Limited Persistence in and Subsequent Elimination of *Pneumocystis carinii* from the Lungs after *P. carinii* Pneumonia

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The purpose of this study was to determine the period of persistence of *Pneumocystis carinii* in the lungs after *P. carinii* pneumonitis (PCP). After primary PCP was induced with dexamethasone, experimental rats were moved to a high-efficiency particulate air–filtered isolator to prevent further exposure to environmental *P. carinii* and allowed to recover. At intervals thereafter, sample groups were transferred to a second isolator and reimmunosuppressed with dexamethasone to provoke PCP if *P. carinii* were present. Reactivation of PCP was assessed by histologic examination, counts of cysts per gram of lung, and DNA amplification using nested polymerase chain reaction. A sequential and progressive decrease in *P. carinii* was detected. Thus, *P. carinii* is cleared from the lungs of ≥75% of animals within 1 year after an episode of PCP, implying that persistence of latent organisms is limited.

The long-standing concept that *Pneumocystis carinii* pneumonia (PCP) results from activation of a latent infection [1, 2] has been challenged by reports of nosocomial clusters of PCP among immunocompromised patients [3–12]. Also, Millard and Heryet [13] could not identify *P. carinii* by a monoclonal antibody technique in the lungs of AIDS patients without PCP and of people who died from accidents or natural causes. More recently, Peters et al. [14] were unable to find evidence of latent *P. carinii* by use of a more sensitive DNA amplification technique with *P. carinii*–specific primers.

This currently accepted view that *P. carinii* remains latent in the lungs after a primary infection is supported by the high prevalence of *P. carinii* antibodies detectable at an early age and persisting for life in normal persons and by the occurrence of *P. carinii* pneumonia with the advent of immunosuppression [1, 2, 15].

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Animals were housed in accordance with standards of laboratory animal care.

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The widely used animal model of PCP takes advantage of the fact that PCP develops in almost 100% of rats after a period of 6–8 weeks of dexamethasone-induced immunosuppression. This PCP is believed to arise from reactivation of latent organisms [2, 16]. Using the dexamethasone-induced rat model of PCP [2, 16, 17], we studied the time *P. carinii* persisted in the lungs after this pneumonia.

Materials and Methods

Experimental plan. The experiments were based on the observation that rats immunosuppressed for ≥6 weeks with a corticosteroid and treated with an antibiotic to prevent bacterial infection will develop overt PCP. Although *P. carinii* can rarely be found by histologic examination of lungs before immunosuppression, extensive PCP is obvious after immunosuppression. As a stringent test to determine if *P. carinii* is cleared from the lungs, animals can be challenged with a corticosteroid to provoke *P. carinii* replication; after 10–12 weeks of immunosuppression, animals are sacrificed and the lungs examined by histopathology, total cyst counts are determined, and polymerase chain reaction (PCR) is used to amplify *P. carinii* DNA. Also important to the experimental plan is the observation that rats can be protected from naturally acquired *P. carinii* infection by containment in high-efficiency particulate air (HEPA)–filtered germfree isolators [17, 18].

The study was designed to provoke PCP in rats with dexamethasone, document the infection histologically, and allow the animals to recover by withdrawal of immunosuppression. Animals were subsequently maintained for up to 1 year in a HEPA-filtered germfree isolator (M50; Isotec, Oxfordshire, UK) to prevent reexposure to *P. carinii* from environmental sources. At intervals of 9, 19, 30, 42, and 53 weeks, subgroups of rats were transferred to a separate sterile isolator, and dexamethasone immunosuppression was restarted and continued for 10–11 weeks to provoke PCP if infection with *P. carinii* persisted (figure 1). The main isolator and supplies were sterilized with paraformaldehyde gas before the

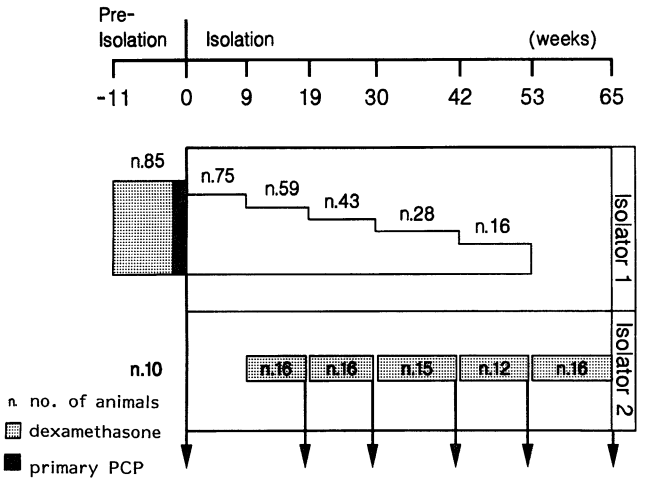

Figure 1. Experimental protocol: Rats received dexamethasone for 10 weeks in nonisolated environment to provoke PCP, were moved, and were allowed to recover inside filtered isolator (week 0). Subgroups were moved at successive intervals into second isolator and rechallenged with dexamethasone to reactivate *P. carinii* if present. Arrows indicate sacrifice.

experiment was begun and the second isolator was sterilized before each new sample group was moved in. Filters were regularly certified. On death or sacrifice, lung tissue obtained by necropsy was studied for *P. carinii* infection histologically, with cyst counts per gram of lung, and by PCR amplification.

Animals. Female Sprague-Dawley virus-free rats, weighing 150–175 g (Harlan Industries, Indianapolis), were shipped in filtered, autoclaved containers via virus-free trucks. Our previous studies showed that rats from this supplier (barrier room 202c) are consistently infected with *P. carinii* when immunosuppressed in open cages. They were housed 2–4 per cage, depending on the weight of the rats, through the experiment. Weight of the rats was recorded at the start of each immunosuppression period and at the time of sacrifice. Rats were given free access to rat chow (Purina Mills, Richmond, IN) and water throughout the study.

Immunosuppression. Dexamethasone sodium phosphate, 2 mg (Decadron; Merck Sharpe & Dohme, West Point, PA), and tetracycline hydrochloride, 500 mg (Sumycin; E. R. Squibb, Princeton, NJ), were added to 1 L of drinking water. The water was made available ad libitum and changed to a fresh solution every 2 or 3 days during the immunosuppression periods.

Dexamethasone-tetracycline was given during 10–11 weeks for each immunosuppression period.

Postmortem analysis. Rats were sacrificed at the end of either the first or second immunosuppression period by asphyxiation with CO_2. Lungs were carefully dissected and removed with attention to avoidance of cross-contamination between animals.

Histologic examination. One-third of the right lung was fixed in 10% formalin, embedded in paraffin, serially sectioned, and stained with Gomori-methenamine-silver. The extent of PCP was scored as described [19], from none, if no *P. carinii* organisms were seen, to 3+, if the lung was diffusely and extensively penetrated by organisms in almost all high-power fields.

Cyst counts. Left lungs were frozen at −70°C until processed for cyst counts. Cysts were counted in parallel at the end of the experiment as described [20, 21]. Briefly, 500–1000 mg of left lung was separated from the main bronchial tree, weighed, passed through a 100-mesh steel sieve (Sigma, St. Louis), washed with 50 mL of PBS, and centrifuged at 800 *g*; the pellet was incubated for 45 min in 5 mL of dithiothreitol (Calbiochem, La Jolla, CA), centrifuged again, and reconstituted in 2 mL of PBS.

Cysts contained in a 2-μL aliquot were counted after staining with Gomori-methenamine-silver. Counts were done in duplicate and the highest count considered for analysis. Counts were converted to cysts per gram of lung.

DNA amplification. About one-third of the right lung was examined for *P. carinii* by DNA amplification as described [14, 22–24]. Briefly, lung tissue was finely minced and homogenized in a microfuge tube with a pellet mixer, and the samples were digested with proteinase K (500 $\mu g/mL^{-1}$) at 50°C in the presence of 10 m*M* EDTA and 0.5% SDS. Total DNA was extracted with phenol-chloroform, purified, concentrated ("clean up" system; Promega, Madison, WI), and recovered in a volume of 50 μL.

For DNA amplification, the *P. carinii*–specific oligonucleotide primers pAZ102-E and pAZ102-H were used [23, 24]. Amplification was done at 94°C for 1.5 min, 55°C for 1.5 min, and 72°C for 2.0 min for 40 cycles. To achieve a greater level of sensitivity, primers pAZ102-X (5′-GTGAAATACAAATCGGACTAGG-3′) and pAZ102-L1R (5′-CTCTCGACTCCTCACCTTAT-3′), which are internal to the first set of primers, were used in a second round of PCR. Thirty-five cycles of amplification were done under the same conditions as for external primers. PCR products were separated on 1.5% agarose gels and visualized without need for oligohybridization. Negative controls with no added template DNA were included after each sample to monitor for cross-contamination. A sample of rat-derived *P. carinii* DNA was used as a positive control in each experiment. All manipulations during DNA extraction and amplification were done in laminar flow cabinets, using disposable pipettes, tubes, and reagent aliquots to avoid contamination.

Transthoracic lung needle aspirates. Transthoracic lung needle aspirates were obtained at week 0 in all rats (before they were put in isolation). The procedure was done under deep anesthesia with 0.1–0.15 mL of a 1:1 mixture (by volume) of ketamine, 100 mg/mL (Fort Dodge Laboratories, Fort Dodge, IA), and xylazine, 20 mg/mL (Mobay, Shawnee, KS), administered subcutaneously. Rats were placed on a surgery board. A 22-gauge needle was introduced up to 1 cm in the midaxillary line at half the distance between the manubrium and xiphoid appendix and withdrawn slowly while negative pressure was applied with a 1-mL tuberculin syringe, previously loaded with 0.02 mL of sterile saline. After the procedure, the needle was flushed and the contents deposited on a glass slide and air-dried until stained with Gomori-methenamine-silver and toluidine blue. Cysts were identified by typical morphology by 2 independent observers blinded to the findings of the other.

Results

Course of the experiment. Of the 101 rats that started the experiment, 16 were lost: 4 died as a result of the transthoracic lung needle aspirate, 2 were sacrificed because they had developed large mammary tumors, and 10 died of undetermined

Table 1. Detection of *P. carinii* in lungs of rats immunosuppressed inside filtered-air isolator at successive intervals after primary PCP.

	Weeks at restart of immunosuppression after primary PCP					
	0	9	19	30	42	53
Histology results (% positive)	10/10 (100)	10/16 (62.5)	7/16 (43.7)	7/15 (46.6)	0/12	0/16
Cysts/g of lung, mean (range)*	7.25×10^5 (3.3×10^5–9.8×10^6)	2.7×10^4 (2.8×10^3–4.6×10^6)	$<4 \times 10^3$ (0–3.9×10^5)	$<4 \times 10^3$ (0–8.4×10^4)	Negative	Negative
DNA amplification (% positive)	10/10 (100)	NT	NT	NT	12/12 (100)	4/16 (25)

NOTE. NT, not tested.
* $P < .001$, Cochran-Armitage test, supports progressive decrease in *P. carinii* load in lungs at successive weeks.

causes. Therefore, 85 rats finished the experiment and were evaluable (figure 1). Immunosuppressive periods lasted ~10 weeks (range, 67–81 days; median, 70.5). Age and average weight of animals at the start of each immunosuppression period were 3 months and 210 g (range, 172–220 g), 9.3 months and 286 g (range, 269–298 g), 12 months and 269 g (range, 246–287 g), 14 months and 299 g (range, 294–300 g), and 17 months and 313 g (range, 290–338 g). This represents a total weight gain of 102 g for the group that spent 17 months in isolator 1 before being moved to isolator 2 for reimmunosuppression. The rats tolerated both the primary and reactivation periods of immunosuppression adequately and presented a consistent mean loss of 29.1% (range, 27%–32%) of their body weight during each period.

Follow-up of P. carinii carriage in the lungs. A summary of the evolution of *P. carinii* load in the lungs (cysts/gram of lung) and the proportion of *P. carinii*–positive rats at different times of the experiment are shown in table 1. The trend for a decreasing number of *P. carinii* cysts per gram of lung was significant ($P < .001$; Cochran-Armitage test for observed proportions). The 10 rats randomly sacrificed at week 0, which was defined as the end of the first immunosuppression period, tested positive for *P. carinii* by histologic evaluation, cyst counts, and amplification of *P. carinii* DNA. The *P. carinii* load ranged from 3.3×10^5 to 9.8×10^6 cysts/g of lung (median, 7.25×10^5) and was consistent with 1+ or 2+ histology scores. This random sample of rats sacrificed at time 0 documented an adequate degree of infection before rats were moved to isolation. A sequential and progressive decrease in the proportion of rats positive for *P. carinii* by histology and in the number of cysts per gram of lung was evident in each sacrificed sample group starting with the first PCP reactivation episode (figure 2).

Rats rechallenged with dexamethasone after week 42 (10 months) tested negative for *P. carinii* by histologic examination and cyst counts. However, *P. carinii* DNA was detected by PCR in 100% of these animals. The proportion of animals remaining positive by PCR decreased to 25% in those with immunosuppression that began at week 53 (1 year after the first PCP episode; figure 3, table 1). Transthoracic needle aspirates at time 0 were negative for all 10 rats sacrificed at that time, despite histologic evidence that all of them had infection. This discrepancy is indicative of the inadequacy of transthoracic needle aspirates to diagnose PCP in rats. Interestingly, aspirates were positive in 3 of 75 rats that entered isolation. One of these (rat 4) was randomly allocated to the group reimmunosuppressed 53 weeks after primary infection. This rat showed no evidence of PCP by DNA amplification after the second period of immunosuppression (figure 3), giving further documentation that the infection was cleared through the course of the experiment.

Discussion

In this study, experimental *P. carinii* was cleared from the lungs in the majority of cases within 1 year after a primary

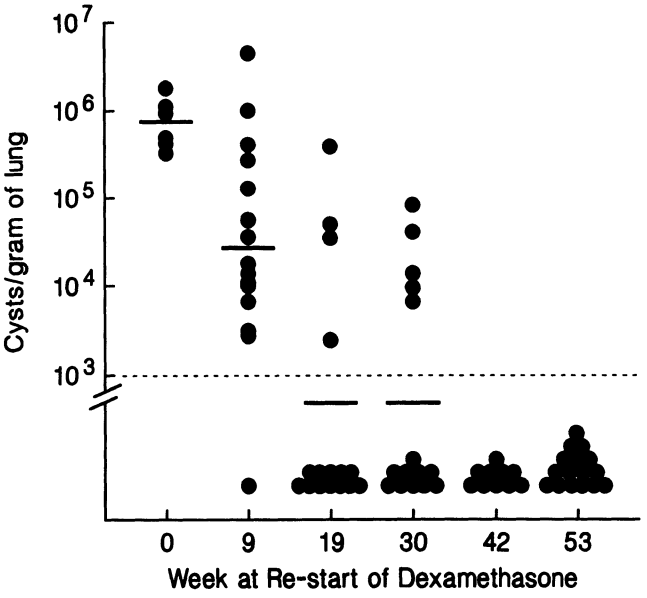

Figure 2. Number of *P. carinii* cysts/g of lung of immunosuppressed rats sacrificed at time of primary PCP (week 0) and progressive decline in counts for rats sacrificed after second period of immunosuppression at successive intervals (weeks 9, 19, 30, 42, and 53). Sensitivity is indicated by dashed line. Each symbol represents individual observation.

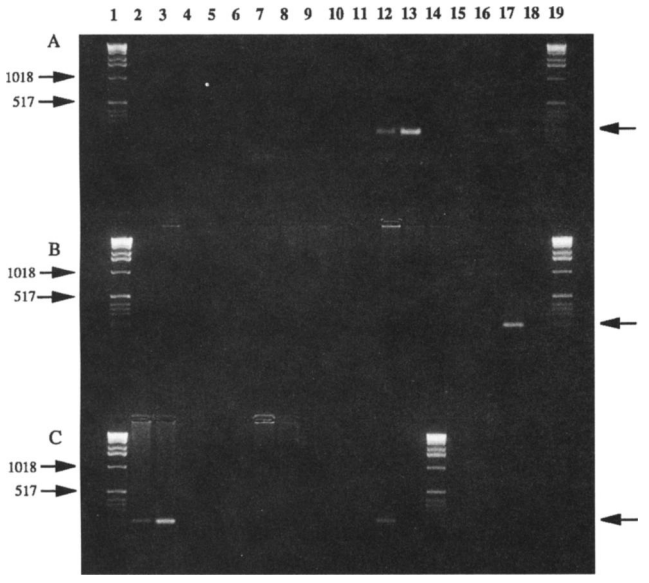

Figure 3. Amplification (by use of *P. carinii*–specific primers) of DNA from lungs of rats sacrificed after second period of immunosuppression 53 weeks after primary PCP. No reactivity was seen in samples from lungs of rats 1, 2, 4, 5, 6, and 8. In each figure part, outside lanes are 1-kb molecular mass markers; paired rat samples are undiluted and ×5 dilution, respectively. **A**, Lanes 2 and 3, rat 1; lanes 7 and 8, rat 2; lanes 12 and 13, rat 3; lane 17, *P. carinii* DNA positive control; lanes 4, 9, and 14, no template DNA—negative control. **B**, Lanes 2 and 3, rat 4; lanes 7 and 8, rat 5; lanes 12 and 13, rat 6; lane 17, *P. carinii* DNA positive control; lanes 4, 9, and 14, no template DNA—negative control. **C**, Lanes 2 and 3, rat 7; lanes 7 and 8, rat 8; lane 12, *P. carinii* DNA positive control; lanes 4, 9, 10, and 13, no template DNA—negative control. Arrows on right indicate position of *P. carinii*–specific amplification product.

episode of PCP. Also, the severity of reactivation declined in the controlled environment, with the second episode decreased sequentially from the primary episode (figure 2). This decrease in *P. carinii* organisms occurred regardless of baseline undernourishment as a consequence of prolonged isolation and the long periods of high-dose dexamethasone, which are both well-documented PCP-provoking factors. The consistent weight loss during the periods of immunosuppression is recognized evidence of the wasting effects of dexamethasone-induced immunosuppression in rats and gives further support to the progressive decline in severity of PCP reactivation.

Furthermore, no histologic evidence of reactivated PCP was found when ⩾10 months had elapsed from the primary infection, although evidence of *P. carinii* DNA was still present for 12 months in one-fourth of the animals after primary infection, as detected by nested PCR. DNA amplification using primers designed for the gene encoding the mitochondrial large subunit ribosomal RNA, which is present in several copies per organism, has been shown to be highly sensitive. Calibration of parasite numbers detected by a single round of DNA amplification followed by oligonucleotide hybridization has demonstrated that parasites can be detected to a lower limit of 1 or 2 organisms [25]. Nested PCR is of equal sensitivity and eliminates the need for Southern blotting and oligohybridization [26]. The absence of histologic evidence of PCP is indicative that no clinically significant PCP occurred at these later stages of recovery from the first PCP episode.

The experimental model of transient, dexamethasone-induced immunosuppression may resemble the transient immunosuppression secondary to anticancer chemotherapy, as cancer patients likely recover their ability to mount an adequate immune response against *P. carinii* within 3 months after chemotherapy has been stopped. AIDS patients, on the other hand, may carry the organism for life once it is established, as a result of the inexorably progressing immune suppression of the disease. AIDS patients would thus benefit from the development of new anti–*P. carinii* therapies that could eradicate the carrier state [27], regardless of their decreasing CD4 lymphocyte count and persistence of their immune suppression.

Reactivation of latent *P. carinii* forms, persisting in the lungs after a primary, usually asymptomatic infection, has been the accepted theory to explain the pathogenesis of PCP since the description of subclinical *Pneumocystis* pneumonitis by Sheldon in 1959 [1]. The subsequent report by Frenkel et al. in 1966 [2] showing that rats almost universally developed PCP after immunosuppression with steroids, gave further support to this hypothesis. However, with increasing frequency, latency has been challenged by reports on nosocomial clusters of PCP that have raised the issue of whether PCP is the result of human-to-human transmission or of reactivation from latent organisms established in the lungs after a primary infection [3–12, 17].

Serologic studies indicate that *P. carinii* infection is highly prevalent both in humans and in other animals worldwide, with evidence for human antibodies in up to 100% at the age of 2 years in some regions [15]. Despite this finding, recent studies using specific monoclonal antibodies or PCR have not been able to demonstrate the presence of *P. carinii* in persons dying of accidental causes or diseases unrelated to PCP [13, 14]. Our studies are in agreement with these previous failed attempts to identify latent forms of *P. carinii* in otherwise healthy adults. The frequency of development of PCP after a period of severe immunosuppression in a variety of mammalian hosts more likely suggests that *P. carinii* organisms are highly prevalent in the environment and thus readily available to proliferate in susceptible individuals [2, 16, 17].

Germfree (*P. carinii*–free) rats are protected from PCP when immunosuppressed inside HEPA-filtered isolators. This has been documented in a previous controlled experiment in which litters of germfree rats, delivered inside a HEPA-filtered isolator, were randomized to receive dexamethasone inside or outside of the isolator. While a high and significant proportion of rats immunosuppressed outside the isolator developed PCP, HEPA filtering protected all of the rats inside the isolator [18], showing that PCP episodes occurring in our model before 10 months from the primary episode were the result of reactivation

of organisms remaining in the lungs after the primary infection. Lack of reactivation in animals immunosuppressed beyond 10 months after primary infection was documented by histology, and clearance of the organisms from the lungs was shown by the lack of amplification of *P. carinii* DNA in 75% of rats after 12 months.

We conclude that *P. carinii* may not establish long-term latency as currently accepted. Carriage of *P. carinii* organisms in the lungs after a primary infection is thus a limited-time phenomenon inversely related to the immunologic recovery of the host. We believe a reasonable hypothesis is that PCP in the compromised host may occur from either an acute acquisition of organisms from the environment or from activation of latent organisms residing in the lungs.

This animal model may also provide a useful experimental tool for comparative chemotherapy trials on the efficiency of anti–*P. carinii* drugs in decreasing the length of carriage of *P. carinii* after an episode of PCP.

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