C.M. 1991/F:22
REF.K

STRATEGY OF RESEARCHES ON MARINE BIVALVE DEFENSE MECHANISMS

Evelyne BACHERE, Eric MIALHE, Thierry NOEL, Vivianne BOULO, Dominique HERVIO, Gyslaine LE GALL and Annie MORVAN

IFREMER, LABEIM, Laboratoire Biologie et Ecologie des Invertébrés Marins, URPIGM, Unité de recherches en Pathologie, Immunologie et Génétique Moléculaire, BP 133, 17390 LA TREMBLADE, FRANCE.

During the last ten years, significative progresses have been made in the knowledge of infectious diseases of economically important marine molluscs. As a matter of fact, after a long period of descriptive works essentially focused on morphology of pathogens, anatomopathology of infections and epidemiology of the main diseases, a new time for pathological researches was subsequent to the establishment of purification protocols of the pathogens, the most being intracellular and consequently non cultivable *in vitro* because of the lack of marine mollusc cell lines.

The availability of purified pathogens (1,2) has quickly opened on the preparation of specific molecular probes and, in a few cases, on the development of immunodiagnostics or nucleic acid based-hybridization diagnostics (3). Because of the potential advantages of such diagnostic methods for efficient zoosanitary controls, it may be hoped that they will be considered by national and international organizations in charge of prophylaxy for marine aquaculture productions. These quantitative and sensitive diagnostic techniques are also very useful in studies about host-pathogen interactions.

Indeed, another chief field of applications of pathogen purification protocols concerns researches based on experimental infections which became possible at laboratory. At the animal level, the injection of purified and quantified pathogens, such as *Bonamia ostreae* the intrahemocytic parasite of the flat oyster, successfully led to reliable reproduction of some diseases and in some first cases to determine the 50% infectious dose (4). From this kind of data, comparisons can be undertaken between mollusc individuals or strains for their respective sensitivity to a pathogen, that led for example to identify *Bonamia*-resistant oysters. However, the immunological processes involved in such an apparent pathogen resistance are not yet understood, but argue for developing researches in the field of mollusc immunology against infectious pathogens. Moreover, the availability of purified pathogens permits *in vitro* experimentations which are better-adapted to investigate the molluscan immune response at the cellular and molecular levels.

Thus, from a few years, numerous immunological methodologies have been specifically adapted to marine bivalve molluscs: special medium avoiding hemocyte activation and aggregation during hemolymph withdrawal has been elaborated; primary cultures of hemocytes can be routinely prepared from hemolymph punctured without contamination and without sacrifying the animals; hemocyte separations by isopycnic centrifugation and counterflow

elutriation (5). Moreover, several biological assays related to the chief types of diseases have yet been established which will be useful for identification of cellular and humoral defense effectors involved in the destruction of pathogens (6,7).
Like in other invertebrate groups, the cellular defense mechanisms in molluscs may certainly be largely ensured by the hemocytes which were until now wrongly characterized in terms of cell types and lineages as well as immune functions. For these purposes, new axis of researches have been developped by preparing monoclonal antibodies against serum components and hemocytes (8).
The exploration of the use of monoclonal antibodies for hemocyte antigenic characterization is in progress with several short term aims: identification of hemocyte sub-populations; antigenic establishment of hemograms and analyses of their individual variability according to physiological, environmental or stress parameters; search of correlations between hemogram characteristics and pathogen resistance; search of antigenic markers of hemocytes with specific microbicide activity.
The best investigated species are the Japanese oyster *Crassostrea gigas* and at a lesser extend the blue mussel *Mytilus edulis*. Particularly, monoclonal antibodies have been proved to be specific of hemocyte sub-populations, of hemocyte receptor involved in phagocytosis and of hemocytes with microbicide activity (8).
The microbicide activity of mollusc hemocytes has been recently investigated by the technique of chemiluminescence after the numerous experimental parameters were precisely established (9). Like for vertebrates, this quantitative technique is suitable for individual estimation of reactive oxygen intermediates produced consecutively to phagocytosis. It may be assumed that chemiluminescence will be useful for analysing the effect of any external (10,11) or internal factor on the phagocytic defense capacity and the related oxygen-dependant killing mechanism. Because of its simplicity and reliability, chemiluminescence is well-adapted for large scale experiments and, since the hemolymph samples can be withdrawn without sacrifying the molluscs, chemiluminescence will permit to look for a genetic origin of the observed variability of microbicidal activity. Such phenotypic immunological marker would be very interesting as selection criterion for quantitative genetics.
The study of the oxygen-dependant microbicidal process must be examined more precisely by considering hemocyte sub-populations identified with monoclonal antibodies and separated by centrifugations or immunopurification, preliminary results leading to suspect one antigenic type as directly involved in chemiluminescence activity (8).
The involvement and the efficiency of oxygen-dependent killing mechanisms against specific molluscan pathogen have begun to be studied, the pathogen-hemocyte interactions being easily investigated *in vitro* on the basis of experimental infections of hemocyte primary cultures with purified pathogens. The most of data yet acquired for protozoan (12) and rickettsia (13) indicated some efficient adaptations of the pathogens for avoiding or counteracting this microbicidal process.

The future of researches on bivalve mollusc immunology will have to consider the enormous knowledge acquired for vertebrates and partially transfered to invertebrates, in particular to insects, without underestimation of their specificities.
The availability of different *in vitro* hemocyte-pathogen models may offer suitable experimental systems to look for other anti-infectious non specific mechanisms, such as the powerful system linked to the generation of nitric oxide (14), and for cytokines, such as TNF or CSF which activate defense cells for killing pathogens (15,16,17).

Another attractive way of investigations will consist to test against molluscan pathogens some heterologous antimicrobial proteins, such as defensins, magainins, cecropins, attacins, which have been isolated, biochemically and genetically characterized. Parallely, by using techniques of molecular biology and by exploiting the sequence homologies inside gene families, corresponding genes of molluscs could be possibly isolated.

The strategy of researches presently proposed for immunology of bivalve molluscs is closely related to infectious pathology and will become strongly linked to genetics for selection of pathogen resistant strains.
By identifying resistant phenotypes, disease-resistant molluscs would be possibly produced by crossing individuals exhibiting this character, but the genes for resistance may be tightly linked to undesirable traits, or the resistance may be multigenic and difficult to fix.
Genetic transformation technologies may be used to overcome some of the factors limiting traditional plant breeding. The understanding of mechanisms underlaying resistance in molluscs may lead to identify and isolate immune genes. Such genes would be appropriate to transfer, but a particular strength of gene transfer is the potential to bypass species barriers, that means heterologous immune genes could be transfered if the corresponding protein was proved efficient against some molluscan pathogens.

1- MIALHE E., E. BACHERE, D. CHAGOT, H. GRIZEL, 1988. Isolation and purification of the protozoan *Bonamia Ostreae* (Pichot et al. 1980) a parasite affecting the flat oyster *Ostrea edulis* L. *Aquacult.*, 71:293-299.

2- LE GALL G. and E. MIALHE, 1991. Purification of Rickettsiales-like procaryotes associated with *Pecten maximus* (Mollusca: Bivalvia). Serological and biochemical characterization. Submit. to *Dis. Aquatic. Org.*.

3- MIALHE E., V. BOULO, E. BACHERE, D. HERVIO, K. COUSIN, D. NOEL, M. OHRESSER, R.M. LE DEUFF, AND S. GENDREAU. 1990. Development of new methodologies for diagnostic of infectious diseases in molluscs and schrimp aquaculture. Submit. to *Aquaculture and Fisheries Management*.

4- HERVIO D., V. VUILLEMIN, E. BACHERE, V. BOULO, N. COCHENNEC, and E. MIALHE, 1990. Establishment of an experimental infection protocol for the flat oyster *Ostrea edulis* with *Bonamia ostreae* parasites : application to parasite resistant oyster selection. Submit. to *Aquaculture*.

5- BACHERE E., D. CHAGOT, H. GRIZEL, 1988. Separation of *Crassostrea gigas* hemocytes by density gradient centrifugation and counterflow centrifugal elutriation. *Develop.comp.Immunol.*, vol. 12(3): 549-559.

6- MOURTON C., V. BOULO , D. CHAGOT, D. HERVIO, E. MIALHE and H. GRIZEL, 1990. Interactions between *Bonamia ostreae* (Protozoa : Ascetospora) and hemocytes of *Ostrea edulis* and *Crassostrea gigas* (Mollusca : Bivalvia) : *In vitro* model establishment. Soumis à *J. Invert. Pathol.*

7- BACHERE E., D. HERVIO, E. MIALHE, H. GRIZEL, 1989. Evidence for a neutralizing activity against T3 coliphage in the *Crassostrea gigas* oyster hemolymph. *Develop. comp. Immunol.* vol. 13(4): 384.

8- MORVAN A., V. BOULO, B. DESPRES, D. HERVIO, E. BACHERE and E. MIALHE, 1991. Monoclonal antibodies against hemocytes of the japaneese oyster Crassostrea gigas (Mollusca: Bivalvia). Fifth ISDCI Congress, Portland, Oregon, USA: 4-9 th August 1991.

9- BACHERE E., D. HERVIO and E. MIALHE, 1990. Luminol-dependent chemiluminescence by hemocytes of two marine bivalve species, *Ostrea edulis* and *Crassostrea gigas*. *Dis. Aquat. Org.*, in press.

10- LARSON K.G., B.S. ROBERSON and F.M. HETRICK , 1989. Effect of environmental pollutants on the chemiluminescence of hemocytes from the American oyster *Crassostrea virginica*. *Dis. Aquat. Org.*,6: 131-136.

11- BACHERE E., T. NOEL, E. MIALHE, 1991. Effects of environmental pollutants and chemotherapeutic agents on the chemiluminescence of the hemocytes from the oyster *Crassostrea gigas*. E.A.S. Dublin, 10-12 juin 1991.

12- HERVIO D., E. BACHERE, E. MIALHE, H. GRIZEL, 1989. Chemiluminescent responses of *Ostrea edulis* and *Crassostrea gigas* hemocytes to *Bonamia ostreae* (Ascetospora). *Develop. comp. Immunol.*, vol. 13 (4): 449.

13- LE GALL G., E. BACHERE and E. MIALHE, 1991. Chemilumines-cent activity of *Pecten maximus* hemocytes stimulated with zymosan and host-specific Rickettsiales-like orbganisms. *Dis. Aquat. Org.*, in press.

14- LIEW F.Y. and F.E.G. COX , 1991. Nonspecific defence mechanism: the role of nitric oxide. Reprint from *Immunoparasitol. Today*, A17-A21, 1991.

15- BERKOW R.L.and M. R. DODSON, 1988. Biochemical mechanisms involved in the priming of neutrophils by Tumor Necrosis Factor. *J. Leukoc. Biol.*, 44:345-352.

16- BOGDAN C., H. MOLL, W. SOLBACH and M. ROLLINGHOFF,1990. Tumor Necrosis Factor-α in combination with interferon-τ, but not with interleukin 4 activates murine macrophages for elimination of *Leishmania major* amastigotes. *Eur. J. Immunol.*, 20:1131-1135

17- KOWANKO I.C., A. FERRANTE, D.P. HARVEY and K.L. CARMAN, 1991. Granulocyte-macrophage colony-stimulating factor augments neutrophil killing of *Torulopsis glabrata* and stimulates neutrophil respiratory burst and degranulation. *Clin. exp. Immunol.*, 83:225-230.