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Monoclonal antibodies against *Bonamia ostreae* (Protozoa: Ascetospora), an intrahaemocytic parasite of flat oyster *Ostrea edulis* (Mollusca: Bivalvia)

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ABSTRACT: The protozoan *Bonamia ostreae* (Ascetospora), a parasite of the flat oyster *Ostrea edulis*, was purified by differential and isopycnic centrifugations. Mice of the strain Balb/c were immunized with purified parasites and hybridomas were prepared by fusion of immunized mouse splenocytes with the mouse myeloma cell line P3-X63-Ag8-653. From the fusion, 12 clones were isolated and saved. The resulting antibodies were characterized with a solid phase radioimmunoassay (RIA). Antibodies from 7 clones reacted only with *B. ostreae* and not with normal host tissue. Although some of the antibodies appeared to react with the same epitopes, at least 4 clearly different epitope specificities were identified. One of these antibodies (20B2-1B12) had an apparent association constant of ca 3×10^8 M^{-1} and bound to a number of different sites on the parasite. These monoclonal antibodies should prove to be of great value as diagnostic and research tools.

INTRODUCTION

The protozoan *Bonamia ostreae* (Ascetospora) is responsible for hemocyte disease of the flat oyster *Ostrea edulis* (Pichot et al. 1980). The parasite is ca 3 µm in diameter and replicates within the parasitophorous vacuoles of the host hemocytes. Infected cells die and liberate additional parasites into the vascular system. Bonamiasis is characterized by branchial ulceration and the accumulation of hemocytes in focal areas of the connective tissue. The disease is usually fatal causing a high mortality rate in a population within 6 mo of infection. Transmission of the disease occurs throughout the year, probably resulting from the continuing liberation of parasites from the tissues of dying and dead oysters (Grizel 1985).

Bonamiasis was first described in south Brittany (France) and since 1979 has spread to numerous oyster farming areas in France, England (Bannister & Key 1982), Holland (Van Banning 1982) and Spain (Polanco et al. 1984). The disease was also recently reported in association with serious oyster mortalities in Ireland (McCardle pers. comm.). The loss of production due to bonamiasis varies in different countries, but the mortality rate can be as high as 90%. Recently, the disease was described in North America (Elston et al. 1986, Farley et al. 1988). In addition, a similar but morphologically different parasite was found associated with mass mortalities of the New Zealand dredge oyster *Tiostrea lutaria* (Dinamani et al. 1987). To guarantee the continuity of oyster farming in several areas of the world, it is essential to develop methods for the prevention and management of bonamiasis. In the absence of oyster strains resistant to the disease, as well as effective treatment methods, it is necessary to develop immunodiagnostic procedures which will support strategies for the management of bonamiasis.

We describe here the production of monoclonal anti-

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bodies against *Bonamia ostreae*. The specificity and affinity we observed for certain of these antibodies will be useful in specific immunodiagnostic tests and as research tools.

MATERIALS AND METHODS

Organism and purification system. Healthy oysters *Ostrea edulis* were obtained from the 2 *Bonamia ostreae* free oyster culture sites in France: Thau Pond (Mediterranean), and natural oyster beds of Belle-Isle (Brittany). These oysters were kept in the laboratory several months before use.

Parasitized oysters were dredged from the Bay of Quiberon (Brittany) where bonamiasis has been enzootic for several years.

Bonamia ostreae was purified from parasitized oysters by differential and isopycnic centrifugations (Mialhe et al. 1988). Purified *B. ostreae* parasites were washed with sterile seawater by centrifugation (2000 × *g*, 30 min) in order to remove residual Percoll, and then resuspended in seawater to a concentration of 2×10^6 cells ml^{-1}. Electron microscopic examination of purified parasites verified the structural integrity of the purified organisms but also indicated the presence of fragments of host membranes on the parasite surface. These membranes are thought to represent portions of parasitophorous vacuole membranes from the host cell.

A single healthy oyster was selected for observation at the laboratory for several weeks and examined histologically to confirm the absence of bonamiasis. This oyster was homogenized with an Ultra-Turrax homogenizer in 500 ml of sterile seawater. The homogenate was passed through a 25 µm mesh sieve and 100 µl were plated into each well of a microtiter plate and treated as described in hybridoma screening procedures.

Immunization protocol. Six mice (Balb/c) were immunized by initial intraperitoneal (i.p.) (500 µl), intravenous (i.v.) (100 µl) and intramuscular (100 µl) injections of purified parasite suspensions (10^7 parasites ml^{-1}) diluted with one part complete Freund's adjuvant to 3 parts parasite suspension. Six subsequent i.p. injections without adjuvant were made at monthly intervals. The mouse with the highest serum titer was reinjected i.v. with 200 µl of parasite suspension at a concentration of 10^7 ml^{-1}, 3 d before the lymphocyte hybridization.

Lymphocyte hybridization protocol. The myeloma cell line P3-X63-Ag8-653 was cultived in RPMI 1640 medium (Gibco laboratories) containing 10% heat-inactivated fetal calf serum and 1 mM glutamine. Spleen cells were fused with the myeloma line by a method adapted from French et al. (1986): splenocytes and myeloma cells were washed and suspended in a serum-free medium. For each fusion, 3×10^8 splenocytes and 1.5×10^8 myeloma cells were mixed and centrifuged at 200 × *g* for 10 min at room temperature. Then, 1 ml 40% polyethyleneglycol 1540 (Riedel-de-Haën AG, Seelze, Hannover, Germany) was added to the cell pellet dropwise over 30 s with gentle stirring. After 1 min at 37 °C the cells were pelleted by centrifugation at 300 × *g* for 1.5 min and incubated for another 2 min at 37 °C. The pellet was then diluted to 15 ml by the addition of serum-free medium (the first 5 ml was added over 5 min). Cells were centrifuged at 150 × *g* for 10 min, resuspended in the medium containing 15% fetal calf serum and distributed (0.1 ml $well^{-1}$) into microculture plates (microplates with 10^5 cells $well^{-1}$ and microplates with 2×10^5 cells $well^{-1}$) containing macrophages as feeder cells (5×10^3 Balb/c peritoneal cells per 0.2 ml $well^{-1}$). One day after the fusion, hybridomas were selected by adding 0.1 ml $well^{-1}$ of double-concentrated hypoxanthine-aminopterin-thymidine medium (HAT) (hypoxanthine, 10^{-4} M; aminopterin, 4×10^{-7} M, thymidine, 1.6×10^{-5} M). Four and 7 d after lymphocyte hybridization, 100 µl of culture supernatant was replaced in each well with fresh normal HAT medium. The frequency of cultures with growing hybrid cells, analyzed according to Poisson's distribution (Paolucci et al. 1986), was determined after 7, 9 and 14 d culture by visual examination with an inverted phase microscope.

Hybridoma screening procedures. The 2 screening tests were indirect solid phase radioimmunoassay (RIA). The control screening test was performed in an identical manner to the positive screening test, except that *Bonamia ostreae* was replaced, in each well, by non-infected oyster homogenate.

The positive screening test was performed with *Bonamia ostreae* as follows: cells were seeded (2×10^5 per 100 µl $well^{-1}$) in microtiter plates which had been pretreated with poly-L-lysine (20 µg ml^{-1}, 50 µl $well^{-1}$, 30 min, 37 °C) to enhance the absorption of *B. ostreae* cells. Afterwards, the plate was centrifuged (2000 × *g*, 30 min), and the parasites were fixed with glutaraldehyde (0.5% in phosphate buffer saline [PBS], 20 µl $well^{-1}$, 8 min, 20 °C) and washed carefully with PBS. Finally, the plates were filled with PBS-BSA (Bovine serum albumine) (2% BSA, 0.75% glycine, 200 µl $well^{-1}$, 30 min, 20 °C) and stored at −20 °C until use.

When needed, the plates were thawed and washed 3 times with PBS. Each hybridoma culture supernatant (50 µl) was diluted with an equal volume of Buffer C (PBS, 5% BSA, 0.5% Tween 20) and placed in one well of *Bonamia ostreae* cells and one well of healthy oyster homogenate for incubation (2 h, 37 °C). After 3 washes

(150 mM NaCl, 0.5% Tween 20), an anti-mouse sheep IgG (Diagnostic pasteur), conjugated with ^{125}I by oxidation with chloramine T (Butt 1984) and diluted in Buffer C to 3×10^6 cpm ml^{-1}, was incubated in each well (100 µl $well^{-1}$, 1 h, 37 °C). Following incubation and 5 washes, the radioactivity of each well was measured with a gamma counter (Kontron).

Production and purification of monoclonal antibodies (MAbs). After cloning by the limiting dilution method, 2×10^6 hybridoma cells from the selected clones were injected i.p. into mice (Balb/c). The mice had been injected 15 d before with 500 µl of pristane (Sigma, ref. T 7640) to avoid developing a solid tumor. About 2 wk after injection of hybridoma cells, the ascite fluids containing MAbs were punctured and clarified by centrifugation. The monoclonal antibodies were then purified by affinity chromatography with Protein-A-Sepharose (Pharmacia). Isotypes and isoelectric points of purified antibodies were identified by the Ouchterlony and electrofocusing methods.

Specificity of monoclonal antibodies. The specificity of the selected MAbs was tested by direct solid phase RIA on nitrocellulose. Purified antibodies were radiolabelled with ^{125}I as described earlier. The specific binding activity of the antibodies was tested as follows. Ten microliters of each type of antigen (either purified parasite suspensions or hemolymph from healthy oysters) was absorbed onto a 6 mm diameter nitrocellulose discs placed in a microtiter well for 15 h at 37 °C. The nitrocellulose discs, which were subsequently dried, were then incubated (2 h, 37 °C) in 100 µl of gelatin (5 mg ml^{-1} in 100 mM phosphate buffer at pH 7.4) in a microtiter well. After 3 washes (NaCl 100 mM), 100 µl of phosphate buffer (100 mM) with 5 % gelatin containing the radiolabelled MAbs (100 ng ml^{-1} and 2.5×10^6 cpm ml^{-1}) was incubated in each well for 3 h at 37 °C. The radioactivity of the filter discs was determined after 5 washes with 100 mM NaCl.

The specificity was confirmed by indirect RIA. The protocol was identical to the direct RIA method with the addition of an incubation (1 h, 37 °C) in radiolabelled rabbit anti-mouse serum (2.5×10^5 cpm 100 μl^{-1} in 100 mM PBS, 5 % gelatin at pH 7.4).

Differentiation of monoclonal antibodies. Differentiation of the anti-*Bonamia ostreae* MAbs was accomplished by competing each radiolabelled antibody with a non-labelled antibody. The protocol was identical to the direct solid-phase RIA on nitrocellulose with *B. ostreae*, except that the labelled MAbs (120 ng ml^{-1}) were simultaneously incubated with a 100-fold excess of non-labelled MAbs (12 µg ml^{-1}).

Affinity of monoclonal antibodies. The apparent equilibrium association constant, k, was determined by direct solid phase RIA on nitrocellulose with parasitized oyster hemolymph and purified *Bonamia ostreae*. Assays were performed exactly as described for complementarity test except that different concentrations of non-labelled 20B2-1B12 were used to inhibit the fixation of a constant amount of ^{125}I labelled 20B2-1B12.

RESULTS

Immunization of mice

Following the 6 immunizations, the serum of each mouse was evaluated by the hybridoma screening method. These sera contained antibodies directed against non-infected oyster cells and *Bonamia ostreae*. The mouse which had the best specific immunologic activity against *Bonamia ostreae* was given a last immunization 3 d before the fusion. The antibody titer of this mouse was determined on the day of the fusion (Fig. 1). For a serum dilution of 1/1000, the reactivity of the serum antibodies with normal cells of the oyster (control screening test) was 3 times stronger than with *B. ostreae* antigens (positive screening test). The results show that the normal oyster tissue is highly immunogenic to the mouse.

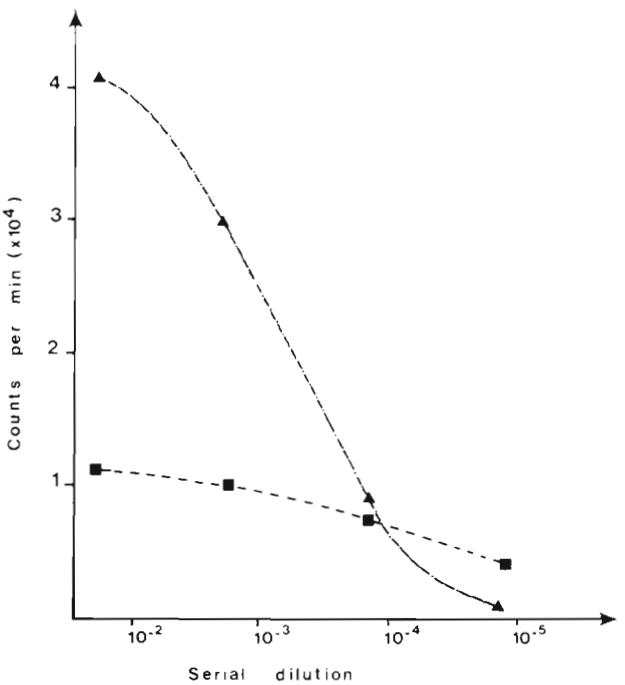

Fig. 1. Monoclonal antibodies against *Bonamia ostreae*. Characteristics of immune serum of the mouse used for the lymphocyte hybridization. Test was performed with solid phase RIA in the presence of purified *B. ostreae* parasites (■) or normal oyster hemocytes (▲). Antibody reactivity with oyster cells or parasite was detected with a radiolabelled anti-mouse immunoglobulin antibody

Hybridoma production

After 7 d culturing, from 1344 wells each receiving ca 10^5 cells, 682 wells (50.7 %) contained one or more types of hybridoma cells. Using the Poisson analysis, 470 wells (35 %) should contain only one type of hybridoma, 162 (12 %) should contain 2 hybridoma types and 50 (3.7 %) should contain 3 types of hybridomas. In contrast, from 170 wells receiving an inoculum of 2×10^5 cells, we expected 138 wells (81 %) to contain one or more hybridomas. Only 54 (31.5 %) wells should contain a single hybridoma, 45 (26.2 %) wells should contain 2 hybridomas and 25 (14.5 %) wells should contain 3 or more hybridomas. These results show the importance of initially inoculating a larger number of wells with a relatively low number of cells (10^5 per cell). The hybridomas recovered under these conditions were precloned.

In addition, 2 microplates were cultivated in the absence of macrophages. Out of 185 wells also receiving 2×10^5 cells, only 64 (34.6 %) wells contained one or more hybridomas, showing the determinant role of feeder cells in the hybridoma growth.

Isolation of hybridomas producing anti-*Bonamia ostreae* antibodies

In order to identify hybridomas producing specific antibodies against *Bonamia ostreae*, a 2-step screening test was used. This was necessary because of the difficulties in purifying large quantities of the parasites, and because of the presence of host membranes on the surface of the parasites. In the first step, the culture supernatants of the precloned hybridomas were tested against healthy oyster cells (control screening test); the strongly positive culture supernatants were eliminated. The other supernatants were subsequently analyzed by a second step against *B. ostreae* cells (positive screening test).

After 3 screenings, conducted 7, 9 and 14 d after the fusion, 166 wells (ca 24.3 %) contained antibodies reacting against normal oyster cells while 23 other wells (3.4 %) contained antibodies reacting specifically against *Bonamia ostreae*. These results are related to those for the serum of the mouse used for the fusion, which showed a strong antibody response against normal oyster antigens (Fig 1).

Subsequent cloning of 8 different *Bonamia ostreae* hybridomas allowed us to obtain 7 clones producing specific anti-*B. ostreae* antibodies as determined by indirect solid phase RIA (the positive screening test using *B. ostreae* cells). Antibodies from 3 other clones (16G5-2E7, 16G5-3D10 and 16F11-2A7) reacted strongly with *B. ostreae* but also to a lesser degree with oyster tissue.

Antibodies produced by these 10 clones were produced in ascite fluids, purified with Protein-A-Sepharose and characterized (Table 1).

Specificity of purified antibodies

Results for immunological and cellular specificities of 2 selected antibodies (20B2-1B12 and 15C2-2F2) are given in Fig. 2. The reactions of these 2 MAbs on purified parasites were only slightly inhibited in the presence of a 100-fold excess (12 $\mu g\ ml^{-1}$) of normal mouse immunoglobulin (Fig. 2A). The binding of radiolabelled antibody was almost totally extinguished in the presence of a 100-fold excess (12 $\mu g\ ml^{-1}$) of the homologous unlabelled antibody (Fig. 2A). The reaction of the radiolabelled monoclonal antibodies with the parasite is due to specific immunological recognition. The minimal reaction of the radiolabelled antibodies with the normal oyster tissue confirms the specificity of these antibodies for *Bonamia ostreae* (Fig. 2B).

Only 4 other MAbs retained their full activity after radiolabelling (10F1-2D4, 16G5-2E7, 16G5-3D10, 20B2-3A8). The specificities of 2 of the radiolabelled antibodies (10F1-2D4 and 20B2-3A8) are in all respects comparable (i.e. competed by the homologous non-labelled antibodies and not reacting with normal oyster tissue) to the antibodies produced by Clones 20B2-1B12 and 15C2-2F2, and display the characteristics shown in Fig. 2.

The 2 clones 20B2-1B12 and 20B2-3A8 originated from the same progenitor wells.

Radiolabelled antibodies 16G5-2E7 and 16G5-3D10 reacted with normal oyster cells and originated from

Table 1. Monoclonal antibodies against *Bonamia ostreae*. Immunological and biochemical characteristics of selected antibodies aigainst oyster parasite. For 2 hybridoma-clones these characteristics were not determined (ND)

Hybridoma-clone	Isotype	Isoelectric point
2 E3 1 F2	Ig G1	6.7–7.2
10 F1 2 D4	Ig G1	6.5–7.0
10 G9 2 D2	Ig G1	7.5–8.0
15 C2 2 F2	Ig G2a	8.2–8.5
16 G5 2 E7	Ig G2a	6.3–6.6
16 G5 3 D10	Ig G2a	ND
16 F11 2 A7	Ig G1	7.0–7.3
16 F11 2 F10	Ig G1	7.0–7.3
20 B2 1 B12	Ig G2a	6.9–7.3
20 B2 3 A8	Ig G3	ND

the same progenitor well. Four other clones (2E3-1F2, 10G9-2D2, 16F11-2A7 and 16F11-2F10) lost their immunoreactivity after radiolabelling.

The specificities of the antibodies were confirmed using an indirect RIA on nitrocellulose. Antibodies 2E3-1F2 and 10G9-2D2 specifically recognized *Bonamia ostreae* antigens. Antibodies 16F11-2A7 and 16F11-2F10 also reacted with normal oyster cells and originated from the same progenitor well.

Relative specificity of monoclonal antibodies

Results of the test of relative specificity (Table 2) showed that the different radiolabelled antibodies recognized 4 different epitopes. Epitope I was recognized by the 2 antibodies from Clones 20B2-1B12 and 20B2-3A8 which both originated from progenitor Clone 20B2. Epitope II was recognized by antibody from Clone 15C2-2F2. Epitope III was recognized by the 2 antibodies 16G5-2E7 and 16G5-3D10, which both originated from progenitor Clone 16G5. Epitope IV was identified only by antibody from Clone 10F1-2D4. These results indicate that antibodies produced by clones originating from the same progenitor well displayed the same characteristics and were probably identical.

The 4 antibodies which we could not radiolabel (2E3-1F2, 10G9-2D2, 16F11-2A7 and 16F11-1F10) did not inhibit the reactivity of any of the radiolabelled antibodies against *Bonamia ostreae*. These 4 antibodies recognized different epitopes than those defined above (Epitopes I to IV).

Affinity constant of Antibody 20B2-1B12

The apparent association constant, k, of Antibody 20B2-1B12 was determined according to Scatchard representation (Walker 1977), with purified *Bonamia ostreae* (Fig. 3A) or with infected hemolymph (Fig. 3B). The k values, $2.7 \times 10^8\ M^{-1}$ and $3.9 \times 10^8\ M^{-1}$ respectively, are not significantly different and show that the antibody is equally efficient in recognizing antigen on either purified parasites or in the hemolymph of the infected oyster. In addition, since the epitope number on each parasite can be calculated from the molar epitope concentration (mc) with an estimated 200 000 purified parasites per well (Fig. 3A) it can be assumed that Antibody 20B2-1B12 recognized ca 800 000 sites on each parasite. We concluded therefore that it should be possible to detect very small quantities of *B. ostreae* with this antibody.

DISCUSSION

Bonamia ostreae is a protozoan provisionally belonging to the phylum Ascetospora which contains several important pathogens of bivalve molluscs. This intracellular parasite has not been cultivated in vitro since permissive cell lines for this and other bivalve pathogens do not exist.

Before it was possible to produce specific antibodies to the parasite, it was necessary to develop a protocol for the purification of *Bonamia ostreae* from infected oyster tissue to obtain the necessary relatively large quantities of purified and concentrated parasite anti-

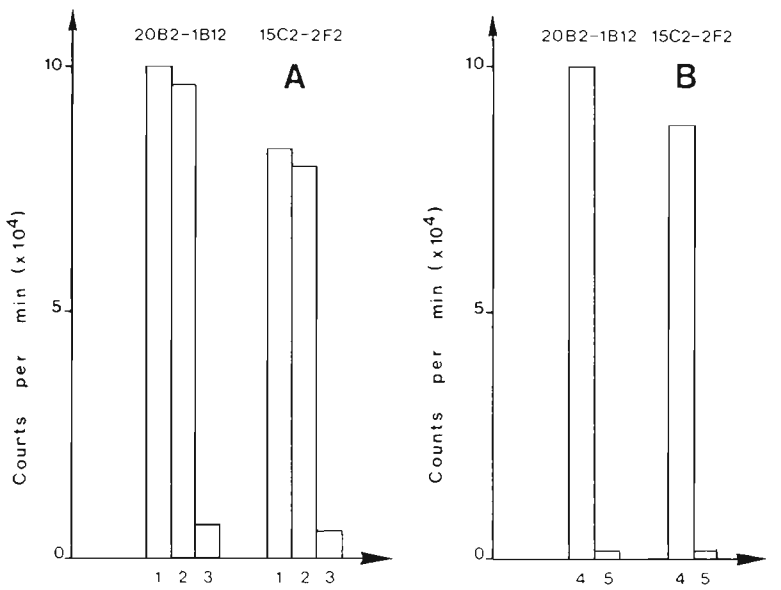

Fig. 2. Monoclonal antibodies against *Bonamia ostreae*. (A) Immunological and (B) cellular specificities of 2 purified antibodies from ascite fluids. (A) Radiolabelled monoclonal antibodies 20B2-1B12 and 15C2-2F2 incubated with purified *B. ostreae* adsorbed to nitrocellulose. Each of the antibodies was incubated alone (1), or in the presence of mouse immunoglobulin (2), or in the presence of the unlabelled homologous antibody (3) at a 100-fold greater concentration. (B) The radiolabelled monoclonal antibodies 20B2-1B12 and 15C2-2F2 were incubated on either purified *B. ostreae* (4) or hemolymph of non-parasitized oysters (5) adsorbed to nitrocellulose

Table 2. Monoclonal antibodies against *Bonamia ostreae*. Comparative specificities of purified monoclonal antibodies. Each radiolabelled antibody (at left) was incubated with purified *B. ostreae* antigens adsorbed to nitrocellulose in the presence of a 100-fold greater concentration of non-labelled antibody. Total (TI) or partial (PI) inhibition of the radioactive signal is representative of the identity or similarity of epitopes recognized by each of the 2 antibodies. NI: no inhibition; NT: no inhibition tested

	2E3–1F2	10F1–2D4	10G9–2D2	15C2–2F2	16G5–2E7	16G5–3D10	16F11–2F10	16F11–2A7	20B2–1B12	20B2–3A8
10F1–2D4	NI	TI	NI	NI	PI	PI	NI	NI	NI	NI
15C2–F2	NI	NI	NI	TI	NI	NI	NI	NI	NI	NI
16G5–2E7	NI	NI	NI	NI	TI	TI	NI	NI	NI	NI
16G5–3D10	NI	NI	NI	NI	TI	TI	NI	NI	NI	NI
20B2–1B12	NI	NI	NI	NI	NI	NI	NI	NI	TI	TI
20B2–3A8	NT	NT	NT	NT	NT	NT	NT	NT	TI	TI

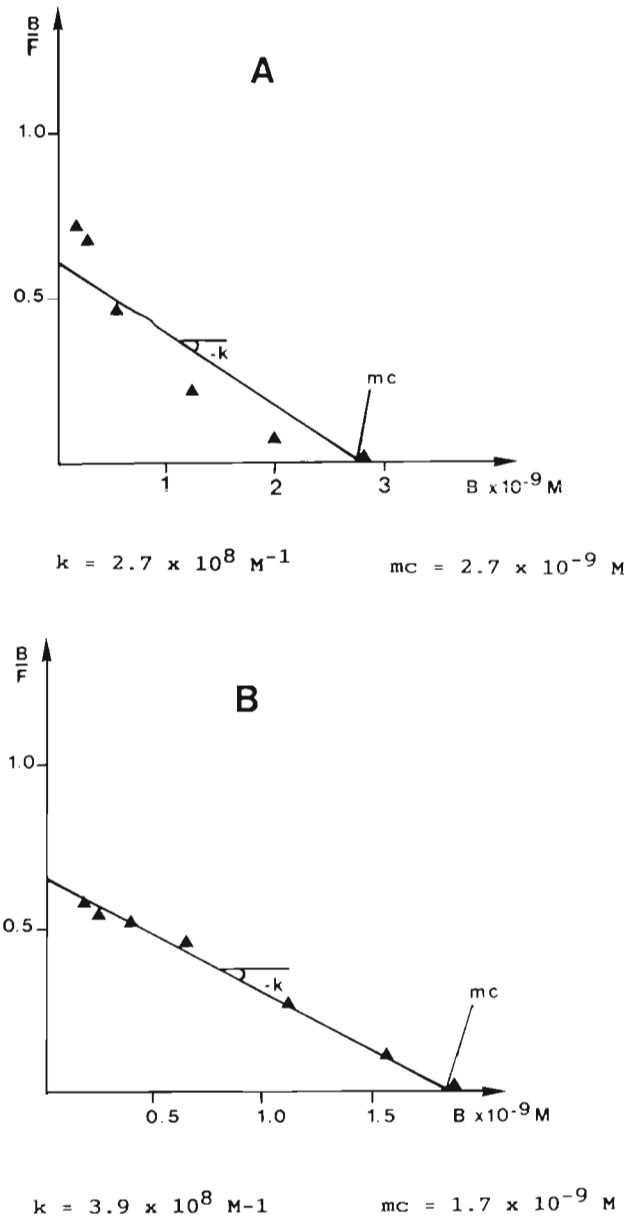

Fig. 3. Monoclonal antibodies against *Bonamia ostreae*. Determination of apparent association constant (k) of Antibody 20B2-1B12 according to the Scatchard representation $B/F = kmc - kB$ where B is molar concentration of bound antibody sites, F is molar concentration of free antibody sites, mc is molar concentration of epitope sites, k is apparent association constant. Experiments were performed in the presence of a constant concentration of radiolabelled antibody and a variable concentration of non-radiolabelled antibody reacting (A) against purified *B. ostreae* at 200 000 cells 100 μl^{-1} or (B) against a highly infected hemolymph sample (100 µl). From the estimated molar concentration value (mc) which is the intercept of the line with the x-axis ($mc = 2.7 \times 10^{-9}$ M for A and 1.7×10^{-9} M for B), the number of epitopes can be calculated as $1.62 \times 10^{14}\ l^{-1}$. Taking into account that 200 000 *B. ostreae* cells were contained in 100 µl the number of epitope parasite cells is ca 800 000

gens. It was then possible to produce polyclonal antibodies with sufficient specificity to the parasite for use in indirect immunofluorescence tests (Mialhe et al. 1988) after absorption with acetone extracts of normal oyster tissue. However, antibodies derived from such immune sera were not suitable for direct or indirect enzymatic immunoassays (ELISA) (Schönherr & Houwink 1984). This was due to a very low concentration of antibodies or to their weak affinity for these parasite antigens. It is essential for an ELISA assay to have antibodies of high specific affinity when the concentration of antigen is very low (Schönherr & Houwink 1984). For a long-term epidemiological survey program, it is necessary to produce a large quantity of antibodies with a standard titer. A large variation of antibody production in mice makes this impossible which led us to production of monoclonal antibodies in order to fulfill our objectives.

The indirect immunofluorescence technique is currently used for the screening of hybridoma supernatants for specificity to infectious disease agents (Danforth et al. 1982, Steiger & Seebeck 1986, Laxer et al. 1987). However the labor intensive immunofluorescent method is not practical, when using the precloning method (advantages of which are discussed by Paolucci 1983), because a very large number of wells must be seeded and screened. The screening method, based on the solid phase direct RIA appeared well adapted since it permitted a quick analysis of several hundreds of hybridomas. Moreover, because even after purification the parasites have fragments of parasitophorous vacuole attached to their surface, the screening was performed in 2 steps, the hybridomas producing antibodies against the interfering host cell antigens being previously eliminated.

According to our available information, this work represents the first production of MAbs against a parasite belonging to the phylum Ascetospora. Previous studies have reported the production of MAbs to protozoan parasites of man and domestic animals belonging to the Apicomplexa and Sarcomastigophora (Mc Mahon-Pratt et al. 1982, Jaffe & McMahon-Pratt 1983, Anthony et al. 1985) protozoan phyla. The results of this study are also important in the field of general marine invertebrate pathology because they demonstrate that relatively simple, specific and sensitive diagnostic tests could be produced and should be useful to prevent and manage the dissemination of infectious diseases in cultivated marine invertebrates.

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