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Monoclonal antibodies against sporangia and spores of *Marteilia* sp. (Protozoa: Ascetospora)

J. A. F. Robledo[1], V. Boulo[2], E. Mialhe[2], B. Després[2], A. Figueras[1,*]

[1]Departamento de Biología y Patología de Organismos Marinos, Instituto de Investigaciones Marinas, CSIC, Eduardo Cabello, 6, E-36208 Vigo, Spain

[2]IFREMER-CNRS, Université de Montpellier 2, UMR 9947 Défence et Résistance chez les Invertébrés Marins (DRIM), 2, Place Eugène Bataillon, CP 71, F-34095 Montpellier Cedex 5, France

ABSTRACT: Digestive glands of mussels *Mytilus edulis* from Brittany, France, infected with *Marteilia* sp. (Ascetospora) were used to purify the parasite. A modification of a previously used purification protocol increased purification efficiency, permitting sporangial primordia and sporangia of *Marteilia* sp. to be obtained. Mouse (Balb/c) monoclonal antibodies were generated against this parasite. From the fusion, 26 monoclonal antibodies against *Marteilia* sp. were obtained. Antibodies from 6 clones reacted only with *Marteilia* sp. cells and not with normal host tissues. Four of these antibodies (1/1-3, 3/1-1, 4/1-1 and 6/2-3) reacted with the sporangia wall and two with the spore cytoplasm (9/1-1 and 12/5-1). Antibodies cross-reacted with *Marteilia refringens* from *Mytilus galloprovincialis* obtained in the Ría de Vigo, Spain.

KEY WORDS: *Marteilia* sp. · *Mytilus edulis* · Monoclonal antibodies

INTRODUCTION

Several species of the genus *Marteilia* have been detected in a variety of hosts and geographical locations: *Marteilia refringens* in flat oysters *Ostrea edulis* from France (Grizel et al. 1974); *Marteilia sydneyi* in the rock oyster *Saccostrea commercialis* from Australia (Perkins & Wolf 1976, Wolf 1979); *Marteilia maurini* in both *Mytilus galloprovincialis* and *Mytilus edulis* from France (Comps et al. 1982, Auffret & Poder 1985); and *Marteilia* sp. in cockles *Cardium edule* (Comps et al. 1975), clams *Tapes rhomboides* and *Tapes pullastra* (Poder et al. 1983) and mussels *Mytilus galloprovincialis* from northwestern Spain (Figueras et al. 1991, Robledo et al. 1992). Recently, the species that infects mussels from Spain was identified as *Marteilia refringens* (Villalba et al. 1993).

Marteilia refringens and *M. sydneyi* can be distinguished with light microscopy. Diagnoses of the 2 *Marteilia* species from Europe (*M. refringens* and *M. maurini*) relied on ultrastructural characteristics (Grizel et al. 1974, Comps et al. 1982); however, there is some doubt as to whether *M. maurini* is a true species. Since *M. refringens* has caused serious mortalities in flat oysters cultured in Europe (Alderman 1979), it is important to establish whether one or several species exist in this genus and to obtain a tool that would make diagnosis accurate and rapid.

Monoclonal antibodies (MAbs) are extensively used for diagnoses in human and veterinary medicine. In aquaculture, MAbs have been obtained and used for serological comparison of *Bonamia* isolates (Mialhe et al. 1988) and to perform enzyme linked immunosorbent assays (ELISA) to diagnose *Bonamia ostreae* infections in flat oysters (Cochennec et al. 1992). In addition, Noël et al. (1991b) used MAbs for diagnosis of 'Brown Ring Disease' in clams *Tapes philippinarum*; Goggin et al. (1991) developed MAbs against *Perkinsus atlanticus* infections in clams *Tapes decussatus*. Noël et al. (1991a) prepared and characterized MAbs against neoplastic hemocytes of *Mytilus edulis*, and Dungan & Roberson (1993) used polyclonal antibodies to compare *Perkinsus* species infecting hosts world-

*Addressee for correspondence

wide. Producing MAbs against *Marteilia* sp. would permit development of tools to make the diagnoses accurate and quick in both host tissues and environmental samples. MAbs specific for unique epitopes could be used for studies of biochemical composition, gene expression and physiology of specific developmental stages in the pathogen life cycle (Dungan & Roberson 1993).

In this paper, we describe a modification of a previous purification protocol (Mialhe et al. 1985) in which centrifugation on sucrose and Percoll gradient for *Marteilia* sp. permits sporangial primordia and sporangia to be obtained. These isolated parasites were used to prepare 6 MAbs against spores and sporangia of *Marteilia* sp.

MATERIALS AND METHODS

Origin of mussels. *Marteilia* sp. was purified from 2-yr-old mussels *Mytilus edulis* obtained from natural beds at Grazu, La Trinité-sur-Mer, France. Uninfected mussels were collected from La Tremblade, France. Smears of digestive gland from mussels were stained using a Hemacolor kit (Merck) to assess the presence of the parasite.

Mussels *Mytilus galloprovincialis* were also obtained from the Ría de Vigo, Spain, from populations where the highest prevalence of *Marteilia* sp. is found.

Purification protocol. To purify *Marteilia* sp., digestive glands from 20 to 40 infected mussels were used. Superficial gonad tissue was removed from the digestive gland in ripe individuals, because sexual products form aggregates and may decrease the efficiency of purification. These glands were suspended in filtered (0.22 µm) sea water (4 ml per digestive gland) with Tween 80 (1%) (filtered sea water with Tween, FSWT) and homogenized in a beaker (Ultra-Turrax; Janke & Kunkel GmbH). The homogenate was then sieved progressively through 250 µm and 75 µm nylon meshes to remove large fragments. The resultant filtrate was clarified by centrifugation (2500 × *g*, 30 min, 4 °C). The pellet was diluted in 6 ml FSWT, layered on a discontinuous 5%, 10%, 15%, 20%, 25% and 30% sucrose gradient in FSWT (w/w) and centrifuged (2500 × *g*, 30 min, 4 °C). Then, the interfaces 15%/20% and 20%/25% were diluted in FSWT (v/v), and centrifuged as above for 10 min. The pellet with sporangial primordia was resuspended in FSW and stored at –20 °C. These interphases were used as a positive control for hybridoma screening (see below). The pellet from the sucrose gradient was gently diluted in 1 ml of FSWT and centrifuged at 2500 × *g* for 30 min at 4 °C in a discontinuous gradient of Percoll (20%, 30%, 40%, 50% and 60%) [Percoll (Pharmacia), 0.5 M NaCl/FSWT]. The interfaces 20%/30% and 30%/40% were collected with a syringe, regrouped and diluted (v/v) in FSW to reduce the density of the Percoll solution, deposited on a cushion of sucrose (20%) and centrifuged (2500 × *g*, 10 min, 4 °C) to pellet parasites and washout. The supernatant was discarded and the pellet resuspended in FSW and centrifuged as above to eliminate sucrose. The pellet was resuspended in FSW and, after counting of *Marteilia* sp. sporangia, stored at –20 °C.

Immunization of mice. Heavily infected digestive glands of mussels containing several *Marteilia* sp. developmental stages were used directly for 3 first immunizations. The digestive glands were homogenized, and the homogenate was sieved through nylon meshes (250 and 75 µm) and centrifuged (2500 × *g*, 20 min). The pellet was resuspended in physiological solution, thawed and sonicated (100 W, 60 s) before injection. Three doses for each mouse were prepared and stored at –20 °C. Four Balb/c mice were immunized by intraperitoneal injections (500 µl). Two subsequent injections were made on Days 4 and 6 after the first immunization. On Day 20 the blood of each mouse was extracted and centrifuged, and the serum containing antibodies against the parasite and the mussel cells was treated with a powder made from healthy mussel tissue (after an acetone extraction process) to eliminate antibodies against mussel cells. The sera, free of antibodies against mussel cells, were tested by indirect immunofluorescence assay (IIFA) to determine the immunization status of the 4 mice against the parasite. The 2 mice with the best specific immune activity against the parasite were given a final intraperitoneal booster with the purified parasite before fusion (Day 37). Splenocytes of these mice were removed and used for lymphocyte hybridization.

Lymphocyte hybridization protocol. The myeloma cell line P3-NS1/1-Ag4-1 was cultured in RPMI 1640 medium (Gibco Laboratories) containing 10% heat-inactived fetal bovine serum (FBS), 1 mM glutamine and antibiotics. Before fusion, these cells were cultivated in complete RPMI supplemented by 0.13 mM azaguanine, which is a lethal analog of hypoxanthine, to eliminate $HGPRT^+$ mutants in the culture. The fusion was carried out by a method adapted from French et al. (1986) and detailed in Rogier et al. (1991). For each fusion, 3×10^6 and 1.5×10^6 splenocytes and 10^6 and 0.5×10^6 myeloma cells respectively (i.e. 3:1 ratio) were mixed and hybridized using polyethylene glycol 1540 (Riedel de Haën AG, Germany). After fusion to obtain precloning hybridomas, the cells were distributed into microculture plates containing HAT medium (15% FBS and hypoxanthine 10^{-4} M, aminopterin 4×10^{-7} M and thymidine $1.6\ 10^{-5}$ M) at the rate of 25×10^3 (20 plates), 50×10^3 (50 plates) and 1×10^5 (10 plates) cells per well. Seven days after lym-

phocyte hybridization, 100 µl of culture supernatant was removed and replaced with HAT medium.

Hybridoma screening procedure. Each precloned hybridoma culture supernatant was assessed for activity against *Marteilia* sp. The hybridoma culture supernatants were tested by IIFA on both purified sporangial primordia and sporangia slides and on smears of digestive gland from infected mussels. A drop of parasite suspension was deposited on a slide, air-dried, and fixed in acetone (5 min). Four hundred slides were prepared and kept at −20°C until used for screening. Healthy mussel digestive gland smears were prepared as negative controls.

Hybridoma culture medium supernatant (30 µl) was deposited on prepared slides and incubated in a moist chamber (30 min, room temperature). After 2 washes (5 min) with immunofluorescence buffer (IF Buffer, Diagnostic Pasteur), slides were covered with a solution of fluorescent isothiocyanate-conjugated goat anti-mouse IgG (Diagnostic Pasteur) diluted (1:100) in the same buffer containing 0.01% Evans blue. After incubation in a moist chamber (30 min, room temperature) in the dark, slides were washed twice with IF buffer and mounted with glycerine buffer (Diagnostic Pasteur). Slides were inspected with a Nikon Optiphot epifluorescence microscope for bright yellowish-green fluorescence.

Hybridoma supernatants which were positive for *Marteilia* sp. were tested against uninfected mussel digestive gland smears. Hybridomas for which the specificity was confirmed were cloned by the limiting dilution technique (1 or 0.3 cells per well) and further selected for preservation according to the results.

RESULTS

Purification. *Marteilia* sp. sporangial primordia were found at the 10%/15%, 15%/20% and 20%/25% sucrose interfaces, with most parasites found at the 15%/20% and 20%/25% interfaces. The pellet of the sucrose gradient had the highest sporangia concentration; however, it also contained cellular debris and small particles. Using the Percoll gradient, the sporangia were found at the 20%/30% and 30%/40% interfaces, being most numerous at the first.

The number of sporangia (determined from the observation of a Malassez cell at 400× magnification) per digestive gland of *Mytilus edulis* was estimated at up to 13 million sporangia (digestive gland mean weight 0.216 g) or 61 million sporangia g^{-1} digestive gland.

Immunization of mice and hybridoma screening. The pattern obtained with the polyclonal antibodies in the 4 sera revealed the presence of antibodies directed against *Mytilus edulis* cells as well as *Marteilia* sp. spores and sporangia. After 14 d of culture, 350 hybridomas were tested. The number of hybridomas obtained from the 2 mice was similar. On the basis of IIFA results, 26 hybridomas were selected and the 6 hybridomas with the most specific immune activity were cloned.

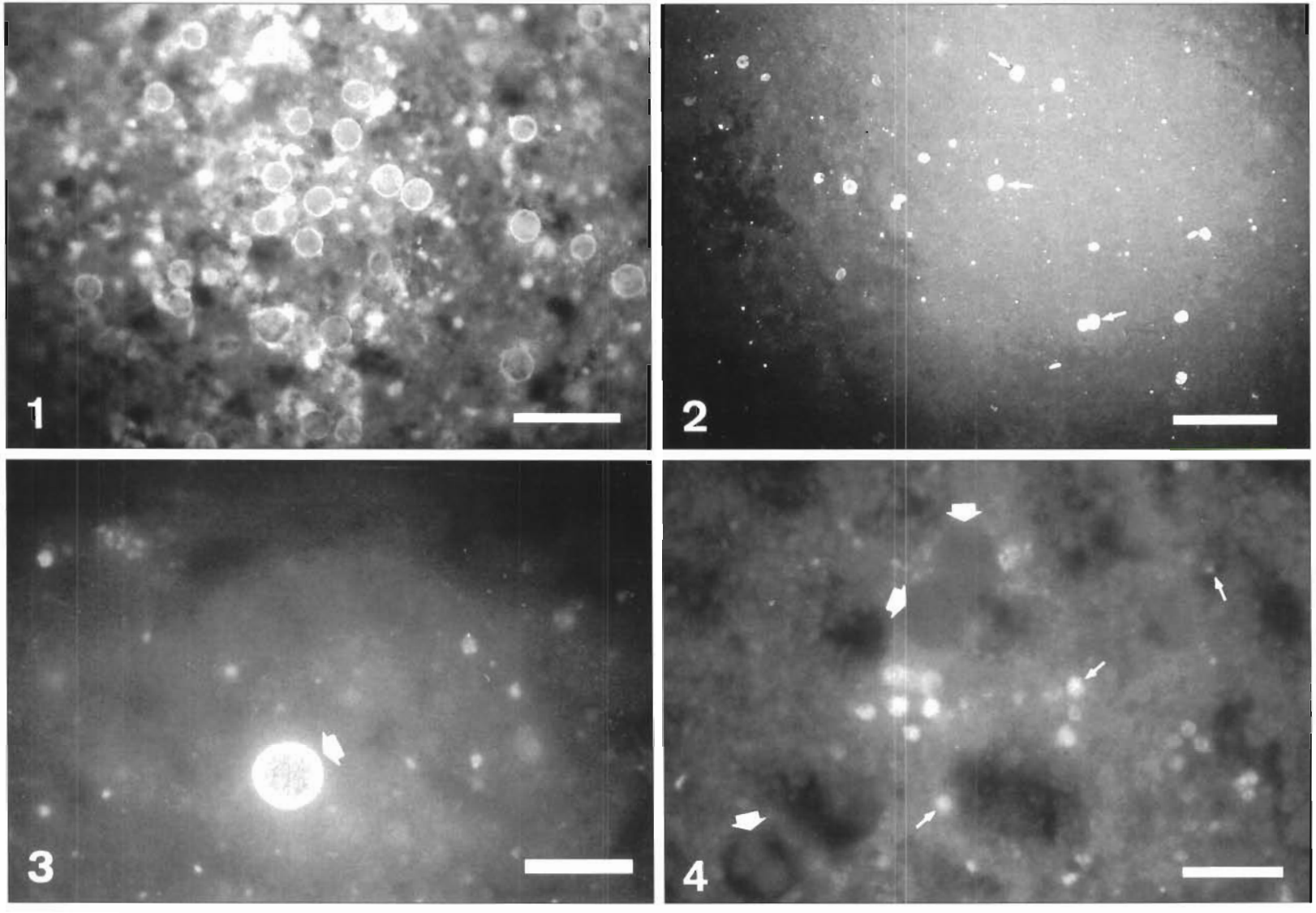
Characterization of monoclonal antibodies against the genus *Marteilia*. The IIFA method directly distinguished 2 main fluorescent patterns for the MAbs raised against *Marteilia* sp. isolated from infected mussels, which corresponded to the sporangia walls and spores. Four of these antibodies (1/1-3, 3/1-1, 4/1-1 and 6/2-3) bound strongly to the sporangia wall. Some sporangia walls did not bind antibodies, and the intensity was not similar for all sporangia; spores and refringent granules within sporangia were unstained (Fig. 1). Low magnification photography shows the fluorescence corresponding to the sporangia wall (yellowish-green, on a red background); small fluorescing particles were seen in the same smear (Figs. 2 & 3). The fluoresence pattern obtained with the 1/1-3, 3/1-1, 4/1-1 and 6/2-3 Mabs specific for the sporangia wall was the same; no differences in fluorescence pattern were found between these antibodies (Fig. 3).

Two monoclonal antibodies (9/1-1 and 12/5-1) produced intense fluorescence in the *Marteilia* sp. spore cytoplasm, particularly 12/5-1 (Fig. 4). Some spores within sporangia did not bind antibodies or emit the same intensity of fluorescence. Free spores from broken sporangia emitted yellowish-green fluorescence.

Antibodies which were produced against *Marteilia* sp. isolates from *Mytilus edulis* obtained in France (1/1-3, 3/1-1, 4/1-1, 6/2-3, 9/1-1 and 12/5-1) and which were tested with IIFA cross-reacted with *Marteilia refringens* from *Mytilus galloprovincialis* obtained in Spain.

DISCUSSION

Our objective was to obtain MAbs against *Marteilia* sp. isolated from *Mytilus edulis*. A modification of the Mialhe et al. (1985) protocol enabled isolation of both sporangia and sporangial primordia (described by Perkins 1976). Presently, *Marteilia refringens* can be transferred from oyster to oyster in the field (Tigé & Rabouin 1976, Balouet et al. 1979), but laboratory attempts were unsuccessful when infected oysters were held with uninfected ones or when healthy oysters were infected with digestive glands of infected oysters (Balouet et al. 1979). Comps & Joly (1980) transmitted *M. refringens* from oysters to mussels experimentally; however, only 2 infected mussels were found. Attempts to transfer *Marteilia sydneyi* among



Figs. 1 to 4. *Marteilia* sp. from *Mytilus edulis*. Indirect immunofluorescence assay in smears of digestive gland from infected mussels from France. Fig. 1. Sporangia showing antibodies binding to the external wall of the parasite (MAb 6/2-3). Some sporangia walls did not bind the antibodies and the fluorescence intensity was not the same for all sporangia. Scale bar = 25 µm. Fig. 2. Low magnification photograph showing fluorescence corresponding to the sporangia wall (arrows). The fluorescence is yellowish-green on a red background. Scale bar = 48 µm. Fig. 3. High magnification photograph of a sporangium (arrow) with MAb 1/1-3, showing sporangial wall and small fluorescing particles. Scale bar = 10 µm. Fig. 4. Sporangia showing antibodies binding to the spores of the parasite (MAb 12/5-1). Free spores from disrupted sporangia emitted yellowish-green fluorescence (small arrows). Some spores within the sporangia did not bind the antibodies (large arrows). Scale bar = 10 µm

infected and uninfected oysters *Saccostrea commercialis* have been unsuccessful (Roubal et al. 1989). Our modified purification protocol will permit the transfer of different stages of *M. refringens* in laboratory trials (after sonication of sporangia, spores and refringent granules can be obtained using a Percoll gradient; author's unpubl. data). Moreover, these different stages could be used for either biochemical or enzymatic characterizations.

Purified parasites were not used for initial immunization of the mice. However, digestive glands infected with *Marteilia* sp. enabled the mice to produce antibodies. Selection of specific hybridomas was based on an indirect immunofluorescent assay which was preferred to ELISA because the former permits direct visual evaluation of MAb reactivity against different life-cycle stages of *Marteilia* sp. Using IIFA, several hundred hybridomas can be screened rapidly and the site where the antibody recognizes and binds the parasite can be seen directly.

Six MAbs specific for either sporangia or spores were selected with 2 fluorescence patterns. These MAbs differentiated several structures of *Marteilia* sp. Four of these antibodies (1/1-3, 3/1-1, 4/1-1 and 6/2-3) bound to the sporangia wall; however, the fluorescence intensity of antibody bound to the wall was not similar in all sporangia, and some sporangial walls did not bind antibodies. Perkins (1976) demonstrated that the sporangia wall is formed around each developing sporangium and consists of a granular layer. Our results indicated that sporangia walls at different stages of maturity, and not all antigens were fully expressed. Perhaps the epitopes of *Marteilia* sp. were modified by purification. Small fluorescent particles observed could have been walls of disrupted sporangia.

Two MAb antisera also bound to the *Marteilia* sp. spore. Some spores within sporangia did not bind to antibodies; however, free spores from disrupted sporangia had yellowish-green fluorescence. Perhaps some antibodies do not pass through the sporangial wall and consequently do not reach spore epitopes.

Positive binding reactions of all MAbs tested in *Marteilia refringens* from Spanish *Mytilus galloprovincialis* indicate the presence of common epitopes. Villalba et al. (1993) identified the species present in *M. galloprovincialis* from Galicia as *Marteilia refringens*; however, in a field experiment Figueras & Robledo (1993) noted that *Marteilia* (then known as *Marteilia* sp.) from mussels did not infect flat oysters *Ostrea edulis* cultured in the same raft. MAbs could be used to extend the knowledge about problematic *Marteilia* species and to better elucidate the developmental cycle of *Marteilia* spp.

Specific localisation of epitopes at the subcellular level should be determined more precisely using the immunogold technique (Noël et al. 1991a). Moreover, isotyping each MAb will be done in the future. MAbs allow taxonomic comparison between Ascetospora isolated from different hosts world-wide.

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