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Responses of *Leishmania* (*Viannia*) *braziliensis* Cutaneous Infection to *N*-Methylglucamine Antimoniate in the Rhesus Monkey (*Macaca mulatta*) Model

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ABSTRACT: The antileishmanial efficacy of the reference drug *N*-methylglucamine antimoniate (Glucantime®) was evaluated in groups of rhesus monkeys with acute and chronic *Leishmania* (*Viannia*) *braziliensis* cutaneous infection. The therapeutic responses in experimental animals to either a low dose (5 mg/kg body wt/day for 28 days) or a routine dose (20 mg/kg/day for 28 days) of pentavalent antimony were similar to those reported in the human disease. Primates were cured of their lesions after treatment, but with cryptic parasitism and/or relapse. The rhesus model of *L.* (*V.*) *braziliensis* cutaneous leishmaniasis therefore provides an additional resource for preclinical trials with newer drugs.

Self-healing cutaneous leishmaniasis (CL) has been associated with all New World *Leishmania* species, but a proportion of nonhealing cases of cutaneous and mucocutaneous disease can be very difficult to cure (Grimaldi and Tesh, 1993). Despite their toxic properties, pentavalent antimony (Sb^v) compounds (meglumine antimoniate and sodium stibogluconate), which must be administered parentally, remain the primary therapy for human leishmaniasis. Should this treatment fail, a number of other drugs and immune modulators may be employed, but toxic properties remain the major obstacle for the most potent antileishmanial agents (Croft and Yardley, 2002).

Cutaneous leishmaniasis resulting from infection with *Leishmania* (*V.*) *braziliensis*, which can cause considerably morbidity and that may result in severe disfigurement (Grimaldi and Tesh, 1993), continues to present serious therapeutic problems (Romero et al., 2001). Current regimes use Sb^v at a dosage of 20 mg/kg body weight/day for 20 days (for cutaneous lesions) or 28 days (for mucosal lesions). Some studies conducted in locales where this pathogen is endemic have suggested that shorter courses of treatment (5 mg/kg/day) may be as efficacious (Oliveira-Neto et al., 1997, 2000). Moderate resistance to Sb^v exists among *Leishmania* strains in nature, and some isolates are innately less or more susceptible to this drug than other strains (Grogl et al., 1992). Whether specific genotypes of this pathogen (Cupolillo et al., 2003) are responsible for antimony resistance remains to be determined. The development of antimony-resistant clones in vitro by discontinuous drug exposure (Grogl et al., 1989) indicates the necessity of finding new therapeutic agents for the treatment of leishmaniasis.

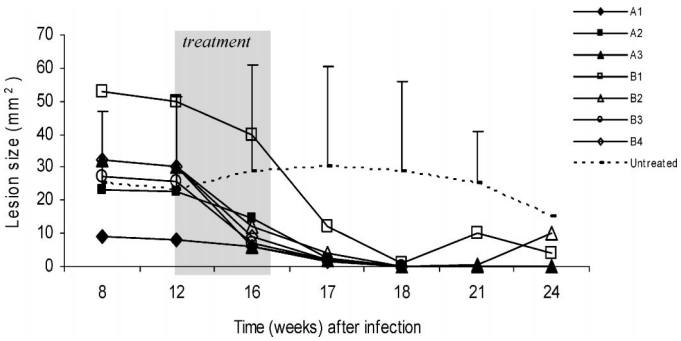

FIGURE 1. Responses of *Leishmania* (*Viannia*) *braziliensis* acute cutaneous infection in rhesus macaques to a low dose of *N*-methylglucamine antimoniate (5 mg/kg body wt/day given intramuscularly for 28 days). Treatment started at week 12 after inoculation with 1×10^7 promastigotes into the skin of either the forearm (monkeys A1 to A3) or eyebrow (monkeys B1 to B4). As an assessment of therapy, the diameter of the lesions was scored weekly following infection and treatment. Primary lesions were measured with a vernier caliper, and lesion size was estimated using the following formula: area (mm^2) = $\pi \times$ greatest radius $\times$ least radius. Data in the untreated group are the mean $\pm$ SD of four infected monkeys.

Nonhuman primates appear to have significant advantages over conventional laboratory animals in terms of modeling CL for purposes of drug evaluation (Kennedy et al., 1997). Rodent models may not be good predictors of human responses, because their metabolism often is different from that of humans for some drug candidates. In contrast, the ability to do efficacy-PK studies in simians is a huge advantage in this regard. In our previous studies, we have shown that *Macaca mulatta* models the clinical presentations of *L.* (*V.*) *braziliensis* infection in humans (Teva et al., 2003). The severity of the resulting lesions induced with different strains of this pathogen was greater compared to those induced with either *L.* (*L.*) *major* or *L.* (*L.*) *amazonensis* (Amaral et al., 1996, 2001). In the present study, we compare the efficacy of a low dose with that of a standard dose of Sb^v in *L.* (*V.*) *braziliensis*–infected rhesus macaques.

Fourteen laboratory-bred and -reared, young adult *M. mulatta* monkeys of mixed sexes were used in the present study. Three of the animals had been previously infected with *L.* (*V.*) *braziliensis* in the forehead above the left eyelid but had not recovered from clinical disease (Teva et al., 2003). The other 11 monkeys were naïve animals that had never been exposed to leishmanial parasites. Their care and maintenance have been described previously (Amaral et al., 1996).

The first experiment (experiment 1) was designed to provide information concerning the effect of a low dose of *N*-methylglucamine antimoniate (Glucantime® [Rhodia Farma, S. Paulo, Brazil] 5 mg/kg/day of Sb^v given intramuscularly for 28 days) on development of a local dermal lesion caused by *L.* (*V.*) *braziliensis* (strain MHOM/BR/1997/SIS) in naïve monkeys inoculated with 1×10^7 infective promastigotes. Treatment employing this dose rapidly reduced the lesion sizes (Figs. 1, 2) in experimental animals (compared with untreated animals). Complete healing was achieved in 5 of the 7 (71.9%) monkeys with acute cutaneous infection, and in the remaining 2, reactivation of the scar lesions was observed by week 5 and week 8, respectively, after the completion of treatment. The satisfactory result observed in our monkeys treated with a low-dose regimen might be related to a nonrefractoriness of the parasite strain causing the disease and/or a distinct host response to this particular parasite. This seems to be the case of *L.* (*V.*) *braziliensis* parasites in Rio de Janeiro (Brazil), where high cure rates for cutaneous and mucosal disease were obtained with low Sb^v doses (Oliveira-Neto et al., 1997, 2000).

In an additional experiment (experiment 2), 3 monkeys with long-lived *L.* (*V.*) *braziliensis* skin lesions (36 mo postinfection) were treated with a routine Sb^v dose (20 mg/kg/day for 28 days). Primates were cured of their lesions, but 1 animal also relapsed 2 mo after the cessation of therapy (data not shown). No antimonial-related side effects in treated animals were apparent during the trials.

Response to treatment with Sb^v varies considerably depending on the parasite as well as host factors (Grogl et al., 1992; Romero et al., 2001). In human leishmaniasis, spontaneous or drug-induced acquired resistance to intracellular *Leishmania* spp. is T-helper 1 (Th1) cell cytokine dependent and largely mediated by interferon (IFN)-γ (Murray, 2000). Likewise, monkeys controlling *L.* (*V.*) *braziliensis* infection developed parasite-specific Th1 immune responses (Teva et al., 2003). In the present study, the DTH reaction to leishmanin skin test (LST) was used as an in vivo correlate of cellular immunity. The LST induration size values (range, 5–13 mm) were larger during active infection (mean $\pm$ SD, 8.7 $\pm$ 3.6 mm) compared with values after drug-induced cure of the

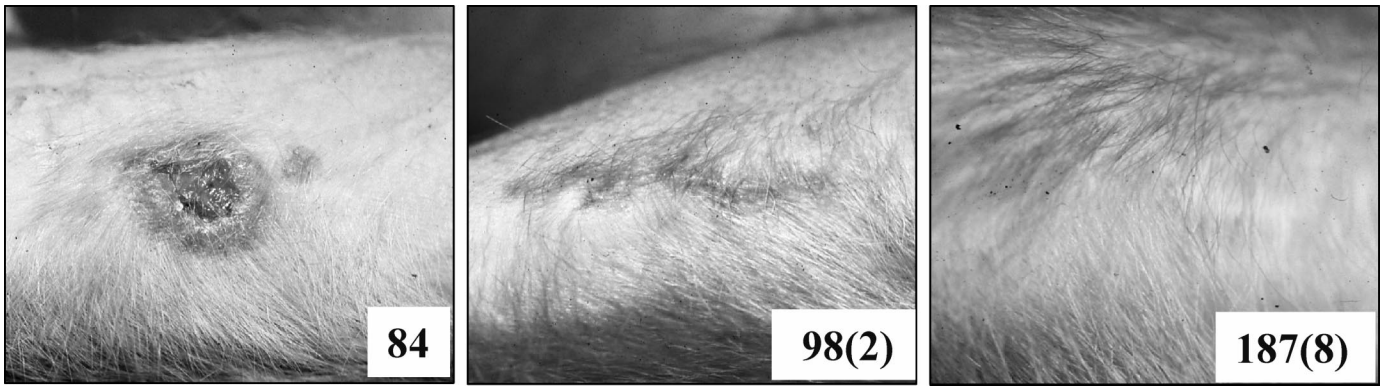

FIGURE 2. Effect of one 28-day course of pentavalent antimony (5 mg/kg body wt/day administered intramuscularly) on *Leishmania* (*Viannia*) *braziliensis* skin lesion development in a rhesus monkey (A2). The numbers are days after infection, whereas the numbers within parentheses are weeks after treatment was initiated.

disease (3.7 ± 2.7 mm). As illustrated in Figure 3, proliferative responses in vitro to soluble leishmanial antigens of peripheral blood leukocytes from infected animals were comparable before and after chemotherapy (mean ± SD of the stimulation index values, 57 ± 38 and 50 ± 37, respectively), but treated monkeys had significantly ($P > 0.005$) lower levels of parasite-specific IFN-γ secreted in culture (mean ± SD, 60 ± 50 pg/ml) compared with levels in untreated monkeys (223 ± 190 pg/ml). The lack of increase of specific T-cell responses obtained in drug-cured monkeys probably reflects the elimination of parasites at the site of infection.

Despite clinical healing, we were able to isolate *L.* (*V.*) *braziliensis* by the culture of biopsy specimens from skin scars of recovered monkeys. Likewise, *L.* (*V.*) *braziliensis* CL scars represented a site of parasite persistence after antimonial therapy and clinical cure (Schubach et al., 1998). The possibility of parasite persistence after clinical cure suggests that the immune response can control, but not fully eliminate, the infection. In experimental murine CL, increased polarization towards a Th2 cytokine profile before the onset of drug therapy leads to an increased frequency of relapse after treatment (Nabors and Farrel, 1996). Although IFN-γ is essential for activating macrophages to kill the pathogen, its action may be blocked or down-regulated by another mediator, the interleukin-10 produced by $CD4^+$ $CD25^+$ T cells, which is required for parasite persistence in the skin after healing and for maintenance of immunity to reinfection (Belkaid et al., 2002).

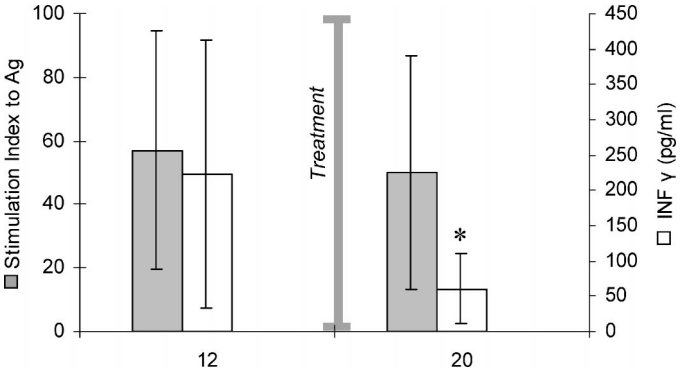

FIGURE 3. Parasite-specific lymphoproliferative and interferon (IFN)-γ responses in rhesus monkeys following primary infection with *Leishmania* (*Viannia*) *braziliensis* and after Glucantime® treatment (experiment 1). Purified peripheral blood leukocytes were adjusted to 2×10^6 cells/ml in complete RPMI medium and stimulated with soluble leishmanial antigens (10 μg/well) for 96 h at 37 C in 5% CO_2. Then, the cells were pulsed for the last 18 h of incubation with 0.5 μCi of [^{3}H]thymidine. Cell proliferation was assessed by measuring [^{3}H]thymidine incorporation; results are expressed as the stimulation index (SI; mean cpm of stimulated cultures/mean cpm of unstimulated cultures). Culture supernatants were collected from duplicate wells after 72 h of stimulation, and the concentration of IFN-γ in the supernatant was determined by ELISA as described previously (Teva et al., 2003). Data are the mean ± SD of seven experimental monkeys tested. *Significant difference ($P < 0.005$) in the specific IFN-γ response of drug-cured monkeys compared with that of untreated animals.

In conclusion, the data strongly support the utility of this primate model of *L.* (*V.*) *braziliensis* CL for drug development against the human disease. Therapeutic strategies and new antileishmanial compounds can be tested in monkeys under more controlled conditions than are possible in clinical studies.

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Kinetics of Antibodies and Antigens in Serum of Mice Experimentally Infected with *Echinostoma caproni* (Trematoda: Echinostomatidae)

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ABSTRACT: The present study reports on the kinetics of antibodies and antigens in serum of mice experimentally infected with 75 metacercariae of *Echinostoma caproni* during the first 12 wk postinfection (wpi). Antibody titers in the serum of mice were determined by an indirect enzyme-linked immunosorbent assay (ELISA) using excretory/secretory (ES) antigens of *E. caproni*. The early detection of antibodies against ES antigens of *E. caproni* is feasible using indirect ELISA. Mice developed significant antibody responses at 2 wpi, and the values progressively increased until the end of the experiment. This may be related to the intestinal absorption of adult worm antigens that induces humoral responses. The presence of *E. caproni* circulating antigens was determined by a capture ELISA based on polyclonal rabbit antibodies against ES antigens of *E. caproni*. High levels of seroantigens in mice were detected by 1–2 wpi, probably because of the local inflammatory responses in mice induced by the adult worms. A drop in circulating antigen levels was observed at 9 wpi, which could reflect changes in the intestinal tissues over the course of the infection.

Echinostoma caproni (Trematoda: Echinostomatidae) is an intestinal trematode that does not undergo tissue migration in its definitive host. This parasite has a wide range of experimental definitive hosts, but its compatibility differs considerably between rodent species. In hosts with high compatibility, such as hamsters, *E. caproni* induces chronic infections, whereas in hosts with low compatibility, such as rats, the worms are rapidly expelled (Toledo, Espert, Carpena et al., 2004). Because of such characteristics, the *E. caproni*/rodent systems are highly suitable models for elucidating aspects of the host-specific components that determine the course of infection with intestinal helminths.

The *E. caproni*/mouse model has often been used as an experimental system to study the immune response to intestinal trematode infections. Most of these studies have been limited to an analysis the host humoral response. The antibody response detected in the serum and affected tissues, such as small intestine, includes IgG, IgM, and IgA (Agger et al., 1993; Graczyk and Fried, 1995). Moreover, intestinal pathology induced by the parasite in the first several weeks postinfection (wpi) has been investigated (Weinstein and Fried, 1991; Fujino and Fried, 1993; Fujino et al., 1996). However, the facts that determine the development of chronic *E. caproni* infections in mice remain unknown. The recent development of capture enzyme-linked immunosorbent assay (ELISA) techniques to detect *E. caproni* antigens in feces and serum of infected rodents has allowed us to gain further insight regarding the immune response of this trematode infection (Toledo, Espert, Muñoz-Antoli et al., 2003, 2004). Toledo, Espert, Muñoz-Antoli et al. (2004) showed that the kinetics of *E. caproni* seroantigens differs markedly between rats and hamsters, and this could be related to the course of the infection in each host species. In the present study, we examined the kinetics of antibodies and antigens in serum of mice experimentally infected with *E. caproni*.

The strain of *E. caproni* used in the present study has been described previously by Fujino and Fried (1993). Encysted metacercariae of *E. caproni* were removed from the kidneys and pericardial cavities of experimentally infected *Biomphalaria glabrata* snails and used to infect ICR mice. Each of 10 male mice (weight, 32–40 g) was infected by stomach tube with 75 metacercariae of *E. caproni*. Moreover, 5 mice were left uninfected and used as controls. All the infected animals were maintained under conventional conditions with food and water ad libitum. The parasite egg release was investigated weekly in each of the infected animals as described by Toledo, Espert, Carpena et al. (2003). To obtain excretory/secretory (ES) antigens of *E. caproni*, we followed the methodology described by Toledo, Espert, Muñoz-Antoli et al. (2003). Blood was collected weekly from each infected and control animal by cardiac puncture under anesthesia. After clotting of the blood overnight at 4 C, serum was separated from the clot by centrifugation at 10,000 rpm for 10 min at room temperature. The antigens and serum samples were stored at −20 C until use.

To detect specific antibodies against *E. caproni* ES products, an indirect ELISA was carried out as described by Toledo, Espert, Muñoz-Antoli et al. (2003). The dilutions of serum samples and horse radish peroxidase-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, Missouri) used were 1:200 and 1:4,000, respectively. The presence of *E. caproni* ES products in serum from mice was evaluated by a polyclonal antibody-based capture ELISA as described by Toledo, Espert, Muñoz-Antoli et al. (2004). The dilutions of serum samples, biotinylated anti-*E. caproni* ES antibody, and labeled streptavidine-peroxidase (Sigma) were 1:20, 1:1,000, and 1:4,000, respectively. Before capture ELISA, the serum samples were heated to dissociate immune complexes (Toledo, Espert, Muñoz-Antoli et al., 2004).

Each ELISA assay was performed in triplicate, and the absorbance readings from wells with the same sample were expressed as the mean ± SD. The cutoff for each ELISA was defined as the mean of the samples from the control mice + 3SD. The difference between the optical density (OD) values for the infection and control specimens at

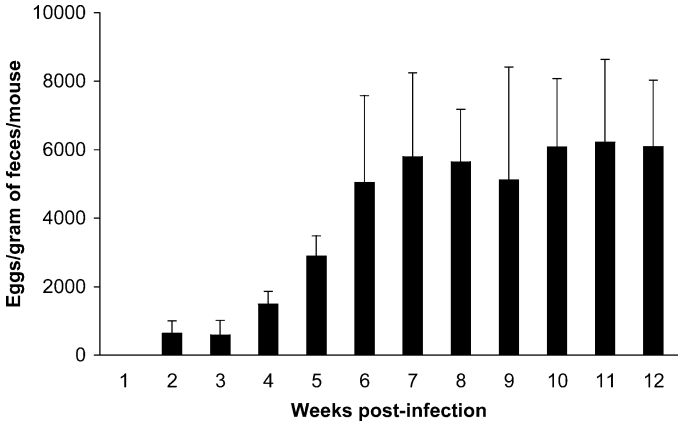

FIGURE 1. Mean values of egg per gram of feces in mice experimentally infected with *Echinostoma caproni* in each week during the experiment. Vertical bars represent the standard deviation.

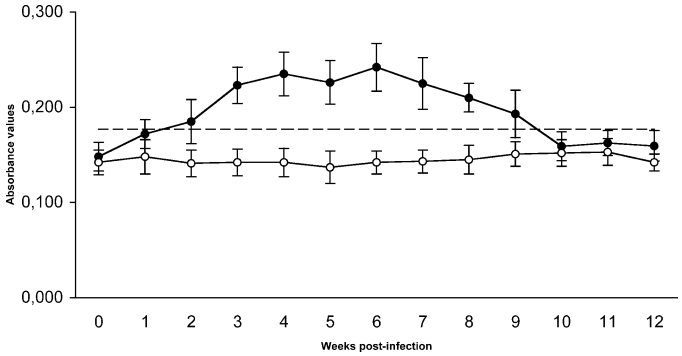

FIGURE 3. Detection of *Echinostoma caproni* excretory/secretory antigens in sera of experimentally infected mice. Mean optical density (OD) values of control (O) and infected (○) animals over the course of the experiment are shown. The cutoff point (—) was defined as the mean OD value of the controls + 3SD. Vertical bars represent the standard deviation.

each point in time was calculated and tested by the use of Student's *t*-test.

All the mice experimentally exposed to 75 metacercariae of *E. caproni* became infected. The duration of the prepatent period was uniform. Egg release began 9–12 (10.0 ± 0.4) days postinfection. The kinetics of egg release was monitored during the first 12 wpi (Fig. 1). Egg output progressively increased, then became relatively stable from 7 wpi, which allowed for a maximum egg release at 11 wpi. The maximum egg release was observed at 11 wpi. All the mice remained positive at the end of the experiment (12 wpi). The number of adult worms recovered per mouse ranged from 27–45 (36.3 ± 8.0).

The results showed that mice develop a significant antibody response against *E. caproni* ES antigens (Fig. 2). The cutoff point differentiating negative from positive sera was an OD value of 0.190. Antibodies to *E. caproni* ES antigens were detected in all infected mice at 2 wpi. Thereafter, antibody levels progressively increased, then were stable from 8 to 12 wpi. The mean OD value during the complete course of the experiment in all the infected mice was 0.408 ± 0.071, whereas the maximum OD value (1.094 ± 0.223) was observed at 10 wpi. Statistically significant differences ($P < 0.05$) between sera from control and infected mice were observed in each sample analyzed from 2 wpi until the end of the experiment.

The detection limit of the capture ELISA to *E. caproni* ES antigens in sample buffer was determined previously and found to be 3 ng/ml (Toledo, Espert, Muñoz-Antoli et al., 2003). The detection limit in sera from mice, determined following the method described by Toledo, Espert, Muñoz-Antoli et al. (2004), was 30 ng/ml. The kinetics of seroantigens in mice experimentally infected with *E. caproni* was followed from 0 to 12 wpi (Fig. 3). The cutoff value to differentiate positive from negative values was 0.177. Four (40%) of the infected mice were positive at seroantigen detection at 1 wpi, whereas at 2 wpi, all of them were positive on capture ELISA. The seroantigen values increased rapidly, reaching a period of high circulating antigen values from 3 to 6 wpi. Thereafter, the absorbance values gradually declined. From 9 wpi, the seroantigen values became negative in 2 (20%) of the infected mice. At 10 wpi, the values reverted to negative in the remainder of the mice. From 10 wpi onward, all the samples were negative. The seroantigen maximal response (0.242 ± 0.025) was observed at 6 wpi, and the mean OD value during the experiment was 0.199 ± 0.031. Statistically significant differences between infected and control mice ($P < 0.05$) were observed for each sample analyzed from 2 to 9 wpi.

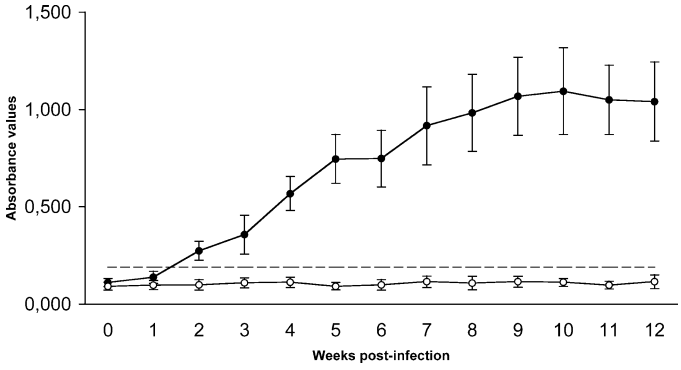

FIGURE 2. Detection of antibodies to *Echinostoma caproni* excretory/secretory antigens by indirect enzyme-linked immunosorbent assay (ELISA) in experimentally infected mice. Mean optical density (OD) values of control (O) and infected (○) animals over the course of the experiment are shown. The cutoff point (—) was defined as the mean OD value of the controls + 3SD. Vertical bars represent the standard deviation.

Mice show a high degree of compatibility with *E. caproni* on the basis of worm establishment and survival (Odaibo et al., 1988). The longevity of *E. caproni* in mice has not been determined exactly, but worms may survive for at least 29 wpi (Hosier and Fried, 1991). This is confirmed by the results obtained herein. The present study demonstrates that early detection of antibodies during *E. caproni* infections in mice is feasible using ES antigens. Positive levels of anti–*E. caproni* IgG were detected at 2 wpi, and the values increased progressively until the end of the experiment. Graczyk and Fried (1994) obtained similar results using glycocalyx membrane crude antigens. However, Graczyk and Fried also reported that antibodies peaked at 14–18 days postinfection, whereas in the present study, the antibody titers reached a maximum at 10 wpi. This may result from the different antigen used in each study. In contrast to these results, Agger et al. (1993) only detected positive levels of *E. caproni* IgG in serum of experimentally infected mice by 4 wpi using crude adult *E. caproni* antigen.

Echinostoma caproni induces systemic humoral responses in those host species in which chronic infections develop (mice and hamsters). In contrast, weaker responses are detected in host species in which the infection is lost earlier (Toledo, Espert, Muñoz-Antoli et al., 2004). The generation of systemic humoral responses in echinostome infections appears to relate to the intestinal absorption of antigens. Significant antibody responses have been detected concurrently with high circulating antigen levels (Toledo, Espert, Muñoz-Antoli et al., 2004). We have observed significant titers of circulating antigens from 1–2 to 9 wpi. The presence of circulating antigens has been demonstrated previously in hamsters experimentally infected with *E. caproni*, whereas low levels of *E. caproni* seroantigens have been observed in rats (Toledo, Espert, Muñoz-Antoli et al., 2004). The presence of circulating antigens has been associated with the intestinal inflammatory responses induced by the parasites. Under inflammatory conditions, maintenance of the epithelial barrier is disrupted, increasing the antigen uptake (Yu and Perdue, 2001). The intestine of mice infected with *E. caproni* has been found to be atrophied, with fused or eroded villi, crypt hyperplasia, and reduced alkaline phosphatase activity (Weinstein and Fried, 1991; Fu-

jino and Fried, 1993; Fujino et al., 1996). Furthermore, Brunet et al. (2000) demonstrated that response against *E. caproni* in mice during the first 3 wpi is consistent with a proinflammatory and Th1 cytokine pattern, with elevated levels of interferon-γ. This pattern in the first week postinfection appeared to be essential for the establishment of chronic infections. This can explain why significant antibody levels and seroantigens are detected only in those host species in which *E. caproni* produces chronic infections. The kinetics of circulating antigens shows a similar pattern in all the host/intestinal helminth combinations studied. After a period of high seroantigen titers, a drop occurs at 9–11 wpi (Avila et al., 2003; Toledo, Espert, Muñoz-Antoli et al., 2004). The drop in circulating antigens could be attributed to a decrease in worm burden. However, we have not observed a decrease in the egg output over the course of the infection, indicating that the worm burden was constant over the experiment. In fact, Hosier and Fried (1991) did not detect significant changes in the worm burden during the fist 24 wpi in mice experimentally infected with *E. caproni*. Furthermore, the decline in circulating antigens could relate to antigen-antibody complex formation, but in the present study, the dissociation of these immune complexes was accomplished by heat treatment before ELISA. With the present data, it is difficult to determine exactly the cause of the decrease in seroantigens observed in several rodent/intestinal helminth models, but it could relate to changes in intestinal inflammation during the later phases of the infection.

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Prevalence and Spatial Distribution of Intraerythrocytic Parasite(s) in Puget Sound Rockfish (*Sebastes emphaeus*) from the San Juan Archipelago, Washington (USA)

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ABSTRACT: Two morphologically distinct forms of an intraerythrocytic parasite(s) were detected by microscopic observation of Giemsa-stained blood films in 45.7% of 119 rockfish (*Sebastes emphaeus*) from the San Juan Archipelago (Washington State, U.S.A.). Infection prevalence for both forms was 53% in males, 44% in females, and 33% in fish of undetermined gender. A binucleate ''ring-stage'' was present at all 4 geographic sites, with a mean prevalence of 45.7%, while mean prevalence of a larger gamont-like form from the same sites was 5.1%. The relationship of the 2 forms to each other could not be determined. Neither schizogony nor binary fission was evident in any of the infected erythrocytes and the parasites contained no obvious pigment. The possibility of the 2 morphologic forms being 2 distinct species is supported by the observation that no difference in parasitemia was seen in the binucleate form among sites (1.6–1.9%), while parasitemia of the gamont-like form varied significantly among sites, ranging from a high of 4% to a low of 0.1%. Taxonomic status of either form could not be determined at this time based on limited existing morphologic data.

Rockfishes are an economically important group in the northeast Pacific Ocean and have experienced significant population declines in recent years (Love et al., 2002). Because of its small size, the Puget Sound

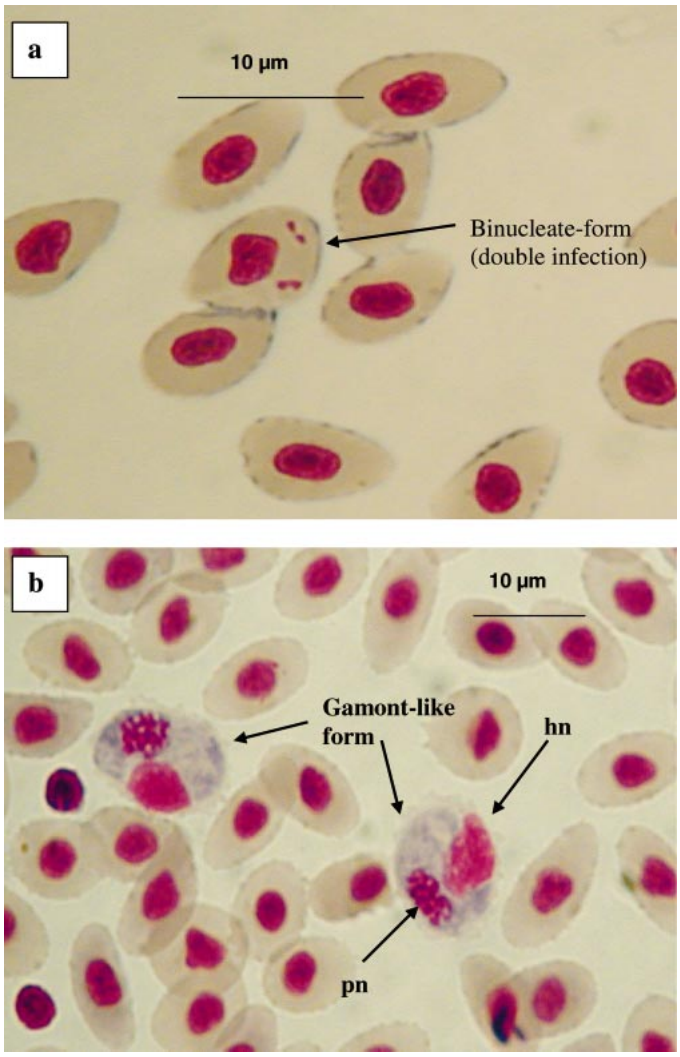

FIGURE 1. Binucleate form (**a**) and gamont-like form (**b**) from Puget Sound rockfish from the San Juan Archipelago, Washington (U.S.A.). pn, parasite nucleus; hn, host nucleus.

rockfish, *Sebastes emphaeus*, Starks 1911, is not a sport fish nor is it economically exploited, so little work has been done to characterize its parasite fauna (Moulton, 1975; Love et al., 2002). It is, however, the most prevalent rockfish species in the Strait of Georgia, possibly the result of dramatic declines in other rockfish and large predator species from the same area (Beckmann et al., 1998). Love et al. (2002) compiled a bibliography of common parasites from 47 species of northeast Pacific rockfish but listed only a single citation for an intraerythrocytic parasite, *Haemogregarina roelofsi* sp.n. (= *Desseria leptocotti*?), in the black rockfish, *S. melanops* Girard 1856 (Hill and Hendrickson, 1991). The authors of the original article state: ''Haematozoa are relatively rare in fishes of the northeast Pacific Ocean.'' However, in the present study, intraerythrocytic blood parasites were readily detected during routine microscopic assessment of Giemsa-stained blood films from Puget Sound rockfish in the San Juan Archipelago, Washington (U.S.A.) in 2003. The only other intraerythrocytic parasite of fish described from the San Juan Archipelago is an unnamed parasite from the spiny dogfish, *Squalus acanthias* L. (Clewly et al., 2002).

The objectives of this study were to determine: (1) the prevalence and intensity of infection in Puget Sound rockfish; (2) the differences in infection prevalence between males and females; and (3) the spatial distribution of the organism(s) within the San Juan Archipelago.

One-hundred and nineteen Puget Sound rockfish were collected by hook-and-line from 4 sites around San Juan Island and Shaw Island. Immediately after capture, the fish were placed into aerated ambient seawater and transported to the Friday Harbor Marine Laboratory (University of Washington) on San Juan Island, where they were placed in flowing seawater until necropsied; blood films were made within 24 hr of capture.

All fish were killed with an overdose of Tricaine methanesulfonate (MS 222) before necropsy. Length, weight, and sex were recorded from each specimen, and triplicate blood smears were made from blood obtained from the dorsal aorta near the caudal peduncle. Slides were air dried, fixed in absolute methanol, and stained in 7% Giemsa stain at pH 6.8 (Accustain GS 500). Stained slides were rinsed in flowing water, air dried, and examined by light microscopy at ×1,000 with an oil immersion lens. Prevalence was determined by assessing the number of infected individuals at each site, divided by the number of individuals examined at that site, while parasitemia (infection intensity) was established by determining the mean number of parasites present in 10^3 red blood cells examined. A standard 2 × 2 chi-square statistic with 1 df was used to compare infection prevalence differences between groups (Leaverton, 1978; Gordis, 2000). Representative voucher specimens (Giemsa-stained blood films) are deposited in the U.S. National Parasite Collection, U.S. Department of Agriculture (Beltsville, Maryland 20705) (USNPC #95252).

Two distinct morphologic forms of intraerythrocytic parasites were observed in infected fish from all sample sites. One form was binucleate, with faintly staining cytoplasm and resembled a ring-stage measuring ~2 μm in diameter (hereafter referred to as ''binucleate form'') (Fig. 1a). A second, larger gamont-like form measured ~10 μm × 4 μm, completely filled the host cell and displaced the host cell nucleus. Parasite cytoplasm stained pale blue and the nucleus appeared reticulated and stained darker than the host cell nucleus (Fig. 1b). No evidence of parasite schizogony or binary fission was observed.

Prevalence in all fish for both morphologic forms combined was 45.4 ± 8.9% for all sites. Prevalence was 53.3% (16 of 30) in males and 43.8% (35 of 80) in females. Three of 9 (33.3%) fish of undetermined gender were also infected (Table I). Prevalence for both forms ranged from a low of 25.8% at Point George to a high of 100% at Pile Point, with Point Caution and Broken Point being intermediate (Table I).

The mean prevalence for the binucleate form was 45.7% for all sites (range = 26.7–100%), while the mean prevalence for the gamont-like form was 5.1% for all sites (range = 3.3–11.1%) (Table II). No statistically significant difference in parasitemia of the binucleate form was observed between the highest and lowest sample sites, i.e., Pile Point (1.9%) and Broken Point (1.6%) ($\chi^2 = 0.11$; $P = 0.74$; n = 49). However, parasitemia for the gamont-like form was significantly higher at Point George (4%) than at Pile Point (0.1%), Broken Point (1%) and Point Caution (1.8%) ($\chi^2 = 18.8$; $P = 0.0001$).

Although blood parasites have been described from northeast Pacific Ocean rockfish, only one intraerythrocytic species has previously been reported (Hill and Hendrickson, 1991). During the present survey of Puget Sound rockfish, 2 distinct morphologic forms of intraerythrocytic parasites were observed in 45.4% of the fish sampled from 4 sites within the San Juan archipelago (Washington, U.S.A.). The parasite(s) appear to be widely distributed throughout the archipelago with prevalence ranging from 25.6% to 100%, with no difference in prevalence between males and females. The most commonly observed intraerythrocytic parasite form was a small binucleate ring stage, similar to one of the many multinucleate forms reported for species of *Haemohormidium* in American plaice (*Hippoglossoides platessoides*) (Siddall et al., 1994). However, unlike *Haemohormidium*, where 100% of infected fish were observed with mono- and tetranucleate forms and >60% had octonucleate forms, no mono-, tetra-, or octonucleate forms were observed in any of the infected Puget Sound rockfish. The second morphologic form (gamont-like form) observed in rockfish in the present study did not resemble any known hematozoan previously reported from fish (Davies and Johnson, 2000). This form completely filled the host cell cytoplasm distorting both the host cell and nucleus (Fig. 1b). No sexual dimorphism was observed and neither the binucleate forms nor gamont-like forms contained visible hematin pigment. At this point, it is not known if the 2 morphologic forms are different life stages of a single species or represent 2 distinct species; however, no intermediate stages between the 2 forms were observed.

The mode of transmission for these organisms is presently unknown, but it seems reasonable that a hematophagous invertebrate, such as a

TABLE I. Sample sites and infection prevalence for Puget Sound rockfish infected with unidentified intraerythrocytic parasites.

Site	GPS coordinates	Capture depth (m)	*n*	Sex	% Infected ± SE
Point Caution	4°33.192′N, 123°00.397′W	20–60	10	m	40.0
			24	f	41.7
			5	?	40.0
Total			39		41.0
Point George*	48°33.529′N, 122°59.323′W	50–75	2	m	50.0
			25	f	24.0
			4	?	25.0
Total			31		25.8
Broken Point	48°35.44′N, 123°57.56′W	30–100	17	m	58.8
			23	f	47.8
			0	?	—
Total			40		52.5
Pile Point†	48°29.823′N, 123°05.823′W	50–75	1	m	100
			8	f	100
			0	?	—
Total			9		100
Subtotal			30	m	53.3 ± 17.8‡
			80	f	43.8 ± 10.9
			9	?	33.3
Total			119	m + f + ?	45.4 ± 8.9

* Significantly lower than Point Caution or Broken Point ($\chi^2 = 5.6$; $P = 0.018$; $n = 70$, and, $\chi^2 = 4.9$; $P = 0.027$; $n = 71$).
† Sample size too small for meaningful statistical comparison.
‡ No significant difference between males and females ($\chi^2 = 0.466$; $P = 0.49$; $n = 110$).

marine leech, may be involved (Khan, 1980). Although the binucleate form of the parasite occurred in both mature erythrocytes and reticulocytes, no clinical signs of disease were observed in infected fish.

Numerous studies on the parasites of rockfish have been published, but none has included the Puget Sound rockfish and none has reported organisms similar those reported here (Love et al., 2002). Because Puget Sound rockfish have not been extensively studied and the prevalence and intensity of infection of these parasites was relatively high, their abundance and ease of detection suggests that they may be unique to Puget Sound rockfish or that they represent an emerging infection (as defined by the Centers for Disease Control, 2003) in rockfish of the San Juan Archipelago.

The difference in prevalence observed at the 4 collection locations may reflect physical or biological differences among sites that influence transmission of the parasite, i.e., the presence or absence of a suitable vector or the proportion of immune and susceptible rockfish at each site. The uniform level of parasitemia exhibited by the binucleate form at all sites suggests that transmission and susceptibility may be uniform throughout the collection sites. The significant difference in parasitemia between sites of the gamont-like form suggests that it is not related to the binucleate form or is perhaps being transmitted under the influence of different factors.

Controlled experimental infections and seasonal sampling are needed to clarify the relationship between the morphologic forms observed and to establish a mechanism for transmission.

TABLE II. Infection prevalence of the binucleate and gamont-like forms of intraerythrocytic parasite seen in Puget Sound rockfish from four sites in the San Juan Archipelago, Washington (USA).

Sample site	*n*	Binucleate form (%)	Gamont-like form (%)
Point Caution	39	43.7	5.2
Point George	31	26.7	3.3
Broken Point	40	52.5	5.0
Pile Point	9	100	11.1

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