Multiplication of human-derived *Pneumocystis carinii* in severe combined immunodeficient (SCID) mice

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Abstract. Clinically healthy SCID mice were infected intratracheally with *Pneumocystis carinii* (PC) of human origin. The data obtained provides unambiguous evidence that progressive multiplication of PC organisms of human origin takes place in the lungs of experimentally infected animals. SCID mice that were infected with human-derived PC also revealed a markedly greater number of mouse PC organisms in their lungs than the controls. All the SCID recipients of human PC died by day 65 post infection, whereas the controls, housed under identical conditions, started dying significantly later due to severe mouse pneumocystosis. This animal model could be used for the maintenance and propagation of human PC, and for evaluating strategies for treating human pneumocystosis.
Key words. SCID mice; *Pneumocystis carinii*; immunodeficiency; pneumonia; monoclonal antibodies.

Pneumocystis carinii (PC) is well recognized as the etiologic agent of a life-threatening pneumonia in the immunosuppressed host[2] and has emerged as the leading cause of morbidity and mortality in patients with AIDS[1,3]. The organism has not been grown in cell-free culture media, and limited success has been achieved in maintaining and/or growing *Pneumocystis* of rat or human origin in certain types of cell cultures[4-6]; to date, continuous culturing of the organism has not been documented. A variety of laboratory animals which have been treated for prolonged periods with immunosuppressive agents such as corticosteroids, or maintained on a low-protein diet, spontaneously develop fulminant pneumocystosis, presumably as a result of the reactivation of latent infection[7-10]. However, such animal models are not suitable for analysing the direct pathogenic effects of PC or for assessing specific histological and immunological findings. One of the disadvantages is that long-term immunosuppression in animals leads to an exacerbation of a variety of other lung infections, besides pneumocystosis[10]. Earlier efforts to establish infection from one animal species to another have failed[7] and experimental transmission of human or rat PC in congenitally athymic (nude) mice or in nude rats has met with only modest success[11].

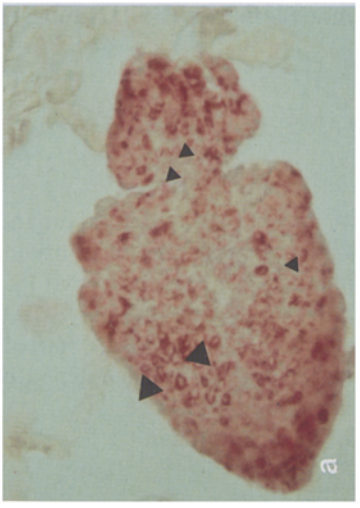
In the present study, human-derived PC organisms were used to infect severe combined immunodeficient (SCID) mutant mice which lack both functional T and B cells[12]. Before experimental infection, the SCID mice showed no signs of clinical illness, but occasional PC cysts were observed, either singly or in small clusters in the lung imprints from 6 of the 10 randomly selected animals. Monoclonal antibodies (Mabs) which could distinguish between PC of human and mouse origin were used as fluoresceinated probes to identify and simultaneously monitor the development/multiplication of human and/or mouse PC in the lung tissues of the infected mice.

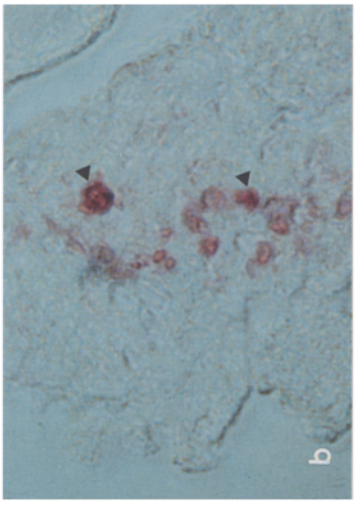
Materials and methods

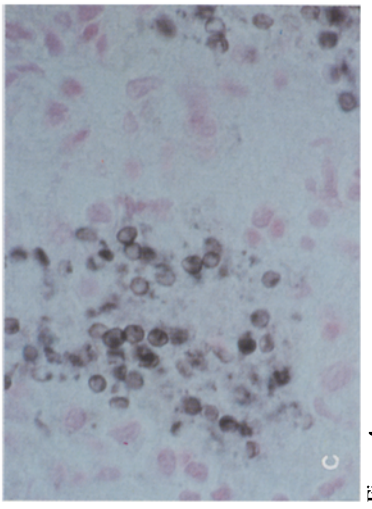
Young, clinically healthy C3H/HeSnJ male SCID mice were obtained from Jackson Laboratory. A total of 30 SCID mice were inoculated intratracheally by a previously-described procedure[15]. Briefly, mice were anesthesized by intraperitoneal injection of pentobarbital (80 mg/kg), a midline incision was made over the trachea and 0.1 ml of concentrated inoculum (approximately 10^5 cystic forms) followed by 0.2 ml of air was injected under direct visualization in the exposed trachea. The incision was sutured and the injected mice allowed to recover. The inoculum used for infecting the mice consisted of human-derived PC organisms harvested from the bronchoalveolar lavage (BAL) fluid of an AIDS patient with PC pneumonia. A freshly-obtained BAL specimen was strained through 2 layers of sterile gauze and then subjected to density gradient centifugation with Ficoll-Paque (Pharmacia Sweden). The interface between the supernatant and the Ficoll was collected and suspended in phosphate buffered saline (PBS) and used for inoculation after vigorous vortexing. When immunostained with human PC specific Mabs (fig. 2), the inoculum showed clumps of PC cysts, intermediate forms and structures corresponding to trophozoites, which were embedded in a stained matrix. The inoculum was free of bacterial and fungal contamination, and attempts to isolate AIDS virus in culture failed.

To simultaneously detect and quantitate human and mouse derived PC in lung smears of infected mice, double immunofluorescence (DIF) staining was performed with conjugates of PC specific Mabs. A mixture of Mabs 2G2 and 6B8 allows the visualization of different developmental stages of human PC[16,18], and 2C Mabs stains exclusively the cystic forms of mouse-derived PC[17]. Mabs 2G2 and 6B8 were conjugated with fluorescein-isothiocyanate (FITC) and Mab 2C11 with rhodamine according to standard procedures[19]. Samples from selected areas of lung tissues were used to prepare impression smears (approximately 1 cm^2) on glass slides.

The smears were fixed in cold acetone for 5 min, and covered with appropriate dilutions of FITC conjugated Mabs reagent for 30 min at 37 °C. The smears were washed 3 times with PBS and then covered with pretitrated dilutions of rhodamine-conjugated anti-mouse PC







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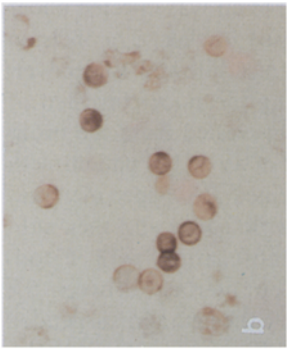

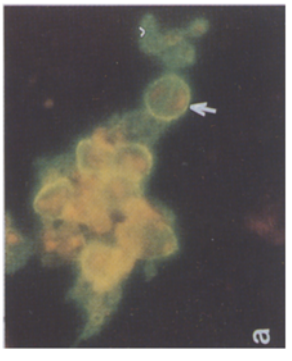

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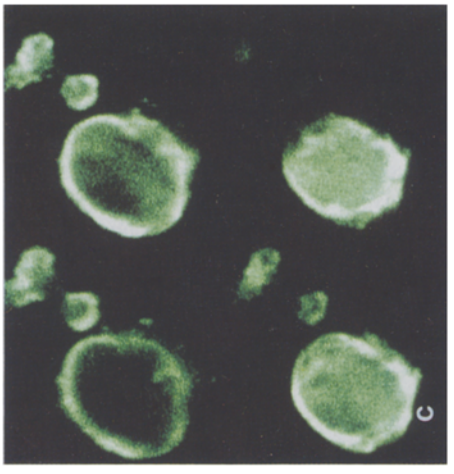

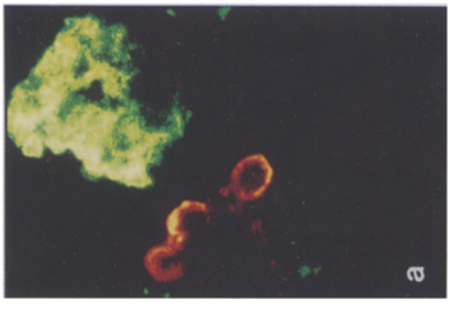

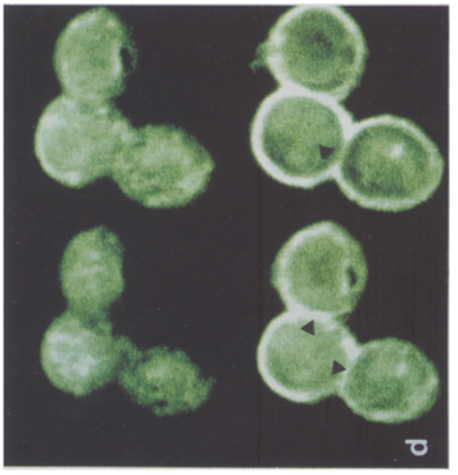

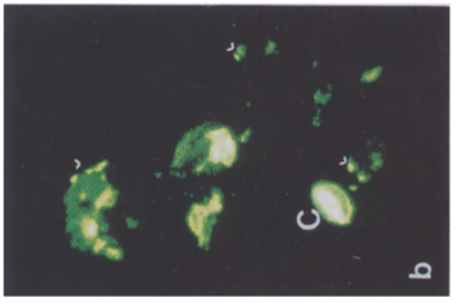

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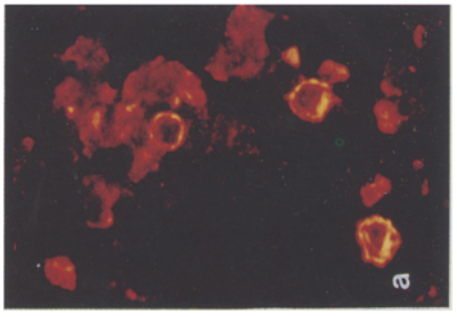

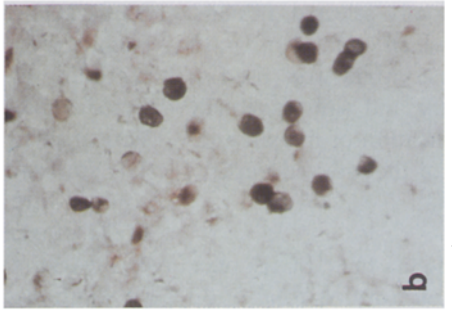

Figure 1

Mabs. After reincubation for 30 min at 37 °C, the smears were washed (3 times) with PBS, mounted under a coverslip in buffered glycerol, and then examined under a fluorescence microscope. In smears which contained both human and mouse PC organisms, the two types could be distinguished; cystic forms of mouse PC stained red and human forms a characteristic apple-green. Lung tissues of mice obtained after autopsy were fixed in 4% buffered formaldehyde, embedded in paraffin and sectioned at 4–8 μm. The sections were stained with GMS stain[20]; only cysts could be evaluated in silver-stained sections. Deparaffinized lung sections were also immunostained by a Mabs-based alkaline phosphatase anti-alkaline phosphatase (APAAP) technique[21]. Briefly, the sections were overlaid with working dilutions of Mabs 2G2 and 6B8 or 2C11 and incubated for 20 min at 37 °C. The sections were washed in PBS at room temperature, incubated with APAAP complex (Dako Patts, Denmark) for 20 min at 37 °C, washed in PBS at room temperature, and developed for 20 min at room temperature using New Fuschin and β-naphthol-AS-Bi-phosphate as substrate. The intensity of infection was scored by evaluation of lung sections originating from at least 3–4 lung blocks from each animal, representing both right and left lung lobes.

Results and discussion

Because SCID mice are known to carry enzootically subclinical PC infections[13, 14], a group of randomly selected mice were sacrified immediately after arrival from the supplier and examined for the presence of PC in their lungs. The lungs and other organs of the necropsied animals appeared normal on gross examination. Histologically, the lung tissues revealed mild or no inflammatory response. However, PC organisms could be identified in the lung sections as well as in lung smears from 6 of the 10 mice both by Gomori's methenamine silver (GMS) staining and by staining with Mabs specific for mouse-derived PC (fig. 1). The organisms were observed as scattered, isolated cysts and very infrequently as small clusters; *Pneumocystis* forms consistent with trophozoites were not identified.

A group of 30 SCID mice received intratracheal injections of human-derived PC organisms (approximately 10^5 cystic forms) and a second group of 10 SCID mice were included as uninoculated controls. All except 4 of the 22 intratracheally infected mice which were sacrificed during the observation period showed unequivocal evidence of the proliferation of human PC in their lung tissues (table). The lung smears of 2 experimentally infected mice sacrificed on day 4 post-infection showed no evidence for the multiplication of human PC when probed with specific Mabs. However, on day 10, a substantial increase in the quantity of human PC organisms, compared to the starting inoculum levels, was observed in the lung smears of sacrificed mice with the aid of Mabs specific for human PC. The organisms appeared in the form of numbers of discrete clumps of thin-walled cyst-like structures embedded in a proteinaceous matrix which was heavily stained by specific Mabs (fig. 3). Additionally, a preponderance of trophozoite forms, either embedded in the matrix or lying separately, was consistently seen. At this stage of infection, no significant changes in the number of PC cysts of mouse origin were observed in the lung smears of inoculated mice or of the age-matched controls. These mice did not appear to be clinically ill, and on necropsy their lungs appeared normal on gross examination.

Mice sacrificed between days 21 and 65 also showed no signs of clinical disease before necropsy. However, in most of them the lungs were enlarged and grayish whereas other organs appeared normal. The lung imprints of these mice exhibited impressively large sticky aggregates of human PC which were teeming with trophozoite forms, but the characteristic cyst forms were fewer in number. In some instances, cysts were not identifiable at all among large aggregates of PC forms stained with human PC specific Mabs. Infected mice which were sacrificed from day 20 onwards also showed markedly increased numbers of PC cysts of mouse origin compared with the controls which did not receive human PC. Eight of the 30 inoculated mice died spontaneously between days 21 and 65 post infection. Clinically, these mice were sick for several days before death and their lungs showed

Figure 1. *a* Mouse PC cysts in lung imprint from a normal (uninoculated) SCID mouse stained with rhodamine conjugated mouse PC specific Mabs (2C11). Note bright red staining of the peripheral rims of isolated cysts (× 1400). *b* GMS stained histologic lung section from a normal (uninoculated) SCID mouse: Note oval and crescent-shaped cysts of mouse PC (× 140).

Figure 2. *a* Human PC organisms stained with Mabs 2G2 and 6B8. Note cluster of cysts with characteristic parenthesis-like bodies (arrow) and forms corresponding to trophozoites (arrow head) embedded in stained matrix (× 840). *b* Human PC organisms stained with GMS stain (× 140).

Figure 3. Confocal laser scanning in photo micrographs (GLSM) of DIF stained lung imprints from a SCID mouse infected with human PC and sacrificed on day 10 p.i. *a* Bright yellow-green fluorescent aggregate representing human PC organisms and structures with red stained peripheral rims are cysts of mouse PC (× 1400). *b* A different microscopic field of the lung imprint showing human PC cyst (C) and small pleomorphic structures either singly or in clumps which correspond to trophozoites (arrow head; × 1400). *c, d* The illustrations show 4 confocal sections at a distance of 3 μm (× 2800). Note the individual trophozoite forms (c) and cystic forms showing evidence of binary division (d).

Figure 4. Lung sections of SCID mouse infected with human PC; the mouse died spontaneously on day 55 p.i. *a* Immunostaining with human PC specific Mabs using APAAP method. Note red-stained intraalveolar mass of human PC organisms showing cyst cell walls (large triangles) and numerous trophozoite forms (small arrows) (× 875). *b* Demonstration of mouse PC cysts (small triangles) in lung tissue by immunostaining using mouse PC specific Mabs (2C11) and APAAP method (× 875). *c* GMS stained lung section showing numerous PC oval and crescent PC cysts which stained brownish black (× 560).

Summary of findings on 30 SCID mutant mice experimentally infected with human derived *P. carinii*

No. of mice sacrificed (S)/spontaneous death (D)[a]	No. of days between inoculation and death	Clinical symptoms[c]	Microscopic findings in lungs: Double immunofluorescence staining of impression smears – Reactivity with: FITC conjugated anti-human PC Mabs 2G2+6B8			Rhodamine conjugated antimouse PC Mabs 2C11	Histological sections[d] Gomori silver staining	Immunostaining (APAAP) with Mabs	
			Fluorescent aggregates[b]	Trophozoites	Precyst/cyst stage	Precyst/cyst stage		Anti-human PC Mabs 2G2+6B8	Anti-mouse PC Mabs 2C11
2 (S)	4	−/−	−/−	M/M	−/1+	0/1+	0/0	0.5/0	0.5/0.5
2 (S)	6	−/−	1+/±	M/E	−/−	1+/0	0/0	0.5/0.5	0.5/0
2 (S)	10	−/−	2+/2+	E/E	−/+	1+/1+	0/0	1+/1+	0/0
2 (S)	15	−/−	1+/2+	E/E	−/−	1+/2+	0/0	1+/1+	0.5/0.5
1 (D)	21	+	2+	VE	−	2+	0.5+	2+/1+	0.5/1+
2 (S)	25	−/−	3+/3	E/E	−/1+	2+/2+	0.5+/0.5+	2+/2+	0.5/1+
2 (S)	30	−/−	4+/3+	M/M	−/−	3+/3+	1+/1+	2+/3+	1+/1+
2 (S)	36	−/−	4+/4+	M/M	−/−	3+/2+	1+/2+	3+/3+	1+/1+
2 (S)	40	−/−	4+/3+	M/M	−/1+	3+/2+	2+/2+	3+/3+	1+/2
2 (S)	45	−/+	2+/2+	VE/VE	−/+	3+/4+	2+/2+	3+/4+	2+/2+
2 (S)	52	−/−	NT	NT	NT	NT/NT	2+/2+	3+/3+	1+/2+
3 (D)	55	+/+/+	3+/3+/4+	M/M/E	−/−/1+	4+/4+/2+	2+/2+/2+	3+/3+	2+/3+
2 (D)	58	+/+	4+/4+	M/M	−/1+	3+/4+	2+/2+	3+/3+	2+/3+
2 (S)	60	−/+	4+/4+	M/M	−/−	4+/4+	2+/2+	3+/3+	4+/3+
2 (D)	65	+/+	3+/4+	M/E	−/−	4+/NT	2+/2+	3+/3+	4+/2+

[a] Mice sacrificed (S) or died spontaneously (D). [b] Characteristic homogeneous/granular fluorescent aggregates (FA) contained various developmental stages of human PC (presumably the trophozoites (TR), sporozoites, precyst-cyst (C) stages). The presence of FA per smear was scored semiquantitatively: 1+ = 1–10 small FA, 2+ = >10 small and large FA, 3+ = 5–10 only large FA and 4+ = >10 large FA. The quantity of morphologically unequivocal TR (individual and/or clumps) per smear was arbitrarily scored as N = no organisms seen, M = minimal, E = extensive and VE = very extensive. At least 10 oil immersion fields (x 63) per smear were scanned for the presence of trophozoites. The number of morphologically unequivocal C/smear was expressed by a score: 0 = no recognizable cysts, 1+ = 2–5 cysts, 3+ = 10–20 cysts and 4+ = >20 cysts mostly in aggregates. Each smear was examined entirely under high power (x 63). The results are based on examination of repeat smears (5–10) from each animal. NT = not tested. [c] Clinical symptoms refer to weight loss, ruffled greasy coat, scaly skin and dyspnea; + sigr = clinical symptoms present and − = clinical symptoms absent. [d] Grading scores for the intensity of infection are indicated as: 0 = absence of cysts and/or APAAP staining. The abbreviations 0–5+, 1+, 2+, 3+ and 4+ denote <1%, 1–25%, 25–50% and 50–70% am. alveoli containing cysts and/or APAAP staining material.

areas of consolidation on cut surfaces. Histologically, large numbers of PC organisms of human as well as of mouse origin could be detected in their lungs (fig. 4). No spontaneous deaths were observed among the control group of mice during a 2-month period. However, eventually all the controls died spontaneously between 4 and 5 months after their arrival in the laboratory. All these mice showed manifestations of severe clinical disease which included loss of body weight, dry skin and dyspnea; the lungs were rubbery and heavily consolidated. Lung smears and histological sections from these mice revealed strikingly large numbers of organisms that were stained by mouse but not by human PC specific Mabs. The above results document that human PC can be maintained and will proliferate in the lung tissues of SCID mice. The availability of Mabs specific for various developmental stages of human PC, together with the distinctive growth characteristics of these organisms when compared to PC of mouse origin, allowed the unambiguous detection and quantitation of human PC in mouse lung tissues. An earlier study[11] failed to obtain evidence of infection in nude (*nu/nu*) mice which received human PC intranasally. However, infection could be demonstrated in mice given intrapulmonary injections of human PC. In the present study, the human PC inoculum used to infect the mice contained virtually all the developmental stages of the organisms, and therefore it is not possible to define which stage(s) actually led to infection.

During the experimental period, remarkably increased numbers of trophozoites and markedly fewer encysted forms of human PC were detectable in the lungs of injected SCID mice. The observed sparsity of the mature cystic forms may be attributable either to the short observation period, during which the transition of trophozoites to cyst stage was not possible, or alternatively to some unknown host mechanism(s) which prevented the encystment of human PC. It is not clear whether the spontaneous deaths observed among recipients of human PC can be attributed to a direct effect of replicating human PC organisms alone, or to coinfection with PC of mouse origin. Of interest, however, is the observation that intratracheal injection of human PC in SCID mice created, in a relatively short time, a milieu in which the multiplication of mouse PC organisms was accelerated. This experimental model may be useful for providing new insights into the mechanism(s) by which human-derived PC can live successfully in the host environment and also for designing treatment strategies for the disease caused by this opportunistic pathogen.

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1 Mills, T., Rev. infect. Dis. *8* (1986) 1001.
2 Salfelder, K., and Schwarz, J., Am. J. Dis. Child. *114* (1967) 693.
3 Murray, J. F. et al., New Engl. J. Med. *310* (1984) 1682.

4 Cushion, M. T., and Walzer, P. D., Infect. Immun. *44* (1984) 245.
5 Bartlett, M. S., Verbanc, P. A., and Smith, J. W., J. clin. Microbiol. *10* (1979) 796.
6 Pifer, L., Hughes, W. T., and Murphy, M. J., Ped. Res. *II* (1977) 305.
7 Frenkel, J. K., Good, J. T., and Schultz, J. A., Lab. Invest. *15* (1966) 1559.
8 Walzer, P. D., in: *Pneumocystis carinii* infections. Ed. L. S. Young. Marcel Dekker Inc., New York 1984.
9 Sheldon, W. H., J. exp. Med. *110* (1959) 147.
10 Sethi, K. K., Salfelder, K., and Schwarz, J., Mycopath. Mycol. appl. (1968) 121.
11 Walzer, P., Schnelle, V., Armstrong, D., and Rosen, P. P., Science *197* (1977) 177.
12 Bosma, G. C., Custer, R. P., and Bosma, M. J., Nature *301* (1983) 527.
13 Walzer, P. D., et al., Infect. Immun. *57* (1989) 62.
14 Sundberg, J. P., Burnstein, T., Shultz, L. D., and Bedigiam, H., Lab. Animal Sci. *39* (1989) 213.
15 Bartlett, M. S., et al., J. clin. Microbiol. *26* (1988) 1100.
16 Kovacs, J. A., et al., J. Immun. *140* (1988) 2023.
17 Sethi, K. K., in: Progress in AIDS Research in the Federal Republic of Germany, pp. 283–289. MMV Medizin Verlag, München 1990.
18 Sethi, K. K., Ärztl. Lab. *36* (1990) 116.
19 Goldman, M., in: Fluorescence Antibody Methods. Ed. M. Goldman. Academic Press, New York.
20 Grocott, R. G., Am. J. clin. Path. *25* (1955) 175.
21 Cordell, J. L., et al., J. Histochem. Cytochem. *32* (1984) 219.

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Transfer of T-cell mediated immunity to *Hymenolepis nana* from mother mice to their neonates

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Abstract. Administration of lymph node cells from *Hymenolepis nana*-infected mice into lactating mothers, or directly suckling neonates successfully transferred immunity to the neonates. The capacity of lymph node cells to transfer immunity was completely abrogated by pretreatment with anti-Thy-1.2 monoclonal antibody and complement.
Key words. Hymenolepis nana; maternal transfer; protection; T-cell mediated immunity.

Immune mediators present in milk protect neonates from microbiological infections at a time when its own defense mechanisms may not be adequately developed. This type of immunity has been also demonstrated for parasitic infections of *Hymenolepis nana* in mice [1], *Taenia taeniaeformis* in mice [2], *Trichinella spiralis* in rats [3, 4] and *Schistosoma mansoni* in rats [5], among others. Although all of these reports showed that humoral factors in milk are important in providing immunity to suckling neonates, the cellular mediators in this immunity have received little attention.

H. nana, the dwarf tape worm of mice, is known to be strongly immunogenic. An oral inoculation with eggs elicits, during worm development in the intestinal villi, strong immune responses which confer protection upon the host by inhibiting larval growth in the intestinal villi: oncospheres from the challenge egg inoculation can invade the intestinal villi but cannot form cysticercoids there [6, 7]. It has been also shown that the immune responses responsible for inhibition of larval growth in the intestine of *H. nana*-infected mice are T-cell dependent. This was substantiated by studies in athymic nude mice [8] and in neonatally thymectomized mice [9, 10]. Furthermore, intravenous injection with mesenteric lymph node cells, especially Lyt-1 positive T cells from immunized donor mice have been shown to be capable of transferring immunity to athymic nude mice [11]. More recently, we reported that intravenous injection of BALB/c mice with monoclonal antibody to L3T4 antigen completely blocked not only development of immunity but also the increase in number of T cells characteristically induced by *H. nana* infection [12].

With regard to humoral antibody, Di Conza [13] indicated that serum from mice that received an oral inoculation of *H. nana* eggs had significant immune activity against subcutaneous larval growth, when the serum was injected into the area where the parasite was developing. Other observations [14, 15] also showed the presence of humoral antibody in serum from *H. nana*-infected mice. Since humoral antibody was detectable in serum only after 2 or more weeks of infection, its development can not account for the fact that the mice acquire immunity very rapidly, within 24 h.

Judging from these reports, the concept may be established that T cells are essential to initiate immunity to *H. nana*. Therefore, the *H. nana*-mouse system seemed to be a particularly good model for investigating maternal transfer of T-cell mediated immunity. We used this system in this study as a model of maternal transmission of immunity and examined whether T-cells responsible for development of immunity were adoptively transferred into suckling neonates via the milk.

Materials and methods

Mouse and parasite. BALB/c mice maintained under specific-pathogen free conditions were purchased from