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Effect of β-Amyloid Protein and IFN-γ on Activation of Microglia

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The senile plaques characteristic of aging and Alzheimer's disease in brain tissue are composed of insoluble aggregates of β-amyloid protein, and infiltrated by reactive microglia. The role of microglia in neuronal damage is not fully understood. It has been suggested that immune responses may lead to damage of brain tissue. The fact that microglia express MHC class I and II molecules suggests that they are able to process protein antigens and present the antigen peptides to T lymphocytes. Thus, the microglia that are the resident macrophages in brain tissue are able to participate in induction of immune responses in the brain. It has been reported that the activated microglial cells produce reactive nitrogen intermediates such as nitric oxide and hydrogen peroxide, which lead to damage of brain tissue. In this study, we examine whether rat microglial cells produce nitric oxide upon stimulation with active fragment of β-amyloid protein(*25-35* amino acids). The results indicate that β-amyloid alone does not stimulate microglia to produce nitric oxide. However, we observed an enhancing effect of β-amyloid and IFN-γ in triggering the production of nitric oxide by microglial culture. In addition, IFN-γ potentiates a synergistic effect with LPS on the production of nitric oxide in microglial culture. These findings suggest that β-amyloid activates microglial cells in the presence of IFN-γ, and this may have a role in the pathogenesis of neuronal degeneration observed in aging and Alzheimer's disease. (Ajou Med J 1997; 2(2): 96~101)

Key Words: *Microglia, Immune response, Nitric oxide, β-amyloid protein(25-35 amino acids), Neurodegeneration*

INTRODUCTION

The degeneration of neuronal cells causes several neurological diseases: Alzheimer's disease (AD), Pick's disease, Shy-drager syndrome, Parkinson's disease (PD) and several other chronic degenerative neurological disorders. Several mechanisms have been suggested for neurodegeneration. One of them would be immune functions in the brain[1~10], but the possible role of the immune system in the brain has not been systematically studied in neuronal degeneration. It has been suspected that the brain is an immunologically privileged organ. This is based on several reasons as follows: 1) the existence of the blood-brain barrier, 2) the absence of conventional lymphatic drainage, 3) the failure of neurons to express major histocompatibility complex (MHC) glycoproteins, and 4) the unusual tolerance of the brain to transplanted tissue. However, it is well known that the immune system responds to acute infections in the brain. In particular, multiple sclerosis and experimental allergic encephalitis are the most studied chronic inflammations[11~15].

The microglia are the residents in brain tissue which may represent the immune system and they express two monocyte markers of leukocyte common antigen (LCA) and Fc γ R1 receptor[16]. Furthermore, microglia express MHC class II antigens[17~21], which are molecules important to active T lymphocytes in association with antigenic peptides. The presence of MHC class II molecules on microglia suggest that these cells are immunocompetent

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and able to initiate a specific immune response by processing protein antigens and presenting antigenic peptides to T lymphocytes[22,23].

A number of investigators have suggested that microglia produce reactive oxygen compounds such as nitric oxide (NO) and hydrogen peroxide (H_2O_2) upon stimulation with LPS[24~27]. The reactive oxygen compounds are toxic to neuronal cells. Furthermore, a synergistic effect between β-amyloid protein (Aβ) and interferon-γ (IFN-γ) has been observed in the production of reactive oxygen intermediates and tumor necrosis factor-α (TNF-α) by microglial culture[28~30]. Aβ is a major component in formation of senile plaques on neuronal cells, which is closely associated with neurodegenerative diseases[31~33].

In the present study, we determined the production of NO in microglial culture incubated separately with Aβ, LPS or IFN-γ. We also studied the effect of IFN-γ and Aβ in the production of NO in microglial cultures. These results suggest one of the possible mechanisms involved in the pathogenesis of neurodegenerative diseases.

MATERIALS AND METHODS

Animals

Pregnant Sprague Dawley (SD) rats were purchased from the Korean Center for Experimental Animals.

Reagent

LPS prepared from E. coli 026:B6 and Aβ (*25-35* amino acids : a.a.) were purchased from Sigma Chemical Co. (St Louise, MO, USA). Recombinant rat IFN-γ was purchased from Biosource (Camarillo, CA, USA).

Preparation of microglia

Microglia and astrocytes were prepared from the cerebral cortice of 1~3 days old SD rats as described[7] with some modification. The cortice were triturated into single cells in minimal essential medium supplemented with 10% fetal bovine serum in a plastic petri dish under the stereomicroscope. The cells were transferred into 15ml tubes and then washed by centrifugation. The single cell suspension in the culture medium was dispensed into $75cm^2$ flasks and 24 well plates. The flasks and plates were incubated at 37°C and 5% CO_2 in saturated humidified air for 7 days. Since these cells are a mixture of microglia and astrocytes, the microglia were isolated from astrocytes by shaking the flasks after 7 days of incubation. Under this condition, microglia are easily detached from the surface of the flask, and the culture medium is then decanted into 50 ml tubes. The microglia were collected and washed by centrifugation. The astrocytes are firmly attached on the plastic surface of the flask. The isolated microglia are suspended in the culture medium and dispensed into 24 well plates with 5×10^4 cells/ml, and incubated until the cells form a confluent monolayer.

Stimulation of microglia

When monolayers of microglia form on the surface of a 24 well plate, various concentrations of LPS, IFN-γ or Aβ (*25-35* a.a.) are added separately to the microglia, and then incubated for the indicated hours. At the end of incubation, the culture supernatants are collected.

Measurement of NO production

The accumulation of NO_2^-, a stable end product of NO formation, in the supernatant of microglia culture supernatant is used as a relative measurement of NO production. The NO_2^- concentrations in the samples are determined by Griess method (7). Briefly, Griess test reagent is prepared with 0.1% naphthylethylene diamine dihydrochloride in 1% sulfanilamide and 2.5% H_3PO_4 in distilled water. Fifty μl of Griess test reagent and 50 μl of the supernatant of microglial culture are added into the well of a 96 well microtiter plate. The mixtures are incubated at 37°C for 10 min and then the optical densities are read at 550nm. The standard curve is constructed by using a sodium nitrite solution of the concentrations ranging from 1.0pM to 25 μM.

RESULTS

Effect of LPS, IFN-γ or Aβ on the production of NO in microglial culture

As shown in Fig. 1, microglia incubated with 100

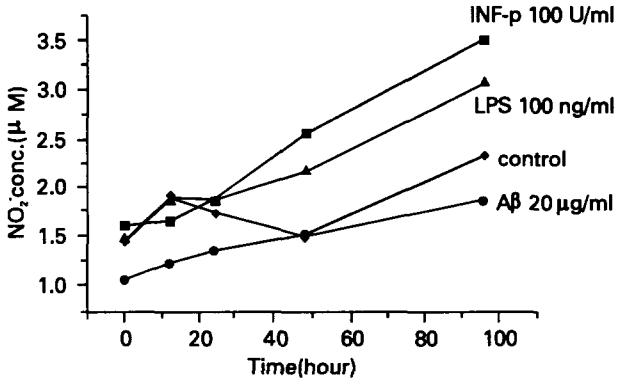

Fig. 1. Effect of IFN-γ, Aβ(25-35 a.a.) and LPS on the production of NO_2^- in microglial cultures.
The microglial cultures(1 ml) in the wells of 24 well plate are incubated with respective agents, LPS, IFN-γ and Aβ(25-35 a.a.) for 0, 12, 24, 48 and 96 hours. At the end of incubation the supernatants are collected and the concentrations of NO_2^- determined by Griess method.

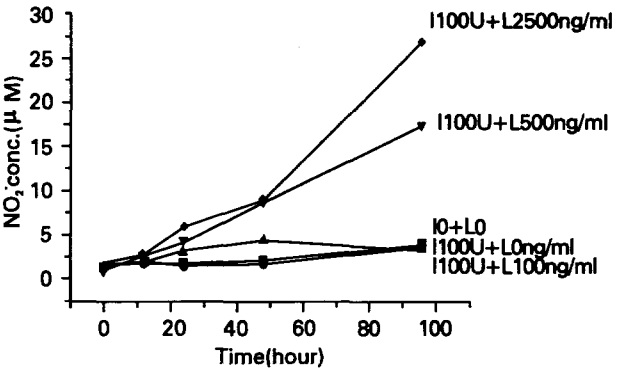

Fig. 3. Effect of IFN-γ, and LPS on the production of NO_2^- in microglial cultures.
The microglial cultures(1 ml) in the wells of 24 well plate are incubated with indicated concentrations of both IFNγ(I) and LPS(L) for 0, 12, 24, 48 and 96 hours. At the end of incubation, the supernatants are collected and the concentrations of NO_2^- determined by Griess method.

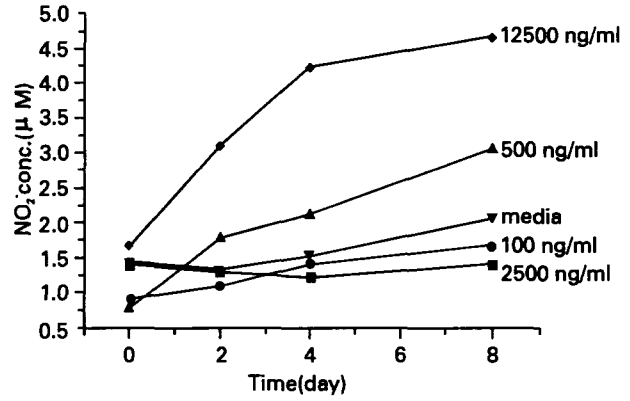

Fig. 2. Effect of various concentrations of LPS on the production of NO_2^- in micrglial culture.
The microglial cultures(1 ml) in the wells of 24 well plate are incubated with various concentrations of LPS for 0, 2, 4 and 8 days. At the end of incubation, the supernatants are collected and the concentrations of NO_2^- determined by Griess method.

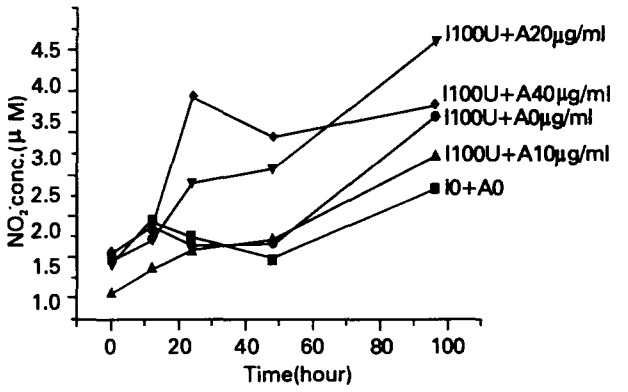

Fig. 4. Effect of IFN-γ, and Aβ(25-35 a.a.) on the production of NO_2^- in microglial cultures.
The microglial cultures(1 ml) in the wells of 24 well plate are incubated with indicated concentrations of both IFNγ(I) and Aβ(25-35 a.a.) (A) for 0, 12, 24, 48 and 96 hours. At the end of incubation, the supernatants are collected and the concentrations of NO_2^- determined by Griess method.

ng/ml LPS or 100 U/ml IFN-γ stimulate the production of NO in a time-dependent manner up to 96 hours. The microglial culture incubated with 20 μg/ml Aβ (25-35 a.a.) alone showed a similar amount of NO_2^- as the control microglial culture throughout 96 hour incubation. In order to determine the optimal concentration of LPS for the production of NO, the microglial cultures were incubated with 100, 500, 2,500, 12,500 ng/ml LPS. As shown in Fig. 2, the concentrations of NO_2^- in the microglial culture were in a time-and dose-dependent manner except 2,500 ng/ml LPS. The microglial culture incubated with 12,500 ng/ml LPS produces the highest concentration of NO_2^-.

Table 1. Synergistic effect of IFN-γ and LPS on the production of NO_2^- in microglia cultures

Time (hour)		Concentration of NO_2^- (μM/ml)					
	IFN-γ (U/ml)	0	0	100	100	100	100
	LPS (ng/ml)	0	500	0	100	500	2500
0		1.44	0.78	1.66	1.35	1.09	1.79
12		1.92	–	1.83	1.88	2.53	2.75
24		1.75	1.78	1.48	3.09	4.01	5.88
48		1.48	2.11	1.75	4.40	8.53	8.84
96		2.35	3.05	3.57	3.27	17.28	26.68

The microglia cultures (1ml) in the wells of 24 well plate are incubated with indicated concentrations of both IFN-γ and LPS for 0, 12, 24, 48 and 96 hours. At the end of incubation, the supernatants are collected and the concentration of NO_2^- determined by the Griess test method.

Table 2. Synergistic effect of IFN-γ and β-amyloid on the production of NO_2^- in microglia cultures

Time (hour)		Concentration of NO_2^- (μM/ml)					
	IFN-γ (U/ml)	0	0	100	100	100	100
	LPS (ng/ml)	0	20	0	10	20	40
0		1.44	1.05	1.53	1.05	1.44	1.53
12		1.92	1.22	1.88	1.35	1.70	1.83
24		1.75	1.35	1.62	1.57	2.40	3.44
48		1.48	1.53	1.66	1.70	2.57	2.96
96		2.35	1.88	3.22	2.75	4.14	3.36

The microglia cultures (1ml) in the wells of 24 well plate are incubated with indicated concentrations of both IFN-γ and β-amyloid for 0, 12, 24, 48 and 96 hours. At the end of incubation, the supernatants are collected and the concentration of NO_2^- determined by the Griess test method.

Effect of LPS and IFN-γ on production of NO in microglial culture

As shown in Fig. 1 and 2, LPS or IFN-γ alone can stimulate the microglia to produce NO. To investigate whether these two reagents show any effect, microglial cultures were incubated with the fixed concentration of IFN-γ (100 U/ml) together with various concentrations of LPS (100, 500, 2,500, 12,500 ng/ml).

As shown in Fig. 3, the microglial culture incubated with 100 U/ml IFN-γ and 12,500 ng/ml LPS showed a significant enhancing effect in the production of NO_2^- at 2 and 4 days of incubation (8.84 and 26.68 μM respectively). Even the microglial culture incubated with 100U IFN-γ and 500 ng/ml LPS showed synergistic effect at 2 and 4 days of incubation (8.53 and 17.28 μM respectively).

Effect of IFN-γ and Aβ on the production of NO in microglial culture

Senile plaques are extracellular deposits on neuronal cells principally composed of insoluble aggregates of Aβ and infiltrated by reactive microglia. As shown in Fig. 1,

Aβ (25-35 a.a.) alone did not stimulate the microglia to produce NO. However, the microglial cultures incubated with various concentrations of Aβ (25-35 a.a.) (10, 20, 40 μg/ml) together with a fixed concentration of IFN-γ (100 U/ml) showed an enhancing effect in the production of NO (Fig. 4). Especially, an enhancing effect was prominent at concentrations of 20, 40 μg/ml Aβ (25-35 a.a.) and 100 U/ml IFN-γ at the 24 (2.4 and 3.4 μM) and 48 hours of incubation as well (2.57 and 2.97 μM).

DISCUSSION

It has been suggested that neural immune reactions are involved in pathogenesis of many neurodegenerative diseases[1~10]. For example, Aβ deposits are associated with the presence of cytokines, complement protein and proteinase inhibitor[35], and are predominantly infiltrated by microglia in Alzheimer's disease. The microglia are involved in amplifying the local response by activation and release of immune mediator[36]. Aβ (25-35 a.a.) has been shown to be chemotactic for murine resident peritoneal macrophages and rat microglia[37], but it is not clear whether the chemotactic activities are due to Aβ themselves or through the soluble mediators produced by the microglia which is activated by Aβ.

One of the mechanisms suggested to be involved in neurodegeneration is that NO plays an important role in toxic processes in neuronal cell death. The role of microglia in neurodegenerative diseases has been suggested by the fact that they express MHC class I and II molecules which are able to process protein antigens and to present antigenic peptides to T lymhocytes in association with MHC class II molecules. Demonstrations of reactive microglia and T cells in brain tissues in conditions such as AD, PD and ALS are consistent with the presence of a cell-mediated immune response. Another possible mechanism is that the stimulation of microglia in culture produces a variety of cytokines as well as reactive oxygen compounds such as NO and H_2O_2. The oxygen-dependent free radical generation by reactive microglia may lead to neuronal damage due to oxidation stress, and some of the cytokines produced by microglia may cause gliosis and upregulation of the APP mRNA in other cells, which may contribute to the deleterious formation of glia scars and senile plaques in AD[38].

In the present study, we demonstrate that LPS or IFN-γ alone stimulates the production of NO in a time- and dose- dependent manner, but Aβ (25-35 a.a.) ranging from 10 to 40 μg/μl did not induce any detectable NO accumulation even after 96 hours of incubation. However, Aβ (25-35 a.a.) potentiated the accumulation of NO from microglial culture in a time- and dose-dependent manner when they are incubated together with IFN-γ. When microglia are cultured together with both IFN-γ and LPS, a significant effect was observed in the production of NO by microglial culture in a LPS dose-dependent manner. In agreement with observation of other investigators the present finding suggests that microglia in brain tissues produce oxygen-dependent free radicals, such as NO, and may lead to neuronal damages with the presence of IFN-γ and Aβ.

In conclusion, our study adds further insight into the spectrum of proinflammatory function modulated by Aβ which might be involved in the pathogenesis of aging and AD.

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