Erythrocyte Agglutinins in the Blood of Certain Ascidians

The coelomic fluid of many invertebrates possesses natural hemagglutinins specific for a variety of erythrocytes[1–8]. Although these substances are proteins and function as opsonins, they differ in many aspects of their molecular structure from vertebrate immunoglobulins[9–12]. Little is known about natural hemagglutinins of protochordates. Recent studies[13] carried out on the plasma of an Ascidian have suggested that hemagglutinins present in the plasma of this animal may be polysaccharides or mucopolysaccharides.

Since it is well known that the Urochordata represent a precocious branch from the evolutionary line of the Chordata[14], the study of natural agglutinins in Ascidian species might supply additional data on the evolutionary origin of immunoglobulins. In the present paper we report on the occurrence and some properties of the hemagglutinins in the plasma from *Ciona intestinalis* L., *Ascidia malaca* Fraust and *Phallusia mamillata* Cuv.

Table I. Hemagglutinating activity of plasma from 3 different species of Ascidian against erythrocytes from various vertebrates

Erythrocytes used for hemagglutination tests	Hemagglutinating activity[a]		
	Ciona intestinalis	*Ascidia malaca*	*Phallusia mamillata*
Anguilla sp.. (Eel)	—	—	+
Uranoscopus scaber (Star-gather fish)	—	—	+
Discoglossus pictus (Frog-like)	—	—	+
Bufo vulgaris (Toad)	—	—	+
Chicken	—	—	+
Ziphius cavirostris (Dolphin)	—	+	+
Rat	+	++	++
Rabbit	+	++	+++
Sheep	—	—	+++
Pig	—	+	+
Horse	—	—	++
Calf	—	+	+
Human-blood group: 0	—	+	++
A	—	+	++
B	—	+	++

[a] Hemagglutination titres obtained from repeated tests and expressed as reciprocal of the last dilution given agglutination; +, includes < 2, 2, 4; ++, includes 8, 16, 32; +++, includes 64, 128, 256.

Table II. Hemagglutinating activity of absorbed plasma from *Phallusia mamillata* and *Ascidia malaca*

Ascidian	Erythrocytes used for absorption	Hemagglutinating activity[a]		
		Rabbit	Sheep	Rat
Phallusia mamillata	no absorption	+++	+++	++
	Rabbit	—	—	—
	Sheep	—	—	—
	Rat	—	—	—
Ascidia malaca	no absorption	++		+
	Rat	—		—
	Rabbit	—		—

[a] Hemagglutination titres obtained from repeated tests and expressed as reciprocal of the last dilution given agglutination; +, includes < 2, 2, 4; ++, includes 8, 16, 32; +++, includes 64, 128, 256.

The animals were collected in the Gulf of Palermo (Sicily). Animals were bled into a beaker by cardiac puncture. The plasma, obtained by centrifugation at $4000 \times g$, was dyalized against phosphate buffered saline pH 7.4 (PBS). Hemagglutination tests were performed in microtitrator plates (Cook Enginering Co., Alexandria, Va.) following the standard methods[15]. Titres were expressed as the reciprocal of the last dilution which gives a clear agglutination.

Different results have been obtained with each species examined: the plasma from *Ph. mamillata* agglutinates erythrocytes from all the species of vertebrates employed and shows the highest hemagglutination titre; plasma from *A. malaca* agglutinates erythrocytes from certain species of mammals, including humans; that from *C. intestinalis* only agglutinates rat and rabbit erythrocytes and shows the lowest hemagglutination titre (Table I).

The specificity of the hemagglutinin fraction was examined by the absorption method of mixing plasma preparations from *Ph. mamillata* or *A. malaca* with an equal volume of packed erythrocytes from rabbit, rat or sheep. It was observed that in both species the activity of the plasma was completely eliminated by incubating 1 h at 37 °C or 2 h at 4 °C (Table II).

Plasma preparations were incubated at temperature of 0 °C, 37 °C, 75 °C and 100 °C for 10, 20 and 30 min. The hemagglutinating activity was completely destroyed at 100 °C for 30 min and decreased by shorter periods of incubation (10 min) and lower temperature (75 °C).

Aliquots of plasma previously dialyzed against 0.15 *M* NaCl were dialyzed at room temperature for 12 h against buffer solutions of various pH values (2 to 10) and then dialyzed against PBS. No change of the activity was observed in the pH range of 2 to 10.

Since it was found that calcium ions may be important in stabilizing the structure of hemagglutinin[9,10], the effect of bivalent cations on hemagglutinating activity of plasma was examined. Plasma preparations were dialyzed against saline (0.15 *M* NaCl) containing 0.01 *M* and 0.05 *M* EDTA or against *Tris*-HCl (pH 7.4)-0.15 *M* NaCl containing 0.002 *M*, 0.005 *M*, 0.01 *M*, and 0.02 *M* $CaCl_2$ or $MgCl_2$. The same results were obtained by using Ca^{++}; Mg^{++} free plasma preparations or plasma containing these cations: the activity did not change as a result of the dialysis.

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Under certain conditions, periodate oxidizes polysaccharides. Consequently plasma of *C. intestinalis*, *A. malaca*, or *Ph. mamillata* was incubated at pH 5.4 with sodium metaperiodate, 0.02 *M* and 0.04 *M* final concentrations. The reaction mixtures were incubated in darkness for several hours at 25 °C and then dialyzed against PBS. The hemagglutinating activity of the plasma preparations was not affected by periodate treatment. The activity of the periodate concentrations was verified by incubating reaction mixtures for 1 h in which the plasma was replaced by 0.01 *M* glucose or saccharose solutions and 1% starch solution. The periodate consumption was traced by reading the absorbance at 223 nm[16].

Since molecules with definite size, shape and charge can be characterized by their solubility in a given medium, attempts were made to fractionate hemagglutinins on the basis of their solubility in ammonium sulphate solutions[17]. The experiments were carried out with plasma from *Ph. mamillata* and *A. malaca* which have shown the highest hemagglutination titres. Aliquots were incubated at 0 °C for several hours with increasing concentrations of ammonium sulphate (25, 50, 75, 100% final concentration) and then centrifuged at 18,000 × *g* for 60 min at 0 °C. The precipitate was dissolved in saline. All the samples were dyalized against PBS and then subjected to a 4-fold concentration in a Diaflo equipped with UM2 membrane (Amicon Corp., Lexington, Mass.). The active fraction precipitates completely at 75 and 100% ammonium sulphate saturation levels and the hemagglutinating activity was found in the precipitate.

In a further experiment, we attempted to see whether or not 2-mercaptoethanol, a reagent that breaks the S-S linkage in protein molecules, affects hemagglutinating activity of the plasma preparations. Aliquots of the plasma were treated for 1 h at room temperature with 2-mercaptoethanol (0.1 *M* and 0.2 *M* final concentration) in saline; the reaction was stopped by addition respectively of 0.2 *M* and 0.4 *M* iodoacetamide, and the reaction mixture were dialyzed against PBS. Both 2-mercaptoethanol concentrations destroyed the hemagglutinating activity of the plasma.

An attempt was made to digest plasma preparations with proteolytic enzyme to see if the fraction carrying the hemagglutinating activity was protein; 0.9 ml plasma preparations were dialyzed overnight against 0.05 *M* KCl-HCl buffer (pH 2) and then incubated with 0.1 ml pepsin (Sigma) solution (4 mg/ml) at 37 °C for 1 h. The reaction mixture was dialyzed against PBS and the hemagglutinating tests were performed at 37 °C or 4 °C. This treatment completely destroyed the hemagglutinating activity of plasma preparation from *Ascidia malaca* and *Phallusia mamillata*. Since the hemagglutinating activity was not influenced when enzyme-free reaction mixture were incubated, possibly hemagglutinin is digested by the enzyme.

Conclusions. Data presented in this paper show that *Ciona intestinalis* L., *Ascidia malaca* Fraust and *Phallusia mamillata* Cuv. possess in their plasma agglutinins for a number of vertebrate erythrocytes. In each Ascidian species, the agglutinins are specific for a given variety of erythrocytes. Absorption tests suggest that the reactive sites of these molecules have similar properties.

Plasma from *Phallusia mamillata* shows the highest activity and agglutinates erythrocytes from all vertebrate employed. By contrast plasma from *Ciona intestinalis* presents the lowest spectrum of hemagglutinating activity. Possibly there are small phylogenetic differences in the molecular structure of the hemagglutinins which could account for this range of specificity.

Other investigators[13] found that hemagglutinin of *Styela plicata* is very heat-stable (140 °C for 30 min), resistant to trypsin digestion and destroyed by periodate. Therefore they suggest that this molecule is a polysaccharide or a mucopolysaccharide.

Our results, obtained by physical and chemical treatments of the plasma, exclude the possibility that hemagglutinins from *Phallusia mamillata* and *Ascidia malaca* are polysaccharides. They are resistant to periodate, sensitive to mercaptoethanol, digested by pepsin, inactivated by heating at 100 °C and precipitated by ammonium sulphate. These data suggest that, at least in these two Ascidian species, the hemagglutinin may be a protein or a protein-like substance in which the molecular structure is characterized by a high molecular weight, high resistance to thermal denaturation and insensitivity to pH and cation action.

Summary. Plasma from *Ciona intestinalis*, *Phallusia mamillata* and *Ascidia malaca* possess hemagglutinin for a variety of erythrocytes. Results obtained by physical and chemical treatments suggest that hemagglutinin for *Phallusia mamillata* and *Ascidia malaca* may be a protein or a protein-like substance.

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Bio-Electrical Changes Induced by a Reaction Between Rabbit Ig and Anti-Rabbit Ig on the Surface of the Rat Cervical Ganglion

The present experiment concerns the initial phase of activation of the cell by immunological means, and is based on the following inferences drawn from immunosciences and neuro-sciences: the recognition of antigen by lymphocytes is mediated by immunoglobulin molecules incorporated into the membrane surface[1]; there is a close relationship between membrane potentials and macromolecules of the neuronal membrane; the anti-brain antibodies combine specifically with antigenic determinants of the neuronal membrane[2], and the basic processes underlying the response of the lymphocyte and the neuron to stimuli are similar[3].

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