

Enzyme Immunoassay of Somatostatin (SS)-like Immunoreactive Substance in Bovine Milk

Masaharu TAKEYAMA,^{*,a} Naoko YANAGA,^a Koji YARIMIZU,^b Junko ONO,^b Ryosaburo TAKAKI,^b Nobutaka FUJII^c and Haruaki YAJIMA^c

Department of Hospital Pharmacy,^a and First Division, Department of Medicine,^b Medical College of Oita, 1 Idaigaoka-1, Hasama-cho, Oita-gun, Oita 879-56, Japan and Faculty of Pharmaceutical Sciences, Kyoto University,^c Sakyo-ku, Kyoto 606, Japan. Received August 11, 1989

A sensitive and specific enzyme immunoassay (EIA) for somatostatin (SS)-like immunoreactivity (SS-LI) was developed with the use of β -D-galactosidase labeled antigen. The minimum amount of SS-like immunoreactive substance (SS-IS) detectable by this method was 1.0 fmol/well (25 pmol/l). The level of SS-IS in bovine foremilk was about 20 pmol/l, and the level was unchanged after delivery. On the other hand, the levels of gastrin releasing peptide (GRP)-IS and vasoactive intestinal polypeptide (VIP)-IS in bovine foremilk were very high, but fell during 1 week after delivery to about 10% of those in foremilk.

Keywords somatostatin (SS)-like immunoreactivity; bovine milk; SS-linked- β -D-galactosidase; highly sensitive EIA; anti-rabbit IgG coated immuno-plate

Somatostatin (SS) is a tetradecapeptide that was isolated from ovine hypothalamus by Brazeau *et al.*^{1,2)} and identified on the basis of its action as an inhibitor of growth hormone (GH) release. SS inhibits the secretion of other polypeptides and hormones, including GH, thyrotropin, insulin, glucagon, gastrin, secretin, motilin and glucose-dependent insulinotropic peptide (GIP).³⁾ In the gastrointestinal tract, gastric acid and pepsin secretion, gastric emptying, duodenal motility, gallbladder contraction, pancreatic exocrine function, and the absorptions of glucose, triglyceride and amino acid are inhibited by SS.³⁾ SS-like immunoreactivity (SS-LI) has been shown to be widely distributed throughout the central nervous system, gastrointestinal tract and pancreas. SS is thought to function as a neurotransmitter, because a substance with SS-LI has been demonstrated in axons and nerve cell bodies of primary sensory neurons,⁴⁾ sympathetic neurons⁵⁾ and intrinsic neurons of the intestine⁶⁾ as well as in neurons in the central nervous system.⁷⁾

At present, sheep,^{1,2)} pig,⁸⁾ pigeon,⁹⁾ angler fish,¹⁰⁾ rat¹¹⁾ and human¹²⁾ SS have been demonstrated to be identical in structure to ovine SS, and an amino-terminal extended form of SS, SS-28, was reported¹³⁾ (Fig. 1). Radioimmunoassay (RIA) of SS has been developed by several authors.^{14,15)} However, in term of safety, sensitivity and easiness of handling, the existing methods are still less than satisfactory.

In 1985, Werner *et al.*¹⁶⁾ detected the SS-like immunoreactive substance (SS-IS) in milk by RIA. We wish to report that such SS-IS can be detected by a highly sensitive enzyme immunoassay (EIA), and also to compare the levels with those of vasoactive intestinal polypeptide (VIP)-IS and gastrin releasing peptide (GRP)-IS in bovine milk and foremilk.

Materials and Methods

Materials Bovine milk and foremilk were kindly supplied by Nakashibetsu Preparation Center, Mitsubishi Kasei Corp. Synthetic SS, substance-P (S-P), human atrial natriuretic peptide (ANP) and gastrin were purchased from Peptide Institute Inc. (Osaka, Japan). Synthetic porcine VIP, porcine secretin, human glucose-dependent insulinotropic peptide (GIP), peptide histidine isoleucine (PHI), human GRP, porcine brain natriuretic peptide (BNP), and neuropeptide Y (NPY)¹⁷⁻²³⁾ were used. Bovine serum albumin (BSA), polyoxyethylene sorbitan (Tween-20), *N*-(ϵ -maleimidocaproyloxy)succinimide (EMC-succinimide) and 4-methylumbelliferyl β -D-galactopyranoside (MUG) were purchased from Sigma Chemical Co. β -D-Galactosidase (β -Gal from *Escherichia coli*) and goat anti-rabbit immunoglobulin G (IgG) (TAGO 4120) were purchased from Boehringer Mannheim Corp. and TAGO Inc. (Burlingame, U.S.A.) respectively.

Antiserum to SS (RA-08-108) was purchased from Cambridge Res. Biochem. (Cambridge, England) and the lyophilized anti-SS-serum was reconstituted to 90 ml with assay buffer (0.05 M phosphate buffer, pH 7.4, containing 0.25% BSA and 250 KIU aprotinin). All other chemicals were of analytical reagent grade.

Preparation of Milk Extract Bovine milk and foremilk were defatted by centrifugation at 10000 $\times g$ at 4°C for 30 min. Casein and other proteins were coagulated by acidification to pH 4 with 4% acetic acid (AcOH) and removed by centrifugation. Defatted/decaseined milk samples were diluted fivefold with 4% AcOH (pH 4) and loaded on a reversed-phase C₁₈ cartridge (Sep-Pack, Waters Co., Inc., Milford, MA). After washing with 4% AcOH (10 ml), the SS was eluted with 70% acetonitrile in 0.5% AcOH (2 ml). Eluates were concentrated by spin-vacuum evaporation and were subjected to EIA. Recovery of SS by this extraction procedure was 96 \pm 7%.

Preparation of Enzyme-Labeled Antigen SS was conjugated onto β -Gal by using EMC-succinimide according to the method of Kitagawa *et al.*²⁴⁾ SS (0.49 mg) was dissolved in 0.05 M phosphate buffer pH 7.0 (1 ml), and an aliquot of tetrahydrofuran (50 μ l) containing EMC-succinimide (0.33 mg) was added. The mixture was stirred at 20°C for 30 min, then applied to a Sephadex G-25 column (1.5 \times 51 cm) pre-equilibrated with 0.05 M phosphate buffer, pH 7.0. The column was eluted with the same buffer. Individual fractions (1.8 ml each) that showed absorbance at 275 nm were collected. The pooled EMC-SS fractions (No. 39—41 in Fig. 2a) were combined with β -Gal (0.79 mg) by stirring at 20°C for 30 min. The β -Gal conjugate was applied to a Sephacryl S-300 column (1.5 \times

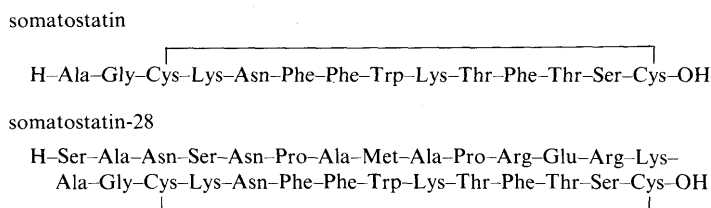


Fig. 1. Structure of Somatostatin

58 cm) pre-equilibrated with 0.05 M phosphate buffer pH 7.0, containing 1 mM MgCl_2 and was eluted with the same buffer. Individual fractions (1.8 ml each, No. 28–32) that showed absorbance at 275 nm were collected and stored at 4°C after addition of 0.2% BSA and 0.1% NaN_3 .

Preparation of Second-Antibody-Coated Immuno-Plate The washing buffer was 0.01 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween-20 and the coating buffer was 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl. Polystyrene plates (Nunc MicroWell Maxisorp F8 plate) were coated with the above coating buffer (100 μl /well) containing goat anti-rabbit IgG (10 $\mu\text{g}/\text{ml}$) at 4°C overnight under Parafilm. The plates were washed 4 times with the above washing buffer and stored at 4°C until use.

Assay Procedure for SS For assay, the above-mentioned assay buffer was used. A test tube containing 100 μl of anti-SS-serum, RA-08-108, and each sample (or standard, 100 μl) was incubated at 4°C for 24 h and then the diluted enzyme-labeled antigen (50 μl) was added. The test tube was further incubated at 4°C for 24 h. The antibody-antigen solution (100 μl) from each test tube was added to the second-antibody-coated immuno-plate. The plate was incubated at 4°C overnight, then washed 4 times with the washing buffer, and 0.1 mM MUG (200 μl) in substrate buffer (0.05 M phosphate buffer, pH 7.0, containing 1 mM MgCl_2) was added to each well. The plate was again incubated at 37°C for 3 h. The resulting fluorescence intensities (emission wavelength 450 nm, excitation wavelength 360 nm) of each well were measured with a Corona MTP-100F microplate reader.

Gel-Filtration of Bovine Foremilk Extract A defatted bovine foremilk sample (2.0 ml) was shaken with 0.1% trifluoroacetic acid (TFA) in methanol (8 ml), then the solution was clarified by centrifugation (10000 $\times g$ for 30 min at 4°C), and the supernatant was concentrated to

dryness under vacuum. The residue was dissolved in 0.1 N AcOH and the solution was applied to a Sephadex G-25 column (0.8 \times 65 cm) pre-equilibrated with 0.1 N AcOH. The SS was eluted with the same solvent. The fractions (1.8 ml each) were lyophilized and submitted to EIA.

EIA for GRP and VIP EIAs for GRP and VIP were performed as previously described.^{25,26} Assay procedures of EIA for GRP and VIP were almost the same as that of SS.

Results and Discussion

Standard Curve A typical standard curve for EIA of SS is shown in Fig. 4. A linear displacement of enzyme-labeled SS by unlabeled SS was obtained, when the data were plotted as a semilogarithmic function, from 20 to 1000 pmol/l of SS (Fig. 4). The minimum amount of SS detectable by the present EIA system was 25 pmol/l (1.0 fmol/

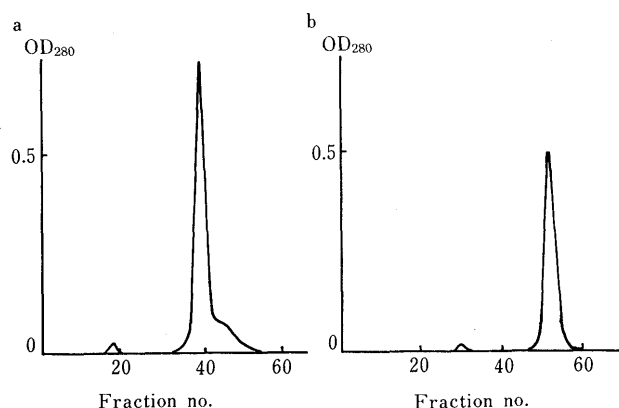


Fig. 2. Purification of SS-Linked- β -Gal

a. Gel-filtration of EMC-SS on Sephadex G-25: column, 1.5 \times 51 cm, fraction, 1.8 ml; elution, 0.05 M phosphate buffer, pH 7.0.
b. Gel-filtration of SS-linked β -Gal on Sephacryl S-300: column, 1.5 \times 57 cm; fraction, 1.8 ml; elution, 0.05 M phosphate buffer, pH 7.0 containing MgCl_2 (0.1 mM).

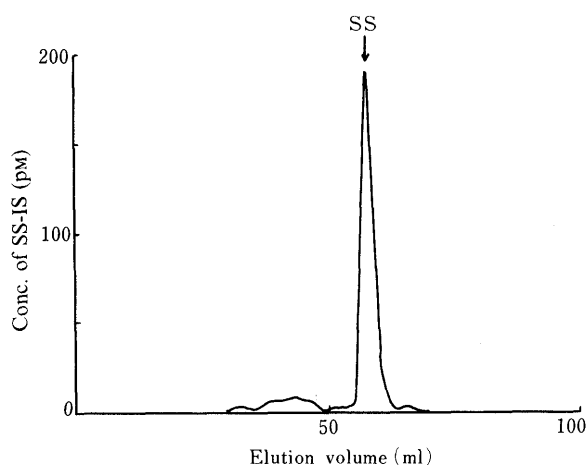


Fig. 3. Gel-Filtration of Bovine Foremilk Extract on Sephadex G-25

Fractions were lyophilized and their content of SS-IS was measured. Synthetic SS was run in a separate chromatography under the same conditions and its elution volume is indicated by the arrow: column, 0.8 \times 66 cm; elution, 0.6% AcOH.

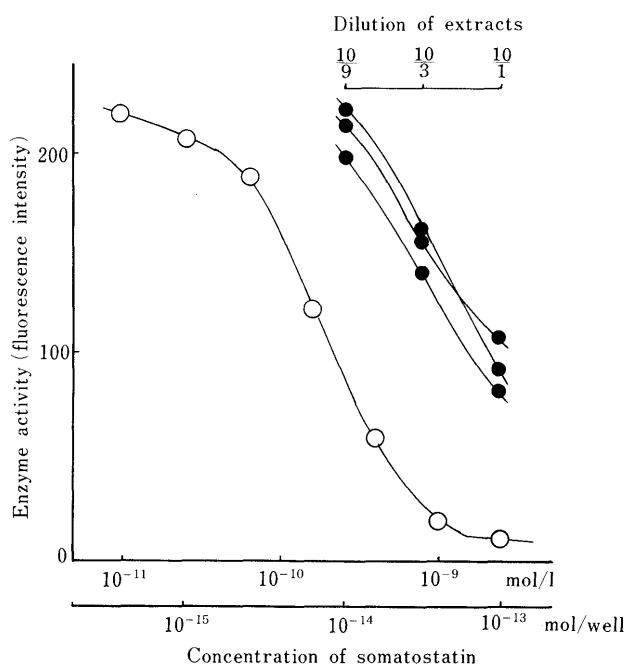


Fig. 4. Standard Curve of Synthetic SS and Dilution Curves of Milk Extracts Obtained with Antiserum RA-08-108 by EIA

Synthetic SS (○), milk extracts: (●).

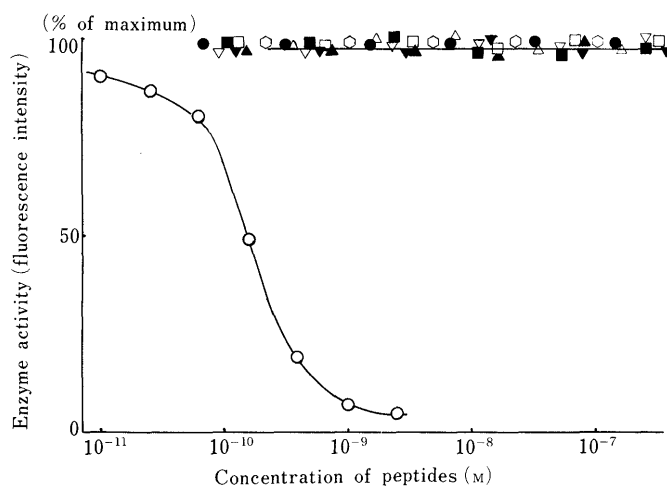


Fig. 5. Inhibition Curves of Various Peptides in EIA by Competition between SS-Linked β -Gal and Various Peptides toward Antiserum RA-08-108

SS (○), VIP (▲), GRP (△), GIP (□), PHI (●), NPY (▼), hANP (▽), BNP (■), S-P (○).

TABLE I. Levels of SS-, VIP- and GRP-like Immunoreactive Substance in Bovine Milk

	SS-LI (pM)	VIP-LI (pM)	GRP-LI (nM)
Foremilk	23 ± 8	139 ± 32	45 ± 25
Normal milk	19 ± 6	16 ± 9	1.4 ± 1.0

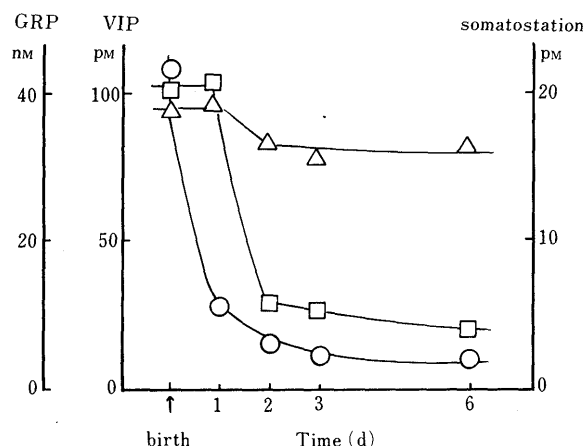


Fig. 6. Time Course of SS-IS, VIP-IS, and GRP-IS Levels in Bovine Milk Samples after Delivery

SS (Δ), VIP (○), GRP (□).

well). An intraassay variation of 6% and interassay variation of 11% were obtained at 160 pmol/l. As also shown in Fig. 5, serial dilution of bovine milk extracts showed a parallel displacement of enzyme-labeled SS to synthetic SS in EIA.

Specificity of the Antiserum (RA-08-108) Examined by EIA Immuno-specificity of the antiserum (RA-08-108) was examined by EIA using SS-linked-β-Gal. As shown in Fig. 5, SS fully inhibited the binding of SS-linked-β-Gal with the anti-SS-body. VIP, BNP, ANP, GIP, PHI, S-P and NPY hardly inhibited the binding of SS-linked-β-Gal with the anti-SS-antibody.

Measurements of SS-like Immunoreactivity in Milk Samples by EIA Concentrations of SS-IS in bovine milk and foremilk were measured by EIA. The level of SS-IS in bovine milk and foremilk was between 10 and 30 pmol/l and was unchanged after delivery (Fig. 6). The levels of GRP-IS and VIP-IS in foremilk were high but dropped within 2 d after delivery.

Gel-Filtration of Bovine Foremilk Extract on Sephadex G-25 Next, we examined the molecular variants of SS-IS in a bovine foremilk sample. Fractionation of bovine foremilk extract on Sephadex G-25 revealed that the main SS-IS was eluted a single peak at the same elution volume as synthetic SS (Fig. 3).

Using β-galactosidase-linked SS as a marker antigen and anti-rabbit IgG coated immuno-plate, the EIA for SS was established, with a detection limit as low as 25 pmol/l (1 fmol/well). Using this EIA system, SS-LI in bovine milk were examined and it was found that the level of SS-IS in bovine foremilk was not significantly different from the mean concentration in normal bovine milk samples. The molecular size of SS-IS in bovine foremilk was the same as that of synthetic SS.

Milk is a rich nutrient and is also known to contain

peptides^{16,27,28)} and steroid hormones²⁷⁾ in addition to a growth factor²⁹⁾ and enzymes.³⁰⁾ Moreover, the peptide hormones and the growth factors retain their biological activities in milk^{27,28)} and when ingested by the neonate, appear intact in plasma.³¹⁾ The levels of SS-IS present in bovine milk were constant, while the levels of VIP-IS and GRP-IS in bovine foremilk were very high, especially GRP-IS, but fell within 3 d after delivery to 10% of that foremilk. GRP has been shown to be mitogenic for bronchial mucosal cells³²⁾ and to have a trophic effect on the stomach and pancreas.³³⁾ VIP has a wide range of actions in the cardiovascular system, respiratory system, digestive system, metabolism, endocrine function, central nervous system³⁴⁾ and immunosystem.³⁵⁾ SS has a predominantly down-regulatory systemic role in the digestive tract, genitourinary tract, endocrine function, central nervous system and immunosystem. Thus, SS, GRP and VIP may have some physiological regulatory functions in bovine fetus and newborn calves.

References

- 1) P. Brazeau, W. Vale, R. Burgus, N. Ling, M. Butcher, J. Rivier and R. Guillemin, *Science*, **179**, 77 (1973).
- 2) N. Ling, R. Burgus, J. Rivier, W. Vale and P. Brazeau, *Biochem. Biophys. Res. Commun.*, **50**, 127 (1973).
- 3) W. Vale, C. Rivier and M. Brown, *Ann. Rev. Physiol.*, **39**, 473 (1977).
- 4) A. Tessler, B. T. Himes, J. Gruber-Bollinger and S. Reichlin, *Brain Res.*, **370**, 232 (1986).
- 5) T. Hökfelt, L. G. Elfvén, R. Elde, M. Schultzberg, M. Goldstein and R. Luft, *Proc. Natl. Acad. Sci. U.S.A.*, **74**, 3587 (1977).
- 6) J. R. Keast, J. B. Furness and M. Costa, *Cell Tissue Res.*, **237**, 299 (1984).
- 7) Y. C. Patel and S. Reichlin, *Endocrinology*, **102**, 523 (1978).
- 8) A. V. Schally, A. Dupont, A. Arimura, T. W. Redding, N. Nishi, G. L. Linthicum and D. H. Schlesinger, *Biochemistry*, **15**, 509 (1976).
- 9) J. Spiess, J. E. Rivier, J. A. Rodkey, C. D. Bennett and W. Vale, *Proc. Natl. Acad. Sci. U.S.A.*, **76**, 2974 (1979).
- 10) B. D. Noe, J. Spiess, J. E. Rivier and W. Vale, *Endocrinology*, **105**, 1410 (1979).
- 11) R. Benoit, P. Böhlen, P. Brazeau, N. Ling and R. Guillemin, *Endocrinology*, **107**, 2127 (1980).
- 12) L. P. Shen, R. L. Pictet and W. J. Rutter, *Proc. Natl. Acad. Sci. U.S.A.*, **79**, 4575 (1982).
- 13) L. Pradayrol, H. Jörnvall, V. Mutt and A. Ribet, *FEBS Lett.*, **109**, 55 (1980).
- 14) A. Arimura, H. Sato, D. H. Coy and A. V. Schally, *Proc. Soc. Exp. Biol. Med.*, **148**, 784 (1975).
- 15) T. Chiba, Y. Seino, Y. Goto, S. Kadowaki, T. Taminato, H. Abe, Y. Kato, S. Matsukura, M. Nozawa and H. Imura, *Biochem. Biophys. Res. Commun.*, **82**, 731 (1978).
- 16) H. Werner, T. Amarant, R. P. Millar, M. Fridkin and Y. Koch, *Eur. J. Biochem.*, **148**, 353 (1985).
- 17) M. Takeyama, K. Koyama, K. Inoue, T. Kawano, H. Adachi, T. Tobe and H. Yajima, *Chem. Pharm. Bull.*, **28**, 1873 (1980).
- 18) S. Kiyama, K. Kitagawa, T. Akita, W. Y. Chey, A. Ayalp, A. Otsuki, S. Funakoshi, N. Fujii and H. Yajima, *Chem. Pharm. Bull.*, **33**, 3205 (1985).
- 19) H. Yajima, N. Fujii, K. Akaji, M. Sakurai, M. Nomizu, K. Mizuta, M. Aono, M. Moriga, K. Inoue, R. Hosotani and T. Tobe, *Chem. Pharm. Bull.*, **33**, 3578 (1985).
- 20) N. Fujii, W. Lee, H. Yajima, M. Moriga and K. Mizuta, *Chem. Pharm. Bull.*, **31**, 3503 (1983).
- 21) S. Kuno, K. Akaji, O. Ikemura, M. Moriga, M. Aono, K. Mizuta, A. Takagi and H. Yajima, *Chem. Pharm. Bull.*, **34**, 2462 (1986).
- 22) A. Otaka, H. Morimoto, N. Fujii and H. Yajima, *Pept. Chem.*, **1988**, 207.
- 23) S. Kiyama, Y. Ooi, K. Kitagawa, T. Nakamura, T. Akita, M. Kogire, R. Hosotani, K. Inoue, T. Tobe and H. Yajima, *Int. J. Pept. Protein Res.*, **29**, 533 (1987).
- 24) T. Kitagawa, T. Shimozono, T. Aikawa, T. Yoshida and H.

- Nishimura, *Chem. Pharm. Bull.*, **29**, 1130 (1981).
- 25) M. Takeyama, K. Kondo, Y. Hayashi and H. Yajima, *Int. J. Pept. Protein Res.*, **34**, 70 (1989).
- 26) M. Takeyama, K. Wakayama, F. Takayama, K. Kondo, N. Fujii and H. Yajima, *Chem. Pharm. Bull.*, in press.
- 27) O. Koldovsky, *Life Sci.*, **26**, 1833 (1980).
- 28) R. R. Gala, I. A. Forsyth and A. Turvey, *Life Sci.*, **26**, 987 (1980).
- 29) G. Carpenter, *Science*, **210**, 198 (1980).
- 30) Z. Moldoveanu, J. Tenovuo, J. Mestecky and K. M. Pruitt, *Biochem. Biophys. Acta*, **718**, 103 (1982).
- 31) N. S. Whitworth and C. E. Grosvenor, *J. Endocrinol.*, **79**, 191 (1978).
- 32) J. C. Willey, J. F. Lechner and C. C. Harris, *Exp. Cell Res.*, **153**, 245 (1984).
- 33) T. Lehy, F. Puccio, J. Chariot and D. Labeille, *Gastroenterology*, **90**, 1942 (1986).
- 34) S. I. Said, "Gastrointestinal Hormones," ed. by George B. Jerzy Glass, Raven Press, New York, 1980, pp. 245—273.
- 35) A. M. Stanisz, D. Befus and J. Bienenstock, *J. Immunol.*, **136**, 152 (1986).