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Antitumor Activity of *Bacillus natto*. II.[1] Formation of Cytolytic Substances on Ehrlich Ascites Carcinoma in *Bacillus natto* KMD 1126[2]

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Bacillus natto KMD 1126 suspension had not remarkable cytolytic activity on Ehrlich ascites carcinoma cells. But when the bacterial suspension was preincubated in the buffer at 37° for 2 hr, cytolytic substances were found out of the cells. These results were observed by eosin stain method, measurement of cell injuring activity and *in vitro–in vivo* test. It was observed that the formation of cytolytic substances were intimately related with bacterial growth phase, and bacterial cells at logarithmic phase and early stationary phase made much amounts of the substances, and cells at lag phase could not form it. On the other hand, it was observed that there are at least two kind of cytolytic substances in the preincubation mixture by Sephadex G 25 gel filtration. One, which is high molecule, had cytolytic and hemolytic activities, but the other, which is low molecule, had cytolytic activity only.

In previous paper,[1] the authors reported that a strain of *Bacillus natto* (tentatively called KMD 1126), which was newly isolated from "Natto" (fermented beans), has a contact antitumor effect on solid type Ehrlich carcinoma cells. Also it was reported that there are several strains in *Bacillus natto* which have different antitumor activity by *in vivo* test.[4] As the continuation of this work, it was now of interest to examine whether this bacteria have direct cytolytic action on Ehrlich ascites carcinoma cells, or not.

With regard to *in vitro* contact test for cytolytic substance, Kikuchi and Matsuzawa[5] reported that eosin stain method is useful for judgement of life or death of tumor cells. Shimizu, *et al.*[6] reported that when tumor cells and bacterial suspension, which has cytolytic activity, were incubated in buffer, absorbance at 260 mμ increased in the buffer by tumor cell injure and this method was easier and useful for screening of cytolytic substance. Also it was observed by Mizuno, *et al.*[7] that the delay in tumor development occurred in animals inoculated with tumor cells which had been incubated *in vitro* with cytolytic substance.

In our experiments, these techniques were employed for screening method of cytolytic antitumor effect. As the results, it was found that *Bacillus natto* KMD 1126 suspension had no remarkable cytolytic effect, but cytolytic substance was formed when the suspension was incubated at 37° for 2 hr in the buffer. The present paper deals with the results of these experiments and condition of the formation of cytolytic substance on Ehrlich ascites carcinoma cell in *Bacillus natto* KMD 1126.

1) Part I: Y. Kameda, S. Kanatomo, Y. Kameda, and Y. Saito, *Chem. Pharm. Bull.* (Tokyo), **16**, 186 (1968).
2) This work was reported at 91st Annual Meeting of Pharmaceutical Society of Japan, Fukuoka, April 1971.
3) Location: *13-1, Takaramachi, Kanazawa.*
4) Y. Kameda and S. Kanatomo, Abstracts papers, The 88th Annual Meeting of Pharmaceutical Society of Japan, Tokyo, April 1968, p. 431.
5) K. Kikuchi and M. Matsuzawa, *J. Antibiotics* (Tokyo) Ser. B, **8**, 170 (1955).
6) R. Shimizu, N. Nishita, K. Banto, S. Koshimura, A. Hayashi, and T. Kobayashi, "Annual Report of the Research Institute of Tuberculosis Kanazawa Uinversity," **22**, 27 (1964); M. Hatano, R. Shimizu, O. Morita, and T. Yamagishi, *Medicine and Biology*, **74**, 293 (1967).
7) D. Mizuno, O. Yoshioka, M. Akamatsu, and T. Kataoka, *Cancer Res.*, **28**, 1531 (1968).

Material and Method

Animal——Female ICR-SLC mice, weighing 18—22 g, were used for all experiments. All animals were fed on standard laboratory diet and given water *ad libitum.*

Tumor——Ehrlich ascites carcinoma was maintained by weekly intraperitoneal transplantation in ICR-SLC mice.

Buffer——Buffer, containing 8 g of NaCl, 0.4 g of KCl, 0.06 g of $Na_2HPO_4 \cdot 12H_2O$, 0.06 g of KH_2PO_4, 0.14 g of $CaCl_2$, and 0.2 g of $MgSO_4 \cdot 7H_2O$, and adjusted to pH 7.2 with $NaHCO_3$, was used for all experiments.

Bacterial Strain and Culture——*Bacillus natto* (*B. natto*) KMD 1126 is the stock strain kept in this laboratory on nutrient agar slants. Culture was carried out by the following manner. To 3 liters shaking flasks, 800 ml of bouillon (pH 7.2) were added, sterilized in an autoclave under 1.0 kg/cm^2 pressure for 20 min, and inoculated with 50 ml of seed culture of the bacteria, which was prepared by shaking culture of the organisms at 37° for 15 hr in the same medium. The culture was carried out at 37° with reciprocal shaking (130 c/min, strock 7 cm) for 6 hr.

Formation of Cytolytic Substance by Preincubation in the Buffer——Living *B. natto* KMD 1126, derived from 800 ml of a 6 hr culture broth, were washed twice with the buffer by centrifugation and suspended in 40 ml of the buffer. This suspension was added in 200 ml of shaking flask, and incubated at 37° for 2 hr with shaking. The resultant mixture was centrifuged at 10000 rpm for 10 min to obtain clear supernatant solution.

Disruption of Bacterial Cells——Bacterial cells were disintegrated by sonication. About 7 g of wet cells were washed twice with the buffer by centrifugation, suspended in 40 ml of the buffer and subjected to sonic occillation (Tominaga Co. Type UR 150 P, power 4) for 10 min. The resultant sonicate was centrifuged at 15000 rpm for 15 min to remove cell wall fragments and unbroken cells.

Preparation of Tumor Cell Suspension——The ascites fluid, aspirated from mice bearing 6—9 day old Ehrlich ascites carcinoma by sterile syringe, was suspended in chilled buffer and centrifuged at 700 rpm for 3 min. The sedimented tumor cells were washed twice with chilled buffer and resuspended in appropriate volume of the buffer and then the tumor cell count was made in a hemocytometer by the standard method for blood leucocytes. The tumor cell suspension was further diluted, if necessary, to give a desired cell concentration (usually $2.5—3.5 \times 10^7$ cells/ml).

***In Vitro* Contact Test of Cytolytic Activity**——1) Eosin Stain Method: The reaction mixture containing 0.2 ml of tumor cell suspension (2×10^7 cells/ml) and 0.2 ml of bacterial suspension or preincubation mixture was incubated in water bath at 37° for 2 hr. One drop of 0.05% eosin in the buffer was added to this mixture and total tumor cells and red stained cells were counted under a microscope. Activity was expressed by percentage of red-stained cells in total cells.

2) Cell Injuring Reaction: The reaction mixtures, containing 0.2 ml of tumor cell suspension and 0.2 ml of appropriately diluted samples, were incubated in water bath at 37° for 2 hr. After incubation they were diluted with 3.6 ml of the buffer, and centrifuged at 3000 rpm for 10 min. Absorbance of the supernatant was measured at 260 mμ after 10 fold dilution (HITACHI Model 101 Spectrophotometer). At the same time, 2 control experiments were run using following 2 mixtures and absorbance of the controls was deducted from that of sample. 1) A mixture of 0.2 ml of tumor cell suspension and 0.2 ml of the buffer. 2) A mixture of 0.2 ml of the sample and 0.2 ml of the buffer. One unit of the cell injuring activity was defined as the amount of cytolytic substance which gave an E_{260} reading of 1 under above conditions. Therefore, total activity was expressed as follows.

Total Activity (unit) $= E_{260} \times$ Dilution $\times 5 \times$ Volume

3) *In Vitro-In Vivo* Test: The reaction mixture containing 0.2 ml of tumor cell suspension (3×10^7 cells/ml) and 0.2 ml of the bacterial suspension or preincubation mixture to be tested was incubated in water bath at 37° for 2 hr. The incubation mixture was diluted with 3.6 ml of the buffer and centrifuged at 1000 rpm for 3 min. Three ml of the resultant supernatant was discarded to remove the bacterial cells. The washing was repeated once more and the remainder was used for intraperitoneal inoculation into mice (1 ml for each mice). In the case of supernatant of preincubation mixture, incubation mixture was inoculated intraperitoneally without washing. At the same time, 1 control experiment was run using the buffer solution instead of sample. Animals which died during the experiment were examined macroscopically for the cause of death, and animals still alive 50 days after inoculation were sacrificed and autopsied.

Hemolytic Test[8]——The incubation mixture containing 0.5 ml of human red blood cells (3% suspension in the buffer) and 0.5 ml of sample was placed in a water bath at 37° for 1 hr, diluted with 4.0 ml of the buffer and centrifuged at 3000 rpm for 5 min to remove the unhemolyzed cells. A control experiment was run in parallel with above measurement using a mixture of 0.5 ml of human red blood cells and 0.5 ml of the buffer. The degree of hemolysis was estimated by comparison of the absorbance at 562 mμ of the

8) A.W. Bernheimer, *J. Exp. Med.*, **80**, 309 (1944).

supernatant fluid in the test tube with that due to total hemolysis. Total hemolysis was obtained by incubation of 0.5 ml of human red blood cells with 4.5 ml of distilled water. The hemolytic unit is the amount of hemolysin which liberated one half of the total hemoglobin contained in 0.5 ml of the red blood cell suspension.

Sephadex G 25 Gel Filtration——Sample was introduced into a column of Sephadex G 25 (2.0×35 cm) equilibrated with the buffer and filtration through the gel bed was performed with the same buffer. The effluents were fractionated in 5 ml portion at a rate of approximately 150 ml per hr. Absorbances of the fractions were determined spectrophotometrically at 280 mμ. Cell injuring and hemolytic activities of each fractions were also measured.

Result

In Vitro Contact Test of Cytolytic Activity with Eosin Stain Method

As shown in Table I, *B. natto* KMD 1126 suspension had low cytolytic activity against Ehrlich ascites carcinoma cells as demonstrated by low percentage of red stained cells. However, if we used the bacterial suspension after preincubation in the buffer at 37° for 2 hr, more cells were stained red. Also, it was observed that morphological change of tumor cells and reduction of tumor cell number occurred. These results suggest that *B. natto* KMD 1126 had no cytolytic antitumor activity, but when it was preincubated in the buffer at 37°, cytolytic substance was formed.

Table I. Assay of Cytolytic Activity of *Bacillus natto* KMD 1126 Suspension and Preincubation Mixture by Eosin Stain Method

	Percentage of eosin stained cells
Control	2— 6
B. natto KMD 1126 suspension	8—20
Preincubation mixture	60—90

In Virto Contact Test of Cytolytic Activity with Cell Injuring Reaction

As shown in Table II, *B. natto* KMD 1126 suspension had low cell injuring activity against Ehrlich ascites carcinoma cells. However, cell injuring activity of the bacterial suspension began rapidly to increase with preincubation at 37° in the buffer. That is, the formation of cytolytic substance was observed with this method similar to eosin stain method, when the organisms were preincubated in the buffer at 37° for 2 hr.

Table II. Assay of Cytolytic Activity of *Bacillus natto* KMD 1126 Suspension and Preincubation Mixture by Cell Injuring Reaction

	Cell injuring activity (u/ml)
B. natto KMD 1126 suspension	0.2—0.3
Preincubation mixture	3.1—4.2

In Vitro Contact Test of Cytolytic Activity by *in Vitro—in Vivo* Test

As shown in Fig. 1, all animals in the control groups died of tumor growth between 9 and 12 days after inoculation, indicating that incubation with the buffer at 37° for 2 hr is entirely harmless for tumor cells. In the group treated with bacterial suspension, 4 animals died of tumor growth between 12 and 15 days after inoculation, but 1 animal died after 30 days. In contrast to above groups, 3 animals died between 25 and 30 days but 2 animals alive 50

days after inoculation of tumor cells which had been incubated with the preincubation mixture. That is, distinct suppression of tumor growth was observed in the group treated with preincubation mixture. Cell injuring activities of the bacterial suspension and the preincubation mixture used in these experiments were 0.4 u/ml and 3.3 u/ml respectively. From these results, it is also suggested that *B. natto* KMD 1126 suspension had no cytolytic activity, but when it was incubated in the buffer at 37° for 2 hr, cytolytic substance was newly formed.

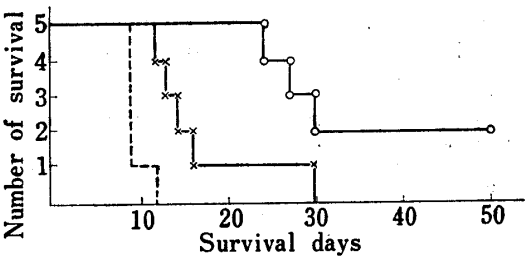

Fig. 1. Assay of Cytolytic Activity of *Bacillus natto* KMD 1126 Suspension and Preincubation Mixture by *In Vitro–In Vivo* Test

Mixture of Ehrlich ascites carcinoma cell suspension and the buffer (——), *B. natto* KMD 1126 suspension (×—×), or its preincubation mixture (O—O) were incubated at 37° for 2 hr. Then the tumor cells were washed twice with the buffer by centrifugation and the cells were inoculated intraperitoneally to mice.

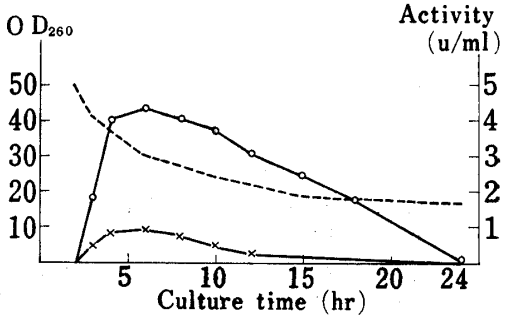

Fig. 2. Relation between Cultural Age and the Formation of Cytolytic Substance

Bacterial cells at desired time intervals grown in bouillon medium with shaking were centrifuged down, washed twice with the buffer and resuspended in the buffer. The formation of cytolytic substance (O—O), was measured by cell injuring reaction after preincubation at 37° for 2 hr. At the same time, hemolytic activity (×—×) and absorbance at 260 mμ (--------) of the supernatant of preincubation mixture were measured.

Relation between Cultural Age of *B, natto* KMD 1126 and Formation of Cytolytic Substance

The formation of cytolytic substance in the bacterial cells were measured at different cultural ages. That is, bacterial cells at desired time intervals grown in bouillon medium with shaking were centrifuged down, washed twice with the buffer and resuspended in the buffer so as to show the standard turbidity. The bacterial suspensions were placed in shaking tube and shaken at 37° for 2 hr. The formation of cytolytic substance was measured by cell injuring reaction. Fig. 2 shows cell injuring and hemolytic activities of preincubation mixture and absorbance at 260 mμ of the supernatant of preincubation mixture with various time cultured cells. At the same time, turbidity of cultures was measured with a photoelectric colorimeter at 610 mμ under the same condition (Fig. 3). From Fig. 2, it was observed that bacterial cells harvested from bouillon medium at 4, 6, and 8 hr culture formed cytolytic substance abundantly after suspended in the buffer followed by incubation with shaking. But cells taken at 2 hr could not form cytolytic substance. That is, cells at logarithmic growth phase (4 hr) and early stationary phase (6 and 8 hr) had an ability to form cytolytic substance in the buffer, and cells at lag phase (2 hr) had not. On the other hand absorbance at 260 mμ of the supernatant of preincubation mixture was increased during 2 hr shaking in the buffer and especially, the cells taken at lag phase showed the highest absorbance. That is, cell lysis occurred in the course of preincubation. The ability to form cytolytic substance was decreased relatively from 8 to 24 hr, and the cells taken at 24 hr had not the ability. In the following, bacterial suspension was heated at 80° for 10 min to destroy vegetative cells and then the resulting mixture was incubated in the buffr at 37° for 2 hr and measured cell injuring activity. But this suspension could not make cytolytic substance. It was demonstrated that there were live heat resistant spores in this suspension by colony count method after dilution and incubation using bouillon agar plate. That is, spore could not make cytolytic substance. This results agree with the result that 24 hr cultured cells could not make cytolytic substance.

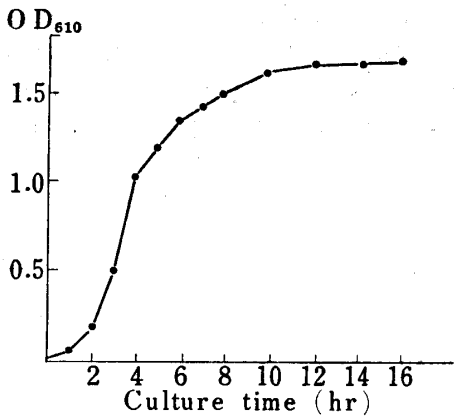

Fig. 3. Growth Curve of *Bacillus natto* KMD 1126

Turbidity of cultures was measured with a photoelectric colorimeter at 610 mμ under standard condition.

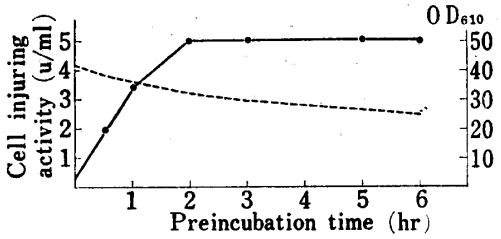

Fig. 4. Relation between Preincubation Time and the Formation of Cytolytic Substance

Cytolytic activities (●—●) in preincubation mixture were measured by cell injuring reaction withdrawn at hourly intervals with 6 hr cultured bacterial cells. Absorbances at 610 mμ (----------) of the preincubation mixture were also measured.

Relation between Preincubation Time and the Formation of Cytolytic Substance

Cytolytic activities in preincubation mixture were measured by cell injuring reaction withdrown at hourly intervals with 6 hr cultured bacterial cells. Also absorbances at 610 mμ of preincubation mixture were measured. As shown in Fig. 4, cell injuring activity was increased in proportion to preincubation time from 0 to 2 hr, but then the activity no more increased with longer preincubation time. On the other hand, absorbance at 610 mμ of the bacterial suspension was decreased with longer preincubation time. That is, cell lysis occurred in these period, but cytolytic substance was not formed. It was concluded from above experiments that 2 hr preincubation was enough to from cytolytic substance.

Cytolytic Activity in Cell Free Extract

As we have seen in above experiments, cytolytic substance was fromed in *B. natto* KMD 1126 when it was preincubated in the buffer at 37° for 2 hr. In order to obtain cell free cytolytic substance, bacterial suspension and its preincubation mixture were disintegrated by sonication and the resultant sonicates were centrifuged to remove cell wall fragments and unbroken cells. Cell injuring activities of these supernatantes were measured. As shown in Table III, *B. natto* KMD 1126 suspension (1126), its sonicate (So), and the supernatant of sonicate (SoS) had no remarkable cell injuring activities. However, *B. natto* KMD 1126 preincubation mixture (I), supernatant of the preincubation mixture (IS), sonicate of the preincubation mixture (ISo), and supernatant of the sonicate (ISoS) showed high activities respectively. It was clear from last 2 experimental results that sonication is entirely harmless for cytolytic substance. Accordingly, it was demonstrated from these experiments that cytolytic substance was not obtained by sonication of the bacterial suspension, but it was newly formed out of the cell while the cells were preincubated in the buffer.

Assay of Cytolytic Activity of the Supernatant of Preincubation Mixture by *In Vitro-In Vivo* Test

In previous section, it was observed that *B. natto* KMD 1126 preincubation mixture had evident cytolytic activity on Ehrlich ascites carcinoma cells by *in vitro–in vivo* test. Since the active substance was found to be produced at cell free state, the effect of cell free active substance was examined by *in vitro–in vivo* test. As shown in Fig. 5, all animals in the control group died of tumor growth between 12 and 13 days after inoculation. On the other hand, delay in tumor development was observed in animals inoculated with tumor cells that had been incubated *in vitro* with supernatant of preincubation mixture. That is, an evident prolongation of life was observed. Cell injuring activity of this supernatant was 3.1 u/ml.

Table III. Comparison of Cell Injuring Activity of *Bacillus natto* KMD 1126

Sample	Cell injuring activity (u/ml)	Sample	Cell injuring activity (u/ml)
1126	0.80	IS	4.44
So	0	ISo	4.16
SoS	0	ISoS	4.56
I	4.04		

1126: *B. natto* KMD 1126 suspension
So: sonicate of *B. natto* KMD 1126 suspension
SoS: supernatant of the sonicate of *B. natto* KMD 1126 suspension
I: preincubation mixture of *B. natto* KMD 1126 suspension
IS: supernatant of the preincubation mixture
ISo: sonicate of the preincubation mixture
ISoS: supernatant of the sonicate of preincubation mixture

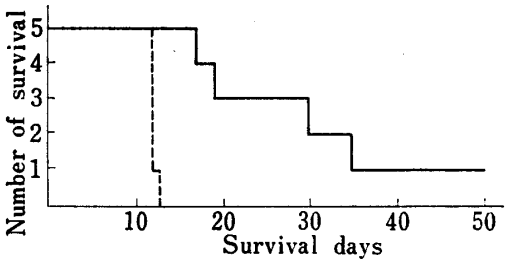

Fig. 5. Assay of Cytolytic Activity of the Supernatant of Preincubation Mixture by *In Vitro–in Vivo* Test

Mixture of Ehrlich ascites carcinoma cell suspension and the buffer (------) or supernatant of preincubation mixture (——) were incubated at 37° for 2 hr. Then, the tumor cells were inoculated intraperitoneally to mice.

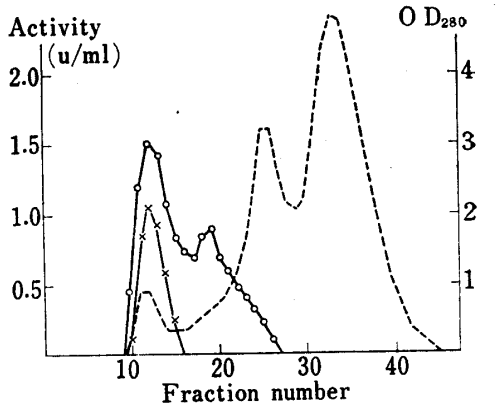

Fig. 6. Sephadex G 25 Gel Filtration of the Supernatant of Preincubation Mixture

Forty ml of the supernatant of preincubation mixture was concentrated by lyophilization and applied to a column of Sephadex G 25 (2.0×35 cm) equilibrated with the buffer. The effluents were fractionated in 5 ml portion and measured absorbance at 280 mμ (--------), cell injuring activity (O—O), and hemolytic activity (×—×).

Sephadex G 25 Gel Filtration of Cytolytic Substance

Forty ml of the supernatant of preincubation mixture was concentrated by lyophilization and applied to Sephadex G 25 column (2.0×35 cm). The effluents were fractionated in 5 ml portion and measured absorbances at 280 mμ, cell injuring activities and hemolytic activities. As shown in Fig. 6, there were at least two kind of cytolytic substances in the supernatant. One, which is large molecule, had cytolytic and hemolytic activities, but the other, which is small molecule, had cytolytic activity only.

Discussion

Bacillus natto KMD 1126 suspension had no remarkable direct cytolytic activity on Ehrlich ascites carcinoma cells. However, when the bacterial suspension was preincubated at 37° for 2 hr in the buffer, the formation of cytolytic substance was observed by eosin stain method, measurement of cell injuring activity, and *in vitro–in vivo* test. The cytolytic substance was not obtained by sonication of the bacterial cells, but it was obtained in cell free state by preincubation in the buffer. It was suggested from these experimental results that cytolytic substance was newly formed while the organisms were preincubated in the buffer.

In the course of studying the formation of cytolytic substance, an interesting fact was found out, which led us to an assumption on existence of a relation between cultural age and ability of the formation of cytolytic substance. That is, the cells at logarithmic growth phase and early stationary phase had an ability to form cytolytic substance, when the organisms were preincubated in the buffer at 37° for 2 hr, but cells at lag phase had not the ability inspite of cell lysis occurred. Spore suspension, which was obtained by heating at 80° for 10 min to destroy vegetative cells, and the cells taken at 24 hr grew in bouillon had not also the ability. Accordingly, the bacterial cell can be defined to two separate stages, one, at the logarithmic and early stationary phase and able to form the cytolytic substance and the other, at the lag phase and spore, and unable to form it. With regard to the capacity of cells to sporulate, Hardwick and Foster[9] and Nakada, *et al.*[10] reported that vegetative cells can be defined to two separate stages, one, at the logarithmic growth phase and able to sporulate in water and the other, at the lag phase and unable to sporulate. If we consider the above experimental results in relation to these publications, it was suggested that the formation of cytolytic substance may be related with spore formation. But a conclusion cannot be drawn due to an insufficient number of experimental results. On the other hand, it was observed that there are at least two kind of cytolytic substances in the preincubation mixture by Sephadex G 25 gel filtration. It was interesting for us that one, which is low molecule, had cytolytic activity but not hemolytic activity. Further investigation is necessary for these purification and identification.

9) W.A. Hardwick and J.W. Foster, *J. Gen. Physiol.*, **35**, 907 (1952).
10) D. Nakada, A. Matsushiro, and T. Miwatani, *Med. J. Osaka Univ.*, **6**, 1047 (1956).