REFERENCES

1. Bertilsson L, Baillie TA and Reviriego J, Factors influencing the metabolism of diazepam. *Pharmacol Ther* **45**: 85–91, 1990.
2. Chung M, Hilbert JM, Gural RP, Radwanski E, Symchowicz S and Zampaglione N, Multiple-dose halazepam kinetics. *Clin Pharmacol Ther* **35**: 838–842, 1984.
3. Comi V, Fossati A and Gervasi GB, Specific metabolic pathway *in vitro* of pinazepam and diazepam by rat liver microsomal enzymes of different animal species. *Il Farmaco — Ed Sc* **32**: 278–285, 1977.
4. Viau J-P, Epps JE and Di Carlo FJ, Prazepam metabolism *in vitro*. *Biochem Pharmacol* **21**: 563–569, 1972.
5. Lu X-L, Guengerich FP and Yang SK, Stereoselective metabolism of prazepam and halazepam by human liver microsomes. *Drug Metab Dispos* **19**: 637–642, 1991.
6. Hooper WD, Watt JA, McKinnon GE and Reilly PEB, Metabolism of diazepam and related benzodiazepines by human liver microsomes. *Eur J Drug Metab Pharmacokinet*, in press.
7. Reilly PEB, Thompson DA, Mason SR and Hooper WD, Cytochrome P450IIIA enzymes in rat liver microsomes: involvement in C_3-hydroxylation of diazepam and nordazepam but not N-dealkylation of diazepam and temazepam. *Mol Pharmacol* **37**: 767–774, 1990.
8. Reilly PEB, Mason SR and Hooper WD, Effects of ethinylestradiol and testosterone implants on hepatic microsomal cytochrome P450 monooxygenases of birth gonadectomized male and female Dark Agouti rats. *J Steroid Biochem Mol Biol* **39**: 741–749, 1991.
9. Trebbi A, Gervasi GB and Comi V, Determination of pinazepam and its metabolites in serum, urine and brain by gas-liquid chromatography and mass spectrometry. *J Chromatogr* **110**: 309–319, 1975.
10. Molteni L, Tenconi F and Tagliabue R, 1-Propargil-1,4-benzodiazepine. *South African Patent* No.747695, 1974.
11. Guengerich FP, Microsomal enzymes involved in toxicology—analysis and separation. In: *Principles and Methods in Toxicology* (Ed. Hayes AW), pp. 609–634. Raven Press, New York, 1982.
12. Estabrook WR, Peterson J, Baron J and Hildebrandt A, The spectrophotometric measurement of turbid suspensions of cytochromes associated with drug metabolism. In: *Methods in Pharmacology* (Ed. Chignell CF), pp. 303–350. Appleton Century Crofts, Meredith Corp., New York, 1972.
13. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
14. Haky JE and Young AM, Evaluation of a simple HPLC correlation method for the estimation of the octanol–water partition coefficients of organic compounds. *J Liquid Chromatogr* **7**: 675–689, 1984.
15. Pietrogrande MC, Bighi C, Borea PA, Barbaro AM Guerra MC and Biagi GL, Relationship between log k' values of benzodiazepines and composition of the mobile phase. *J Liquid Chromatogr* **8**: 1711–1729, 1985.
16. Wala EP, Sloan JW, Martin WR and Pruitt T, Pharmacokinetics and metabolism of halazepam in naive and dependent dogs. *Pharmacol Biochem Behav* **38**: 561–567, 1991.
17. Marcucci F, Airoldi L, Zavattini G and Mussini E, Metabolism of pinazepam by rat liver microsomes. *Eur J Drug Metab Pharmacokinet* **6**: 109–114, 1981.
18. Inaba T, Tait A, Nakano M, Mahon WA and Kalow W, Metabolism of diazepam *in vitro* by human liver: independent variability of N-demethylation and C_3-hydroxylation. *Drug Metab Dispos* **16**: 605–608, 1988.
19. Faibushevich AA, Guliaeva LF, Grishanova AI, Mishin VM and Liakhovich VV, Diazepam metabolism by multiple forms of cytochrome P450 in rat liver microsomes. *Biokhimiia* **55**: 1210–1215, 1990.
20. Schwartz MA, Koechlin BA, Postma E, Palmer S and Krol G, Metabolism of diazepam in rat, dog, and man. *J Pharmacol Exp Ther* **149**: 423–435, 1965.

Biochemical Pharmacology, Vol. 43, No. 6, pp. 1380–1383, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00
Pergamon Press plc

Effects of deoxyspergualin on dipeptidyl peptidase-II and -IV in the spleen of BXSB mice and MRL/*lpr* mice during the development of the lupus erythematosus-like syndrome

(*Received* 23 *April* 1991; *accepted* 10 *December* 1991)

Several murine strains such as (NZB × NZW)F_1 mice, MRL/Mp-*lpr*/*lpr* (MRL/*lpr*) mice or male BXSB mice are known as animal models of systemic lupus erythematosus (SLE*) [1, 2]. The etiopathogenesis of SLE in these strains of mice has been a subject of extensive study. Significant changes in some peptidases in tissues or serum have been demonstrated in these animal models of SLE. In our previous studies [3, 4], we found increased activity of DPP II (EC 3.4.14.2) and decreased activity of DPP IV (EC 3.4.14.5) in the spleen of NZB mice, male BXSB mice and male MRL/*lpr* mice with lupus erythematosus-like syndrome as compared with the activities of male MRL/++ mice, female BXSB mice and female BALB/c mice, as controls without SLE. As a result, an increase in the ratio of DPP II/DPP IV activities in the spleen of these lupus mice was noted as compared to the activities of control mice [3, 4]. As in lupus mice, serum DPP II activity in patients with rheumatoid arthritis or SLE was increased

* Abbreviations: SLE, systemic lupus erythematous; DPP, dipeptidyl peptidase; DSP, 15-deoxyspergualin (1-amino-19-guanidino -11- hydroxy-4,9,12-triazanadecane -10,13 - dione); Lys - Ala - MCA, 7 - (Lys - Ala) - 4 - methylcoumarinamide; Gly-Pro-MCA, 7-(Gly-Pro) -4-methylcoumarinamide.

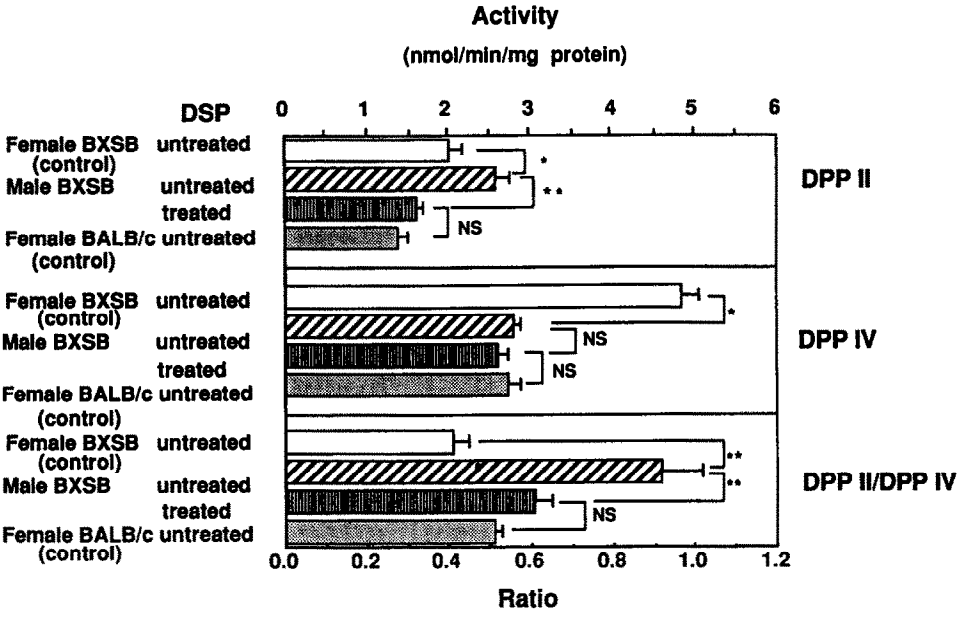

Fig. 1. Effect of DSP on DPP activities in the spleen of BXSB mice with SLE-like syndrome. Means ± SD (N = 5); *P < 0.005; **P < 0.001; NS, not significant. DSP-untreated, female BXSB mice and BALB/c mice did not show SLE syndrome.

and that of DPP IV was decreased resulting in the increased DPP II/DPP IV ratio [3].

Immunological abnormalities were observed in male BXSB mice and male MRL/*lpr* mice at the onset of lupus nephritis at 13 weeks of age [5]. In contrast, no immunological abnormalities were found in control female BXSB mice and male MRL/++ mice at even 20 weeks of age. The changes in peptidases paralleled with these immunological abnormalities [4]. Thus, there is a possibility that these peptidases may be linked to the development of immunological abnormalities in SLE or rheumatoid arthritis.

This study was undertaken to confirm our hypothesis that an increase in the ratio of DPP II/DPP IV activities can be a biochemical index of lupus erythematosus-like syndrome. A newly developed therapeutic agent, DSP, is one of the analogues of spergualin which was isolated by Umezawa *et al.* [6] and shown to have immunosuppressive effects as well as anti-tumor activities against murine leukemia L1210, EL4 lymphoma, etc. [7]. In this study, we examined the prophylactic and therapeutic effects of DSP on the changes of activities of DPPs in male BXSB mice and male MRL/*lpr* mice.

Materials and Methods

DSP was supplied by Takara Shuzo Co. Ltd. The drug was dissolved in physiological saline shortly before use and injected intraperitoneally into mice. Male BXSB mice and male MRL/*lpr* mice were used. The mice were originally obtained from the pedigree stock of the Jackson Laboratory (Bar Harbor, ME, U.S.A.) and maintained at our animal facility under sterile conditions. Administration of DSP was started from 13 weeks of age. DSP was administered every day for 2 weeks and again administered for 2 weeks after a 1 week interval. Mice were killed by decapitation at 20 weeks of age, and the spleens were removed and stored at −80° until assay. The organs were homogenized in 0.25 mol/L sucrose solution, the homogenates were centrifuged at 3000 *g* for 20 min, and enzymatic activities in the supernatant solutions were analysed. 7-Amino-4-methylcoumarin, Lys-Ala-MCA and Gly-Pro-MCA were obtained from the Peptide Institute, Protein Research Foundation (Minoh, Osaka, Japan). Universal buffer (0.2 mol/L sodium borate and 0.05 mol citrate/L adjusted to pH 5.3 with 0.1 mol/L sodium phosphate buffer) was used for the DPP II assay, and Gly-NaOH buffer (0.15 mol/L each, pH 8.7) for DPP IV. Enzyme activities were assayed fluorometrically by measuring the enzymatic formation of 7-amino-4-methylcoumarin. DPP II activity was measured by HPLC with fluorometric detection with Lys-Ala-MCA as substrate [8]. DPP IV activity was measured fluorometrically with Gly-Pro-MCA as substrate, as described previously [9]. Protein was assayed by the method of Lowry *et al.* [10]. Statistical significance was evaluated by a one-tail *t*-test.

Symptoms of SLE were confirmed histologically in the spleen by the previously reported method [11, 12].

Results

Figure 1 shows a comparison between the enzyme activities in the spleen of DSP-treated male BXSB mice without SLE symptoms vs DSP-untreated male BXSB mice with SLE symptoms and DSP-untreated female BXSB mice as SLE-free control mice and DSP-untreated female BALB/c mice as normal control mice. DPP II activity and DPP II/DPP IV ratio in the spleen of DSP-treated male BXSB mice that did not develop SLE symptoms were significantly lower than those of DSP-untreated male BXSB mice that developed SLE symptoms ($P < 0.001$). In the spleen of DSP-treated male BXSB mice the activity of DPP II and DPP II/DPP IV ratio were not significantly different from those of DSP-untreated normal female BALB/c mice.

Figure 2 similarly compares the changes between the two DPP activates in the spleen of DSP-treated male MRL/*lpr* mice without SLE symptoms vs DSP-untreated male MRL/*lpr* mice with SLE symptoms and DSP-untreated female BALB/c mice as normal controls. In the spleen of DSP-treated male MRL/*lpr* mice that did not develop SLE symptoms, DPP II activity and the DPP II/DPP IV ratio were lower than those of DSP-untreated male MRL/*lpr* mice that developed SLE symptoms, and were not significantly different from those of DSP-untreated normal female BALB/c mice.

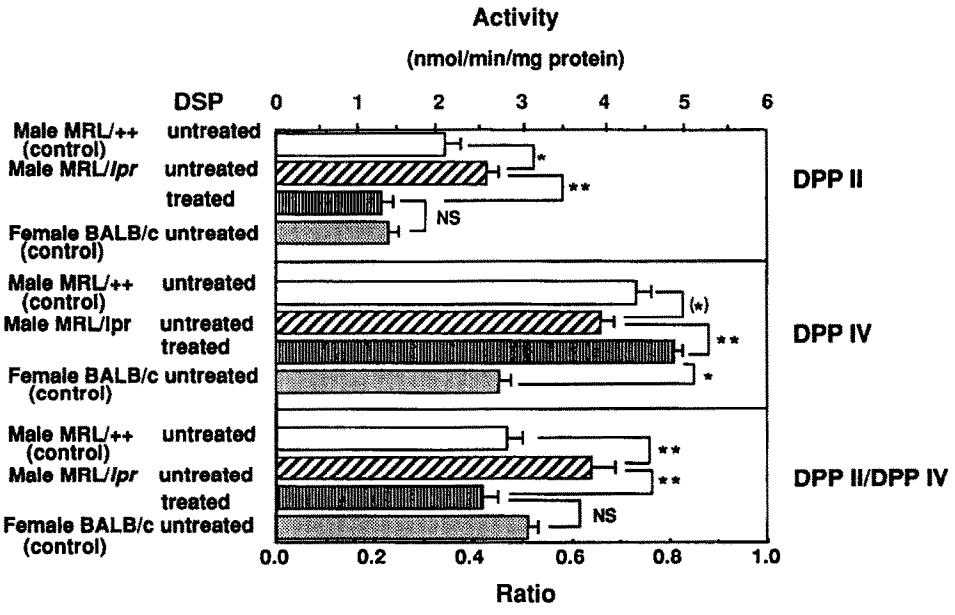

Fig. 2. Effect of DSP on DPP activities in the spleen of MRL/*lpr* mice with SLE-like syndrome. Means ± SD (N = 5); *P < 0.05; *P < 0.01; **P < 0.001; NS, not significant. DSP-untreated, male MRL/++ mice and female BALB/c mice did not show SLE syndrome.

The results in Figs 1 and 2 suggest that DSP treatment may prevent the increases in the activity of DPP II and in the ratio of DPP II/DPP IV in two experimental models of SLE, male BXSB mice and MRL/*lpr* mice, in parallel with the prevention of the development of SLE symptoms.

Discussion

We had earlier reported increased DPP II activity in female NZB mice with SLE-like symptoms as compared to that in control female Balb/c mice without the symptoms [3]. An interesting finding in this study was the reciprocal changes in the activities of DPP II and DPP IV, which was similar to our previous findings on tumor-bearing animals and cancer patients [13]. In our following study [4], DPP II activity increased and DPP IV activity decreased in the spleen with age in male BXSB mice or male MRL/*lpr* mice with SLE-like symptoms as compared to the activities in the control female BXSB mice or male MRL/++ mice. Since there seems to be a male to female difference in BXSB mice, we also examined male MRL/*lpr* mice vs control male MRL/++ mice. The two control mice, female BXSB mice and male MRL/++ mice also developed SLE-like symptoms at a later age, but were normal till 20 weeks of age. Other studies also indicate the participation of hydrolytic enzymes in pathogenesis of immunoallergic disturbances [14–18].

The specific roles of these peptidases in immunological abnormality have not been elucidated yet. DPP IV was shown to modulate T-lymphocyte-mediated lymphokine [19]. Moreover, specific inhibition of DPP IV with an irreversible suicide inhibitor blocks T cell activation by lectins [20]. The role of DPP II in immunological reactions is not clear, but as a lysosomal enzyme, DPP II may participate in degradation of intracellular peptides related to immunological response.

DSP is an anti-tumor agent newly developed by Umezawa and co-workers [6, 7] and has been shown to have an immunosuppressive function. It is one of the analogues of spergualin which was previously reported to suppress various types of immune response [6].

In our previous study, DSP effectively prevented the progress of spontaneous lupus glomerulonephritis in both male BXSB and MRL/*lpr* strains of mice [11, 12]. DSP suppressed the development of the polyclonal B cell activation in autoimmune male BXSB and MRL/*lpr* mice and also showed therapeutic effects in these mice. Thus, the drug was assumed to act on both B and T cells.

In this paper, we examined the effect of DSP on changes of DPP II activity and the DPP II/DPP IV ratio in male BXSB and MRL/*lpr* mice. The present results on the effect of DSP on DPP II activity and the DPP II/DPP IV ratio coincide with the therapeutic effect of DSP on the lupus symptoms in these mice [11, 12], and may further support that the ratio of DPP II/DPP IV could be hypothesized as a biochemical index of some autoimmune diseases, and that prevention of SLE-like symptoms by DSP may be related to the changes in DPP activities.

‖ Correspondence: Professor T. Nagatsu, Institute for Comprehensive Medical Sciences, School of Medicine, Fujita Health University, Toyoake, Aichi 470-11, Japan.

*Department of Biochemistry
Nagoya University School of Medicine
Nagoya 466, Japan
†Animal Center for Biomedical Research
Faculty of Medicine
University of Tokyo
Tokyo 113, Japan
‡Institute of Microbial Chemistry
Tokyo 141, Japan
§Institute for Comprehensive Medical Sciences
School of Medicine
Fujita Health University
Toyoake
Aichi 470-11, Japan

Masako Hagihara*
Kaneo Sekiguchi*
Michio Fujiwara†
Takaaki Aoyagi‡
Tomio Takeuchi‡
Toshiharu Nagatsu§‖

REFERENCES

1. Murphy ED and Roths JB, Autoimmunity and lymphoproliferation: induction by mutant gene *lpr*, and acceleration by a male-associated factor in strain BXSB mice. In: *Genetic Control of Autoimmune Disease* (Eds. Rose N, Bgazzii P and Wanner N), pp. 207–221. Elsevier North Holland, New York, 1978.
2. Theofilopoulos AN and Dixon FJ, Murine models of systemic lupus erythematosus. *Adv Immunol* **37**: 269–390, 1985.
3. Hagihara M, Ohhashi M and Nagatsu T, Activities of dipeptidyl peptidase II and dipeptidyl peptidase IV in mice with lupus erythematosus-like syndrome and in patients with lupus erythematosus and rheumatoid arthritis. *Clin Chem* **33**: 1463–1465, 1987.
4. Hagihara M, Fujiwara M and Nagatsu T, Activities of dipeptidyl peptidases in BXSB mice and MRL/*lpr* mice with lupus erythematosus-like syndrome. *Biochem Med Metab Biol* **42**: 224–231, 1989.
5. Makino M, Fujiwara M and Watanabe H, Studies on the mechanisms of the development of lupus nephritis in BXSB mice. I. Analysis of immunological abnormalities at the onset period. *J Clin Lab Immunol* **22**: 127–131, 1987.
6. Umezawa H, Ishizuka M, Takeuchi T, Abe F, Nemoto K, Shibuya K and Nakamura T, Suppression of tissue graft rejection by spergualin. *J Antibiot* **18**: 283–284, 1985.
7. Takeuchi T, Iimura H, Kunimoto S, Masuda T, Ishizuka, M, Takeuchi M, Mamada M, Naganawa H, Kondo S and Umezawa H, A new antitumor antibiotics, spergualin: isolation and antitumor activity. *J Antibiot* **34**: 1619–1621, 1981.
8. Nagatsu T, Sakai T, Kojima K, Araki E, Sakakibara S, Fukasawa K and Harada M, A sensitive and specific assay for dipeptidyl-aminopeptidase II in serum and tissues by liquid chromatography-fluorometry. *Anal Biochem* **147**: 80–85, 1985.
9. Kato T, Nagatsu T, Kimura T and Sakakibara S, Fluorescence assay of X-prolyl dipeptidyl-aminopeptidase activity with new fluorogenic substrate. *Biochem Med* **19**: 351–359, 1978.
10. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
11. Makino M, Fujiwara M, Aoyagi T and Umezawa H, Immunosuppressive activities of deoxyspergualin. I. Effect of long-term administration of the drug on the development of murine lupus. *Immunopharmacology* **14**: 107–114, 1987.
12. Ito S, Ueno M, Arakawa M, Saito T, Aoyagi T and Fujiwara M, Therapeutic effect of 15-deoxyspergualin on the progression of lupus nephritis in MRL mice I. Immunopathological analyses. *Clin Exp Immunol* **81**: 446–453, 1990.
13. Kojima K, Mihara R, Sakai T, Togari A, Matsui M, Shinpo K, Fujita K, Fukasawa K, Harada M and Nagatsu T, Serum activities of dipetidyl-aminopeptidase II and dipeptidyl-aminopeptidase IV in tumor-bearing animals and cancer patients. *Biochem Med Metab Biol* **37**: 35–41, 1987.
14. Aoyagi T, Wada T, Ishikawa Y, Kojima F, Nagai M, Osanai T, Nagai Y and Umezawa H, Systemic enzymatic changes in guinea pig suffering from experimental allergic encephalomyelities. *J Pharm Dyn* **6**: 963–973, 1983.
15. Aoyagi T, Wada T, Tanaka T, Tanimoto K, Aikawa T, Hara T, Umeda T, Niijima T and Umezawa H, Decreased serum levels of various hydrolytic enzymes in patients with rheumatoid arthritis. *Biochem Int* **8**: 529–535, 1984.
16. Aoyagi T, Wada T, Kojima F, Nagai M, Okubo M, Masaki Y and Umezawa H, Abnormality of the post-proline-cleaving enzyme activity in mice with systemic lupus erythematosus-like syndrome. *J Appl Biochem* **7**: 273–281, 1985.
17. Bennet RM and Stosey JL, Loctoferrin and lysozyme levels in synovial fluid: differential indices of articular inflammation and degradation. *Arthritis Rheum* **20**: 84–90, 1977.
18. Iwase-Okada K, Kojima K, Kato T, Haku H, Okazaki T, Wago K, Sakakibara S and Nagatsu T, Collagenase-like peptidase activity in serum from patients with rheumatoid arthritis. *Experientia* **41**: 487–488, 1985.
19. Absorge S and Schon E, Dipeptidyl peptidase IV (DAP IV), a functional marker of the T lymphocyte system. *Acta Histochem* **82**: 41–46, 1987.
20. Schon E, Jahn S, Kissig ST, Demuth HU, Neubert K, Barth A and Ansorge S, The role of dipeptidyl peptidase IV in human T lymphocyte activation. Inhibitor and antibodies against dipeptidyl peptidase IV suppress lymphocyte proliferation and immunoglobulin synthesis *in vitro*. *Eur J Immunol* **17**: 1821–1826, 1987.