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Purification of *Sarcophaga* (fleshfly) lectin and detection of sarcotoxins in the culture medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina*

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An established cell line originating from a *Sarcophaga peregrina* (fleshfly) embryo, NIH-Sape-4, was found to synthesize mRNAs for *Sarcophaga* lectin and sarcotoxin IA, but not those for storage protein or 25 kDa protein. These four proteins are known to be synthesized in the fat-body of third-instar larvae, and the two former in particular are known to participate in the defence mechanism of this insect and to be induced in response to injury of the body wall. Thus the embryonic cell line NIH-Sape-4 synthesizes certain defence proteins constitutively. This cell line will be useful for large-scale purification of *Sarcophaga* lectin, since 50 μg of purified *Sarcophaga* lectin could be obtained from about 400 ml of culture medium.

INTRODUCTION

We have reported that a galactose-specific lectin (*Sarcophaga* lectin) and at least three groups of antibacterial proteins are synthesized by the fat-body, and eventually secreted into the haemolymph, of third-instar larvae of *Sarcophaga peregrina* when their body wall is pricked with a hypodermic needle (Komano *et al.*, 1980; Okada & Natori, 1983). These proteins are supposed to participate in the defence mechanism of this insect, since such a mechanism would be expected to be enhanced under conditions of injury to eliminate pathogens invading through the damaged body wall. In fact, *Sarcophaga* lectin was shown to be essential for the elimination of sheep red blood cells introduced into the abdominal cavity of third-instar larvae (Komano & Natori, 1985). We have isolated cDNA clones for *Sarcophaga* lectin and one of the antibacterial proteins termed 'sarcotoxin IA' (Takahashi *et al.*, 1985; Matsumoto *et al.*, 1986*a*). Northern-blot-hybridization experiments using these cDNA clones as probes showed that the *Sarcophaga*-lectin gene is transiently expressed in the early embryonic and early pupal stages (Takahashi *et al.*, 1986), and that the sarcotoxin IA gene is also expressed in the early pupal stage, although its expression in the early embryonic stage is not certain (Y. Nakajima, unpublished work). These results strongly suggest that, in *Sarcophaga*, certain proteins participating in the defence mechanism are also indispensable for the ontogenetic process.

Since we are interested in the function of these proteins in ontogeny, we investigated their production by NIH-Sape-4 cells, an established cell line derived from a *Sarcophaga peregrina* embryo (Takahashi *et al.*, 1980). We found that this cell line synthesizes *Sarcophaga* lectin and also various antibacterial proteins, including sarcotoxin I and II, and secretes them into the culture medium. mRNAs for two other marker proteins of the larval fat-body were not detected in the cells, indicating that this line synthesizes constitutively only proteins that are produced by the larval fat-body in response to injury of the body.

MATERIALS AND METHODS

Cells and culture medium

The *Sarcophaga peregrina* embryonic cell line NIH-Sape-4 was routinely cultured in 30 ml of M-M medium (Mitsuhashi & Maramorasch, 1964) at 25 °C by the procedure reported by Takahashi *et al.* (1980). Its doubling time was about 24 h, and its saturation density was $(1–2) \times 10^7$ cells/ml under these conditions. Cells were usually harvested when they reached a density of $(1–4) \times 10^6$ cells/ml and were used for preparation of RNA. The resulting culture medium was immediately frozen and kept at −20 °C.

DNA probes

The DNA probes used were pLE10 for *Sarcophaga* lectin (Takahashi *et al.*, 1985), pTO19 for sarcotoxin IA (Matsumoto *et al.*, 1986*a*), pS25 for 25 kDa protein (Matsumoto *et al.*, 1985) and pSP2 for storage protein (Matsumoto *et al.*, 1986*b*). Each DNA was labelled with ^{32}P by nick-translation (Weinstock *et al.*, 1978). The specific radioactivity was usually $(1–1.5) \times 10^8$ c.p.m./μg of DNA.

Preparation of RNA and analysis by blot hybridization

RNA was obtained by successive extractions with phenol/chloroform (1:1, v/v) and chloroform/3-methylbutan-1-ol (1:1, v/v) from cultured cells or the fat-body of larvae whose body wall had been pricked with a hypodermic needle 6 h previously. About 1.3 mg of RNA was obtained from 2×10^8 cells. Poly(A)$^+$ RNA was prepared from total RNA by oligo(dT)–cellulose column chromatography.

Northern blot analysis was done by the method of Thomas (1980), with minor modifications.

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Assay of antibacterial activity

Antibacterial activity was assayed by two independent methods. One method was assay of inhibition of growth of *Escherichia coli* in culture medium containing the test sample as described previously (Okada & Natori, 1983). The other method was location of proteins able to inhibit the growth of *E. coli* on polyacrylamide slab gels as described by Hultmark *et al.* (1980). For this, the gel was overlaid with soft agar (0.6%) medium containing 2×10^5 *E. coli* cells/ml, and incubated for 18 h at 37 °C.

Assay of haemagglutinating activity

Haemagglutinating activity was assayed essentially as described previously with commercially available sheep red blood cells as an indicator (Komano *et al.*, 1980). Unagglutinated cells formed a clear dot, whereas agglutinated cells formed a diffuse mat on the bottom of wells of microtitre V-plates. The end point of transition from a diffuse mat to a dot was distinct on dilution of the sample. Haemagglutinating activity was defined as the reciprocal of the maximum dilution of the test sample causing haemagglutination (titre).

Radioimmunoassay of *Sarcophaga* lectin

Radioimmunoassay was done essentially as described previously by using antibody raised against *Sarcophaga* lectin (Komano *et al.*, 1983). The assay was done in 1 ml of phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4) containing 0.2 M-galactose, 0.1% Triton X-100 and 0.2% bovine serum albumin. A mixture of a fixed amount of radioiodinated *Sarcophaga* lectin, 0–1000 ng of unlabelled *Sarcophaga* lectin and 20 μl of diluted antiserum was incubated at 4 °C for 12 h. Then 20 μl of Cowan I solution, prepared by the method of Kessler (1975), was added. After 60 min, the reaction mixture was centrifuged for 15 min at 8800 ***g***, and the radioactivity of a 900 μl sample of the resulting clear supernatant was determined. The amount of *Sarcophaga* lectin in the test sample was determined from a dose–response curve obtained with unlabelled lectin.

Polyacrylamide-gel electrophoresis and protein determination

Electrophoresis under non-denaturing conditions was carried out in 15%(w/v)-polyacrylamide slab gel by the method of Gabriel (1971). Electrophoresis on 12.5%-(w/v)-polyacrylamide/SDS slab gels was carried out by the method of Laemmli (1970). Proteins (1–5 μg per lane) were denatured by heating them in 1% SDS solution containing 2% (v/v) 2-mercaptoethanol for 20 min at 75 °C. Gels were stained with Coomassie Brilliant Blue R250 by the method of Fairbanks *et al.* (1971). Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Fractionation of culture medium of NIH-Sape-4 containing *Sarcophaga* lectin

For purification of *Sarcophaga* lectin, about 400 ml of culture medium was mixed with 4 vol. of 10 mM-phosphate buffer, pH 6.0, and passed through a column of CM-cellulose (2 cm × 10 cm) equilibrated with the same buffer. Most of the *Sarcophaga* lectin, detected by radioimmunoassay, was recovered in the flow-through fraction, free from antibacterial proteins. To 1200 ml of flow-through fraction was added 523 g of $(NH_4)_2SO_4$ and the mixture was stirred for 3 h at 4 °C. The precipitate was collected by centrifugation and dissolved in 5 ml of 10 mM-phosphate buffer, pH 8.0, containing 30% (w/v) $(NH_4)_2SO_4$. The resulting clear solution was applied to a column (2.6 cm × 8 cm) of butyl-Toyopearl 630 M (Toyo Soda, Tokyo, Japan) equilibrated with the same phosphate buffer. The column was washed extensively, and then developed with 50 ml of a decreasing linear gradient of 30–10% satd. $(NH_4)_2SO_4$.

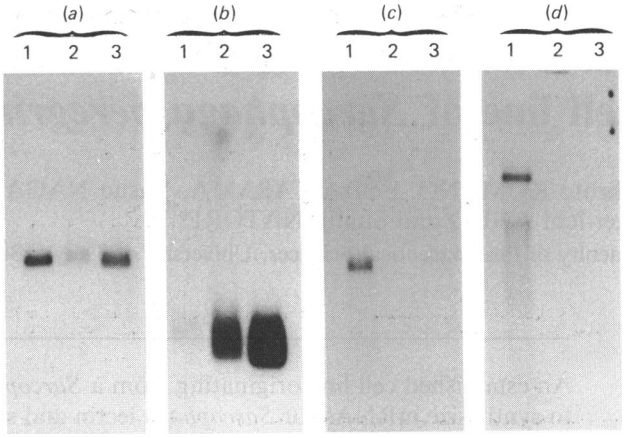

Fig. 1. Detection of *Sarcophaga* lectin and sarcotoxin IA mRNA by RNA blot hybridization

RNA was extracted from the fat-body of injured larvae or NIH-Sape-4 cells, and samples (1–15 μg) were subjected to electrophoresis in agarose gel under denaturing conditions. After electrophoresis, RNA was blotted on to a nitrocellulose filter, and the filter was incubated with nick-translated cDNA probes. Then the filter was washed thoroughly and autoradiographed. The DNA probes used were (*a*) pLE10 (*Sarcophaga* lectin), (*b*) pTO19 (sarcotoxin IA), (*c*) pS25 (25 kDa protein) and (*d*) pSP2 (storage protein). Lane 1, total RNA from the fat-body; lane 2, total RNA from cultured cells; lane 3, poly(A)$^+$ RNA from cultured cells.

Galactose–agarose chromatography

Fractions, from the butyl-Toyopearl chromatography described above, containing haemagglutinating activity were pooled, dialysed against phosphate-buffered saline and subjected to affinity chromatography on a column (1 cm × 6.7 cm) of galactose–agarose (Pierce Chemical Co., Rockford, IL, U.S.A.) Elution was with 0.2 M-galactose.

RESULTS

Sarcophaga lectin was originally found to be synthesized in the fat-body of third-instar larvae of *Sarcophaga peregrina* in response to injury of the body wall and to be secreted into the haemolymph (Komano *et al.*, 1980), and more recently it was also found to be synthesized in the early-embryonic and early-pupal stages (Takahashi *et al.*, 1986). It has the interesting effect of activating mammalian immune cells (Itoh *et al.*, 1984*a*,*b*; Tamura

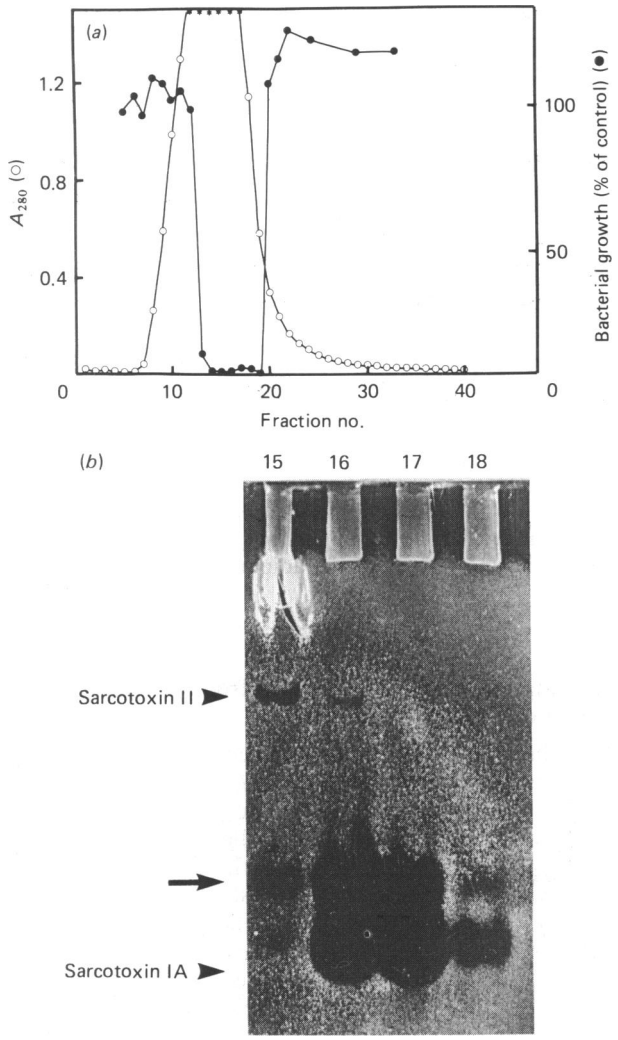

Fig. 2. (*a*) Chromatogram of antibacterial activity on a column of CM-cellulose, and (*b*) polyacrylamide-gel electrophoresis of each fraction

(*a*) The culture medium (400 ml) was mixed with 4 vol. of 10 mM-phosphate buffer, pH 6.0, and applied to a column of CM-cellulose (4 cm × 6 cm). Adsorbed material was eluted with 260 mM-NaCl in the same buffer; fractions (5 ml) were collected, and the antibacterial activity of each fraction was assayed. ●, Bacterial growth; ○, A_{280} (*b*) A sample (40 μl) of each fraction was subjected to polyacrylamide-gel electrophoresis, and antibacterial activity (►) was located by examination of growth of *E. coli* overlaid on the gel. Lane numbers correspond to fraction numbers in (*a*). The arrow (➞) indicates a spot of unidentified material.

& Natori, 1984). For examination of its biological activity, it needs to be obtained in sufficient quantity; so far, however, the only source for its purification is larval haemolymph. We therefore examined whether NIH-Sape-4, the only embryonic cell line of *Sarcophaga peregrina* so far established, can synthesize this lectin.

Northern blot analysis of NIH-Sape-4 RNA with four fat-body cDNA clones

First, using Northern blot hybridization with four cloned fat-body-protein cDNAs as probes, we investigated whether this cell line synthesizes mRNA for fat-body proteins. Of these proteins, *Sarcophaga* lectin and sarcotoxin IA are synthesized in response to injury of the body wall (Komano *et al.*, 1980; Okada & Natori, 1983), whereas the storage protein and 25 kDa protein are developmentally regulated and are synthesized in the fat-body of third-instar larvae during normal development (Tahara *et al.*, 1982; Tamura *et al.*, 1983).

As shown in Figs. 1(*a*) and 1(*b*), the genes for both *Sarcophaga* lectin and sarcotoxin IA were expressed in the cultured cells, whereas those for the storage protein and 25 kDa protein were not expressed significantly (*c* and *d*). In this experiment, we used RNA from the fat-body of injured larvae as a positive control; expressions of the genes for *Sarcophaga* lectin and sarcotoxin IA in the larval fat-body were not detected unless the body wall of the larvae was injured (results not shown). These results suggest that this embryonic cell line constitutively synthesizes the defence proteins that are synthesized in the larval fat-body in response to body injury, but does not synthesize other fat-body proteins.

Detection of antibacterial proteins in the culture medium of NIH-Sape-4

As reported previously, three groups of antibacterial proteins that differ in mobility on polyacrylamide-gel electrophoresis under non-denaturing conditions are induced in the haemolymph when the body wall of third-instar larvae is pricked with a hypodermic needle (Okada & Natori, 1983). Two of these groups of proteins have been purified. Sarcotoxin I is a group of proteins with a molecular mass of about 4000 Da, and consists of three proteins, sarcotoxins IA, IB and IC, which have almost the same primary structure but differ by a few amino acid residues (Okada & Natori, 1985). Sarcotoxin II, another group of proteins, consists of three similar proteins, sarcotoxin IIA, IIB and IIC, with molecular masses of about 24000 (Andoh *et al.*, 1987). Since mRNA for sarcotoxin IA was detected in NIH-Sape-4 cells, we examined the antibacterial activity of the culture medium.

For this purpose we subjected the culture medium to CM-cellulose chromatography and measured the antibacterial activity in the eluate, since the antibacterial proteins are known to be adsorbed to this resin under the conditions used (Okada & Natori, 1983). As shown in Fig. 2(*a*), significant antibacterial activity was eluted from the resin, and its elution profile was almost identical with that of the activity of the haemolymph of injured larvae. We then subjected fractions containing antibacterial activity to polyacrylamide-gel electrophoresis and located the antibacterial activity on the gel. As is evident from Fig. 2(*b*), three distinct main bands of material inhibiting the growth of *E. coli* were detected. The sharp band of low mobility detected in fractions 15 and 16 was identified as sarcotoxin II, by comparison of its mobility with that of an authentic sample (Andoh *et al.*, 1987). The band of material showing highest mobility is likely to be one of the group of sarcotoxin I proteins (Okada & Natori, 1983), because its mobility is similar to that of authentic sarcotoxin IA. Probably this cell line produces a sarcotoxin IA-like protein, but not sarcotoxin IA itself, and mRNA for this protein may hybridize with cDNA for sarcotoxin IA. The culture medium also contained another unidentified antibacterial component (shown by an arrow in Fig. 2*b*).

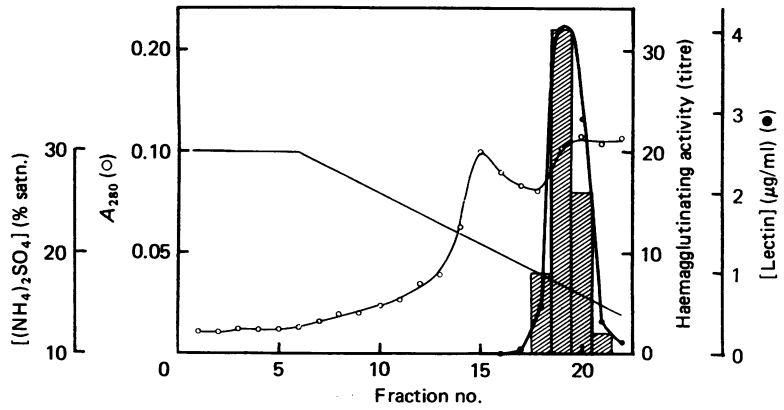

Fig. 3. Chromatogram of *Sarcophaga* lectin on a column of butyl-Toyopearl

Material cross-reacting with antibody against *Sarcophaga* lectin in the fraction from CM-cellulose was precipitated with $(NH_4)_2SO_4$ and applied to a hydrophobic column (2.6 cm × 8 cm) of butyl-Toyopearl 630 M. Adsorbed material was eluted with a decreasing linear gradient of 30–10%-satd. $(NH_4)_2SO_4$. After dialysis, the haemagglutinating activity of each fraction was assayed. ▨, Haemagglutinating activity (titre); ●, amount of *Sarcophaga* lectin determined by radioimmunoassay; ○, A_{280}; ——, percentage saturation of $(NH_4)_2SO_4$.

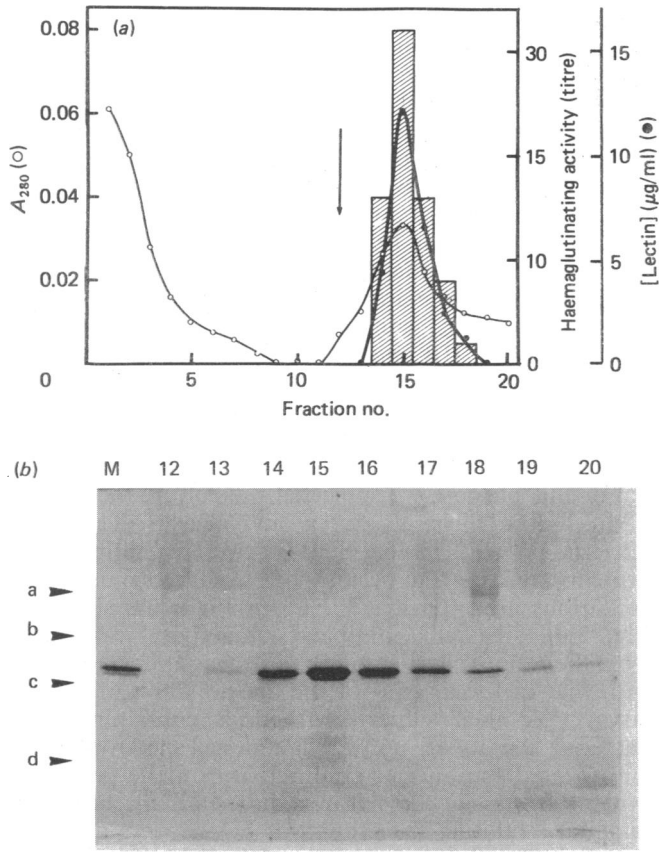

Fig. 4. (*a*) Chromatogram of *Sarcophaga* lectin on a column of galactose–agarose, and (*b*) SDS/polyacrylamide-gel electrophoresis of *Sarcophaga* lectin eluted from the column

Fractions from the butyl-Toyopearl column were pooled and applied to an affinity column (1 cm × 6.7 cm) of galactose–agarose equilibrated with phosphate-buffered saline (137 mM-NaCl/2.7 mM-KCl/8.1 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.4. The column was washed thoroughly, and then the adsorbed material was eluted with 0.2 M-galactose in the same buffer by changing the buffer at the point indicated by an arrow. ▨, Haemagglutinating activity (titre); ○, A_{280}; ●, amount of *Sarcophaga* lectin determined by radioimmunoassay. (*b*) Each fraction (200 μl) was subjected to SDS/polyacrylamide-gel electrophoresis after precipitation with 10% (w/v) trichloroacetic acid. Lanes 1–6 correspond to fractions 12–20 in (*a*); lane M, authentic *Sarcophaga* lectin purified from the haemolymph. The following market proteins were used to calibrate the gel: a, bovine serum albumin (66 kDa); b, ovalbumin (45 kDa); c, chymotrypsinogen (25 kDa); d, cytochrome *c* (12.5 kDa).

Purification of *Sarcophaga* lectin from the culture medium of NIH-Sape-4

Northern blot analysis showed that this cell line synthesized mRNA for *Sarcophaga* lectin. This protein can be purified from the haemolymph of injured larvae; it is, however, very laborious to collect haemolymph, so if NIH-Sape-4 secretes this lectin, the culture medium of these cells should be a better starting material than haemolymph for large-scale purification of this lectin. The culture medium did not show appreciable haemagglutinating activity, but contained a protein that cross-reacted with antibody against *Sarcophaga* lectin. We purified this protein and examined its cross-reactivity by radioimmunoassay, finding that it was in fact *Sarcophaga* lectin. We also found that the culture medium contained a substance that inhibited the haemagglutinating activity of *Sarcophaga* lectin and that *Sarcophaga* lectin could be separated from this inhibitor on a column of butyl-Toyopearl.

About 400 ml of culture medium was fractionated by the procedure described in the Materials and methods section. The elution profile of *Sarcophaga* lectin from a column of butyl-Toyopearl is shown in Fig. 3. Until this step, no appreciable haemagglutinating activity was detected, but at this step a haemagglutinating activity was eluted from the column in the same position as the peak of *Sarcophaga* lectin determined by radioimmunoassay. When haemagglutinating fractions from butyl-Toyopearl chromatography were subjected to galactose–

Table 1. Summary of the purification of *Sarcophaga* lectin from the culture medium of NIH-Sape-4 cells

Step	Total protein (mg)	*Sarcophaga* lectin (μg)*	Recovery (%)	Specific activity (titre/mg)
Culture medium	67	398		—
CM-cellulose	48	294	100	—
$(NH_4)_2SO_4$ precipitation	29	132	46	—
Butyl-Toyopearl	1.3	81	29	446†
Galactose–agarose	0.053	53	19	2792†

* The amount of protein was determined by radioimmunoassay.
† Haemagglutinating activity was measured as described in the Materials and methods section.

agarose chromatography, a single peak of haem-agglutinating activity was eluted from the column with 0.2 M-galactose solution, as shown in Fig. 4(*a*), and the electrophoretic profile of protein in this peak coincided with that of *Sarcophaga* lectin purified from the haemolymph of injured larvae, as shown in Fig. 4(*b*). *Sarcophaga* lectin was almost homogenous at this step. The specific activity of the purified lectin was about the same as that of authentic *Sarcophaga* lectin from the haemolymph. About 50 μg of purified *Sarcophaga* lectin could be obtained from about 400 ml of the culture medium of NIH-Sape-4 cells. The results of a typical purification are summarized in Table 1.

DISCUSSION

In the present study we have demonstrated that NIH-Sape-4 cells secrete antibacterial proteins and a galactose-specific agglutinin, *Sarcophaga* lectin. Originally, these proteins were found to be synthesized in the fat-body of *Sarcophaga* larvae after injury of their body wall with consequent stimulation of the larval defence mechanism. This suggested that they might participate in the defence mechanism of this insect (Komano *et al.*, 1980; Okada & Natori, 1985; Andoh *et al.*, 1987). However, later, Northern blot analysis with a cDNA clone for *Sarcophaga* lectin showed that this lectin also transiently appears in the early-embryonic and early-pupal stages (Takahashi *et al.*, 1986). Thus this lectin has dual functions, in the defence mechanism and in development, although its physiological role in development is unknown.

The present results demonstrated that NIH-Sape-4 cells synthesize *Sarcophaga* lectin constitutively. In the embryo, expression of the *Sarcophaga* lectin gene is only transient (Takahashi *et al.*, 1986). So there must be some mechanism to shut off its expression in the embryo that is missing in NIH-Sape-4 cell.

This cell line produces antibacterial proteins such as sarcotoxin I and II, in addition to *Sarcophaga* lectin. But since it does not express the genes for two other typical fat-body proteins, storage protein and 25 kDa protein, it seems to synthesize selectively the fat-body proteins that are produced in response to injury of the body wall. The present results suggest that in common with *Sarcophaga* lectin, sarcotoxin I and II are synthesized in the early embryonic stage and play an essential role in the ontogeny of this insect as well as in the defence mechanism.

NIH-Sape-4 cells can be maintained in culture, but they are a heterogeneous population of embryonic cells and have not yet been cloned (Takahashi *et al.*, 1980). Probably the co-operative effects of multiple cells are needed to keep them in culture. All the cells originated from a *Sarcophaga* embryo, but we do not know whether the same cells produce both *Sarcophaga* lectin and sarcotoxins.

In the present study we found that the culture medium of NIH-Sape-4 cells is good starting material for purification of *Sarcophaga* lectin. This lectin has the interesting property of activating mammalian immune cells (Itoh *et al.*, 1984*a*,*b*; Tamura & Natori, 1984), so immunological studies on it should be useful. For these studies, a sufficient quantity of purified lectin will be necessary, and the present work shows that this can be obtained from the culture medium of NIH-Sape-4 cells.

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