Jpn. J. Cancer Res. 87, 1259–1262, December 1996

Presence in *Pieris rapae* of Cytotoxic Activity against Human Carcinoma Cells

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Cytotoxic activity in extracts of pupae and adults of various kinds of butterflies and moths was tested *in vitro* against the human gastric carcinoma cell line, TMK-1, which was chosen as an example of human carcinoma cells. Among the species examined, cytotoxicity was limited to *Pieris rapae, Pieris napi* and *Pieris brassicae*. Activity was found down to a dilution of $1/10^4$, while with the other butterflies and moths no activity was observed, even at $1/10^2$. When the cytotoxicity of the three developmental stages, larvae, pupae and adults, of *Pieris rapae* was compared, the pupae showed the strongest activity, the IC_{50} against TMK-1 cells being at the $1/10^6$ dilution. For larvae and adults, the respective IC_{50} values were at the $1/10^5$ and $5/10^5$ dilutions. The active principle in the pupae of *Pieris rapae* was found to be heat-labile and not extractable with organic solvents, but precipitated with ammonium sulfate and digested by proteases, suggesting that it is a protein. This cytotoxic factor was named pierisin.

Key words: Cytotoxicity — Gastric carcinoma cell line — Butterfly — *Pieris rapae* — Pierisin

Insects do not possess lymphocytes or immunoglobulins, and their immune systems are very different from those of vertebrates. As a defense against bacterial infection, insects produce several antibacterial proteins.[1] Insect lysozyme is a well known antibacterial protein, although it is only bactericidal towards a few Gram-positive species such as *Bacillus megaterium* and *Micrococcus luteus*. Melittin, the main lytic component in bee venom, has been demonstrated to lyse both bacteria and eukaryotic cells. A series of cecropins has been isolated from the Cecropia moth, *Hyalophora cecropia*, as potent antibacterial proteins with molecular weights of around 4×10^3.[2-4] These cecropins have been detected in several kinds of insects[1,5,6] and found to be effective against a variety of Gram-positive and Gram-negative bacterial strains. Another kind of antibacterial protein, defensin, has also been isolated from various insect species.[7-9]

In addition to these antibacterial substances, invertebrate lectins are thought to play an important role in self-defense mechanisms. *Sarcophaga* lectin, purified from the hemolymph of *Sarcophaga peregrina*, has been suggested to have several biological functions, including the elimination of invading foreign substances and unnecessary cells produced during embryogenesis and pupation.[10,11] The galactosyl-binding lectin in *Periplaneta americana* has been reported to be involved in biological defense.[12,13] Moreover, *Sarcophaga* lectin was found to show *in vivo* cytotoxic activity against murine ascitic and solid tumors.[14]

The above observations suggest that insects could be a rich source of new drugs. In the present study, we therefore examined whether extracts of larvae, pupae and adults of butterflies and moths exhibit cytotoxicity against the human gastric carcinoma cell line TMK-1, chosen as an example of human carcinoma cells. We report here the presence of a cytotoxic agent(s) in three butterflies, *Pieris rapae*, *Pieris napi* and *Pieris brassicae*, and also some details of its nature based on studies of extracts from *Pieris rapae* pupae.

MATERIALS AND METHODS

Species of butterflies and moths All the samples except *Bombyx mori*, a moth, were collected from farming areas and forests in Japan. *Bombyx mori* was kindly provided by Dr. M Yamakawa (National Institute of Sericultural and Entomological Science, Tsukuba). Adults of 19 kinds of butterflies and moths, *Bombyx mori, Celastrina argiolus, Colias erate, Dichorragia nesimachus, Eurema hecabe, Hebomoia glaucippe, Hestina japonica, Lycaena phlaeas, Mamestra brassicae, Papilio bianor, Papilio helenus, Papilio maackii, Papilio machaon, Papilio protenor, Papilio xuthus, Pieris napi, Pieris rapae, Sasakia charonda* and *Vanessa indica* were examined for cytotoxicity in their body fluids. Pupae from 14 of the above butterflies and moths, excluding *Celastrina argiolus, Colias erate, Eurema hecabe, Lycaena phlaeas* and *Vanessa indica*, and from *Pieris brassicae* were also tested for cytotoxicity. Larvae of *Pieris rapae* were also examined. Samples were stored at $-50°C$ until extraction.

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Preparation of extracts from butterflies and moths Frozen whole bodies of pupae and larvae, and abdomens of the adults were weighed and squeezed through a syringe. The ejected and residual materials were combined and mixed with nine volumes (v/w) of phosphate-buffered saline, thoroughly agitated, then centrifuged at 5000 rpm for 15 min at 4°C. The supernatant was filtered through a Millex-GV filter (0.22 μm, Millipore Corp., Bedford, MA). The resulting filtrate, termed the extract, was stored at −50°C.

Cytotoxicity test The human gastric carcinoma cell line TMK-1[15] was used for cytotoxicity testing. Near-confluent cells were trypsinized, collected and diluted to 5×10^4 cells/ml with RDF medium[16] containing 10% fetal calf serum (Gibco BRL, Gaithersburg, MD). One hundred microliter aliquots of the cell suspension were dispensed into the wells of 96-well plates and combined with diluted samples of the extract. After 48 h incubation at 37°C in 5% CO_2 in air, cytotoxic effects were measured with an XTT cell proliferation assay kit (Boehringer Mannheim, Mannheim, Germany).

Stability of the cytotoxic factor in the "extract" The "extract" was kept at 20, 40 or 60°C for 1 h at pH 7.4, or at 37°C for 1 h at pH 1.0, 3.0, 5.0, 7.0, 9.0 or 11.0 and the cytotoxicity was measured using the method described above.

Enzyme treatment of the extract The extract was diluted ten times, to $1/10^2$, then treated with pronase E, proteinase K, micrococcal nuclease plus spleen phosphodiesterase II or nuclease P1 at 37°C for 1 h, and the cytotoxicity was measured after appropriate dilution. Pronase E (Kaken Seiyaku, Tokyo) was applied at a final concentration of 1 mg/ml in 50 m*M* NaCl, 10 m*M* Tris-HCl pH 8.0, 5 m*M* $CaCl_2$; proteinase K (Merck, Darmstadt,

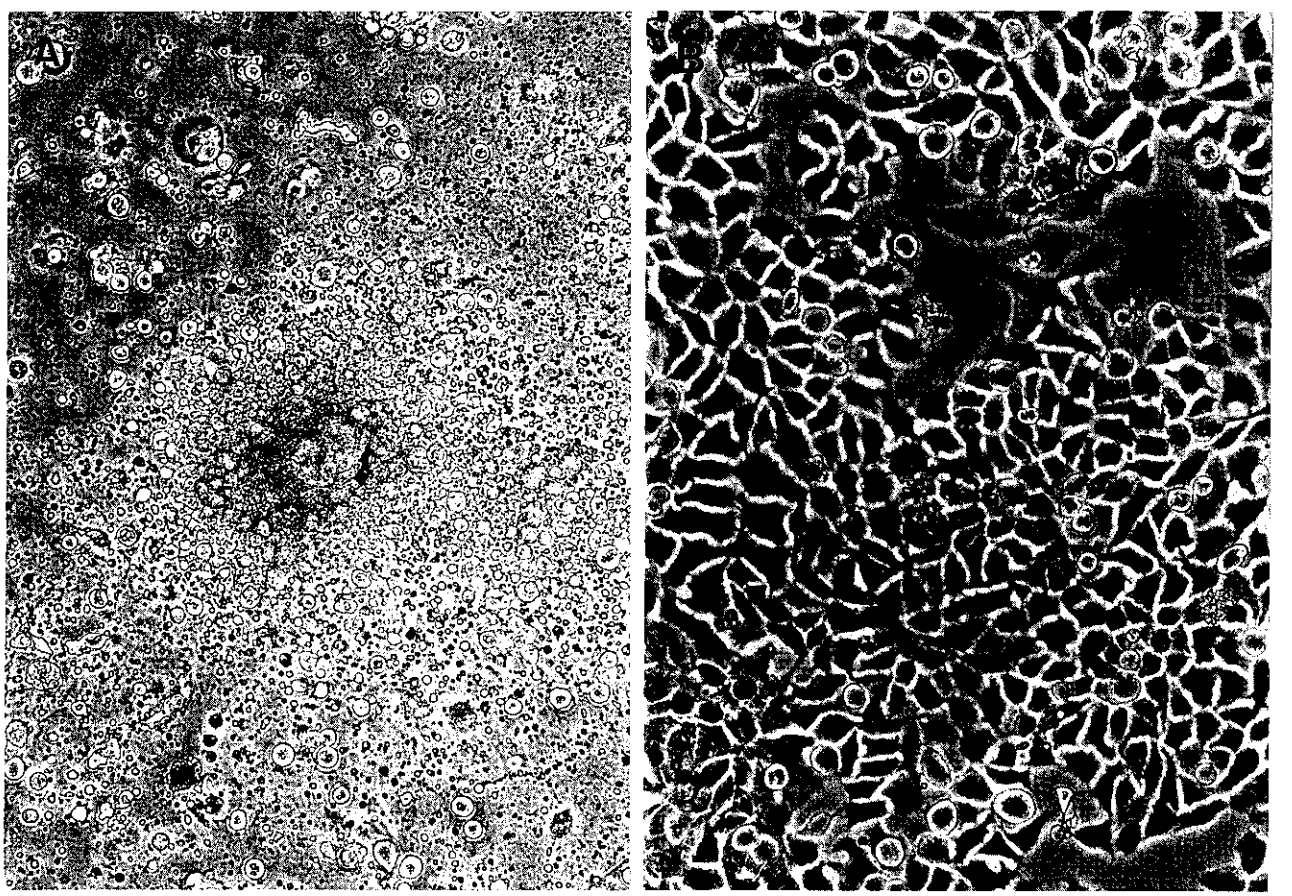

Fig. 1. Phase-contrast micrographs of TMK-1 cells treated with the extract from *Pieris rapae* pupae. The extract sample was diluted ten-fold, to $1/10^2$, and 0.1 ml of the diluted sample was mixed with TMK-1 cells, which were grown to near-confluence in a 10 cm dish containing 9.9 ml of medium as described in the "Materials and Methods." The final dilution of the original pupae body fluid was $1/10^4$. TMK-1 cells after treatment with the extract for 20 h at 37°C are shown in (A) and those not treated with the extract in (B).

Germany) at a final concentration of 1 mg/ml in 50 m*M* NaCl, 10 m*M* Tris-HCl, 5 m*M* EDTA pH 8.0; micrococcal nuclease (Worthington, Freehold, NJ) plus spleen phosphodiesterase II (Worthington) at final concentrations of 200 units/ml and 2 units/ml, respectively, in 20 m*M* sodium succinate buffer pH 6.0, 10 m*M* $CaCl_2$; nuclease P1 (Yamasa, Cho-shi) at a final concentration of 0.5 mg/ml in 53 m*M* sodium acetate buffer pH 5.3, 0.12 m*M* $ZnCl_2$.

Recovery of the cytotoxic factor by ammonium sulfate precipitation and organic solvent extraction The extract was diluted to $1/10^2$, mixed with saturated ammonium sulfate solution to give 20%, 50% or 80% saturation, allowed to stand for 24 h at 4°C and centrifuged. The precipitates were washed with the same concentration of ammonium sulfate, dissolved in phosphate-buffered saline, and examined for cytotoxicity.

The extract was diluted to $1/10^2$, mixed with the same volume of chloroform or diethyl ether and shaken for 5 min at room temperature. The separated organic solvent layer was dried and evaporated, and the residue was dissolved in phosphate-buffered saline and examined for cytotoxicity.

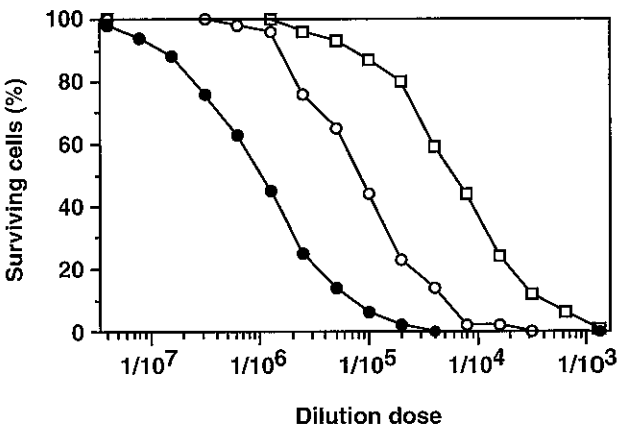

Fig. 2. Dose-dependent cytotoxic effects of extracts from three developmental stages (larva, pupa, adult) of *Pieris rapae* on TMK-1 cells. Extract samples from larvae (○), pupae (●) and adults (□) at various dilutions were incubated with TMK-1 cells and numbers of living cells were measured with the XTT cell proliferation assay. The value shown at each point represents the mean for four wells.

RESULTS AND DISCUSSION

Samples of 34 extracts, prepared from pupae and adults of 18 kinds of butterflies and 2 kinds of moths, were diluted step-wise, the concentration at each step being one-tenth that of the previous one. At each stage, cytotoxic activity against the human gastric carcinoma cell line, TMK-1, was examined. Among the samples, the extracts of pupae and adults from *Pieris rapae*, *Pieris napi* and *Pieris brassicae* were found to show cytotoxicity down to dilutions of $1/10^4$, reducing the numbers of surviving cells to less than 50%. None of the other butterflies and moths exhibited any cytotoxicity, even at the $1/10^2$ dilution. Fig. 1 illustrates the microscopic appearance of TMK-1 cells after treatment with the extract from *Pieris rapae* pupae.

As compared to *Pieris napi* and *Pieris brassicae*, *Pieris rapae* is very commonly distributed in Japan, and the following experiments were therefore carried out using this species. First, the dose-dependence of the cytotoxicity of extract samples from the three developmental stages of *Pieris rapae*, larvae, pupae and adults, was tested. As shown in Fig. 2, the pupae exhibited the strongest cytotoxic activity of the three, killing 50% of TMK-1 cells at the $1/10^6$ dilution (IC_{50}). The larvae had the second strongest toxicity, with the IC_{50} at the $1/10^5$ dilution, and the adults showed the IC_{50} at the $5/10^5$ dilution.

To understand the nature of the cytotoxic factor in pupae of *Pieris rapae*, the extract was subjected to various treatments. When the extract was heated to 60°C for 1 h at pH 7.4, cytotoxic activity was completely abolished, but when the extract was maintained at 20°C for 1 h at pH 7.4, the original activity was retained. Exposure to 40°C for 1 h caused a 30% loss of activity. When the extract was kept at pH 5 or 7 for 1 h at 37°C, about 15% of the cytotoxic activity was lost. About 50% loss of activity was observed at pH 3 or pH 9 for 1 h at 37°C, whereas the activity was completely abolished after exposure to pH 1 or pH 11. The cytotoxic factor in the "extract" was not precipitated at 20% saturation of ammonium sulfate, but all the activity was recovered by precipitation with ammonium sulfate at saturation levels of 50% or more. The factor was not extractable with either chloroform or diethyl ether and most of the activity remained in the aqueous phase. Enzyme digestion experiments showed that both pronase E and proteinase K caused more than 95% inactivation. On the other hand, micrococcal nuclease, spleen phosphodiesterase II and nuclease P1 did not affect the cytotoxic activity. From the above observations, the cytotoxic factor(s) in the pupae of *Pieris rapae*, active against the human gastric carcinoma cell line TMK-1, was suggested to be proteinaceous in nature. Preliminary studies with Superose gel filtration chromatography suggested the molecular weight of the cytotoxic factor to be around 150,000. As described above, the extract from the pupae of *Pieris rapae* showed the IC_{50} for TMK-1 cells at a dilution of $1/10^6$. Based on this value for the potency of the activity and the suggested molecular weight, this

cytotoxic factor appears to show activity at a concentration of less than 1 n*M*. We propose to name this cytotoxic factor in *Pieris rapae* pierisin.

Butterflies of the *Pieris* family may all express this cytotoxic factor. The reason why the pupae of *Pieris rapae* contain stronger activity than the larvae or adults is not clear at present, and the distribution of the activity in the bodies of pupae, larvae and adults still needs to be studied. The cytotoxic effects of pierisin should be examined on various kinds of cells, including normal cells, in order to see whether there is any selectivity for malignant cells. It would also be interesting to see whether or not the cytotoxic activity is more pronounced in log phase cells or in resting cells. To facilitate investigation of the mechanism of cell death, purification of pierisin, from the pupae of *Pieris rapae* is under way in our laboratory.

ACKNOWLEDGMENTS

We thank Dr. M. Yamakawa (National Institute of Sericultural and Entomological Science, Tsukuba) for providing pupae and adults of *Bombyx mori*. This study was supported by Grants-in-Aid for Cancer Research from the Ministry of Health and Welfare, for Scientific Research from the Ministry of Education, Science, Sports and Culture, and for the Second-Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health and Welfare, Japan.

(Received September 30, 1996/Accepted October 16, 1996)

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