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Pneumocystis carinii Is Not Universally Transmissible between Mammalian Species

FRANCIS GIGLIOTTI,[1,2]* ALLEN G. HARMSEN,[3] CONSTANTINE G. HAIDARIS,[2,4] AND PATRICIA J. HAIDARIS[2,5,6]

Departments of Pediatrics,[1] Microbiology and Immunology,[2] Medicine,[5] Pathology and Laboratory Medicine,[6] and Dental Research,[4] University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, and Trudeau Institute, Inc., Saranac Lake, New York 12983[3]

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In a series of five experiments, we attempted to transmit *Pneumocystis carinii* from ferrets to SCID mice by intratracheal inoculation. Using highly specific and sensitive assay techniques, we could not document infection of SCID mice by *P. carinii* isolated from ferrets. In contrast, under identical inoculation conditions, *P. carinii* was easily transmissible from one SCID mouse to another. These results indicate that *P. carinii* organisms, at least those isolated from ferrets, have a restricted host range. The finding of restricted transmission of *P. carinii* is consistent with the increasing evidence for host species-specific antigenic variation among isolates of *P. carinii*. If restricted host range is a consistent biological feature of animal-derived *P. carinii*, it would suggest that *P. carinii* pneumonitis in humans may not be a zoonosis as previously speculated.

It has been 50 years since the first clear pathological description of *Pneumocystis carinii* pneumonitis (PCP) in humans (14); however, the reservoir of organisms responsible for human disease is unknown. Early epidemiological observations led to the speculation that *P. carinii* pneumonitis in humans was a zoonosis (15). *P. carinii* is clearly transmissible between animals of the same species by the airborne route under controlled experimental conditions (14). However, the ability of *P. carinii* from one mammalian host species to infect members of another species is unclear. Published experimental data are conflicting. Attempts to transmit *P. carinii*, by either intranasal or intrapulmonary inoculation, from dogs to immunosuppressed guinea pigs, from rats to immunosuppressed mice or hamsters, from mice to nude rats, and from humans to nude or immunosuppressed rats have all been unsuccessful (3–5, 16). In contrast, intranasal or intrapulmonary inoculation of humans or rat *P. carinii* into nude mice has been reported to result in infection of the mice (5, 23). However, subsequent experiments by one of these investigators under more controlled conditions failed to demonstrate infection of nude mice by rat *P. carinii* (22). Most recently, intratracheal inoculation of human *P. carinii* into SCID mice already infected with *P. carinii* is reported to have resulted in a simultaneous infection by human and mouse *P. carinii*, as determined by staining of the organisms with monoclonal antibodies (MAbs) (17). The reasons for the conflicting results are unclear; however, most of these studies (except the one described in reference 17) were performed before reagents capable of distinguishing between *P. carinii* isolated from various host species were available. Therefore, even though control animals were used in many of these experiments, the development of natural (or endogenous) PCP would have been difficult, if not impossible, to identify in the inoculated animals, had it occurred.

Many laboratories, including our own, have been actively involved in basic studies of *P. carinii* (reviewed in references 2 and 19). These studies have generated reagents which can be used to study specific antigens and genes of *P. carinii*. For example, using MAbs specific for *P. carinii* isolated from diverse mammalian hosts (6) and molecular probes based on antigen-specific cDNA (10, 18), we have demonstrated both phenotypic and genotypic variation among antigens of *P. carinii* which are specific to the host of origin (6, 8).

The availability of reagents with which we could unequivocally distinguish between ferret and SCID mouse *P. carinii* prompted us to reexamine the issue of transmission of *P. carinii* from one host species to another. Using the SCID mouse model of PCP, in which *P. carinii* is easily and reproducibly transmitted from mouse to mouse by direct intratracheal inoculation, we examined the ability of ferret *P. carinii* to produce pneumonitis after inoculation into susceptible SCID mice.

MATERIALS AND METHODS

Animals. Male ferrets weighing 500 to 750 g were purchased from Marshall Farms, North Rose, N.Y. To induce PCP, ferrets were given drinking water containing 4 mg of dexamethasone (Lypho Med Inc., Rosemont, Calif.) per liter and 500 mg of tetracycline (Butler, Columbus, Ohio) per liter ad libitum. We obtained 4- to 11-week-old C.B-17 *scid/scid* mice from the Trudeau Animal Breeding Facility. These animals were bred from a foundation stock of SCID mice originally obtained from Leonard Schultz, Jackson Laboratory, Bar Harbor, Maine. They were bred and housed in microisolator cages into which air was introduced through high-efficiency particulate filters. The mice were given autoclaved food and water, and all animal maintenance procedures were performed in a clean hood. Two colonies of SCID mice are maintained at the Trudeau Institute. One colony is infected with *P. carinii*, whereas the other is free of *P. carinii*. The *P. carinii*-free colony consists of SCID mouse breeders that are the offspring of SCID mice that were reconstituted with 5×10^7 C.B-17 spleen cells intravenously. This is a convenient means to derive *P. carinii*-free SCID mice.

Transmission studies. The development of PCP in the ferrets was documented by lavage of the airway of a sentinel

* Corresponding author.

animal with 5 ml of saline after administration of light anesthesia (Metofane; Pitman-Moore, Inc., Washington Crossing, N.J.) and by examination of the lavage fluid after staining it with toluidine blue O (14). The sentinel animal was not used as a source of organisms for the inoculation studies. Once PCP had developed in the sentinel animal, a second ferret was sacrificed by an overdose (1 ml intraperitoneally) of barbiturate (Buthenasia; Schering Corp., Kenilworth, N.J.). The lungs were then removed, cut into small pieces, and pushed through a stainless steel screen into Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (GIBCO, Grand Island, N.Y.). The number of *P. carinii* nuclei in the resulting suspension was determined microscopically as described below. The homogenates were then washed once and resuspended to the concentrations of *P. carinii* indicated in individual experiments. SCID mice were inoculated intratracheally with 0.1 ml of the lung homogenates as previously described (12). *P. carinii*-infected SCID mouse lungs were also processed in parallel with the ferret lungs, and additional *P. carinii*-free SCID mice were inoculated with numbers of mouse *P. carinii* cells similar to the ferret *P. carinii* dose given the other mice. Throughout the procedure, the inocula were kept at 4°C and were used within 1 h of sacrifice of the donor animal. Each experiment was done with a ferret purchased from the vendor at a different time. The experiments took place over a 2-year period.

Analysis of transmission of *P. carinii*. At various times, SCID mice inoculated with ferret- or mouse-derived *P. carinii* and uninoculated controls were sacrificed, and the number of *P. carinii* nuclei in their lungs was determined as previously described (13). Briefly, lungs were pushed through stainless steel screens into 5 ml of Mg^{2+}- and Ca^{2+}-free Hanks' balanced salt solution and cytospin preparations of diluted homogenates were stained with Diff Quik (American Scientific Products, McGaw Park, Ill.). The number of *P. carinii* nuclei per 20 to 50 oil immersion fields was determined and was used to calculate the total number of *P. carinii* nuclei per lung. With this method, the presence of one nucleus in 50 fields corresponds to a limit of detectability of $10^{3.8}$ *P. carinii* nuclei per lung.

Characterization of *P. carinii* recovered from SCID mice. *P. carinii* cells isolated from inoculated SCID mice were characterized as being of either mouse or ferret origin by two methods. First, indirect immunofluorescence analysis (IFA) was performed as previously described (6, 9). For the IFA, we used five MAbs: 85-1-5E12, which binds to *P. carinii* regardless of its host of origin; 88-2-1D1 and 88-2-3E1, which specifically recognize ferret *P. carinii*; and 90-3-2B5 and 90-3-5B1, which are specific for mouse *P. carinii*. Second, we performed polymerase chain reaction (PCR) analysis with two sets of primers. Primers 903-S (5'-CCAGAAG GAATAGATCCACTGGTG-3') and 904-AS (5'-GTGCGT TGCATTTCGTTACAG-3') specifically amplify a portion of the gene encoding glycoprotein A of ferret *P. carinii* but not that encoding the homologous molecule in *P. carinii* from mice, rats, or humans (8). Primers pAZ102-E (5'-GATGGC TGTTTCCAAGCCCA-3') and pAZ102-H (5'-GTGTACGT TGCAAAGTACTC-3') amplify a portion of the gene encoding the mitochondrial rRNA of *P. carinii* isolated from all hosts studied to date (8, 20). PCR was performed by using Gene Amp PCR Core reagents and the hot start method, with AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.) as described previously (10). Reactions were performed in a volume of 100 μl containing 0.2 mM deoxynucleoside triphosphates, 50 mM KCl, 3 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 1 μM each primer, and 2.5 U of AmpliTaq. Samples were prepared by boiling the lung homogenates for 10 min, followed by centrifugation at 16,000 × *g* for 3 min. A 2-μl volume of the clarified supernatant was used in each reaction mix. Samples were denatured at 94°C for 90 s, annealed at 65°C for 90 s, and extended at 72°C for 120 s. A total of 35 cycles were performed. Amplified DNA products were separated by agarose gel electrophoresis (1.4% agarose), stained with ethidium bromide, and visualized by UV transillumination.

RESULTS

A summary of the results of five experiments is shown in Table 1. In experiments 1 to 3, a total of 28 SCID mice were inoculated intratracheally with ferret *P. carinii*. The size of the inoculum varied from 8×10^5 to 1×10^7 *P. carinii* nuclei. When sacrificed 27 to 84 days postinoculation, none of the animals had detectable *P. carinii* in their lungs as determined by staining with Diff Quik. The failure to detect *P. carinii* by Diff Quik staining was confirmed in animals from experiment 3 by IFA, using several *P. carinii*-specific MAbs (Table 2, group 3a). Likewise, none of the 28 uninoculated controls had any evidence of infection with *P. carinii*. In contrast, 92% (24 of 26) of SCID mice inoculated with mouse *P. carinii* developed PCP (Table 1, experiments 1 to 3).

While these studies were under way, it was reported that SCID mice naturally infected with *P. carinii* could be inoculated with human-derived *P. carinii*, resulting in a dual infection (17). To determine whether similar results could be achieved with ferret *P. carinii*, *P. carinii*-free SCID mice and SCID mice naturally infected with *P. carinii* were inoculated with 5×10^6 ferret *P. carinii* nuclei (Table 1, experiments 4a and 4d). The *P. carinii*-free SCID mice, inoculated with ferret *P. carinii*, were free of *P. carinii* when examined by Diff Quik staining and IFA 9 weeks after inoculation (Tables 1 and 2). As expected, the SCID mice naturally infected with *P. carinii* and inoculated with ferret *P. carinii* were heavily infected with *P. carinii* when sacrificed 8 weeks after inoculation. Control *P. carinii*-free SCID mice inoculated with mouse *P. carinii* also developed PCP; the uninoculated mice were again free of demonstrable organisms.

The *P. carinii* organisms recovered from the animals in experiment 4 were analyzed by IFA and PCR to determine whether they were of ferret or mouse origin. IFA demonstrated that the *P. carinii* organisms isolated from group 4b and 4d animals reacted with mouse *P. carinii*-specific MAbs and not with ferret *P. carinii*-specific MAbs, indicating they were typical mouse *P. carinii* organisms (Table 2). PCR was performed on lung homogenates from the same animals by using both ferret *P. carinii*-specific primers and primers which amplify *P. carinii* DNA from all sources (Fig. 1A). The universal *P. carinii* primers were able to amplify *P. carinii* DNA from the ferret *P. carinii* inoculum, the mouse *P. carinii* inoculum, the *P. carinii* organisms recovered from the *P. carinii*-free SCID mice inoculated with mouse *P. carinii* (experiment 4b), and the *P. carinii* organisms recovered from the naturally infected SCID mice inoculated with ferret *P. carinii* (experiment 4d). In contrast, the ferret *P. carinii*-specific primers were able to amplify DNA from the ferret *P. carinii* inoculum only. These results indicated that inoculation of naturally *P. carinii*-infected SCID mice with ferret *P. carinii* did not result in a dual infection.

PCR analysis of the lung homogenates provided further evidence that the failure to identify ferret *P. carinii* inoculated into the SCID mice was because of a lack of infection rather than because the numbers of organisms were below

TABLE 1. Summary of the results of inoculating SCID mice with mouse- or ferret-derived *P. carinii*

Expt	Age (wk)[a]	No. of *P. carinii* inoculated		Time (days)[b]	No. of *P. carinii* organisms recovered (logs)[c]	No. infected/no. inoculated
		Ferret	Mouse			
1a	4–6	8×10^6	0	27	<3.80	0/8
1b	4–6	8×10^6	0	43	<3.80	0/6
1c	4–6	0	8×10^6	27	6.40 ± 0.62	8/8
1d	4–6	0	8×10^6	43	6.80 ± 0.69	8/8
1e	4–6	0	0	27	<3.80	0/8
1f	4–6	0	0	43	<3.80	0/8
2a	8–10	1×10^7	0	56	<3.80	0/10
2b	8–10	0	1×10^7	56	5.74 ± 1.39	5/7
2c	8–10	0	0	56	<3.80	0/8
3a	10	8×10^5	0	84	<3.80	0/4
3b	10	0	8×10^5	84	6.77 ± 0.53	3/3
3c	10	0	0	84	<3.80	0/4
4a	4–6	5×10^6	0	63	<3.80	0/4
4b	4–6	0	5×10^6	63	6.73 ± 0.96	4/4
4c	4–6	0	0	63	<3.80	0/4
4d	6[d]	5×10^6	0[d]	56	7.71 ± 0.32[e]	3/3
5a	11	7×10^6	0	28	3.31 ± 0.01	1/4
5b	1	7×10^6	0	55	4.94 ± 1.67	3/4
5c	1	0	0	28	<3.80	0/1
5d	1	0	0	55	<3.80	0/3

[a] At time of inoculation.
[b] Time from inoculation to sacrifice.
[c] Limit of detection is 3.80 $\log_{10}$ units by Diff Quik staining; the number is the mean count per mouse.
[d] These mice had PCP at the time of inoculation with ferret *P. carinii*.
[e] Mouse *P. carinii* by phenotype and genotype (see text).

the limit of microscopic detection. Homogenates from animals in experiment 4a were negative for *P. carinii* DNA by PCR with both sets of primers: 903-S plus 904-AS and pAZ102-E plus pAZ102-H (Fig. 1A). In titer determinations with the pAZ102-E plus pAZ102-H primer pair, we were able to detect mouse *P. carinii* at a concentration of 10 or fewer nuclei per ml of lung homogenates.

Experiment 5 demonstrated the potential for "breakthrough" natural infection during the performance of these types of studies. Four of eight *P. carinii*-free SCID mice that were inoculated with ferret *P. carinii* were noted to have organisms in their lungs (Table 1), although the number of nuclei was approximately 1 to 2 log units lower than noted in the other experiments. However, when examined by IFA, these organisms were recognized only by the mouse *P. carinii*-specific MAb (Table 2). In addition, when analyzed by Western immunoblot, *P. carinii* glycoprotein A from these organisms demonstrated the characteristic size and migration pattern seen with mouse *P. carinii* (6) (data not shown). PCR with the ferret *P. carinii*-specific primers failed to detect any target DNA in the inoculated animals (Fig. 1B, lane 2). In contrast, PCR with the pAZ102 primer pair confirmed the presence of *P. carinii* DNA not only in three of four inoculated mice (group 5b) in which *P. carinii* were demonstrated by microscopy but also in one of the three uninoculated control mice (group 5d). A lung homogenate

TABLE 2. Results of IFA of lung homogenates obtained from various groups of mice

Group[a]	Visualization of *P. carinii* with MAb specific for:					
	All *P. carinii* (85-1-5E12)	None (PBS)[b]	Mouse *P. carinii*		Ferret *P. carinii*	
			90-3-2B5	90-3-5B1	88-2-3E1	88-2-1D1
3a	−	−	−	−	−	−
3b	+	−	+	+	−	−
3c	−	−	−	−	−	−
4a	−	−	−	−	−	−
4b	+	−	+	+	−	−
4c	−	−	−	−	−	−
4d	+	−	+	+	−	−
5b	+	−	+	+	−	−
Ferret *P. carinii*	+	−	−	−	+	+
Mouse *P. carinii*	+	−	+	+	−	−

[a] Groups correspond to the experimental groups listed in Table 1.
[b] PBS, phosphate-buffered saline control.

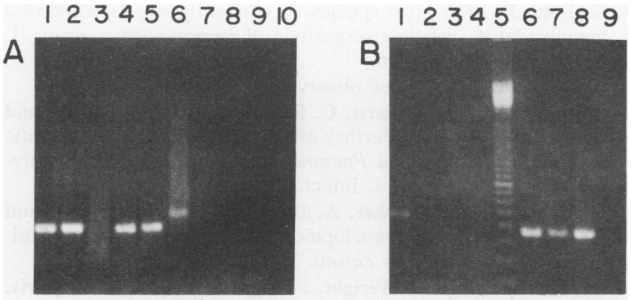

FIG. 1. (A) PCR analysis of representative lung homogenates from experiment 4 in which ferret or mouse *P. carinii* organisms were inoculated into *P. carinii*-free SCID mice or SCID mice naturally infected with *P. carinii*. Samples in lanes 1 to 5 were amplified with the "universal" *P. carinii* rRNA primers pAZ102-E and pAZ102-H, whereas those in lanes 6 to 10 were amplified with ferret *P. carinii* specific primers 903-S and 904-AS. Reaction product-sizing standards in 100-bp increments for both panels are shown in panel B, lane 5. Lanes: 1 and 6, ferret *P. carinii* inoculum; 2 and 7, mouse *P. carinii* inoculum; 3 and 8, *P. carinii*-free SCID mouse inoculated with ferret *P. carinii* (experiment 4a); 4 and 9, *P. carinii*-free SCID mouse inoculated with mouse *P. carinii* (experiment 4b); 5 and 10, *P. carinii*-infected SCID mouse inoculated with ferret *P. carinii* (experiment 4d). (B) PCR analysis of representative lung homogenates from experiment 5. Samples in lanes 1 to 4 were amplified with ferret *P. carinii*-specific primers, whereas those in lanes 6 to 9 were amplified with the universal *P. carinii* rRNA primers. Lanes: 1 and 6, ferret *P. carinii* inoculum; 2 and 7, SCID mouse inoculated with ferret *P. carinii* (experiment 5b); 3 and 8, uninoculated control SCID mouse (experiment 5d); 4 and 9, *P. carinii*-free SCID mouse control obtained from a different litter; 5, sizing standards in 100-bp increments.

from a *P. carinii*-free SCID mouse taken from a different group of animals was negative for *P. carinii* DNA in PCR with both primer pairs (Fig. 1B, lanes 4 and 9). We have analyzed samples from numerous SCID mice by PCR, and no reaction product was detectable in animals truly free of *P. carinii* when the pAZ102 primer pair was used. Therefore, our interpretation of these results is that the PCP which occurred in group 5a and 5b animals was not a result of inoculation with ferret *P. carinii* but, rather, resulted from an inadvertent break in the containment of the *P. carinii*-free SCID mice.

DISCUSSION

Direct inoculation of SCID mice with ferret *P. carinii* was used to show that *P. carinii* obtained from one mammalian host is not necessarily transmissible to a different mammalian host. Forty-three SCID mice were inoculated with *P. carinii* isolated from five different ferrets over a 2-year period; none of the mice ever became infected with ferret *P. carinii*. Four methods were used to examine the lungs of the recipient animals to ensure that no organisms were transmitted: Diff Quik and toluidine blue O staining, IFA, and PCR. In marked contrast, similar experiments with mouse-derived *P. carinii* as the inoculum consistently resulted in infection of the *P. carinii*-free SCID mice (28 of 30 mice). The ability to easily transmit *P. carinii* from mouse to mouse or from rat to rat (1) by intratracheal inoculation is strong evidence that our failure to transmit ferret-derived *P. carinii* to SCID mice was due to true host range restriction by *P. carinii* rather than to the artificial mode of transmission used in our experiments.

Our results confirm and extend the observations by other investigators who were unable to transmit *P. carinii* from one mammalian host to another (3–5, 16). There are three possible explanations for our results: (i) the inoculated organisms were not viable, (ii) the infectious form of the organism was not present in the lungs of the donor ferrets, or (iii) the SCID mice could not support the growth of ferret *P. carinii*. Although there is no unequivocal method to prove whether *P. carinii* organisms are viable, we took precautions to ensure that organisms were removed and processed quickly and gently to protect their viability. In control experiments, mouse *P. carinii* was successfully transmitted to *P. carinii*-free SCID mice, indicating that the processing involved in these experiments did not kill the *P. carinii* organisms and that the infectious form of the organisms is present in the lungs during active PCP. Because specific-pathogen-free (*P. carinii*-free) ferrets are not available, we were not able to include the additional control experiment of ferret-to-ferret transmission. Therefore, our interpretation of the experimental results was that SCID mice were unable to support the growth and replication of ferret-derived *P. carinii*.

In contrast to our inability to transmit ferret *P. carinii* to immunosuppressed mice, human *P. carinii* organisms have been reported to infect susceptible mice (5, 17, 23). It is possible that the biology of human-derived *P. carinii* is different than that of ferret-derived organisms, thereby enabling human *P. carinii* to infect other mammals. It is also possible that the interpretation that human *P. carinii* was transmitted was complicated by a breakthrough natural infection. As shown in experiment 5, breakthrough infection with mouse *P. carinii* occurred, even through precautions were taken to prevent natural infection in the mice. Breakthrough infections in colonies of susceptible mice have been noted by other investigators (21). It was only because of the availability of specific reagents, which allowed us to distinguish ferret *P. carinii* from mouse *P. carinii*, that we were able to make the distinction between transmission and breakthrough infection. One study did use MAbs to analyze the transmission of human *P. carinii* to SCID mice (17). However, the design of that study was unconventional in that human *P. carinii* organisms were inoculated into SCID mice which were already actively infected with naturally acquired *P. carinii*. The organisms recovered from these animals appeared to be a mixed population of human and mouse *P. carinii* organisms on the basis of staining with MAbs. However, we were unable to obtain a similar result when using ferret *P. carinii* inoculated into *P. carinii*-infected SCID mice (experiment 4). It will be important to repeat studies of transmission of human *P. carinii* with specific reagents and *P. carinii*-free SCID mice to definitively answer the question of transmissibility of human *P. carinii* to susceptible animals.

PCR analysis demonstrated the presence of *P. carinii* DNA in both ferret *P. carinii*-inoculated and uninoculated SCID mice used in experiment 5. However, only in mice which were inoculated with ferret *P. carinii* were the organisms numerous enough to be detected by light microscopy. It is not known why this difference occurred, but it is possible that the inoculation with ferret lung homogenate in some way exacerbated the development of PCP in these animals. Indeed, we have found that nonspecific inflammatory responses in the lungs of SCID mice can exacerbate PCP, and it is reasonable to presume that the inoculation with ferret lung homogenates did induce an inflammatory response (11).

In summary, our results indicate that there can be a biological barrier to the transmission of *P. carinii* from one

mammalian host species to another. They are also consistent with the increasing evidence for antigenic diversity among *P. carinii* organisms isolated from different mammalian hosts (2, 6, 8, 19). These results should not be interpreted as indicating that *P. carinii* from one species can never infect another species, since the potential number of donor and recipient species combinations is enormous and has not been evaluated.

Two important questions are raised by our studies: first, the mechanism by which the host range of *P. carinii* is restricted, and, second, the location of the reservoir for organisms responsible for human disease. We have shown significant phenotypic and genotypic variation between two different surface antigens of *P. carinii* (7, 8). It is possible that the variation in these or other *P. carinii* surface molecules plays a role in defining host specificity. If this restricted host range extends to organisms responsible for human disease, the possibility must be considered that PCP in humans is not a zoonosis but, rather, is spread directly from human to human or from humans to the inanimate environment and then back to humans. As we continue to extend our understanding of this significant opportunistic pathogen and develop additional reagents for its study, identification of the reservoir of organisms responsible for human disease will have important epidemiological implications.

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