The data provided from our results indicate that differences in the central cholinergic system occur between the obese Zucker rat and its lean littermate. It may therefore be postulated that one possible etiology of obesity in the Zucker rats is related to the changes in the brain cholinergic function, which might lead to the increase in the vagus nerve activity, which in turn leads to both hyperinsulinemia and thus obesity.

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Administration of D-alanine did not cause increase of D-amino acid oxidase activity in mice

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Abstract. D-amino acid oxidase (DAAO) activity was not altered in the liver and kidney by oral administration of D-alanine to adult mice. The enzyme was apparently not induced by the enteric microflora either, since the enzyme activity in the liver and kidney of germ-free mice was not different from that of specific-pathogen-free mice. The times of appearance of DAAO activity and of free D-amino acids in the kidney were elucidated using suckling mice. DAAO activity started to increase 7 days after birth, and reached almost the adult level by 28 days. The content of free neutral D-amino acids also increased with age, in a similar fashion. A possible conclusion is that the enzyme activity normally increases during this period, to eliminate the free D-amino acids which have increased with age in the suckling mice. Consequently, the administration of D-alanine had no further effect in increasing enzyme activity.
Key words. D-amino acid oxidase; D-amino acids; D-alanine; microflora; induction; germ free.

D-amino acid oxidase (DAAO, EC 1.4.3.3) is a flavoprotein that catalyses the oxidative deamination of neutral free D-amino acids to the corresponding 2-oxo acids. The presence of DAAO has been reported in peroxisomes of many organs such as kidney, liver, brain, etc., but its physiological function is still unclear [1]. We have suggested that the physiological role of DAAO is, in part, to eliminate D-amino acids present in the body. This sug-

gestion is based on studies using mutant mice lacking DAAO[2,3], and plasma from patients with renal diseases[4].

Concerning the inducible nature of the enzyme, which may be closely related to its physiological function, there are two contradictory communications. One, by Lyle and Jutila[5], states that the kidneys of most germ-free (GF) mice showed no DAAO activity. The activity was stimulated in the kidneys of GF mice by D-alanine injection or by bacteria. The other report is by Meister et al.[6], stating that kidneys of GF mice exhibited appreciable DAAO activity, and the activity was comparable to that of the kidneys of mice raised under non-sterile conditions. Recently, Yamada et al.[7] found that liver D-aspartate oxidase activity increased after the administration of D-aspartate to mice, and that the increase depended specifically on D-aspartate. Therefore it was suggested that D-aspartate was a physiological substrate of D-aspartate oxidase. The enzyme resembles DAAO in that it is a peroxisomal flavoenzyme and is distributed over a variety of organs.

The finding prompted us to re-examine the inducible nature of DAAO, and we administered D-alanine, which is a substrate of the enzyme in vitro, to mice.

Materials and methods

Mice. Littermate ICR mice (male, 8 weeks old), purchased from Japan SLC (Shizuoka, Japan) were divided into two groups. The animals of one group (D-alanine group, n = 5) were given free access to drinking water containing 1 g/l saccharin and 20 g/l D-alanine in distilled water for 2 weeks before sacrifice, and the other group (control group, n = 5) was supplied with water containing 1 g/l saccharin in distilled water, in the same way[7]. Each group was fed normal diet chow (Type MF, Oriental Yeast, Tokyo, Japan), and housed in a cage with a mesh floor to prevent the mice from touching their feces, which are contaminated with bacteria which contain D-alanine and D-glutamic acid as cell wall components[8]. The volume of the water intake and the weight gain during the 2-week treatment were not different in the two groups (table 1). The amount of D-alanine ingested by individual animals of the D-alanine group was calculated to be 54.8 mmol/kg b.wt/d on average. Liver and kidney weights showed no difference between the two groups. Urine was collected a day before sacrifice. Blood was obtained by decapitation of the mice under anesthesia with diethyl ether.

Table 1. Water intake, weight gain and organ weight of D-alanine-administered mice. The volume of water taken by each animal was estimated based on the volume change of the drinking water in the water bottle during the 2-week treatment (values are mean of 5 animals). The body weight was measured at the beginning and the end of the 2-week treatment. The values are means ± SD for 5 animals.

	D-Alanine group	Control group
Water intake (ml)	122	144
Weight gain (g)	8.1 ± 0.6	9.4 ± 1.3
Liver weight (g)	2.50 ± 0.06	2.52 ± 0.06
Kidney weight (g)	0.80 ± 0.02	0.79 ± 0.03

Male germ-free (GF) mice (ICR, 6 weeks old) and age- and sex-matched specific pathogen-free (SPF) mice of the same strain were obtained from Japan Clea (Tokyo, Japan). Each animal was housed alone in a cage with a mesh floor, and fed 'NIH-improved type' chow (Oriental Yeast) which included no supplemental D-amino acids, for 2 weeks under sterilized condition. The GF mice were kept sterile without infection with bacteria or fungi[9].

Male ddY/DAO$^-$ mutant mice lacking DAAO activity[10,11] and normal ddY/DAO$^+$ mice were raised at Dokkyo University School of Medicine, and sacrificed on day 1, 7, 14 or 28 after birth.

Methods for enzyme and D-amino acid determination. The mice were starved, but allowed water, for 16–19 h before sacrifice. The serum was separated by centrifugation after the blood had clotted at room temperature. After being rinsed with phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4) to remove blood, each organ was minced into small pieces, homogenized at 1000 rpm for 1 min with 4 vols of the phosphate-buffered saline in a glass homogenizer in an ice bucket, and centrifuged at 160,000 × g for 10 min at 4 °C. The supernatants were used in the assays for DAAO activity and free D-amino acid content.

Determination of DAAO activity was performed by a previously-described method[12], i.e., D-alanine was utilized as the substrate of DAAO, and pyruvate production was assayed by measuring the absorbance at 445 nm. The content of neutral free D-amino acids in the supernatants of tissue homogenates was measured with an enzymic method[13], as follows. Firstly, 5% trichloroacetic acid was added and the mixture was centrifuged. Trichloroacetic acid was removed from the supernatants by chromatography on a Dowex 1x8 column, and the solutions then assayed for the D-amino acids. 2-oxo acids derived from D-amino acids by DAAO were measured as hydrazones at 445 nm. The method is not affected by the presence of a large excess of L-amino acids owing to the rigid stereospecificity of DAAO[13], and measures only neutral D-amino acids. Urinary creatinine was determined with a kit (Serotec, Sapporo, Japan). DAAO from pig kidney was purchased from Sigma (St. Louis, MO, USA), and all other chemicals used were analytical-grade products.

Results

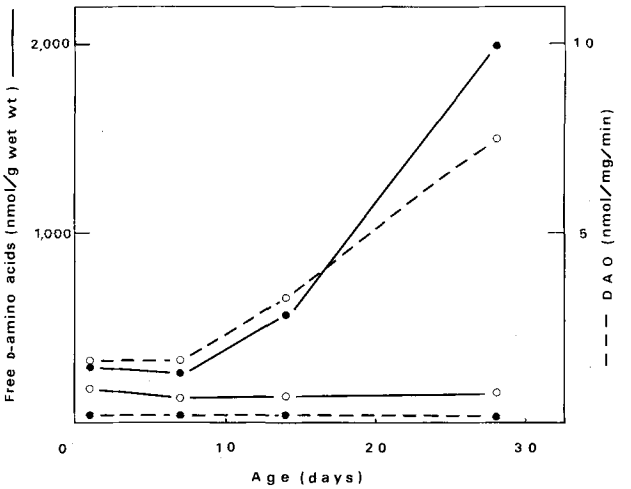
The contents of neutral free D-amino acids in the liver, kidney, serum and urine of control mice, and mice given D-alanine, was measured in order to confirm the ingestion of D-alanine (table 2). This was shown by the fact that in the D-alanine group the D-amino acid concentration in serum and urine was significantly higher than in the control group. The liver is the organ that responds to an exogenous invader. In the mouse liver, DAAO activity is not high[14]. It was therefore expected that the DAAO

Table 2. Free D-amino acid content of D-alanine-administered mice. Content of free D-amino acid in several tissues from D-alanine-treated and the control mice were measured as described under the Materials and Methods section. The values are means ± SD for 5 animals. Data were subjected to t-test to assess the significance of any difference between the two groups. a) $p < 0.0005$, b) $p < 0.01$.

	Free D-amino acids		
	D-Alanine group	Control group	
Liver (nmol/g wet wt)	291 ± 60	242 ± 10	
Kidney (nmol/g wet wt)	184 ± 57	195 ± 44	
Serum (nmol/ml)	81 ± 7	32 ± 12	a)
Urine (nmol/mg of creatinine)	12,949 ± 4,905	1,008 ± 239	b)

Table 3. D-Amino acid oxidase activity of D-alanine-administered mice (A), and GF and SPF mice (B). Liver and kidney DAAO activity was determined by the method described in the materials and methods section. The tissue extracts from D-alanine-treated and control mice, as well as GF and SPF mice were assayed. The values are means ± SD for 5 (A) or 4 (B) animals.

		DAAO activity (nmol D-Ala catalyzed/mg/min)	
		Liver	Kidney
A	D-Alanine group	0.162 ± 0.047	7.33 ± 2.66
	Control group	0.176 ± 0.041	8.34 ± 3.43
B	GF mice	0.084 ± 0.043	5.70 ± 0.79
	SPF mice	0.127 ± 0.054	5.86 ± 0.80



Kidney D-amino acid oxidase activity and neutral free D-amino acid content of suckling mice. Eight kidneys (4 animals) of each age were pooled and homogenized as described under Materials and Methods. The extract was used for the assay. ○– – –○, DAAO activity of normal mice (ddY/DAO$^+$); ●– – –●, DAAO activity of DAAO-lacking mutant mice (ddY/DAO$^-$); ○——○, free D-amino acid content of normal mice; ●——●, free D-amino acid content of mutant mice.

activity would increase in the liver in response to the exogenous substrate, D-alanine. As shown in table 3 A, however, no difference was observed between the two groups in the liver or in the kidney. The result clearly revealed that DAAO was not inducible in adult mice even with a high dose of D-alanine.

In order to examine the possibility that DAAO may have been produced in the adult control mice in response to D-amino acids derived from the cell walls of the enteric bacteria, the DAAO activity of GF mice was compared to that of mice of the same strain raised under specific pathogen-free conditions. During the experiment the GF mice were free from infection with bacteria and fungi. As indicated in table 3 B, the DAAO activity in the liver and kidney did not differ significantly between GF and SPF mice. The result demonstrated that the DAAO activity observed in the adult mice was not related to the enteric microflora.

To find out at what stage DAAO and free D-amino acids appeared, DAAO activity and the free neutral D-amino acid content were measured in the kidneys both of suckling normal mice (ddY/DAO$^+$) and of mutant mice lacking DAAO (ddY/DAO$^-$) (fig.). Since both DAAO activity[14] and free D-amino acid content[2,15] are high in the kidney in comparison with other organs, the kidney was chosen for the examination. In the normal mice, DAAO activity started to increase 7 days after birth, and almost reached the adult level in 28 days, while the D-amino acid content remained low. In the mutant mice, in contrast, the DAAO activity was negligible throughout the period, whereas the free D-amino acid level started to increase 7 days after birth, and reached the adult level by 28 days.

Discussion

Meister et al.[6] observed no difference between GF mice and nonsterile control animals in the kidney DAAO activity, nor did they find any alteration of DAAO activity after injection or oral administration of D-amino acids in very young rats. The present results agree well with the above observations but not with the report by Lyle and Jutila[5], which states that GF mice did not possess DAAO activity and that they gained the enzyme activity after bacterial infection or injection with D-alanine. It is difficult to explain the reason for the discrepancy. According to Lyle and Jutila, the discrepancy might be caused by a difference in the mouse strain used in the experiments. It is also possible that the precent experiments gave different results because: (1) more accurate methods for microdetermination of DAAO activity[12] and of free D-amino acid content[13] were used; (2) great care was taken to prevent the mice from eating their feces, by housing them in cages with a mesh floor, and in the maintenance of the sterile conditions for the GF mice, i.e., by provision of autoclaved diet chow, water and cages; and (3) a diet was selected which contained no supplemental D-amino acids: the mice obtained only 0.256 mmol/kg b.wt/d D-alanine from the diet chow in the D-alanine feeding experiments. Lyle and Jutila's view that DAAO was induced in mice by contamination with bacteria is based on the communication of Hoeprich[16] that D-alanine present in the serum of conventionally-raised mice was from the enteric microflora, and that

only a negligible amount of D-alanine was detected in the GF mice.

However, we have found that the free D-amino acid content in the serum, kidney, liver, brain and small intestine[9], of GF mice was not different from that of SPF mice. This suggests that the free D-amino acids in those tissues are not microbial origin.

The time of appearance and the rate of increase of D-amino acids were examined using suckling mice (fig.). Mutant mice lacking DAAO were used in the experiment, since the amounts of free D-amino acids were not large enough for the accurate detection of a slight quantitative alteration in normal animals. The mutant mice look normal, and have a life span similar to other mouse strains. It seems probable, therefore, that the free D-amino acid content in the mutant mice may show what the D-amino acid level in normal mice would be if it were not for the presence of DAAO. DAAO activity also increased with age in the normal suckling mice. Thus a possible interpretation for the DAAO activity observed in the present experiment may be that the enzyme activity had increased to eliminate the free D-amino acids which had increased with age in the young normal mice, and the D-alanine administration altered the enzyme activity no further in the adult animals.

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Distribution of the 1,25 dihydroxy-vitamin D_3 receptor in the bursa of Fabricius of chicken

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Abstract. The vitamin D_3 metabolite $1,25(OH)_2D_3$ is probably involved in B lymphocyte ontogeny. We therefore determined the distribution of the $1,25(OH)_2D_3$ receptor in the bursa of Fabricius and spleen cells of 7-day-old chicks, by immunohistochemistry using a monoclonal antibody against the chick intestinal cell $1,25(OH)_2D_3$ receptor. The bursa cells of young (7-day-old) chicks contained large amounts of receptor while the spleen cells did not. The bursa cells of older (35-day-old) chicks contained fewer receptors, but the number of receptors in the spleen increased.
Key words. Vitamin D_3 receptor; dihydroxy-vitamin D_3; bursa of Fabricius; ontogeny of immune system; B lymphocytes.

Since the first reports suggesting a role for 1,25 dihydroxy-vitamin D_3 ($1,25(OH)_2D_3$)[1,2] and its receptor[3] in lymphocyte functions, the vitamin D_3 metabolite $1,25(OH)_2D_3$ has been considered to be an immunomodulating hormone. A specific receptor for $1,25(OH)_2D_3$ has been shown by radiobinding[1] to be present in activated human T and B lymphocytes. Vitamin D_3 affects activated lymphocytes in various ways: it inhibits lymphocyte proliferation, the production of gamma interferon[1], and the production of antibodies[4,5]. Inhibition of IL-2 production by activated lymphocytes has also been reported[6]. However, the antiproliferative activity of $1,25(OH)_2D_3$ seems to be related to the mitogens used and to the presence of monocytes in lymphocyte culture[7]. The production of colony-stimulating factors (CSF) by lymphocytes is also modulated by $1,25(OH)_2D_3$: CSF production is reduced when lymphocytes are activated by PHA[8] but it seems to be increased when lymphocytes are activated by Con A[9]. These data suggest that $1,25(OH)_2D_3$ plays a role in the physiology of the immune system and that it may be considered to be an immunomodulating hormone[2].

The chicken bursa of Fabricius is a spherical organ connected to the cloaca by a duct. 12 to 14 longitudinal plicae of lymphoid tissue surrounded by epithelial cells protrude into the bursal lumen. The organ contains