American Journal of Immunology, 2012, 8 (4), 191-195
ISSN 1553-619X

doi:10.3844/ajisp.2012.191.195 Published Online 8 (4) 2012 (http://www.thescipub.com/aji.toc)

Cell-Mediated Immune Responses in the Sea-Star *Asterias rubens* (Echinoderm)

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Received 2012-09-21, Revised 2012-09-26; Accepted 2012-11-27

ABSTRACT

Cell-mediated immune responses occur in sea star system. In *Asterias rubens* it is said that B sea star lymphocytes and T sea star lymphocytes exist in the axial organ which can be considered as an ancestral lymphoid organ. In the same manner the origin of lymphocytes can be found in Invertebrates such as Echinodermal.

Keywords: Invertebrate, Immunogenesis, Phylogenesis, Sea-Star Axial Organ, Echinoderma, Review

1. INTRODUCTION

In Vertebrates, immunity is characterized by physiological mechanisms mediated by variuos types of cells and various soluble proteins, whose complex interplay results in a defence response which is specific and anamnesic for each particular immunogen (antigen) and which is greatly amplified in the presence of such antigen.

B and T lymphocytes, phagocytes and other cells of the reticuloendothelial system, leukines secreted by these cells, complement and humoral antibodies have been studied in great detail in vertebrates, especially in mouse and man, but little information is available on the presence(or absence) of them, especially humoral antibodies, in invertebrates.

Thus, although it is largely admitted that the origin of the immune response resides among invertebrates, no definite evidence exists concerning the phylogeny of such response or the evolutionary homologies between the various components of the immune system and the components of the «eventual immune system» of invertebrates.

The works of Cooper (1969) and Pasquier and Duprat (1968) have first demonstrated the existence of cell-mediated immune reactions in annelids. It has also been shown that tunicates can recognize and react to alloantigenic determinants and Hildeman and Dix (1972) have presented strong evidence of highly discriminating immunocompetence in echinoderms.

Millott (1966) proposed that the axial organ of the sea urchin was implicated in defence mechanisms.

Cells present in this organ are morphologically very similar to vertebrate lymphocytes and this similarity prompted the idea that the axial organ could be an ancestral Leclerc *et al*. (1980).

A great number of experiments performed in our laboratory in recent years have confirmed this hypothesis, showing that the axial organ cells of the sea-star are able to mount a cellular and humoral response having many of the characteristics of the vertebrate immune system.

The invertebrates consist of a variety of phyla whose exact evolutionary relationships and genealogy are difficult to assess.

1.1. Echinoderma

The position of the echinoderms, in particular, has been a matter of doubt and controversy in the studies of the vertebrate ancestors.

Two evolutionary trees proposed by Leclerc *et al*. (1980) and Kampmeier (1969) are well-known.

Embryological, anatomical and biochemical evidence seem now to indicate that the first one is wrong: Echinoderms, as proposed Kampmeier, are more likely to have been the ancestors of the invertebrates than the annelids.

An essential point appears to be the fact that echinoderms are deuterostomia, like the vertebrates, while annelids are protostomia, like arthropods and mollusks.

In the ontogeny of protostomia, the « blastopore » becomes the mouth; in the deuterostomia, on the contrary, the anus arises from the blastopore, the mouth being a new acquisition.

It must also be remarked that although, in the phylum Echinodermata, the adult animal has a radial symetry, the larva is bilateral.

1.2. The Sea-Star *Asterias Rubens*

Sea-stars belong to the class Asteroidea of the phylum Echinodermata.

They are marine animals, also named starfishes. They have a pentaradiate structure with a calcareous endoskeleton.

The Asteroidea do not possess a definite head or brain but an oral and an aboral face. On the aboral face, the madreporite (Leclerc, 1970) takes place as a circular grooved plate, situated in the interradius CD or Carpenter's system. Just under the madreporic plate is the axial complex.

The axial organ lies along the stone canal (Leclerc, 1970).

The structure of the axial organ is glandular, spongy and crossed by connective tissue.

Many follicles of heterogeneous size with clear zones are lined by cellular cords.

The various types of cells present in the axial organ have been studied in our laboratory, by optical and electron microscopy (S.E.M and T.E.M).

Essentially two types of cells are found:

- Cells which morphologically resemble mammal lymphocytes
- Cells which are phagocytes

The species *Asterias rubens* (also named *Asterias vulgaris*) is frequently found on the Atlantic coast of France, on the channel too. It leaves on the sea floor at a depth of about 5 m, where the temperature of the water is between 7 and 15°C according to the season.It can attain a size of 25 cm or more in diameter.

A.rubens was obtained from different marine institutes (Wimereux, Arcachon, Roscoff, Luc-sur-mer. France).

After collection, the sea-stars were maintained in aquariums with running sea water, at 10°C.

1.3. Properties of the Axial Organ Cells

1.3.1. Obtention and Separation of Cells

Axial organs were removed, pooled, washed with sterile buffer and teased.

The cells were finally filtered through a sterile fine-mesh nylon cloth (pore diameter: 20 µm).

The buffer used throughout contained a high concentration of NaCl, similar to that of the sea water.

The total population could be separated into two subpopulations according to the method of Julius *et al*. (1973).

Sterile nylon wool purchased from Fenwal Laboratories (Morton Grove III) was washed and dried as described by the above authors for *in vitro* experiments.

Aliquots of about 100 mg of washed and dried nylon wool were packed into the barrels of 5 mL plastic syringes. After autoclaving, the sterile nylon columns were rinsed with 50 mL of high molarity buffer and incubated at room temperature for 30 min.

Cell suspensions containing about 50.000.000 cells/mL were loaded on the columns so that the total suspension was absorbed by the nylon wool. After 45 min., at room temperature, the non-adherent cells were eluted by slowly adding 5 to 10 mL of 0.6 M PBS/NaCl buffer.

The eluted cells were collected by centrifugation at 250 g for 10 min.

The columns were then washed with 20 mL of buffer at room temperature and the effluent was discarded.

In order to recover the adherent cells, 5-10 mL of sterile buffer, prewarmed to 30°C, was loaded on the nylon wool columns and the latter were submitted to two gentle pressures with the piston.

The eluates were collected and the cells spun at 250 g for 10 min.

The eluates, i.e., Adherent cells, represented about 20% of the whole axial organ population and non-adherent cells 80%. Cell viability was checked by trypan blue exclusion.

The percentage of adherent cells was enhanced after antigenic stimulation and grew up to 35% in some cases.

1.4. Morphology

Adherent and non adherent cells were studied by electron microscopy after fixing in 2% glutaraldehyde in cacodylate buffer 0.4 M, 3 mM (Anteunis *et al*., 1985).

Ultrastructural cytochemistry was performed to detect endogenous peroxidase by the well-known diaminobenzidine method

Results were summarized in the study of Anteunis *et al*. (1985).

1.5. Non-Adherent Population

Cellular types found in the non-adherent population resembled the Th lymphocytes and mononuclear

phagocytes of the vertebrates. Lymphocyte-like cells were small, average diameter 4 µm and had a high nucleocytoplasmic ratio.

The second cellular type observed was a phagocytic cell whose diameter was 7-8 µm.

Its nucleus wax excentric and could have two aspects: In some cells, the diffuse chromatin was predominant, while others were principally composed of condensed chromatin.

The cytochemical DAB procedure for visualization of endogenous peroxidase showed a negative reaction in the lymphocyte-like cells and a positive staining in some phagocytic cells.

This reaction was located in the endoplasmic reticulum and in the phagosomes. The experimental conditions used did not permit detection of any positivity in the perinuclear space or in the Golgi apparatus.

1.6. Adherent Population

Two types of cells were also observed in the adherent population.

One type resembled lymphoplasmocytes. These cells had a nucleocytoplasmatic ratio lower than that of the non dherent cells and a more highly developed vacuolar system, i.e., Smooth- and rough-surfaced endoplasmic reticulum in variable quantities and large multivesicular bodies.

These cells represented 20% of the adherent population and 5-10% of the whole cell population.

The diameter of these cells was also greater than that of the non-adherent cells, namely 5 to 6 µm.

Within this cellular type, some were characterized by substantial development of the rough endoplasmic reticulum and by the presence of lipids and dense bodies of irregular form and heterogeneous density.

The other type found was a phagocytic cell similar to that found in the non-adherent population.

These cells also had a positive endogenous peroxidase reaction localized in the endoplasmic reticulum and in the phagosomes.

1.7. Effect of Mitogens

To test the action of mitogens, cell cultures were performed.

After pooling and removal of axial organs, they were rinsed in sterile 0.6 M PBS/NaCl buffer (pH 7.2). Then they were washed seven times for 10 min each time on 0.6 M PBS/NaCl supplemented with penicillin streptomycin at 125 U/mL in order to avoid bacterial contamination.

After teasing, a cell suspension was obtained and filtered through a sterile fine-mesh nylon cloth (pore diameter: 20 µm).

Cell viability was checked by trypan blue exclusion.

The culture medium was Eagle ‘s Minimal Essential Medium (MEM) supplemented with L- glutamine and 5% foetal calf serum, pH 7.2 containing penicillin and steptomycin.

Sterile plastic tubes were used (Falcon tubes 12.75 mm, Flow Lab., USA).

Depending on the experiments, each tube contained 500; 000 to 1.500.000 cells per milliliter.

All the operations were carried out under a laminar flow cabinet (Microflow).

In all experiments, the level of stimulation was estimated by incorporation of tritiated H-methyl thymidine (CEA 91190, Gif-sur-Yvette, France. Labelled thymidine was added 4 h after the beginning of culture. The initial specific activity was 28 Ci/mM and the thymidine was diluted to 1.5 µCi per 20 µL per tube.

Cells were counted 24 h after the beginning of cultures. Cell suspensions were harvested and filtered through glass fibre «Whatman GF/C» filters (Whatman Inc., Clifton, NJ, USA) on a vacuum funnel.

Filters were washed twice with 5 mL of cool PBS/NaCl 0.6 M buffer, thoroughly dried at 56°C and placed in plastic scintillation counting vials filled with 10 ml of «Organic Counting Scintillant Liquid» (OCS, Amersham, France, SA, 78000 Versailles, France).

Counting was done in a « Beckmann LS 133 P » liquid scintillation counting system.

The index of stimulation was calculated by dividing the mean number of cpm obtained in the stimulated cultures by the mean number of cpm obtained in control cultures.

The mitogens tested were PWM (pokeweed), ConA(Concavalin A), LPS (Lipopoysaccharide) Limulin, PHA (phytohaemagglutinin) (Sigma products) Nocardia opaca delipidated.

Different axial organ cellular populations (total, adherent and non-adherent to nylon wool) were cultured for 24, 48 and 72 h in the presence of various concentrations of these mitogens (Brillouet *et al*., 1981) and the stimulation indexes were calculated. A summary of the results obtained, is presented in **Table 1**, where it can be seen that non-adherent cells where stimulated by PWM, ConA, Limulin and PHA, but not by LPS and Nocardia opaca delipidated. Adherent cells were stimulated by LPS and Nocardia opaca delipidated (Leclerc *et al*., 1988). When the total population was tested, considerable stimulation was observed only in

the presence of PWM, a weak stimulation was observed in the presence only of foetal calf serum. In general stimulation was greatest after 48 h of contact and then diminished.

A dose-effect was also observed with high concentations of mitogens producing less stimulation (Brillouet *et al*., 1981; Leclerc *et al*., 1981). The conclusion that can be drawn from these experiments is that non-adherent and adherent cellular subpopulations, respectively, were stimulated by the same mitogens which stimulate mammal T and B lymphocytes. This analogy is further reinforced by the fact that the procedure employed to separate adherent from non-adherent cells is identical to that used to separate B from T lymphocytes.

1.8. Agglutination by Lectins

It was found that axial organ cells were selectively agglutinated by Redziniak *et al*. (1978). This property allowed fractionation of the cells into two subpopulations. Soybean Agglutinin (SBA) and Wheat Germ Agglutinin (WGA) agglutinated about 10°/° of the cells. In contrast, Con A agglutinated a very large number of cells. The agglutinated cells could be dissociated with appropriate concentrations of sugar specific for the lectin and they were viable after dissociation by use of trypan blue.

1.9. Lymphokine Substances

The method of Leiper and Solomon (1977) was used, with some modifications. Cells were separated by nylon wool and were cultured for 24 h at 10°C in the presence of PWM-coated Sepharose beads. Supernatants from these cultures were centrifuged to eliminate beads and debris, sterilized by membrane filtration (0.22 µm Millipore) and concentrated 5- or 10-fold with an ultrafiltration device (Amicon) fitted with «PM-10 Diaflo» membranes. Supernatant controls were obtained from cells cultured with non-coated beads, from cells cultured alone and from beads cultured alone. The supernatants were added to cultures of total axial organ cells which had been started 24 h before and 24 h later, the cells were harvested and mitogenic stimulation was evaluated by measuring tritiated-H-methyl- thymidine incorporation.

It was found that the supernatant from the non-adherent cells cultured in the presence of PWM beads stimulated the total axial organ cells (Leclerc *et al*., 1981). No stimulation was observed in the various controls. This result indicates that non-adherent cells produce a lymphokine mediator which is able to stimulate the total axial organ cells.

Table 1. Effect of various mitogens on axial organ cells

Mitogen	Total population	Adherent cells	Non-adherent cells
PWM	+	–	+
ConA	–	–	+
Limulin	–	–	+
PHA	–	–	+
LPS	–	+	–
Nocardia-opaca	–	+	–

The active substance was inactivated by trypsin and chemotrypsin, or by heating for 1 h at 70°C, but it resisted at 56°C.

Recently, cytokines and cytokine-receptors were discovered indirectly by the use of Cytofluorometry (Legac *et al*., 1996) and directly by genomic studies.

Immune reactions induced by axial organ cells.

Attempts were made to demonstrate the capability of axial organ cells to induce cellular immune reactions in various systems currently employed with vertebrate cells.

1.10. Angiogenesis

It was found that angiogenesis was induced when irradiated mice were injected subcutaneously with a suspension of axial organ cells. The technique employed was that described by Sidky and Auerbach (1975) who presented it as a graft-versus-host reaction. A study of the effector cells, inducing this phenomenon showed that they were non-adherent to nylon wool, agglutinated by ConA and non-agglutinated by WGA or SBA. In this respect, the effector cells resembled mammal T lymphocytes.

1.11. Splenomegaly

Injection of axial organ cells into 16-day chicken embryos induced significant splenomegaly. Appropriate controls using killed cells, sea star ovocytes or sea star brachial digestive cells were all negative (Leclerc *et al*., 1977a; 1977b).

1.12. Mixed Lymphocyte Reaction

The mixed lymphocyte reaction between axial organ cells from *A.rubens* and another sea star, *Marthasterias glacialis*, gave a positive response at the fifth day of culture (Luquet *et al*., 1984).

1.13. Cytotoxicity toward Tumour Cells

Axial organ cells, had spontaneous cytotoxicity toward malignant mouse target cells (MBL2 cells) (Luquet and Leclerc, 1983), an induced cytotoxicity toward mouse SP2 cells. That study was performed according to the method of Brunner *et al*. (1968).

2. CONCLUSION

The general idea that emerges from the experiments reported in the present review is that Echinoderma, as exemplified by the sea star: *Asterias rubens*, possess an ancestral lymphoïd organ: The axial organ. It appears that all the elements necessary to immune responses (phagocytic cells, T-lymphocytes, B lymphocytes, NK cells) are present in this organ which initiates the antibody factor, the lymphokines of the sea star.

3. REFERENCES

Anteunis, A., M. Leclerc, M. Vial, C. Brillouet and G. Luquet *et al*., 1985. Immunocompetent cells in the starfish *Asterias rubens*. An ultrastructural study. Cell Biol. Int. Rep., 9: 663-670. PMID: 4028193

Brillouet, C., M. Leclerc, J. Panijel and R. Binaghi, 1981. *In vitro* effect of various mitogens on starfish (*Asterias rubens*) axial organ cells. Cell Immunol., 57: 136-144. PMID: 7214530

Brunner, K.T., J. Mauel, J.C. Cerrottini and B. Chapuis, 1968. Quantitative assay of the lytic action of immune lymphoid cells on 51-Cr-labelled allogeneic target cells *in vitro*; inhibition by isoantibody and by drugs. Immunology, 14: 181-196. PMID: 4966657

Cooper, E.L., 1969. Specific tissue graft rejection in earthworms. Science, 166: 1414-1415. DOI: 10.1126/science.166.3911.1414

Hildeman, W.H. and T.G. Dix, 1972. Transplantation reactions of tropical Australian echinoderms. Transplantation, 14: 624-633. PMID: 4563201

Julius, M.H., E. Simpson and L.A. Herzenberg, 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. Eur. J. Immunol., 3: 645-649. DOI: 10.1002/eji.1830031011

Kampmeier, O.F., 1969. Evolution and Comparative Morphology of the Lymphatic System. 1st Edn., Thomas, Springfield, pp: 620.

Leclerc, M., 1970. Bull. Soc. Fr. Mineral. Cristallogr, 94: 86-88.

Leclerc, M., C. Brillouet and G. Luquet, 1980. The starfish axial organ: An ancestral lymphoid organ. Dev. Comp. Immunol., 4: 605-615. DOI: 10.1016/S0145-305X(80)80063-2

Leclerc, M., C. Brillouet, G. Luquet, P. Agogue and R.A. Binaghi, 1981. Properties of cell subpopulations of starfish axial organ: *In vitro* effect of pokeweed mitogen and evidence of lymphokine-like substances. Scand. J. Immunol., 14: 281-284. DOI: 10.1111/j.1365-3083.1981.tb00565.x

Leclerc, M., G. Redziniak, J. Panijel and M. El Lababidi, 1977a. Reactions induced in vertebrates by invertebrate cell suspensions I/ specific effects of sea star axial organ cells injection. Dev. Comp. Immunol., 1: 299-310. DOI: 10.1016/S0145-305X(77)80013-X

Leclerc, M., G. Redziniak, J. Panijel and M. El Lababidi, 1977b. Reactions induced in vertebrates by invertebrate cell suspensions II/ non adherent axial organ cells as effector cells. Dev. Comp. Immunol., 1: 311-319. DOI: 10.1016/S0145-305X(77)80014-1

Leclerc, M., T. Bahmaoud, C. Brillouet, H. Tlaskalova-Hogenova and J.F. Petit *et al*., 1988. Stimulation of sea star *Asterias rubens* axial organ B-like cells by Nocardia-delipidated cell mitogen and derived fractions. Folia Biol., 34: 182-191. PMID: 3203818

Legac, E., G.L. Vaugier, F. Bousquet, M. Bajelan and M. Leclerc, 1996. Primitive cytokines and cytokine receptors in invertebrates: The sea star *asterias rubens* as a model of study. Scand. J. Immunol., 44: 375-380. DOI: 10.1046/j.1365-3083.1996.d01-322.x

Leiper, J.B. and J.B. Solomon, 1977. Ontogeny of mitogenic responsiveness to PHA and sheep erythrocytes and lymphokine production in foetal guinea-pigs. Immunology, 32: 215-226. PMID: 849876

Luquet, G. and M. Leclerc, 1983. Spontaneous and induced cytotoxicity of axial organ cells from *Asterias rubens* (Asterid--echinoderm). Immunol. Lett., 6: 339-342. PMID: 6629429

Luquet, G., C. Brillouet and M. Leclerc, 1984. MLR-like reaction between axial organ cells from asteroids. Immunol. Lett., 7: 235-238. DOI: 10.1016/0165-2478(84)90027-0

Millott, N., 1966. A possible function for the axial organ of echinoids. Nature, 209: 549-596. DOI: 10.1038/209594a0

Pasquier, L.D. and P. Duprat, 1968. Aspects humoraux et cellulaires d'une immunite naturelle non specifique chez l'oligochete Eisenia fetida (SAV.). C.R. Acad. Sci., 266: 538-541.

Redziniak, G., M. Leclerc, J. Panijel and M. Monsigny, 1978. Separation of two different populations of axial organ cells of *Asterias rubens* by the use of lectins. Biochimie, 60: 525-527. PMID: 29670

Sidky, Y.A. and R. Auerbach, 1975. Lymphocyte-induced angiogenesis: A quantitative and sensitive assay of the graft-vs.-host reaction J. Exp. Med., 141: 1084-1100. DOI: 10.1084/jem.141.5.1084