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Relation of cyst counts with numbers of total nuclei of *Pneumocystis carinii* in rats

Sung-Tae Hong*, Jee Suk Yu, and Mejeong Lee

Department of Parasitology and Institute of Endemic Diseases
Seoul National University, College of Medicine, Seoul 110-799 Korea

Abstract: Wistar rats were induced of *Pneumocystis carinii* infection by injection with methyl-prednisolone to correlate the cyst counts and numbers of nuclei. Seven sections of the lungs were examined by impression smears and also whole lung homogenates were screened for nucleus counting for each rat. At the first week of the experiment, all of the impression smears except one were cyst negative but trophic forms were counted around 10^6. At the third week, the cysts appeared one per 20 immersion oil lens fields. The nuclei were on the order of 10^7 at this period, and this amount of Pc is regarded as the limit of cyst detection on impression smears. When the nuclei were over 10^9 in the lungs, the cysts were counted about 50 in 20 microscopic fields. The organisms were distributed in the lungs without any predilection focus. The present data suggest that the trophic forms, proliferate first and the cysts appear later in the lungs.
Key words: *Pneumocystis carinii*, cyst, trophic form, count

INTRODUCTION

Purification and quantitation is an important procedure in experiments with *Pneumocystis carinii*. Since *in vitro* cultivation of *P. carinii* is not satisfactory, the organisms should be supplied from animal models (Cushion and Ebbets, 1990; Sloand *et al.*, 1993). The primary living focus is the alveolar lumen of the lungs. Therefore, any required amount of the organisms should be collected from the lung tissue. Purification of *P. carinii* from host cells or other microorganisms is the most laborious step, and usually membrane filtration or gradient centrifugation is applied.

Various methods have been developed for quantitation of *P. carinii*, but they are grossly classified into two. Counting cysts by area (mm^2) on histological specimens is the classical one (Kim *et al.*, 1987) and the other is quantitation of cells after homogenation (Ruffolo *et al.*, 1986). Estimation of total number of *P. carinii* nuclei in lung homogenates is now the standard method for various preparations of this protist (Hong *et al.*, 1990). However, both methods are time-consuming and tricky, and thus sensitive but handy technique is required for quantitative analysis.

The present study was carried out to evaluate counting *P. carinii* cysts on impression smears as a quantitation technique in experimental rats. Also whether any quantitative difference of *P. carinii* infection was present by the regions in the rat lungs was screened.

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* Corresponding author

MATERIALS AND METHODS

1. Induction of *P. carinii* infection

Albino rats of Wistar strain were suppressed of their immunity by weekly subcutaneous injection of 2 mg methyl-prednisolone (Depomedrol®, Upjohn Korea Co.). The rats were kept in conventional animal rooms with regular diet and pipe water. Table 1 summarized the scheme of rats.

2. Counting cysts

The lungs were removed after death of the rats by neck dislocation. The lungs were separated into right, left, and accessory lobes. Right or left lobes were divided into 3 parts, upper, middle, and lower. Impression smears were made with the 7 cut surfaces of each rat, and stained with modified Giemsa solution (Diff-Quik®, Fisher Scientific Co., U.S.A.). *P. carinii* cysts with intracystic bodies were counted through the immersion oil lens. Two persons counted same specimens and their mean counts were used.

3. Quantitation of nuclei

The lung fragments of individual rats were chopped after impression smear, and homogenized in a laboratory blender (Stomacher®, U.K.). The host cells were removed by filtration through 10 μm pore membrane, and remaining cells were lysed in hypotonic solution. After washing and resuspension in saline, the *P. carinii* solution was diluted with saline. Ten μl of the diluted suspensions were smeared on slides and stained with Diff-Quik solution. The number of *P. carinii* nuclei was counted under immersion oil lens power in duplicate, and total number of nuclei was estimated by multiplying nucleus number per field with the converting constant, 4.45×10^5, dilution titer and volume of the final suspension (Ruffolo *et al.*, 1986).

RESULTS

Relation of cyst counts with number of nuclei of *P. carinii*: The numbers of cysts and nuclei are presented by the experimental period in Table 1. In the first week of immune suppression, only 2 cysts in 20 immersion oil lens fields (IOF) were detected from one smear of a rat. All of remaining smears were cyst negative microscopically (Fig. 1A). The lung homogenates were counted for *P. carinii* nuclei, and 4 of 10 rats harboured Pc nuclei around 10^6 (Fig. 1B). The cysts appeared in 7 out of 8 rats 3 weeks after the steroid injection. The mean number of the cysts was about 1 per 20 IOF in the 8 rats, and total number of nuclei also increased slightly. At 4 weeks, mean number of cysts increased to 8.5 but much jumped to 19.4 per 20 IOF at 5 weeks. The nuclei became on the order of 10^8 at 4 weeks. The cysts increased to their maximum mean number, 58.9 per 20 IOF and the average nuclei 1.9×10^9 at 6 weeks (Fig. 2A & 2B). At 9 weeks, the cysts were 30.0 in average and the nuclei were 4.2×10^9. The number of cysts became maximum at 6 weeks, but the nuclei were maintained at the order of 10^9

Table 1. Numbers of cysts and nuclei of *Pneumocystis carinii* from rats

Group of rats	No. of rats		No. of cysts[a]		No. of nuclei[b]	
	Exam.	Cyst posi.	Range	Mean	Range	Mean
1 week	10	1	0-0.3	0.03	2.9×10^5-8.9×10^6	5.8×10^6
2 weeks	3	1	0-0.2	0.06	ND[c]	
3 weeks	8	7	0-4.1	1.4	4.0×10^6-1.7×10^9	3.5×10^8
4 weeks	9	9	0.4-29.1	8.5	3.0×10^7-4.3×10^8	1.5×10^8
5 weeks	10	10	0.6-80.6	19.4	ND	
6 weeks	15	15	4.7-132.9	58.9	1.1×10^8-4.9×10^9	1.9×10^9
9 weeks	8	8	6.0-97.1	30.0	3.7×10^8-1.3×10^{10}	4.2×10^9

[a] Numbers of nucleated cysts per 20 immersion oil lens fields; [b] Number of nuclei from the whole lungs of individual rats; [c] Not done. The cyst counts are well correlated with No. of nuclei, r = 0.53950, P = 0.0001 by Pearson Correlation Coefficients.

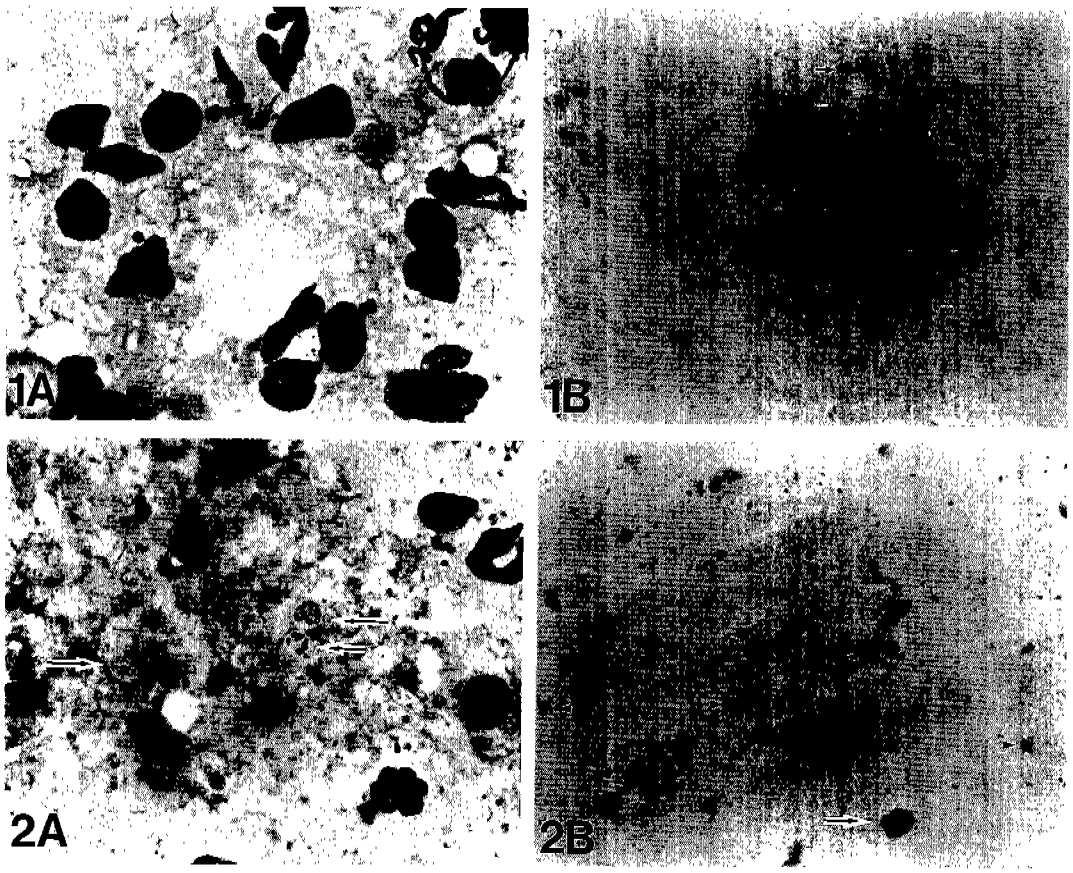

Fig. 1. A. Impression smear of the right upper lobe of the lungs, W28-5 rat, 1 week after immune suppression. No cysts were observed, Diff-Quik stained, original magnification × 1500. **B.** Smear of the whole lung homogenate (10 fold dilution) of the rat W28-5. Two nuclei (arrows) of *P. carinii* were found in this field. Diff-Quik stained, original magnification × 1500. **Fig. 2. A.** Impression smear of the right upper lobe of the lungs, W29-6 rat, 6 week after immune suppression. Three cysts (arrows) and many trophic forms (arrow heads) were observed in this field, Diff-Quik stained, original magnification × 1500. **B.** Smear of the whole lung homogenate (100 fold dilution) of the rat W29-6. Numerous nuclei of Pc trophic forms (arrow heads) and a cyst (arrow) were found in this field. Diff-Quik stained, original magnification × 1500.

from 6 to 9 weeks. Individual rats were plotted on a figure to represent correlation between numbers of cysts and nuclei (Fig. 3).

Number of *P. carinii* cysts by regions of the lungs: As shown in Figs. 1 and 2, *P. carinii* cysts were counted on impression smears of 7 different sites in the lungs. Mean number of the cysts is summarized by the locations and period of the experiment in Table 2. Throughout the whole groups of different *P. carinii* burdens, any predilection of the organism was not noticed.

DISCUSSION

Accurate enumeration of *P. carinii* organisms has based on morphological identification and counting them under microscopy. Because the trophic form of this organism is too small to identify (Cushion *et al.*, 1988), counting of *P. carinii* nuclei requires lots of experience. Differentiation of other microorganisms in the lung homogenates is most important and difficult. Practically it is not easy to differentiate trophic forms on impression smears even done by an expert. In the present

study, therefore, nuclei of *P. carinii* were counted only in the homogenates. As presented in Figs. 1 and 2, the eukaryotic nucleus associated with blue cytoplasm by Diff-Quik staining was regarded as a trophic form of *P. carinii* (Cushion *et al.*, 1988).

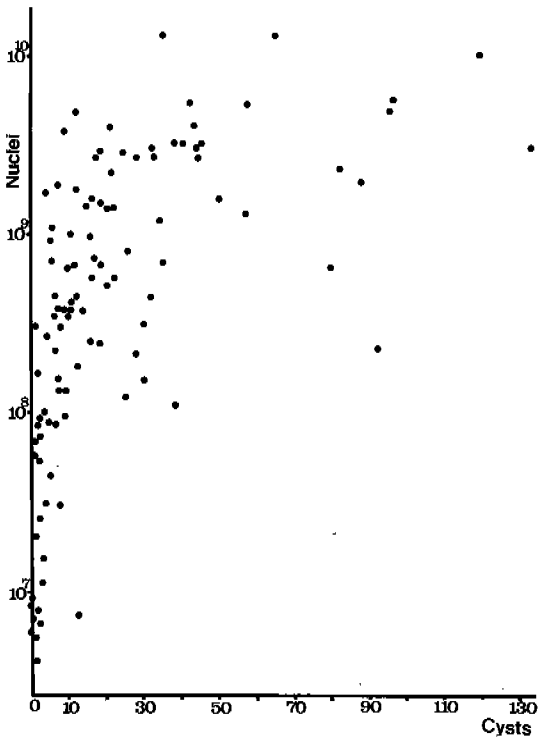

Fig. 3. Correlation of the cyst counts with numbers of nuclei of *P. carinii* from the rat lungs. Each dot represents one rat. The number of cysts is the count in 20 immersion oil lens fields, and the number of nuclei is the total count of nuclei in the lungs of a rat marked on the logarithmic scale.

Severity of *P. carinii* pneumonia was evaluated by lung weight, histological scores, and counting organisms in the tissue (Kim *et al.*, 1987; Bray *et al.*, 1993). Lung weight is rather a crude index of the disease and significant only in the lungs with far advanced lesions. The histological score or cyst counts on the histopathological specimens are a good index for estimating the degree of pneumonia and number of the organisms. The more the organisms, the more the disease progressed, and *vise versa*. Histology-based estimation of the organisms, however, is too complicated in the whole procedure.

Counting the cysts in BAL is rather easy for quantitation of human *P. carinii* by Diff-Quik staining of the standardized BAL solution after cytocentrifugation (Baughman *et al.*, 1990). The number *P. carinii* was based on the number of 200 or 300 nucleated host cells. This may be very useful for clinical materials, if BAL is carried out optimally. Also the number of neutrophils in BAL specimens is indicative for severity of the *P. carinii* pneumonia (Sadaghdar *et al.*, 1992). In advanced AIDS patients with pulmonary complication, more neutrophils were observed in BAL fluid.

For laboratory purpose of Pc quantitation, counting Diff-Quik stained nuclei on a certain volume of diluted lung homogenates has long been used (Ruffolo *et al.*, 1986). This technique also requires too much works. Simpler but accurate quantitation method may much help

Table 2. Number of cysts of *Pneumocystis carinii* on impression smears by the lung locations

Group of rats	No. of rats		Mean no. of cysts by locations[a]							
	Exam.	Cyst posi.	LU	LM	LL	RU	RM	RL	A	Total
1 week	10	1	0	0	0	0	0	0	0.2	0.03
2 weeks	3	1	0	0.3	0	0	0	0	0	0.06
3 weeks	8	7	1.7	1.9	1.5	1.3	1.1	1.9	1.3	1.4
4 weeks	9	9	13.4	5.8	4.6	8.4	15.0	7.6	8.5	5.0
5 weeks	10	10	34.2	20.0	19.8	15.1	15.9	13.6	16.7	19.4
6 weeks	15	15	68.4	56.1	46.5	58.3	72.2	58.8	58.2	58.9
9 weeks	8	8	18.6	27.8	33.5	30.3	30.1	35.0	36.5	30.0

[a] Cut surfaces of the lungs were impressed on slides by the location; LU, left upper; LM, left middle; LL, left lower; RU, right upper; RM, right middle; RL, right lower; A, accessory lobe. The numbers are mean of the examined rats by the group. The cyst counts are not significant by the 7 locations, by ANOVA $F = 0.41$, $P = 0.8699$, $df = 6, 417$; by Krustal-Wallis test $P = 0.9920$.

handling *P. carinii*.

From the present findings, it is definite that trophic forms appeared first in the lungs at the early phase of immune suppression (Table 1). The cysts were not detectable at the beginning stage. As the host immunity becomes worse, trophic forms of *P. carinii* proliferate for a few weeks. The fact that trophic forms develop into the cysts is compatible with previously proposed life cycles of *P. carinii* (Vavra and Kucera, 1970; Cushion *et al.*, 1988). When the number of nuclei in a rat reaches around 10^7, the cysts are detectable on impression smears. That was the third week of prednisolone injection in this study scheme. One to 3 cysts were found in 20 IOF, and mean number of cysts of the 7 chosen sites in a rat was 1.4. This number of the cysts may be the limit of microscopical detection. The routine screening of *P. carinii* by impression smears depends upon observation of the cysts not trophic forms due to the difficulty of identification. The correlation equation between cyst counts and numbers of nuclei was $y = 2.918 \times 10^8 + 5.619 \times 10^7$ $\chi(n = 107, \rho = 0.53950, p = 0.0001)$

The number of *P. carinii* reached at maximum 6 weeks after immune suppression in this animal model. After then the nucleated cyst decreased slowly but the number of nuclei were maintained. As far as the hosts are immunocompromised, the infection is known to progress. The lungs of a rat may harbour *P. carinii* of 10^{10} as the maximum capacity. The rat should die with such a burden of *P. carinii*. After 9 weeks of the experiment, the number of cysts or nuclei decreased remarkably. That means death of severely infected rats.

Collecting numerous organisms is definitely essential for most experiments of *P. carinii*. The rat with cyst counts more than 50 per 20 IOF can be regarded as infected with heavy burden, more than 10^9 organisms. Screening of the lung impression smears with *P. carinii* cysts may be a good index to pick out well-infected animals.

The primary focus of *P. carinii* infection is known to be the apical lobe or the hilar region in the lungs in humans (Walzer *et al.*, 1989). Table 2 revealed no differences of the cysts in the lungs, among the right, left, and accessory lobes. Also no preference was noticed by the upper, middle, and lower regions in a lobe. Since *P. carinii* is transmitted by air (Hong *et al.*, 1992; Powles *et al.*, 1992), the apex or perihilar region may be the site of primary settlement of *P. carinii* in the lungs of human. In the present study, however, any skewed distribution was not recognized in the lung of rats. Of course the lungs are small and the physiologic position is not upright in rats, and this may be the reason of no significant differences of the numbers in the 7 divided parts of the lungs. Still presence of the preferable primary site of *P. carinii* proliferation in rats is not excluded, and it may be one of further studies.

Of course the recovered *P. carinii* from rats are regarded as recurrent organisms from latent infection. Still little is known for the latent stage, however, trophic forms are much more abundant in the early phase of infection. The trophic forms of *P. carinii* are expected different from cysts in several points (Chatterton *et al.*, 1990). The present findings suggest that sensitive and specific detection of the trophic forms should make better diagnosis of *P. carinii* infection. Most morphological diagnosis is based upon screening of the cysts after various staining at present. It should be persued for earlier and more sensitive diagnosis of *P. carinii* infection.

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=국문초록=

흰쥐 주폐포자충에 있어서 도말표본상 포낭의 수와 핵의 총 수와의 상관성

서울대학교 의과대학 기생충학교실 및 풍토병연구소

홍성태, 유지숙, 이미정

Wistar 계통 흰쥐를 면역억제하여 주폐포자충을 발현시킨 후, 기간 별로 폐단면 도말검사 표본에서 포낭의 수를 세고, 폐 전체를 균질액으로 만들어 주폐포자충의 핵을 세었다. 또한 폐의 7개 부위에 따라서 포낭의 수를 비교하였다. 포낭은 실험 3주가 되어서야 도말표본에서 20개의 1000배 확대 시야에서 평균 1개 이상이 검출될 정도로 나타났으나, 폐 균질액에서 주폐포자충의 영양형은 실험 1주 후부터 10^6 정도의 수가 계산되었다. 각 개체 별로 비교한 결과 도말검사에서 포낭형을 검출하려면 폐 전체에 적어도 10^7 정도의 병원체가 있어야 된다는 사실을 확인하였다. 또한 20 개 시야에서 50 개 이상의 포낭을 관찰하는 경우에는 10^9 이상의 병원체가 있다고 평가할 수 있었다. 좌우엽의 상, 중, 하 세 부분과 소엽(accessory lobe)의 부위에 따른 포낭의 수에서 어떠한 의미있는 경향을 인정할 수 없었다. 이 실험 자료를 통하여 폐 단면 도말표본에서 포낭의 수를 세는 것을 전체 병원체의 수를 파악하는 방법으로 사용할 수 있다고 판단하였다. 또한 주폐포자충은 감염 초기에 영양형으로 시작하여 일정 수가 되어야 포낭형이 출현하는 것으로 추측하였다.

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