BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **251,** 277–282 (1998)
ARTICLE NO. RC989457

Astrocytes Modulate Nitric Oxide Production by Microglial Cells through Secretion of Serine and Glycine

Lihua Yang,* Junya Tanaka,†,[1] Bo Zhang,* Masahiro Sakanaka,* and Nobuji Maeda†
**Department of Anatomy and †Department of Physiology, School of Medicine, Ehime University Shigenobu, Ehime 791-0295, Japan*

Received August 25, 1998

We investigated lipopolysaccharide (LPS)-induced nitric oxide (NO) production by rat microglia in neuron-microglia and astrocyte-microglia cocultures to evaluate the influence of neurons and astrocytes on microglial activity. Microglial cells solely cultured in medium devoid of serine (Ser), glycine (Gly) hardly expressed inducible NO synthase (iNOS), while those cocultured with neurons and astrocytes expressed iNOS. When microglial cells and astrocytes were separately cultured by using tissue culture inserts, which allowed the microglial cells to be exposed to only diffusible factors arising from astrocytes, NO production was significantly enhanced. On the other hand, neurons, when separated from microglial cells by the inserts, could not activate microglial cells possibly due to lacking of direct contact between neurons and microglial cells. NO production in pure microglial cultures was significantly enhanced in the presence of Ser/Gly at concentrations higher than 25 μM. Conditioned media obtained from microglia culture and neuron-microglia coculture contained less than 10 μM of Ser and Gly, while media from astrocyte culture and astrocyte-microglia coculture contained 33–41 μM Ser and 20–26 μM Gly. Accordingly, astrocytes modulate the activity of microglial cells by secreting Ser and Gly. The present study proposes a novel metabolic coupling between astrocytes and microglial cells via amino acids.

Microglial cells, one type of resident glia in the brain, are well known to become activated under a variety of pathologic conditions. The microglial activation is accompanied by morphological changes from ramified to ameboid shape (1, 2, 3). Such morphological and functional changes can also be observed during embryonic development; at early developmental stages, microglia display activated properties and ameboid shape, and later they become resting cells with ramified morphology. Although the detailed mechanisms underlying the physiological and pathological transformation of microglial cells are yet to be determined, astrocytes, another type of resident glia, are believed to affect the activity and morphology of microglia by secreting both diffusible and nondiffusible factors (4, 5, 6, 7). For example, granulocyte macrophage colony-stimulating factor (GM-CSF), which is secreted possibly from astrocytes, has been shown to induce microglial ramification (8). Transforming growth factor-β derived from astrocytes appears to inhibit nitric oxide (NO) production by microglial cells (9). Moreover, astrocytes may modulate the morphology of microglia by secreting extracellular matrix proteins such as fibronectin and laminin (7, 10, 11, 12).

Recently, we have demonstrated that microglial activity is highly dependent on the concentrations of amino acids serine (Ser) and glycine (Gly) (12). When microglial cells are cultured in medium containing less than 10 μM of Ser and Gly, microglial NO production is suppressed. Ser and Gly activate microglial cells in a concentration-dependent manner. Thus, resting microglial cells can be induced in Ser, Gly-free culture medium, and they can be activated by high concentrations of Ser and Gly. In the present study, we compared the influence of neurons and astrocytes on inducible NO synthase (iNOS) activity of cultured microglia. Consequently, it was shown that when cocultured with neurons or astrocytes, NO production by microglial cells was enhanced even in culture medium devoid of Ser and Gly. We further found that astrocytes but not neurons enhanced microglial iNOS activity by secreting diffusible factors and that astrocytic conditioned medium contained Ser and Gly of enough concentrations to activate microglial cells.

MATERIALS AND METHODS

Culture of cortical neurons, astrocytes, and microglial cells. Cortical neurons prepared from the cerebral cortices of 17-day-old rat embryos were cultured as previously described (13). Astrocytes from

[1] Corresponding author. Fax: +81-89-960-5246. E-mail: jtanaka@m.ehime-u.ac.jp.

0006-291X/98 $25.00

the forebrains of newborn rats were cultured as described elsewhere (8, 12). Microglial cells were obtained from mixed cell cultures of the newborn rat forebrains according to the method of Suzumura et al. (14) with some modifications (15). The purity of cortical neurons, astrocytes and microglia in their cultures was 86–94 %, 93–96 %, and 98–100%, respectively, as determined by the previously described techniques (7).

Mixed-coculture of microglial cells with cortical neurons or astrocytes. In coculture experiments, neurons were first seeded at a density of 2.6×10^5 cells/cm^2 on poly-L-lysine (PLL)-coated glass coverslips (Matsunami, Kishiwada, Japan) placed in 24-well culture plates for immunocytochemistry or on PLL-coated 24-well culture plates for biochemical analyses. In cases of astrocyte-microglia coculture, astrocytes suspended in Dalbecco's modified Eagle's medium (DMEM; Iwaki glass, Funahashi, Japan) containing 10% FCS were seeded at a density of 7.4×10^4 cells/cm^2 beforehand. After neurons or astrocytes were firmly attached to the PLL-coated coverslips or culture plates, microglial cells suspended in DMEM containing 3% FCS were seeded on the same coverslips or plates at a density of 3.7×10^4 cells/cm^2. Two hours later, the medium was replaced with Eagle's minimum essential medium (MEM; Iwaki glass) containing 1 mg/ml of bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, MO) and 10 mM HEPES (pH 7.3; Boehringer-Mannheim, Mannheim, Germany), which was termed as serum-free MEM in the present report, and the cells were cultured at 37°C for 48–72 h in the presence of 1 μg/ml lipopolysaccharide (LPS; from *Escherichia coli* serotype 055:B5; Sigma).

iNOS immunocytochemistry. Microglial cells in pure culture and cocultures on PLL-coated glass coverslips were fixed with a fixative containing 4 % paraformaldehyde. Subsequently, the microglial cells were immunostained with an antibody to mouse iNOS fragment (Transduction Laboratories, Lexington, KY) employing the avidin-biotin-peroxidase complex (ABC) method (16). The iNOS-positive immunoreaction products were identified as brown due to the oxidation of diaminobenzidine, a substrate for peroxidase. In the cases of cocultures, to identify astrocytes and neurons, immunocytochemistry for glial fibrillary acidic protein (GFAP) and microtubule-associated protein 2 (MAP2), specific markers for astrocytes and neurons, respectively, was further carried out after iNOS immunostaining. Rabbit anti-GFAP antibody and mouse anti-MAP2 antibody were purchased from Sigma and Sternberger Monoclonals, Inc. (Baltimore, MD), respectively. After the incubation with these antibodies, the cells were subjected to the secondary immunoreaction with alkaline phosphatase-labelled antibodies against rabbit or mouse IgG (Promega, Madison, WI). The immunoreactions for GFAP and MAP2 were visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Sigma) as color development substrates (17). The immunoreaction products for GFAP and MAP2 were blue or purple. The cells were observed through Nomarski optics using an inverted microscope (Olympus, Tokyo, Japan).

Cultures with tissue culture inserts. To evaluate the effect(s) of diffusible factors derived from astrocytes and neurons on the activity of microglial cells, microglial cells were seeded on PLL-coated tissue culture inserts (Falcon 3090 with pores 0.4 μm in diameter; Falcon, Franklin Lakes, NJ) that were placed in wells of PLL-coated 6-well plates (Falcon) (18). On the bottom of the wells below the inserts, astrocytes, neurons or no cells were cultured in serum-free MEM. The conditioned media were subjected to the measurement of nitrite at 72 h after the onset of culture.

Measurement of nitrite. To evaluate the iNOS activity of microglia, nitrite concentrations in conditioned media were determined using the Griess reaction as described elsewhere (8).

Analyses of amino acids in conditioned media. The conditioned media from pure cultures and cocultures in 24-well plates were subjected to amino acid analyses. One ml of each conditioned medium was treated with 2 ml of 2 % sulfosalicylic acid on ice for 5 min, and the mixture was centrifuged at 3,000 g for 10 min followed by filtration with membrane filters with 0.2 μm pores. The solutions were analyzed with an amino acid analyzer (JEM JLC-555/V; JEOL, Tokyo, Japan).

Statistics. The numerical data were evaluated by the analysis of variance followed by a *post hoc* test (Fisher's protected least significant difference).

RESULTS

iNOS expression in microglial cells in pure and cocultures. In serum-free MEM, microglial cells in pure culture did not significantly express iNOS even in the presence of lipopolysaccharide (LPS) (Fig. 1). In contrast, microglial cells expressed iNOS in cocultures with astrocytes or neurons in the same culture medium. In the neuron-microglia coculture, some microglial cells rapidly began to express iNOS within 12 h after the onset of coculture, while there was a significant number of iNOS-negative microglial cells even at 48 h after the onset. On the other hand, in the astrocyte-microglia coculture, it took longer period for microglial cells to fully express iNOS. In agreement of the study of Vincent et al. (9), iNOS-immunoreactivity was not detected in neurons or in astrocytes (Fig. 1). Many microglial cells in the pure culture extended fine processes on PLL-coated coverslips, while those in the neuron-microglia coculture displayed round morphology. In the astrocyte-microglia coculture, microglial cells gradually extended thick processes, which were distinct from the fine processes observed in the pure microglial culture.

NO production by microglial cells on tissue culture insert membranes. To evaluate the effects of soluble factors derived from neurons and astrocytes, microglial cells were cultured on tissue culture insert membranes while neurons or astrocytes were cultured below the membranes. Since the pore size of the membranes was 0.4 μm, microglial cells could not get out of the upper chamber. Neurons and astrocytes cultured on the bottoms of 6-well culture plates in the absence of microglial cells produced 1.3 ± 0.14 (means $\pm$ SEM) and 6.9 ± 1.2 μM of nitrite, an oxidized product of NO, respectively. Microglial cells on insert membranes without any cells on the bottoms of 6-well plates produced 9.4 ± 0.71 μM of nitrite, which was not significantly different from the nitrite level (12 ± 1.6 μM) in the medium of microglial culture with neurons on the bottom. When astrocytes were seeded on the bottom and microglial cells on insert membranes, 25 ± 2.0 μM of nitrite was detected in the medium (Fig. 2). The nitrite concentration 25 μM was significantly higher than the sum of nitrite (16 μM) produced by independently cultured microglial cells and astrocytes ($P<0.005$). These results together with those shown in Figure 1 suggest that astrocytes stimulated microglial NO production by secreting soluble factors, while neu-

rons required direct contact to induce iNOS expression in microglial cells.

Dependency of microglial NO production on Ser and Gly. Ser and Gly stimulated microglial NO production in a concentration-dependent manner (Fig. 3 A). Simultaneous addition of both amino acids with the same concentration to pure microglial cultures significantly enhanced NO production at concentrations higher than 25 μM (Fig. 3 B). In contrast, when microglial cells were cocultured with neurons or astrocytes, NO production was apparently enhanced even in Ser, Gly-free culture medium, and the addition of Ser and Gly into the cocultures no longer caused significant changes in NO production (Fig. 3 B). Microglial cells in pure culture produced 20 μM (mean value) of nitrite in the absence of Ser and Gly, and they produced 32 μM of nitrite (12 μM increase in nitrite) in the presence of 25 μM of each amino acid. Microglial cells in coculture with astrocytes produced 39 μM of nitrite (19 μM increase in nitrite) in Ser, Gly-free medium.

Concentrations of Ser and Gly in conditioned media. We determined the concentrations of Ser and Gly in conditioned media obtained from pure cultures and cocultures using an amino acid analyzer (Table 1). The control medium consisting of serum-free MEM and LPS contained less than 10 μM each of Ser and Gly. The conditioned media from microglial and neuronal pure cultures, and from neuron-microglia coculture contained less than 10 μM of both amino acids. In contrast, the astrocyte-conditioned medium contained 41 μM of Ser and 20μM of Gly. The medium from astrocyte-microglia coculture contained 33 μM Ser and 26 μM Gly. These concentrations of Ser and Gly were enough to enhance microglial NO production as shown in Figure 3 B.

DISCUSSION

Dalbecco's modified Eagle's medium (DMEM) containing 400 μM of Ser and Gly is frequently used to culture microglial cells. However, the majority of microdialysis studies have shown that only $\sim$5 μM of both amino acids are present in the extracellular milieu in the normal mature brain parenchyma (19, 20, 21), where microglial cells are in resting state with ramified morphology. Cultured microglial cells often display round morphology with activated functions resembling peripheral tissue macrophages (7, 8), because they are frequently cultured in DMEM containing unphysiologically high concentrations of Ser and Gly together with serum, another activator for microglial cells (8, 12). On the other hand, microglial cells maintain resting state and ramified morphology as shown in Figure 1, when they are cultured on PLL-coated substrate in the absence of Ser, Gly and serum as reported previously (12, 22). Thus, the establishment of a method for keeping microglial cells in resting state is a prerequisite to investigate the mechanisms for microglial activation. In addition, Ser, Gly-free, serum-free medium does not affect the viability of microglial cells (12), and therefore the medium seems to be appropriate for resting microglial cells.

In neuron-microglia cocultures, the majority of microglial cells making direct contact with neurons displayed round morphology and high ability to produce NO (Fig. 1). The morphological changes observed in neuron-microglia cocultures has been described elsewhere (22, 23). It has been shown that direct contact between neurons and microglial cells is essential for microglial activation, and that not only viable neurons but the membrane fraction prepared from cultured neurons can activate microglial cells (22). In line with the notion that the direct contact with neurons triggers microglial activation, microglial cells on tissue culture insert membranes were not activated, even if the cells were exposed to soluble factors arising from neurons (Fig. 2).

Although microglial cells were activated also in astrocyte-microglia cocultures, there were several differences between the neuron- and astrocyte-induced microglial activations. Microglial cells in neuron-microglia cocultures became activated within 12 h after the onset of coculture while displaying round morphology. In contrast, microglial cells cocultured with astrocytes bore processes and they were activated later than 12 h after the onset of coculture. Furthermore, NO production by microglial cells on tissue culture insert membranes was enhanced when astrocytes but not neurons were present below the inserts. The conditioned media from astrocyte culture or microglia-astrocyte coculture contained enough Ser and Gly to enhance microglial NO production. These findings suggest that astrocytes can activate resting microglial cells by secreting Ser and Gly.

The reason why microglial cells require Ser and Gly to become activated is unclear at present. Ser is not an essential amino acid, and it is synthesized from 3-phosphoglycerate, an intermediate metabolite in the glycolysis pathway. Serine hydroxymethyltransferase synthesizes Gly from Ser. Taken the fact that peripheral macrophages cannot be viable in culture medium lacking Ser and Gly (data not shown), microglial cells, whose origin is considered to be the same as that of macrophages, might not have sufficient ability to synthesize Ser and Gly. Since macrophages are usually distributed in the Ser and Gly-rich environment surrounded by blood plasma containing more than 100 μM of each amino acid (24), they may be able to absorb sufficient amounts of both amino acids even though not endowed with enzymes to synthesize Ser and Gly. On the other hand, astrocytes appear to synthesize large amounts of Ser and Gly. The cellular demand for Ser

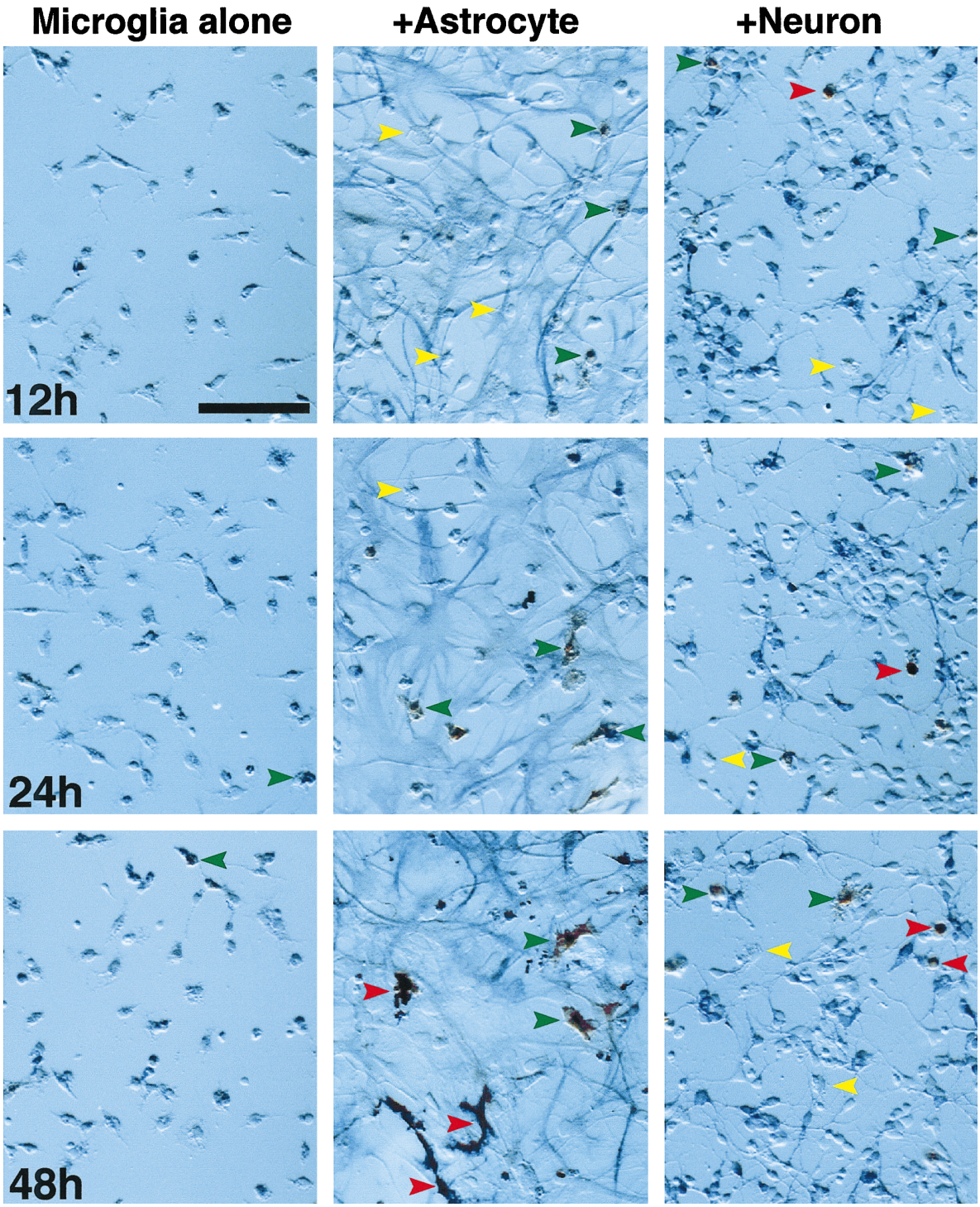

FIG. 1. iNOS immunocytochemistry for microglial cells in pure culture and in astrocyte- or neuron-microglia cocultures. Cells were cultured in serum-free MEM containing LPS. Each culture was fixed at 12, 24, and 48 h after the addition of LPS to the medium. Yellow arrowheads indicate iNOS-negative microglial cells. Green and red ones indicate cells with moderate and strong iNOS-immunoreactivity, respectively. Astrocytes and neurons immunoreactive for GFAP and MAP2, respectively, turned blue or purple (see Materials and Methods). Ramification of microglial cells in pure culture is the most prominent at 24 h after the onset of culture, although LPS obscured the ramification to some extent. Scale bar = 100 μm.

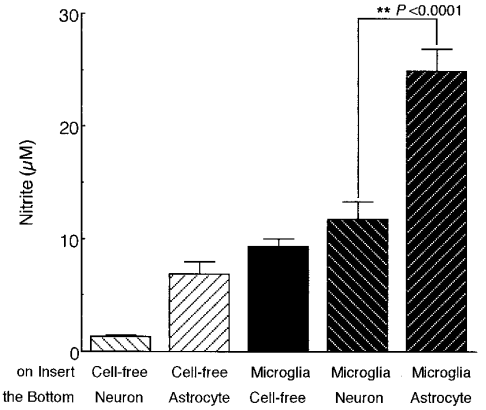

FIG. 2. NO production by microglial cells under the influences of soluble factors derived from astrocytes and neurons in serum-free MEM containing LPS. Microglial cells were cultured for 72 h on tissue culture insert membranes, while astrocytes or neurons were cultured on the bottoms of the culture plate wells. The insert membranes with pores of 0.4 μm in diameter enabled soluble factors to diffuse between upper and lower chambers but did not cell processes to pass the membranes. The conditioned media were used for nitrite measurements. Neurons alone and astrocytes alone produced less than 10 μM of nitrite. Microglia alone and microglia/neuron culture produced ~10 μM of nitrite. However, microglia/astrocyte culture produced significantly higher concentration of nitrite, as compared with that in the other cases. Data expressed as means ± SEM (n=5) were obtained by using 5 independent astrocyte and microglia cultures, and 3 independent neuronal cultures.

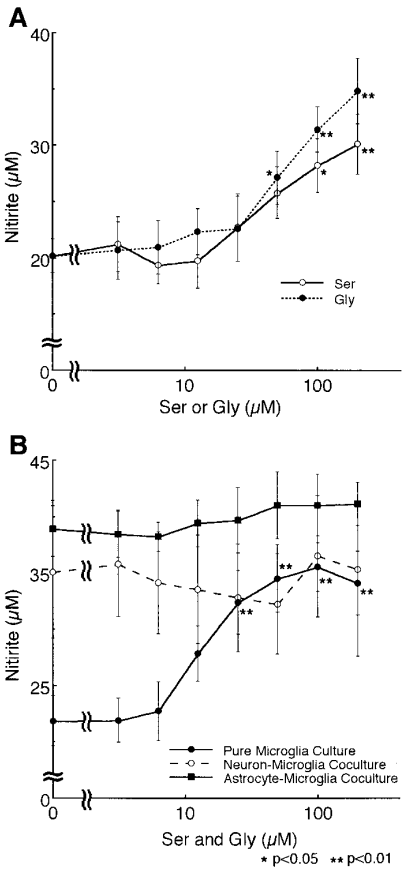

FIG. 3. (A) The dependency of NO production by microglial cells on Ser and Gly. Ser and Gly enhanced microglial NO producing activity in a concentration-dependent manner. (B) The effects of simultaneous addition of Ser and Gly into pure microglial cultures, astrocyte-microglia and neuron-microglia mixed cocultures. Ser/Gly enhanced the NO producing activity of microglia in the pure cultures in a concentration-dependent manner. In contrast, in cases of the cocultures, Ser/Gly did not elicit significant effects. Data from 6 independent cultures are expressed as means ± SEM.

and Gly may be rather high, because [1] Ser is essential for the synthesis of phosphatidyl serine, an abundant component of plasma membrane, [2] Gly is required to make purine and glutathione, and [3] the both amino acids are, of course, important materials for protein synthesis. Taken together, it is plausible that microglial activity is suppressed, if the supply of Ser and Gly is insufficient.

In conclusion, the present study showed the novel interactions between astrocytes and microglial cells via Ser and Gly. Astrocytes have been considered to control microglial cells, but the majority of studies have dealt with growth factors, cytokines (5, 8, 25, 26) or non-diffusible factors secreted by astrocytes (7, 27). However, according to the present results (Fig. 3 B), 25 μM Ser and Gly increased microglial NO production by 12 μM as compared with that of microglial cells in pure culture in Ser, Gly-free medium. On the other hand, the coculture of microglia with astrocytes increased the NO production by 19 μM in comparison with the NO production by microglial cells in pure culture in Ser, Gly-free medium. As the media from the astrocyte-microglia coculture contained more than 25 μM of both amino acids, Ser and Gly secreted by the cocultured astrocytes may have yielded ~12 μM out of 19 μM nitrite. This estimation, therefore, suggests that the effects of astrocytes on microglial activity are mainly elicited by Ser and Gly rather than by growth factors, cytokines and extracellular matrix proteins in Ser, Gly-free and serum-free medium.

TABLE 1

Concentrations of Ser and Gly in Conditioned Media

Media conditioned by	Ser (μM)	Gly (μM)
Cell-free	8.3 ± 6.9	4.2 ± 4.3
Microglia	7.6 ± 4.7	9.7 ± 5.8
Astrocyte	41.2 ± 20.4**	20.3 ± 2.5*
Neuron	6.7 ± 5.8	7.3 ± 6.4
Microglia + Astrocyte	33.1 ± 4.0*	26.3 ± 7.6*
Microglia + Neuron	8.0 ± 5.5	8.6 ± 6.2

Note. Data obtained from 5 separate experiments are expressed means ± S.D. *$P < 0.05$, **$P < 0.01$ vs cell-free medium.

ACKNOWLEDGMENTS

The authors thank Mr. T. Takaku for helping with measurements of amino acid concentrations in culture media. We are grateful to Drs. K. Ishihara and K. Ikoma for their fruitful suggestions and encouragement throughout this work. This study was partly supported by grants from the Ministry of Education, Science, Sports, and Culture of Japan, from the Ministry of Health and Welfare, and from the Ichiro Kanehara Foundation.

REFERENCES

1. Kreutzberg, G. W. (1996) *Trends. Neurosci.* **19,** 312–318.
2. Ling, E. A., and Wong, W. C. (1993) *Glia* **7,** 9–18.
3. Nakajima, K., and Kohsaka, S. (1993) *Neurosci. Res.* **17,** 187–203.
4. DeWitt, D. A., Perry, G., Cohen, M., Doller, C., and Silver, J. (1998) *Exp. Neurol.* **149,** 329–340.
5. Kloss, C. U., Kreutzberg, G. W., and Raivich, G. (1997) *J. Neurosci. Res.* **49,** 248–254.
6. Sievers, J., Parwaresch, R., and Wottge, H. U. (1994) *Glia* **12,** 245–258.
7. Tanaka, J., and Maeda, N. (1996) *Exp. Neurol.* **137,** 367–375.
8. Fujita, H., Tanaka, J., Toku, K., Tateishi, N., Suzuki, Y., Matsuda, S., Sakanaka, M., and Maeda, N. (1996) *Glia* **18,** 269–281.
9. Vincent, V. A., Tilders, F. J., and Van Dam, A. M. (1997) *Glia* **19,** 190–198.
10. Chamak, B., and Mallat, M. (1991) *Neuroscience* **45,** 513–527.
11. Oh, L. Y., and Yong, V. W. (1996) *Glia* **17,** 237–253.
12. Tanaka, J., Toku, K., Matsuda, S., Sudo, S., Fujita, H., Sakanaka, M., and Maeda, N. (1998) *Glia* **24,** 198–215.
13. Zhang, B., Matsuda, S., Tanaka, J., Tateishi, N., Maeda, N., Wen, T.-C., Peng, H., and Sakanaka, M. (1998) *J. Stroke Cerebrovasc. Dis.* **7,** 1–9.
14. Suzumura, A., Mezitis, S. G., Gonatas, N. K., and Silberberg, D. H. (1987) *J. Neuroimmunol.* **15,** 263–278.
15. Tanaka, J., Fujita, H., Matsuda, S., Toku, K., Sakanaka, M., and Maeda, N. (1997) *Glia* **20,** 23–37.
16. Tanaka, J., and Sobue, K. (1994) *J. Neurosci.* **14,** 1038–1052.
17. Wen, T.-C., Tanaka, J., Peng, H., Desaki, J., Matsuda, S., Maeda, N., Fujita, H., Sato, K., and Sakanaka, M. (1998) *J. Exp. Med.* **188,** 635–649.
18. Hayashi, Y., Nomura, M., Yamagishi, S., Harada, S., Yamashita, J., and Yamamoto, H. (1997) *Glia* **19,** 13–26.
19. Baker, A. J., Zornow, M. H., Sheller, M. S., Yaksh, T. L., Skilling, S. R., Smullin, D. H., Larson, A. A., and Kuczenski, R. (1991) *J. Neurochem.* **57,** 1370–1379.
20. Benveniste, H., Drejer, J., Schousboe, A., and Diermer, N. H. (1984) *J. Neurochem.* **43,** 1369–1374.
21. Korf, J., Klein, H. C., Venema, K., and Postema, F. (1988) *J. Neurochem.* **50,** 1087–1096.
22. Sudo, S., Tanaka, J., Toku, K., Desaki, J., Matsuda, S., Arai, T., Sakanaka, M., and Maeda, N. (1998) *Exp. Neurol.* in press
23. Toku, K., Tanaka, J., Yano, H., Desaki, J., Zhang, B., Yang, L., Ishihara, K., Sakanaka, M., and Maeda, N. (1998) *J. Neurosci. Res.* **53,** 415–425.
24. Perry, T. L., Hansen, S., and Kennedy, J. (1975) *J. Neurochem.* **24,** 587–589.
25. Ohsawa, K., Imai, Y., Nakajima, K., and Kohsaka, S. (1997) *Glia* **21,** 285–298.
26. Sawada, M., Suzumura, A., and Marunouchi, T. (1995) *Int. J. Dev. Neurosci.* **13,** 253–264.
27. Giulian, D., Li, J., Bartel, S., Broker, J., Li, X., and Kirkpatrick, J. B. (1995) *J. Neurosci.* **15,** 7712–7726.