The Generation of Macrophages from Precursor Cells Incubated with Brain Endothelial Cells — A Release of CSF-1 Like Factor from Endothelial Cells —

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TANAKA, M., KISHI, K., MCCARRON, R.M. and MIYATAKE, T. *The Generation of Macrophages from Precursor Cells Incubated with Brain Endothelial Cells — A Release of CSF-1 Like Factor from Endothelial Cells —* Tohoku J. Exp. Med., 1993, 171 (3), 211-220 — The perivascular macrophages in the brain are thought to be derived from bone marrow precursor cells. Results presented here demonstrate that immature macrophages obtained from nonadherent spleen cell populations can adhere to cerebrovascular endothelial cell (EC) monolayers and proliferate. The proliferation of these cells can be stimulated by either purified murine granulocyte-macrophage colony-stimulating factor (GM-CSF) or EC conditioned medium but not by IL-3. No proliferation of the GM-CSF- or IL-3-dependent murine cell line IC-2 was observed in the presence of EC conditioned medium. Macrophage-like cells could also be derived from murine bone marrow cells but inhibited by anti-macrophage-CSF (M-CSF or CSF-1). The capacity of EC conditioned medium to induce the proliferation of macrophage-like cells from the spleen is therefore not due to the release of GM-CSF but CSF-1. In concomitant experiments using mature macrophages incubated on EC monolayers, no proliferation was observed. These findings suggest that the growth of perivascular macrophages in the brain may be stimulated by cerebrovascular EC. ——— macrophage; endothelial cell; experimental allergic encephalomyelitis; macrophage colony-stimulating factor

Macrophages cause tissue injury by releasing some mediators such as interleu-

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Abbreviations: EC, endothelial cells; GM-CSF, granulocyte-macrophage colony-stimulating factor; CNS, central nervous system; LNC, lymph node cells; ; CM, conditioned medium; FITC, fluorescence isothiocyanate; FCS, fetal calf serum; PBS, phosphate buffer saline.

kin (IL) 1, tumor necrosis factor- α , prostaglandins, neutral proteases like plasminogen activator or other myelin degrading enzymes (Perry and Gordon 1988; Perry et al. 1993). Macrophages also might be necessary for the removal of the debris from damaged tissue and macrophage-derived IL-1 or tumor necrosis factor may be necessary for proliferation of astrocytes (Giulian et al. 1988; Selmaj et al. 1990). An initial event of inflammation involves adhesion of monocytes to the endothelial cell (EC) surface followed by penetration under the influence of chemotactic factors (DiCorieto and de la Motte 1985; Migliorisi et al. 1987). Monocytes are found to bind selectively to the intercellular borders of individual EC and migrate without disruption of the EC monolayers (Pawlowski et al. 1988).

The microvascular unit consisting of endothelial cells and macrophages is reported to be seen in the brain (Hickey and Kimura 1988). Using chimeric rats, the perivascular macrophages in the central nervous system (CNS) were shown to be derived from bone marrow precursor cells (Hickey and Kimura 1988).

In this communication, we present evidence that macrophages can be generated from precursor cells such as adult murine non-adherent spleen cells or bone marrow cells by co-culturing with microvascular EC isolated from SJL brains.

METHODS

Reagents

Monoclonal antibodies used in this study include anti-Mac-1 (Hybritech, San Diego, CA, USA), anti-Thy 1.2 (Becton Dickinson, Mountain View, CA, USA), anti-33D₁ reacting with murine splenic dendritic cells (ATCC, Rockville, MD, USA), anti-NK reacting with murine NK cells (clone PK136, ATCC), anti-Ia^P (clone 7-16.17; Cedarlane Lab., Ontario, Canada) and anti-mouse CSF-1 (clone YYG-106.17.23, Oncogene Science, Uniondale, NY, USA).

Cell culture

Female SJL mice, aged 6–12 weeks, were used. The EC line generated from SJL brain microvessels used in this study was established at NIH and maintained in vitro for more than six months (Tanaka and McCarron 1990). Granulocyte and macrophage colony forming cells have been found in murine bone marrow as well as spleen (Metcalf 1977). Non-adherent spleen cells were prepared using fetal calf serum (FCS)-coated flasks (Kumagai et al. 1979) and incubated on EC monolayers which had been irradiated (500 rad). Floating cells and medium (RPMI 1640 containing 10% FCS) were removed and fresh media was added daily for the first five days of culture and then changed every other day.

Murine lymph node cells (LNC) or thioglycollate-stimulated peritoneal exudate cells were prepared as reported previously (Mishell et al. 1980). Cells (5×10^5 spleen cells/dish or 10^4 bone marrow cells/dish) were cultured with 1.2% methylcellulose (1,500 cps, Sigma, St. Louis, MO, USA), 30% FCS, 1% bovine serum albumin, 10^{-4} M 2-mercaptethanol, and α -MEM (Flow Laboratories, Inc., McLean, VA, USA). After seven days of culture, colony numbers were counted (Kishi et al. 1989). Murine bone marrow cells of 5×10^5 were incubated with 1×10^4 500 rad irradiated EC per 100 μ l on 96 well plate in RPMI 1640 containing 10% FCS with or without 0.5 μ g/well anti-CSF-1.

Assay for growth factor dependence

EC-binding cells obtained after 7 days incubation on EC monolayers were seeded in 96

well culture plates at a density of 1×10^4 per well. Cells were incubated for 72 hr in RPMI 1640 containing 10% FCS with or without EC conditioned medium (CM), purified murine granulocyte-macrophage colony-stimulating factor (GM-CSF) (Genzyme, Boston, MA, USA) or purified murine IL-3 (Genzyme). All wells were pulsed for the last 16 hr with $1 \mu\text{Ci}$ of [^3H]thymidine (74.0 GBq/mmol; Du Pont, Wilmington, DE, USA) per well. The cells were then collected by cell harvester and radioactivity was measured by a scintillation counter.

To investigate GM-CSF activity in EC CM, the proliferation of IC-2, GM-CSF or IL-3 dependent murine cell line (Dr. Akio Suzumura, Dept. of Neurology, School of Medicine, Fijita Health University, Toyoake), was examined by [^3H]thymidine uptake. IC-2 cells, $1 \times 10^4/200 \mu\text{l}$, were incubated with several kinds of culture conditioned media or recombinant murine GM-CSF (Genzyme) in RPMI 1640 containing 10% FCS for 4 days. Cells were pulsed with thymidine and counted as described above. EC CM (RPMI 1640 containing 10% FCS) was collected after 4 days culture of EC following 500 rad irradiation. WEHI-3 CM was also collected and stored at -70°C until use.

Assay for the growth of mature or immature macrophages with EC monolayers

The culture conditions used to study macrophages on irradiated EC monolayers are the same as described above. Non-adherent spleen cells of 1×10^6 , PEC or LNC were incubated with 3.5×10^4 EC on collagen type 1 (Collaborative Res., Bedford, MA, USA) coated 96 well plate in RPMI 1640 containing 10% FCS. On the seventh day, cells were pulsed for 16 hr with $1 \mu\text{Ci}$ of [^3H]thymidine per well and collected by cell harvester.

FACS analysis

EC-binding cells were removed from EC monolayers after 7 days of culture by gentle tapping of flask. Cells were reacted with several monoclonal antibodies for 30 min at 4°C in RPMI 1640. After washing, cells were incubated with fluorescence isothiocyanate (FITC)-conjugated anti-murine IgG goat F(ab')₂ (TAGO, Burlingame, CA, USA) or FITC-conjugated anti-rat IgG goat F(ab')₂ (TAGO) for 30 min at 4°C . Before analysis of cells by FACS IV analyzer (FACS System; Becton Dickinson), cells were washed with phosphate buffer saline (PBS) containing 0.01 M NaN₃ as reported previously (Tanaka et al. 1985).

RESULTS

After addition of nonadherent spleen cells on EC monolayers, small colonies consisting of a few round cells associated with EC monolayers could be seen after a few days of incubation with EC (Fig. 1). These cells loosely adhered to EC and were easily separated from EC layers by gently tapping flasks. Maximal proliferation was observed seven days after incubation of non-adherent spleen cells with EC. These cells could not be maintained for more than one month even with incubation on freshly irradiated EC. The characteristics of these EC-binding cells were examined. Since EC-binding cells were much smaller than EC and EC were tightly adhered to flasks, these cells could be examined separately using FACS even in samples contaminated with a few EC. FACS analysis revealed that 90% of the EC-binding cells were Mac-1 positive. No staining was observed with antibodies detecting Thy-1, 33D₁ (murine spleen dendritic cells), NK (clone PK136), Ia, or surface IgG. In addition, EC-binding cells did not demonstrate cytotoxicity against YAC-1 cells, targets of murine NK cells (data not shown).

Fig. 1. A small colony consisting of a few round cells on endothelial cell (EC) monolayers ($\times 200$). They can be found after a few days of incubation with EC and the maximal proliferation is seen on the seventh day after incubation of non-adherent spleen cells with EC.

These cells adhered to the hydrophobic plastic surfaces within 30 min incubation. They were round shaped on the EC monolayers or in floating condition but they produced foot processes similar to macrophages upon incubation on hydrophobic plastic surfaces. All cells could phagocytize latex beads and were positive for non-specific esterases (data not shown) which suggest that they are macrophages. EC CM supported the formation of granulocyte-macrophage colonies in cultures of bone marrow cells as well as spleen cells (Table 1).

These macrophage-like cells revealed increased thymidine uptake when incubated with EC CM or GM-CSF but not with IL-3 (Table 2). They could grow for a few days with EC CM or GM-CSF alone but the presence of EC monolayers was required to sustain growth for more than 7 days. The capacity of EC to secrete GM-CSF was examined. The data in Fig. 2 shows that the proliferation of GM-CSF or IL-3 dependent murine cell line, IC-2, is stimulated with WEHI-3 CM (containing IL-3) or recombinant murine GM-CSF but not with EC CM or L-929 CM (containing M-CSF). Although not shown, L-929 CM was also able to support proliferation of macrophage-like cells. Macrophage-like cells could also be generated from bone marrow cells incubated with EC (data not shown). We examined whether CSF-1 was involved in the induction of macrophage-like cells. Fig. 3 shows the inhibition by anti-CSF-1 in the generation of macrophage-like cells from bone marrow cells incubated with irradiated EC.

We also examined whether similar proliferative responses could be generated by mature macrophage populations incubated on EC monolayers. The data in

TABLE 1. *Number of CFU-GM from murine spleen and bone marrow cells stimulated with EC conditioned medium*

Factors		Spleen cells ^a (5×10^5 cells)	Bone marrow cells ^a (10^4 cells)
None		0	0
EC CM	1%	28	28
	10%	78	54
	100%	93	63
WEHI-3 CM 5%		132	59
hM-CSF 100 u		26	27

^aCells (5×10^5 spleen cells/dish or 10^4 bone marrow cells/dish) were cultured in α -MEM containing 1.2% methylcellulose, 30% fetal calf serum, 1% bovine serum albumin, and 10^{-4} M 2-mercaptethanol.

CFU-GM, colony forming unit-granulocyte macrophage.

TABLE 2. *Effect of EC conditioned medium, GM-CSF or IL-3 on ^3H -TdR uptake by EC-binding cells*

Addition ^a		^3H -TdR incorporation ^b
Exp. 1		
None		1029 ± 444
1% EC CM		904 ± 143
5% EC CM		1188 ± 167
25% EC CM		$11072 \pm 1629^{**}$
Exp. 2		
None		1224 ± 552
1 u/ml GM-CSF		1956 ± 583
10 u/ml GM-CSF		$5265 \pm 1947^*$
50 u/ml GM-CSF		$14757 \pm 1740^{**}$
100 u/ml GM-CSF		$13697 \pm 1362^{**}$
10 u/ml IL-3		1025 ± 204
100 u/ml IL-3		1286 ± 667

^aEC-binding cells obtained from non-adherent SPC populations were incubated in media containing the indicated additions as described in the materials and methods section.

^bAll assays were performed in quadruplicate; data represent the incorporation of ^3H -TdR (mean cpm $\pm$ s.d.)

* $p < 0.05$, ** $p < 0.01$; CM, conditioned medium.

Table 3 shows that macrophage like cells which bind EC monolayers could be grown only from non-adherent spleen cell populations which contained immature macrophage precursor cells. No proliferation of either peritoneal exudate cells or lymph node cell populations as sources of mature macrophage populations was

Fig. 2. The effects of various conditioned medium (CM) and recombinant murine GM-CSF on the proliferation of IC-2 (GM-CSF or IL-3 dependent murine cell line). IC-2 can grow with WEHI-3 containing IL-3 or recombinant GM-CSF but not with EC CM or L-929 CM (containing CSF-1). Assay conditions were described in methods section and results are presented as mean of quadruplicate samples $\pm$ S.D.

observed when incubated on EC monolayers.

DISCUSSION

The concept of the immunologically privileged status of the brain refers to the empirical finding that allogeneic or xenogeneic tissues exhibit a prolonged survival, or a higher rate of survival after a given time, when grafted into the brain parenchyma in comparison with those grafted to peripheral sites (Widner and Brundin 1988). This does not mean that immune reactions cannot take place in the brain. There are a number of studies suggesting that under appropriate conditions, cells in the CNS express Class II MHC molecules and present antigens to T cells (McCarron et al. 1985, 1991). Macrophages in the brain or microglia are examples of these antigen presenting cells in the CNS (Frei et al. 1987).

Small numbers of macrophages are found in the cerebrospinal fluid of normal individuals which indicates traffic of macrophages through the choroid plexus to the cerebrospinal fluid (Perry and Gordon 1988). The new expression of myelomonocytic antigens on microglia and perivascular cells in the CNS lesions following lethal motor neuron injury may imply that these cells are of blood origin

Fig. 3. The induction of macrophage-like cells from murine bone marrow cells incubated with EC (A) and inhibition by anti-CSF-1 (B) after 4 days culture ($\times 100$).

TABLE 3. *Proliferative response of EC-binding cells from non-adherent SPC, PEC or LNC populations*

Cells cultured on EC ^a	³ H-TdR incorporation ^b
None	6505 $\pm$ 1339
Non-adherent SPC ^c	13526 $\pm$ 1620**
PEC ^d	7591 $\pm$ 502
Whole LNC ^e	9308 $\pm$ 2677

^aEC culture were irradiated (500 R) and cells were prepared and cultured on EC monolayers as described in the materials and methods section.

^bAll assays were performed in quadruplicate; data represent the incorporation of ³H-TdR (mean cpm $\pm$ S.D.)

^cSPC, spleen cells; ^dPEC, peritoneal exudate cells;

^eLNC, lymph node cells.

** $p < 0.01$.

and have recently infiltrated into the CNS (Graeber et al. 1990). MHC Class II positive monocyte-like cells are observed in the experimental metastatic tumors of the brain that preserve intact blood-brain barrier (Schackert et al. 1988). These studies suggest that brain phagocytes can be derived from circulating monocytes

in adult brains. Bone marrow-derived brain macrophages have been also shown to be located in the leptomeninges, the ventricles, and the blood vessel walls in the developing murine brains using in situ hybridization and immunoperoxidase techniques (De Groot et al. 1992).

Murine resident macrophages have been shown to proliferate on a layer of mesothelial feeder cells after 3–4 weeks of culture (Lombard et al. 1988). As was observed with our EC-binding cells, direct contact with the layer was necessary (Lombard et al. 1988). However, unlike the nonadherent spleen cells described here, the macrophage-like cell lines from peritoneum, spleen, or lung described by Lombard et al. seemed to be derived from mature macrophages. In addition, the macrophages utilized here were not cell line derived and could not continue to proliferate after one month in culture. GM-CSF or CSF-1 can be produced by EC (Bonnem and Morstyn 1988) and can stimulate the proliferation of immature macrophages (Hogan and Vogel 1988). EC CM in this study may contain GM-CSF or CSF-1 because EC CM could support the induction of CFU-GM (Table 1). The data presented indicate that EC CM induces the proliferation of the macrophage-like cells described here (Table 2) but not the proliferation of the GM-CSF-dependent cell line IC-2 (Fig. 2). Since macrophage-like cells proliferated in the presence of L-929 CM containing CSF-1 (data not shown) and anti-CSF-1 could inhibit the induction of macrophage-like cells from bone marrow cells (Fig. 3), the capacity of EC to support proliferation of macrophage-like cells is probably due primarily to the release of CSF-1. Our data show that immature macrophages from spleens could grow on monolayers of EC derived from brain microvessels, whereas mature macrophages and peritoneal exudate cells could not proliferate under identical conditions. The data also infer that perivascular macrophages in the CNS may be of hematogenous origin and their proliferation can be supported by EC lining the cerebral vasculature. Because CSF-1 produced by astrocytes, microglia (Lee et al. 1993) and neurons (Nohava et al. 1992) is increased in the CNS at the height of clinical disease of experimental allergic encephalomyelitis (EAE) (Hulkower et al. 1993), macrophages seem to be an important component of the disease process in EAE.

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