Agglutinins of Frog Eggs: a New Class of Proteins Causing Preferential Agglutination of Tumor Cells

There is growing evidence that cancer cells are aberrant in cell surface structure and function (see for a review[1] and [2]). The agglutinability of cells by lectins has been hypothesized as being related to regulatory mechanisms of the cell cycle and proliferation[2], although the molecular basis has not been conclusively decided.

The altered reactivity of malignant cell surfaces has not only been demonstrated by phytohemagglutinin, but also by a certain invertebrate protein[3], as well as by antibodies against some glycolipids[4]. The enhanced reactivity of cells by these reagents is not only observed in transformed cells, but also in fetal cells[5,6]. The present paper reports our recent observation that some proteins released from eggs of some frog species showed a distinctive preferential agglutination of various tumor cells. The tumor-distinctive agglutination caused by frog egg agglutinin was inhibited by a new type of glucosamine-containing ganglioside of human erythrocytes. Inhibition by various monosaccharides is so far unsuccessful.

Agglutinins from eggs of *Rana japonica* Guenther, *Rana nigromaculata nigromaculata Hallowell*, and other frogs, as listed in Table I, were prepared from homogenizing eggs with 10 volumes of 0.85% sodium chloride for 10 min in a Waring blender followed by centrifugation at 10,000 rpm for 60 min. A bulky precipitate occurring on dialysis of the supernatant fluid in distilled water was centrifuged, and the water-clear supernatant was lyophilized (Stage 1). The dried residue was added with 100 volumes of distilled water; some insoluble precipitate was removed by centrifugation, and the supernatant was added with ethanol up to 20% under stirring. The non-precipitable part was concentrated and lyophilized to yield a fluffy powder (450 mg from 250 g of eggs, Stage 2). Agglutination activity was found exclusively in this fraction. The active fraction was further separated by chromatography on a column of Sephadex G-75 (Pharmacia, Uppsala, Sweden) with monitoring effluent by ultraviolet scanning. The agglutinating activity was found only in peak III.

Cell suspensions (0.1 ml) were incubated with 0.1 ml of agglutinin solution that contained varying concentrations (1 µg to 100 µg/0.1 ml), and incubated at room temperature with occasional shaking. Alternatively, the cell suspension was mixed with agglutinin solution on a glass plate, and the agglutination was observed under illuminated light against a dark background. For inhibition of agglutination, aliquots of agglutinin solution (10–20 µg/0.05 ml) were incubated first with 0.05 ml of phosphate-buffered saline solution that contained varying concentrations of sugars or gangliosides. Final sugar concentration was 0.1 *M*.

As shown in Table I, only eggs of *Rana japonica* Guenther and of *Rana nigromaculata nigromaculata* Hallowell showed a distinctive agglutination characteristic for ascites hepatoma cells or transformed cell in vitro. The *Rana japonica* agglutinins had no effect on erythrocytes of humans and rats and had much less effect on non-transformed normal cultured cells. The *Rana nigromaculata nigromaculata* agglutinin showed effects on tumor cells and on blood group A erythrocytes but had no effect on erythrocytes with other blood types. The agglutinins of *Rana tigrina tigrina* and of *Rana rugosa* showed 'panagglutination' of various erythrocytes but had no effect on tumor cells, whereas the agglutinin of *Rana catesbiana* preferentially agglutinated blood group A erythrocytes. Nevertheless, this A-like agglutination

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Table II. Agglutinability of various cells to the agglutinin of *Rana japonica Guenther*

	Agglutinability expressed by the minimum amount of purified agglutinin in µg (Stage III) to cause obvious agglutination (per ml)
Ascites hepatoma cells (AH109A) [a]	1–2
Ascites hepatoma cells (AH130) [a]	2–5
Polyoma-transformed NIL cells [b]	5–10
Polyoma-transformed BHK cells [b]	5–10
SV40 virus-transformed 3T3 cells	2–5
Rat erythrocytes	>2000
Human erythrocytes	>2000
Normal NIL cells	500
Normal BHK cells	250
Normal 3T3 cells	1000

[a] Cells prepared in the same way as footnote to Table I. [b] Liberated from cultured flasks by 0.1% EDTA, washed and suspended in phosphate-buffered saline pH 7.2; cell number 10^6/0.2 ml.

Table I. Distribution and reactivities of agglutinins in eggs of various frogs [a]

	Tumor cells [b]	Erythrocytes of blood type		
		A	B	0
Rana japonica Guenther	+	−	−	−
Rana nigromaculata nigromaculata Hallowell	+	+	−	−
Rana tigrina tigrina Daudin	−	+	+	+
Rana catesbiana Shaw	−	+	−	−
Rana rugosa Shlegel	−	+	+	+
Bufo vulgaris formosus Boulenger	−	−	−	−
Hyla arborea japonica Guenther	−	−	−	−

[a] Comparative agglutination tested with a crude preparation at 'Stage 2'. [b] Ascites hepatoma cells (AH109A) grown in Donryu rats, washed and suspended in phosphate-buffered saline pH 7.5: concentration adjusted as 10^6/0.1 ml.

Table III. Inhibition of frog egg agglutinin by monosaccharides, oligosaccharides and glycolipids

Minimum amount of inhibitor (in μmole) to inhibit hemagglutination caused by 50 μg of *Rana japonica* agglutinin	
	μmole per 0.1 ml
D-Glc; D-Gal; D-Mann; D-Xyl; L-Fuc; L-Arab; D-GlcNAc; D-GalNAc; N-acetylneuraminic acid; lactose; melibiose; globoside	All these sugars and glycolipids do not inhibit even at 10–20 μg
Erythrocyte ganglioside[a]	1
Desialylated erythrocyte ganglioside	3–5
Ovomucoid; bovine albumin; egg albumin	Do not inhibit even at 50 μg

[a] A gift from Professor S. HAKOMORI; structure and properties, see[7,8].

was not inhibited by N-acetylgalactosamine, which will be described in detail elsewhere. Normal cultured cells showed some agglutination by as much as 250–1000 μg of the agglutinin, whereas the transformed counterpart was agglutinated by as little as 5–10 μg of the agglutinin (Table II).

Inhibition of tumor cell agglutination by simple monosaccharides or by oligosaccharides have been unsuccessful so far (see Table III). This is in rather striking contrast to the fact that a number of agglutinations caused by plant agglutinins were inhibited by simple sugars. Hemagglutination caused by anti-carbohydrate antibodies is, however, difficult to inhibit by simple sugars or oligosaccharides. It may be that the frog egg agglutinin differs from lectins but rather resembles the antibodies directed to carbohydrates. It is noteworthy that a ganglioside fraction of human erythrocyte membrane[7,8] was capable of inhibiting the agglutination.

In preliminary analysis of the agglutinin fraction, all proteins showed cathodic migration on cellulose acetate electrophoresis at various pH's, including pH 9, indicating that the agglutinin seems to be classidied as a basic protein or proteins[9].

Zusammenfassung. Nachweis, dass der Auszug einer basischen Eiweissfraktion aus Eiern verschiedener Froscharten (*Rana japonica* Guenther, *Rana nigromaculata nigromaculata* Hallowell) mit isotonischer Salzlösung bei diversen Tumorzellen zu besonderer Agglutination führt, während diese Wirkung auf transformierte, normale Zellen oder Erythrozyten ausbleibt. Mit Gangliosiden menschlicher Erythrozyten konnte die Agglutination gehemmt werden, nicht aber mit den bisher geprüften Zuckern.

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Ultrastructure of Cytoplasmic Hemosiderin Inclusion Bodies in Malignant Phagocytic Lymphocytes

During 15 years of routine observations on various lymphoma-leukemia group blood samples, a patient in whom cytoplasmic iron-containing inclusions could be detected in about 20% of the peripheral lymphoid cells, was found for the first time. An earlier study of KOZEWSKI in 1955[1] on the storage of hemosiderin in normal lymphocytes and monocytes of anemic patients suffering from spontaneous hemochromatosis led us to study similar inclusions in malignant lymphoid cells. An ultrastructural analysis of previously unstudied inclusion material was also carried out.

Whole blood from patients diagnosed as having chronic lymphatic leukemia and lymphosarcoma was processed for cytochemical and electron microscopic observations. Several staining procedures employed included reactions for phospholipids[2], alkaline phosphatase[3] and hemosiderin[4]. Toluidin blue, Nile blue, polysacharides and luxol fast blue[5] were employed. Serum analysis revealed low-normal levels of ferrum-content (68 γ%) and ferrum-binding capacity (292 γ%). Bone marrow aspiration and bone marrow biopsy showed the presence of myelofibrosis and aplasia. No signs of hemochromatosis were found in liver and bone marrow biopsy.

Electron microscopy was carried out on unstained and double stained (uranyl acetate and lead citrate) sections at different magnifications from 2,000 to 50,000 with the Jem T7 and E-200 Philips electron microscopes[6–10].

Routine observations of peripheral blood smears (stained with MGG) revealed the presence of basophilic cytoplasmic inclusion bodies in about 20% of the lymphoid cells that comprised 85% of the peripheral leukocytes

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