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BRONCHOALVEOLAR LAVAGE AND PULMONARY HISTOPATHOLOGY IN HARP SEALS (*PHOCA GROENLANDICA*) EXPERIMENTALLY INFECTED WITH *OTOSTRONGYLUS CIRCUMLITUS*

Caroline Piché,[1] Lena Measures,[2] Christian Bédard,[1] and Stéphane Lair[1,3]

[1] Faculté de Médecine Vétérinaire, Université de Montréal, 3200 Sicotte St., St-Hyacinthe, Québec J2S 2M2, Canada
[2] Fisheries and Oceans Canada, Maurice Lamontagne Institute, 850 Route de la mer, Mont-Joli, Québec G5H 3Z4, Canada
[3] Corresponding author (email: stephane.lair@umontreal.ca)

ABSTRACT: The objective of this study was to characterize pathologic changes associated with experimental infection of harp seals (*Phoca groenlandica*) with the lungworm *Otostrongylus circumlitus* (Metastrongyloidea: Crenosomatidae). The leukocyte differential cell count in samples obtained by unguided bronchoalveolar lavage (BAL) and the intensity of the histologic lesions in the lungs were assessed in seven harp seals experimentally exposed to 300 infective, third-stage *O. circumlitus* larvae. Seven unexposed harp seals were used as controls. First-stage larvae were observed in the feces of three of the seven exposed seals at 38, 42, and 45 days postexposure (dpe). Adult nematodes were found in the right primary bronchi of two of these three seals at necropsy 53 dpe. Fifty-six BALs were performed on the 14 seals. No statistical difference was observed between the exposed and control seals and among the four sampling times in percentage of neutrophils and macrophages in the BAL fluid. A significant difference was observed between the exposed and control seal groups in the percentage of eosinophils ($P<0.0001$), the count of eosinophils having increased by a factor of 70.4 in exposed seals. Significant statistical differences were observed between exposed and control seals in intensity of interstitial inflammation ($P=0.001$), bronchitis ($P=0.02$), bronchiolitis ($P=0.04$), alveolitis ($P=0.03$), and interstitial granulomatous inflammation ($P=0.04$). Our findings showed that harp seals are susceptible to infection with *O. circumlitus*. However, parasitic infections were transient and of low intensity, at least under our experimental conditions.

Key words: Bronchoalveolar lavage, harp seal (*Phoca groenlandica*), lungworm, nematode, *Otostrongylus circumlitus*, pathology, pneumonia.

INTRODUCTION

Pneumonia is a common cause of morbidity and mortality in wild and captive pinnipeds (Sweeney, 1974; Howard, 1983; Van Der Kamp, 1987). Diseases of the respiratory system in seals and sea lions have been associated with various parasitic (Stroud, 1978; Howard, 1983; Bergeron et al., 1997b), bacterial, viral, and fungal agents (Gulland et al., 2001). Numerous bacterial species have been incriminated in pneumonia of pinnipeds (Howard, 1983), either as the primary etiologic agent or as opportunistic agents following viral infection (Howard, 1983; Gulland et al., 2001). Verminous pneumonia is a frequent cause of morbidity and mortality in marine mammals (Sweeney, 1974; Van Der Kamp, 1987). In pinnipeds, at least seven species of Metastrongyloidea are recognized. All but one of these nematodes belong to the Filaroididae family (genus *Parafilaroides*), whereas the genus *Otostrongylus* is classified in the Crenosomatidae family (Gosselin and Measures, 1997; Dailey, 2006). The most frequently reported lungworms in seals are species of *Parafilaroides* and *Otostrongylus circumlitus* (Stroud, 1978; Van Der Kamp, 1987). *Parafilaroides* spp., often referred to as the small parenchymal lungworm, is generally observed in the lumen of bronchioles and in pulmonary alveoli (Fleischman and Squire, 1970). The large lungworm, *O. circumlitus*, resides in the bronchi and in the bronchioles (Stroud, 1978; Onderka, 1989). *Otostrongylus circumlitus* is also reported in the pulmonary arteries and right ventricle

of northern elephant seals (*Mirounga angustirostris*; Gulland et al., 1997) and one California sea lion (*Zalophus californianus*; Kelly et al., 2005).

Infection of seals with *O. circumlitus* occurs after the ingestion of an obligate intermediate host containing infective third-stage larvae (Bergeron et al., 1997a). It is believed that these larvae reach the pulmonary tissue through the portal or lymphatic circulation and pulmonary arteries. Adult, female nematodes, which are ovoviviparous, release first-stage larvae into pulmonary tissues. Larvae passively move up the bronchial escalator by mucociliary action into the pharynx, where they are swallowed and finally excreted with feces (Measures, 2001).

Otostrongylus circumlitus has a Holarctic and circumpolar distribution and has been reported in many seal species, including harbor seal (*Phoca vitulina*; Gosselin et al., 1998), ringed seal (*Phoca hispida*; Onderka, 1989), grey seal (*Halichoerus grypus*; Baker, 1989), northern elephant seal (Stroud, 1978), and harp seal (*Phoca groenlandica*; Lucas et al., 2003). Infection with *O. circumlitus* has also been described in a California sea lion (Kelly et al., 2005). The prevalence and intensity of parasitic infections are highly variable between seal species and among studies (Measures, 2001). In general, the prevalence of infection with *O. circumlitus* is higher in young seals, especially young-of-the-year (YOY; Onderka, 1989). Infections of adult seals are observed less frequently. One study reported a prevalence of 81% in YOY ringed seals from eastern Arctic Canada, compared with an overall prevalence of 9.5% when all age groups were included (Bergeron et al., 1997b). In that study, the mean intensity of infection was 9.4 adult nematodes, with a range of one to 32 parasites per animal (Bergeron et al., 1997b); however, Measures and Gosselin (1994) reported 77 nematodes in one ringed seal. Overall prevalence in grey seals and in harbor seals from the Canadian east coast was reported as 5% and 6%, respectively, but in YOY, prevalence reached 82% (Gosselin et al., 1998).

Clinical signs reported in harbor seals infected with *O. circumlitus* are of variable intensity and include cough, dyspnea, anorexia, emaciation, dehydration, and depression (Vercruysse et al., 2003). Onderka (1989) suggested that infected seals may have shorter and more superficial dives compared with uninfected seals, which may lead to a reduced capacity to forage. A negative correlation between sternal blubber thickness and the intensity of *O. circumlitus* infection has been shown in ringed seals, suggesting a negative effect of this parasite on energy reserves (Bergeron et al., 1997b). Lesions reported with *O. circumlitus* ranged from excessive production of mucus in the airways to obstructive bronchitis as well as bronchiolitis (Measures and Gosselin, 1994). Bacterial concomitant infections are frequent and suppurative tracheobronchitis, and pulmonary abscesses have also been reported (Onderka, 1989).

Antemortem diagnosis of lungworm infection is based on the identification of first-stage larvae in bronchial mucus and in fresh feces using the Baermann technique (Measures, 2001). However, the absence of these larvae in sputum or feces cannot rule out a verminous pneumonia. Causes of false-negative Baermann results include sampling during the prepatent period (PPP), infection with parasites of the same sex or only one nematode, intermittent shedding of larvae or too few larvae being passed in feces to detect. An assay based on the detection of immunoglobulins against *O. circumlitus* has been recently developed for the northern elephant seal and for the Pacific harbor seal (*Phoca vitulina richardii*) but is not yet available commercially (Elson-Riggins et al., 2004). Bronchoalveolar lavage (BAL), which is a commonly used diagnostic method to assess pulmonary disease in domestic animals, has also been

used in pinnipeds. However, to the best of our knowledge, this technique has not been thoroughly described as a viable diagnostic tool for phocids in the veterinary literature.

The objective of this study was to characterize pulmonary changes in YOY harp seals associated with experimental infection with *O. circumlitus.* We were also interested in assessing the usefulness and safety of unguided BAL as an antemortem diagnostic procedure for this parasitic infection.

MATERIALS AND METHODS

Care of animals

This project was carried out according to animal care protocols approved by the animal care committees of the institutions involved in this project, both of which operate under the auspices of the Canadian Council on Animal Care. Fourteen recently weaned, female, harp seal pups, aged approximately 12 days, were live captured with scientific permit from Fisheries and Oceans Canada on the pack ice in the Gulf of St-Lawrence in March 2004 and transported by air to the Maurice Lamontagne Institute (Mont-Joli, Québec, Canada). The seals weighed, on average, 28.0±4.7 kg (mean±SD) during the experiment. The animals were kept in two indoor fiberglass tanks measuring 3.71 m×3.55 m×1.36 m, with a capacity of 17,900 l each, filled with salt water (flow rate, 20 l/min; salinity, 24–30%). Each tank had a haul-out ledge of 1.82 m×3.55 m. Thermoneutral conditions were maintained (air, 10 C; water, 0 C) with a complete air exchange every 5 min. Seals initially fasted in dry tanks to facilitate completion of the molting process. After 10 days, they were fed ad libitum daily, first with defrosted frozen shrimp, then capelin (*Mallotus villosus*) and herring (*Clupea harengus*). Vitamin and mineral supplements (SeaTab®, Pacific Research Laboratories, San Diego, California, USA) were given daily with fish as part of the diet.

Exposure to *Otostrongylus circumlitus*

Infective, third-stage larvae used in this study were obtained from eight American plaice (*Hippoglossoides platessoides*) infected in October 1999 with first-stage *O. circumlitus* larvae obtained from naturally infected ringed seals (handling of first-stage larvae as described by Bergeron et al., 1997b). Larvae developed to the infective third stage and remained encapsulated in American plaice until used in the present experiment in March 2004. Third-stage larvae were obtained from plaice as described by Bergeron et al. (1997b).

A double-blinded design was used in this experiment. Seals were divided into a control group (seals 1 to 7) and an exposed group (seals 8 to 14). Following a 12-hr fast, seals from the exposed group were given, by gastric intubation, 5 ml of saline containing approximately 300 *O. circumlitus* infective third-stage larvae. Control seals received 5 ml of saline by gastric intubation.

Sample collection

During the 10 wk of the experiment, each seal was anesthetized four times for the unguided BAL procedure. The first BAL was conducted just before exposure to *O. circumlitus* infective larvae, that is, on 0 day postexposure (dpe). The three other BALs were conducted 20, 34, and 53 dpe, respectively. The anesthesia protocol used has been described elsewhere (Pang et al., 2006). Briefly, food was withheld for 12 hr before anesthesia. Each seal was premedicated with 2 mg/kg of aminophylline (Hospira Healthcare, Saint-Laurent, Québec, Canada) intramuscularly. Midazolam hydrochloride (Versed® 5 mg/ml, Hoffmann-La Roche, Mississauga, Ontario, Canada) was administered (0.1–0.2 mg/kg) intramuscularly at the same time in selected animals. Mask induction with isoflurane (Aerrane®, Baxter, Mississauga, Ontario, Canada) using a handmade mask (semirigid, modified traffic cone) with a Bain nonrebreathing system was performed to achieve muscle relaxation. When obtained, the animal was intubated with a cuffed endotracheal tube of 6–7 mm internal diameter. Before intubation, endotracheal tubes were soaked in a solution of 1% w/v Virkon® (Vetoquinol, Lavaltrie, Québec, Canada) for 20 min and then rinsed with sterile saline. The BAL was performed after stabilization of vital signs and level of anesthesia. A sterile Sovereign® urinary catheter (external diameter, 2.7 mm; length, 56 cm; Sherwood Medical, St-Louis, Missouri, USA) was inserted into the endotracheal tube and gently advanced until resistance was encountered or up to full insertion of the catheter. A total of 9–17 ml of warm sterile saline was then slowly injected and immediately withdrawn using a 60-ml syringe catheter tip (Terumo, Elkton, Maryland, USA) directly attached to the urinary catheter. At the end of the lavage procedure, isoflurane was removed. Extuba-

tion followed return to spontaneous ventilation. Animals were returned to the tank after complete recovery from anesthesia. After the fourth BAL procedure, seals were euthanized using sodium pentobarbital (Euthanyl Strong, Bimeda-MTC Animal Health, Cambridge, Ontario, Canada) injected intravenously while still under general anesthesia. A complete necropsy was performed on each seal, including histopathology of major organs and tissues. Six samples of lung tissue were taken from each seal for histopathologic examination, three samples from each lung (from the cranial third, from the medial third, and from the caudal third). Sampled tissues were fixed in 10% neutral-buffered formalin and later processed using standard histologic techniques, including embedding in paraffin wax, cutting sections to 5–7 μm thick, and staining with hematoxylin, phloxine, and saffron (HPS; Luna, 1968). From 33 dpe to 53 dpe, feces of exposed seals were collected by gently inserting a lubricated soft plastic tube into the rectum. Feces were placed in a modified Baermann apparatus, as previously described (Bergeron et al., 1997b), and the presence of first-stage larvae was noted.

Sample preparation and evaluation

The BAL fluid was measured and characterized by color, turbidity, and presence of mucus based on a predetermined semiquantitative scoring system (score 0, completely clear, colorless fluid with mucus absent; score 1, almost completely clear, slightly whitish fluid, with light mucus; score 2, partially cloudy, moderately whitish fluid, with moderate mucus; score 3, completely cloudy, white fluid, with abundant mucus).

The fluid obtained was poured into a 3 ml (7%) and a 7 ml (15%) ethylenediaminetetraacetic acid (EDTA) K_3 tubes (Monoject®, Tyco Health Care Group, Mansfield, Massachusetts, USA). Two to three drops of autologous serum were added to the 3 ml of bronchoalveolar fluid, which was then refrigerated within 1 hr of collection. The 3-ml tube was used to prepare two 200-μl cytospins (Cytospin2®, Shandon Southern Instruments, Sewickley, Pennsylvania, USA; Zinkl, 2002) and centrifuged at 157 × G for 10 min within 12–36 hr of collection. Within 2 hr of collection, the 7-ml tube was used to measure the BAL fluid density with a refractometer (Leica TS Meter Refractometer®, Leica Microsystems, Buffalo, New York, USA). The fluid was then centrifuged (Dynac II centrifuge, BD, Parsippany, New Jersey, USA) in a tube with a conical base (Sarstedt, Newton, North Carolina, USA) at 179 × G for 10 min. The supernatant was removed and one drop of the sediment, taken with a 1-ml glass Pasteur pipette (Wheaton, Millville, New Jersey, USA), was placed on a slide to prepare a line smear (Zinkl, 2002). Four to six line smears were prepared from each sample. The cytospins and the line smears were stained with a modified Wright stain (7120 Aerospray® slide stainer, Wescor, Logan, Utah, USA). Microscopic examination of the BAL fluid was performed by the same observer (C.P.), who was blinded for the seal and the period at which the sample was taken. Each BAL sample was scored for the presence of inflammatory cells and noninflammatory cells (squamous and ciliated columnar cells) on line smears (10 random fields; magnification, 10×). The smears were scored as follows: 0 =absent; 1=cells on one field; 2=cells on two to five fields; 3=cells on six to ten fields; 4= numerous cells in every field. Differential leukocyte counts were determined manually on cytospins or line smears by counting 200 cells when available. If fewer than 200 cells were observed, the number of cells counted was recorded. Samples containing fewer than 15 cells were rejected. The presence of phagocytized bacteria, microscopic mucus, and Curschmann's spirals (coiled mucinous fibrils) were recorded.

Histopathologic examination of all six samples of lung tissue was performed by two blinded observers (C.P. and S.L.). When results differed between observers, slides were reexamined jointly until a consensus was reached. Each slide was assessed for the presence of pleuritis, interstitial inflammation, presence of mucus in the airways, bronchitis, bronchiolitis, bronchial glandular hyperplasia, alveolar atelectasia, alveolitis, and interstitial granulomatous inflammation. The scoring system used for this evaluation is presented in Table 1. The presence of larvae in tissue sections was also assessed. For each lesion category, the sum of all pulmonary tissue scores was calculated and used for statistical analysis.

Statistical analysis

Repeated-measure analysis of variance (ANOVA), with period as a within-subject factor and group as a between-subject factor, was used to evaluate statistically the percentage of BAL fluid recovery, the fluid density, and the percentage of neutrophils and macrophages in the differential leukocyte counts. A priori contrast was used to examine differences between groups at each sampling time. The

TABLE 1. Scores used in the semiquantitative evaluation of histopathologic changes in the lungs of harp seals experimentally infected with *Otostrongylus circumlitus* and in control seals.

Histopathologic lesions	Scores				
	0	1	2	3	4
Pleuritis	Absent	Focal	Multifocal	Extensive	nu[c]
Interstitial inflammation	Absent	Focal	Multifocal	Extensive	nu
Granulomatous inflammation	Absent	Focal	Multifocal	Extensive	nu
Presence of mucus in the airways (%)[a]	0	1–25	25–50	>50	nu
Bronchitis (%)[a]	0	1–25	25–50	>50	nu
Bronchiolitis (%)[a]	0	1–25	25–50	>50	nu
Bronchial glandular hyperplasia (%)[a]	0	1–25	25–50	>50	nu
Alveolar atelectasia (%)[b]	0	1–10	10–30	30–50	>50
Alveolitis (%)[a]	0	1–25	25–50	50–75	>75

[a] Percentage of the examined structure (bronchi, bronchioles, or alveoli) with histopathologic lesions.

[b] Percentage of surface area with atelectasia.

[c] nu = not used.

percentage of eosinophils and lymphocytes observed in the differential leukocyte counts were evaluated with a binomial regression model, with period as a within-subject factor, group as a between-subject factor, and number of available cells as an offset. A Cochran-Mantel-Haenszel statistical test was used for the ordinally scaled variables (BAL fluid color, turbidity, presence of mucus, and cell scores) to examine whether the average score within each group differed at each sampling and between groups. For prevalence variables, such as the presence of phagocytized bacteria, microscopic mucus, and Curschmann's spirals, an exact chi-square statistic test was used. A Wilcoxon two-sample test was used to compare the median score among groups for each histopathologic lesion category. A $P<0.05$ was considered to be statistically significant. Statistical analyses were performed using SAS version 9.1 (SAS Institute, Cary, North Carolina, USA).

RESULTS

Infection with *Otostrongylus circumlitus*

Infection was confirmed in three of the seven harp seals (seals 10, 11, and 12) experimentally exposed to infective larvae of *O. circumlitus*. First-stage larvae were found in the feces of these three animals at 42, 38, and 45 dpe, respectively. Adult nematodes (two males and two females) were found at necropsy in the lumen of the right primary bronchi of seal 11 and 12 (Fig. 1), but none was found in seal 10. Neither larvae in feces nor adult nematodes were observed in the remaining four exposed seals (seals 8, 9, 13, and 14). Seal 9 was euthanized 5 days before the end of the experiment because of a subcutaneous abscess unrelated to exposure to *O. circumlitus*.

Bronchoalveolar lavage

On average, 56.6±18.2% (mean±SD) of the injected BAL fluid was recovered. BAL fluids were generally colorless to slightly whitish, clear to slightly cloudy, and often contained a small quantity of mucus. The gross appearance of the BAL fluid did not differ between exposed and control animals or among sampling times. The density of BAL fluid was greater in exposed seals (mean±SD, 1.0058±0.0007) compared with control seals (mean±SD, 1.0053±0.0005) with all sampling times combined ($P=0.03$).

In control seals, the mean leukocyte score was higher 34 dpe compared with the baseline value from 0 dpe ($P=0.04$). In exposed seals, no significant difference was found in the leukocyte score between sampling times. The mean noninflammatory cell score was lower at 20 dpe compared with the baseline value from 0 dpe in exposed seals ($P=0.048$) but did not vary over time in control seals. No statistically significant differences were observed between control and exposed

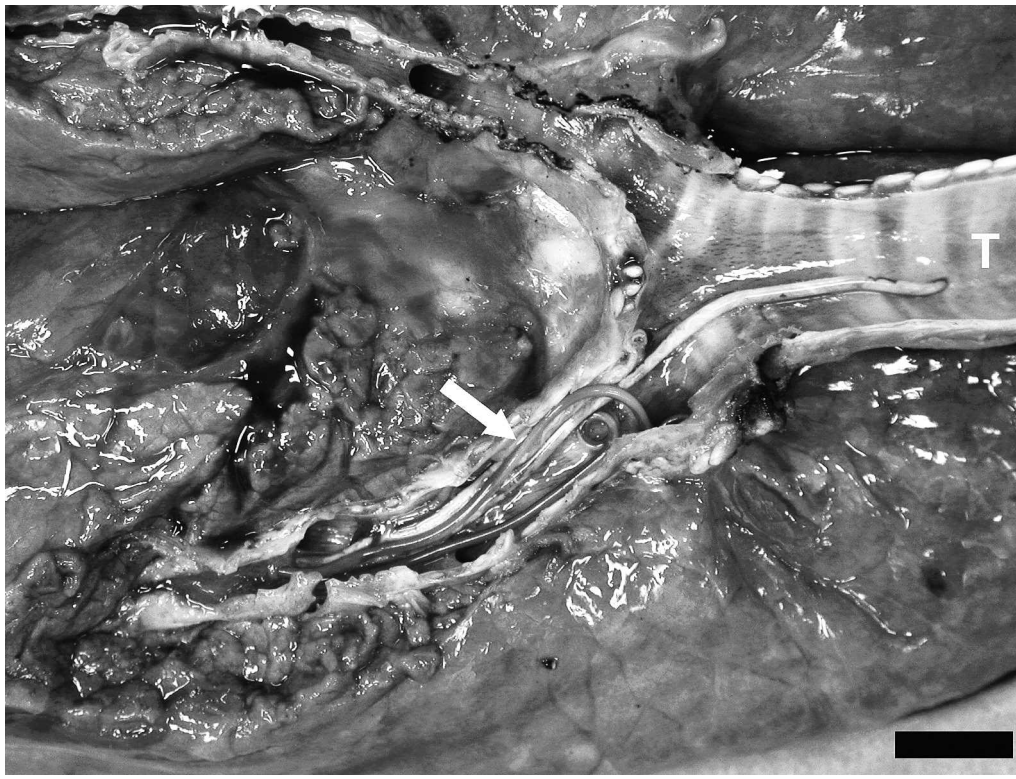

FIGURE 1. Ventral aspect of the lung from a harp seal (seal 12) experimentally infected with *Otostrongylus circumlitus*. Adult nematodes (arrow) are visible in the opened primary bronchus and extend into the tracheal lumen (T). Bar=1 cm.

seal groups for the presence of phagocytized bacteria ($P=0.26$), microscopic mucus ($P=0.35$), and Curschmann's spirals ($P=0.37$) with all sampling times combined.

Eight of the 56 BAL samples, five of which came from the control group, were excluded from the leukocyte differential cell count because of low inflammatory cell density (Table 2). Percentages of neutrophils, macrophages, and lymphocytes for each group on each sampling day are presented in Table 3. The only statistically significant difference between the BAL of the exposed and the control groups was the percentage of lymphocytes at 20 dpe ($P<0.01$; Table 3). The large proportion of BAL samples without eosinophils (30 of 48 of the samples) precluded comparison of the percentage of eosinophils between exposed and control seals at each sampling time. Therefore, all samples were combined based on exposure or not to *O. circumlitus*. A significant difference was observed between the exposed and nonexposed seals (nonexposed seals in this case were the exposed seal group at 0 dpe, i.e., before exposure, and the control seal group) in the percentage of eosinophils ($P<0.0001$), the count of eosinophils having increased in exposed seals by a factor of 70.4. The percentages of eosinophils in all BAL samples from the nonexposed seals ranged between 0% and 1%. All seven seals exposed to *O. circumlitus* larvae had at least one value of eosinophils equal or greater than 5% at one or more postexposure BAL sampling times, except seal 9 (Table 2).

Lung pathology

The summary of the lesions observed at necropsy are presented in Table 4. Le-

TABLE 2. Differential counts expressed as the percentage of eosinophils from bronchoalveolar lavage fluid obtained from seals exposed to *Otostrongylus circumlitus* larvae ($n=7$) and control seals ($n=7$).

Seal no.	Exposure group	% of eosinophils[a]			
		Preexposure	Day 20	Day 34	Day 53
1	Control	0	1	0	0
2	Control	N/A	N/A	0	0
3	Control	0	1	1	0
4	Control	0	0	0	0
5	Control	N/A	0	0	0
6	Control	0	N/A	0	0
7	Control	1	N/A	1	0
8	Exposed	0	0	7	0
9	Exposed	0	0	0	0
10[b]	Exposed	0	11	7	0
11[b]	Exposed	N/A	37	89	7
12[b]	Exposed	1	N/A	7	10
13	Exposed	0	0	19	72
14	Exposed	0	N/A	6	5

[a] N/A=no data available for that sampling time because cellularity of the samples too low.

[b] First-stage larvae detected in feces of these seals.

sions of bronchitis were characterized by the presence of a variable quantity of mucus, frequently infiltrated by granulocytes, in the bronchiolar lumens and by a mild to moderate infiltration of the bronchial mucosa and submucosa by a mixed population of inflammatory cells composed of lymphocytes, small macrophages, and granulocytes. Hyperplasic bronchial glands were formed of swollen, tall, cuboidal epithelial cells centered on markedly dilated lumens filled with mucus. The lesions of obstructive bronchitis observed in two animals (seals 11 and 12) were associated with marked glandular hyperplasia and complete occlusion of the bronchial lumen by a large quantity of mucus containing a large number of

TABLE 3. Differential counts expressed as the percentage of neutrophils, macrophages, and lymphocytes from bronchoalveolar lavage fluid obtained from seals exposed to *Otostrongylus circumlitus* larvae ($n=7$) and control seals ($n=7$).[a]

Leukocytes	No. of days postexposure	Exposed seals		Control seals		*P* values
		Mean±SD (%)	Min.–max.	Mean±SD (%)	Min.–max.	
Macrophages	0	30.9±24.6	6–63	35.6±24.5	17–71	0.95
	20	22.1±11.0	5–33	44.3±37.6	1–80	0.16
	34	22.8±20.6	5–63	30.9±18.1	8–57	0.59
	53	45.0±40.4	6–100	44.8±30.2	6–89	0.99
Neutrophils	0	66.3±25.3	35–94	62.0±27.2	2–81	0.93
	20	67.8±17.8	40–84	52.1±35.7	16–92	0.31
	34	52.8±29.7	5–82	61.3±17.6	37–88	0.59
	53	39.3±40.2	0–87	53.3±31.0	11–94	0.38
Lymphocytes	0	2.7±3.0	0–7	2.1±3.1	0–8	0.73
	20	0.6±1.1	0–3	3.25±3.1	0–8	<0.01[b]
	34	5.4±4.1	1–13	7.6±7.7	0–23	0.45
	53	2.4±3.2	0–8	2.0±2.9	0–8	0.94

[a] Min.=minimum; max=maximum.

[b] Significant difference between control and exposed seals.

TABLE 4. Intensity of different histopathologic pulmonary lesions (sum of the pulmonary histopathologic scores for each of the sections examined) observed in seals exposed to *Otostrongylus circumlitus* larvae ($n=7$) and control seals ($n=7$).[a]

Histopathologic lesions	Control		Exposed		*P* values
	Median	Min.–max.	Median	Min–max	
Pleuritis	0	0–0	0	0–2	0.09
Interstitial inflammation	0	0–2	6	3–16	0.001[b]
Presence of mucus in the airways	0	0–1	1	0–8	0.07
Bronchitis	0	0–0	1	0–11	0.02[b]
Bronchial glandular hyperplasia	0	0–1	0	0–5	0.23
Bronchiolitis	0	0–1	1	0–6	0.04[b]
Alveolar atelectasia	0	0–0	0	0–2	0.09
Alveolitis	7	4–15	12	7–24	0.03[b]
Granulomatous inflammation	0	0–0	0	0–5	0.04[b]

[a] Min.=minimum; max.=maximum.

[b] Significant difference between control and exposed seals.

granulocytes (neutrophils or eosinophils or both). The pulmonary parenchyma surrounding the occluded bronchi was extensively collapsed (Fig. 2). The alveolitis, which was characterized by a mild to moderate multifocal infiltration of the alveolar lumen with a small number of foamy macrophages, neutrophils, and eosinophils, was usually observed throughout the six sections of lung tissue examined and generally more extensive in exposed seals than in control seals. In one of the exposed seals (seal 12), the alveolar cellular infiltrate was focally extensive and associated with fibrinous material and a marked focally extensive hyperplasia of type II pneumocytes. The granulomatous inflammatory changes, which were present in two of the exposed seals (seals 11 and 12), were formed of well-demarcated and small, interstitial aggregates of macrophages, eosinophils, and lymphocytes, and multinucleated cells, and in one of the two seals (seal 11), multinucleated cells were often centered on cross-sections of larval nematodes surrounded by degenerate eosinophils (Fig. 3). Statis-

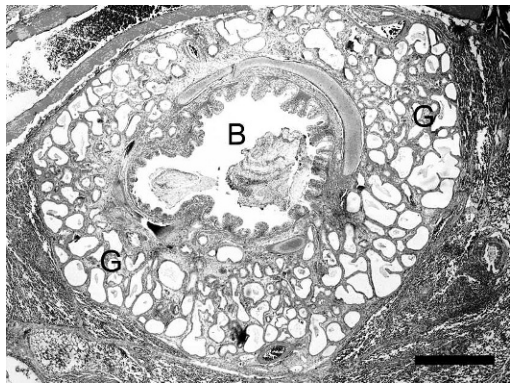

FIGURE 2. Histologic section of lung from a harp seal (seal 11) experimentally infected with *Otostrongylus circumlitus*. Note the extensive hyperplasia of the bronchial glands (G). The lumen of the bronchus (B) contains a large amount of mucus infiltrated by numerous neutrophils and eosinophils. Hematoxylin-phloxine-saffron stain. Bar=1 mm.

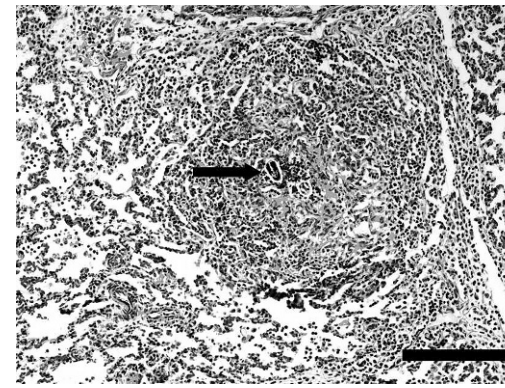

FIGURE 3. Histologic section of lung from a harp seal (seal 11) experimentally infected with *Otostrongylus circumlitus*. Note granulomatous inflammatory reaction surrounding a larval *O. circumlitus* (arrow). Hematoxylin-phloxine-saffron stain. Bar=200 μm.

TABLE 5. Predominant inflammatory cells in antemortem bronchoalveolar lavage (BAL) fluid (53 days postexposure) and histopathologic examination (histopathology) of lung tissue sections from exposed and control seal.

	Predominant inflammatory cell	No. of antemortem BAL cases			
		Macrophage	Neutrophil	Eosinophil	Total
Histopathology	Macrophage	7	5	1	13
	Neutrophil	0	0	0	0
	Eosinophil	0	1	0	1
	Total	7	6	1	14

tically significant differences were detected between exposed and control seals in the severity of interstitial inflammation, bronchitis, bronchiolitis, alveolitis, and interstitial granulomatous inflammation (Table 4).

Histopathologic examination of other organs, such as liver and thoracic lymph nodes, failed to detect larvae of nematodes. Marked tracheobronchial lymphadenomegaly was, however, observed in all the exposed seals, except seals 9 and 14. Concordance between the predominant type of leukocytes observed in the BAL fluid, collected just before euthanasia at 53 dpe, and the predominant type of leukocyte observed in the respiratory airways on histologic examination are presented in Table 5.

DISCUSSION

We successfully induced verminous pneumonia in at least three of the seven harp seals exposed to infective, third-stage *O. circumlitus* larvae obtained from experimentally infected American plaice. We are confident that the source of these infections is the administered larvae because the experimental animals were brought into captivity shortly after weaning and during their natural fasting period (Lavigne and Kovacs, 1988). Vertical transmission in utero or during lactation is unlikely because no infection with *O. circumlitus* has been reported in seals younger than 3.5 mo old (Onderka, 1989). In addition, all food given to seals during this study was frozen, which destroys larvae in invertebrates or fish tissue (Measures, 2001).

We performed 56 BALs without complication or mortality. This shows that this diagnostic procedure can be safely performed using the technique described here, at least in healthy seals or seals with only moderate pulmonary diseases. For BAL, we elected to inject a small amount of saline in only one aliquot to improve the safety of the lavage and to reduce the duration of the procedure. However, a posteriori, by extrapolating recommendations for dogs (Burkhard et al., 2001), a total of 140 ml of saline (on average) could have been used safely in our seals. Thus, the injection of 40 to 55 ml, if the lavage was repeated three times, or 30 ml if the lavage was repeated five times, could yield a good quality lavage. Five aliquots of 30 ml each were used in the BALs of three species of pinnipeds by Spragg et al. (2004).

In our study, percentages of neutrophils and macrophages were not different between exposed and control groups (Table 3). This suggests either that exposure to this parasite does not result in significant variation of these two types of leukocytes in the BAL fluid or that the number of seals from the exposed group that developed verminous pneumonia was too low to detect a statistically significant difference. In addition, there were no significant differences in the percentages of neutrophils and macrophages by the time of sampling, with all groups com-

bined. Based on this finding, it seems that repeating the BAL at 2-wk to 3-wk intervals does not significantly influence percentages of these two cell types, which are the two most abundant leukocytes in BAL fluid.

We did not observe age-related differences in leukocyte differentials. This differs from piglets, in which an age-related increase of neutrophils in the BAL fluid during the first 10 wk of age has been reported (Jolie et al., 2000).

In our study, percentages of neutrophils present in the BAL fluid of control seals were higher than reference ranges reported for domestic species, including dogs, cats (Cowell et al., 1998), and horses (Fogarty, 1990). To our knowledge, only one study reported reference ranges of leukocyte differential counts in BAL fluid from pinnipeds (Spragg et al., 2004). Percentages of neutrophils obtained from three California sea lions, five northern elephant seals, and one harbor seal were (mean±SD) 0.4±0.4%, 7.0±2.6%, and 4%, respectively (Spragg et al., 2004). The relatively high percentages of neutrophils observed in our study may be attributed, at least in part, to the segment of the respiratory tree sampled via a blind BAL, which is not necessarily homogeneous from one sample to another. It is known that higher percentages of neutrophils should be expected in the proximal respiratory tract (trachea), compared with the distal airways (Mair et al., 1987). The high percentages of neutrophils observed in our control seals, as well as the presence of numerous squamous and ciliated epithelial cells, suggest that blind placement of the catheter did not prevent saline injection into the tracheal lumen, and therefore, cells in the recovered BAL fluid may be more representative of the tracheobronchial region. Only one aliquot of saline was injected, which might also account for the higher percentage of neutrophils observed (King, 2004). Indoor environmental conditions, including air and water quality, could also play a role in the unusually high percentage of neutrophils observed in our study because exposure to aerosolized particles is known to be a cause of neutrophil increase in BAL fluid (Mair et al., 1987; Jolie et al., 2000). Interestingly, histopathologic examination of lung tissues revealed a mild alveolar infiltration by leukocytes in both control seals and exposed seals. These lesions of diffuse, low-grade alveolitis in our experimental animals support the possibility of exposure to a low level of environmental irritants.

Most of the seals exposed to *O. circumlitus* larvae had a statistically higher percentage of eosinophils in BAL fluid compared with that from control seals (Table 2). Consequently, exposure to *O. circumlitus* larvae was associated with a relative increase of eosinophils in the airways of exposed seals. This finding is not surprising given that parasitic infection of tissues is commonly associated with accumulation of eosinophils in affected tissues (Williams, 2004). Our results suggest that, in a clinical context, verminous pneumonia should be considered in a seal in which the percentage of eosinophils in the BAL fluid is higher than 2%. However, because other nonparasitic conditions, such as allergic reactions, eosinophilic granulomas, and fungal infections, can also be associated with an increase in the number of eosinophils, this finding is not specific to verminous pneumonia (Burkhard et al., 2001). Percentages of eosinophils greater than 7% were observed in at least two postexposure BAL fluid samples in three seals that passed larvae in their feces. A transient increased percentage of eosinophils in the BAL fluid was documented in the seal (seal 10) that had a positive Baermann test for the presence of larvae in the feces but no observed adult lungworm during the postmortem examination. Increased percentages of eosinophils in the BAL fluid were also observed in three of the other exposed seals (seals 8, 13, and 14) in which neither larvae in feces nor adult lungworms in the airways were

observed. The remaining exposed seal (seal 9) did not show an increase in the percentage of eosinophils in any BAL fluid samples. Interestingly, regurgitation occurred in seals 9 and 14 just after administration of infective larvae by gastric intubation followed by a saline injection of about 5 ml to rinse the tube. The possible expulsion of a small portion of the administered larvae could account for the lack or the low intensity of infection in those two seals. These observations suggest that at least four seals (seals 8, 10, 13, and 14) had a transient infection of *O. circumlitus* of short duration and eliminated the infection before the end of the experiment. If we assume that the infectivity of the larvae used in this study is similar to the infectivity encountered in naturally infected fish, our results suggest that harp seals are susceptible to experimental infection with *O. circumlitus* but that they are capable of limiting infections to a transient nature (i.e., of short duration) or to low-intensity infections with associated mild or subclinical disease. Harp seals may be generally refractory to infection with *O. circumlitus*, which is supported by field observations in YOY harp seals (Gosselin et al., 1998; Measures, 2001; Lucas et al., 2003).

Gross and histopathologic lesions observed in seals with active infections were similar to those described in ringed seals with natural infections (Onderka, 1989) and other verminous pneumonia in domestic animals (Stockdale and Hulland, 1970) and wild canids (Bourque et al., 2005). The main pathologic feature of these infections is the presence of focal to multifocal bronchitis usually associated with a marked glandular hyperplasia most likely induced by the irritating or immunogenic nature of these parasites. The hypersecretion of bronchial and bronchiolar glands results in the obliteration of the bronchiolar lumen preventing ventilation of the associated pulmonary lobule and causing the well-demarcated areas of lobular atelectasia observed grossly in these seals in the present study.

Otostrongylus circumlitus infections observed in our study were limited to the airways and pulmonary interstitial tissues. Neither parasites nor lesions were observed in the cardiovascular system. This distribution is similar to that reported in ringed seals in which immature *O. circumlitus* were found in blood vessels in only two of the 39 seals with *O. circumlitus* infections (Onderka, 1989), but this differs from natural infections seen in northern elephant seals in which immature, adult, female *O. circumlitus* were often present in the right cardiac ventricle and the lumen of the pulmonary arteries (Gulland et al., 1997).

There was 50% agreement between results of antemortem BAL and those of histopathologic examination of lung tissue sections with respect to the predominant cellular infiltrate in the airways of seven of the 14 seals (Table 5). The 50% disagreement may be explained by the relatively limited volume of pulmonary tissue examined histologically, which may not be representative of the entire organ (Norris et al., 2002). The BAL has the advantage of more easily sampling a larger surface and multiple regions of the lungs (Norris et al., 2002). Therefore, BAL and pulmonary histopathology can be complementary diagnostic tools used to diagnose pulmonary diseases (Norris et al., 2002), albeit the former is an antemortem and the latter is usually a postmortem method.

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