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Agglutinins from aquatic insects—tumor cell agglutination activity

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Abstract. Agglutinins were identified in whole body extracts of aquatic insects by means of murine tumor cell agglutination, using sarcoma 180 ascites, Ehrlich, and MM-46 cells. Screening revealed agglutinins in 5 of 10 of the larvae tested, and in 2 of 6 of the water-dwelling adult insects; *Gerris paludum insularis* and *Gyrinus japonicus*. Only the agglutinin from adult *G. paludum* also agglutinated human erythrocytes. An ascites tumor was converted into a solid form in vivo after administration of *G. paludum* agglutinin. The observation that these aquatic insect agglutinins preferentially agglutinate tumor cells has considerable implications in terms of anti-tumor effects such as inhibition of cell proliferation and metastasis.
Key words. Agglutinin; aquatic insect; tumor cell agglutination; hemagglutination; antiproliferative effect; *Gerris paludum*.

Lectins (including agglutinins) from various sea and land-dwelling species of invertebrates have been extensively studied[1,2]. Invertebrate lectins are thought to participate in cell development and in host defense mechanisms, by inducing cell to cell interaction[3]. The amino acid sequences of lectins from *Limulus polyphemus*[4], *Megabalanus rosa*[5], *Sarcophaga peregrina*[6], *Polyandrocarpa misakiensiss*[7] and *Anthocidaris crassispina*[8] have been determined recently. They were found to have about 20% identical sequences. These lectins contain 4 tryptophan residues and 2 disulfide loops in common, and their disulfide loops may constitute the sugar recognition domain[9,10]. It is possible that this domain is conserved in these lectins, which are associated with a self-defense mechanism.

The cytoagglutination activity of lectins has been studied using several species of mammalian erythrocytes. A few investigators have studied animal lectins (either invertebrate or vertebrate) which agglutinate only tumor cells. We reported sialic acid-binding lectins from frog eggs which selectively agglutinated ascites tumor cells of mice and rats, and transformed cells infected with polyoma virus[11–13]. The primary structures of these lectins revealed that they are homologous with frog liver RNase, bovine pancreatic RNase and angiogenin[14–16].

In this resport, we describe tumor cell agglutination induced by agglutinins from aquatic insects, and the conversion of an ascites tumor into the solid form in vivo by an agglutinin from *Gerris paludum*.

Materials and methods

Collection of aquatic insects: Various insects were collected in the rivers, ponds and lakes of Miyagi Prefecture (Japan) during the period from March to September 1991. The larvae of *Protohermes grandis* were collected in the rivers of Ibaraki Prefecture. The samples varied in size but were sufficient for study.

Agglutinin extraction from insects: The insects were kept on ice, washed with ice-cold water, homogenized with 5 volumes of cold 0.15 M NaCl using a Waring blender and placed in 10 volumes of acetone. The acetone-dried powder obtained was stored at −20 °C until extracted. The powder (1.0 g) was homogenized with 0.15 M NaCl (20 ml) in a Potter-Elvehjem glass homogenizer, then centrifuged at 9000 × *g* for 30 min. The precipitate was reextracted with 0.15 M NaCl as described above. The combined supernatants were dialyzed against distilled water for 3 days and then lyophilized (crude extract). The crude extract (2 mg) was dissolved in 0.15 M NaCl (0.1 ml) and used for the screening test.

Agglutination of cancer cells and its inhibition: Sarcoma 180 (S-180) ascites tumor and Ehrlich cells were inoculated into the peritoneal cavity of ddY mice. MM-46 cells were propagated as ascites in C3H/He mice in the same manner. The ascitic fluid was collected and the cells were washed with 0.15 M NaCl and diluted to 1×10^6 cells per 0.1 ml of 0.15 M NaCl.

To screen for tumor cell agglutination, the sample solution (0.02 ml) was mixed with the same volume of tumor cell suspension (final concentration, 2×10^5 cells/0.04 ml) on glass plates. The agglutination reaction was observed under light against a black background, as described previously[11]. Agglutination was measured using the percent total particle number (%TPN). When the %TPN fell below 90 compared with the initial particle number (2×10^5 cells/0.04 ml, 100%TPN) because of the formation of cell aggregates, the extract

Table 1. Distribution of agglutinins in whole body extracts of aquatic insects

Species	Stage[a]	Agglutination[b] Tumor cells Ehrlich	S-180	MM-46	Human erythrocytes
Trichoptera					
Stenopsyche marmorata Navas	L	+	+	+	−
Nemotaulius admorsus McLachlan	L	−	−	−	−
Plecoptera					
Oyamia gibba Klapalek	L	−	−	−	−
Megaloptera					
Protohermes grandis Thunberg	L	+	+	+	−
Ephemeroptera					
Ephemerella cryptomeria Imanishi	L	−	−	−	−
Rhithrogena japonica Ueno	L	+	+	+	−
Hemiptera					
Gerris paludum insularis Motschulsky	A	+	+	+	+
Hesperocorixa distanti Kirkaldy	A	−	−	−	−
Notonecta triguttata Motschulsky	A	−	−	−	−
Ranatra chinensis Mayr	A	−	−	−	−
Coleoptera					
Gyrinus japonicus Sharp	A	−	+	−	−
Mataeopsephus japonicus Ishida	L	−	−	−	−
Rhantus pulverosus Stephens	A	−	−	−	−
Odonata					
Anax parthenope julius Brauer	L	++	++	++	−
Sympetrum frequens Selys	L	++	++	++	−
Diptera					
Chironomus yoshimatsui Martin	L	−	−	−	−

[a]L, larva; A, adult.
[b]The tumor cells were treated with extracts at room temperature for 5–10 min, and the decrease of the percent total particle number (%TPN) was measured. The initial particle number (100%TPN) was 2×10^5 per 0.04 ml. −, 100%TPN; +, 51–90%TPN; ++, ~50%TPN.

was described as having positive agglutination activity (−, 100%TPN; +, 51–90%TPN; ++, ~50%TPN, see table 1).

The agglutination reaction was also performed in small test tubes as described previously[12]. Serial doubling dilutions of the sample solution were made, starting with a concentration of 1000 μg/100 μl. 100 μl portions of the agglutinin solutions were placed in small test-tubes, and to each tube 100 μl of a cell suspension containing 1×10^6 tumor cells was added, and the tube shaken for 10 min. The agglutination activity was expressed as the reciprocal of the highest dilution of the original sample solution that showed agglutinating activity (titre $^{-1}$).

To study the inhibition of agglutination, sugars (0.1 ml) were serially diluted in small test tubes, then incubated with three times the minimum concentration that showed agglutination activity of the 10 mM-eluted fraction (D10) derived from DEAE-cellulose, as described below (0.01 ml). The tubes were left to stand for 30 min, then tumor cell suspension (0.1 ml) was added, and then the tubes shaken at room temperature for 10 min and then left for 30 min. Agglutination was scored as described previously[12].

Hemagglutination of human erythrocytes: Human erythrocytes were washed 3 times with 0.15 M NaCl and diluted to give a 2% suspension. The extract (0.05 ml) was placed on the hemagglutination U-plate, and the 2% erythrocyte suspension (0.05 ml) added. The hemagglutination reaction was performed as described previously[17]. The erythrocytes did not agglutinate in 0.15 M NaCl by themselves under these conditions.

DEAE-cellulose column chromatography of the crude extracts from G.paludum insularis and Stenopsyche marmorata: The crude extract (1.0 g) from *G.paludum* was dissolved in 1 mM phosphate buffer (PB) (pH 6.8, 30 ml) and applied to a column (2.4 × 40 cm) of DEAE-cellulose (DE23, Whatman) equilibrated with 1 mM PB. Samples were eluted with a stepwise increase of the molarity of PB (pH 6.8) (10, 30, 50, 100 and 200 mM). Each fraction was concentrated to a small volume, dialyzed against distilled water and lyophilized. Ion-exchange chromatography of the crude extract from *S.marmorata* was performed, and the agglutination activities of the DEAE fractions assayed, as described above.

In vivo studies: Sarcoma 180 tumor cells (1×10^6 cells) were inoculated into the peritoneal cavity of ddY mice as described above. D10 (5 mg/kg) derived from *G.paludum* was injected 5 times intraperitoneally at 24 h-intervals, starting 24 h after inoculation. On day 12, the % solid tumor formation and the % increase in body weight were measured.

Results and discussion

Invertebrate lectins and agglutinins have been found in hemolymph[18] and coelomic fluid[19,20], and in secretions[21]. They have been detected using agglutination of mammalian erythrocytes, microorganisms or tumor cells[22]. All of the larvae or pupae of aquatic insects live in the water, while the habitat of adult insects except for Hemiptera and Coleoptera is the land. The agglutination of the whole body extract from 16 aquatic insects (larvae, and adults which live in water: e.g. *G.paludum*, *Gyrinus japonicus*) against three ascites mouse tumor cell lines and human erythrocytes is shown in table 1. An agglutinin from *G.paludum* adults agglutinated tumor cells potently and human erythrocytes weakly. Agglutinins from larvae of *S.marmorata*, *P.grandis*, *Rhithrogena japonica*, *Anax parthenope julius* and *Sympetrum frequens* agglutinated all the tumor cell lines shown in table 1, but did not agglutinate human erythrocytes. *A.parthenope* and *S.frequens* agglutinins agglutinated tumor cells more potently than any of the others. An agglutinin from *G.japonicus* adults agglutinated only S-180 cells.

Agglutinins were present in 5 out of 10 of the larvae and in 2 of the 6 adults, *G.paludum* and *G.japonicus*. After agglutination had been induced by the active crude extract, the supernatant from this mixture failed to agglutinate fresh cells. The presence of agglutinins was independent of the order the insects belonged to. These results suggest that agglutination activities can be detected more efficiently using tumor cells than using human erythrocytes.

The active crude extracts of *G.paludum* and *S.marmorata* were fractionated on DEAE-cellulose (fig. 1). Agglutination activity was detected in fraction D10 in both *S.marmorata* and *G.paludum*. The titers^{-1} of tumor cell agglutination activity and yield of D10 derived were 24 and 32–64, and 1.6 and 1.3%, from *G.paludum* and *S.marmorata*, respectively. Agglutination activities were also detected in the 100 mM PB-eluted fraction from *G.paludum* and the 200 mM PB-eluted fraction from *S.marmorata*.

The D10-induced agglutination of Ehrlich and S-180 ascites cells was not inhibited by the following mono- and oligosaccharides even at 100 mM: D-glucose, D-galactose, D-mannose, L-rhamnose, L-fucose, D-fructose, D-xylose, N-acetyl-D-glucosamine, N-acetyl-D-galactosamine, melibiose, lactose, lactulose, raffinose and stachyose. Further studies are necessary to purify the agglutinins and to determine their sugar specificities. Many investigators have described the varied serological and immunological properties of invertebrate agglutinins. Prokop et al.[23] studied cytoagglutination induced by garden snail (*Helix pomatia*) agglutinin in rodent tumor cell lines and human erythrocytes. An anti-A agglutinin from *H.pomatia* caused remarkable agglutination in Zajdela hepatic rat tumor cells and

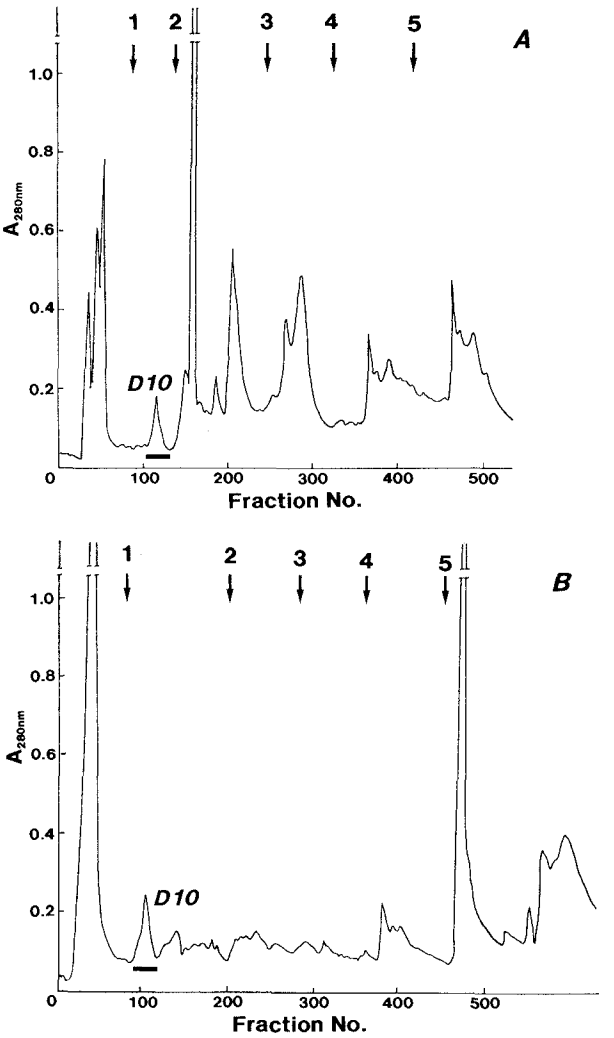

Figure 1. DEAE-cellulose column chromatography of crude extracts from *G.paludum A* and *S.marmorata B*.
Samples were eluted with a stepwise increase of the molarity of PB (pH 6.8) as indicated with arrows from 1 to 5. Eluent 1, 0.01 M PB; eluent 2, 0.03 M PB; eluent 3, 0.05 M PB; eluent 4, 0.1 M PB; eluent 5, 0.2 M PB. Five milliliter fractions were collected and the absorbance was monitored at 280 nm. D10 was obtained from the fractions indicated with bars.

mouse bone marrow leukocytes, but did not agglutinate erythrocytes of normal or cancer-bearing animals, Walker carcinoma and polyoma sarcoma of rats, rat germ cells, or liver, kidney and heart cells of young rats. Pardoe et al.[24] have demonstrated that *L.polyphemus* agglutinin recognized sialidase-sensitive N-acetylsialic acid residues of human Burkitt's lymphoma cell surface glycoconjugates. Lectins from *Balanus resesus* and *B.balanoides*, which are marine invertebrates, agglutinated normal splenic cells and erythrocytes of mice, as well as MM-46, MH134 and Lewis tumor cells[25].

When D10 derived from *G.paludum* was administered 5 times intraperitoneally at 24 h-intervals from 24 h after tumor inoculation, sarcoma 180 cells were propagated in a solid form with 56% probability (10/18) (table 2).

Table 2. Effect of *Gerris paludum* agglutinin on S-180 tumor cell growth

	G.paludum agglutinin treatment	untreated control
Formation of solid form tumor[a] (%)	56	0
Increase of body weight[b] (%)	4.4	32.7

[a]The positive ratios (%) of three separate experiments were averaged on day 12. Six mice (one group) were used in each experiment. [b]Increase % was calculated from the following formula: (Increments of body weight on day 12/body weight on day 0) × 100

In one experiment, all untreated control tumor-bearing mice (n = 6 mice) died between days 12 to 18, whereas 4 mice (4/6) treated with D10 survived for up to 30 days. Mice bearing the solid form tumor which were treated later with D10 died, as a result of the accumulation of ascitic fluid. Conversion of the ascites form into the solid form of the tumor in vivo also occurred after administration of the agglutination-active fractions derived from *S.marmorata* and *P.grandis* (data not shown). Further work is necessary to determine whether or not the *G.paludum* agglutinin agglutinated S-180 ascites tumor cells in the peritoneal cavity of mice, and this resulted in the formation of the solid form of the tumor. These findings, that insect agglutinins preferentially agglutinate tumor cells, are of interest in relation to their potential anti-tumor activity, for example in the inhibition of cell proliferation and metastasis.

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