ANTICANCER RESEARCH *24*: 2331-2336 (2004)

Somatostatin Controls LFA-1 Gene Expression by Altering Neuraminidase Expression in Spleen Cells

WON-KEE YOON[1], HO-JUN KIM[2], HWA-YOUNG SON[2], KYU-SHIK JEONG[3], SANG-JOON PARK[3], TAE-HWAN KIM[3], SUNG-HO KIM[4], SE-RA KIM[4] and SI-YUN RYU[2]

[1]*Biopotency Evaluation Laboratory, Korea Research Institute of Bioscience and Biotechnology, Daejeon, 305-333;*
[2]*Laboratory of Veterinary Anatomy, College of Veterinary Medicine, Chungnam National University, Daejon, 305-764;*
[3]*Laboratory of Veterinary Pathology, College of Veterinary Medicine, Kyungpook National University, Daegu, 702-701;*
[4]*Laboratory of Veterinary Anatomy, College of Veterinary Medicine, Chonnam National Univeristy, Kwangju, 500-757, Korea*

Abstract. *This study investigated whether neuraminidase (Neu) affects LFA-1 mRNA expression in spleen cells and whether somatostatin (SOM) and substance P (SP) treatment induce changes in the Neu mRNA expression level in spleen cells. Neu treatments down-regulated the LFA-1 mRNA levels after culturing for 2 h. SOM increased the Neu mRNA level slightly after 24-h culture and strongly after 48-h culture. These results suggest that prolonged exposure to SOM may regulate the Neu activation pathway, which in turn impairs the regulation of LFA-1 expression.*

The maintenance of leukocytes in circulation during the normal state and their adhesion to the endothelium and subsequent emigration from the bloodstream into inflammation sites are tightly regulated phenomena. At least part of the control lies in the regulation of the cell adhesion molecules on the surface of both the leukocytes and the endothelial cells (1). The leukocyte function-associated antigen-1 (LFA-1) is one of the adhesion molecules expressed on T- and B-lymphocytes, which plays an important role in the cell-cell interaction mechanisms and is involved in the homing phenomenon (2, 3).

A previous study reported that lymphocyte adhesion is influenced by the sialic acid content of the glycocalyx (4). The degree of sialylation possessed by the particular glycoconjugate species can vary greatly, and is regulated through the combined enzymatic activities of the cell-associated sialotransferases and the cell-associated sialidases. Neu-1 sialidase has been reported to play a major role in determining the sialylation levels of some cell-associated and secreted sialoconjugates (5, 6).

Substance P (SP) is a pro-inflammatory neuropeptide while somatostatin (SOM) is an anti-inflammatory substance (7-12). Recent studies have shown that SP increases neutrophil adhesion to the bronchial epithelial cells (13) and up-regulates splenocyte adhesion to the endothelial cells by acting both as an up-regulator of intercellular adhesion molecule-1 (ICAM-1) expression on the endothelial cells and as a leukocyte function-associated antigen 1 (LFA-1; CD11a/CD18) activator on the lymphocytes (14). SOM inhibits the migration of circulating leukocytes to inflammatory sites (10) and leads to the down-regulation of LFA-1 mRNA expression in mice splenocytes (15).

This study investigated the possibility that neuraminidase (Neu) may be linked to the modulation of LFA-1 mRNA expression in spleen cells, and that SOM and SP treatment may induce neuraminidase mRNA expression in spleen cells.

Materials and Methods

Animals. C3H mice (10-14 weeks of age) were housed in cages. The room temperature was maintained at 22±1˚C and the relative humidity ranged from 40 to 60%. Standard laboratory rodent chow and tap water were provided *ad libitum*. Pooled splenocytes from 5-10 mice were used for each experiment.

Cell culture. Single cell suspensions of splenocytes were prepared from groups of age- and sex-matched mice. The cells were washed twice with RPMI 1640 (Gibco BRL, Grand Island, NY, USA), and suspended at a density of $5x10^6$ cells/ml in RPMI 1640 supplemented with 1% Nutridoma (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA), 10 μg/ml gentamicin (Gibco BRL), $5x10^{-5}$ M 2-mercaptoethanol (Sigma, St. Louis, MO, USA), 100units/penicillin (Sigma) and 100 μg/ml streptomycin (Sigma). The cell suspension was added to the culture plates coated or uncoated with the immobilized anti-CD3ε (PharMingen, San Diego, CA, USA) and incubated with either Neu (20mU/ml Vibrio cholera sialidase, Sigma), SOM (10^{-10}M, Sigma), or SP (10^{-10}M, Sigma) in a

Correspondence to: Si-Yun Ryu, Laboratory of Veterinary Anatomy, College of Veterinary Medicine, Chungnam National University, Daejon 305-764, Korea. Tel: 82-42-821-6758, Fax: 82-42-822-4216, e-mail: syryu@cnu.ac.kr

Key Words: Somatostatin, substance P, LFA-1, neuraminidase.

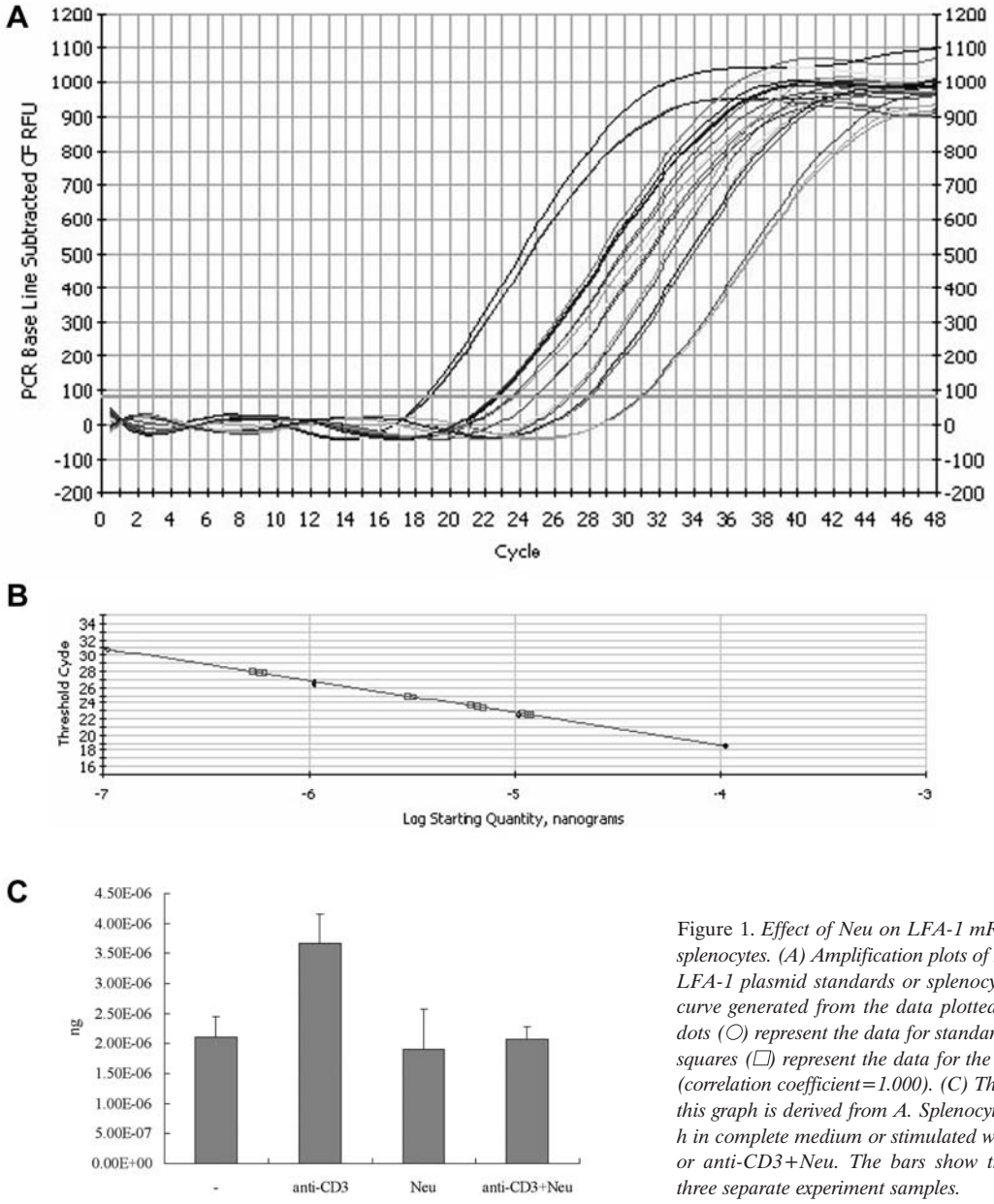

Figure 1. *Effect of Neu on LFA-1 mRNA expression on splenocytes. (A) Amplification plots of DNA derived from LFA-1 plasmid standards or splenocytes. (B) Standard curve generated from the data plotted in A. The round dots (○) represent the data for standard samples and the squares (□) represent the data for the unknown samples (correlation coefficient=1.000). (C) The quantification in this graph is derived from A. Splenocytes incubated for 2 h in complete medium or stimulated with anti-CD3, Neu or anti-CD3+Neu. The bars show the means±SD of three separate experiment samples.*

humidified atmosphere of 5% CO_2 at 37˚C (16, 17). The immobilized anti-CD3ε plates were prepared by coating the tissue culture plates with a 10mg/ml solution of anti-CD3e dissolved in 0.01 M Tris-HCl (pH 9.6) in an overnight incubation at 4˚C. The plates were extensively washed with D-PBS prior to use.

Quantitative PCR using the real-time system. This study was performed using the SYBR Green I dye, which is a highly specific double-stranded DNA binding dye that allows the detection of a product accumulation during each PCR cycle (18). Following incubation for 2 h, 24 h or 48 h, the total cellular RNAs were extracted from the splenocytes using the TRIzol Reagent (Invitrogen Life Technologies, Carlabad, CA, USA). cDNA was synthesized from the total cellular RNAs by reverse transcription using AccuPower® RT PreMix (Bioneer, Cheongwon-Kun, Korea). For a real-time PCR assay, the 20 μl PCR mixture contained a 10 μl premix (QuantiTect SYBR Green PCR kit, Qiagen GmbH, Hilden, Germany), 2 μl of each primer (10pmol), 2 μl of cDNA (100ng) and 4 μl of dd-H_2O. After 1 min at 95˚C, the amplification conditions were 30 sec at 94˚C, 30 sec at 50˚C for LFA-1 or 56˚C for Neu, and 30 sec at 72˚C. The sequences for the primers used are as follows: LFA-1 (108bp): sense primer, CCGACAACTCCAACCAGTTT, and antisense primer, GCAATGCAACTTGCATT; Neu (112bp): sense primer, AAAACGGCACGGATGGAAAG, and antisense primer, GTGCCGTAGACGCTGATTTT. The amplification and detection were performed using the iCycler iQ™ real-time PCR

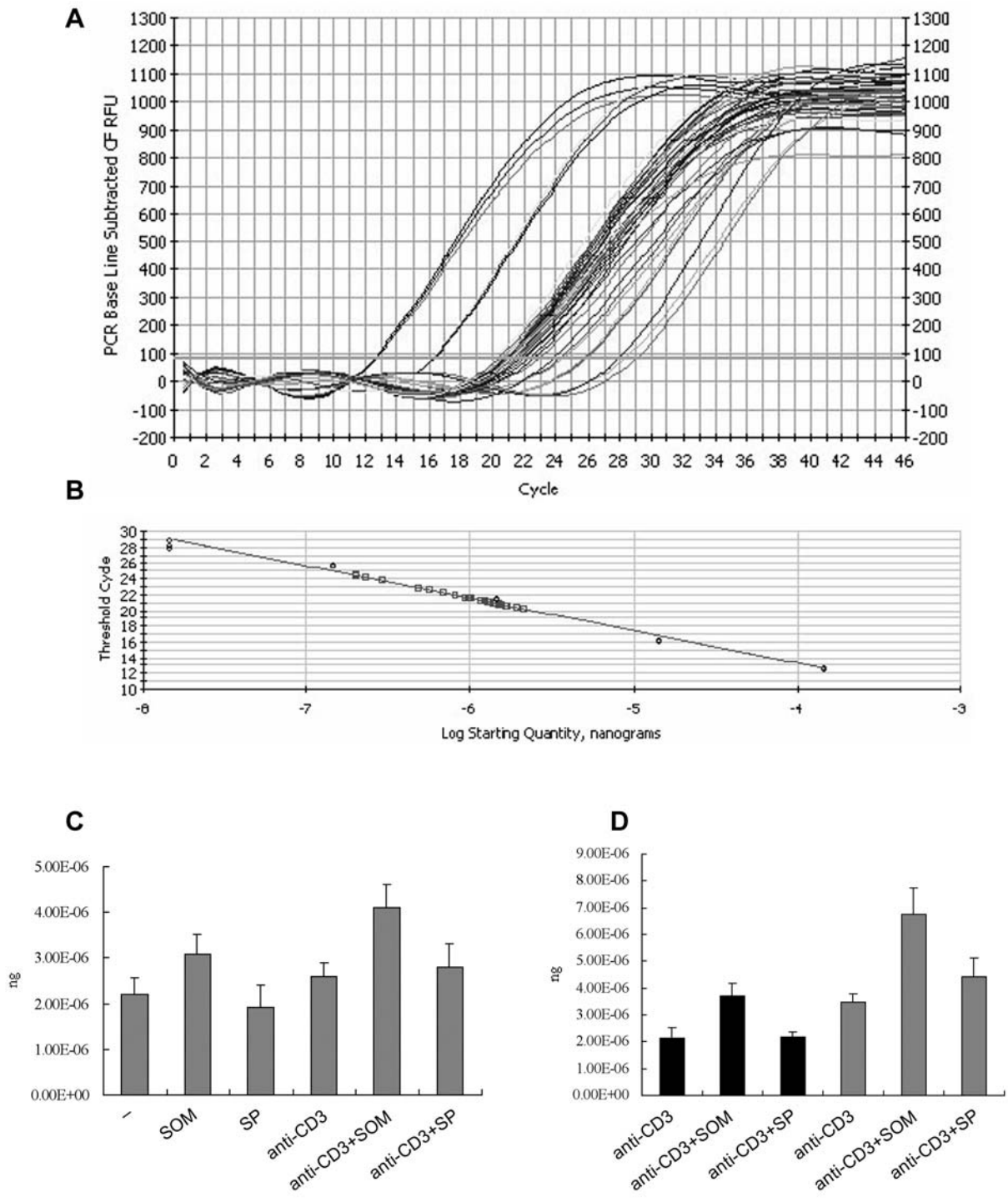

Figure 2. *Effect of SOM and SP on Neu mRNA expression on splenocytes. (A) Amplification plots of DNA derived from Neu plasmid standards or splenocytes. (B) Standard curve generated from the data plotted in A. The round dots (○) represent the data for standard samples and the squares (□) represent the data for the unknown samples (correlation coefficient=0.994). (C) The quantification in this graph was derived from A. The splenocytes were incubated for 24 h in complete medium or stimulated with SOM, SP, anti-CD3, anti-CD3+SOM or anti-CD3+SP. The bars show the means±SD of three separate experimental samples. (D) The quantification in this graph is derived from A. Splenocytes stimulated with anti-CD3, anti-CD3+SOM or anti-CD3+SP for 24 h (■) or 48 h (■). The bars show the means±SD of three separate experimental samples.*

detection system (Bio-Rad, San Diego, CA, USA). The number of targets in unknown samples was quantified by measuring the threshold cycle, Ct, using the standard curve. Each PCR product showed a single band upon gel electrophoresis. The Student's *t*-test was used to compare the treatment effects. The differences were considered statistically significant at $p<0.05$.

LFA-1 and Neu cloning and plasmid preparation. The PCR products of LFA-1 and Neu, whose sequences were verified, were cloned using the pCR2.1-TOPO vector and One-Shot *E. coli* according to the manufacturer's instructions. X-gal in the LB agar plates was used to identify the positive colonies. The positive colonies were further verified, using LFA-1- and Neu-specific PCR. The LFA-1 and Neu plasmids were then extracted from the *E. coli* using a plasmid mini purification kit according to the manufacturer's guidelines. The DNA concentration of the plasmid was determined using UV spectrophotometry. Subsequently, the copy number concentration of the plasmid (copy numbers per ml) was calculated using the known molecular weight of the plasmid plus the insert.

Results

To first determine whether Neu affects LFA-1 mRNA expression, the spleen cells were either nonstimulated or stimulated with either anti-CD3, Neu or anti-CD3+Neu, and assayed by real-time PCR. Figure 1A shows the increase in the fluorescence intensity with the increasing number of cycles and Figure 1B shows the resulting standard curve. As shown in Figure 1C, anti-CD3 significantly induced LFA-1 mRNA expression when compared to the non-stimulated group ($p<0.05$). However, anti-CD3+Neu after incubation for 2 h significantly inhibited LFA-1 mRNA expression when compared to the anti-CD3-stimulated group ($p<0.05$).

Next, SOM or SP were investigated to determine if they might affect the Neu mRNA expression level in cultured splenocytes. Previous studies have shown that a 48-h period of time after activation *in vitro* is required to induce the Neu-1 sialidase activities in conventional T cells, in which the maximum sialidase activity was detected at 72 h (16, 19). Based on these reports, the splenocytes were incubated with anti-CD3, anti-CD3+SOM, anti-CD3+SP or without stimulation for either 24 or 48 h to observe the changes in the Neu mRNA levels. As shown in Figure 2A, 2B and 2C, the anti-CD3+SOM after 24 h of incubation induced Neu mRNA expression markedly ($p<0.05$) when compared to the anti-CD3-treated group. However, the Neu mRNA expression level in the anti-CD3+SP group did not exceed that of the anti-CD3-stimulated group. The next experiments were designed to determine the effect of SOM or SP on the modulation of Neu mRNA expression by the splenocytes with the culture time. As shown in Figure 2D, the Neu mRNA expression level in the anti-CD3+SOM-treated group after incubation for 48 h was further enhanced beyond that of the group incubated for 24 h ($p=0.0172$, $p=0.0447$, respectively).

Discussion

Integrin, particularly LFA-1, is essential for most adhesion-dependent lymphocyte functions, including antigen- and APC-induced helper T cell stimulation, the CTL-mediated killing of target cells and lymphocyte adhesion to the endothelium (20). Pretreatment with anti-LFA-1 caused a decrease in the number of both migrated and bound T cells, approximately 50% and 70%, respectively (21). These results suggest that LFA-1 is involved in the transendothelial migration of the T cells by mediating their binding to the endothelium.

Gangliosides are sialic acid-containing glycosphingolipids, which are present in the plasma membrane of all eukaryotic cells. They are involved in several biological events, such as proliferation, differentiation, signal transduction and modulation of the immune responses (22-26). Recently Homma *et al.* (21) reported that disialoganglioside 6C2/GD3 molecules on the surface of T cells are crucial for transendothelial migration, which allows the T cells to extravagate and accumulate in inflammation sites. The degree of sialylation possessed by particular glycoconjugate species can vary greatly and is regulated through the combined enzymatic activities of the cell-associated sialotransferases and the cell-associated sialidases (6). Neu-1 sialidase has been reported to play a major role in determining the sialylation levels of some cell-associated and secreted sialoconjugates (5). Neu-1 sialidase, a glycosidase that resides in the plasma membrane of activated T cells, is encoded by the Neu-1 gene, which is located in the class III region of the murine MHC. The T cell expression of Neu-1 sialidase can be induced after cell activation (reviewed in 27).

A previous report did not determine the relationships between the gangliosides and the expression and function of the adhesion molecules, and the molecular mechanism through which the 6C2/GD3 molecule participates in the transendothelial migration (21). However, because the removal of sialic acid residues from GD3 with neuraminidase resulted in a loss of the transendothelial migratory capacity of the T cells (21), and T lymphocytes have higher total sialic acid and neuraminidase susceptible sialic acid levels on their surface than B lymphocytes (6), it is possible that the gangliosides play a role in T cell adhesion.

The nervous and immune systems are involved in bi-directional communication through soluble products such as neuropeptides and cytokines (28). Immune cells could be influenced by neuropeptides derived locally from the nerve fibers at local sites of infection, and/or be affected by their endogenously-produced peptides such as SOM and SP. Previous studies have shown that SP functions as an early signal, and SOM functions later in the inflammatory process as a normal feedback mechanism that regulates the degree of inflammation in tissues (17). In addition, it was reported that SOM decreases the adhesion of splenocytes to the

ICAM-1-coated plate by down-regulating the LFA-1 expression level (15). Overall, this study showed, using a real-time quantitative PCR assay, that Neu treatment down-regulates the LFA-1 expression level in splenocytes, and that anti-CD3+SOM treatment for 24 h and 48 h up-regulates the Neu expression level in splenocytes. Taken together with the previous results, these results suggest that prolonged exposure of splenocytes to SOM may induce Neu activity, which then leads to a decrease in the LFA-1 expression level.

Acknowledgements

This work was supported by a grant from the Korean Research Foundation (KRF-2001-041-G00079).

References

1 Khew-Goodall Y Jr, Gamble JR and Vadas MA: Regulation of adhesion and adhesion molecules in endothelium by transforming growth factor-β. Curr Top Microbiol Immunol *184*: 187-198, 1993.
2 Guerin V, Bene MC, Amiel C, Hartemann P, Leclere J and Faure G: Decreased lymphocyte function-associated antigen-1 molecule expression on peripheral blood lymphocytes from patients with Graves' disease. J Clin Endocrinol Metab *69*: 648-653, 1989.
3 Katagiri K, Kinashi T, Irie S and Katagiri T: Differential regulation of leukocyte function-associated antigen-1/intercellular adhesion molecules-1-dependent adhesion and aggregation in HL-60 cells. Blood *87*: 4276-4285, 1996.
4 Springer T: Adhesion receptors of the immune system. Nature *346*: 425-434, 1990.
5 Potier MD, Yan LS and Womack JE: Neuraminidase deficiency in the mouse. FEBS Lett *108*: 345-348, 1979.
6 Steimer F and Despont JP: Cell surface changes in human normal and abnormal lymphoid cells detected by sialic acid content and sialyltransferase activity. Leuk Res *4*: 265-269 1980.
7 Berczi I, Chalmers IM, Nagy E and Warrington RJ: The immune effects of neuropeptides. Baillières Clin Rheumatol *10*: 227-257, 1996.
8 Berman AS, Chancellor-Freeland C, Zhu G and Black PH: Substance P primes murine peritoneal macrophages for an augmented proinflammatory cytokine response to lipopolysaccharide. Neuroimmunomodulation *3*: 141-149, 1996.
9 De Giorgio R, Tazzari PL, Barbara G, Stanghellini V and Corinaldesi R: Detection of substance P immunoreactivity in human peripheral leukocytes. Neuroimmunol *82*: 175-181, 1994.
10 Karalis K, Mastorakos G, Chrousos GP and Tolis G: Somatostatin analogues suppress the inflammatory reaction *in vivo*. J Clin Invest *93*: 2000-2006, 1994.
11 Karalis K, Mastorakos G, Sano H, Wilder RL and Chrousos GP: Somatostatin may participate in the anti-inflammatory actions of glucocorticoids. Endocrinology *136*: 4133-4138, 1995.
12 Madden KS and Felten DL: Experimental basis for neural-immune interactions. Physiol Rev *75*: 77-106, 1995.
13 DeRose V, Robbins RA, Snide RM, Spurzem JR, Thiele GM, Rennard SI and Rubinstein I: Substance P increases neurophil adhesion to bronchial epithelial cells. J Immunol *152*: 1339-1346, 1994.
14 Vishwanath R and Mukherjee R: Substance P promotes lymphocyte-endothelial cell adhesion preferentially *via* LFA-1/ICAM-1 interactions. J Neuroimmunol *71*: 163-171, 1996.
15 Kang BN, Kim HJ, Jeong KS, Park SJ, Kim SH, Kim SR, Kim TH and Ryu SY: Regulation of LFA-1-mediated adhesion by somatostatin and substance P in mice spleen cells. Neuroimmunomodulation *11*: 84-92, 2004.
16 Chen X-P, Enioutina EY and Daynes RA: The control of IL-4 gene expression in activated murine T lymphocytes. A novel role for neu-1 sialidase. J Immunol *158*: 3070-3080, 1997.
17 Ryu SY, Jeong KS, Yoon WK, Park SJ, Kang BN, Kim SH, Park BK and Cho SW: Somatostatin and substance P induced *in vivo* by lipopolysaccharide and in peritoneal macrophages stimulated with lipopolysaccharide or interferon-gamma have differential effects on murine cytokine production. Neuroimmunomodulation *8*: 25-30, 2000.
18 Simpson DA, Feeney S, Boyle C and Stitt AW: Retinal VEGF mRNA measured by SYBR green I fluorescence: A versatile approach to quantitative PCR. Mol Vision *6*: 178-183, 2000.
19 Landolfi NF, Leone J, Womack JE and Cook RG: Activation of T lymphocyte results in an increase in H-2 encoded neuraminidase. Immunogenetics *22*: 159-167, 1985.
20 Abbas AK, Lichtmann AH and Pober JS: Chapter 6. Antigen receptors and accessory molecules of T lymphocytes. *In*: Cellular and Molecular Immunology. Philadelphia, London, New York, St. Louis, Sydney, Toronto, W.B.Saunders Co., 2000; pp.102-121
21 Homma T, Hosono O, Iwata S, Ando S, Sasaki K, Nishi T, Kawasaki H, Tanaka H and Morimoto C: Recognition of cell surface GD3 by monoclonal antibody anti-6C2 in rheumatoid arthritis synovial fluid. Expression on human T cells with transendothelial migratory activity. Arthritis Rheum *44*: 296-306, 2001.
22 Hakomori S, Yamamura S and Handa AK: Signal transduction through glyco(sphingo)lipid: introduction and recent studies on glyco(sphingo)lipid-enriched microdomains. Ann NY Acad Sci *845*: 1-10, 1998.
23 Kleinman HK, Martin GR and Fishman PH: Ganglioside inhibition of fibronectin-mediated cell adhesion to collagen. Proc Natl Acad Sci USA *76*: 3367-3371, 1979.
24 Nagai Y and Iwamori M: Chapter 6. Cellular biology of gangliosides. *In*: Biology of Sialic Acid (Resenberg A, ed). New York, Plenum Press, 1995, pp.197-240.
25 Nohara K, Suzuki M, Inagaki F, Sano T and Kaya K: A novel disialoganglioside in rat spleen lymphocytes. J Biol Chem *267*: 14982-14986, 1992.
26 Taira S and Nariuchi H: Possible role of neuraminidase in activated T cells in the recognition of allogeneic Ia. J Immunol *141*: 440-446, 1988.
27 Chen X-P, Ding X and Daynes RA: Ganglioside control over IL-4 priming and cytokine production in activated T cells. Cytokine *12*: 972-985, 2000.
28 Weigent DA and Blalock JE: Associations between the neuroendocrine and immune systems. J Leukoc Biol *57*: 137-150, 1995.

Received March 1, 2004
Accepted May 25, 2004