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Effect of Bovine Thymic Hypocalcemic Factor on Increasing Antibody-producing Cells in Mice[1)]

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Previously we reported on the lymphocyte-stimulating activity of the hypocalcemic substances (TP_1, TP_2) which were isolated from the bovine thymic extracts, and on their possibility of promoting immune competence (A. Mizutani, *et al.*, *Ann. N. Y. Acad. Sci.*, **249**, 220 (1975)). To acquire the direct evidence for promoting immune competence, both substances and several fractions, which were obtained from the thymic extracts in the course of purification, were examined for the increase in antibody-producing cells (PFC activity) in mice by the Jerne method with a minor change. Roughly, it was recognized that there was a tendency for the promotion of PFC activity of the fractions to be dependent on that of their hypocalcemic activity accompanied by lymphocyte-stimulating activity. In the administration of both TP_1 and TP_2, in a dose of 1 μg/mouse, to neonatal mice, significant PFC activity was found. Thus it gives a clearer understanding of the action of promoting immune competence taking the lymphocyte-stimulating action into account.

Keywords——bovine thymic hypocalcemic factor; lymphocytes/polymorphs ratios; immunoactive component; gel chromatography; preparative polyacrylamide gel electrophoresis; single band in analytical polyacrylamide gel electrophoresis; neonatal mice; intraperitoneal injection; increase of antibody-producing cells; lymphocyte-stimulating action

It has recently been found that the function of the thymus gland is very complex and diverse, and that it plays an important role in promoting immune competence. In 1961, Miller[3)] found that hardly any transplantation immunity is produced in mice thymectomized soon after birth. Metcalf[4)] reported an assay method for the lymphocytosis, produced in the newborn mice a few days after the injection of a thymus extract, based on the increases in lymphocytes/polymorphs ratios (L/P activity). Hand, *et al.*[5)] isolated the lymphocyte-stimulating hormone (LSH) from the calf thymus. Hooper, *et al.*[6)] obtained an immunoactive component, Thymosin, from bovine thymus gland. In the previous experiment, we isolated two hypocalcemic substances, TP_1 and TP_2 from bovine thymus, and found that these substances have L/P activity.[7)] However, we could not conclude that the substances might promote immune competence because they merely produced L/P activity. Therefore, an experiment was carried out to obtain direct evidence for immune competence, in which the substances TP_1, TP_2, and several other fractions which were obtained in the course of purification were examined for increasing plaque forming cells (PFC activity) by the Jerne method, with a minor change.[8,9)] This paper reports the PFC activity of the fractions ob-

1) I. Suzuki, Y. Matsushita, K. Hayakawa, and A. Mizutani, *Seikagaku* (in Japanese), **47**, 672 (1975).
2) Location: *Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) J.F.A.P. Miller, *Lancet*, **2**, 748 (1961).
4) D. Metcalf, *Ann. N. Y. Acad. Sci.*, **73**, 113 (1958).
5) T. Hand, P. Caster, and T.D. Luckey, *Biochem. Biophys. Res. Commun.*, **26**, 125 (1967).
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7) A. Mizutani, M. Shimizu, I. Suzuki, T. Mizutani, and S. Hayase, *Ann. N. Y. Acad. Sci.*, **249**, 220 (1975).
8) N.K. Jerne and A.A. Nordin, *Science*, **140**, 405 (1963).
9) W.S. Ceglowski, T.L. Hand, and H. Friedman, "Thymic Hormones," ed. T.D. Luckey, University Park Press, Baltimore, 1973, p. 185.

tained from bovine thymus extracts, and the correlation of this activity with L/P and hypocalcemic activity (Ca activity) is discussed herein.

Materials and Methods

Materials——Fractions TP_1 and TP_2 were obtained from bovine thymus gland by the method reported[10] previously or by the method with a minor change. Several fractions which were obtained during the steps of purification were used for reference.

Acetone-dried powder, T-1, was prepared from thymus gland of a steer in a yield of about 3.8%. The saline extract from the powder was fractionated with $(NH_4)_2SO_4$, and the fraction, T-1. A-15, precipitated at 15% (w/w) of $(NH_4)_2SO_4$ concentration was obtained in a yield of about 1.8%. The fraction T-1. A-15 was further fractionated by chromatography on DEAE-cellulose, and thereby four fractions, DE-I, DE-II, DE-III, and DE-IV, were obtained in yields of 11.9%, 29.2%, 8.4%, and 6.0%, respectively, by elution with the following four solvents: 0.007 M phosphate buffer, pH 7.90, $\mu=0.02$; 0.067 M phosphate buffer, pH 6.80, $\mu=0.13$; 0.067 M phosphate buffer +0.1 M NaCl, pH 6.50, $\mu=0.23$; 0.067 M phosphate buffer +0.2 M NaCl, pH 6.30, $\mu=0.33$. In the assay on Ca activity, both the fractions DE-III and DE-IV showed a high activity compared with the other fractions. The fraction DE-III was subjected to the purification for TP_1. The fraction $G_{4B}\cdot G_{6B}$-2 was obtained from the fraction DE-III by gel chromatography on Sepharose 4B and second gel chromatography on Sepharose 6B (Kav: 0.50), in a yield of about 41.1%. Subsequently, this fraction was purified by preparative polyacrylamide gel disc electrophoresis using Toyo CD-50 with some modifications in our laboratory.[7] During electrophoresis, the fraction TP_1 was pumped out from the elution chamber of the apparatus, and its yield was about 1.3%. The fraction TP_1 gave a single band in analytical disc electrophoresis (7.5% gel, pH 8.9)[11] (Fig. 4a).

Purification of TP_2 was as follows. The fraction DE-IV was loaded on a column of Sepharose 6B (2.9×117 cm) followed by elution with 0.025 M phosphate buffer (pH 7.0, $\mu=0.06$), which resulted in the pattern showed in Fig. 1. This eluate was divided into 6 fractions, whose yields and Ca activity are shown in Table I. The fraction G_{6B}-4, shaded area in the graph, had the highest Ca activity, and gave rise to several bands in disc electrophoresis (Fig. 2). Thus, the fraction was purified further by preparative disc electrophoresis.

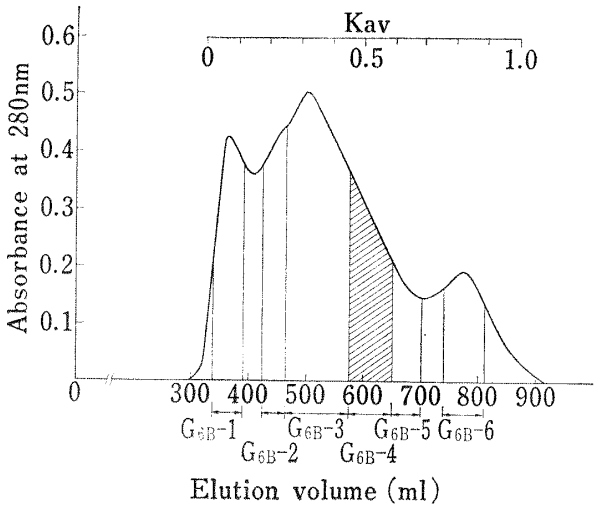

Fig. 1. Gel Chromatography of the Fraction DE-IV on Sepharose 6B

sample; 171 mg in 8 ml of 0.025 M phosphate buffer (pH 7.0, $\mu=0.06$), column size; 2.9×117 cm, flow rate; 32.4 ml/hr.

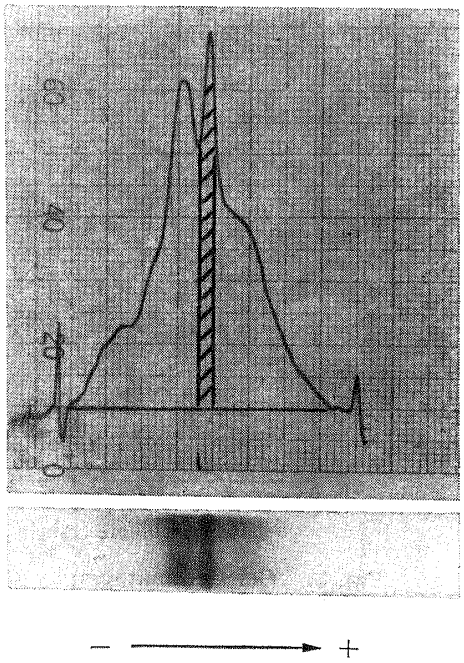

Fig. 2. Upper: Densitometric Tracting of Disc Electrophorogram of the Fraction G_{6B}-4.

Bottom: Disc Electrophoretic Pattern of the Fraction G_{6B}-4.

7.5% gel, pH 8.9.

10) A. Mizutani, K. Yamamoto, H. Sato, Y. Saito, H. Kitsugawa, and H. Yamamoto, *Yakugaku Zasshi*, **91**, 297 (1971).
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Table I. Hypocalcemic Activities and Yields of Fractions obtained from Gel Chromatography of the Fraction (DE-IV) on Sepharose 6B

Fraction	Dose mg/kg	Percent decrease in serum Ca Mean ± S.E. ($N=6$)	Yield (%)
G_{6B}-1	0.1	5.91±0.25	14.6
G_{6B}-2	0.1	2.71±0.10	12.6
G_{6B}-3	0.1	9.45±3.02	22.1
G_{6B}-4	0.1	11.33±1.41[a)]	16.3
G_{6B}-5	0.1	8.53±1.33[b)]	9.6
G_{6B}-6	0.1	2.40±0.21	5.1

a) Significantly different from control $p<0.01$.
b) Significantly different from control $p<0.05$.

Initially, the fraction TP_2 was obtained by pumping out from the elution chamber of the same apparatus as used for the purification of TP_1, but on account of low mobility of TP_2 compared with TP_1, a long period of time was required for the electrophoresis, and thereby the effect of separation was poor. Therefore, we used an alternative apparatus which was manufactured in our laboratory.[12] After the electrophoresis, the gel was taken out from the apparatus and subsequently sliced into pieces. Appropriate gel slices were extracted with water followed by dialysis and lyophilization of the aqueous extract. The final TP_2 product was obtained from the lyophilized powder through the removal of the polyacrylamide gel particles by means of gel chromatography on Sepharose 6B (Kav: 0.45, Fig. 3) in a yield of about 2.5%, giving single band as shown in Fig. 4b. Bovine serum albumin (BSA) used for control was obtained from Armour Co., U.S.A.

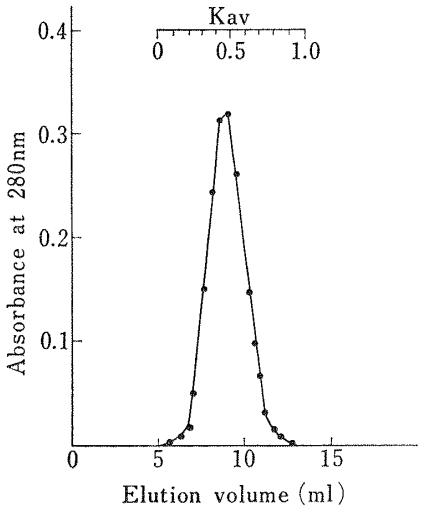

Fig. 3. Gel Chromatography of the Fraction obtained by preparative Disc Electrophoresis on Sepharose 6B

Sample; 24.7 mg in 1.0 ml of 0.025 M phosphate buffer (pH 7.0, $\mu=0.06$), column size; 0.75×30 cm, flow rate;3.5 ml/hr, one tube; 0.5 ml.

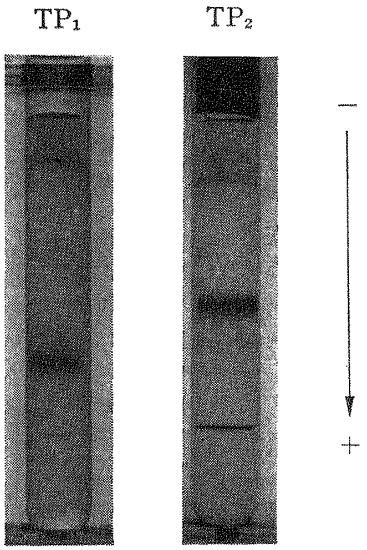

Fig. 4. Disc Electrophoretic Patterns of the Fractions TP_1 and TP_2.

(a) TP_1, (b) TP_2, 7.5% gel, pH 8.9.

Measurements of Biological Activities

1) **PFC Activity**——The Jerne method with a minor change[8,9] was employed for this assay. The littermates of neonatal mice of ICR strain were divided into two groups. At 6—12 hr after birth, one group

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of the mice was injected intraperitoneally with the sample dissolved in saline and the other group with saline as a control. After 14 days, the mice were injected with 20% sheep red blood cells, and the spleen was excised 4 days later. The plaque formation on agar plates was performed using the cell suspension which was prepared from the spleen of the mice according to the method mentioned above, and resulting plaques were counted. Meanwhile, the number of cells in the cell suspension was counted by using a melangeur for leucocytes and Bürker-Türk hemocytometer.

The number of PFC per 10^6 cells was calculated, and Student's *t*-test was made on the difference between the mean of the test group and that of the control group, and the sample tested was considered effective when the difference was significant at below 5% level of significance.

2) **L/P Activity**——The method of Hand, *et al.*[5] based on the original method of Metcalf[4] was used. The littermates of neonatal mice of Swiss-Webster strain was divided into two groups; one group was intraperitoneally injected with the sample dissolved in saline at 6—12 hr after birth and the other group with saline as a control. Blood was drawn from the tail of the mice before the injection and 6, 10, and 14 days after the injection, smear preparations of the blood were made, stained by the Wright staining method, and the number of lymphocytes and polymorphs were counted for a total of 100 cells and the ratio of that to this (L/P) was calculated.

In both the test and the control group, the increment of the L/P ratios at 6, 10, and 14 days after the injection to the L/P ratio before injection were calculated, and the difference in the mean of the increment between the both groups was examined by the *t*-test on each bleeding day. If one of three blood samples showed a significant difference at the 5% probability level, the sample tested was considered to be effective.

3) **Ca Activity**——Ca activity was assayed by the method described previously.[13] Groups of six normal mature male rabbits, deprived of diet for about 24 hr, were used. The sample dissolved in saline was intravenously injected into rabbits in a dose of 0.5 ml/kg. Blood was drawn prior to the injection and 4, 5, and 6 hr after the injection, and the amounts of serum calcium were determined by the chelate titration method of Bachra, *et al.*,[14] using Dotite-NN as an indicator or by the aid of an atomic absorption spectrophotometer. From three serum samples taken after the injection, the maximum percent decrease in serum calcium was calculated in each animal, then averaged for the six animals. The difference between the mean values of the test group and the control group injected with saline was examined by the *t*-test. The sample injected was considered effective when the difference was significant at below 5% level of significance.

Results

Values obtained for PFC, L/P and Ca activities are summarized in Table II. Mean values for the test group are given in the upper line and those of the control group in the lower line. Treatment of the mice with 50 μg/mouse of the acetone-dried powder T–1 gave a PFC activity of 138.66, which was significantly different from the control at 1% level, and L/P activity was also significant at 1% level in a dose of 20 μg/mouse, while Ca activity showed an about 9% decrease in serum calcium, which was also significant. Treatment of the mice with T–1. A–15, ammonium sulfate fractionation product, gave values which were significantly different from those of the control: PFC activity at a dose of 20 μg/mouse, L/P activity at 10 μg/mouse, and Ca activity at 1 mg/kg dose. In the cases of the fractions DE–I and DE–II, products of DEAE-cellulose chromatography, the values obtained by their use were insignificant: PFC activity at a dose of 10 μg/mouse, L/P activity at 5 μg/mouse, and Ca actvity at 0.5 mg/kg dose. Both the fractions DE–III and DE–IV gave significant values: PFC activity at a dose of 10 μg/mouse: L/P activity at 5 μg/mouse, and Ca activity at 0.5 mg/kg dose. The gel chromatography product $G_{4B}\cdot G_{6B}$–2 also gave significant values: PFC activity at 5 μg/mouse, L/P activity at 0.2 μg/mouse, and Ca activity at 0.01 mg/kg dose. TP_1 showed a PFC activity at a dose of 1 μg/mouse, but the value was insignificant at a dose of 0.5 μg/mouse. The values were significant for L/P activity at a dose of 0.2 μg/mouse and for Ca activity at 0.005 mg/kg dose. The gel chromatography product G_{6B}–4 gave significant values for PFC activity at 20 μg/mouse, for L/P activity at 1 μg/mouse, and for Ca activity at 0.1 mg/kg. TP_2 gave significant values for PFC activity at 1 μg/mouse, for L/P activity at 0.1

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14) B.N. Bachra, A. Dauer, and A.E. Sobel, *Clin. Chem.*, 4, 107 (1958).

Table II. PFC, L/P, and Hypocalcemic Activities of the Fractions obtained from Thymus Extract

Material injected	PFC activity (ICR mice) No. of animals N	Dose (μg/mouse)	No. of PFC/10^6 cells Mean ± S.E. Upper: Sample Lower: Control	L/P activity (Swiss-Webster mice) No. of animals N	Dose (μg/mouse)	Ratio Mean ± S.E. Upper: Sample Lower: Control	Hypocalcemic activity (rabbits) Dose (mg/kg)	Percent decrease in serum Ca Mean ±S.E. (N=6)
T-1	7	50	138.66±31.49[a]	4	20	2.56±0.10[a]	10	8.9±0.9[a]
	6		3.92± 0.22	5		1.27±0.07		
	5	20	44.68± 9.26	3	10	2.11±0.17	5	7.3±1.1
	4		25.91±14.09	3		1.66±0.06		
T-1.A-15	5	20	463.67±35.05[a]	4	10	3.10±0.30[a]	1	14.8±2.2[a]
	4		27.10± 3.82	4		1.80±0.10		
DE-I	6	10	35.11±10.20	3	5	1.21±0.17	0.5	5.8±0.8
	5		17.24± 2.60	3		1.06±0.06		
DE-II	5	10	38.14± 8.53	4	5	1.86±0.13	0.5	3.7±1.8
	3		30.25± 3.68			1.20±0.07		
DE-III	4	10	209.34±73.85[b]	3	5	2.37±0.24[a]	0.5	9.9±1.9[a]
	4		30.25± 3.60	3		1.26±0.06		
DE-IV	4	10	142.85±43.07[b]	3	5	2.43±0.17[a]	0.5	9.0±1.4[b]
	5		10.21± 1.55	3		1.48±0.05		
G_{4B}.G_{6B}-2	5	5	24.91± 3.12[a]	5	0.2	2.74±0.08[a]	0.01	19.1±1.8[a]
	4		5.05± 1.03	5		1.44±0.09		
TP_1	7	1	48.28±18.39[b]	4	0.2	2.09±0.27[b]	0.005	8.3±1.4[b]
	6		6.85± 1.91	4		1.26±0.09		
	5	0.5	28.74±18.97	4	0.1	1.95±0.09		
	4		6.60± 2.04	4		1.93±0.10		
G_{6B}-4	6	20	73.97±13.65[b]	6	1	3.17±0.22[a]	0.1	11.3±1.4[a]
	6		29.79± 2.51	5		2.37±0.10		
TP_2	5	5	144.90±37.60[a]	5	0.1	3.09±0.14[a]	0.05	8.0±0.6[a]
	5		10.33± 1.09	4		1.52±0.10		
	8	1	75.17±13.69[b]				0.025	5.1±1.2
	7		9.33± 1.99					
	4	0.5	9.37± 1.74					
	4		5.74± 1.44					
B.S.A.[c]	5	20	14.94± 3.55	4	20	1.64±0.08	1	6.3±0.9
	6		8.25± 1.56	5		1.61±0.05		
							4	5.5±0.9

a) Significantly different from control $p<0.01$.
b) Significantly different from control $p<0.05$.
c) Bovine serum albumin.
T-1; acetone-dried powder.
T-1.A-15; 15% $(NH_4)_2SO_4$ fraction.
DE-I—DE-IV; chromatography of 15% $(NH_4)_2SO_4$ fraction on DEAE-cellulose.
G_{4B}·G_{6B}-2; gel chromatography of DE-III on Sepharose 4B and 6B.
TP_1; preparative polyacrylamide gel electrophoresis of G_{4B}·G_{6B}-2.
G_{6B}-4; gel chromatography of DE-IV on Sepharose 6B.
TP_2; preparative polyacrylamide gel electrophoresis and gel electrophoresis of G_{6B}-4.

μg/mouse, and for Ca activity at 0.05 mg/kg dose. Bovine serum albumin did not give any significant values for PFC activity and L/P activity at 20 μg/mouse, and for Ca activity at 4 mg/kg dose.

Discussion

The PFC activities of both TP_1 and TP_2 were effective at a dose of 1 μg/mouse, and hence their potencies were enhanced by about 50 times that of the acetone-dried powder (Table II). The L/P activity of TP_1, effective at 0.2 μg/mouse, was lower than that of TP_2 which

was effective at 0.1 μg/mouse, and the potencies of TP_1 and TP_2 were enhanced by about 100 times and 200 times, respectively, over the acetone-dried powder (Table II). On the other hand, the Ca activity of TP_1, effective at 0.005 mg/kg, was higher than that of TP_2, 0.05 mg/kg, in contrast to the case of L/P activity, and the increments of activity of TP_1 and TP_2 were about 2000 times and 200 times, respectively, compared with that of the original powder. However, a fair difference was found among the rates of three kinds of the promoting activity. For this reason, it is considered that 1) both factors have originally retained the different levels of the three activities, and 2) the crude material, such as acetone-dried powder, was mixed with some other factors which were separated from the factors by fractionations. The difference in assay methods utilized including the sensitivity of response varied with the strain of animal may be an additional reason. The partially purified preparations, obtained in the course of purification, showed lower immune activity compared with both factors. Thus the increases in both activities of PFC and L/P seem to be dependent roughly on the increase in Ca activity of the preparations. Both the fractions DE–I and DE–II having no significant Ca activity showed also no activity in the assays on immunity at the doses administered. Bovine serum albumin was examined as a control, but any activity was not found, that is, Ca activity was insignificant at a dose of 4 mg/kg, 80 to 800 fold over the doses of TP_2 or TP_1, L/P activity at 20 μg/mouse, 100 to 200 fold over the dose of TP_1 or TP_2, and PFC activity at 20 μg/mouse, 20 fold over the dose of TP_1 or TP_2. On the basis of these facts, the substances lacking in Ca activity appear to be ineffective on immunity. Nevertheless, we hesitate to ignore the presence of substance with immune activities but without Ca activity. At any rate, it was revealed that both the hypocalcemic substances TP_1 and TP_2 have PFC and L/P activities though their potencies are somewhat different from each other.

LSHh showed L/P activity at 0.1 μg/mouse,[5] comparable to that of TP_2, but LSHr has a lower activity.[15] PFC activity was observed in 5 out of 9 mice of the experimental group in a dose of 0.1 μg/mouse,[15] but the results can hardly be compared with our results because of the different method of evaluation. Molecular weight of TP_1 was found to be 68000 and that of TP_2 as 57000 from SDS-polyacrylamide gel electrophoresis.[7,16] The molecular weight of LSHr is reported to be 79950[17] and that of LSHh as 17000.[5] The amino acid composition of LSHr was found to be different from either TP_1[7] or TP_2 (Mizutani, *et al.*, unpublished results). Both TP_1[18] and TP_2 are heat-labile proteins (56°, 30 min),[19] while LSHr is heat-stable (60°, 30 min).[17] Thymosin has a molecular weight of 12000 and is stable on heating,[6] and is apparently different from the products we obtained. Ca activities of LSH and Thymosin have never been reported. Administration of LSH resulted in marked splenomegaly,[15] but the change was slight in the treatment with TP_1 or TP_2.

It is known that an endotoxin causes a leukocytosis, hypocalcemia,[20] and enlargement of spleen and liver due to their swelling,[21] but Hand, *et al.*[15] reported that the injection of the endotoxin in newborn mice did not promote their PFC response. There was a fear that the preparations of TP_1 and TP_2 were contaminated with the endotoxin because they had Ca activity, but the endotoxin was not detected in the preparations, as tested by the method stipulated in Japanese Pharmacopoeia (dose: 0.01 mg/kg)[22] for TP_1 and by the Pregel method,[20] said to be an extremely sensitive method using the lysate from Amebocyte of *Tachy-*

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16) A. Mizutani and S. Hayase, *Abstr. Papers 94th Annu. Meet. Pharm. Soc. Japan*, p. 61 (1974).
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21) M. Tamakuma, K. Ishiyama, and M. Usui, *Naika*, **30**, 629 (1972).
22) Japan Pharmacopoeia Ed. VIII, Part 1, Explanatory Notes, B-160, Hirokawa Shoten, Tokyo (1971).

pleus tridentatus (Pregel: a product of Teikoku Hormone Mfg. Co.) for TP_2. These facts suggest that the Ca activities of TP_1 and TP_2 are not due to the endotoxin. Consequently, both TP_1 and TP_2 were found to be the hypocalcemic substances promoting immune competence, different from LSHr, LSHh, or Thymosin, but the relationship between immune activity and Ca activity still remains obscure at present.

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