophage (CFU-GM) cells—i.e., the progenitor common for the granulocytic and monocytic lineages—as well as the progeny of the CFU-GM cells (10). F4/80 binds to macrophage-committed monocytes and to mature macrophages (11). It has been shown by others that Mac-1 labels cells located in the brain parenchyma from embryonic day 16 and that the number of Mac-1^+ and F4/80^+ cells increases drastically between embryonic days 16 and 19 (2).

In preliminary experiments, we have investigated the adhesion to poly(lysine)-coated glass coverslips of cells dissociated from the forebrain, the cerebellum, and the brainstem of 16- to 18-day embryos. The vast majority of the cells adhered within a few hours. To determine the proportion of microglial cells, cultures were double labeled with Mac-1 and 2.4G2 or F4/80 at various times. Mac-1^+ cells were clearly seen 2–3 days after seeding. All Mac-1^+ cells were also immunolabeled by 2.4G2, a monoclonal antibody to the FcR (data not shown). The number of adhering Mac-1^+ cells was much higher in the brainstem than in the forebrain or cerebellum. However, only very few of the Mac-1^+ cells were also labeled by F4/80. These data show that the vast majority of adhering Mac-1^+ cells originating from perfused brains are not macrophage or microglial cells. In addition, we also observed that survival and proliferation of Mac-1^+ cells were dependent upon the seeding density of the brain cell suspensions. Indeed, when 3×10^4 or more brain cells were plated per cm^2, Mac-1^+ cells—as well as astrocytes—began to proliferate after 2 weeks, and later the majority of Mac-1^+ cells were also immunolabeled by F4/80. Conversely, when 1×10^4 or fewer brain cells were seeded per cm^2, no cell type survived beyond 1 week. Taken together, these results suggested that the Mac-1^+, F4/80^- cells might be progenitors for microglial cells and that their survival and proliferation were dependent upon the other brain cell types that had adhered to poly(lysine)-coated glass coverslips. Since astrocytes and, in particular, large flat astrocytes represent the majority of these cells, we next investigated the role of astrocytic monolayers on the survival of Mac-1^+ brain cells. We used an astroglial clone, D19, derived from C57BL mouse cerebellar cultures, whose cells have a large flat soma with no processes and thus resemble the velate protoplasmic astrocytes of the cerebellar cortex (5, 6). D19 astroglial cells never express the Mac-1 or F4/80 epitope. When confluent, they form an almost perfect translucid monolayer onto which seeded cells can be easily distinguished.

Single cell suspensions, comprising all cell types present in embryonic day 16–18 brains, were seeded at various densities on D19 monolayers and double immunolabeled with Mac-1 and 2.4G2 or F4/80 antibodies after 1, 2, and 3 days of coculture. After 24 hr, no cell was clearly immunoreactive to any of these antibodies. However, after 2–3 days of coculture, Mac-1^+, 2.4G2^+ cells could be seen and counted. In contrast, only very few Mac-1^+ cells were labeled by F4/80 (Table 1). The vast majority of the other brain cells that adhered to the D19 monolayers were astrocytes, a few neuroblasts, and oligodendrocytes identified by specific markers. The Mac-1^+ cells plated on the D19 astrocytes did survive even when the brain suspensions were seeded at a concentration of 10^4 or fewer cells per cm^2. Although the number of Mac-1^+, 2.4G2^+ cells began to increase after 3–4 days of coculture, the number of F4/80^+ cells remained very low and even decreased (data not shown). After 10–15 days of coculture, multiplying round cells were seen on top of the D19 monolayers. These cells were Mac-1^+, 2.4G2^+ and some of them were F4/80^+. Four weeks later, when proliferation had stopped, the vast majority of the Mac-1^+, 2.4G2^+ cells were also labeled by F4/80. These results suggested that the proliferating cells originate from Mac-1^+, F4/80^- progenitors.

Clonal Proliferation of Mac-1^+ Cells from Embryonic Brain. These data prompted further experiments to determine whether all embryonic Mac-1^+ brain cells are capable of proliferating in coculture with D19 astrocytes and whether all multiplying cells have the same proliferative capacity and acquire the F4/80 epitope. To answer these questions, it was necessary to investigate the progeny of single Mac-1^+ cells. The ideal strategy would have been to obtain, immediately after brain dissociation, a pure population of single Mac-1^+ cells by a method such as fluorescence-activated cell sorting. However, as mentioned above, the Mac-1 epitope is not detected on fresh cell suspensions. Therefore, in order to distribute the Mac-1^+ cells according to the Poisson law, we had to take into account the number of Mac-1^+ cells present after 2 days of coculture (Table 1). Thus, 140 single brainstem, 475 forebrain, or 985 cerebellum cells were plated onto each well of multiwell plates covered with D19 astrocytes. Under these conditions, we observed that when cocultures were immunolabeled after 2–3 days, one-third of the wells contained no Mac-1^+ cell, whereas the other wells contained a single (and exceptionally two or three) Mac-1^+ cell.

We then investigated the proliferative capacity of the seeded brain cells. The first foci of multiplying cells that could be distinguished from the D19 monolayers under phase-

Table 1. Mac-1$^+$ cells, F4/80$^+$ cells, and microglial progenitors in different brain regions of embryonic and adult mice

Brain region	Total number of cells × 10^6*	Mac-1$^+$ cells		F4/80$^+$ cells,†	Microglial progenitors,§
		No.†	Ratio‡	no.	no.
Brainstem					
E	0.445	2,125 ± 550	1:210	160 ± 45	470 ± 60
A	14	1,800 ± 470	1:7780	140 ± 40	1800 ± 170
Cerebellum					
E	0.830	560 ± 140	1:1480	8 ± 3	100 ± 15
A	16	500 ± 130	1:32,000	53 ± 12	500 ± 45
Forebrain					
E	7.450	10,435 ± 2,700	1:710	75 ± 15	4135 ± 520
A	50	7,000 ± 2,050	1:7140	330 ± 90	7000 ± 690

E, embryonic; A, adult. For each type of cell suspension, data represent the average of six independent experiments.
*The total number of cells per region was counted after complete dissociation.
†Cell suspensions were obtained as described in the text. Single cells were plated onto D19 monolayers. Mac-1$^+$ cells and F4/80$^+$ cells were counted after 2 days. Results are expressed as the total number of adhering Mac-1$^+$ or F4/80$^+$ cells per brain region.
‡Results are expressed as the ratio of adhering Mac-1$^+$ cells to the total number of cells.
§To determine the number of microglial progenitors in brain cell suspensions—i.e., of cell that give rise in our cocultures to clones of about 2 × 10^5 Mac-1$^+$, F4/80$^+$ cells—single Mac-1$^+$ brain cells were seeded onto D19 monolayers in multiwell plates at dilutions at which these clones appeared in at most two-thirds of the wells (see text).

contrast microscopy were seen after 12 days of coculture. They proliferated vigorously and 8–15 days later covered the D19 astrocytes with small round cells that had a tendency to detach. Cells that gave rise to these large clones—i.e., microglial progenitors—represented only a fraction of the number of seeded Mac-1$^+$ cells (Table 1). After 1 month of coculture, the number of Mac-1$^+$ cells per well was measured as described in *Materials and Methods*. Each well contained, on average, 2 × 10^5 Mac-1$^+$ cells independent of the brain region from which the cell suspension was prepared. That the colonies were indeed the progeny of single cells was further indicated by the fact that the number of colonies decreased proportionally to the number of seeded cells, although the number of cells per colony remained constant at about 2 × 10^5. Two types of data indicate the existence of an upper limit for the proliferative capacity of these cells. (*i*) When a single Mac-1$^+$ brain cell was seeded onto 7-cm^2 coverslips, the maximal number of cells per clone was similar to that found when a single Mac-1$^+$ cell was plated onto 2-cm^2 wells. (*ii*) When Mac-1$^+$ cells, collected after proliferation had reached a plateau (i.e., about 2 × 10^5 cells per 2-cm^2 wells), were replated in clonal conditions on fresh D19 monolayers, no further multiplication of these Mac-1$^+$ cells was observed. These data suggest that the proliferative capacity of the cells that comprise these large microglial clones is restricted by an internal clock.

To determine the phenotype of these proliferative brain cells, they were immunolabeled with Mac-1, 2.4G2, and F4/80 monoclonal antibodies. All clones were Mac-1$^+$ and 2.4G2$^+$ during the multiplication period and after proliferation had ceased. At the end of the proliferative stage, the vast majority of Mac-1$^+$ cells were also labeled by the F4/80 antibody. Fig. 1 shows two distinct morphologies of Mac-1$^+$, F4/80$^+$ cells, which can be either spread or round. Mac-1 binds to the whole surface of spread cells as well as to round cells, whereas F4/80 labels only the perinuclear zone of flat cells but labels the whole surface of round cells. These cells became Ia$^+$ after interferon γ treatment (unpublished results). Thus, cells of the large clones expressed markers typical of microglia.

Taken together, these data indicate that the mouse embryonic brain contains cells that are potential microglia progenitors and whose proportion differs in distinct embryonic brain regions (Table 1). In addition to these large Mac-1$^+$, F4/80$^+$ microglial clones, an equal number of clones composed of <5 × 10^3 Mac-1$^+$ cells was also observed. This was the upper proliferative limit for this type of clone under our culture conditions. In contrast to the large microglial clones, cells that made up these small clones never acquired the F4/80 epitope. It is noteworthy that there were apparently no cells capable of giving rise to clones comprising between 5 × 10^3 and 2 × 10^5 Mac-1$^+$ cells.

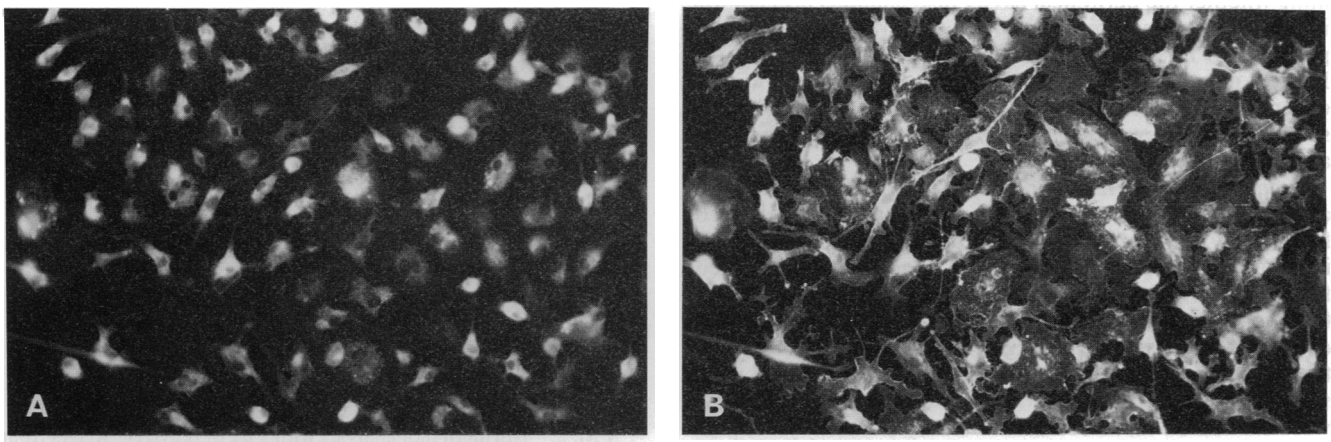

FIG. 1. Double immunolabeling of a microglial clone with F4/80 and Mac-1 monoclonal antibodies. The same field was labeled by F4/80 (*A*) and Mac-1 (*B*) antibodies. (×270.)

Adult Brain Contains Microglial Progenitors. We next asked whether microglial progenitors are also present in the adult brain. Single cell suspensions from adult brainstem, forebrain, and cerebellum were seeded at 10^5 cells per cm^2 onto D19 astrocytes and counted after 1, 2, or 3 days. No Mac-1^+ cell was detected after 24 hr. Table 1 shows the number of Mac-1^+ cells counted 2–3 days after plating; as for embryonic cells, almost no F4/80^+ cell adhered to the D19 astrocytes. However, the number of Mac-1^+, 2.4G2^+ cells increased progressively. During the first days of coculture these cells were F4/80^-; they began to acquire the F4/80 epitope only after 12–15 days. To determine the proliferative capacity of the Mac-1^+ cells from adult brain, we used the same strategy as for embryonic cells—i.e., we observed their progeny in clonal conditions, after distribution onto D19 monolayers in multiwell plates, according to the Poisson law. Actively multiplying cells progressively covered the D19 monolayers in two-thirds of the wells. At the end of the proliferation period—i.e., after 4–5 weeks—the majority of clones comprised each about 2×10^5 cells. All multiplying cells were Mac-1^+ and remained Mac-1^+ after proliferation had ceased. Furthermore, >90% of the Mac-1^+ cells were also labeled by F4/80, indicating that these are microglial cells (Table 1). In addition to these large Mac-1^+, F4/80^+ microglial clones, foci made of $<5 \times 10^3$ cells, which remained Mac-1^+ and F4/80^-, were also observed. The proportion of these clones was greater in cocultures of cell suspensions derived from the brainstem than from other CNS regions (data not shown). Thus, the combined number of large microglial clones and of small foci was superior to the number of seeded Mac-1^+ cells.

Comparison of the number of the Mac-1^+ cells as well as of microglial progenitors between embryonic day 16–18 embryos and adult (Table 1) shows that (*i*) the number of Mac-1^+ cells does not increase and (*ii*) the number of microglial progenitors increases four to five times in the brainstem and the cerebellum.

Effect of D19 Astrocytes on Bone Marrow Proliferation. These results led us to investigate whether bone marrow macrophage progenitors have the same proliferative capacity as microglial progenitors when cocultured on D19 astroglial monolayers. Bone marrow is a very heterogeneous cell population. Indeed, when unfractionated bone marrow cells from adult mouse were plated on D19 monolayers, only about 2% of the adhering cells were Mac-1^+ and 0.1% were F4/80^+. At limiting dilutions, 2.5% of bone marrow cells gave rise, within 10–12 days, to colonies of $3–4 \times 10^5$ Mac-1^+, 2.4G2^+, F4/80^+ cells. It thus appears that brain microglia and bone marrow macrophage progenitors have a very close proliferative capacity when cocultured on D19 astrocytes. To determine whether the peritoneal cavity also contains progenitors in addition to mature macrophages, peritoneal fluid was plated onto D19 monolayers. Although numerous Mac-1^+, F4/80^+ macrophages were seen, no cell proliferation was observed, except for a few foci made up of at most 20 cells, showing that the peritoneal cavity does not contain macrophage progenitors.

Properties of the Mitogenic Activity. The D19 astroglial cell clone used here is a spontaneously immortalized line derived from 8-day postnatal mouse cerebellar explants. We thus investigated whether astrocyte-enriched cultures derived from various regions of the mouse CNS were also mitogenic for brain microglial progenitors. Secondary cultures that contain essentially large flat astrocytes devoid of other cell types were prepared from the forebrain, cerebellar, or brainstem of postnatal mice (12). When brain cell suspensions were seeded on these confluent astrocyte monolayers, proliferation of microglial progenitors that gave rise to clones of about 2×10^5 cells was also observed. However, proliferation of microglial progenitors was delayed up to 3 weeks and its extent varied between experiments. This may be accounted for by the heterogeneity of astroglial cell types in secondary brain cultures. To determine whether the mitogenic signal is cell bound or released into the culture medium, brainstem cells were plated at a concentration of 1×10^4 cells per cm^2 onto glass coverslips and cultured in control medium or in conditioned medium collected from 4-day D19 cultures. Although the vast majority of cells (including astrocytes) in control medium (BME/10% fetal bovine serum) did not survive when seeded at this low density, in cultures fed initially with D19 CM proliferating cells appeared that gave rise within 2–3 weeks to large colonies of Mac-1^+, F4/80^+ cells. It is noteworthy that D19 CM was present only during the first week, suggesting that it may be only necessary for the very first cell divisions of microglial progenitors. These results indicated that the D19 mitogenic signal(s) might be a soluble factor(s). In addition, D19 CM induced the proliferation of bone marrow cells up to a dilution of 1:50 (not shown).

We next investigated whether the D19 cells express the CSFs that might be involved in the proliferation of macrophage precursors (13–15). Therefore we performed Northern blot experiments with cDNA probes for interleukins 1, 3, and 6, granulocyte CSF, granulocyte/macrophage CSF, and CSF-1. Fig. 2 shows that the macrophage CSF (CSF-1) is expressed at a high level. In contrast, the other probes gave no detectable signal. This suggests that the corresponding proteins may not be produced by the D19 cells, although it is known that a very low rate of transcription may sometimes lead to abundant protein synthesis. Astrocyte-enriched cultures also express the CSF-1 gene at a high level (not shown). These data prompted experiments to determine whether CSF-1 is a mitogenic signal for proliferation of the microglial progenitors. To this end, we used an antiserum specific for CSF-1, which blocked completely (at a concentration of 1:1000) the proliferation of bone marrow cells induced by medium conditioned by the D19 astroglial cells as well as by secondary confluent astrocyte cultures (data not shown). Brainstem cells (1×10^4) were plated onto 1.5-cm^2 glass coverslips and cultured in D19 CM alone or preincubated with various concentrations of the CSF-1 antiserum. In five independent experiments, the number of proliferating foci was reduced by almost half and the appearance of the other foci was clearly delayed in cultures fed with D19 CM preincubated with anti-CSF-1 diluted 1:300 or 1:100 (not shown). In this context, it is noteworthy that CSF-1 receptors can be detected at the surface of proliferating Mac-1^+ cells but not

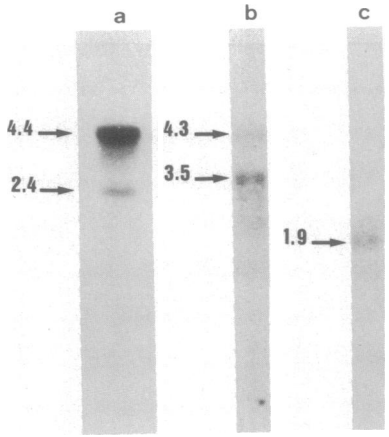

FIG. 2. Northern blot analysis with 2 μg of poly(A)$^+$ RNAs from the D19 astroglial cell line probed with CSF-1 (lane a), platelet-derived growth factor B (lane b), and platelet-derived growth factor A (lane c) cDNAs. Sizes are given in kilobase pairs.

of astrocytes with a specific anti-CSF-1 receptor antiserum (gift of E. R. Stanley) (data not shown).

Furthermore, although D19 astrocytes express platelet-derived growth factors A and B (Fig. 2) as reported for primary astrocytes (16), a specific antibody to PDGF had no effect on proliferation of microglial progenitors.

DISCUSSION

The data reported here show that the brain of embryonic as well as adult mice contains intraparenchymatous cells that have the capacity to proliferate very actively *in vitro* and give rise to clones of about 2×10^5 Mac-1^+, 2.4G2^+, F4/80^+ cells. In this respect, these brain cells resemble the bone marrow progenitors that, under the same *in vitro* conditions, give rise to clones of $3–4 \times 10^5$ cells expressing macrophage markers. Clonal proliferation of microglial and macrophage progenitors was dependent upon coculture on astroglial monolayers. These conclusions can be drawn because the experimental procedures permitted us to ascertain that Mac-1^+, F4/80^+ microglial colonies were the progeny of single cells.

In which brain compartment are the microglial progenitors located? They are found in the absence of circulating blood cells and of meninges. Moreover, the number of microglial progenitors obtained from nonperfused animals was comparable to that of carefully perfused brains, indicating that contamination of brain cell suspensions by blood circulating microglial progenitors, if any, was negligible. It is thus clear that the microglial progenitors belong to the brain parenchyma.

What are these microglial progenitors? Our data indicate that they are *not* mature F4-80^+ cells that acquire the capacity to multiply in culture since the number of F4/80^+ microglial cells from embryonic and adult brain that adhere *in vitro* to astrocytic monolayers is 10–50 times lower than that of the microglial progenitors, except for the embryonic brainstem. It is known that the progenitors of the macrophages are the CFU-GM cells, which give rise to the granulocytic and monocytic lineages, and it appears that the Mac-1 epitope first becomes expressed by the CFU-GM cells (17). Thus, it is reasonable to assume that microglial progenitors represent a subset of the Mac-1^+ cells. However, since in the adult brain the number of Mac-1^+ cells adhering to the D19 monolayers is inferior to the number of Mac-1^+ colonies, it is possible that some microglial clones do not derive from Mac-1^+ cells but rather from earlier cells that have not yet acquired the Mac-1 epitope. The microglial progenitors described in the present study are reminiscent of the pluripotential hemopoietic stem cells, as detected by the spleen colony-forming assay, previously reported in the adult mouse brain (18). It is possible that the experimental conditions used in our study have driven these cells along the microglial lineage.

Where do these microglial progenitors come from? The fact that the numbers of Mac-1^+ cells found at embryonic day 16–18 and in adults are similar suggests that these cells may have migrated into the brain during development and are restricted from leaving the brain after formation of the blood–brain and the meningeal barriers. In contrast, increase in the number of microglial progenitors between late embryos and adults may indicate that these cells can penetrate the blood–brain or meningeal barrier or else the fenestrated capillaries present in various brain regions (19). It is possible, however, that these microglial progenitors are Mac-1^+ cells that later acquire the capacity to proliferate.

The search for the mitogenic signal responsible for the *in vitro* proliferation of the microglial progenitors has been complicated by the fact that it is not yet possible to culture them as a purified cell population. Our results show that CSF-1 present in D19 CM can initiate division of the quiescent microglial progenitors. Taken together, the data reported here indicate that although CSF-1 appears sufficient for the multiplication of bone marrow macrophage progenitors, sustained proliferation of microglial progenitors requires perhaps another growth factor in addition to CSF-1.

An intriguing and important question raised by these data concerns the control of the proliferation of the microglial progenitors in the normal brain. Although they may be responsible for the slow renewal of microglia, it is obvious that microglial progenitors have a markedly restrained proliferative potential in normal conditions. This may be due to the lack of availability of growth factor(s) or of receptor(s). An alternative but not exclusive possibility is that a growth inhibitor may be responsible for the relative quiescent state of these cells *in vivo*. The recent observation that microglial cells produce tumor necrosis factor α may be relevant to this question (20). Proliferation of microglial progenitors in infectious diseases, such as AIDS, or after traumas may contribute directly or indirectly to their pathologies.

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