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Parasitological examination of wasting disease in black abalone, Haliotis cracherodii : final report

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PARASITOLOGICAL EXAMINATION OF WASTING DISEASE IN BLACK ABALONE, *HALIOTIS CRACHERODII*

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Abstract: Black abalone in southern California are afflicted with chronic intestinal infections of a rickettsia-like organism that causes a debilitating and fatal withering syndrome. The hematology of withered animals indicated that cellular degradation and apoptosis occurred in tandem with the decline and catabolism of abalone tissues. Two types of hemocytes were found in the hemolymph. Type I and Type II Hyalinocytes were distinguished by subtle differences in their cytoplasmic vesicles. Densities of both types of hemocytes declined in abundance, and small, presumptive stem cells increased in abundance with the progression of the disease. No circulating granulocytes were present in hemolymph; but serous cells were present as fixed granulocytes in hemal spaces. Cellular inclusions, dying cells, and vacuolate cells increased in abundance with the disease. An evaluation of cellular immunity resulted in disparate findings associated with serum levels and types of buffer used. In the presence of micronutrients, and divalent metal ions, however, hemocytes from infected abalone showed increased degrees of phagocytosis (percent phagocytosis, and number of yeast particles per hemocyte) compared to hemocytes from uninfected animals. Experimental transmission of the disease was effected in healthy, unexposed abalone held together with asymptomatic, exposed abalone. Clinical signs of infection appeared after 180 days of cohabitation. The chronic nature of the disease progressed relatively unnoticed until 6 to 8 months when infected abalone began to show signs of withering. The withered condition represented an end stage of the disease and probably resulted from starvation caused by rickettsial disruption of the digestive processes.

Executive Summary

Black abalone in southern California are afflicted with chronic intestinal infections of rickettsia-like organisms that cause a debilitating and fatal withering syndrome. At the time the proposal was funded, the causative agent was unknown. We, therefore, examined the blood of abalone in an attempt to find the causative agent of the disease. The hematology of withered animals indicated that cellular degradation and apoptosis occurred in tandem with the decline and catabolism of abalone tissues. Two types of blood cells (hemocytes) were found in the hemolymph. Type I and Type II Hyalinocytes were characterized by subtle differences in their cytoplasmic vesicles. Densities of both types of hyalinocytes declined in abundance, and small, presumptive stem cells increased in abundance with the progression of the disease. Circulating granulocytes were not present in hemolymph, but fixed granulocytes (serous cells) were present in hemal spaces, and connective tissues. Cellular inclusions, dying cells, and vacuolate cells increased in abundance with the disease. While, hemocyte densities were highly variable through time, hemocytes decreased in abundance, and the proportion of dead cells increased in abundance with the progression of the disease.

We evaluated the ability of abalone hemocytes to phagocytize yeast cells as an assessment of the cellular defenses (immune capability) of the host. Serum levels and the type of buffer used in the assessments caused significant, and, in some cases, radical variations in the percentage of phagocytic hemocytes, and in the number of yeast cells phagocytized. In Hanks balanced salt solution adjusted with NaCl (HBSS-NaCl) phagocytosis decreased with decreasing serum levels. In 100% serum mixed 1:1 with yeast suspended in HBSS-NaCl, a majority (50%+) of hemocytes

from healthy, unexposed abalone were capable of phagocytosis. In buffer alone (O% serum) fewer cells (<5%) were phagocytic; and they possessed fewer particles. Phagocytosis was markedly different between cells suspended in HBSS-NaCl and those in HBSS adjusted with artificial seawater (HBSS-Sea). Hemocytes from healthy abalone showed a decreased response in HBSS-Sea, while those from symptomatic abalone showed enhanced phagocytic response. The presence of opsonins and divalent metal ions may explain the marked differences between experimental treatments. In the presence of micronutrients, and divalent metal ions (HBSS-Sea), hemocytes from infected abalone showed increased degrees of phagocytosis (percent phagocytosis, and number of yeast particles per hemocyte) compared to hemocytes from uninfected animals. The results indicate that infected abalone may have a heightened phagocytic response that could ultimately impair their cellular defenses to other disease agents. The general use of phagocytosis as an indicator of cellular immunity in invertebrates may require standardization. Standardization of buffers, and, possibly, serum components may improve the utility of the assay in the future.

Three experiments were undertaken to analyze transmission of the disease in the laboratory. Dr. Carolyn Friedman undertook the successful Cohabitation Experiment. Experimental transmission of the disease was effected in healthy, unexposed abalone held together with asymptomatic, exposed abalone. Clinical signs of infection appeared after 180 days of cohabitation. The chronic nature of the disease progressed relatively unnoticed until 6 to 8 months when infected abalone began to show signs of withering. The withered condition represented an end stage of the disease and probably resulted from starvation caused by rickettsial disruption of the digestive processes.

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INTRODUCTION

Withering syndrome is a recently described condition of the black abalone, *Haliotis cracherodii.* The syndrome is a debilitating and fatal disease caused by a putative rickettsia-like organism (RLO) (Gardner et al., 1995). The pathology of the disease fulminates as the RLOs infect the digestive gland, digestive tubules and columnar epithelial cells of the intestine. Chronic infection with presumed autoinfection result in malabsorption which ultimately leads to starvation of the afflicted abalone. Externally, the withering syndrome is characterized by a small body mass to shell size ratio (i.e., an atrophied foot), abnormal coloration and flaccid tissue mass (Haaker et al., 1992). Atrophy of the foot is marked by reduction in the overall facies of the muscular tissue, and reduction in body mass. The wasted and weakened foot is an end-product of the disease.

The disease syndrome was first noticed in 1985 by commercial abalone divers off Santa Cruz Island. It has since spread to all of the Channel Islands, California (Lafferty and Kuris, 1993; VanBlaricom et al., 1993), Vandenberg Airforce Base in Central California, north to Cayucos (present study). Prior to the epizootic, the black abalone was a major component of the abalone fishery in California, comprising 32 to 51% of the total landings (Oliphant et al., 1990). With the spread of the disease, the black abalone fishery has collapsed in Southern California (Figure 1, Haaker et al., 1992; Steinbeck et al., 1992), and is now closed to commercial harvesting. The disease has now spread to other species of abalone but it is markedly less prevalent in their populations.

Withering syndrome has caused dramatic declines in the abundance of abalone. Decreases of 80 to 90% in abundance have occurred within 5 years of the first signs of the disease (Richards and Davis, 1993; Hakker et al., 1995) with one study reporting a losss of as much as 99% of the population over 6 years (Richards and Davis, 1993). Temperature has been implicated as a contributory factor in withering syndrome. Increased temperatures are known to facilitate the onset of the syndrome (Steinbeck et al., 1992). The increasing temperatures off the California coast have probably facilitated in the catastrophic decline of the stocks (Tissot, 1995), and may have contributed to the spread of the disease.

PURPOSE

At the beginning of this study the causative agent was as yet undetermined. An epizootiological study had ruled out a variety of physical, chemical and ecological factors as agents of the syndrome (Lafferty and Kuris, 1993). The causative agent, a rickettsia-like organism, had been observed in the digestive tract of abalone (VanBlaricom et al., 1993), but since rickettsial organisms rarely cause pathology in their molluscan hosts (e.g., Lauckner, 1983), little attention was paid to the pathogen. We, therefore, undertook parasitological examination of the hemolymph and tissues in an effort to (1) identify the causative agent, and (2) further characterize pathological changes in the hemolymph and tissues as a result of the syndrome. In addition, we attempted transmission of the disease in three separate experiments.

APPROACH

The main approach is detailed in Attachments A and B. Briefly, we examined the hemolymph and tissues of abalone from the Channel Islands, Vandenburg Air Force Base, Cayucos Beach, and Año Nuevo Island. We undertook a detailed examination of the hematology of abalone, and performed a series of experiments to gauge the cellular defenses of abalone hemocytes from abalone with and without the syndrome. We also examined tissues in some detail, but other studies have reported on the pathological changes in relation to the disease (Gardner et al., 1995). Lastly, after joint discussions, the cohabitation experiment was performed by Dr. Carolyn Friedman (Calif. Fish & Game). We analyzed the hematology of abalone from the cohabitation study, and have contributed to the analysis of that experiment.

FINDINGS

Prior to this study, the causative agent of withering syndrome was as yet unknown. We, therefore, reviewed the literature in more depth in an effort to uncover potential relations between withering syndrome and other diseases, and to give perspective to the diseases that afflict abalone.

GENERAL REVIEW OF ABALONE DISEASES

Considering the size and importance of abalone fisheries, there are few reported diseases of abalone (Table 1). Of the infectious pathogens, a withering-like syndrome has been observed in infections of *Vibrio* spp., and an unidentified trematode[1] (Crofts, 1929; Elston and Lockwood,

[1] Trematodes require molluscs as their first intermediate hosts. Hundreds to thousands of abalone are landed every year as a gourmet food, yet no other trematode infections have been reported. Since many trematode infections in snails are host specific (at least to the family level), Croft's record appears as an isolated example of infection in an accidental host.

1983). A withering-like condition has also been reported for starving pink and black abalone (Cox, 1962), and for cultured disk abalone infected with a putative viral infection (Nakatsugawa et al., 1988; Harada et al., 1993). Hence, the withering of the foot is not specific to any single disease agent.

Elston & Lockwood (1983) described the pathology of *Vibrio* spp. infections in juvenile red abalone, *H. rufescens*. In general, diseased abalone were less active and had a reduced ability to remain attached to substrates. Infections began primarily in the foot and the epipodia where there was an exfoliation of the epithelial tissues. Subsequently, the infections spread to the vascular sinuses and the nerves of the hosts (Elston and Lockwood, 1983). Bacteria were observed in all of the affected tissues.

In contrast, Dixon et al. (1991) had difficulty isolating *Clostridium lituseberense* and *Vibrio alginolyticus* from the tissues of the South African abalone, *H. midae*. Infected abalone were presumably affected by toxins produced by the anaerobic *Clostridium*. The foot of infected abalone was swollen, and the digestive gland was often distended (Dixon et al., 1991). The abalone were successfully treated with ozone and/or sulphadimidine.

Cultures of the Japanese disk abalone recently experienced outbreaks of a putative virus that caused severe atrophy of the foot (Nakatsugawa et al., 1988; Harada et al., 1993). The virus was associated with tumor-like lesions in the nerves and muscles of afflicted abalone. Viral particles were observed in infiltrating hemocytes which presumably spread the disease to surrounding

tissues. The disease was successfully transmitted via exposure to homogenized tissue filtrate (220 nm) and to contact with contaminated water (Nakatsugawa, 1990). Tumor-like lesions have not been observed in black abalone afflicted with withering syndrome. Harada et al. (1993) speculate that due to the nature of the infection, some abalone may have an unusual lysosomal disease similar to that reported from mammals (Glew et al., 1985).

A recently described coccidian parasite of black abalone, *Pseudoklossia haliotis*, was thought to be related to the withering syndrome (VanBlaricom et al., 1993), but subsequent studies discounted its pathogenicity (Friedman et al., 1993; Friedman et al., 1995). The parasite is transmitted via the water column and resides primarily in the left kidney of infected abalone. While there is focal cellular destruction arising from intracellular infection, there is no apparent host response. There is no correlation between the withering syndrome and infection by the coccidian (Friedman et al., 1995). Heavily infected individuals are found in populations of black abalone from Año Nuevo a location that is not affected by withering syndrome, and the coccidian can be transmitted to other species of abalone that are outside the range of withering syndrome, with no apparent signs of disease (Friedman et al., 1993).

Other agents cause observable pathological signs in infected abalone. The protozoan *Perkinsus olseni* forms pustules in the tissue of the Australian blacklip and greenlip abalone (*H. rubra*, and *H. laevigata*) (Lester and Davis, 1981; Lester, 1986; O'Donoghue et al., 1991). Infections with *P. olseni* typically result in mortality at temperatures above 20º C, but infected animals held at 15º C may survive the infection (Lester and Davis, 1981). Indeed, the disease is transmitted during the

summer months when water temperatures in South Australia are above 20º C (Lester et al., 1990). Several potential reservoir hosts have been identified including the noncommercial *H. cyclobates* (Lester et al., 1990; Goggin et al., 1989). The disease is of concern to the Australian abalone fishery as the large yellowish pustules cause rejection of the meat, and circumstantial evidence suggests that the disease has caused significant mortalities to local populations of *H. laevigata* (Lewis et al., 1987; O'Donoghue et al., 1991). An undescribed haplosporidan reportedly caused pustules in the connective tissues of the European ormer by Crofts (1929). The low prevalence of the disease (1/400) made it difficult to study further.

An epizootic of *Labyrinthuloides haliotidis* caused mortalities to cultures of juvenile pinto and red abalone (*H. kamtschatkana* and *H. rufescens*) (Bower, 1987; Bower, 1987). The parasite grows in the nerves and muscles of the foot and head of infected abalone where it multiplies rapidly via binary fission. Tissues of the abalone swell with the infection and there is a noticeable loss of integrity (Bower, 1987). The disease is fatal to juvenile abalone (<2mm shell length) but adults are resistant to infection. Transmission is effected via zoospores that develop within 2-3 days of infection (Bower et al., 1989).

The nematode *Echinocephalus pseudouncinatus* causes blisters on the foot of infected abalone (Milleman, 1951). The nematode was found in "old" pink and green abalone (*H. corrugata*, and *H. fulgens*) where it burrowed through the foot musculature. Infection by the nematode resulted in a weakened foot such that commercial divers were able to distinguish between healthy and infected abalone in the field. Definitive hosts for the nematode include the horn shark,

Heterodontus francisci, and the bat ray, *Myliobatis californicus (Milleman, 1963)*. Blisters can also occur when abalone are forcibly removed from the substrate (Shields, pers. obs.). Pycnogonid sea spiders can cause localized swellings on the foot and epipodia of infested abalone (Yamaguti, 1936; Shields pers. comm). While it is interesting to speculate that parasitic copepods or opportunistic micropredators like pycnogonids may vector rickettsial agents, there is evidence that marine rickettsiae are transmitted from water-borne contact (Gulka and Chang, 1985; Le Gall et al., 1991), i.e., transmission does not require an arthropod vector.

Other pests occur as ectoparasites on abalone. The fungus *Pharcidia balani* lives on the calcareous shells of barnacles and molluscs where it forms associations with algae much like a lichen (Kohlmeyer and Kohlmeyer, 1979). Shell deformations have occasionally been noted in cultured juvenile abalone and may be the result of infections with the fungus (Friedman, and Shields, pers. obs.). The organism is considered a saprobe and is probably not pathogenic. An undescribed fungus has recently been observed on abalone shells from New Zealand (Friedman, pers. comm). It penetrates the shell and in severe cases causes severe blistering of the shell at the interface with the foot. Increased nacrezation and some melanization of the surrounding foot gives the shell a blackened appearance, and the foot may be scarred by a heavy infection.

Radical shell deformations have been reported in cultured juvenile abalone from California. The culprit is a sabellid polychaete that infests the leading edge of shell growth (Oakes and Fields, 1996). The tubes of the worm interfere with growth. The worm undergoes rapid direct development thus causing low infestations to blossom over the shell. Infested abalone exhibit

poor shell growth or aberrant shell growth, and can become stunted from the infection and susceptible to mortality from the increased stress of the crowded growing conditions. The problem has thus far only been reported in cultured stocks. In contrast, other shell dwellers like the boring sponges, *Cliona* spp., and another polychaete, *Polydora* spp., cause little damage to abalone (Clavier, 1989).

While abalone host a diverse fouling community of shell inhabitants (e.g., Clavier, 1989, and others), only the pyramidellid snails appear to feed on their hosts' tissues. Pyramidellid snails are known ectoparasites of molluscs with several species reported from chitons, limpets, and abalone (see Dall and Bartsch, 1909; Fretter and Graham, 1949). The pyramidellids that occur on abalone were once thought to prey on the abundant microfauna present on the shell (Robertson and Orr, 1961), but more recent evidence suggest that the snails are feeding directly on abalone tissues (Thomas and Day, 1995). While the micropredatory snails are potential vectors for diseases (White et al., 1987), their role in the transmission of withering syndrome is doubtful (Friedman, unpubl. data).

Few physical and chemical factors have been implicated in abalone diseases (Table 1). A mass mortality of the donkey ear abalone, *H. asinina*, was presumably related to sedimentation following a severe storm event (Sungthong et al., 1993). Mortalities of red and black abalone adjacent to a discharge area from a power plant were caused by the flushing of the cooling system (Martin and Stephenson, 1977). The leaching of copper from copper-nickel tubing was shown to be the culprit in the die off. The histopathology of oxygen poisoning was experimentally

examined in juvenile red abalone (Elston, 1983), but there is little indication that oxygen toxicity is a problem in most aquaculture settings. Lastly, a stunted condition was reported for the Australian blacklip abalone, *H. rubra* (Shepherd and Branden, 1991), but little was reported about the condition.

Several studies have reported estimates of natural mortality from tagging experiments in the field. While not reviewed here, most of the studies have focused on the ecological and fisheries implications of mortality (i.e., estimates of instantaneous mortality) as opposed to pathological effects, or estimates of disease mortality.

Task 1. Primary Objective: Potential agents

The results of the investigation for pathogens are found in Table 2. A manuscript has also been prepared for publication. See Attachment A.

Task 2. Secondary Objective: Pathology of syndrome

The results of our pathological study are included in Attachments A and B.

Task 3. Secondary Objective: Mode of Transmission

We undertook a series of experiments to designed to transmit withering syndrome in the laboratory. Prior to the transmission experiments, we did a sequential analysis of hemolymph changes over time by sampling abalone hemolymph every day for up to 3 months. The results

pointed to episodic changes in relative hemocyte abundances with the onset of the disease (see Attachment A).

Experiment #1 - Blood borne transfer

In this experiment we injected 100 ul of blood from several withered abalone into the foot of several healthy red abalone. Red abalone from a nearby culture facility were used so as to insure a large sample size, and an uninfected status. The experiment failed due to a collapse in the water flow in the system which resulted in most of the abalone dying from poor water quality.

Experiment #2 - Injection/transfection - needle transfer

The objective was to determine if withering syndrome could be transmitted to healthy red and black abalone via needle injection (i.e., is the disease tranmissible?). Abalone in Treatment A were injected with filter-sterilized Ringer's solution. Abalone in Treatment B were injected with 0.1 cc of tissue homogenate (from two symptomatic abalone) passed through a 0.2 um filter. Abalone in Treatment C were injected with 0.1 cc of the tissue homogenate passed through a 0.45 um filter, and those in Treatment D were injected with 0.1 cc of 5.0 um filtrate. Abalone in Treatment E were injected with whole tissue homogenate. Homogenate was passed through cheesecloth prior to the injections. Each treatment consisted of 25 juvenile (2.0 inch) red abalone. Treatment A controlled for the injection and handling process. Treatment B screened for viruses; Treatment C for viruses, rickettsia, and small bacteria; Treatment D for larger bacteria and small protozoans; and Treatment E screened for transfer among all the potential infectious groups. We

used cultured red abalone (50-60 mm shell length) in the experiments due to the difficulty in obtaining large sample sizes of healthy black abalone.

The experiment was unsuccessful. A laboratory mishap resulted in the loss of 25 abalone approximately 1.5 months after the experiment was started. While the experiment failed due to a design flaw and culture conditions, it is interesting to note that the majority of the mortalities occurred in abalone given the 0.45 um filtrate. This is the size range of the RLOs that are responsible for the disease. Regardless, maintenance issues clouded the results. Maintenance of aquaria was a primary issue for most of the work done at UCSB. While abalone are relatively easy to culture, they require regular monitoring and maintenance. Since the principal investigator was stationed in Virginia, it made management and oversight of the culture issues problematic. Other experiments were carried out at the Bodega Marine Laboratory (see below).

Experiment #3: Cohabitation experiment

After many detailed discussions of withering syndrome, Dr. Carolyn Friedman kindly invited us to assist in ongoing transmission experiments in her laboratory. The cohabitation experiment was designed to assess disease transmission and progression in the laboratory. The experiment placed 12 healthy, unexposed animals from Año Nuevo in aquaria with equal numbers of healthy, asymptomatic animals from Vandenburg Air Force Base or Cayucos Beach. Controls consisted of aquaria housing healthy, unexposed abalone (negative controls) separate from healthy, asymptomatic abalone (positive controls). Asymptomatic animals that became withered and died during the experiment were replaced. Additional animals were held in separate aquaria. The

experiment had two replicates of each treatment. Hemolymph was drawn at 40 to 50 day intervals for up to 330 days after the start of the experiment (Attachment A). Abalone were fed kelp *ad libitum*. Ambient water and water temperatures off Bodega Marine Laboratory were used. The water in which abalone were held was chlorinated prior to release.

As abalone from the cohabitation experiment became moribund, they were removed from the experiment and processed for histology. The results indicate that the rickettsia-like organism (RLO) was transmitted to healthy, unexposed abalone in cohabitation with asymptomatic (and symptomatic with the progression of the experiment) abalone (Table 3). Transmission was via either contact, contamination with fecal materials, or via water-borne spread of the disease. Abalone in the negative controls were not infected by the RLO, while those in the experimental exposure and in the positive controls were infected. A manuscript is currently being prepared to report on these findings.

FINAL EVALUATION

The goals of the project were obtained. We have confirmed that the RLO is the putative agent responsible for withering syndrome. Histological studies and the cohabitation experiment support Gardner et al.'s (1995) contention that the RLO is the primary agent for the disease syndrome. We have also contributed to the hematology of the abalone. As far as we're aware, there have been no prior studies on the hemocytes of abalone. We have documented several important features of the hematology and its relation to the disease process. For conclusions with respect to the hematological pathology see the Discussions in Attachments A and B.

One significant problem came up. Transmission experiments with abalone are not simple undertakings. In the cohabitation experiment, previously uninfected abalone didn't show signs of the disease until over 180 days post exposure. The length of time involved and maintenance issues of holding abalone for such periods were partially responsible for the tardiness of the completion of this grant. The issues have, however, been resolved.

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Table 1. Diseases, conditions, and parasites reported for abalone, *Haliotis* spp.

DISEASE AGENTS

Host species	Agent	Reference
H. (Nordotis) discus	withering syndrome in culture	Nakatsugawa et al., 1988
	virus/tumor-like lesions	Harada et al., 1993
H. cracherodii	withering syndrome	Haaker et al., 1992
	rickettsia-like organism	Gardner et al., 1995
		Steinbeck et al., 1992
H. rufescens	*Vibrio alginolyticus*	Elston & Lockwood, 1983
	Vibriosis	Ebert & Houk, 1989
H. midae	*Clostridium lituseberense*	Dixon et al., 1991
H. rufescens	cf. *Pharcidia balani*	Kohlmyer and Kohlmeyer, 1981
H. iris	boring fungus in shell	Friedman, pers. obs.
H. kamtschatkana	*Labyrinthuloides haliotidis*	Bower, 1987a; 1987b
H. rufescens		
H. tuberculata	Haplosporidia cysts?	Crofts, 1929
H. rubra	*Perkinsus olseni*	Lester & Davis, 1981
H. laevigata		O'Donoghue et al., 1991
H. cyclobates		Goggin et al. 1989
H. cracherodii	*Pseudoklossia haliotis*	VanBlaricom et al., 1993
		Friedman et al., 1995
H. tuberculata	larval trematode	Crofts, 1929
H. corrugata	*Echinocephalus pseudouncinatus*	Millemann, 1951, 1963
H. fulgens		
H. cracherodii		Kuris, pers. comm.
H. gigantea	*Panaietis haliotis*	Yamaguti, 1936
H. cracherodii	*Achelia chelata*	Kuris, unpubl. data
		Shields, pers. obs.
Haliotis spp.	Pyramidellid snails	Robert and Orr, 1961
H rufescens	Sabellid worm	Oakes and Fields, 1996
H. rufescens	absence of respiratory aperture	Leighton, 1960
Haliotis spp.	copper toxicity	Martin et al., 1977
H. rufescens	gas-bubble disease	Elston, 1983

Table 2. Diseases and conditions observed in the present study.

Parasites/Observed Conditions

Parasite	Location	Association with disease?
Bacteria	Hemolymph	ns, secondary infections?
Rickettsia	Intestinal villi	implicated in syndrome
Ciliates	Intestinal lumen	ns
Ciliates	gills	ns
Gregarine	Various tissues	ns
Pseudoklossia haliotidis	Kidneys	ns
intracelluar sporozoites	Hemolymph	ns, probably *Pseudoklossia*
Cytoplasmic inclusions	Hemolymph	P<0.01 (phagocytosis)
Knobby cells	Hemolymph	P<0.025 (apoptosis)

Other observed symbionts

cf. *Pharcidia balani* (fungus)	Shell	aquaculture pest
Sabellid polychaete	Shell	aquaculture pest
Odostomia spp. (snails)	Shell	Possible vectors but probably feeding only on epibionts
*Pycnogonids (*Achelia* sp.)	Mantle	Possible vector, but uncommon in enzootic locations

Table 3. Transmission of withering syndrome to unexposed, healthy abalone in the Cohabitation Experiment (Friedman, et al., unpubl. data).

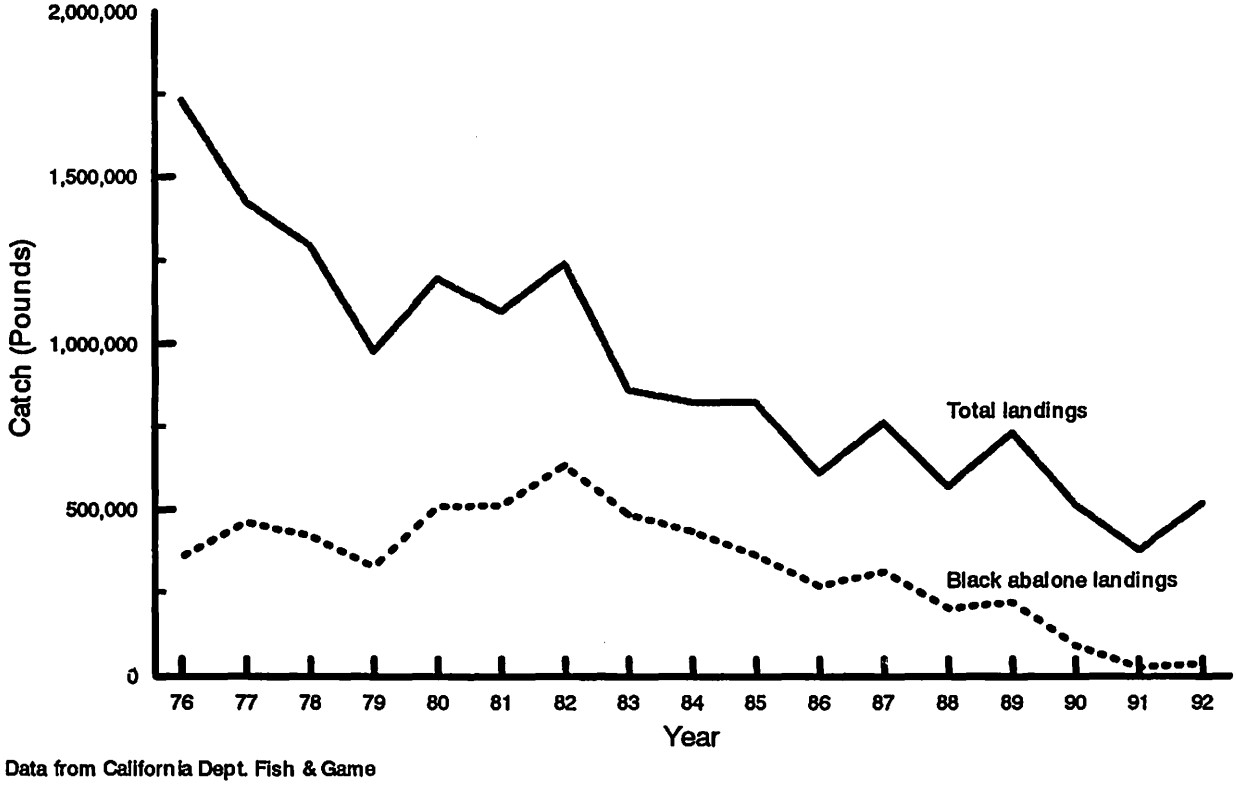
		Status at Termination of Experiment			
Status at Start	Treatment	Healthy	Very Slightly Shrunken[1]	Slightly Shrunken	Shrunken
Healthy, exposed	Experimental exposure	4	3	7	10
Asymptomatic	Experimental exposure	0	0	5	22
Asymptomatic	Positive control	0	0	3	20[2]
Healthy, unexposed	Negative control	17	4	3[3]	0

[1] This stage is frequently difficult to distinguish from the Healthy stage.

[2] One abalone died prior to the onset of the gross signs of infection.

[3] Some deterioration in condition was noted due to the experimental effects of the lengthy holding time in crowded aquaria. These abalone were not infected by the rickettsial agent.

Figure 1. Landings of abalone in California.



Data from California Dept. Fish & Game
Marine Resources Division, Statistical Data
Peter Haaker, Nearshore Invertebrate Project

ATTACHMENT A

For: Diseases of Aquatic Organisms
March 25, 1997

THE HEMATOLOGY OF BLACK ABALONE, *HALIOTIS CRACHERODII*, AFFLICTED WITH WITHERING SYNDROME

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Abstract: The hematology of black abalone, *Haliotis cracherodii,* afflicted with withering syndrome. Diseases of Aquatic Organisms ##: ###-###. Black abalone in southern California are afflicted with chronic intestinal infections of rickettsia-like organisms that cause a debilitating and fatal withering syndrome. The hematology of withered animals indicated that cellular degradation and apoptosis occurred in tandem with the decline and catabolism of abalone tissues. Two types of hyalinocytes were found in the hemolymph. Densities of both types of hyalinocytes declined in abundance, and small, presumptive stem cells increase in abundance with the progression of the disease. No circulating granulocytes were present in hemolymph; serous cells were present as fixed granulocytes in hemal spaces. Cellular inclusions, dying cells, and vacuolate cells increased in abundance with the disease. Hemocyte densities were highly variable through time, but hemocyte densities decreased and the proportion of dead cells increased with disease progression. The withered condition represents an end stage of the disease and probably results from starvation caused by rickettsial disruption of the digestive processes.

Introduction

Withering syndrome is a recently described condition of the black abalone, *Haliotis cracherodii.* The syndrome is a debilitating and fatal disease caused by a presumptive rickettsia-like organism (RLO) (Gardner et al., 1995). The pathology of the disease fulminates as rickettsial bodies occur in chronic infections of the columnar epithelial cells of the intestine, with episodic infections of the epithelial cells of the digestive gland. Externally, the withering syndrome is characterized by a small body mass to shell size ratio (i.e., an atrophied foot), abnormal coloration and flaccid tissue mass (Haaker et al., 1992). Atrophy, shrinking, or withering of the foot is marked by reduction in the overall facies of the muscular tissue, and reduction in body mass. The wasted and weakened foot is an end-product of the disease; abalone presumably starve to death from malabsorption caused by the RLO.

The disease syndrome was first noticed in 1985 by commercial abalone divers off of Santa Cruz Island. It has since spread to all of the Channel Islands, California (Lafferty and Kuris, 1993; VanBlaricom et al., 1993), Vandenberg Airforce Base in Central California, and north to Cayucos Beach (present study). Prior to the epizootic, the black abalone was a major component of the abalone fishery in California, comprising 32 to 51% of the total landings (Oliphant et al., 1990). With the spread of the disease, the black abalone fishery has collapsed in Southern California (Haaker et al., 1992; Steinbeck et al., 1992), and is now closed to commercial harvesting. Other species of abalone are occasionally found with the syndrome but it is markedly less prevalent in their populations. Nonetheless, fisheries for the pink and green abalone (*H. corrugata,* and *H. fulgens*) have recently closed primarily as a result of overfishing and potential losses from disease.

At the beginning of this study the causative agent was as yet undetermined. An epizootiological study had ruled out a variety of physical, chemical and ecological factors as agents of the syndrome (Lafferty and Kuris, 1993). The causative agent, a rickettsia-like organism, had been observed in the digestive tract of abalone (VanBlaricom et al., 1993), but since rickettsial organisms rarely cause pathology in their molluscan hosts (see Otto et al., 1979; Lauckner, 1983; Gulka and Chang, 1984), little attention was paid to the pathogen. We, therefore, undertook a parasitological examination of abalone hemolymph in an effort to (1) identify the potential causative agent, and (2) further characterize pathological changes in the hemolymph as a result of the syndrome.

Materials and Methods

Abalone were collected by hand (California Fish & Game personnel) with the aid of an abalone iron. They were transported to the laboratory in clean, disinfected (bleach or betadine) coolers packed with seaweed. Animals were collected from intertidal rocks at various locations on the Channel Islands (Santa Cruz, San Miguel, and San Nicolas Islands), Vandenberg Airforce Base (near Lompoc), Cayucos Beach, and Año Nuevo Island. The following data from captured abalones were recorded to examine possible host-disease relationships: sex (when possible), size, weight, general condition (shell damage, obvious disease status), location, and in most cases, shell and foot mass (volumetric displacement). Collections were, in most cases, biased toward sampling diseased animals or animals from endemic locations.

Data on the prevalence of withering, disease status, and other variables were analyzed using analysis of variance and *a posteriori* tests, the Kruskal-Wallis test, and the Chi-square test for homogeneity (Sokal and Rohlf, 1981). The status of each animal was rated as either Healthy-unexposed (abalone from Año Nuevo), Healthy-asymptomatic (from other locations), Very Slightly Shrunken (slight but noticeable tissue loss (<10%) from the foot and/or epipodium), Slightly Shrunken (general reduction in foot and epipodial mass (10-25%), withering quite evident, reduced ability to hold to substrate), Shrunken (withering of the foot to approximately 50% of the shell diameter, reduced or lost ability to hold to substrate). The healthy, unexposed category did not include animals from within the endemic region, several of which had subclinical infections.

Abalone were either held in 50 gallon aquaria for observations, or they were necropsied upon their arrival at the laboratory. Hemolymph was drawn from the pallial sinus with a sterile syringe (25-27 ga. needle). Two to three drops of hemolymph were placed on a microslide and allowed to sit for 5 min. before fixation in Davidson's fixative, Bouin's fixative, or 1G:4F fixative (50 ml glutaraldehyde, 300 ml formaldehyde, 2150 ml 0.2um-filtered seawater). In some cases, hemolymph was allowed to dry on the microslide before fixation. Hemolymph slides were then processed through the Harris hematoxylin and eosin staining procedure (Humason, 1979, p. 120). Abalone tissues were fixed as above, dehydrated through an alcohol series, cut with a microtome, and the sections processed and stained with Harris hematoxylin and eosin as above.

A hemocytometer (Improved Neubauer) was used to estimate cell density in freshly drawn hemolymph. For each animal, two chambers were loaded, and the number of cells enumerated on each of 5 large grids (10 grids total). The statistics were performed on the untransformed data. (To transform the counts into cells/ml multiply by $5x10^4$.) For vital staining, 0.25% trypan blue in 0.2 um filter-sterilized phosphate-buffered saline was mixed 1:1 with fresh hemolymph. Live and dead hemocytes were counted either directly on the smear, or with a hemocytometer. In prepared smears, the relative abundances of cell types were subjectively categorized on a scale of 0 to 3. For Type I hyalinocytes, small hemocytes (stem cells), cells with inclusions, etc., the scale was approximately: 0, none present; 1, 1-3 present in 3 fields at 1000x; 2, 4-10 present in 3 fields; and 3, >10 present in 3 fields. For Type II hyalinocytes, the scale was approximately: 0, none present; 1, 1-10 present in 3 fields; 2, 11-100 present in 3 fields; and 3, >100 present in 3 fields. A double blind assessment of the subjective estimation was performed on a subset of 25 smears, with insignificant differences between readers (JDS vs. CSF).

For hemolymph, standard electron microscopy fixations did not give adequate results. A combination primary-secondary fixative was used instead that consisted of 2.5% glutaraldehyde, 2% paraformaldehyde, and 1% osmium tetroxide in a 0.1 M sodium cacodylate buffer (pH 7.4) with 0.2 M sodium chloride. Equal parts hemolymph and combination fixative were gently mixed in a microcentrifuge tube, placed on ice for 30 - 45 min., spun at 30 g for 2 min., rinsed in buffer, spun, rinsed in distilled water, spun, then dehydrated through an acetone series (10 min. per step), embedded in a series of Spurr's resin mixed with acetone prior to final embedding in the standard Spurr's formulation at 70 C overnight (or at least 12 hours). Abalone tissues were fixed on ice

for one hour in 2.5% glutaraldehyde, 2% paraformaldehyde, 0.1 M sodium cacodylate (pH 7.4), and 0.2 M sodium chloride. Tissues were then rinsed twice (10 min) in the sodium cacodylate - sodium chloride buffer, post-fixed for one hour in 1% osmium tetroxide in buffer then processed as above. Sections were cut with an ultramicrotome, stained with uranyl acetate, and lead citrate, and viewed with an electron microscope.

RESULTS

Hemocytometry

Hemocyte densities reflected the changes in the progression of the disease in abalone (Table 1). Healthy, unexposed abalone and healthy, asymptomatic abalone had significantly higher numbers of hemocytes than did animals showing signs of the disease (very slightly shrunken, slightly shrunken, and shrunken categories). Withered abalone (slightly shrunken and shrunken) possessed significantly fewer hemocytes; many individuals possessed less than 5 cells per grid ($<2.5 \times 10^5$ cells/ml of hemolymph) (Figure 1). With the progression of the disease, the distribution of the hemocyte population shifted to the left as the hemocytes declined in abundance (Figure 1).

Hemocyte counts were taken from abalone in a cohabitation experiment designed to assess disease transmission and progression in the laboratory. The experiment placed 12 healthy, unexposed animals from Año Nuevo in aquaria with equal numbers of healthy, asymptomatic animals from Vandenburg Air Force Base or Cayucos Beach. Controls consisted of aquaria housing healthy, unexposed abalone (negative controls) separate from healthy, asymptomatic

abalone (positive controls). Asymptomatic animals that became withered and died during the experiment were replaced. Additional animals were held in separate aquaria. The experiment had two replicates of each treatment. Hemolymph was drawn at 40 to 50 day intervals for up to 330 days after the start of the experiment. Abalone were fed kelp *ad libitum*. Ambient water and water temperatures off Bodega Marine Laboratory were used. The water in which abalone were held was chlorinated prior to release.

Hemocyte densities from animals in the cohabitation experiment were highly variable (Figure 2). Healthy, unexposed abalone exhibited large fluctations in their hemocyte densities with occasional peaks in abundance. Healthy abalone that cohabited with diseased animals exhibited significant declines in hemocyte populations only late in the time course of the exposure, after the onset of the infection (Figure 2B). Asymptomatic animals also exhibited dramatic declines in hemocyte densities in the late stages of the disease (Figure 2C). The peaks in abundance of hemocyte populations indicated that abalone experience occasional episodic increases in hemocyte densities regardless of disease status.

Relative changes in hemocyte populations

We identified three different subpopulations of free hemocytes. Small hemocytes had a small cytoplasm to nucleus ratio and were rarely found in healthy individuals (<2% of the cells). They were most likely stem cells, that would develop into hyalinocytes. The Type I hyalinocyte (Figure) was rounded with thin pseudopodia when present in hemolymph sinuses, but in hemolymph pellets they frequently possessed moderate to large pseudopodia. The Type I hyalinocyte had a

large irregular or binucleate-like nucleus, with the chromatin condensed irregularly around the inner circumference of the nucleus. The cytoplasm contained several small, electron-lucent granules, small, rounded mitochondria, copious amounts of smooth endoplasmic reticulum and vesicles, and vacuoles (phagosomes) filled with electron-lucent material or membranous, fibrillar components (Figure 4). Type I hyalinocytes were common in the hemolymph (approx. 20% of the cells).

Type II hyalinocytes were rounded, with small, thin pseudopodia when not in contact with a surface (Figures 3 and 5). Type II hyalinocytes had a large cytoplasm to nucleus ratio, a rounded nucleus (sometimes with a binucleate appearance), sausage-shaped mitochondria, large vacuoles (phagosomes), and a few small, electron-dense granules (Type 4 acidophillic granules of Auffret, 1988). Type II hyalinocytes had less apparent quantity of smooth endoplasmic reticulum, and vesicles in the cytoplasm than Type I hyalinocytes. Upon contact with a surface, Type II hyalinocytes would spread quickly on the surface. In slightly shrunken and shrunken abalone the Type II hyalinocytes spread out almost immediately upon contact with a microslide (Figure 3). Those from from healthy animals typically spread out after more than 5 minutes. Type II hyalinocytes comprised the greatest proportion of the cell types found in abalone hemolymph (>80% of the cells).

Serous cells (granulocytes) were not free in the hemolymph; they were present as fixed cells attached to the tissues lining the hemolymph sinuses (Figure 6). Theys were large, irregular cells with pseudopodial interdigitations with adjacent tissues. Serous cells possessed several large,

irregular, electron-dense granules in the cytoplasm (cf. Type 5 granules of Auffret, 1988). The granules were frequently acidophilic or stained dark brown when examined with the light microscope. No effort was made to quantify populations of serous cells in abalone tissues.

The relative abundances of the different hemocytes changed in relation to the progression of the disease (Figure 7). Both types of hyalinocytes exhibited marked declines in relative abundance in shrunken animals (Chi-square; Type II hyalinocyte, d.f. = 8, $P<0.001$; Type I hyalinocyte, d.f.=12, $P<0.001$). The presence and relative abundance of small hemocytes increased markedly with infection and indicated the degree of disease stress to the afflicted host (Figure 7). Healthy, unexposed abalone did not typically have (66.7%) or had only few small hemocytes (33.3% prevalence) in their hemolymph. Slightly shrunken and shrunken abalone often had significant populations of small hemocytes (65-69% prevalence). In studies with trypan blue (see below), a proportion of these lymphocytes were dead, or were nuclei that had escaped from ruptured hyalinocytes, but many were indeed viable cells.

The presence and abundance of apparently dying cells (knobby cells), vacuolate hyalinocytes (cells with large, electron-lucent vacuoles, Figure 4 and 8), and cellular inclusions (phagosomes, Figure 4 and 8) increased with the progression of the disease (Figure 7). Knobby cells were found in 38.8% of shrunken abalone compared with 11.0% in healthy, unexposed and healthy, asymptomatic hosts (Chi-square, df=12, $P<0.001$). Vacuolate hyalinocytes were uncommon (8.8%) in the healthy, unexposed abalone, but were quite common in the hemolymph of slightly shrunken, and shrunken animals (37.6, and 27.6%, respectively; Chi-square, d.f.=12, $P<0.01$).

Gross cellular inclusions, phagosomes that included engulfed nuclei, cytoplasmic material, etc., were found in 36.5-39% of slightly shrunken or shrunken abalone compared with 11.1% in healthy, unexposed abalone (Chi square, df=12, $P<0.05$). In addition, a strong positive relationship was found between the presence of knobby cells and the presence of cellular inclusions. The relationship was most noticeable among diseased animals (very slightly shrunken to shrunken) (Chi-square, d.f. =4, $P<0.01$).

Living vs. dead hemocytes

Dead and dying hemocytes were common in diseased abalone (Figure 10, Table 1). Healthy, unexposed abalone had a mean of 1.0% dead cells in their blood compared to a mean of 5.9% in slightly shrunken abalone, and 15.6% in the blood of wasted/shrunken abalone. Statistical analyses were hindered by the high level of heteroscedasticity in the data (Bartlett's Chi-square, $P<0.001$), but the trend was clear (Kruskal-Wallis, $P<0.001$); diseased abalone possessed significantly more dead hemocytes in their hemolymph than did healthy animals. Note that several of the healthy, asymptomatic abalone were experiencing hemolysis (Figure 10). These animals had presumptive subclinical infections.

Other parasites

Other parasites were occasionally noted in abalone hemolymph and tissues. The presence of coccidian sporozoites, and bacteria in the hemolymph were not significantly associated with disease status (Chi-square, $P>0.10$). Neither were found at high enough prevalences or intensities to warrant further treatment. Coccidian parasites (*Pseudoklossia haliotidis*) and gregarine cysts

were noted in the renal tissues, and other tissues, respectively, but they evoked a minimal pathology. Ciliates were observed on the gills and within the lumen of the intestine; no pathology was associated with their presence.

Discussion

The overall findings are consistent with a change in cell populations due to apoptosis resulting from the general starvation of the host. The following scenario is thought to occur. Abalone withering syndrome is caused by a chronic, fulminating rickettsial infection of the digestive gland and intestinal tract (Gardner et al., 1995). Healthy abalone acquire the infections and maintain moderate to high levels of rickettsial foci in their digestive tracts (intestine and tubules leading from digestive glands). Starvation of the afflicted abalone apparently leads to the depletion of the glycogen and lipid reserves in the digestive gland within 30 days (Takami et al., 1995), and may contribute to the malabsorption of the intestinal tract with the further degradation and catabolism of the animal's nutrient resources. The hematology supports the chronic decline in the general health of infected abalone. Episodes of cellular apoptosis and tissue degradation, coupled with normal phagocytosis by the circulating hemocytes appear in concert with the decline and withering of the diseased abalone.

Circulating hemocytes in molluscs show varied responses to different infectious agents. *Biomphalaria glabrata* infected with *Schistosoma mansoni* exhibit a significant, two-fold increase in the density of granulocytes but not in hyalinocytes (Stumpf and Gilbertson, 1980). *Lymnaea stagnalis* infected with *Trichobilharzia ocellata* experience increases in hemocyte density in

relation to the development of the daughter sporocysts and release of cercariae (Amen et al., 1991). Susceptible oysters (*Crassostrea virginica*) infected with *Haplosporidium nelsoni* show decreasing densities of hemocytes in early and moderate infections, while resistant oysters evoke increasing densities in relation to the intensity of infection (Ford et al., 1993). Oysters infected with *Perkinsus marinus* showed significant increases in hemocyte density in relation to the intensity of the infection (Anderson et al., 1995), but controlled experiments found little relationship in hemocyte densities, or the percentage of granulocytes between infected and uninfected oysters (Chu et al., 1993).

Declines in hemocyte densities have been reported for *Ruditapes phillipinarum* exposed to the infectious *Vibrio* sp. that causes Brown Ring Disease (Oubella et al., 1994). In exposed clams the hemocytes increased in density over two weeks post exposure, then declined with the onset of the syndrome. Hemocyte populations in abalone, *H. cracherodii*, infected with the wasting syndrome experience general declines with episodic increases presumbably resulting from bouts of catabolic degradation.

The hematology of black abalone is distinctly different from that of other well studied molluscan systems. Abalone lack circulating granulocytes. Functional granulocytes (serous cells) were affixed to tissues or were only rarely found in hemolymph smears. In contrast, granulocytes comprise the majority cell type found in pulmonate gastropods (Cheng, 1975; Yssel and Wolmarans, 1989; Shozawa and Suto, 1990), oysters (Cheng, 1975; Ruddell, 1971; Feng et al., 1971; Auffret, 1988, Auffret, 1989), venerids (Cheng, 1975; Auffret, 1988), and mytilids (Moore

and Lowe, 1977; Rasmussen et al., 1985). Pectinids, like the abalone, do not possess free granulocytes (Auffret, 1988, Auffret, 1989), but the patellids, which are closely related to abalone do have circulating granulocytes (Davies and Partridge, 1972). Granulocytes are known to function in wound repair via clumping (Foley and Cheng, 1975). Even though abalone hemocytes can form clumps, it is interesting to speculate that the lack of granulocytes may be related to the lack of an effective clotting mechanism in abalone.

Most molluscan hemocytes appear capable of phagocytosis. In abalone, both Type I and II hyalinocytes phagocytize foreign particles (Friedman and Shields, unpubl. data). In oysters, phagocytosis occurs in hyalinocytes and granulocytes, but it occurs with far more frequency in granulocytes (Foley and Cheng, 1975; Renwrantz et al., 1979). The increased prevalence of cellular inclusions, and small, presumptive stem cells in withered abalone indicates that the cellular immune response is functioning at a stressed level, and may compromise animals to bacterial infections that are occasionally observed in shrunken abalone.

Infections of rickettsia-like organisms (RLOs) are generally benign in molluscs. Relatively harmless RLOs infect the gill epithelia of the Pacific oyster, *Crassostrea gigas* (Renault and Cochennec, 1994), the gill epithelia and plicate membrane of the edible mussel, *Mytilus edulis* (Gulka and Chang, 1984), the gill and digestive gland of the hard clam, *Meretrix lusoria* (Chiou-Ming et al., 1994), gill epithelia of the scallop, *Placopecten magellanicus* (Gulka and Chang, 1985), and many other bivalves (e.g., Lauckner, 1983, pp. 503-510). Most RLOs in bivalves

elicit little, if any, host response, but the granulocytes of the mussel, *M. edulis*, are capable of encapsulating infected cells (Gulka and Chang, 1984).

Nonetheless, there are several reports of serious rickettsial pathogens in bivalves. Pathogenic RLOs have been reported in the tellin, *Tellina tenuis* (Buchanan, 1978, Buchanan, 1979), and more recently, the scallop, *Pecten maximus* (Le Gall et al., 1988, Le Gall et al., 1991), and several giant clams, *Hippopus hippopus* (Norton et al., 1993a), *Tridacna crocea* Goggin and Lester, 1990), and *T. gigas* (Norton et al., 1993b). The RLO from the tellin infects the secretory cells of the digestive gland epithelia and presumably alters digestive functions similarly to that infecting the black abalone.

The withered condition is an end stage of the syndrome. It has also been reported with other abalone diseases, hence it is not specific to any single disease agent. Two infectious pathogens have been observed to cause a withering-like syndrome, *Vibrio* spp., and an unidentifed trematode (Crofts, 1929; Elston and Lockwood, 1983); and a withered condition has been reported for starving pink and black abalone (Cox, 1962). A putative viral infection of the nervous tissue can also result in withering (Nakatsugawa et al., 1990; Harada et al., 1993). Elston & Lockwood (1983) described the pathology of *Vibrio* spp. infections in juvenile red abalone, *H. rufescens*. In general, the diseased abalone were less active and had a reduced ability to remain attached to substrates. Infections began primarily in the foot and the epipodia with the exfoliation of the epithelial tissues. Subsequently, the infections spread to the vascular sinuses and the nerves of the hosts (Elston & Lockwood, 1983). Bacteria were observed in all of the affected tissues. In contrast, *Clostridium lituseberense* and *Vibrio alginolyticus* elicit a swelling of the foot in the

South African abalone, *H. midae* (Dixon et al., 1991). Infected abalone were presumably affected by toxins produced by the anaerobic *Clostridium*. None of the other reported disease agents of abalone produce a withering syndrome.

Acknowledgements

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Table 1. Mean counts of hemocytes and proportion of dead cells in hemolymph in relation to the disease status of the host. (N = sample size; for hemocytes, the mean represents the mean number of cells per large grid per chamber; multiply by 5x104 to convert to cells/ml). Values with different letters are significantly different among means (hemocyte counts = a, b, c; proportion dead = y, z). (For hemocyte counts, ANOVA, $P < 0.05$, d.f.= 4, 405, Tukey's HSD; for proportion dead cells, ANOVA, $P<0.001$, d.f. = 4, 85, Tukey's HSD; and Kruskal-Wallis, $P<0.001$).

		Hemocytes		% Dead cells
Status	N	Mean + se	N	Mean + se
Healthy, unexposed	43	59.6 + 5.2 a	21	1.0 + 0.4 y
Healthy, asymptomatic	135	48.1 + 2.5 ab	23	2.6 + 1.0 y
Very slightly shrunken	60	41.8 + 3.6 b	13	2.7 + 1.0 y
Slightly shrunken	87	17.4 + 1.7 c	22	5.9 + 2.5 y
Shrunken	85	13.8 + 1.7 c	11	15.6 + 4.6 z

Figure 1. Frequency distribution of hemocyte populations in relation to withering syndrome: (A) healthy, unexposed abalone from Año Nuevo Island; (B) healthy, asymptomatic abalone from various endemic locations; (C) abalone experiencing onset of the withering condition (very slightly shrunken); (D) abalone with noticeable loss of muscle mass (slightly shrunken); and (E) severely withered abalone (N = 45 healthy unexposed, 100 healthy asymptomatic, 60 very slightly shrunken, 87 slightly shrunken, and 85 shrunken abalone, respectively

Figure 2. Hemocyte counts from abalone held in a cohabitation experiment: (A) healthy, unexposed abalone (negative controls); (B) healthy, unexposed abalone experimentally exposed to disease via cohabitation; (C) healthy, asymptomatic abalone from endemic locations (Vandenburg Air Force Base, and Cayucos Beach, CA). To reduce clutter, only 5 animals are shown in each treatment. Note that all of the abalone in the experimental exposures had acquired the condition by day 250.

Figure 3. Light micrographs of various cell types (bars = 10 um). (A) Type II hyalinocyte with large inclusions (arrow). (B) Hemocyte with an intracellular sporozoite presumably of Pseudoklossia haliotis (arrow). Rounded cells are Type I hyalinocytes. (C) Note the different sizes between Type II hyalinocyte (H), and Type I hyalinocytes (arrow). (D) Type II hyalinocytes spread out on the microslide. Harris hematoxylin and eosin.

Figure 4. Electron micrographs of Type I Hyalinocytes. (A) Hyalinocyte with mostly circular mitochondria, several large vacuoles, and quantity of small vesicles. N=nucleus, bar = 1 um. (B) Hyalinocyte with binucleate appearance. Note the electron-lucent vacuoles (star), and the quantity of vesicles and smooth endoplasmic reticulum (arrows). N =

nucleus, P=pseudopod, bar = 1 um. (C) Hyalinocyte with a large phagosome (arrow). N=nucleus, bar = 1 um. (D) Vacuolate hyalinocyte possessing numerous large electron-lucent vacuoles (V).

Figure 5. Electron micrographs of Type II Hyalinocytes. (A) Hyalinocyte with copius quantities of vesicles and smooth endoplasmic reticulum (small arrows). Note the small, electron dense vacuole (large arrow). N = nucleus, bar = 1 um. (B) Hyalinocyte with binucleate appearance. Note the presence of several electron dense vacuoles (arrows), sausage-shaped mitochondria (star), and enlarged pseudopod (P). Bar = 1 um. (C) Hyalinocytes with several phagosomes (star). N = nucleus, bar = 2 um. (D) Hyalinocyte with several membrane-filled phagosomes (star), and an electron-dense granule (arrow). N = nucleus, bar = 1 um.

Figure 6. Electron micrographs of serous cells (fixed granulocytes). (A) Serous cell from the hemolymph sinus of the heart. Note the several large, electron dense, irregular granules (star) and the interdigitation (arrows) with the adjacent tissue. Bar = 2 um. (B) Detail of the irregular granules (G). N = nucleus, bar = 0.5 um. (C) Serous cell embedded in the basal lining of the digestive tract. Note the peripheral location of the nucleus (arrow) and the numerous electron-dense, irregular granules (G). Bar = 3 um. (D) Serous cell with large electron-dense granules (arrows) from the hemolymph sinus of the heart. Bar = 1 um.

Figure 7. Hemogram frequencies of typical cell types in relation to disease. Significant shifts in cell subpopulations were noted for each cell type (Chi-square, d.f. = 8 or 12, $P<0.01$; N = 45 healthy unexposed, 100 healthy asymptomatic, 55 very slightly shrunken, 67 slightly shrunken, and 59 shrunken abalone, respectively).

Figure 8. Light micrographs of unusual cell types (bars == 10 um). (A) Type I hyalinocyte with nuclear inclusions (arrow). (B) Knobby cell (arrow) that is presumably undergoing cell death. (C) Hyalinocyte with large basophilic inclusions (arrow). (D) Hyalinocyte with large basophilic inclusion. Note the nucleus pushed to the side of the cell. Harris hematoxylin and eosin.

Figure 9. Frequencies of unusual cell types in relation to disease. Significant increases in each cell type were noted for each subpopulation (Chi-square, d.f. =12, $P<0.01$; see Figure 7 for sample sizes).

Figure 10. Frequency of dead cells (stained with trypan blue) from hemocyte counts in relation to disease. Note the shift in frequency of dead cells in shrunken abalone. For sample sizes, see Figure 7.

Figure 1.

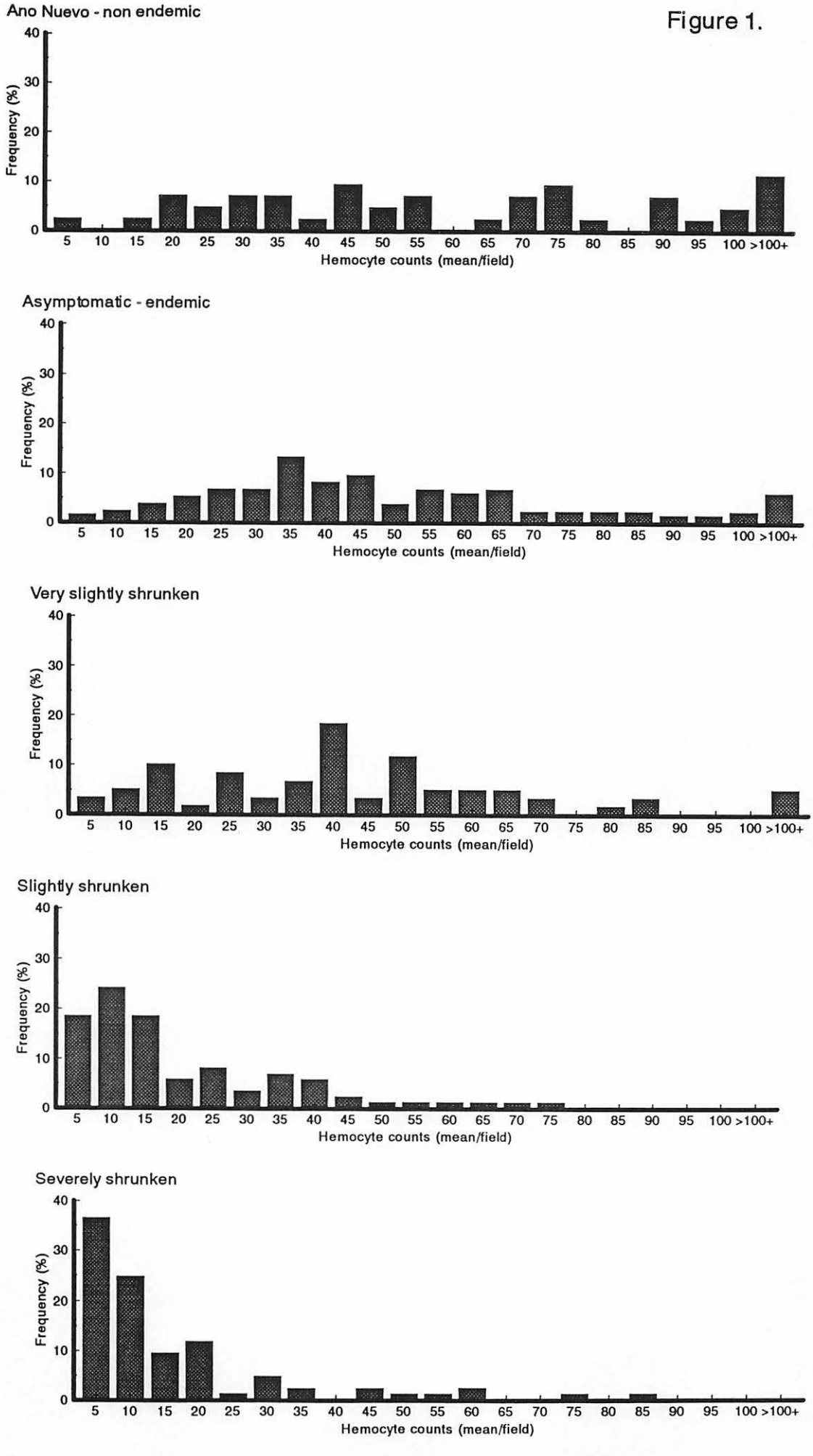
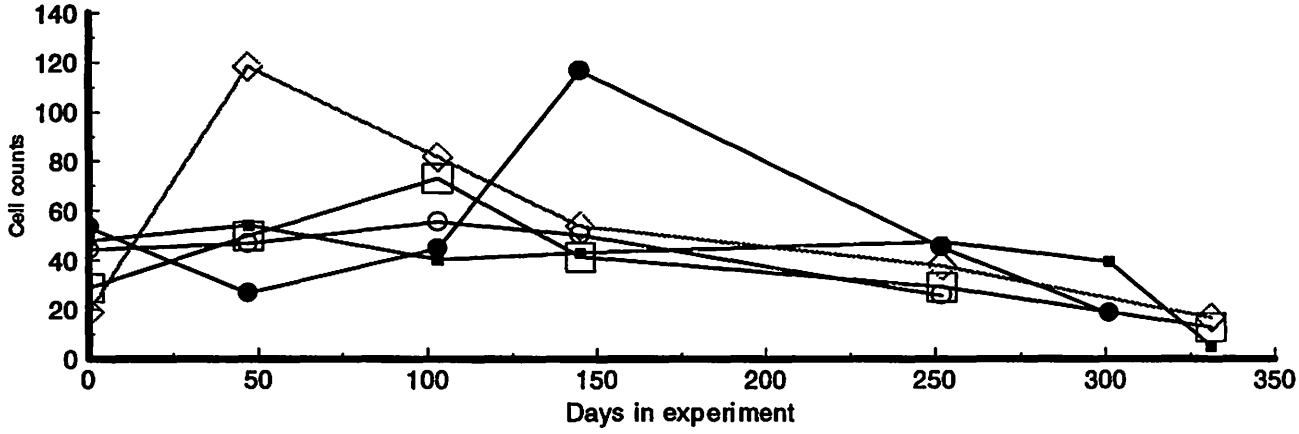
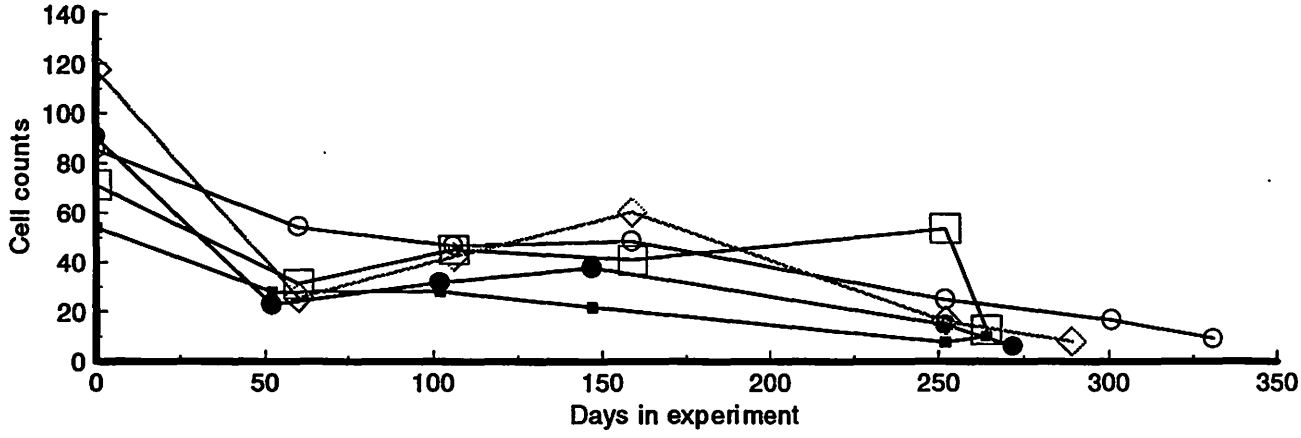

Ano Nuevo - non endemic
Asymptomatic - endemic
Very slightly shrunken
Slightly shrunken
Severely shrunken
Frequency (%)
0
10
20
30
40
5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 >100+
Hemocyte counts (mean/field)

Figure 2.

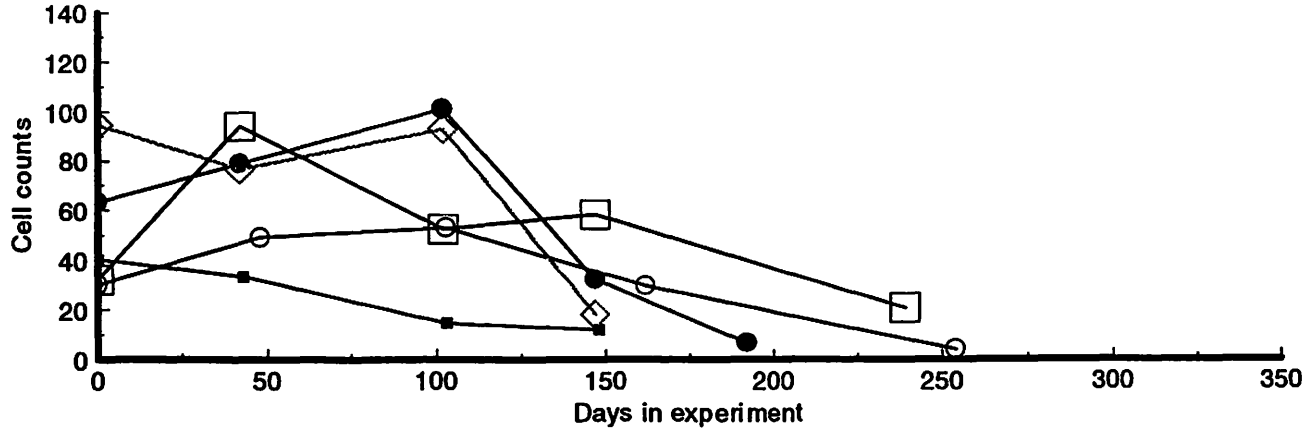
Healthy, unexposed
From Ano Nuevo

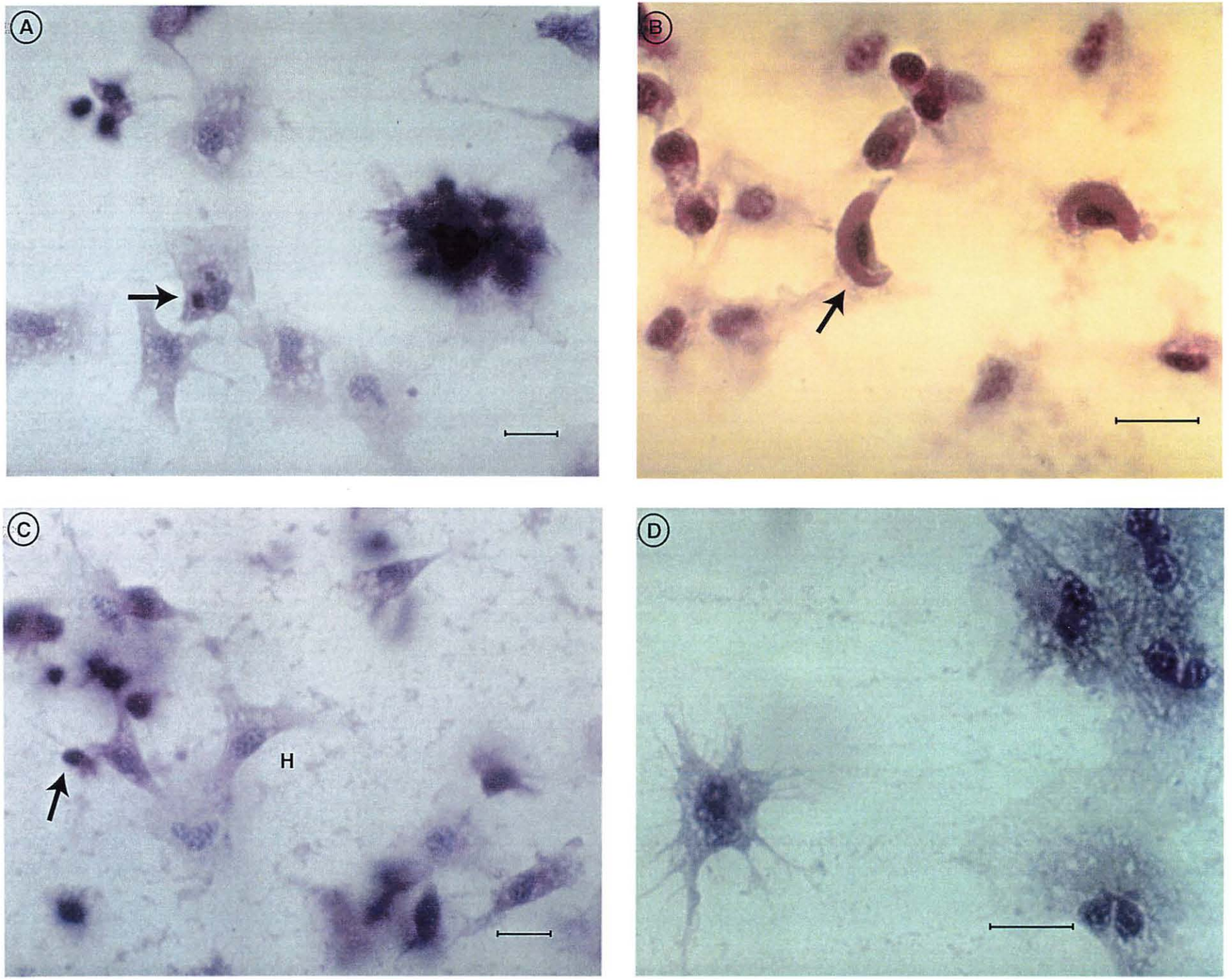


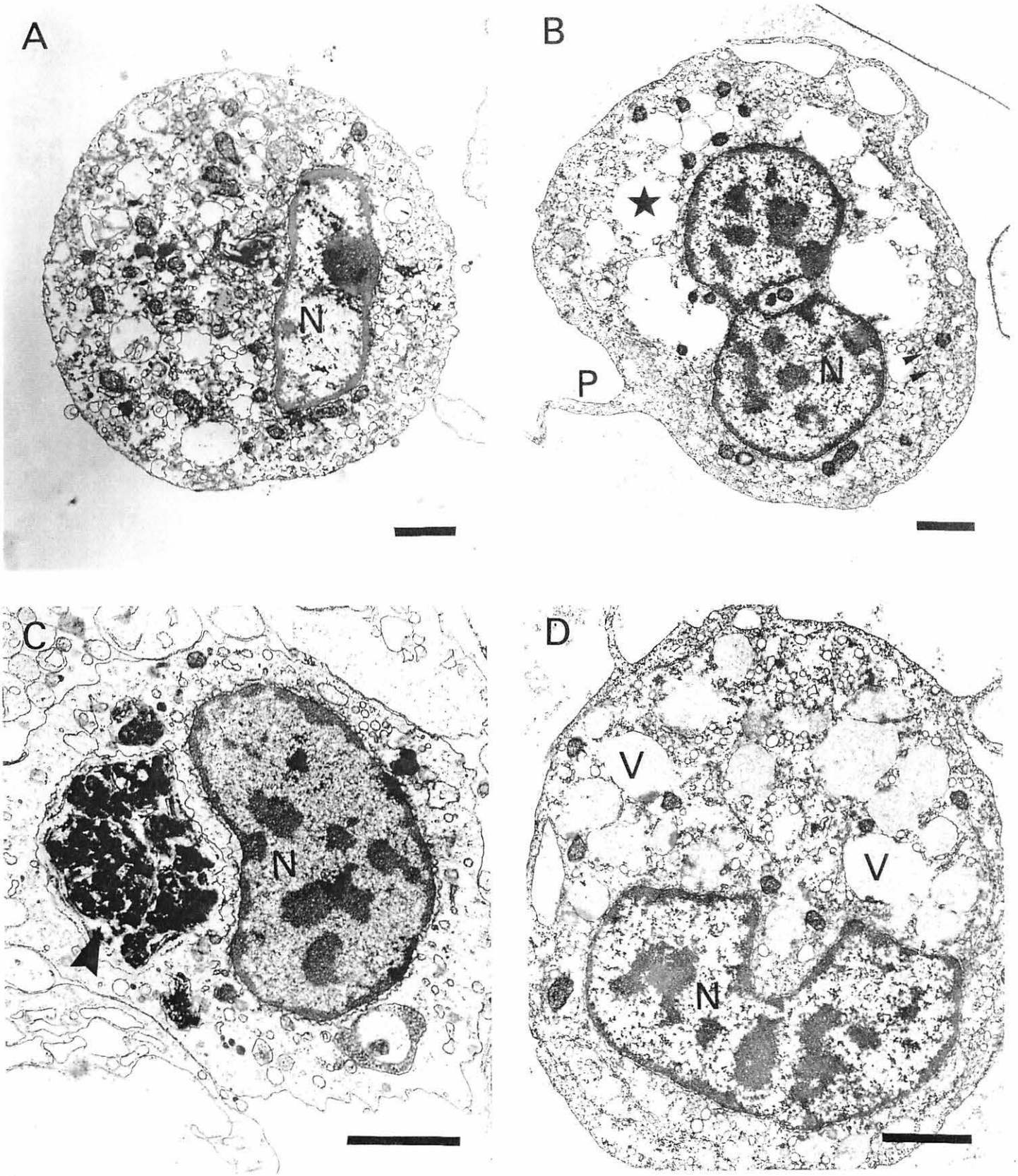
Experimental exposure
From Ano Nuevo

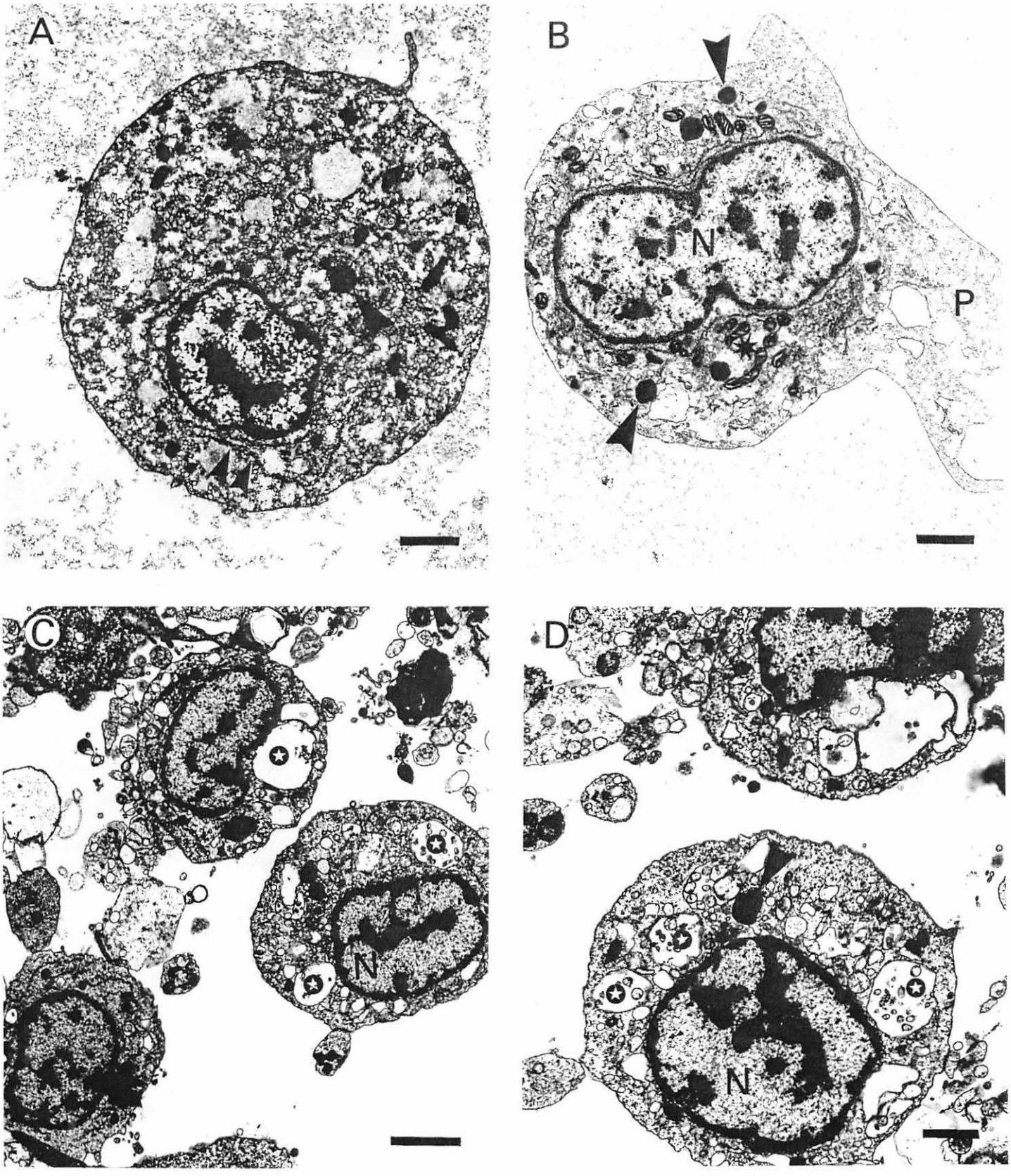


Healthy, asymptomatic
From endemic region




A
B
C
H
D


A
N
B
P
N
C
N
D
V
V
N


A
B
N
P
C
N
D
N

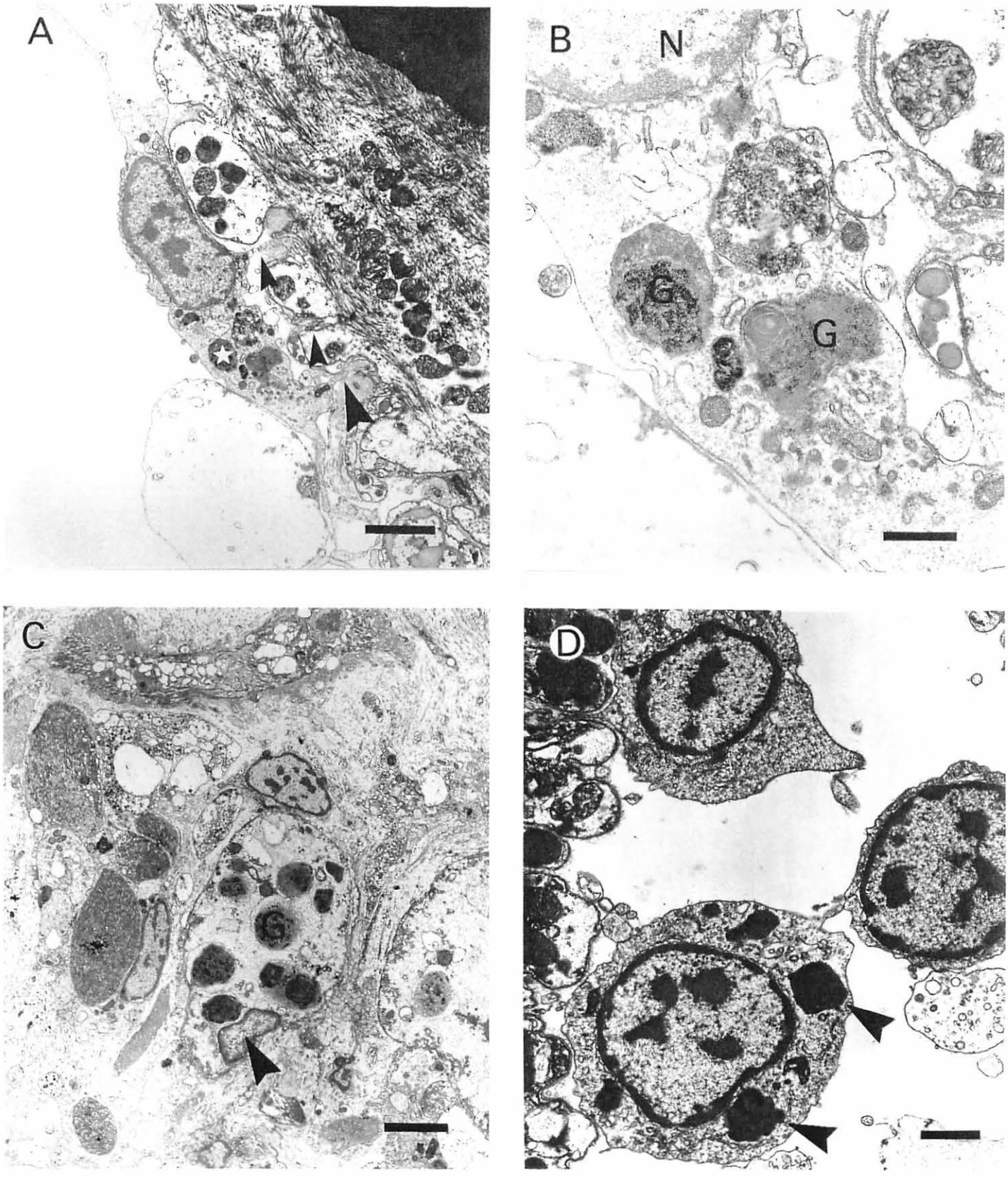
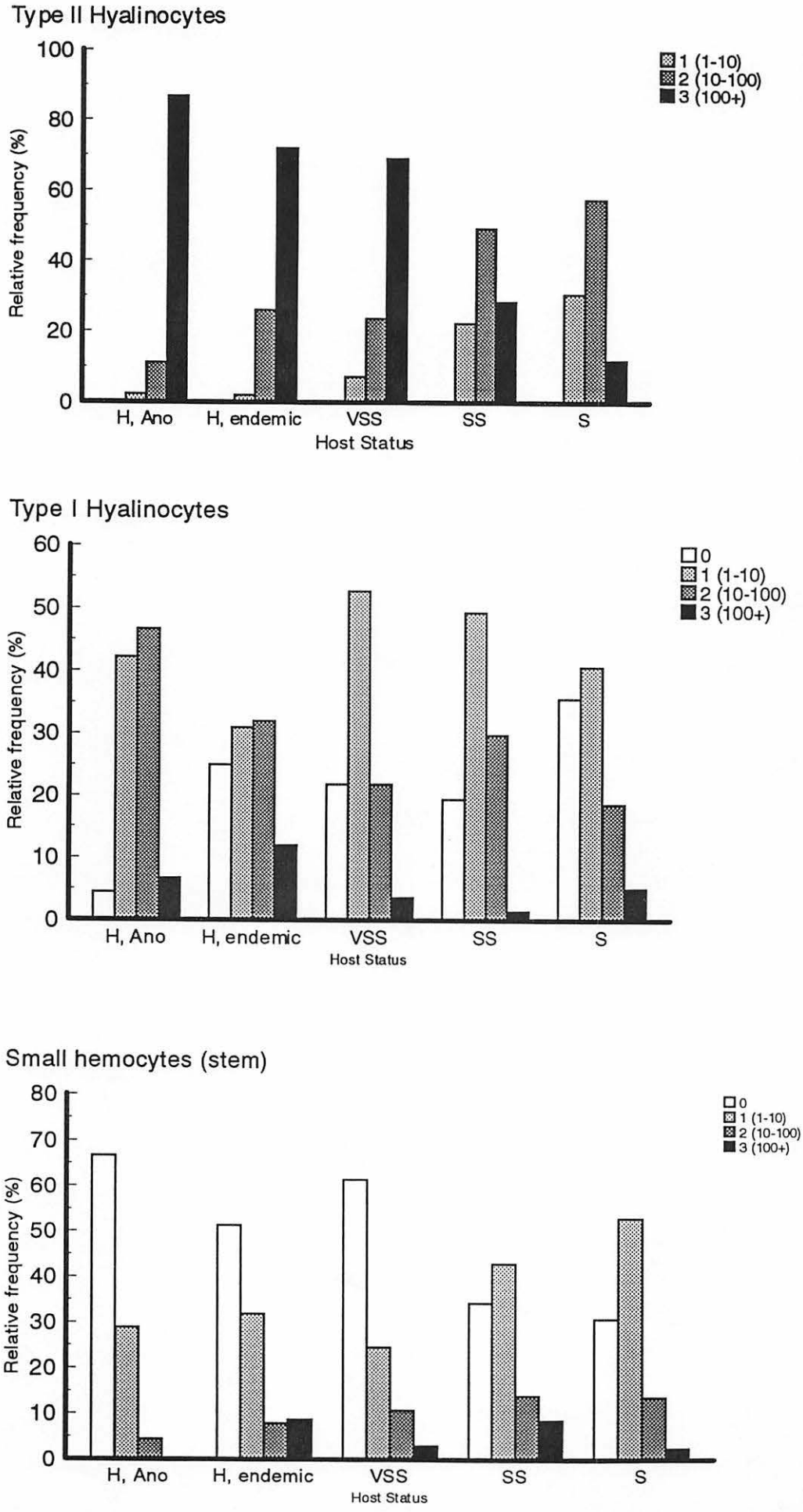

A
B
N
G
G
C
G
D

Figure 7.


Type II Hyalinocytes
100
80
60
40
20
0
Relative frequency (%)
1 (1-10)
2 (10-100)
3 (100+)
H, Ano
H, endemic
VSS
SS
S
Host Status
Type I Hyalinocytes
60
50
40
30
20
10
0
Relative frequency (%)
0
1 (1-10)
2 (10-100)
3 (100+)
H, Ano
H, endemic
VSS
SS
S
Host Status
Small hemocytes (stem)
80
70
60
50
40
30
20
10
0
Relative frequency (%)
0
1 (1-10)
2 (10-100)
3 (100+)
H, Ano
H, endemic
VSS
SS
S
Host Status

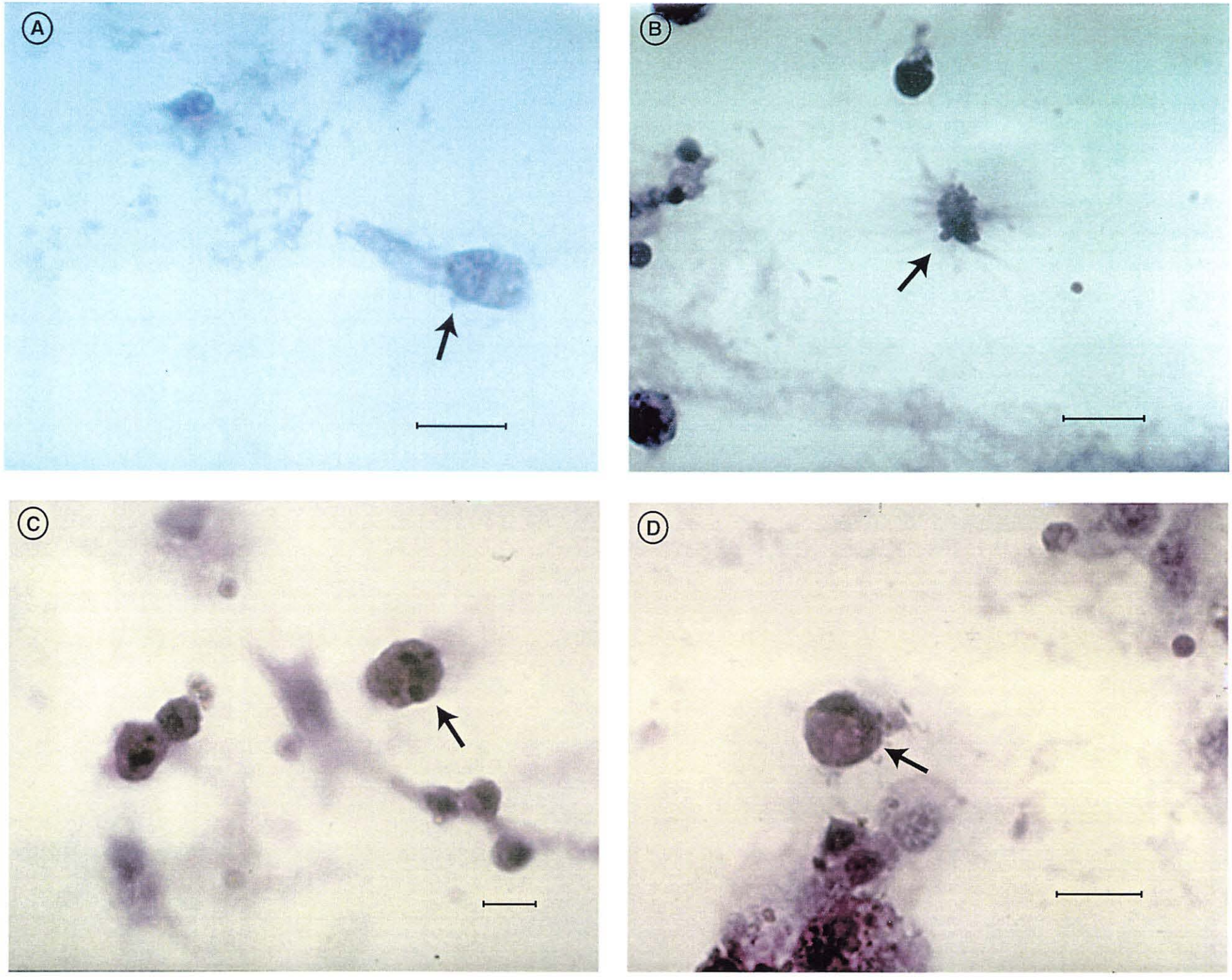
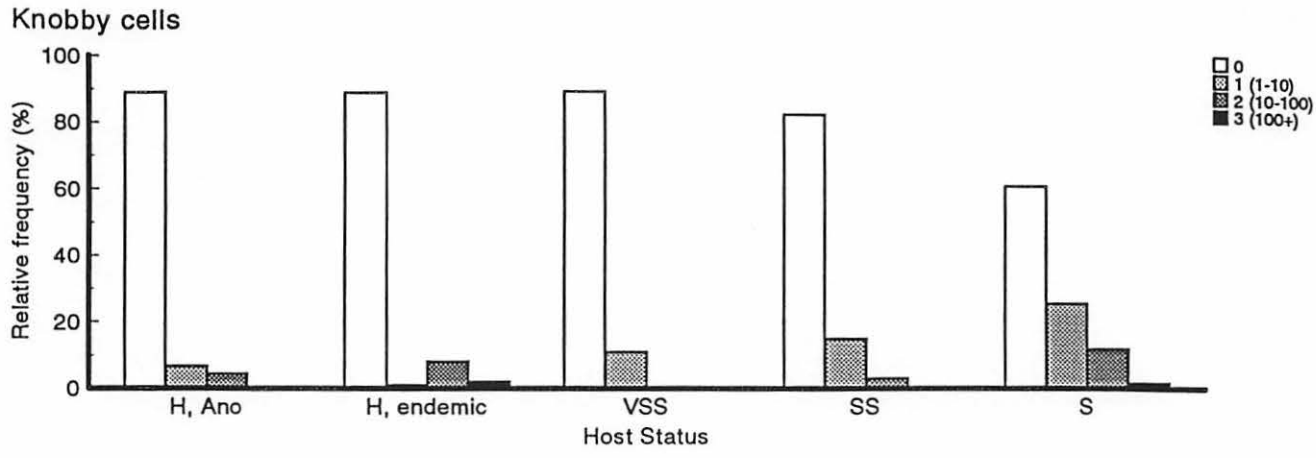
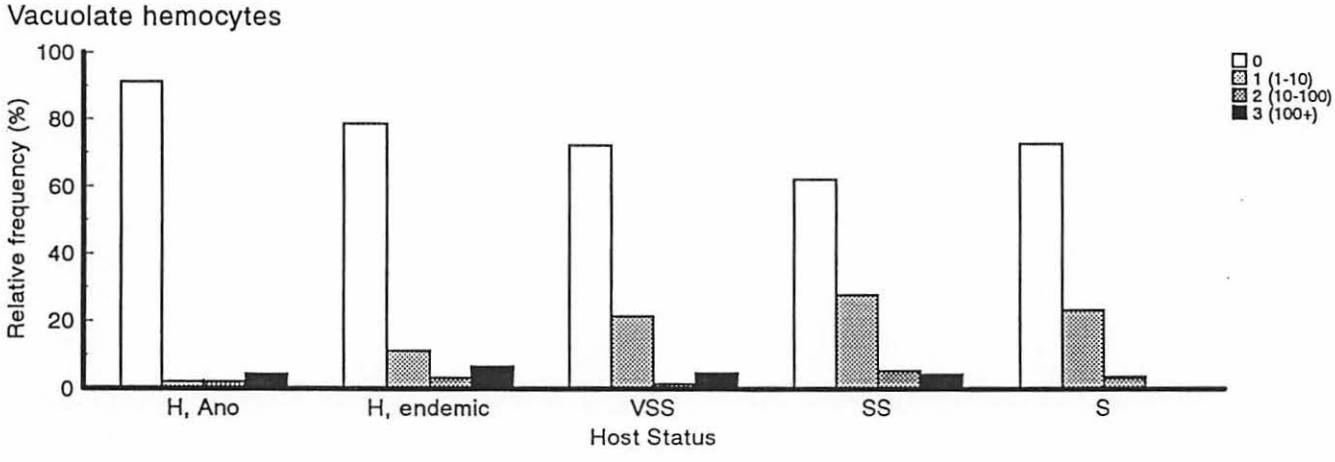

A
B
C
D

Figure 9.


Knobby cells
Relative frequency (%)
100
80
60
40
20
0
H, Ano
H, endemic
VSS
SS
S
Host Status
0
1 (1-10)
2 (10-100)
3 (100+)


Vacuolate hemocytes
Relative frequency (%)
100
80
60
40
20
0
H, Ano
H, endemic
VSS
SS
S
Host Status
0
1 (1-10)
2 (10-100)
3 (100+)

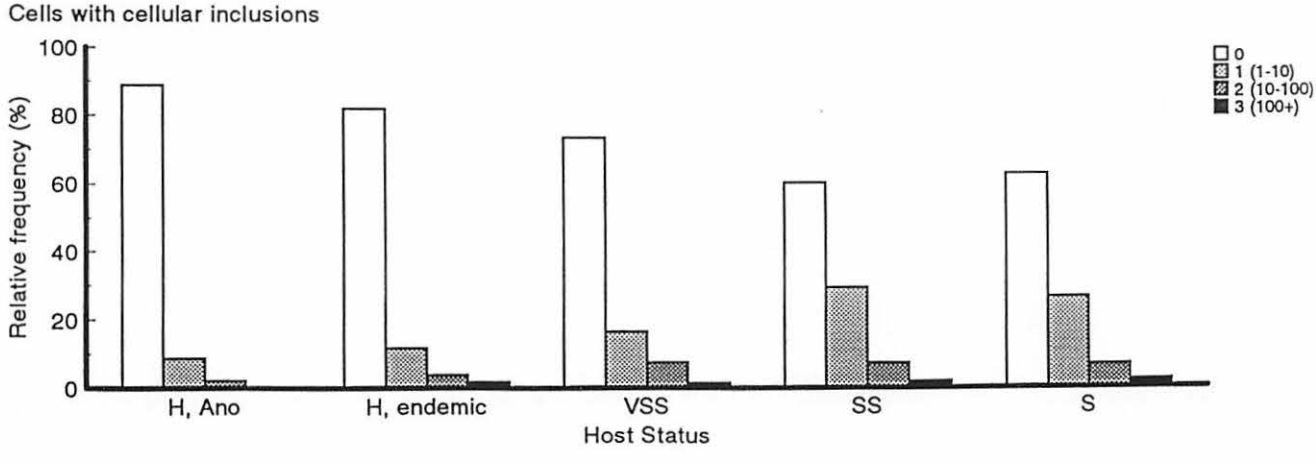
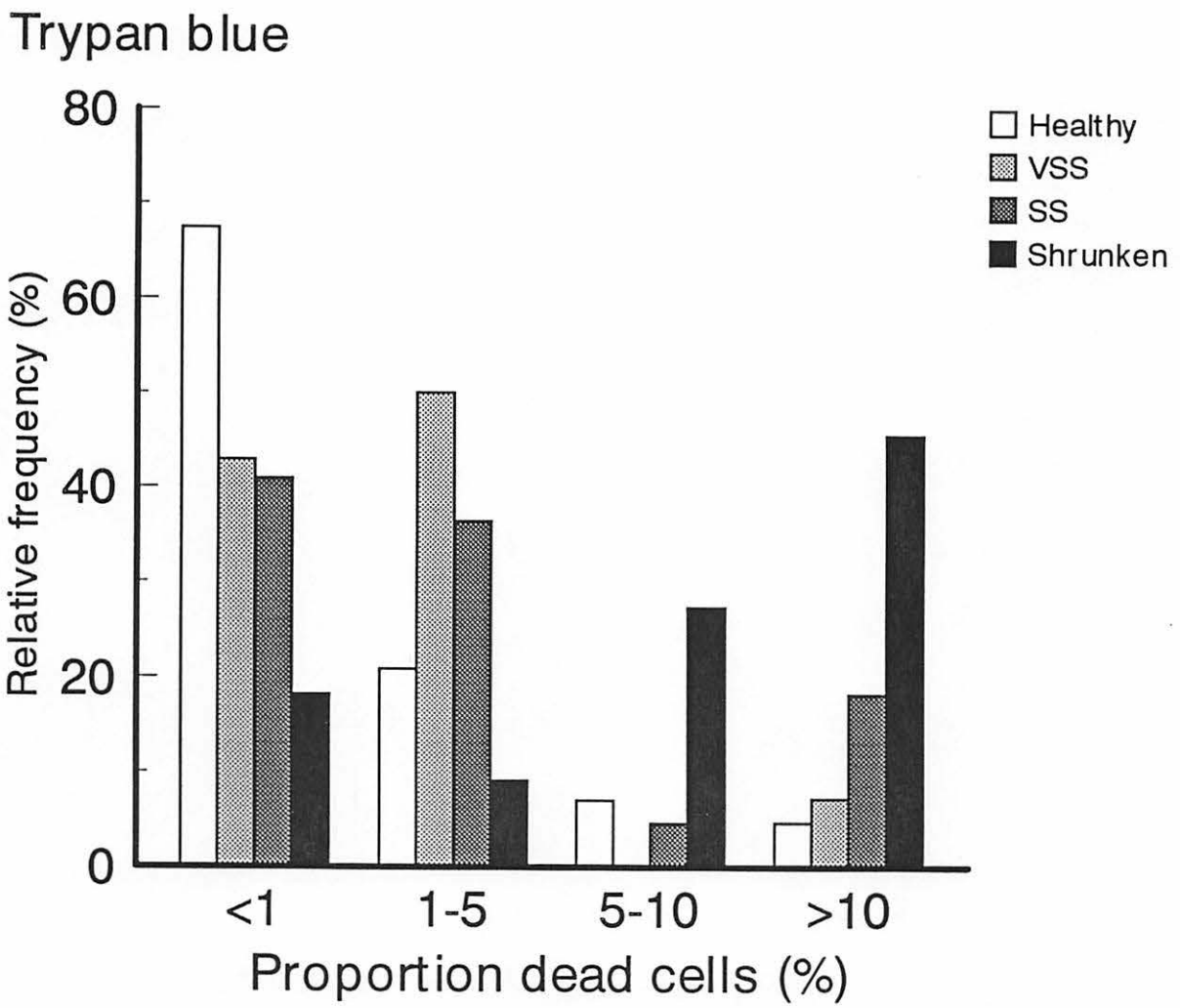

Cells with cellular inclusions
Relative frequency (%)
100
80
60
40
20
0
H, Ano
H, endemic
VSS
SS
S
Host Status
0
1 (1-10)
2 (10-100)
3 (100+)

Figure 10.


Trypan blue
80
60
40
20
0
Relative frequency (%)
Healthy
VSS
SS
Shrunken
<1
1-5
5-10
>10
Proportion dead cells (%)

ATTACHMENT B

FOR: Journal of Invertebrate Pathology

March 18, 1997

AN EVALUATION OF PHAGOCYTOSIS IN THE HEMOCYTES OF BLACK ABALONE, *HALIOTIS CRACHERODII*, AFFLICTED WITH WITHERING SYNDROME

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RH: EVALUATION OF PHAGOCTOSIS IN ABALONE HEMOCYTES

Abstract: An evaluation of phagocytosis in the hemocytes of black abalone, *Haliotis cracherodii,* afflicted with withering syndrome. Journal of Invertebrate Pathology ##: ###-###. Black abalone have recently suffered an epizootic of a rickettsia-like organism that causes a debilitating and fatal withering syndrome. We evaluated the ability of abalone hemocytes to phagocytize yeast cells as an assessment of the immune status of the host. Serum levels and the type of buffer used in the assessments caused significant, and, in some cases, radical variations in the percentage of phagocytic hemocytes, and in the number of yeast cells phagocytized. In Hanks balanced salt solution adjusted with NaCl (HBSS-NaCl) phagocytosis decreased with decreasing serum levels. In 100% serum mixed 1:1 with yeast suspended in HBSS-NaCl, a majority (50%+) of hemocytes from healthy, unexposed abalone were capable of phagocytosis. In buffer alone (O% serum) fewer cells (<5%) were phagocytic; and they possessed fewer particles. Phagocytosis was markedly different between cells suspended in HBSS-NaCl and those in HBSS adjusted with artificial seawater (HBSS-Sea). Hemocytes from healthy abalone showed a decreased response in HBSS-Sea, while those from symptomatic abalone showed enhanced phagocytic response. The presence of opsonins and divalent metal ions may explain the marked differences between experimental treatments. The general use of phagocytosis as an indicator of cellular immunity in invertebrates may require standardization. Standardization of buffers, and, possibly, serum components may improve the utility of the assay in the future.

Introduction

Abalone withering syndrome is caused by a chronic, fulminating rickettsial infection of the digestive gland and intestinal tract (Gardner et al., 1995). Healthy abalone acquire the infection through the water column and maintain moderate to high levels of rickettsial foci in their digestive tracts (intestine and tubules leading from digestive glands) (Friedman et al., in prep.). The rickettsial population may change over the course of the infection since fewer foci are found in the digestive tract of severely shrunken abalone. The decline of the intestinal tract apparently leads to malabsorption, which leads to the starvation of the host with a degradation of host's metabolic resources. The hematology supports this view of the pathology, in that apoptosis (cell death) occurs episodically in withering animals (Shields et al., in prep.).

Chemotaxis, phagocytosis, and cellular digestion are the main line of the cellular defense response in most invertebrates. Changes in the cellular response are typically indicative of the general health of an organism. A weakened response may indicate a sick or immuno-compromised animal, while a heightened response is interpreted as an active defense against disease. The hemocytes of molluscs are all actively phagocytic (see review by Ratcliffe et al., 1985) and respond to many different parasitic and microbial diseases (invertebrate blood cells). Phagocytic hemocytes are found in increasing densities in abalone afflicted with withering syndrome, but hemocyte densities decline in shrunken animals (Shields et al., in prep..). The episodic hemolysis and tissue degradation results in large numbers of dead and dying hemocytes and detritus in the blood. We evaluated phagocytosis as a component of the cellular defense of abalone hemocytes in relation to the withering disease with the understanding that cell lysis and catabolic degradation could further stimulate phagocytosis. Our initial results indicated that serum components were

required for active phagocytosis; hence, the evaluation included experimental examination of the effect of serum concentration and the type of buffer on phagocytosis.

MATERIALS AND METHODS

Abalone were be collected by hand (California Fish & Game personnel) with the aid of an abalone iron. Animals were collected beneath intertidal rocks at Vandenberg Airforce Base (near Lompoc), Cayucos beach, and Año Nuevo Island, transported to the laboratory in clean, disinfected (bleach or betadine) coolers packed with kelp, and held in 50 gallon aquaria for experimental observations. Animals in the experiments were either healthy-unexposed (from Año Nuevo Island), or symptomatic (very slightly shrunken to slightly shrunken per Shields et al., in prep). Data on the various parameters and variables were analyzed with ANOVA, Scheffe's inequality for post hoc comparisons, and the Chi-square test for homogeneity (Sokal and Rohlf, 1981).

Hemocyte preparation

Hemolymph (600 ul) was drawn from the pallial sinus with a sterile syringe (25-27 ga. needle). The hemolymph was split into two aliquots of 300 ul, and set on ice prior to centrifugation at 87 g for 10 min. We removed the sera from hemocytes to examine potential differences between opsonized and unopsonized cells, and the effects of serum on phagocytosis. In Treatment A (serum present), serum (supernatant) was removed from one aliquot, then returned to the same aliquot, and the cells were resuspended via gentle aspiration. In Treatment B (serum absent), the serum from the other aliquot was removed, and the cells were resuspended in either Hanks balanced salt solution adjusted to the osmolarity of seawater with sodium chloride (HBSS -

NaCl), or HBSS adjusted to the osmolarity of seawater with artificial seawater (HBSS - Sea). All of the treatments were spun again at 87 g for 10 min, and the cells were resuspended in either serum or buffer as above. The resuspended cells were then counted in a hemocytometer, and the volume was adjusted to give a final cell density of $1x10^6$ cells/ml. A control treatment consisted of resuspending hemocytes back into serum after having been washed and incubated with the HBSS-NaCl buffer.

Yeast cell preparation

FITC-conjugated yeast cells were prepared as in Anderson et al. (Anderson and Mora, 1995). Briefly, 2 g of yeast were boiled in 50 ml distilled water for 30 min. The yeast cells were washed six times (200 g, 10 min., room temp.) in distilled water, then resuspended in either HBSS-NaCl or HBSS-Sea. Cells were then incubated in the dark in a fluorescein isocyathanate solution (0.1 mg/ml in 0.1 M sodium carbonate buffer, pH 9.5) at 37 C for 30 min. The cells were washed five times (120 g, 10 min, room temp.), resuspended in the dark in one of the above buffers, counted with a hemocytometer, and stored in a stock concentration of $1x10^9$ yeast cells/ml (in individual 300-500 ul aliquots) at -70 C. The best working concentration for the working concentration of hemocytes was determined to be 10:1 to host cells or $1x10^7$ yeast cells/ml.

Fluorescence analysis

Aliquots of 50 ul of hemocyte preparations were placed on individual coverslips and let stand 30 min. An aliquot of 50 ul of yeast preparaion was then added to each coverslip preparation, mixed via gentle aspiration, and let stand 45 min. An aliquot of 50 ul of trypan blue quenching solution (1 mg/ml trypan blue in 0.02 M sodium citrate with 35 mg/ml sodium chloride, pH 4.5, made fresh daily) was added to each coverslip, and let stand 8 min. Each coverslip was then mounted an a microslide, and read with an epifluorescent microscope. Engulfed yeast cells fluoresced whereas those outside the cell were quenched by the trypan blue (Figure 1). Three replicates were examined from each animal for each treatment. In most cases at least 200 hemocytes per replicate were counted and characterized as having phagocytized or not phagocytized. The number of yeast cells phagocytized was enumerated for approximately 25 phagocytic hemocytes per replicate. An additional control was implemented for the quenching of the yeast cells. Aliquots of the yeast preparation were placed on individual coverslips, and let stand 45 min. The trypan blue solution was added (8 min.), the the slide was examined for fluorescence. In the few cases where the fluorescence was not quenched properly, fresh trypan blue solution was added. Note that serum concentrations are reported prior to the addition of yeast particles in their respective buffers. Actual concentrations are therefore halved.

RESULTS

Serum Experiment

The proportion of serum present in the experimental treatments was a critical factor in the ability of the hemocytes to phagocytize yeast cells (Figures 2 - 4) While individual abalone exhibited variation in the degree of phagocytosis, both the percentage of phagocytic hemocytes and the mean number of particles phagocytized increased significantly with serum concentration. Significant differences were found between treatments and between individual abalone (Figures 3 and 4, ANOVA, $P<0.05$). The control of resuspending hemocytes in serum after washes in HBSS-NaCl buffer was intermediate between the 0% serum and 100% serum treatments for both the percentage of phagocytosis and mean number of particles phagocytized.

Buffer Experiment

The type of buffer used had a radical effect on the two phagocytic indices. The presence of HBSS-NaCl apparently hindered phagocytosis in the unopsonized treatments (0% serum) but enhanced phagocytosis in the opsonized treatments (100% serum) (Figure 5 A and B).[1] In addition, hemocytes from symptomatic abalone exhibited significantly less phagocytic ability than their healthy counterparts. In contrast, when the experiment was carried out with cells washed with HBSS-Sea, the results were entirely opposite to that from the HBSS-NaCl series (Figure 5). Not only did the hemocytes of symptomatic abalone exhibit a significant increase in their

[1] Note that serum concentration is given prior to the addition of the yeast suspension; hence, the actual concentration is half that given.

phagocytic capability, but the 0% serum treatment was either not significantly different than the 100% serum treatment, or it was even greater (cf. symptomatic, 0% serum in Figure 5A and C).

Furthermore, the different buffers elicited remarkably different responses in the phagocytic ability of the hemocytes from healthy abalone (see Unexposed in Figures 5A and C). HBSS-NaCl appeared to enhance phagocytosis in hemocytes from healthy animals, while HBSS-Sea appeared to inhibit it (Threeway-ANOVA on serum, buffer, and disease status, $P<0.05$, Scheffe contrast).

The type of buffer also had an effect on the frequency distribution of yeast particles in phagocytic hemocytes (Figure 6). For the HBSS-NaCl treatments, hemocytes in 100% serum from healthy abalone phagocytized a consistently large number of particles, with a relatively even distribution out to 6 yeast cells per phagocyte. Hemocytes in the 100% serum from symptomatic abalone phagocytized significantly fewer particles per cell. For hemocytes in buffer alone (0% serum), most cells (60-80%) phagocytized only single yeast particles. For the HBSS-Sea treatments, hemocytes in 100% serum from healthy abalone phagocytized few yeast particles, while their infected counterparts showed little change in distribution (compare Figure 5C and 6C).

DISCUSSION

We explored a possible artifact in a signficant and widely used test of cellular immunity. Our results indicate that the level of serum and the type of buffer present in phagocytosis tests may require strict control and standardization if they are to be compared within and between other experimental findings. Bayne (1990) suggests that serum not be used in the experimental

examination of specific opsonins in phagocytosis. He argues that the test particles should be incubated in serum then washed free of it. We agree with Bayne (1990) that for experimental studies of specific opsonins or lectin binding characters the phagocytosis test requires buffer-washed, serum-free cells. However, the phagocytosis assay has been developed as an index of environmental stress for fish macrophages (e.g., Loose et al., 1981; Blazer et al., 1989; Wishkovsky et al., 1989) and oysters (Kent et al., 1989; Larson et al., 1989; Alvarez et al., 1991); and as such, a brief review of the literature indicates a general lack of standardization of serum levels, or buffer type among researchers.

Serum levels and buffer type were significant factors affecting phagocytosis in abalone hemocytes. Several factors are known to affect phagocytosis *in vitro*. Serum is known to enhance phagocytosis of yeast cells in oyster hemocytes (Anderson and Good, 1976), but there are few studies of its *in vitro* effects. Low temperatures inhibited phagocytosis in hemocytes of oysters (Fisher and Tamplin, 1988; Alvarez et al., 1989), and clams (Foley and Cheng, 1975), and lowered salinity inhibited phagocytosis in the hemocytes of oysters (Fisher and Tamplin, 1988; Fisher et al., 1987). Unlike vertebrate systems, oxygen may not be required for phagocytosis in the oyster (Cheng, 1976; Alvarez et al., , 1989), but it may be essential for phagocytosis in a scallop (Nakamura et al., 1985) and a pulmonate snail (Dikkeboom et al., 1987). In addition, various drugs (Cheng and Howland, 1982), pollutants (Anderson et al., 1981; Wishkovsky et al., , 1989; Alvarez et al., , 1991) and heavy metals (Cheng, 1988) are known to impair or inhibit phagocytosis.

The equivocal results with abalone may be explained by the presence of opsonins and requirements for specific metal ions. Hemocytes of healthy abalone showed an enhanced phagocytic activity in the presence of serum, and hemocytes of symptomatic abalone showed enhanced phagocytic activity in the presence of components in artificial seawater. Serum factors like opsonins or agglutinins are known to enhance phagocytosis in invertebrates (see Vasta et al., 1982). In addition, specific metal ions, primarily calcium, have a dramatic effect on phagocytosis in molluscs. Phagocytosis of yeast was heightened by mussel hemocytes in the presence of agglutinin, and calcium ions (Renwrantz and Stahmer, 1983), and calcium ions are required to initiate cellular aggregation in pulmonate snails (Tuan and Yoshino, 1987; Shozawa and Suto, 1990). For abalone hemocytes, the serum experiment indicated the potential presence of opsonins. The buffer experiment highlighted the presence of a chemical enhancement (presumably a divalent metal ion), with potential oversaturation or inhibition in the presence of serum, and a significant increase in phagocytic activity in hemocytes from symptomatic animals.

In conclusion, the general use of phagocytosis as an indicator of cellular immunity in invertebrates requires standardization. Standardization of buffers, and, possibly, serum components may improve the utility of the assay in the future. In the presence of divalent cations, hemocytes of symptomatic abalone exhibited an enhanced phagocytic response potentially indicative of their heightened activity against the catabolic degradation resulting from disease.

Acknowledgements

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Figure 1. Micrographs of phagocytized and unphagocytized yeast particles with abalone hemocytes. (A) Epifluorescent micrograph of hemocytes in 100% serum (yeast in Hank's balanced salt solution adjusted with NaCl); bar = 10 um. (B) Transmitted light micrograph of (A).

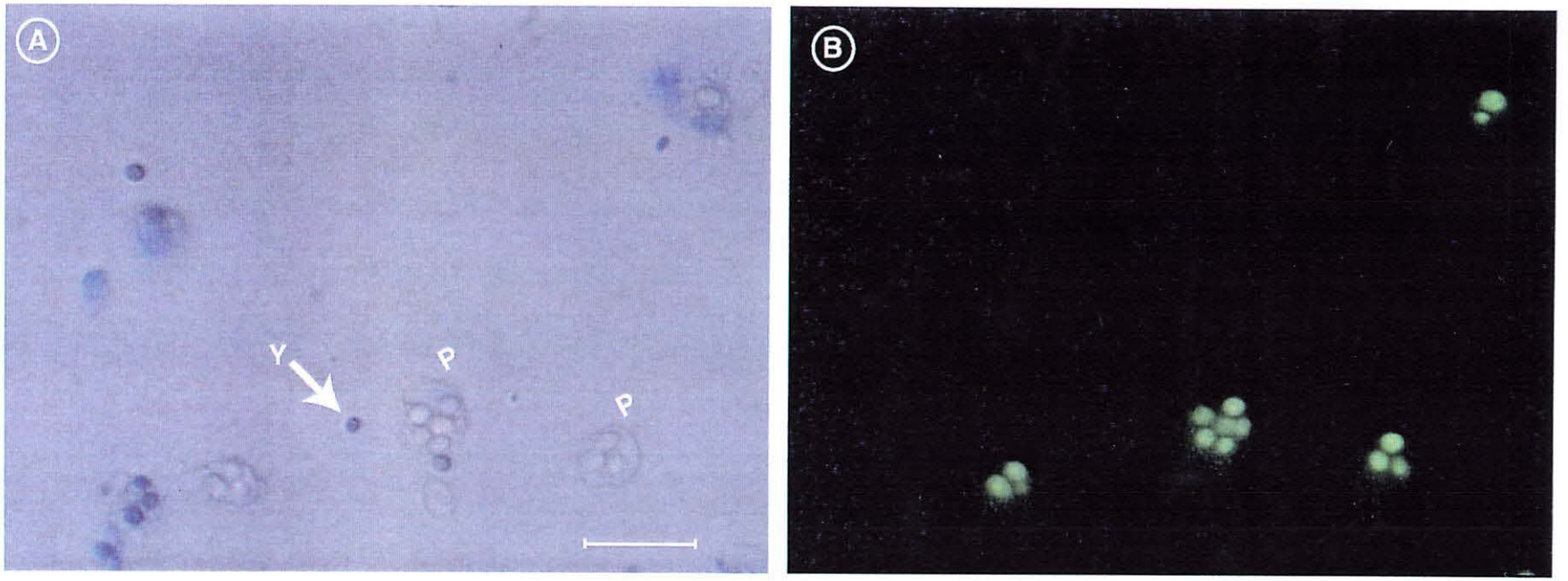
Figure 2. Micrographs of phagocytized and unphagocytized yeast particles with abalone hemocytes. (A) Epifluorescent micrograph of hemocytes in 0% serum (yeast in Hank's balanced salt solution with NaCl); bar = 10 um. (B) Transmitted light micrograph of (A).

Figure 3. The proportion (%) of phagocytic hemocytes from healthy abalone in relation to serum concentration. Serum concentrations are shown prior to mixing 1:1 with the yeast suspended in Hank's balanced salt solution adjusted with NaCl. Note the variation between individuals and the significant declines in phagocytosis with each reduction in serum concentration. Cell counts are from triplicate preparations of individuals at each concentration.

Figure 4. The effect of serum concentration on the mean number of phagocytized yeast particles per phagocytic cell from healthy abalone. Serum concentrations are shown prior to mixing 1:1 with the yeast suspended in Hank's balanced salt solution adjusted with NaCl. Note the variation between individuals and the significant declines in phagocytosis with reductions in serum concentrations. Error bars = sd; particle counts are from approximately 25 hemocytes per replicate.

Figure 5. The effect of serum level (0 and 100%), buffer (Hank's balanced salt solution adjusted for osmolarity with NaCl, or artificial seawater), and infection status on the percentage of phagocytic cells, and the mean number of phagocytized yeast particles; (A) Mean proportion (%) of phagocytic hemocytes in serum or HBSS-NaCl; (B) Mean number of phagocytized yeast particles per phagocytic hemocyte in serum or HBSS-NaCl; (C) Mean proportion (%) of phagocytic hemocytes in serum or HBSS-Sea; (D) Mean number of phagocytized yeast particles per phagocytic hemocyte in serum or HBSS-Sea. Serum concentrations are shown prior to mixing 1:1 with the yeast suspended in HBSS-NaCl or HBSS-Sea. Cell counts are from triplicate preparations at each concentration. Particle counts are from approximately 25 hemocytes per replicate; N = 4 healthy, 6 symptomatic, 4 healthy, and 4 symptomatic, respectively; bar=sd.

Figure 6. Distribution of the proportion (%) of yeast particles per phagocytic hemocyte. Note the difference in the shape of the distributions between serum and buffer (A vs. B) and between healthy and symptomatic animals (A vs. D). (A) Hemocytes from healthy, unexposed abalone suspended in 100% serum prior to inoculation with yeast in Hank's balanced salt solution adjusted with NaCl (HBSS-NaCl). (B) Hemocytes from healthy, unexposed abalone suspended in HBSS-NaCl (black bars). Controls consisted of hemocytes resuspended in serum after buffer washes (light bars). (C) Hemocytes from symptomatic abalone suspended in 100% serum prior to inoculation with yeast in HBSS-NaCl. (D) Hemocytes from symptomatic abalone suspended in HBSS-NaCl (black bars) and controls resuspended in serum after HBSS-NaCl wash (light bars). N = 4 healthy and 6 symptomatic abalone. Cell counts are from triplicates of individual animals. Particle counts are from approximately 25 hemocytes per replicate.


A
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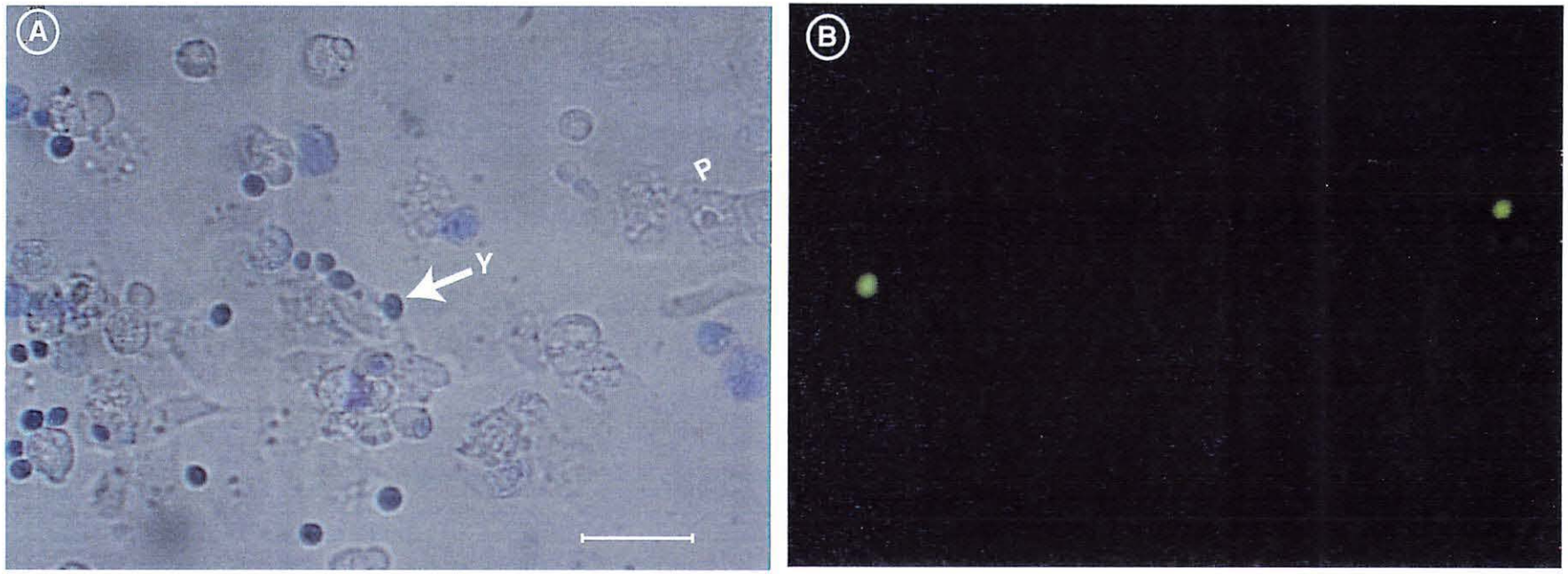
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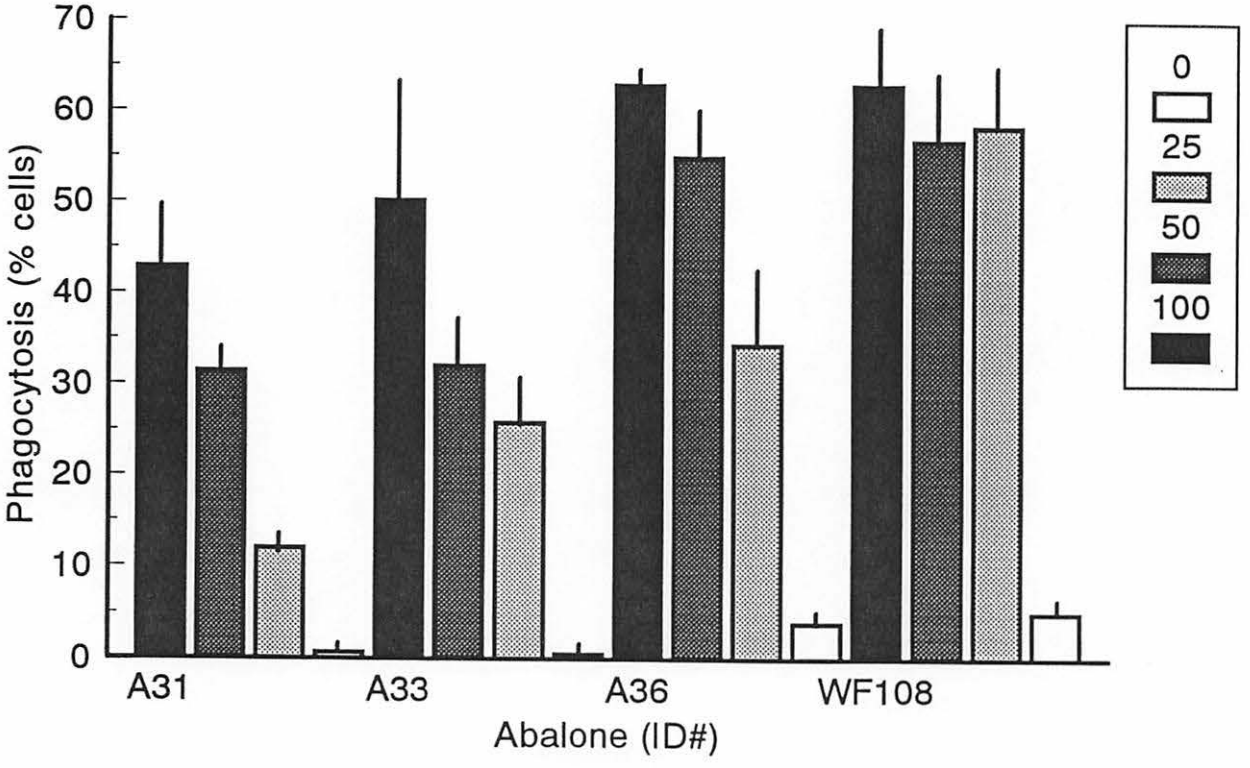
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60
50
40
30
20
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A33
A36
WF108
Abalone (ID#)
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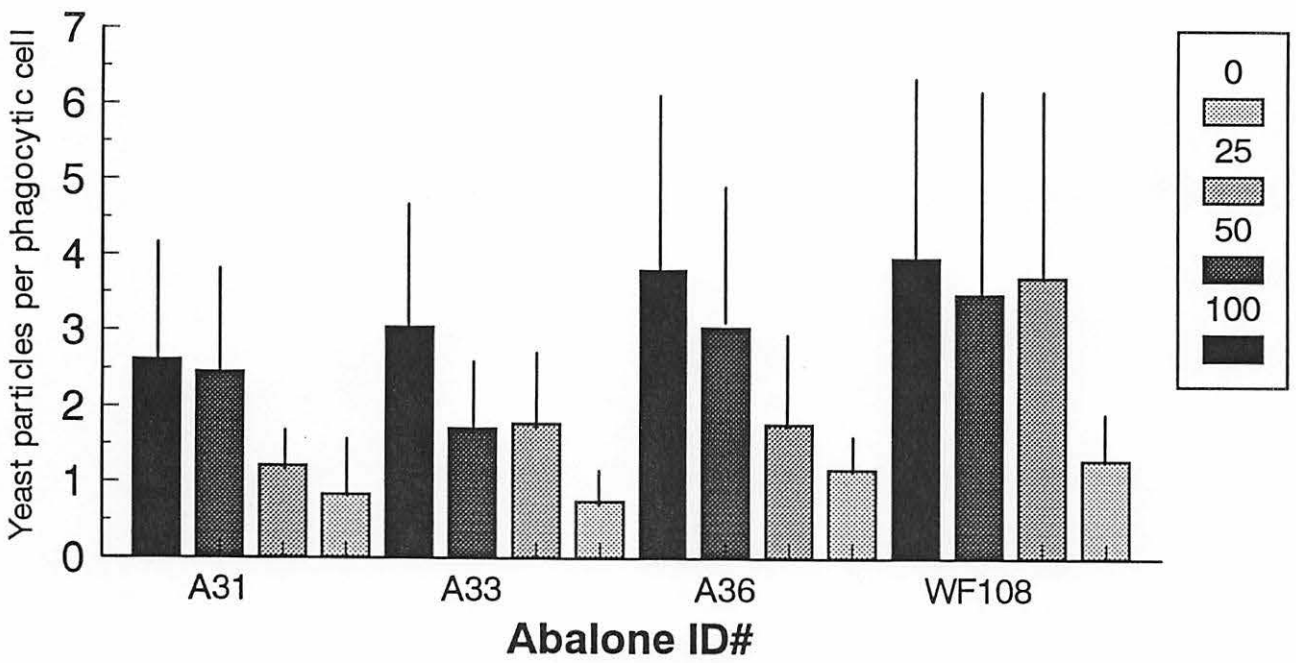
25
50
100

Figure 4.


Yeast particles per phagocytic cell
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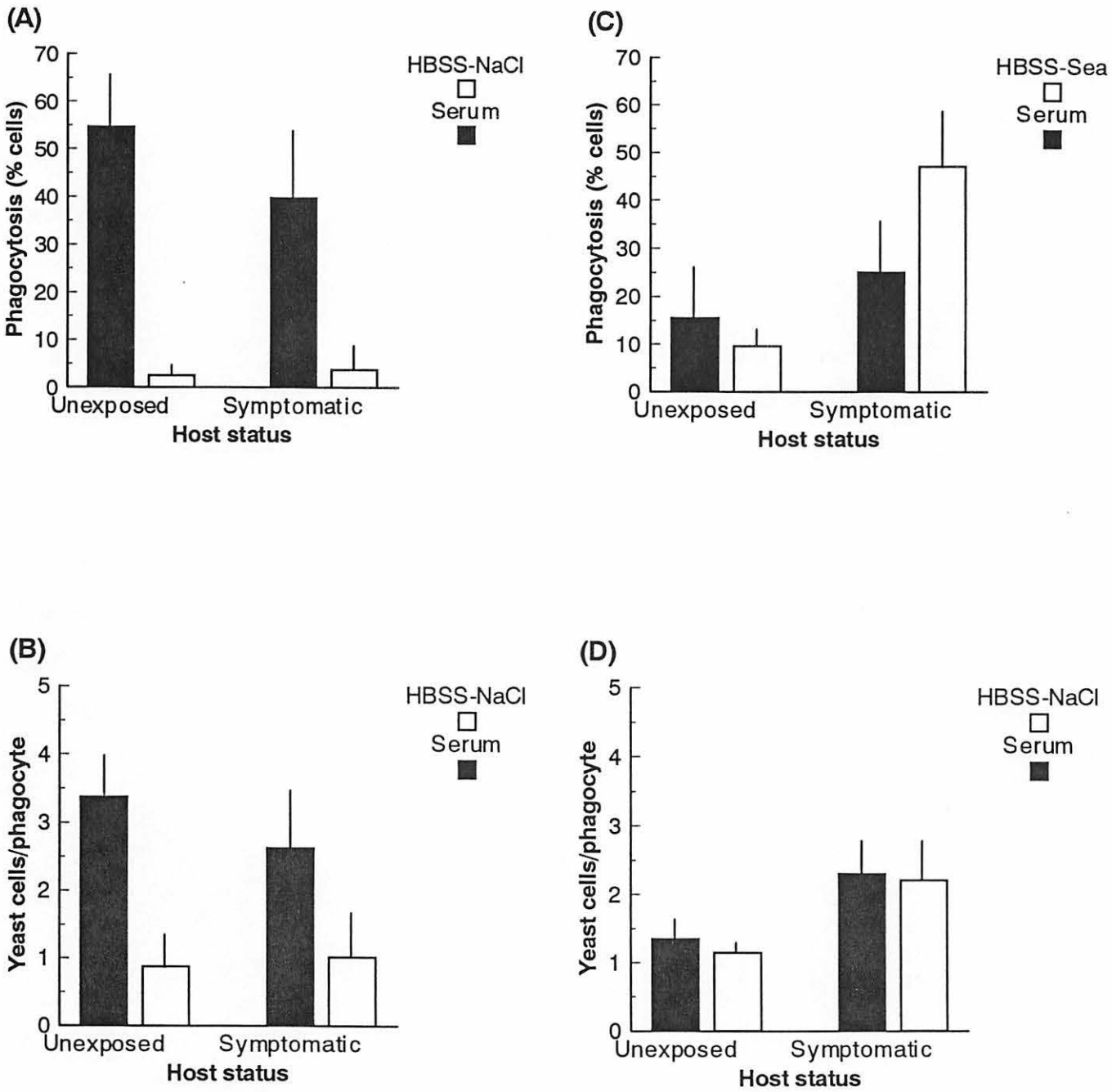
3
4
5
6
7
A31
A33
A36
WF108
Abalone ID#
0
25
50
100

Figure 5.


(A)
Phagocytosis (% cells)
70
60
50
40
30
20
10
0
Unexposed
Symptomatic
Host status
HBSS-NaCl
Serum
(C)
Phagocytosis (% cells)
70
60
50
40
30
20
10
0
Unexposed
Symptomatic
Host status
HBSS-Sea
Serum
(B)
Yeast cells/phagocyte
5
4
3
2
1
0
Unexposed
Symptomatic
Host status
HBSS-NaCl
Serum
(D)
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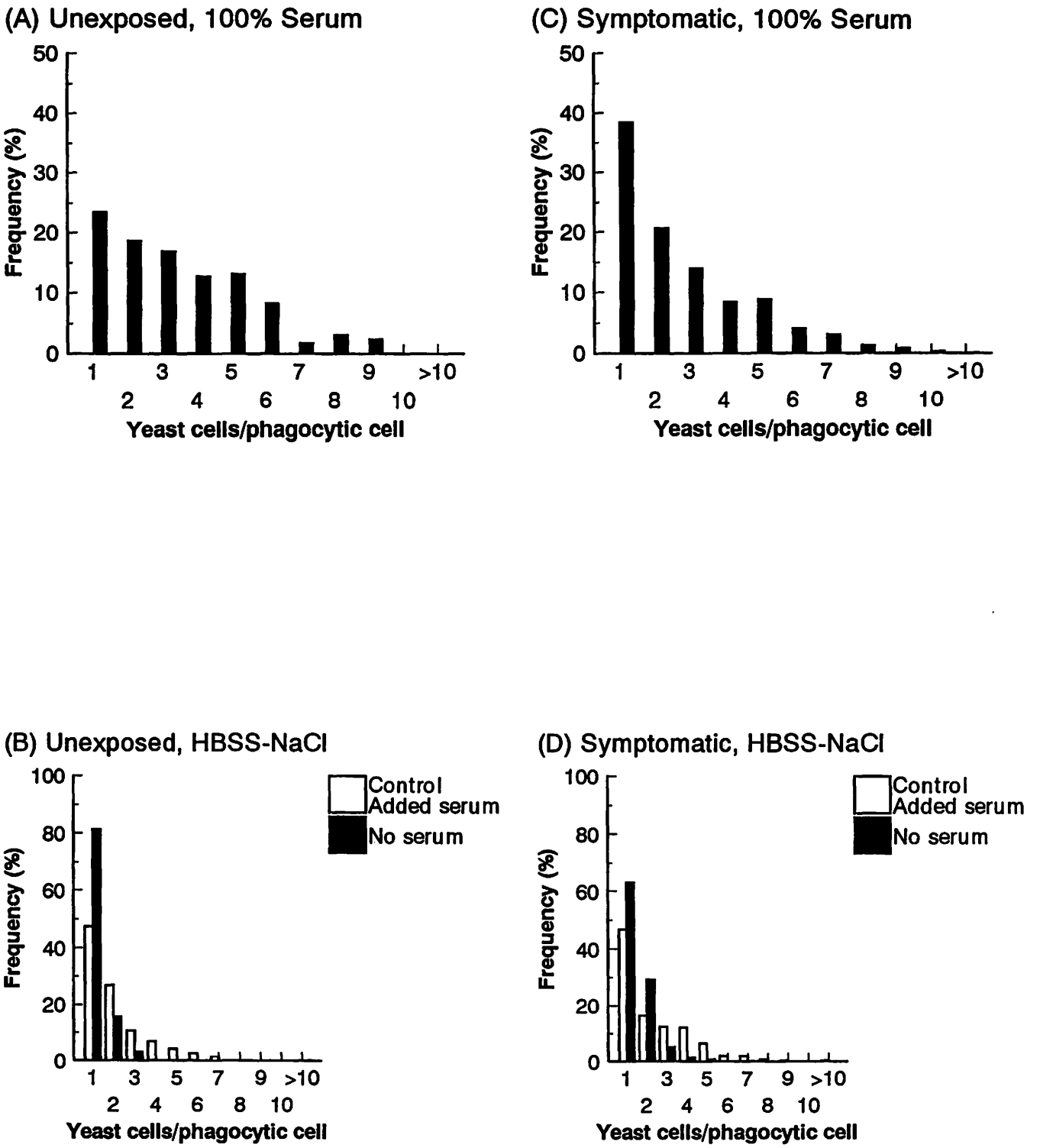
Unexposed
Symptomatic
Host status
HBSS-NaCl
Serum

Figure 6.


(A) Unexposed, 100% Serum
Frequency (%)
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40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
>10
Yeast cells/phagocytic cell
(C) Symptomatic, 100% Serum
Frequency (%)
Yeast cells/phagocytic cell
(B) Unexposed, HBSS-NaCl
Control
Added serum
No serum
Frequency (%)
100
80
60
40
20
0
Yeast cells/phagocytic cell
(D) Symptomatic, HBSS-NaCl
Control
Added serum
No serum
Frequency (%)
Yeast cells/phagocytic cell