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IN VITRO BINDING OF PARASITES (*BONAMIA OSTREAE*) AND LATEX PARTICLES BY HEMOCYTES OF SUSCEPTIBLE AND INSUSCEPTIBLE OYSTERS

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ABSTRACT

Bonamia ostreae is a protozoan parasite that has caused severe losses in the flat oyster (*Ostrea edulis*) industry in Europe. The cupped oyster (*Crassostrea gigas*), recently introduced and cultured in Europe, is not infected by the disease. *In vitro* tests were conducted to determine whether there was a difference in the ability of hemocytes from each species to recognize and bind inert foreign particles (fluorescent latex beads) and purified, infective *B. ostreae*. The results indicated no difference in their ability to bind latex beads, but *C. gigas* were able to bind more *B. ostreae* than were *O. edulis*. The relative inability of the *O. edulis* hemocytes to recognize the parasite is discussed as a possible factor in flat oyster susceptibility.

INTRODUCTION

The protozooan parasite *Bonamia ostreae* has devastated the European flat oyster (*Ostrea edulis*) industry over the last seven years, particularly in its major growing region of Brittany, France (1). The disease was first described in oysters from Tudy Isle in southern Brittany (2), and quickly spread along the Breton coast (3,4,5). Balouet and Poder (6) suggested that the parasite was introduced to France from North America since a similar pathogen, called "microcell" organism, was described in flat oysters from California (7), and flat oyster spat were transported from California to France in 1978 and 1979. Recent studies in North America (8) support this hypothesis.

O. edulis is highly susceptible to *B. ostreae* and can be experimentally infected by injection, feeding, or proximity, implying that the gills, mouth and digestive tract are portals of entry (1,9,10). In early stages of the infection, there is hemocytic infiltration of connective, mantle, stomach,

and especially gill tissues (11). Bachere and co-workers (9) noted lesions on gill filaments for 77% of O. edulis with the disease. C. gigas, on the other hand, is not affected by the parasite in the laboratory (10) or in the field (11). The basis of invulnerability for C. gigas is not clear.

Although gill lesions are a clinical sign for Bonamiasis (4), it is often considered an intrahemocytic disease because viable parasites are routinely found within the cytoplasm of infected O. edulis hemocytes. Balouet and co-workers (11) have presented ultrastructual evidence to suggest that the parasites can multiply within the hemocytes and eventually cause them to lyse. This is thought to be one mechanism used by the parasite to spread throughout the oyster tissue. The presence of viable B. ostreae in the hemocytes could be the result of active parasite penetration or of parasites that are not killed after they have been phagocytosed by the hemocytes. The lack of infection in neoplastic hemocytes, which are unable to phagocytose, and the presence of an endocytotic membrane surrounding the parasite (11) imply that the mode of entry into the hemocyte is phagocytosis.

The hemocytes of O. edulis and C. gigas have been studied (12) and compared histochemically and structurally (13), but their ability to recognize and phagocytose foreign particles has not been assessed. With certain exceptions (14,15), transmission electron microscopy is required to determine whether a foreign particle is phagocytosed or merely adhered to the surface of a phagocyte. However, it is generally believed that particles which bind to the surfaces of phagocytes will be phagocytosed (16). Recognition and binding of foreign particles by oyster hemocytes may be facilitated by agglutinins (lectins) bound to the cell surface (17,18). Once bound, particles are ingested by endocytotic mechanisms and either destroyed by enzymic action from the fusion of lysosomes with the phagosome (19), or eliminated from the oyster by diapedetic migration of the hemocyte into excretory ducts (20,21).

In vitro techniques have recently been employed to quantify hemocyte ability to bind latex particles (fluorescent beads) after test oysters were held in a range of environmental conditions (22,23). The same techniques were used in this study, but isolated B. ostreae parasites were tested in addition to the latex beads. The parasites were isolated by a modification of the techniques of Bachere and Comps (24) and were infective to O. edulis adults after purification (25). The results showed that C. gigas hemocytes were more able than O. edulis hemocytes to bind the purified B. ostreae under a variety of environmental conditions, although there was no difference in their ability to bind latex beads.

MATERIALS AND METHODS

In vitro binding of B. ostreae and latex beads

Three separate experiments measured the ability of O. edulis and C. gigas hemocytes to bind purified B. ostreae. These were conducted on February 26 (Experiment I), April 23 (II) and June 20 (III) 1986. In each case, the parasites were obtained from approximately 30 O. edulis diagnosed as heavily infected with the disease. The parasites were isolated by differential centrifugation according to methods developed by Miahle and co-workers (25)

which does not destroy the parasites infectivity or virulence. O. edulis and C. gigas were obtained from neighboring oyster parks in the Bay of Quiberon in southern Brittany, France. They were always maintained under identical conditions in the laboratory and tested in an alternating pattern to offset any procedural effects. Hemolymph smears were made of each animal to diagnose the natural occurrence of the parasite after each experiment was completed. Those cases where B. ostreae were found associated with hemocytes were eliminated from the data set. Samples were also deleted for technical difficulties, such as tissue contamination of the drawn hemolymph.

In Experiment I, oysters of both species were collected from natural conditions (5°C, 30 ppt salinity) and placed in laboratory tanks for 1 day at 9°C. Then, 6 hours before withdrawing hemocytes, half of the oysters from each species were placed into either control (15°C, 30ppt salinity) or stress (25°C, 15 ppt salinity) conditions. These conditions were selected to emphasize the stress response previously exhibited by O. edulis hemocytes (22). After 6 hours in these conditions, the shells of the oysters were notched and hemolymph withdrawn from the pericardia with a syringe and needle. One drop of hemolymph was added to a glass slide and the hemocytes allowed to settle for 15 minutes at room temperature (16°C). Then a single drop of purified B. ostreae was added. The B. ostreae had been previously diluted in 30 ppt sea water to a concentration of about 10^6 ml^{-1} as measured by a Coulter particle counter. After 30 minutes of contact time, the slides were rinsed in sea water, fixed in 37% formaldehyde vapors and stained with a modified Giemsa stain. Slides were later examined with oil immersion microscopy and 200 hemocytes from each sample were examined for adhering B. ostreae parasites. The results were used to calculate the percentage of hemocytes associated with B. ostreae (%H) and the average number of adherent B. ostreae per counted hemocyte (B.o./H).

Experiment II examined only the effects of temperature on the hemocytes of the two species. O. edulis and C. gigas were collected from a water temperature of 9°C and held in the laboratory for two days at 15°C. Six hours before withdrawal of hemolymph, oysters of each species were moved to aquaria at 15°C, 20°C, or 25°C. Hemocyte withdrawal, settling, and exposure to B. ostreae were all completed at these temperatures using the methods described above. The concentration of B. ostreae was not measured, but equal aliquots of the same sample were added to all slides. Simultaneously, replicate slides of hemolymph were tested for hemocyte ability to bind 2 µm latex beads (fluoresbrite latex microspheres, Polysciences Inc, Warrington, Pennsylvania 18976 USA). The beads were diluted in 30 ppt salinity sea water to a concentration of 10^6 ml^{-1} and were tested under the same conditions as the purified B. ostreae. Two fields-of-view, each with 30-80 hemocytes, were examined with epifluorescence microscopy for bead adherence. Background levels were determined and subtracted from the total according to previous methods (22). The results were used to calculate the percent of hemocytes associated with latex beads (%H) and the average number of adherent beads per counted hemocyte (b/H).

Experiment III also examined the effects of temperature on the ability of hemocytes to bind foreign particles. Both species of animals were collected from natural waters at 14°C and maintained in the laboratory for 1 day at 15°C. They were then distributed equally into aquaria at 15, 20 and 25°C where they were maintained for one day. Oysters were taken from the aquaria in pairs, one of each species, and hemolymph withdrawn, hemocytes settled and

tested for adherence of B. ostreae parasites and latex beads as described above. The concentration of B. ostreae was not measured, but the concentration of beads was 1.5 x 10^6 ml^{-1} by hemocytometer count. Slides were numbered to ensure unbiased counting, and decoded only after the counts and calculations had been completed.

Additional in vitro binding tests with latex beads

In addition to the bead tests described in Experiments II and III, three separate latex bead adherence tests were made during the winter of 1986 to compare the binding abilities of hemocytes from O. edulis and C. gigas oysters. Binding of B. ostreae was not measured. The oysters of both species were collected from the same sites as those in the previous experiments, and the testing protocol was as described above. The concentration of latex beads in the three experiments ranged from 1 - 2 million beads per ml. In each experiment, slides were numbered and decoded only after bead counts were completed.

Oysters of both species sampled on January 24 at a temperature of 9°C were held one day in the laboratory system at 16-18°C before testing. A second sampling was completed on February 26 using oysters collected and held with those used in Experiment I. The oysters were collected at 5°C, held for one day at 9°C, and tested at 16°C. Although from the same sample, the bead tests were not run simultaneously with the B. ostreae adherence tests of Experiment I, nor were the hemocytes from the same animals. Oysters were again collected on March 25 from a temperature of 6°C and brought up to 15°C in the laboratory. After one day, animals were divided into aquaria at 15, 20 and 25°C where they were held 4 hours before sampling. Settling and exposure to latex beads were conducted at the appropriate test temperatures.

RESULTS

In vitro binding of B. ostreae and latex beads

The results from Experiment I showed C. gigas hemocytes to have a greater ability than O. edulis hemocytes to bind purified B. ostreae in both stress and control conditions. Values for %H (Table 1) were not significantly different when analyzed as a 4-group analysis of variance (ANOVA; $F = 2.5$, $df = 16$), but differences were highly significant ($p < .01$) when species were compared without regard for stress or control conditions (2-group ANOVA; $F = 8.3$, $df = 18$). A Student's t-test found binding by C. gigas to be significantly greater ($p < .05$) than O. edulis in the control samples, but the difference in the stress samples was not significant ($p > .05$). The results for B.o./H (Fig. 1) also showed no significant differences for 4-group ANOVA ($F = 2.5$, $df = 16$), but differences were highly significant when O. edulis and C. gigas were compared in a 2-group ANOVA, ($F = 8.6$, $df = 18$). The t-test again showed C. gigas to have significantly greater binding in the control conditions.

Experiment II showed similar results. The percent of hemocytes binding B. ostreae (%H, Table 1) was significantly higher for C. gigas when analyzed by 3 temperatures and 2 species (6-group ANOVA; $F = 2.5$, $df = 33$) and the

TABLE 1

A. Binding of *Bonamia ostreae*:

	O. edulis	*C. gigas*
Experiment I		
Control	5.5 ± 1.1 (6)	11.9 ± 2.6 (5)
Stress	6.0 ± 1.0 (5)	13.3 ± 4.9 (4)
Experiment II		
15°C	41.3 ± 3.3 (8)	58.7 ± 1.9 (7)
20°C	48.7 ± 6.0 (8)	57.4 ± 1.5 (7)
25°C	42.8 ± 4.8 (4)	61.2 ± 1.9 (5)
Experiment III		
15°C	14.5 ± 3.1 (9)	28.3 ± 5.1 (9)
20°C	14.7 ± 1.8 (10)	23.0 ± 3.1 (10)
25°C	12.4 ± 2.1 (9)	28.3 ± 4.4 (9)

B. Binding of latex beads:

	O. edulis	*C. gigas*
Experiment II		
15°C	15.9 ± 2.0 (8)	13.3 ± 0.8 (8)
20°C	22.0 ± 3.4 (11)	16.3 ± 1.1 (8)
25°C	20.5 ± 4.5 (8)	20.4 ± 3.2 (6)
Experiment III		
15°C	20.1 ± 4.0 (8)	16.9 ± 4.0 (7)
20°C	17.6 ± 2.7 (11)	18.5 ± 4.2 (9)
25°C	14.4 ± 4.0 (10)	20.2 ± 3.6 (10)

Binding of *B. ostreae* and Latex Beads

Percent hemocytes associated with *B. ostreae* (A) or latex beads (B) were counted for *O. edulis* and *C. gigas*. Concentrations of beads and *B. ostreae* varied between experiments. Statistical comparisons are presented in the text. Standard errors are noted and sample size given in parentheses.

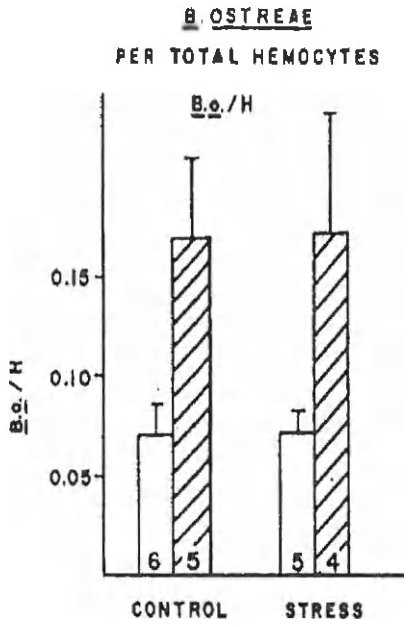

FIG. 1
The average number of B. ostreae per hemocyte (B.o./H) for O. edulis (open bars) and C. gigas (hatched bars) in Experiment I. Control conditions were 30 ppt salinity and 15°C and stress conditions were 15 ppt salinity and 25°C for six hours. Standard errors are shown and sample size is designated at the bottom of each bar.

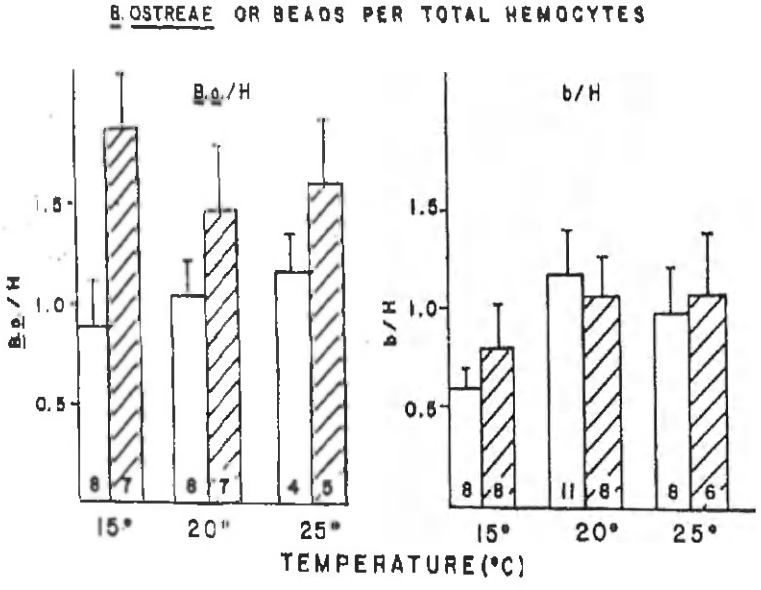

FIG. 2
The average number of B. ostreae per hemocyte examined (B.o./H) and the average number of latex beads per hemocyte (b/H) for O. edulis (open bars) and C. gigas (hatched bars) at three temperatures in Experiment II. Standard errors are shown and sample size is designated at the bottom of each bar.

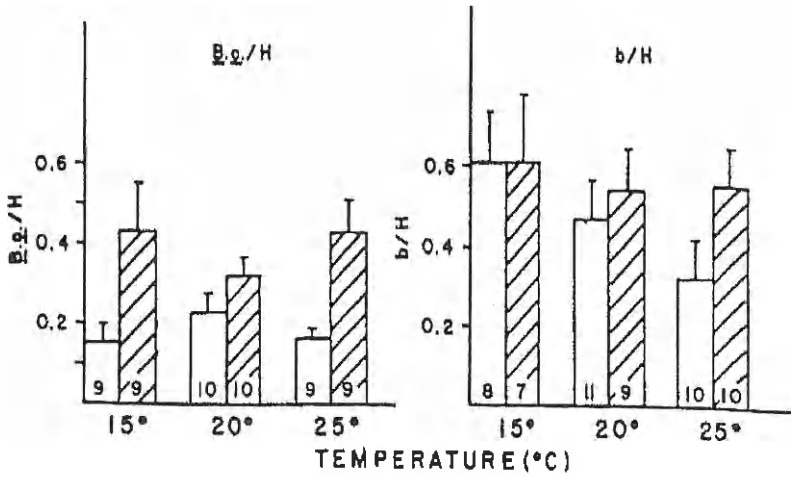

FIG. 3
The average number of B. ostreae per hemocyte (B.o./H) and the average number of latex beads per hemocyte (b/H) for O. edulis (open bars) and C. gigas (hatched bars) at three temperatures in Experiment III. Standard errors are shown and sample size is designated at the bottom of the bars.

TABLE 2

	%H		b/H	
	O. edulis	C. gigas	O. edulis	C. gigas
January	33.6 ± 4.0 (11)	24.6 ± 4.2 (12)	0.80 ± .14 (12)	0.66 ± .20 (12)
February	6.3 ± 1.6 (8)	9.2 ± 2.8 (6)	0.11 ± .02 (8)	0.23 ± .08 (6)
March 20°C	5.3 ± 1.9 (4)	14.3 ± 5.2 (3)	0.06 ± .03 (4)	0.80 ± .63 (3)
March 25°C	20.0 ± 4.8 (4)	19.5 ± 2.5 (4)	0.58 ± .16 (4)	0.80 ± .38 (4)

Binding of Latex Beads

Additional experiments compared the ability of O. edulis and C. gigas hemocytes to bind latex beads. Bead concentrations ranged from 1-2 million ml^{-1} but were constant within each experiment. Percent hemocytes associated with beads (%H) and the average number of beads bound per hemocyte (b/H) were calculated. There were no significant differences detected between the two species (see text). Standard errors are shown and sample sizes are noted within parentheses.

difference was highly significant when the 2 species alone were compared (2-group ANOVA; F = 11.9, df = 37). B.o./H values (Fig. 2) were also significantly higher for C. gigas in the 6-group ANOVA (F = 3.3, df = 33) and the difference was highly significant with a 2-group ANOVA (F = 14.0, df = 37). A t-test applied to the 15°C data found the greater binding of B. ostreae by C. gigas hemocytes to be highly significant for both %H and B.o./H, but showed no significant differences at 20° and 25°C. The latex bead portion of experiment II demonstrated no significant differences in %H (Table 1) or b/H (Figure 2), regardless of grouping.

The results for Experiment III further supported the pattern of greater B. ostreae binding by C. gigas hemocytes. The difference for %H (Table 1) was highly significant ($p < 0.01$) for both a 6-group (F = 4.5, df = 50) and a 2-group (F = 21.2, df = 54) ANOVA. Differences determined by the t-test were significant ($p < .05$) at 15° and 20°C, and highly significant at 25°C. In the bead portion of the experiment, also shown in Figure 3, there were no significant differences between the two species in %H.

The difference for B.o./H in Experiment III (Fig. 3) was significant for the 6-group ANOVA (F = 2.9, df = 50) and highly significant for the 2-group ANOVA (F = 12.9, df = 54). The t-test was highly significant at 25°C, but there was no significance at 15° and 20°C. There were no significant differences between the two species in the bead portion of the experiment, regardless of grouping (Table 1; Fig. 3).

Additional in vitro bead adherence tests

The %H and b/H values for the three additional latex bead experiments are given in Table 2. There were no significant differences due to species (ANOVA) in any of the experiments. In the March 25 survey, there was a significant difference detected for %H, but a t-test revealed the statistical difference was between the 20° and 25°C O. edulis values and did not reflect any differences between the two species.

DISCUSSION

In a variety of experimental test conditions, in vitro binding of purified B. ostreae was consistently greater for C. gigas hemocytes than for O. edulis hemocytes, whereas there were no differences between the two species in their ability to bind latex beads. These results indicate that hemocyte activity is similar for both species yet their ability to recognize and bind the parasite is not. Such a difference may play a role in the relative susceptibilities of these two species to Bonamiasis.

Balouet and co-workers (11) have suggested that the vulnerability of O. edulis to B. ostreae may be due to the inability of O. edulis hemocytes to digest the parasites once they have been ingested. They felt that the parasites could divide within the hemocyte, disrupt, and eventually lyse the cell membrane. The release of parasites from lysed hemocytes would then account

for B. ostreae found in extracellular tissues and the disease could be systemically spread via the hemolymph.

The results presented here raise the possibility that O. edulis is susceptible to B. ostreae because of its relative inability to recognize and bind the parasite for phagocytosis. This would not, however, explain the degeneration and eventual lysis of hemocytes with internalized parasites. An alternate hypothesis is that the relatively poor recognition of B. ostreae occurs within the cytoplasm as well as on the surface of the hemocyte. Although little is known of intracellular phagocytic events, there may be an internal mechanism of recognition necessary for lysosomal enzyme synthesis, phagolysosome formation, and the introduction of digestive enzymes into the phagocytic vacuole. Cheng (19) suggested that three recognition sites were required for lysosomal synthesis; at the plasmalemma, the nuclear envelope, and the lysosomal granule. O. edulis hemocytes, less able than C. gigas hemocytes to recognize and bind B. ostreae parasites to their surfaces, may also have less ability to initiate digestion once they have ingested the parasites.

Bead and parasite binding were not significantly affected by changes in temperature and/or salinity, although the ability of C. gigas hemocytes to bind latex beads appeared to increase at warmer temperatures (Table 1). The lack of an environmental effect may be due to acclimation of hemocytes to the new conditions as found in previous experiments (22). The high variability between experiments found for %H (Table 1) was more likely due to the different concentrations of B. ostreae added than to seasonal differences in hemocyte capability.

The results of this study imply potential differences in parasite recognition between hemocytes of susceptible and insusceptible species. Thus, investigation of cell surface receptors and membrane-bound lectins could reveal differences in the hemocytes of O. edulis and C. gigas. A potential obstacle for such studies is that the differences observed here were relative, i.e. there was parasite binding by O. edulis hemocytes, but not as often as by C. gigas hemocytes. It would be interesting to know whether hemocytes from resistant O. edulis recently reported from Washington State (26), are more able than hemocytes from susceptible strains to bind B. ostreae.

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