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α-MSH Peptides Inhibit Production of Nitric Oxide and Tumor Necrosis Factor-α by Microglial Cells Activated with β-Amyloid and Interferon γ

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α-Melanocyte stimulating hormone (α-MSH) is an ancient tridecapeptide with potent inhibitory activity in all major forms of inflammation. The anti-inflammatory message sequence of α-MSH resides in the COOH-terminal tripeptide α-MSH[11–13]. We tested the influence of α-MSH[1–13] and of α-MSH[11–13] in a cultured murine microglia cell line known to produce nitric oxide (NO_2^-) and tumor necrosis factor (TNFα) when stimulated with β-amyloid protein (Aβ). Melanocortin peptides significantly inhibited release of both NO_2^- and TNFα into cell-free supernatants from microglia stimulated with Aβ[1–42] or Aβ[25–35] peptides and interferon γ (IFNγ). Northern blot analysis demonstrated that α-MSH[1–13] and α-MSH[11–13] inhibited accumulation of inducible nitric oxide synthase (iNOS) and TNFα mRNA was triggered by Aβ stimulation. Aβ/microglial interaction is believed to promote the progression of inflammatory and neurodegenerative changes in senile plaques in Alzheimer's disease. Our data indicate that α-MSH peptides might be used to modulate the local response of the brain to Aβ deposition in this neurodegenerative disease.

α-Melanocyte-stimulating hormone (α-MSH) is an endogenous neuroimmunomodulatory tridecapeptide that is derived from the cleavage of a larger precursor molecule, pro-opiomelanocortin (POMC). α-MSH is widely distributed in tissues of higher organisms; it has been identified in the pituitary, various brain regions, skin, circulation and other sites (1, 2). Although named for its influence on pigmentation, α-MSH mediates other biologic functions such as control of inflammation and fever (3). Antipyretic and antiinflammatory activities of α-MSH have been traced to a "message sequence" contained within the COOH-terminal region: the tripeptide Lys-Pro-Val (α-MSH[11–13]) (4, 5). With regard to its underlying influence in inflammation, the peptide is known to inhibit leukocyte production of proinflammatory mediators such as cytokines and nitric oxide (NO) (6, 7, 8).

The role of inflammation in Alzheimer's disease has recently gained prominence and examination of proinflammatory mediators in the lesions characteristic of this condition is receiving renewed and deserved attention. Classical markers of immune-mediated injury, including cytokines, free radicals and components of the complement cascade, are associated with β-amyloid (Aβ)-containing plaques (for a review, see 9).

Synthetic peptides homologus to Aβ have been utilized to investigate the mechanisms of cerebral deposit formation and the role played by Aβ in Alzheimer's disease. Aβ peptides trigger microglial activation under experimental conditions and numerous studies have characterized the phagocytic response induced by exposure to these peptides (10, 11, 12, 13, 14). The evidence supports a model of Alzheimer's disease in which microglia are activated by Aβ deposition, and the associated cytokine expression promotes neuronal degeneration, astrocyte proliferation, further microglial response and subsequent additional Aβ production leading to plaque maturation. Activated microglia and proinflammatory mediators are therefore likely to be critical in development of neuronal degeneration associated with senile plaques and dementia (15).

Although it has been suggested that anti-inflammatory therapy might suppress the inflammation associated with senile plaques (16), no published studies have directly evaluated the role of anti-inflammatory molecules on Aβ-activated microglia. In the present report we examined the influence of anti-

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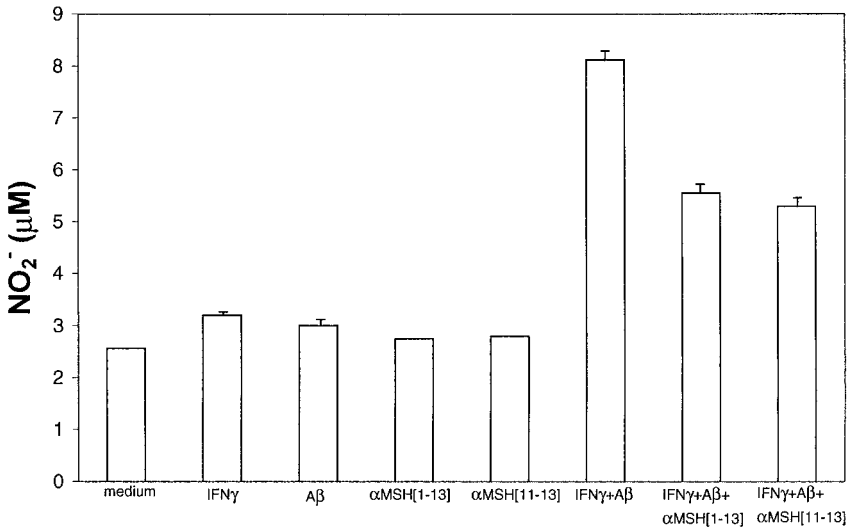

FIG. 1. Effect of αMSH[1–13] and αMSH[11–13] on Aβ[25–35]/IFNγ-induced nitrite production. Microglial cells were cultured in 96-well plates and stimulated with Aβ[25–35] 50 μg/ml and IFNγ 1 U/ml, in the presence or absence of αMSH[1–13] and αMSH[11–13] 10 μM. After 48 h of incubation, supernatants were harvested and assayed for NO_2^- accumulation. Mean values ± S.E.M. of assays performed with supernatants collected from triplicate wells for each condition are shown. The figure depicts a representative experiment out of five performed with similar results.

inflammatory α-MSH[1–13] and its COOH-terminal tripeptide sequence α-MSH[11–13] on cultured murine microglia known to produce TNFα and NO when stimulated with peptides homologus to Aβ in the presence of IFNγ.

MATERIALS AND METHODS

Materials. Peptides Aβ[1–42] and Aβ[25–35] (Bachem, Hannover, Germany) were resuspended in sterile H_2O, aliquoted at 1 mg/ml, and kept at −20°C. Recombinant murine IFNγ was purchased from Sigma Chemical Company (St. Louis, MO), and resuspended in phosphate buffered saline supplemented with 1% fetal bovine serum (GIBCO BRL). α-MSH[1–13] and α-MSH[11–13] were provided by Dr. R. Longhi (CNR, Milano), and dissolved in RPMI-1640 medium.

Cultures of microglial cells. The N9 clone of murine microglial cell lines, obtained by immortalization of embryonic brain cultures with the 3RV retrovirus carrying an activated v-myc oncogene (17), was kindly provided by P. Ricciardi Castagnoli (Centro di CitoFarmacologia, CNR, Milano, Italy). Cells were plated in 96-well plates at a concentration of 4×10^4/100 μl or in 6-well plates (Costar) at a concentration of 1×10^6/ml, and cultured in RPMI-1640 medium (GIBCO BRL) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone Lab. Inc., Logan, UT). After incubation with stimuli for the indicated times, cell-free supernatants were collected and stored at −70°C for subsequent assays. Total RNA was extracted from adherent cells cultured in 6-well plates.

Nitrite assay. Accumulation of NO_2^-, a stable end-product extensively used as an indicator of NO production by cultured cell, was assayed by the Griess reaction, according to the method previously described (18). Briefly, cell-free supernatants were mixed with equal amounts of Griess reagent (p-aminobenzene sulfonilamide 1%, Naphtylethylenediamide 0.1% in phosphoric acid 2.5%), in 96-well plates. Samples were incubated at room temperature for 10 min, and subsequently absorbance was read at 540 nm using a microplate reader. NO_2^- concentrations were calculated in accordance with a sodium nitrite standard curve.

TNFα assay. TNFα release in culture medium was measured with a murine TNFα ELISA kit (Amersham, Little Chalfont, Bucks, UK), based on the quantitative "sandwich" enzyme immunoassay technique. The sensitivity of this assay was 10 pg/ml.

RNA extraction and Northern blot analysis. Microglial cells were plated in 6-well plates at a concentration of 1×10^6/ml and then incubated with stimuli. At the indicated times, total RNA was extracted and analyzed as previously described (19). The cDNA fragments encoding murine TNFα and mouse macrophage iNOS (20) were ^{32}P-labeled using a Ready-To-Go Labeling kit (Pharmacia, Uppsala, Sweden) before hybridization of nylon filters and autoradiography. Blots were subsequently rehybridized with a human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe (Clontech Lab. Inc., Palo Alto, CA), as an internal control.

Statistical analysis. Data are expressed as means ± S.E.M. Statistical evaluation was performed by repeated measures analysis of variance (ANOVA) followed by Dunnet's test for specific comparisons. Statistical significance was set at $P < 0.05$.

RESULTS

Effect of α-MSH[1–13] and α-MSH[11–13] on NO Production from Microglia Stimulated with Aβ and IFNγ

Stimulation of microglial cells for 48 h with 50 μg/ml Aβ[25–35] plus 1 U/ml IFNγ released a significant amount of NO_2^-, as previously described (11). However, addition of α-MSH[1–13] to incubation medium decreased NO production by 32 ± 7% ($P < 0.05$; n = 5). The COOH-terminal tripeptide α-MSH[11–13] was slightly more effective in reducing NO production (38 ± 2%, $P < 0.05$; n = 5). For both α-MSH[1–13] and α-MSH[11–13] the inhibitory influences were observed with 10 μM concentration (Fig. 1). Other concentra-

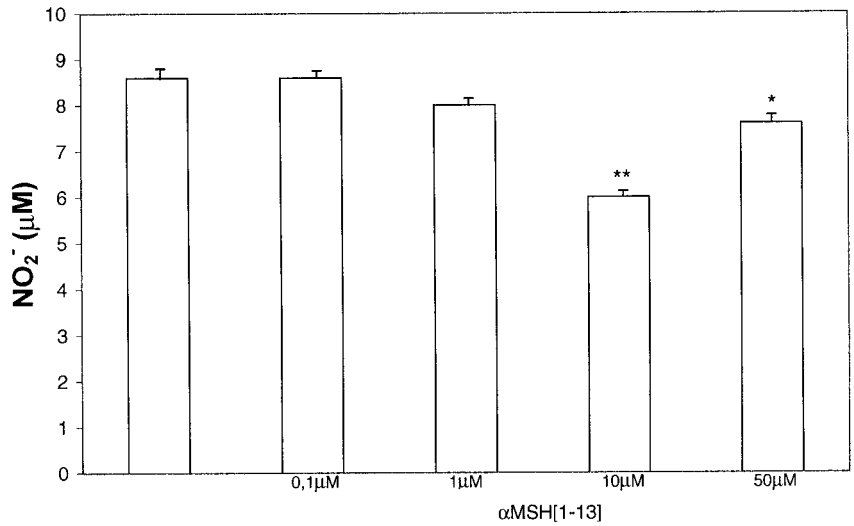

FIG. 2. Dose-dependent effect of αMSH[1–13] on Aβ[25–35]/IFNγ-induced nitrite production. Microglial cells were cultured in 96-well plates and challenged for 48 h with Aβ[25–35] 50 μg/ml plus IFNγ 1 U/ml in the presence or absence of increasing concentrations of αMSH[1–13]. Subsequently, cell-free supernatants were harvested and assayed for NO_2^- accumulation. Mean values ± S.E.M. of assays performed with supernatants collected from triplicate wells for each condition are shown. The figure depicts a representative experiment out of three performed with similar results. *$P < 0.05$; **$P < 0.01$.

tions of melanocortin peptides had lesser or no effect on NO production; the peptide concentration/inhibition pattern was U-shaped (Fig. 2). Similar results were also observed when microglial cells were activated with 100 μg/ml Aβ[1–42] (Fig. 3).

To determine if the inhibitory effect of the melanocortin peptides results from inhibition of gene expression of inducible nitric oxide synthase (iNOS), microglia were cultured for up to 4 h with Aβ[25–35], in the presence or absence of α-MSH[1–13] or α-MSH[11–13], and then total RNA was extracted. Northern blot analysis (Fig. 4) indicated that treatment with Aβ[25–35] induced accumulation of iNOS mRNA and that this accumulation was significantly decreased in the presence of α-MSH[1–13] or α-MSH[11–13]. The inhibition caused by the two peptides, evaluated by densitometric analysis, was 30% and 37%, respectively.

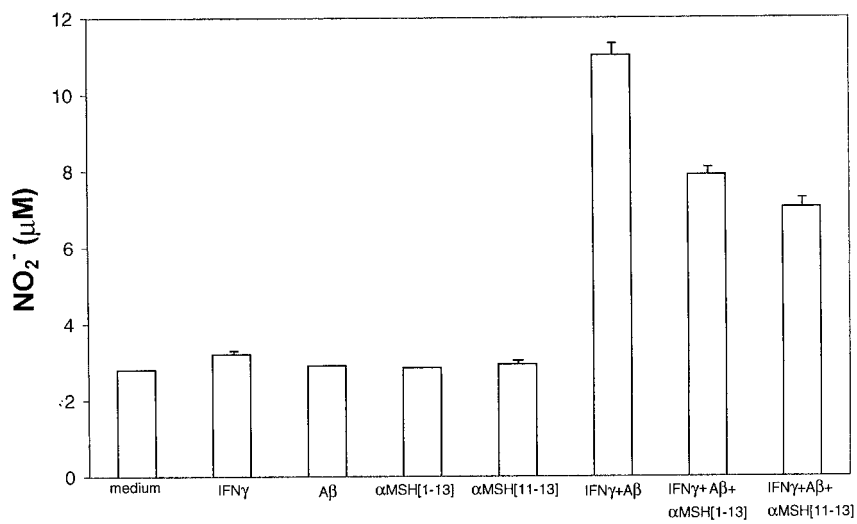

FIG. 3. Effect of αMSH[1–13] and αMSH[11–13] on Aβ[1–42]/IFNγ-induced nitrite production. Microglial cells were cultured in 96-well plates and stimulated with Aβ[1-42] 100 μg/ml and IFNγ 1 U/ml, in the presence or absence of αMSH[1–13] and αMSH[11–13] 10 μM. After 48 h of incubation, supernatants were harvested and assayed for NO_2^- accumulation. Mean values ± S.E.M. of assays performed with supernatants collected from triplicate wells for each condition are shown. The figure depicts a representative experiment out of three performed with similar results.

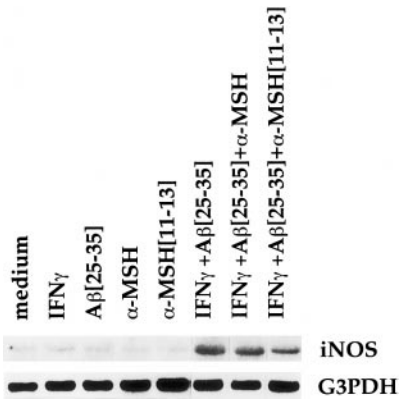

FIG. 4. Effect of αMSH[1–13] and αMSH[11–13] on iNOS mRNA expression in Aβ[25–35]/IFNγ-activated microglia. Microglial cells, cultured in 6-well plates, were stimulated for 4 h with Aβ[25–35] 50 μg/ml and IFNγ 1 U/ml, in the presence or absence of αMSH[1–13] and αMSH[11–13] 10 μM. Total RNA was purified and analyzed by Northern blot analysis using iNOS and G3PDH cDNAs. Shown is a representative experiment out of three performed with similar results.

Effect of α-MSH[1–13] and α-MSH[11–13] on TNFα Production by Microglia Stimulated with Aβ[25–35]

Determination of antigenic TNFα in cell-free supernatants harvested from cultured microglia revealed that cells stimulated for 24 h with 50 μg/ml Aβ[25–35] released high levels of TNFα. These increases in TNFα were significantly lower when the cells were coincubated with 10 μM α-MSH[1–13] (Fig. 5) or α-MSH[11–13] (not shown). Similar inhibitory effects of both melanocortin peptides were observed when TNFα production was induced by 100 μg/ml Aβ[1–42] (not shown).

To examine whether α-MSH[1–13] also affected TNFα gene transcription, we performed Northern blot analysis on total RNA extracted from microglia stimulated for 4 h with Aβ[25–35], in the presence or absence of α-MSH[1–13]. As shown in Fig. 6, 10 μM α-MSH[1–13] significantly (35%) reduced microglial accumulation of TNFα mRNA triggered by Aβ[25–35].

DISCUSSION

In this study we demonstrate that α-MSH[1–13] and α-MSH[11–13] reduced the production of NO and TNFα by cultured microglia stimulated with Aβ[1–42] or Aβ[25–35]. The greatest inhibitory effect was observed with the smaller amino acid sequence α-MSH[11–13]. The peptide concentration/inhibition pattern was U-shaped, a characteristic result with many peptides that are ineffective in very high and very low concentrations (7). That both α-MSH[1–13] and α-MSH[11–13] did not completely inhibit NO and TNFα production triggered by Aβ is consistent with previous studies which have shown that αMSH peptides generally modulate the production of proinflammatory mediators and do not completely block them (2, 8, 21).

α-MSH[1–13] also inhibited Aβ-induced accumulation of iNOS and TNFα mRNA, indicating that α-MSH[1–13] prevents production of those proinflammatory molecules with the primary locus of action at the mRNA level. However, whether this effect is related to inhibition of transcription or message stability is presently unknown. It is likely that the effects of the melanocortin peptides on microglial

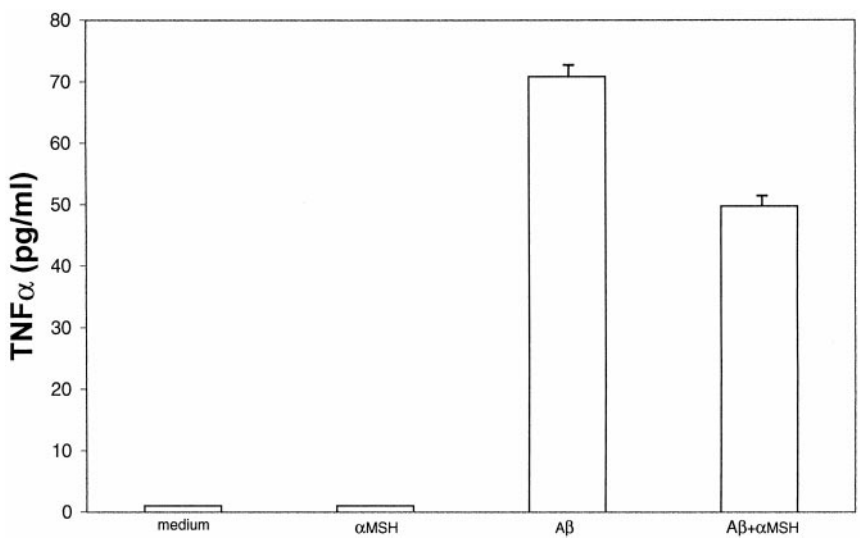

FIG. 5. Effect of αMSH[1–13] on Aβ[25–35]-induced TNFα production. Microglial cells were cultured in 96-well plates and stimulated with Aβ[25–35] 50 μg/ml in the presence or absence of αMSH[1–13] 10 μM. After 24 h of incubation, supernatants were collected and assayed for TNFα production. Mean values ± S.E.M. of duplicate assays performed with supernatants collected and pooled from three wells for each condition are shown. The figure depicts a representative experiment out of three performed with similar results.

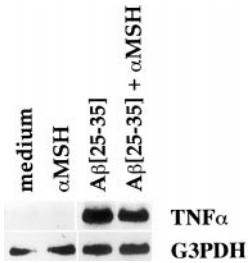

FIG. 6. Effect of αMSH[1–13] on TNFα mRNA expression in Aβ[25–35]-activated microglia. Microglial cells, cultured in 6-well plates, were stimulated for 4 h with Aβ[25–35] 50 μg/ml, in the presence or absence of αMSH 10 μM. Total RNA was purified and analyzed by Northern blot analysis using TNFα and G3PDH cDNAs. A representative experiment out of three performed with similar results is shown.

cells were mediated by melanocortin receptors (MCR) 1, 3, 4 and 5 found in brain. These receptors have been detected in brain tissues, where they may be stimulated by circulating α-MSH (2, 22). Future studies will address which subtypes of MCR are expressed by microglia and mediate the function of α-MSH on central phagocytes. Evidence that Aβ deposition is a crucial event in the pathogenesis of senile plaques in Alzheimer's disease is growing (10). These plaques represent one of the neuropathological hallmarks of Alzheimer's disease and are characterized by Aβ deposition, degenerated neurites, astroglial proliferation and microglia activation (23). Senile plaques are also sites of local immune-mediated inflammatory responses mounted by cytokines, free radicals and virtually all components of the classical complement pathway (for a review, see 9). Previous studies have shown that interaction of Aβ/microglia may lead to biological events involved in the process of plaque evolution since Aβ can activate microglial cells and trigger the production of proinflammatory and potentially cytotoxic mediators such as NO and TNFα (11, 12). These findings suggest that Aβ may induce local neurotoxicity not only via a direct effect on the neuronal plasma membrane (24, 25) but also indirectly with aggregation of Aβ deposits serving as nidus for a local inflammation (10, 11, 12, 13, 14).

NO is synthesized from L-arginine by the enzyme NO-Synthase (NOS), which exists in three isotypes. Two isotypes of them are constitutive isoforms found in neurons and endothelial cells, and they function in calcium-dependent ways. The third, NO calcium Independent Synthase (iNOS) results in much larger quantities of NO expressed by macrophages in the presence of cytokines or bacterial products (26, 27). Even though NO exerts neuroregulatory functions in the brain, its over production can be toxic to neuronal cells (28).

TNFα is a pleiotrophic cytokine that plays a significant role in brain immune and inflammatory activities. Produced in the brain in response to various pathological processes, it causes expression of adhesion molecules, leukocyte migration, and activation of glial cells (29). Though TNFα serves a primary function in "setting the stage" for inflammatory reactions, no clear evidence has demonstrated that TNFα may induce direct neurotoxicity. However, by virtue of its central role as an autocrine mediator in the production of potentially noxious molecules such as NO, TNFα has been suggested to indirectly take part in the modulation of neurotoxic events (30, 31).

Our data on inhibitory influences of melanocortin peptides on microglia products triggered by Aβ indicate that α-MSH might exert an endogenous regulation in the local response of the brain to Aβ deposition in Alzheimer's disease. We suggest that α-MSH might influence Aβ bioactivity in senile plaques and we believe that impairment of α-MSH production in Alzheimer's disease might reduce the potential inhibitory mechanism of the brain against microglia activation induced by Aβ deposition and therefore contribute to neurodegenerative and inflammatory changes in Alzheimer's disease brain. This notion is supported by lower concentrations of α-MSH immunoreactivity in the brain and in the CSF of Alzheimer's disease patients (31, 32), and in aged squirrel monkey brain (33). That α-MSH is presumably involved in cognitive functions (34) is also consistent with evidence that the administration of POMC-derived peptides increases Ach synthesis and turnover in the rat brain (35).

In conclusion, the present study indicates that melanocortin peptides modulate production of proinflammatory cytokines and related mediators by Aβ-activated microglia. These findings suggest that α-MSH could modulate inflammation in senile plaques of Alzheimer's disease brain. Currently, it is unclear whether these inflammatory reactions are primarily responsible for neuronal degeneration or are simply ephiphenomenon of Aβ deposition. However, α-MSH[1–13] and particularly its COOH-terminal amino acid sequence α-MSH[11–13], might be useful in the treatment of Alzheimer's disease given their inhibitory properties on Aβ-mediated inflammatory processes.

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REFERENCES

1. Eberle, A. N. (1988) *Karger Basel* 1–556.
2. Catania, A., and Lipton, J. M. (1993) *Endocrine Reviews* **14,** 564–573.
3. Lipton, J. M. (1988) *in* The Melanotrophic Peptides (Hadley, M., Ed.), pp. 97–113, CRC Press, Boca Raton, FL.

4. Hiltz, M. E., and Lipton, J. M. (1989) *FASEB J.* **3,** 2282–2284.
5. Hiltz, M. E., Catania, A., and Lipton, J. M. (1991) *Peptides* **12,** 767–771.
6. Rajora, N., Boccoli, G., Burns, D., Sharma, S., Catania, A., and Lipton, J. M. (1997) *J. Neurosci.* **17,** 2181–2186.
7. Star, R. A., Rajora, N., Huang, J., Stock, R. C., Catania, A., and Lipton, J. M. (1995) *Proc. Natl. Acad. Sci. USA* **92**(17), 8016–8020.
8. Lipton, J. M., and Catania, A. (1997) *Immunol. Today* **18,** 140–145.
9. Rogers, J. (1992) *Drug Res.* **45,** 439–442.
10. Eikelenboom, P., Zhan, S. S., Van Gool, W. A., and Allsop, D. (1994) *Trends Pharmacol. Sci.* **15,** 447–450.
11. Meda, L., Cassatella, M. A., Szendrei, G. I., Otvos, L., Baron, P. L., Villalba, M., Ferrari, D., and Rossi, F. (1995a) *Nature* **374,** 647–650.
12. Goodwin, J. L., Uemura, E., and Cunnick, J. E. (1996) *Brain Res.* **692,** 207–214.
13. Meda, L., Bernasconi, S., Bonaiuto, C., Sozzani, S., Zhou, D., Otvos, L., Mantovani, A., Rossi, F., and Cassatella, M. A. (1996) *J. Immunol.* **157,** 1213–1218.
14. Meda, L., Bonaiuto, C., Baron, P. L., Otvos, L., Rossi, F., and Cassatella, M. A. (1996b) *Neurosci. Lett.* **219,** 1–4.
15. Lue, L. F., Brachova, L., Civin, W. H., and Rogers, J. (1996) *J. Neuropathol.*
16. *Exp. Neurol.* **55,** 1083–1088.
17. Mackenzie, I. R. A., and Munoz, D. G. (1988) *Neurology* **50,** 986–990.
18. Righi, M., Mori, L., De Libero, G., Sironi, M., Biondi, A., Mantovani, A., Donini, S. D., and Ricciardi-Castagnoli, P. (1989) *Eur. J. Immunol.* **19,** 1443–1448.
19. Rockett, K. A., Awburn, M. M., Aggarwal, B. B., Cowden, W. B., and Clark, I. A. (1992) *Infect. Immun.* **60,** 3725–373.
20. Meda, L., Bonaiuto, C., Szendrei, G. I., Ceska, M., Rossi, F., and Cassatella, M. A. (1995b) *J. Neuroimmunol.* **59,** 29–34.
21. Lyons, C. R., Orloff, G. J., and Cunningham, J. M. (1992) *J. Biol. Chem.* **267,** 6370–6374.
22. Delgado, R., Carlin, A., Airaghi, L., Demitri, M. T., Meda, L., Galimberti, D., Baron, P. L., Lipton, J. M., and Catania, A. (1998) *J. Leukoc. Biol.* **63,** 740–745.
23. Tatro, J. B. (1996) *Neuroimmunomodulation* **3,** 259–284.
24. Maury, C. P. J. (1995) *Lab. Invest.* **72,** 4–16.
25. Yankner, B. A., Duffy, L. K., and Kirschner, D. A. (1990) *Science* **250,** 279–282.
26. Pike, C. J., Burdick, D., Walencewicz, A. J., Glabe, C. G., and Cotman, C. W. (1993) *J. Neurosci.* **13,** 1676–1687.
27. Moncada, S., and Higgs, A. (1993) *New Engl. J. Med.* **329,** 2002–2012.
28. Nathan, C. F. (1992) *FASEB J.* **6,** 3051–3064.
29. Snyder, S. H. (1992) *Curr. Opin. Neurobiol.* **2,** 323–327.
30. Feuerstein, G. Z., Liu, T., and Barone, F. C. (1994) *Cerebrovas. Brain Metab. Rev.* **6,** 341–360.
31. Chao, C. C., Molitor, T. W., and Hu, S. (1993) *J. Immunol.* **151,** 1473–1481.
32. Arai, H., Moroji, T., Kosaka, K., and Iizuka, R. (1986) *Brain Res.* **377,** 305–310.
33. Rainero, I., May, C., Kaye, J. A., Friedland, R. P., and Rapoport, S. I. (1988) *Neurology* **38,** 1281–1284.
34. Bell, R. C., and Lipton, J. M. (1987) *Brain Res. Bull.* **18**(4), 577–579.
35. Sandman, C. A., Barron, J. L., and Parker, L. (1985) *Pharmacol. Biochem. Behav.* **23**(1), 21–26.
36. Wood, P. L., Cheney, D. L., and Costa, E. (1978) *Life Sci.* **22,** 673–678.