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A Bactericidal Substance in Insect Hemolymph Strongly Suppresses Lectin-Induced Mammalian Lymphocyte DNA Synthesis

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ABSTRACT—A bactericidal substance in the *Bombyx mori* hemolymph strongly suppressed lectin-induced lymphocyte DNA synthesis. This substance was purified by ion exchange, gel filtration and thin-layer chromatography and characterized to be a 1 K daltons peptide. The specificity of this peptide for target cells was analyzed and the physiological and phylogenetic significances of this peptide are discussed.

INTRODUCTION

Previously, some bactericidal substances have been reported from the insect hemolymph. Amongst these substances, the relatively low-molecular-weight peptides have been known [1–3]. However, the effects by these peptides on the immunocompetent cells from higher animals have not been elucidated.

In this communication, the author describes a bactericidal substance in moth hemolymph, which exhibits a strong inhibitory effect on murine lymphocyte DNA synthesis. Other biological properties of this substance were analyzed and its significance is discussed.

MATERIALS AND METHODS

Purification of DNA synthesis inhibitory peptide from insect hemolymph

Bombyx mori hemolymph was kindly donated from Dr. H. Fujii (Faculty of Agriculture, Kyushu University). The hemolymph (100 ml) was concentrated with 60% saturated ammonium sulphate (pH 7.5) and the precipitate was dissolved with 10 ml of 10 mM phosphate-buffered saline (PBS, pH 7.2), applied on a Sephadex G–100 column (Pharmacia, 1.8×96 cm) and eluted with the same buffer. The low-molecular-weight fractions of Sephadex G–100 chromatography were applied on a CM Sephadex column (Pharmacia, 1.2×2 cm) and the activities were eluted with water containing various NaCl concentrations after washing the column with PBS. The eluate at 0.8 M NaCl was concentrated by use of a rotary evaporator, applied on a Sephadex G–25 column (Pharmacia, 1.8 ×24 cm) and eluted with water containing 50 mM NaCl. The active fraction (20 μl) of Sephadex G–25 chromatography was spotted on a silica gel-coated plate (Merck, 60×60 cm) and developed with a solvent system of ethanol and water (3: 1) for 2.5 hr. The plate was sprayed with a ninhydrin-solution and heated for 3 min. For preparative thin-layer chromatography, 200 μl of the active fraction of Sephadex G–25 chromatography was spotted along the origin line of the gel plate. After development with the same solvent system, the identical portion corresponding to the ninhydrin-positive portion was recovered and the active

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peptide was extracted with water. Assays for DNA synthesis were performed according to the previous method [4].

Assay for bacterial DNA synthesis

Ten μl of bacterial suspension ($A_{600\,nm}$: 0.2) (*E. coil, P. maltophilia* and *B. subtilis*) was mixed with 0.1 ml of RPMI 1640 medium (Nissui Seiyaku, Tokyo) and 0.1 ml of test solution diluted successively with water and incubated at 37°C for 8 hr in the presence of 1 μCi of [^{3}H] thymidine (5 Ci/mmole, Radiochemical Centre, England) in a microwell plate (Nuncron-R, Nunc, Denmark). The incorporation of [^{3}H] thymidine was terminated by the addition of cold 10% trichloroacetic acid (TCA) and TCA-insoluble fraction was trapped on GF/C membrane filter (Whatman) and washed with 5% TCA and ethanol. The radioactivity was counted by a Beckman liquid scintillation counter.

Assay for lymphocyte DNA synthesis

Splenocytes or thymocytes (5×10^5 cells) from C3H/He mice (7–8 weeks old) were suspended in 0.1 ml of RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, Gibco), 5 μg/ml Concanavalin A (Con A, Sigma) and 0.1 ml of test solution diluted successively in a microtiter well. After culturing the cells at 37°C in 5% CO_2 and 95% air for 2 days, the cells were labeled with 1 μCi of [^{3}H] thymidine for further 24 hr. The labeling was stopped with 10% TCA and the radioactivity in the TCA-insoluble fraction was counted as described above.

Assay for DNA synthesis of other mammalian cells

Mammalian cells (2×10^4 Balb/c 3T3, NIH 3T3, IMR 90, SV40-transformed fibroblast, KB and Hela cells) were suspended in RPMI 1640 medium supplemented with 10% FCS and 0.1 ml of test solution. After culturing for 12 hr at 37°C in 5% CO_2 and 95% air, the cells were labeled with 1 μCi of [^{3}H] thymidine for further 12 hr under the same conditions. The radioactivity was counted as described above.

Effects of the peptide on lymphocyte viability

Splenocytes or thymocytes (5×10^5 cells) from C3H/He mice were cultured in 0.1 ml of RPMI 1640 medium supplemented with 10% FCS and 5 μg/ml Con A. The lymphocyte viability was assayed by an exclusion test of 0.1% trypan blue in the presence or absence of the peptide.

Physicochemical and biochemical properties of the factor

The active fraction of Sephadex G–25 chromatography was digested with DNase I, RNase A, lipase C or bovine pancreas trypsin (50 μg/ml, Sigma) for 1 hr at 37°C and applied on a Sephadex G–25 column. The peptide was treated at 60°C for 1 hr or by freezing at −20°C and thawing. Analysis on pH stability was performed in 10 mM acetate buffer (pH 4.0) or 10 mM Tris-HCl buffer (pH 9.5).

RESULTS

As a preliminary experiment, the effects of *B. mori* hemolymph on *E. coli* DNA synthesis were studied. When the hemolymph was concentrated

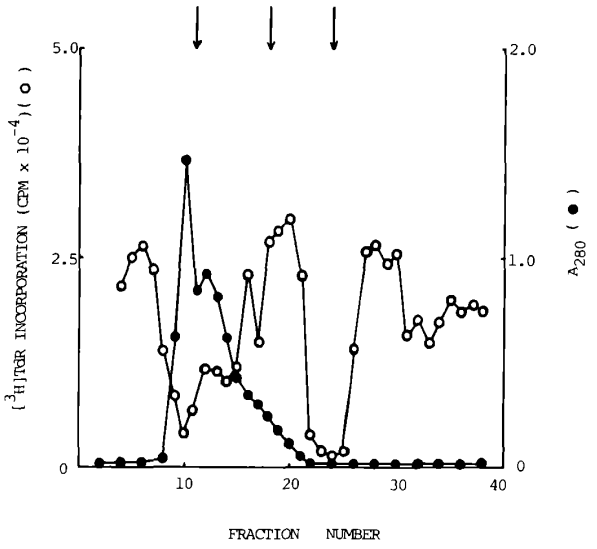

FIG. 1. Effects by *B. mori* hemolymph on *E. coli* DNA synthesis in Sephadex G–100 column chromatography. *B. mori* hemolymph was concentrated and applied onto a Sephadex G–100 column as described in Materials and Methods. Open and closed circles represent DNA synthesis activities of *E. coli* and the absorbance at 280 nm in the fractions. Upper arrows show the elution positions of molecular weight markers; bovine serum albumin (67 K daltons), cytochrome C (13 K daltons) and bacitracin (1.5 K daltons) from the left to the right.

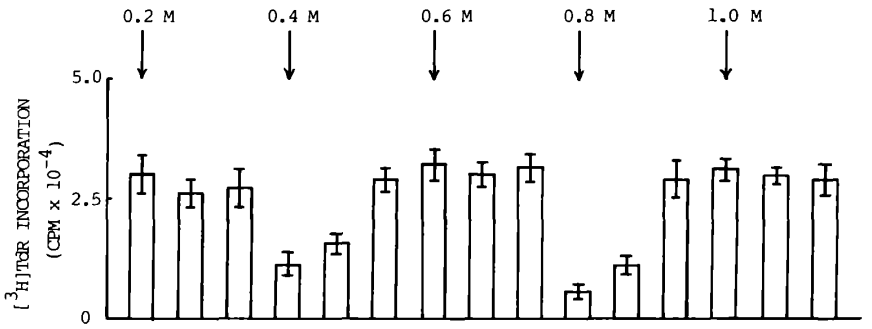

FIG. 2. Separation of inhibitory activities of *E. coli* DNA synthesis by CM Sephadex column chromatography. Active low-molecular-weight fractions of Sephadex G–100 chromatography (fraction 22–25 in Fig. 1) were applied on a CM Sephadex column. After washing the column with PBS, the activities were eluted with water containing various NaCl concentrations as shown in figure. The column and bar represent the mean and standard error of triplicate assays. In order to reduce the effect of NaCl on DNA synthesis assay, each fraction was diluted at 10-fold and assayed.

and fractionated by a Sephadex G–100 column, the inhibitory activities for *E. coli* DNA synthesis were eluted in several peaks corresponding to high and low molecular weight principles. Active principles eluted at low-molecular-weight region (less than 5 K daltons) were separated into different fractions by CM Sephadex chromatography eluting at 0.4 M and 0.8 M NaCl, respectively (Fig. 2). The former activity at 0.4 M was characterized and described elsewhere [4]. In this paper, the active principle eluted at 0.8 M NaCl was investigated. When this principle was applied onto a Sephadex G–25 column, the major inhibitory activity for *E. coli* DNA synthesis was eluted at the position corresponding to about 1 K daltons as judged by the positions of molecular weight markers (open circles in Fig. 3). This fraction also strongly inhibited lectin-induced DNA synthesis in murine lymphocytes (closed circles in Fig. 3). In order to analyze the relationship between the activities for *E. coli* and murine lymphocytes, the active 1 K dalton principle was further purified by silica gel thin-layer chromatography. As shown in Figure 4, a ninhydrin-positive spot was detected and the extract from the portion of silica gels corresponding to the ninhydrin-positive region represented remarkable inhibitory activities of lymphocytes and *E. coli* DNA synthesis (Table 1). This result strongly suggests that this ninhydrin-positive material has dual inhibitory effects on *E. coli* and murine lymphocyte DNA synthesis.

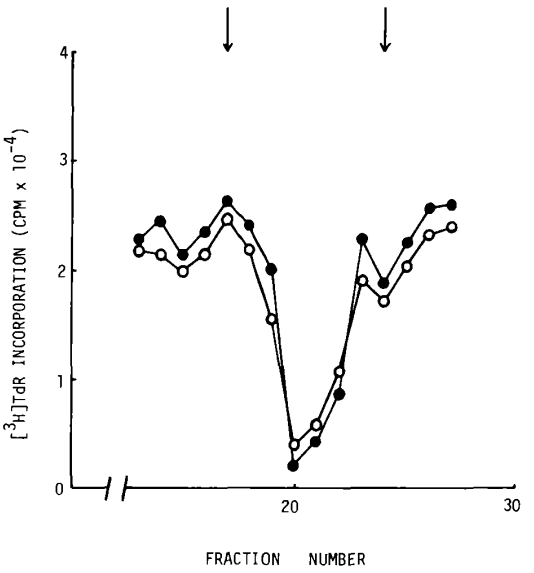

FIG. 3. *E. coli* and lymphocyte DNA synthetic activities of *B. mori* hemolymph in Sephadex G–25 column chromatography. Active fractions eluted at 0.8 M NaCl from CM Sephadex chromatography in Fig. 2 were applied on a Sephadex G–25 column as described in Materials and Methods. Open and closed circles represent DNA synthetic activities of *E. coli* and murine lymphocytes, respectively. Upper arrows in the figure show the positions of molecular weight markers; bacitracin (1.5 K daltons) and phenol red (350 daltons) from the left to the right.

Furthermore, the biochemical properties of this activity were examined. When the active fraction from Sephadex G–25 chromatography was digested with bovine pancreas trypsin, the 1 K daltons activity for lymphocyte DNA sythesis was

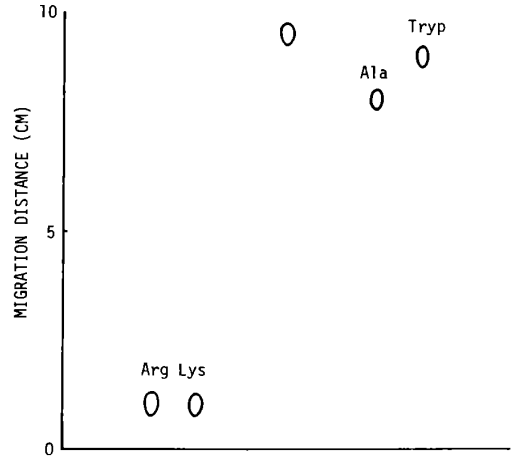

FIG. 4. Silica gel thin-layer chromatography of the peptide. Active fractions at 1 K daltons in Sephadex G–25 chromatography (fraction 20–22 in Fig. 3) were spotted on a silica gel-coated plate. Lys, Arg, Ala and Tryp show the amino acid markers.

markedly decreased (Fig. 5). In addition, the activity for E. coli was also destroyed (not shown). However, such effect did not occur by other enzymes (DNase I, RNase A and lipase C). As shown in Table 2, this activity was resistant to a heat treatment or freezing and thawing and stable in an acidic (pH 4.0) or basic (pH 9.5) environment. These results suggest that the inhibitory activity seems to be derived from heat-stable 1 K dalton peptide.

Then, the modes of action by this peptide against lymphocytes were studied. The strong inhibition on DNA synthesis occurred when added at early periods (0–10 hr) of the cell culture, but significant effects were not detected when added at 30 hr (Table 3). These results indicate that this peptide acts on lymphocytes at early periods after lectin-stimulation and blocks concomitant DNA synthesis. Interestingly, although the strong inhibitory effect on lymphocyte DNA synthesis was observed, the cell viability was not changed significantly as compared with the control experiment (Table 4). Furthermore, when the lymphocytes were washed extensively after treatment of this peptide, lectin-induced activation was restored (not shown). The effect of this peptide on lymphocytes seems to be non-toxic and reversible. However, in *E. coli*, after washing *E. coli* pretreated with this peptide, their growth was not restored and they were lyzed, which seems to be similar effect to that of the bactericidal peptides reported previously [1–3]. The reason of such different effects on both cells are not clear at

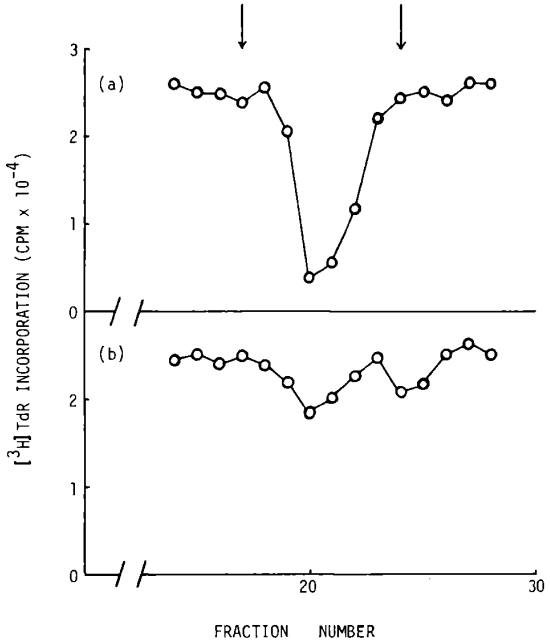

FIG. 5. Effect of trypsin treatment on the inhibitory activity. After the active fraction in Fig. 3 (fraction 20–22) was treated with (a) none or (b) trypsin (50 μg/ml) for 1 hr at 37°C, the Sephadex G–25 rechromatography was carried out. Arrows in the figure are same as in Fig. 3. Open circles represent murine lymphocyte DNA synthetic activities.

TABLE 1. Purification of the factor by thin-layer chromatography

	[^{3}H]TdR Incorporation (CPM)	
	Ninhydrin-negative	Ninhydrin-positive
Murine splenocytes	22980±1795	1235±142
E. coli	21375±1819	1085±196

The radioactivity represents the mean and standard error of triplicate assays.

TABLE 2. Physicochemical properties of the peptide

	[^{3}H]TdR Incorporation (CPM)
Control	21517±1486
+Factor treated with	
None	1296±130
Heat (60°C, 1 hr)	1543±196
Freezing and thawing	1356±151
pH 4.0	1481± 59
pH 9.5	1377±238

The radioactivity shows the mean and standard error of triplicate assays for splenocyte DNA synthesis. The peptide using in this experiment was derived from the combined fractions from No. 20–22 in Fig. 3.

TABLE 3. Effects of the peptide when added at different times after the cell culture

Added at (hr)	[^{3}H]TdR Incorporation (CPM)	Relative Activity (%)
—	18065±2015	100
0	1915± 87	10.6
5	2221± 137	12.3
10	2908± 258	16.1
30	16548±3851	91.6

The value in the table is the average of triplicate assays for lectin-induced splenocyte DNA synthesis. The peptide used is same as in Table 1.

TABLE 4. Effects of the peptide on the lymphocyte viability

	Control		+Peptide	
	No. of Cells ($\times 10^5$)	Viability (%)	No. of Cells ($\times 10^5$)	Viability (%)
Exp. 1	3.0	60	3.1	62
Exp. 2	2.7	54	2.9	58

Murine splenocytes were cultured for 2 days and the viability was assayed as described in Materials and Methods. The peptide is same in Table 1. The value in the table is the average of duplicate assays.

TABLE 5. Effects of the peptide on DNA synthesis of mammlian and bacterial cells

	[^{3}H]TdR Incorporation (CPM)		
	Control	+Peptide	$\frac{+\text{Peptide}}{\text{Control}}$ (%)
Balb/c 3T3	2825± 188	1679± 143	59.4
NIH 3T3	2360± 206	1480± 168	62.7
IMR 90	2968± 195	1986± 87	56.8
SV40-transformed cells (3T3 fibroblast)	6850± 523	6425± 481	93.8
SV40-transformed cells (human fibroblast)	8231± 326	7926± 502	96.3
KB	5963± 613	5868± 187	98.4
Hela	5049± 511	4686± 671	92.8
murine splenocytes	19020± 889	2301± 144	12.1
murine thymocytes	3876± 293	450± 93	11.6
E. coli	23685±1614	2558± 196	10.8
P. maltophilia	19351±2473	8959± 815	46.3
B. subtilis	20715±1602	20425±2390	98.6

The radioactivity shows the mean and standard error of triplicate assays.

present.

The effects of other target cells were then studied. The significant inhibitory effects were observed on some mammalian cells (Balb/c 3T3, NIH 3T3 and IMR 90), but not on malignant cells (SV40-transformed cells, KB and HeLa cells) (Table 5). Especially, the lymphocytes such as splenocytes and thymocytes were very sensitive (Table 5). On the other hand, the effects on some bacteria were studied. As shown in Table 5, *E. coli* was most sensitive and *P. maltophilia* was considerably affected, but not significant on *B. subtilis*.

Thus, results mentioned above indicate that this peptide causes strong inhibitory effects on DNA synthesis of *E. coli* and murine lymphocytes.

DISCUSSION

Results mentioned above suggest that *B. mori* hemolymph contains a basic peptide which strongly suppresses DNA synthesis of murine lymphocytes and *E. coli*.

On the other hand, thymidine and polyamines are well known as low-molecular-weight nonspecific inhibitors of DNA synthesis [5]. However, the inhibitory factor in this paper is not thymidine and polyamines by the following reasons. This factor is eluted at 0.8 M NaCl from CM Sephadex column. Thymidine was easily eluted at 10 mM NaCl from the same column and polyamines (spermine and spermidine) were not even at 1.5 M NaCl, but eluted by 0.25 M NaOH (not shown). Furthermore, the major inhibitory activity was destroyed by trypsin treatment (Fig. 5), which indicates that the active portion of this factor is associated with the peptide structure. In addition, in order to analyze the possibility of the glycopeptide, when the factor was treated with periodate, the activity was not affected, and the factor did not bind some lectin-affinity columns (Concanavalin A, wheat germ agglutinin and so on) (not shown). Thus, the author considered that the major activity by this factor is derived from the peptide structure. The bactericidal peptides reported previously exhibit much higher molecular weights (4–5 K daltons) than that of the peptide in this paper. In *B. mori* hemolymph in this paper, although about 5 K dalton activity can be detected, this activity was relatively weak as compared with 1 K dalton activity. Therefore, the author focused the 1 K dalton activity. Furthermore, in the previous reports [1–3], the effects of the bactericidal peptides on higher animal cells have not been elucidated. As mentioned in the results, 1 K dalton peptide in *B. mori* showed the strong inhibition on lectin-induced lymphocyte DNA synthesis and considerable effects on some fibroblasts, but malignant transformed cells were not affected (Table 5). Interestingly, in spite of the irreversible effect on *E. coli*, which was also confirmed in the previous reports [1–3], the peptide did not change lymphocyte viability (Table 3).

The author found the basic peptide with similar biological activity in fetal calf serum [6], mouse, rat and human sera (not shown). Furthermore, the other low-molecular-weight peptide exists in *B. mori* hemolymph, which was eluted at 0.4 M NaCl from CM Sephadex (Fig. 2). Although this peptide has a different charge property, it represents the similar biological properties to those of the peptide in this paper and is also observed in mammalian serum [4]. As an alternative experiment, the author investigated the cells producing these peptides. The peptides with similar inhibitory activity are detected in the serum-free culture medium conditioned by murine splenocytes [7], fibroblasts [8] and insect fibroblastic cells [9]. Recently, the author found that these peptides affect some functions of T and B lymphocytes (not shown). This finding suggests that such bactericidal substances in insect hemolymph have a potential ability for regulating immune functions of mammalian lymphocytes. Anyhow, these peptides seem to have physiological significance in invertebrates and vertebrates.

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