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CHARACTERIZATION OF *PNEUMOCYSTIS CARINII* INFECTION IN *SOREX ARANEUS* FROM SOUTHERN FINLAND

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ABSTRACT: Histologic and quantitative techniques were compared in an evaluation of the intensity of *Pneumocystis carinii* infection in common shrews (*Sorex araneus*) at Espoo, southern Finland, from September 1992 to May 1993. The histological scores were comparable to the results of the cyst count technique. The number of *P. carinii* cysts found in common shrews was low compared to those reported by others in clinically ill laboratory rats. The inflammatory changes detected in the lung sections had no significant relation to the presence of *P. carinii* infection.

Key words: *Pneumocystis carinii*, *Sorex araneus*, histology, cyst count, histopathology.

INTRODUCTION

Pneumocystis carinii is a pulmonary pathogen causing a fatal pneumonia in immunocompromised hosts (Hopkin, 1991). Subclinical *P. carinii* infection occurs in apparently healthy laboratory rats. The infection develops into pneumonia 6 to 8 wk after administration of corticosteroids (Walzer et al., 1980). *Pneumocystis carinii* infection also has been provoked in laboratory rats by dietary protein deprivation alone (Hughes et al., 1974). Pulmonary infestation by *Pneumocystis* sp. has been observed in various wild mammal species (Settnes and Lodal, 1980; Shiota et al., 1986). More recently, Laakkonen et al. (1993) reported a high prevalence of *P. carinii* in wild populations of common shrews (*Sorex araneus*) as compared to other wild mammals, but no signs of *P. carinii* pneumonia (PCP) were detected. Since the intensity of low-grade *P. carinii* infection is difficult to assess by histological examination, we evaluated the organism burden of common shrews by comparing the scores of histological and cyst count methods. For comparison with the common shrews, we used the cyst count method to assess the intensity of *P. carinii* infection in field voles (*Microtus agrestis*), which have a relatively high prevalence of *P. carinii* in late fall in Finland (J. Laakkonen, unpubl.). Finally, we examined the lungs of *S. araneus* histopathologically for possible inflammatory changes. Since the respiratory rates of shrews are high, even minor inflammatory process may impair the gas exchange of infected shrews. The objective of these analyses was to determine whether the frequent pulmonary infestation by pneumocystis in *S. araneus* is harmful to the apparently healthy host.

MATERIALS AND METHODS

Ninety-two common shrews were collected at Espoo (60°14′N, 24°46′E), southern Finland, during several trapping periods from September 1992 to May 1993. Snap-traps baited with cheese were checked at 6-hr intervals; shrews were taken immediately after trapping to the laboratory for necropsy. The shrews were classified as adults and juveniles according to tooth wear and condition of the pelage (Crowcroft, 1957).

Lungs of 44 shrews were assessed histologically. Lung tissue first was fixed in 10% buffered formalin; the right lobes (cranial, medial, caudal and accessory) of each sample were treated separately from the only lung lobe on the left side. Fixed tissues were dehydrated, embedded in paraffin and sectioned at 5 μm. Ten horizontal sections of each sample were cut at 20 μm intervals so that all lobes were represented, placed on one slide in the order cut, and stained with Grocott's modification of Gomori's Methenamine Silver (GMS) stain (Grocott, 1955). Using a blind study, slides were examined by light microscopy at 200× and 400×. The following scoring system was used to evaluate the intensity of *P. carinii* infection: 0 = no cysts on entire slide, 1 = <20 cysts per slide, 2 = 20 to 80 cysts per slide, 3 = >80 cysts per slide. The distribution of the cysts in each section was recorded.

The other 48 shrews were evaluated by the

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cyst concentration method. The right lung lobes of the shrews were prepared for histology as described previously. The left lung lobe of each sample was frozen at −20 C and used within 1 wk for counting the cysts. In four cases the concentration sample was prepared immediately after necropsy without freezing. For counting cysts, the wet weight ($\bar{x} \pm SD = 0.036 \pm 0.012$ g) of 48 left lobes was recorded after thawing. These lobes were cut into small pieces with razor blades, and digested with 0.2% collagenase (Sigma Chemical Co., St. Louis, Missouri, USA) by incubating the samples for 1 hr with constant stirring at 37 C. The digested fluid was centrifuged at 3,000 rpm for 7 min at 23 C, and the pellet was washed three times with 0.9% NaCl and suspended in 1 ml of 0.9% NaCl. Two 5 μl drops of this suspension were placed on a slide; two slides were made of each sample. The slides were air-dried, fixed in ethanol and stained with toluidine blue O (Settnes and Larsen, 1979). All cysts in each crop were counted, and the mean of the four counts was taken as the final cyst count per microliter. For comparisons with other studies, the number of cysts per microliter was transformed to the number of cysts per gram of lung tissue. The Spearman Rank Test (Sokal and Rohlf, 1981) was used to determine the correlation between the histologic assessment of the intensity of *P. carinii* infection and the quantitative cyst count. The Kruskal-Wallis test (Sokal and Rohlf, 1981) was used to analyze the mean ranks for the cysts count groups.

Pneumocystis carinii positive ($n = 24$) and negative ($n = 23$) samples were examined for histopathological changes. All samples of the histological assessment were included in this examination, and also the three samples with the highest cyst count. From the samples prepared earlier for histologic assessment, three sections of the right lung were placed on the same slide, the slides were stained with hematoxylin and eosin, and each slide was examined by light microscopy. If the number of mononuclear cells (lymphocytes or macrophages) was increased slightly in one or some of the visual fields of lungs the increase was categorized as small, but if their amount was highly increased in some of the visual fields or evenly (although slightly) through the section, the increase was categorized as noticeable. The Pearson's Chi-square test or Fisher's exact test was used to analyze the null hypothesis that *P. carinii* cysts were not more often associated with the observed histopathological changes than can be expected by chance (Sokal and Rohlf, 1981). This was done twice; first the samples of lungs with no changes were analyzed against the samples of small and noticeable changes (analysis 1), and secondly, only the samples with noticeable changes were analyzed against samples of lungs without changes (analysis 2).

For comparison with the common shrews, the left lung lobes of two field voles known to be infected with *P. carinii*, were evaluated by the same cyst concentration method as used for shrews. These voles were caught in the same area as the shrews in November 1992.

RESULTS

Twenty-one (48%) of the 44 shrews assessed histologically were infected with *P. carinii*. Ten of these had <20 cysts per slide (category 1), five had 20 to 80 cysts per slide (category 2) and six shrews had >80 cysts per slide (category 3). No significant ($P > 0.05$) differences were found in prevalence between sexes in either age group (adults, $P = 0.72$, Fisher's exact test; juveniles, $P = 0.26$, Pearson's Chi-square test; $n = 92$). The cysts usually were found individually or in small clusters around the alveolar spaces. In most cases only one cluster in a section was found. Besides clusters, cysts occasionally were scattered around the entire section in the more heavily infected shrews. Generally, the approximate parasite burden was the same in each of the ten sections examined from a given slide; but in the very light infections (category 1) the number of cysts appeared to be greater in the sections close to the surface of the lungs. Generally, the intensity of the infection was similar between the left and the right lung lobes of each individual host.

Twenty (42%) of the 48 lung samples examined by the cyst concentration method had *P. carinii*. These samples were divided into three groups based on the histological assessment of the same samples. The arithmetic mean (±SD) of the cyst count was $3.10\ (\pm 1.51) \times 10^4$ ($n = 8$) for the first group (histological score <20 cysts per slide), $5.52\ (\pm 2.80) \times 10^4$ ($n = 8$) for the second group (histological score 20 to 80 cysts per slide), and $101.4\ (\pm 76.9) \times 10^4$ ($n = 3$) in the third group (histological score >80 cysts per slide). Using the Spearman Rank test a significant ($P < 0.001$) correlation ($r_s = 0.85$) was found between

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the quantitative cyst count and the histological assessment of the intensity of *P. carinii* infection. However, in one case the cyst concentration count was zero, but cysts later were found in the first (19 cysts) and second (four cysts) sections of the same sample by the histological assessment. The rest of the sections of this sample were negative. In another case, no cysts were found by the histologic assessment but the cyst count score was the lowest (4.29×10^3) detected in this study. The highest cyst count score was 1.91×10^6. The size and the appearance of cysts in two samples examined without being frozen were the same as in the ones found in samples stored up to 1 wk at 20 C before concentration.

No significant differences ($P = 0.082$) were found between the cyst counts grouped for shrews with a histological assessment of category 1, 2, or 3, respectively.

The occurrence of *P. carinii* cysts had no significant relation to the histopathological changes observed ($P = 0.18$, analyses 1, and $P = 0.29$, analyses 2). Mononuclear cells either were increased homogeneously in the interstitial space or in clusters peribronchially.

Based on the quantitative cyst count, the two field vole samples had 1.75×10^5 and 9.60×10^4 cysts per gram of lung tissue, respectively.

DISCUSSION

Based on both histological examination and the quantitative cyst count, the *P. carinii* infections found in shrews were mild compared to those of clinically ill laboratory rats. In most concentration samples, the number of cysts found was at the minimum detectable cyst count for laboratory rats (Walzer et al., 1980). Our minimum detectable cyst count was less than 10^4/g (≤ 1 cyst per 5 μl drop) of lung tissue. Such low numbers of cysts cannot be found by histological examination with certainty even when several sections of the sample are studied. However, when the cyst number per slide is >20, *P. carinii* infections in *Sorex araneus* could be demonstrated by histological study if several sections of each sample were examined.

In rats, a highly significant correlation has been found between the histological assessment of the intensity of *P. carinii* infection and quantitative cyst count (Walzer et al., 1980; Kim et al., 1987). Despite the similar correlation found in this study between the two methods, we did not find significant differences between the cyst count groups of shrews because of one very low cyst count score in the small group of the most heavily infected shrews. However, because the arithmetic mean of this group differed clearly from the other groups, we believe that histological examination can be used for the evaluation of the intensity of *P. carinii* infection in ecological studies which require large samples sizes, and in which samples often are collected in the field without proper laboratory facilities. However, the evaluation of the intensity of *P. carinii* infection should be done only on a rough scale: positive or exceptionally positive. Also, because of the differences in the sizes of lungs, an interspecific comparison of the intensity of the infection is difficult without a quantitative method.

Based on the quantitative cyst count of the two *M. agrestis*, the maximum number of cysts found in field voles was lower than those of the shrews. This difference is consistent with earlier findings (J. Laakkonen, unpubl.) among a large number of field voles that the intensity of *P. carinii* infection seldom is as high as was observed in the two field voles examined in this study. Since the prevalence also is much lower in voles than in shrews (Sukura et al., 1992; Laakkonen et al., 1993), we believe that both prevalence and the average number of cysts found in *S. araneus* is higher than those found in field voles.

In the histopathological assessment, we found an increase in mononuclear cell count associated with a small organism burden. The peribronchial clusters of mononuclear cells, mainly lymphocytes,

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resembled bronchus-associated lymphoid tissue which Bienenstock et al. (1973) described in several laboratory animals and which increased in respiratory infections caused by bacteria (Lindsey et al., 1971; Wangxue et al., 1989). However, the histological changes detected in this study had no significant relation to the presence of *P. carinii* cysts.

Without a constant supply of food, *Sorex* sp. shrews will starve within a few hours because of their exceptionally high metabolic rate, relatively low energy reserves, and the lack of capacity to enter torpor (Vogel, 1976, 1980). We propose that shrews of the genus *Sorex* become infected with *P. carinii* without having a symptomatic disease because the hosts would die from this disease and starvation long before PCP develops. The frequent pulmonary infestation of *P. carinii* cysts in *S. araneus* found in this and in the earlier study (Laakkonen et al., 1993) is evidence that *P. carinii* infection is not usually very harmful in this host species. However, as in all disease studies with wild animals it is possible that the animals with acute infections remain in their nests and burrows, and thus are never caught. Starvation experiments in captivity might help in determining whether the observed mild infections develop into PCP. Such studies would give detailed information about the variation of the number of cysts and trophozoites, the proliferating form of *P. carinii*, during the various phases of the infection.

Since *P. carinii* does not seem to be as common in the smaller *Sorex* species (*S. caecutiens*, *S. minutus*) than in *S. araneus* (Laakkonen et al., 1993; J. Laakkonen, unpubl.), the high prevalence of *P. carinii* in *S. araneus* may be a sign of a good adaptation of the host to its parasite. Because *S. araneus* is the numerically dominant shrew species in Europe and western Siberia, and has a longer starvation time than the smaller *Sorex* spp. (Hanski, 1992), it may have had a higher number of encounters, and more time to evolve a more permanent host-parasite relationship with *P. carinii*. *Pneumocystis carinii* of *Sorex araneus* is genetically distinct from isolates of other hosts (Peters et al., 1994). Whether this species-specific variation of *P. carinii* has led to differences in infectivity and pathogenity between isolates awaits to be studied.

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