

## HUMORAL IMMUNE RESPONSES TO VARIOUS ANTIGENS IN THE ASTERIDS: *A.GIBBOSA* AND *A.RUBENS*

Michel Leclerc

Immunologie des Invertébrés, U.E.R Sciences, Université d'Orléans, France

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### ABSTRACT

Immunizations with various antigens of the sea stars *Asterina gibbosa* and *Asterias rubens* produce specific humoral immune reactions which initiate the antibody factor. This antibody factor is correlated with kappa genes and could be composed of 4 kappa chains. It is the first time that such phenomenon was described in an invertebrate.

**Keywords:** Invertebrate, Immunogenesis, Phylogenesis, Sea-Star, Model, Echinoderma

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### 1. INTRODUCTION

In invertebrates, we say now that immunity is characterized by physiological mechanisms mediated by various types of cells, especially lymphocytes and various soluble proteins which recall strongly, in sea star system, vertebrate immunity. In this study, we have tried to show, in an invertebrate, that specific immune reactions, as compared to mammal system, occurred in the asterid one.

### 2. MATERIALS AND METHODS

#### 2.1. Materials

##### 2.2.1. The Sea-Stars

Sea-stars belong to the class Asteroidea of the phylum Echinodermata. They are marine animals, also named starfishes.

The species *Asterina gibbosa* is frequently found on the Channel (France). It lives on the sea floor at a depth of about 2 -5 meters, where the temperature of the water is between 7 and 15°C according to the season. It can attain a size of 3-5 cm in diameter. It is hermaphrodite.

The species *Asterias rubens* also named (*A.vulgaris*) is frequently found on the Atlantic coast of France and the channel. It can attain a size of 30 cm or more in diameter. It is a predator of shellfish (scallops).

Sea-stars were obtained from the « Institut de Biologie Marine de l'Université de Lille »

(Wimereux, France). After collection, the sea-stars were kept in sea water and immediately, sent to the laboratory, or were maintained in aquariums with running sea water at 10°C.

#### 2.2. Methods

Injections of various antigens were performed in the coelomic cavity with a fine needle and a syringe, near the axial organ: it is an original challenge. We inject 0,1ml soluble antigen (horseradish peroxidase, TNP-PAA, FITC-PAA) per animal, per injection near the madreporic plate. In certain cases, following « immunizations », sea star lymphocytes (B and T sea star lymphocytes) + sea star phagocytes were cultured *in vitro* and restimulated with the same antigen.

### 3. RESULTS AND DISCUSSION

#### 3.1. *In vivo* Experiments

In a first study in (Leclerc, 1973), it was shown that the axial organ cells of the sea star *Asterina gibbosa* previously injected with horseradish peroxidase were able to react specifically with the same enzyme. This observation was made by electron microscopy using an immunocytochemical technique. A similar result was later observed with another enzyme, the alkaline phosphatase of *E.coli*. These observations suggested that, following stimulation with foreign antigens, some cells of the axial organ were able to synthesize substances which reacted

specifically with the injected antigen in a manner comparable to the immune response of vertebrates.

So a variety of antigens were injected in the coelomic cavity, near the madreporic plate such as horseradish peroxidase, *E.coli* alkaline phosphatase, bovine serum albumin, rat IgG, human lambda Bence-Jones protein, myoglobin and more recently, the haptens TNP and FITC coupled to polyacrylamide beads.

In a first series of experiments, the animals received three or four injections one week apart and the axial organs were taken one week after the last injection. Electron microscopy cytochemical observations were made with the enzymatic antigens and with peroxidase labelled protein antigens.

In other experiments, the axial organs of the treated animals were pooled and teased and cell suspensions were prepared and examined using fluorescent antigens. The results of these experiments can be summarized as follows:

- When examined by electron microscopy, enzymatic activity was observed in the ergastoplasmic cisternae (and) in the perinuclear spaces of some cells. Exocytose of « bullets » was observed
- The reactions were specific, that is, they were only observed in the cells of the animals treated with the corresponding enzyme or protein; cells from non-treated animals did not show any reaction

### 3.2. *In vitro* Experiments

A technique was developed in recent years which allowed a more detailed study of the nature of the cells and production of soluble factors following immunization of the sea stars. Animals were inoculated *in vivo* and kept in aquariums; 7 days later, their axial organs were dissected, washed and teased and the cells obtained were cultured *in vitro* and restimulated with the same antigen.

With this technique, it was possible to show that the cells of the immunized animals secreted a soluble factor with properties that resembled those of the vertebrate antibodies. This factor could be detected and quantitated by its capacity to lyse red cells labelled with the same hapten used to treat the animals.

The antigens employed were TNP and FITC coupled to PAA beads (Bio-Gel P30, 100-200 mesh) (Brillouet *et al.*, 1984). Coupling was made by the method of Kagedal and Akerstrom (1971) using TNP-sulfonic acid (Sigma, USA) and FITC (Biomérieux, France). Each animal received 4.000 haptenated beads in a volume of 0.1 mL

in sterile high molarity buffer which contains in particular: (35g NaCl; 1litre water) pH 7.0. After a week the axial organs were removed, pooled, washed with sterile high-molarity buffer and teased. The cells were finally filtered through a sterile fine-mesh nylon cloth, pore diameter 20 µm. Cells were cultured according to Mishell and Dutton (1967) in Eagle's MEM supplemented medium (Gibco, England) containing 36 g/litre NaCl and mercaptoethanol. Costar plastic plates were used (Costar, USA), each vial containing 5.000.000 cells in 0.5 mL medium. Plates were gently agitated and gassed with 5% CO<sub>2</sub>. At the beginning of culture the cells were restimulated by adding to each vial 2.000 PAA beads haptenated with the same hapten used to inoculate the animals.

Cultures were daily supplemented with 100 µL of Mishell and Dutton supplementation medium containing dextrose. After 5 days, the cells were harvested, washed by centrifugation with Hanks' medium and resuspended in the desired volume.

The response was evaluated by haemolytic titration. Sheep red cells were haptenated with TNP-sulphonic or with FITC according to Rittenberg and Pratt (1969) and Moller *et al.* (1976)

The haemolytic test was performed by mixing 50 µL of haptenated red cells, 2.000.000 axial organ cells in 0.1 mL, or 0.1 mL of various dilutions of culture supernatants, 0.1 mL of fresh rabbit or guinea-pig serum and Hanks' solution up to a final volume of 0.5 mL.

After 30-min incubation at 37 or 20°C, the tubes were centrifuged, 50 µL of the supernatant was taken and diluted 1/60 and the optical density at 415 nm was measured. The results were expressed as percentage lysis, taking as 100% the complete lysis provoked by distilled water. Control cultures were incubated with normal red cells and with axial organ cells from non-treated sea stars.

In some experiments, the cells were fractionated according to their adherence to nylon wool, as described before (Julius *et al.*, 1973). The fractionations was made immediately after dissecting the axial organs and the adherent and non-adherent populations were cultured separately.

It is seen that the lysis is specific and that is necessitates the presence of fresh rabbit serum.

No lysis was ever observed if the rabbit serum had been previously heated for 1 h at 56°C.

When subpopulations of cells were used, no lysis occurred.

But if the subpopulations of cells were mixed before the test, lysis was obtained. The proportion of cells

mixed in these experiments was 80% of non-adherent cells (T-lymphocytes + Phagocytes) and 20% of adherent cells (B lymphocytes+ Phagocytes) which is the average proportion found in the non-treated axial organ.

Specific haemolytic activity was also found in the supernatants of the cell cultures; this indicates that a soluble antibody factor was released during culture done in triplicate.

Some activity was found after 1 day of culture and it increased up to 5 days, the maximal time tested. Addition of increasing quantities of the monovalent ligand (trinitrophenol) gradually inhibited lysis.

### 3.3. Cell types Involved

Various experiments were performed with cells disrupted by sonication (Leclerc *et al.*, 1986)

### 3.4. Disruption of Cells

The cells were sonicated at the end of culture at 30 KHz for 3 min. The suspension obtained was either used directly or was fractionated by centrifugation at 400g for 15 min: the supernatant contained the cellular membranes (S.4000). In some cases, a further centrifugation was performed at 85.000g for 35 min and the pellet containing the cell membranes was taken in sterile buffer, sonicated at 30KHz for 45 s and tested (Leclerc *et al.*, 1986)

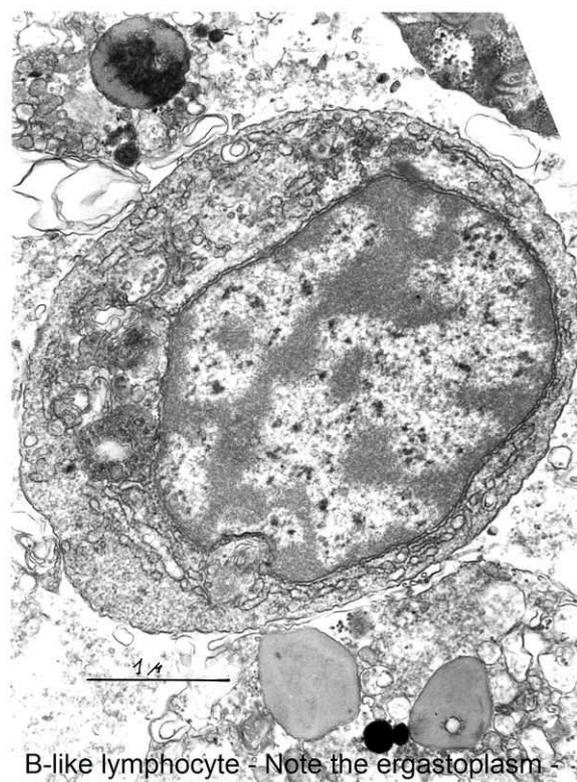
It was found that the haemolytic factor was produced only when the sea star B-lymphocytes (Fig. 1) were intact. On the contrary, sea star T-lymphocytes were effective whether they were intact or disrupted.

Treatment of silica, which is known to destroy phagocytes (Allison *et al.*, 1966), completely inhibited the production of the antibody factor. It was also seen that the addition of mercaptoethanol (always in sterile solution, like for silica) to the cultures was essential. Mercaptoethanol could be SUBSTITUTED by culturing the axial organ cells WITH CELLS OF LATERAL APPENDICES situated in the aboral part of the axial organ (Leclerc: personal communication).

In summary, a kind of cooperation occurred between sea star T and B lymphocytes and sea star phagocytes. The mechanism of this cooperation is not yet understood completely.

Purification of the antibody factor (Delmotte *et al.*, 1986).

The raw material consisted of supernatants harvested from cultures of TNP-PAA-stimulated axial organ cells. Supernatants were first precipitated at 50% ammonium sulphate saturation: the precipitate did not show any haemolytic activity.



**Fig. 1.** B sea star lymphocyte in T.E.M

A second precipitation at 75% ammonium sulphate produced a precipitate with lytic activity.

This 50-75% ammonium sulphate precipitate was dissolved in high molarity buffer and fractionated on a column of « Ultrogel AcA 44 ». The active fraction was further purified by affinity chromatography using agarose coupled to paranitrobenzoic acid as the affinity ligand.

Elution of the retained material was made with potassium thiocyanate 2M.

The final product gave a single peak in crossed immunoelectrophoresis against an antiserum from rabbit injected with the same fraction.

Electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate showed a single band corresponding to a molecular weight of 30.000. This result compared with the value of 132.000 obtained by gel filtration suggests that the molecule may be composed of 4 units.

In 2000 leclerc showed that this molecule had a human-kappa like activity (Leclerc, 2000); in 2011, it was correlated with kappa genes (Leclerc *et al.*, 2011).

So the antibody factor compared with the value of 132.000 could be composed of 4 kappa chains (Leclerc *et al.*, 2011)

#### 4. CONCLUSION

It was already shown that the sea star T lymphocytes were able to release soluble lymphokine mediators with mitogenic properties.

In addition to these properties, the axial organ cells possessed immunocompetent cells and were able to bind the specific antigen after *in vivo* stimulation of the animals. This immunocompetence of the axial organ cells was more clearly demonstrated by the production of a soluble substance which appears to be similar in many respects to the humoral antibodies of the vertebrates after *in vivo* and *in vitro stimulations* of the animals.

The sea-star antibody factor could be correlated to kappa genes and so composed of 4 Kappa chains (Leclerc *et al.*, 2011). It is a great novelty in the invertebrate world.

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