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Effect of Parotid Gland Hypocalcemic Factor on Increasing Antibody-producing Cells in Mice[1]

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The hypocalcemic substance isolated from bovine parotid gland and the fractions obtained in process of purification were examined for increasing antibody-producing cells (PFC activity) in the spleen of ICR mice by the method of Jerne and Nordin, and for lymphocyte-increasing action in mice. The intraperitoneal injection of the final purified sample in a dose of 10 μg/mouse produced 79.20±15.73 of PFC number per 10^6 cells in the spleen of neonatal mice. The difference between the mean of PFC number in the test group and that in the control group was significant at 5% level of significance. The final purified product was increased in PFC activity by 10 times that of the acetone-dried powder (starting material). A tendency to the increase in PFC activity and in lymphocytes increasing activity was found in proportion to the increase in hypocalcemic activity. Therefore, it was found that there was an action of promoting immune competence in the hypocalcemic substance. Further, the immunoactivities of parotid hypocalcemic substance were discussed in comparison with the thymic hypocalcemic substances.

Keywords——parotid gland; hypocalcemic protein; lymphocyte; parotin; leucocyte; immune competence; purification; plaque forming cell (PFC); lymphocyte-increasing action; antibody-producing cells

It was reported that a hypocalcemic substance was isolated from the bovine parotid gland extracts.[3] The hypocalcemic substance was the protein of 48000 in molecular weight, of 54% in α-helix content by circular dichroism (CD) spectra,[4] and of having presumably a rigid structure. It was also shown that the hypocalcemic response of the substance was retarded as compared with that of calcitonin on the basis of the time course of the response.[5] On the other hand, it was found that the hypocalcemic substance obtained from bovine thymic extracts increased in the lymphocytes/polymorphs ratios (L/P activity) in peripheral blood of neonatal mice.[6] Based on the facts that parotin obtained from bovine parotid gland stimulated the mesenchymal system[7] and also had hypocalcemic activity, we have studied on the L/P activity of the parotid hypocalcemic substances.[8] The intraperitoneal injection of the purified sample in a dose of 0.5 μg/mouse elevated L/P ratio to 3.84 in neonatal mice. Therefore, we suggested that it might have an ability in promoting immune competence. However, L/P activity is not the direct evidence for promoting immune competence, thus in the present experiment we further examine for increasing antibody-producing cells (PFC activity) by the method of Jerne and Nordin.[9] Having examined the L/P, hypocalcemic, and PFC activities, we studied on their interrelationship, and discussed the parotid substance in comparison with the thymic hypocalcemic substances (TP_1, TP_2).[6]

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Materials and Methods

Materials——The materials used were a product purified from the bovine parotid gland extracts and the fractions obtained during the steps of purification.[3] The methods of purification are outlined in Figs. 1 and 2 including the marks of products. Percent yields of the fractions obtained from each step of purification are given in the flow sheets.

The fraction precipitating at pH 5.4 from the aqueous extract of bovine parotid gland was prepared as an acetone-dried powder (BC-20). This powder was extracted with physiological saline and the fraction precipitated from the extract at the concentration of 7—15% of ammonium sulfate (BP-20) was collected. Fraction BP-20 was further fractionated into four fractions (D-I, D-II, D-III, and D-IV) by the chromatography on diethylaminoethyl (DEAE)-cellulose. Fraction D-I was eluted with the first buffer (0.05 M phosphate buffer, $\mu=0.13$, pH 7.38), Fraction D-II with the second buffer (0.05 M phosphate buffer+0.15 M NaCl, $\mu=0.28$, pH 7.28), Fraction D-III with the third buffer (0.05 M phosphete buffer+0.27 M NaCl, $\mu=0.40$, pH 7.14), and Fraction D-IV with the fourth buffer (0.05M phosphate buffer+0.47 M NaCl, $\mu=0.60$, pH 6.10). Fraction D-III had the most potent hypocalcemic activity and was the highest yield of these four fractions, then it was subjected to further purification by gel filtration on Sepharose 6B (0.05 M phosphate buffer,

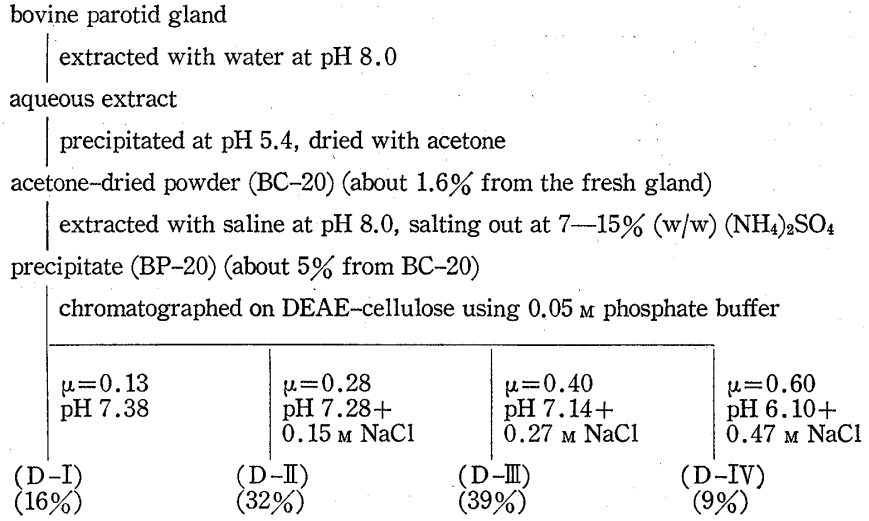

Fig. 1. Fractionation of Bovine Parotid Extract by Salting out with $(NH_4)_2SO_4$ and by Chromatography on DEAE-cellulose Column

The yield of the fraction is expressed in term of percent yield to the last fraction at each step of purification.

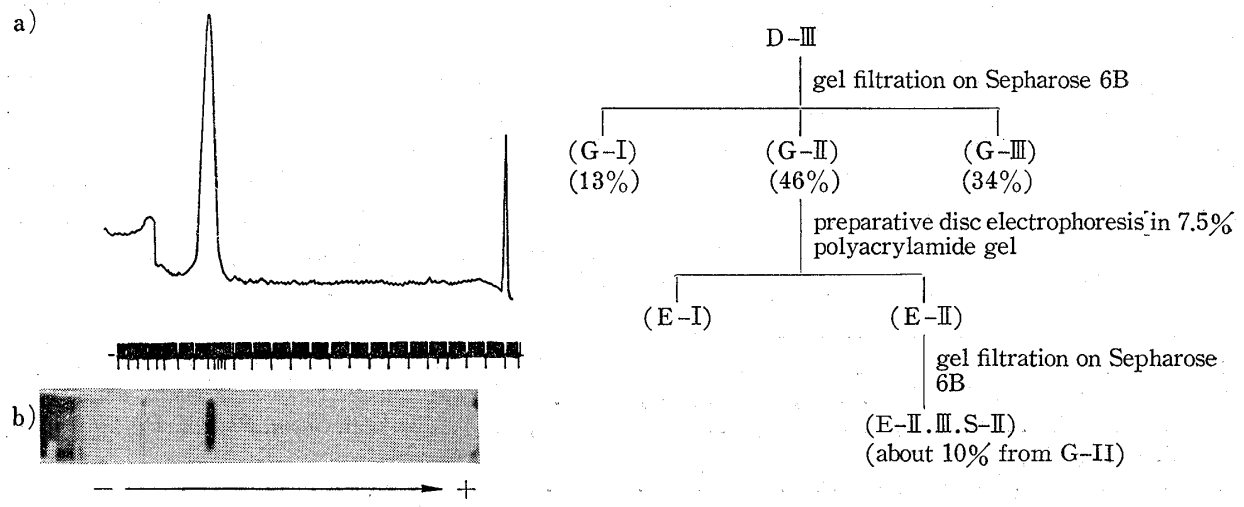

Fig. 2. Purification of D-III by Gel Filtration and Preparative Disc Electrophoresis (7.5% gel, pH8.9)

The percent yields were estimated on the basis of last intermediate fraction.
a) Densitometric tracing of disc electropherogram of the purified sample (E-II.III.S-II).
b) Disc electrophoresis of the purified sample (E-II.III.S-II).

μ=0.13, pH 7.38), and thereby Fraction G-II eluted at Kav=0.3 was obtained. Fraction G-II was purified by preparative disc electrophoresis (7.5% gel, pH 8.9) and successive re-gel filtration on Sepharose 6B to obtain Fraction E-II·III·S-II, giving a single band (Fig. 2) in analytical disc electrophoresis (7.5% gel, pH 8.6). Ovalbumin (SIGMA CHEMICAL COMPANY) was used as a material for control experiment.

Measurements of Biological Activities——1) PFC Activity: The Jerne and Nordin method[9] was employed with a modification by Ceglowski, *et al.*[10,11] The littermates of neonatal mice of ICR strain were divided into two groups. At 6—12 hr after birth, one group of the mice was injected with the sample intraperitoneally and the other group was given physiological 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 cell suspension prepared from the spleen was used for the developing PFC in the usual way. The resulting plaques of hemolysis 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.*[12] based on the original method of Metcalf[13] was used. Experimental animals were newborn Swiss-Webster strain mice within 6—12 hr after birth. The litter was divided into two groups; one group was given test sample and the other physiological saline as a control, both by intraperitoneal injection. Blood was drawn from the tail 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 lymphocytes to polymorphs (L/P) was calculated.

In both the test and the control group, the increments of the L/P ratios at 6, 10, and 14 days after the injection against the L/P ratio before the injection were calculated, and the difference in the mean of the increment between test and control group was examined by the *t*-test on each day bled according to the method in our previous paper.[8] If one of these blood samples showed a significant difference at the 5% probability level, the sample tested was considered to be effective.

3) Hypocalcemic Activity: Hypocalcemic activity was assayed by the method described previously.[14] Groups of six normal mature male rabbits, deprived of food for about 24 hr, were used. The saline solution of a sample 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 amount of serum calcium was determined by atomic absorption spectrophotometer.

Out of three serum samples taken after the injection, the maximum percent decrease in serum calcium was taken. The difference between the mean of the test group and that of the control group injected with physiological 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

The PFC and hypocalcemic activities of the fractions of each purification steps are given in Table I. For the sake of comparison, the maximum L/P ratios obtained among each set of three values for 6, 10, and 14 days are also shown in Table I. The upper rows present the mean values of samples, and the lower rows that of controls in both the cases of PFC and L/P activities. The administration of the acetone-dried powder in a dose of 100 μg/mouse gave 111.48/10^6 cells PFC number, and the effect was significant at the 1% probability compared to the control group. At the same dose of the sample L/P activity was significant. Hypocalcemic activity was significant in a dose of 50 mg/kg in rabbits. Fraction BP-20 (precipitate at 7—15% $(NH_4)_2SO_4$) caused the significant effect on PFC activity in a dose of 80 μg/mouse, and produced significant value 2.96 of L/P ratio in a dose of 10 μg/mouse. Both the fractions D-I and D-II were ineffective on PFC activity in a dose of 40 μg/mouse. The L/P and hypocalcemic activities of D-I and D-II were also insignificant in a dose of 5 μg/mouse and 0.5

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Table I. PFC, L/P, and Hypocalcemic Activities of the Fractions Obtained from Parotid Extract

Materials 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)	L/P ratio Mean ± S.E. Upper: sample Lower: control	Hypocalcemic activity (Rabbits) Dose (mg/kg)	Percent decrease in serum Ca Mean ± S.E. ($N=6$)
BC-20	4	100	111.48± 9.86[a]	7	100	2.45±0.16[b,c]	50	9.89±0.88[a]
	4		10.50± 0.81	5		1.78±0.13		
BP-20	7	80	86.72±15.72[a]	5	10	2.96±0.33[b,c]	1	8.01±0.71[b]
	6		11.99± 5.37	4		2.01±0.16		
D-I	5	40	26.92± 9.09	5	5	0.90±0.10[d]	0.5	2.30±1.35
	5		18.82± 1.91	5		0.97±0.10		
D-II	4	40	50.48±16.31	6	5	1.24±0.09[d]	0.5	3.59±1.41
	5		18.82± 1.91	5		1.24±0.15		
D-III	6	40	58.02±14.68[b]	7	5	4.39±0.43[a,c]	0.5	7.12±0.95[b]
	6		19.48± 8.58	6		2.30±0.34		
D-IV	4	40	154.38±26.54[a]	6	5	3.15±0.21[a,c]	0.5	7.44±1.47[b]
	5		47.38± 7.51	5		2.05±0.11		
G-II	5	20	41.19± 5.06[a]	4	2.5	3.49±0.25[a,c]	0.1	7.38±0.64[b]
	4		6.60± 2.03	4		1.97±0.23		
E-II.III.S-II	4	10	79.20±15.73[b]	4	0.5	3.84±0.19[a,c]	0.03	7.07±0.80[b]
	4		10.35± 0.59	5		2.21±0.28		
	6	5	31.47± 2.79	3	0.1	1.90±0.18[c]	0.01	3.65±1.20
	5		19.08± 1.21	3		1.70±0.16		
Ovalbumin	5	20	15.98± 6.95	5	20	1.22±0.03[d]	1	3.12±1.16
	4		18.20±12.73	4		1.18±0.06		
							4	4.81±1.20

a) Significantly different from control, $p<0.01$.
b) Significantly different from control, $p<0.05$.
c) The data were obtained at 14 days after birth.
d) The data were obtained at 10 days after birth.

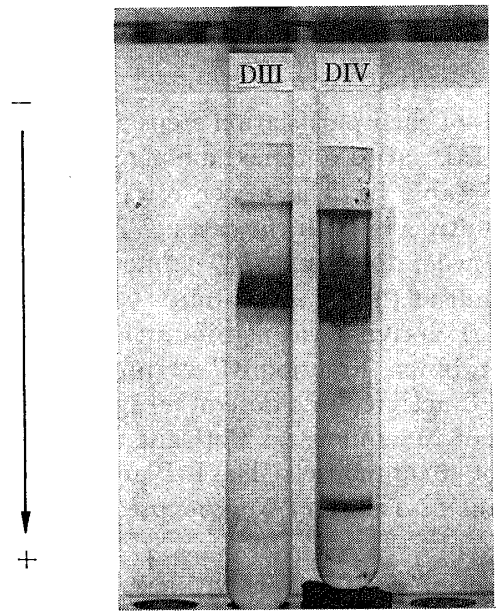

Fig. 3. Disc Electrophoresis of D-III and D-IV

The electrophoresis was carried out with use of 200 μg of the sample, 7.5% gel for separation (pH 8.9), Tris-glycine buffer (pH 8.6, $\mu=0.05$), and Amido black 10B for staining.

mg/kg, respectively. The PFC, L/P, and hypocalcemic activities of both D-III and D-IV were significant in a dose of 40 μg/mouse, 5 μg/mouse, and 0.5 mg/kg, respectively. The results of analytical disc gel electrophoresis of D-III and D-IV are shown in Fig. 3. The PFC activity of G-II obtained by gel filtration was found in a dose of 20 μg/mouse. The L/P and hypocalcemic activities of G-II were also significant in a dose of 2.5 μg/mouse and of 0.1 mg/kg, respectively. The PFC activity of the purified product, E-II.III. S-II, was significant in a dose of 10 μg/mouse but insignificant in a dose of 1—5 μg/mouse. The L/P activity of the purified product was also found in a dose of 0.5 μg/mouse. The hypocalcemic activity of the fraction was significant in a dose of 0.03 mg/kg. The results of analytical disc electrophoresis and the densitometric tracing are shown in Fig. 2. In the case of ovalbumin, both the activities of L/P and PFC were undetected in a dose of 20 μg/mouse, and hypocalcemic activity also not in a dose of 4 mg/kg.

Discussion

In the earlier studies, we reported that the L/P activity of the parotid products rose approximately in parallel with the rise of hypocalcemic activity accompanied by progress in purification, and suggested that both activities may be involved in the same protein component. In this paper, we studied whether the purified hypocalcemic substance and the by-product obtained during the purification steps had PFC, L/P, and hypocalcemic activities. Fraction D-III and D-IV obtained by the chromatography on DEAE-cellulose had potent hypocalcemic and immune response promoting activities. Fraction D-IV contains the main component in Fraction D-III as shown in Fig. 3, and thereby Fraction D-IV may be effective on hypocalcemic activity. However, the yield of D–IV was lower than that of D–III as described in our previous paper, thus further purification was given up.

In view of the increase in activities induced by the purification, it was found that the increase in L/P and PFC activities followed the increase in hypocalcemic activity. The hypocalcemic activity of the purified product raised by about 1700 times that of acetone-dried powder and L/P activity by 200 times. However, the rise of PFC activity was by 10 times and this value was lower than that of L/P and hypocalcemic activities, even if these values obtained by present assay are not absolute but rough estimate. The differences in the rise of PFC and L/P activities must be caused by the heterogeneity of the strain of mice used or the variations in sensitivity of the response on which the assays based. Moreover, the differences may be in response to the following facts that L/P activity based on the changes in the total number of lymphocytes in peripheral blood (T-cell was 50—70% of total lymphocyte), whereas PFC activity was attributed to the changes in the number of IgM producing cell in B-cell localized in spleen, therefore the differences in the rises of both activities depend on the influence of circulating T-cell[15] on the PFC (B-cell). Meanwhile, we suggested in our previous paper the possibility of the presence of a hypocalcemic substance lacking any L/P activity or a substance having opposite nature. LSHh, purified from thymus extracts by Luckey, *et al.*, had a high activity of L/P, but low of PFC, and LSHr oppositely behaved. In the case of purification of the parotid hypocalcemic substance, it is possible that the additional substances having potent PFC activity were contained in crude preparation, and the substances were excluded out and discarded.

We reported that two substances (TP_1 and TP_2) having potent hypocalcemic activity were isolated from bovine thymus extracts in our laboratory and they showed both L/P and

Table II. Comparison of Bovine Parotid and Bovine Thymic Substances

Materials	Mol. wt.	PFC activity (ICR mice)		L/P activity (S.W. mice)		Ca activity (Rabbits)	
		Dose (μg/mouse)	No. of PFC/10^6 cells Mean ± S.E.	Dose (μg/mouse)	L/P ratio Mean ± S.E.	Dose (mg/kg)	Percent decrease in serum Ca Mean ± S.E.
E-Ⅱ.Ⅲ.S-Ⅱ	48000	10	79.20±15.73[a] (×10)	0.5	3.84±0.19[b] (×200)	0.03	7.07±0.80[a] (×1700)
TP_1	68000	1	48.28±18.39[a] (×50)	0.2	2.09±0.27[a] (×100)	0.005	8.3 ±1.4[a] (×2000)
TP_2	57000	1	75.17±13.69[a] (×50)	0.1	3.09±0.14[b] (×200)	0.05	8.0 ±0.6[b] (×200)

a) Significantly different from control, $p<0.05$.
b) Significantly different from control, $p<0.01$.
The × number parenthesized represents roughly the number of times by which the potency of sample rose over that of acetone-dried powder (BC-20 or T-1).

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PFC activities.[6),16)] Therefore, the activities of TP_1, TP_2, and purified parotid product E-II. III.S-II are summarized in Table II for comparison. The parotid product showed a significant effect on PFC activity in a dose of 10 μg/mouse and the number of PFC produced was 79.20/10^6 cells, though TP_1 and TP_2 also significantly effected in a dose of 1 μg/mouse, and gave 48.28 and 75.17/10^6 cells of PFC, respectively. On the other hand, PFC activity was not found in purified parotid preparation in a dose of 5 μg/mouse, hence the activity was lower than that of thymus. The purified parotid product produced 3.84 of L/P ratio and the activity was significant at the 1% probability in a dose of 0.5 μg/mouse but L/P activity was insignificant in a dose of 0.1 μg/mouse, though the thymic substance was significantly effective at the same dose. On the basis of these results, it is considered that L/P activity of the parotid product was lower than that of thymic products which was effective in a dose of 0.1 or 0.2 μg/mouse. The administration of the purified parotid product significantly lowered the serum Ca level by 7% of the initial level in a dose of 0.03 mg/kg, whereas the thymic products were also significantly effective at the similar dose of 0.005—0.05 mg/kg. In comparison with LSH which was isolated from calf thymus by Luckey, *et al.*, the PFC and L/P activities of the parotid product was comparably lower even if definite comparison was impossible.[17)] At any rate, these observations indicate that the pure parotid hypocalcemic substance possesses the ability in promoting immune competence. The facts that the parotid product stimulated the lymphocytes originated from the mesenchymal system supported the theory of that parotin stimulates the mesenchymal system.[7)] However, the relation of hypocalcemic and immunological activities has not been established.

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