The effect of heterogeneous hyperimmune IgG antibody on prophylaxis and treatment of *Pneumocystis carinii* infection in rats

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Abstract: Immunotherapy has been used in support of trimethoprim-sulfamethoxazole treatment for *Pneumocystis carinii* pneumonia. The present study investigated the therapeutic or preventive effects of heterogeneous hyperimmune IgG antibody (HIA) in experimental rats. Their immunity was suppressed by steroid injection, and they were also injected peritoneally with HIA which reacted with 40-55, 92, 116, and 200 kDa bands of the crude antigen. All rats were infected by *P. carinii* and the cystic forms on lung impression smears were counted. The count was 20.5-76.5 (mean 52.5 ± 19.3) in those which received steroid only, but decreased to 6.0-21.0 (mean 13.5 ± 10.6) in those of group 3 which received HIA for the same duration. In other groups, the mean count ranged from 29.9 ± 32.9 to 54.1 ± 47.7, and in those which received 13.7 mg HIA the reduction effect was greater than in those which received 6.8 mg or 20.5 mg HIA. The present finding confirmed that in rats during the early stage of infection, the heterogeneous HIA to MSG antigen bands had a partial effect on *P. carinii* pneumonia, both prophylactically and therapeutically.

Key words: *Pneumocystis carinii*, rats, hyperimmune serum, IgG antibody, prophylaxis, treatment

INTRODUCTION

Pneumocystis carinii is a well-known opportunistic pathogen of pneumonia among immunocompromised hosts. Because of the increasing number of AIDS cases and cancers

as well as instances of organ transplantation, the clinical importance of this pneumonia has expanded. At present, it is controlled in high-risk patients by prophylaxis with trimethoprim-sulfamethoxazole medication or pentamidine aerosol. Prophylaxis or treatment with these two agents is, however, frequently interrupted due to toxicity or resistance, and other alternative measures should replace them; a suitable candidate is immunoprophylaxis or immunotherapy.

In 1985, Furuta *et al.* (1985) demonstrated that loss of cellular or humoral immunity is closely related with *P. carinii* infection, and Gigliotti and Hughes (1988) observed the

partial protective effect of monoclonal antibodies. Roths and Sidman (1993) confirmed that in scid mice, passive transfer of immunized lymphocytes, monoclonal antibodies, and hyperimmune serum effectively treated this infection. These previous studies involved the passive transfer of homogeneous lymphocytes, monoclonal antibodies, and immune serum, and the partial effects of prophylaxis or treatment were observed.

The present study investigated the effect of purified IgG antibody of heterogeneous hyperimmune serum on *P. carinii* infection in rats.

MATERIALS AND METHODS

Induction of *P. carinii* infection of rats

Wistar rats bred simultaneously by the Laboratory Animal Center, Seoul National University, were immunosuppressed by weekly subcutaneous injections of 10 mg/kg methylprednisolone (Depomedrol®, Upjohn Korea Co.). They were kept in conventional animal rooms for three weeks before the experiment and grew until their body weight was over 150 g. During immunosuppression, they were fed a regular diet and tetracycline supplemented tap water.

Production of hyperimmune serum and purification of IgG antibody

The immunosuppressed rats were sacrificed and *P. carinii* was isolated from their lungs. A soluble extract of the organism was prepared as described by Hong et al. (1995) and was used as crude antigen for the immunization of three rabbits (Fig. 1). The protein content of the antigen was 500 µg/ml. After mixing with complete Freund's adjuvant for the primary immunization and with incomplete Freund's adjuvant for 2 successive immunizations, this was peritoneally injected every other week. Two weeks after the last immunization, sera of the immunized rabbits were collected, pooled, and passed through the protein A column (Pharmacia Biotech, Sweden). The column eluate was collected for use as the hyperimmune IgG antibody (HIA) to crude antigen of *P. carinii*.

Injection of HIA for immunotherapy

The protein content of the HIA was 2.44 mg/ml, and the standard dose of one injection was 0.976 mg (0.4 ml) HIA. The rats were divided into nine groups, each consisting of 15 animals; this was according to different regimens of duration or dosage of HIA, as summarized in Table 1. Group 1 rats received sterile saline instead of HIA.

Examination of cystic forms of *P. carinii*

The left lungs of rats that survived the experiment were divided into three pieces, each of which was impression smeared on glass slides. The smears were stained with modified Giemsa (Diff-Quik, Fisher Scientific Co., U.S.A.) and examined through a microscope. Cystic forms of *P. carinii* were counted in 20 fields of immersion oil lens magni-

Table 1. Duration of immunosuppression (IS) and timing and dose of hyperimmune IgG antibody (HIA) by experimental groups[a]

Group	Duration of IS (weeks)	Timing of HIA injection	Duration of HIA injection	Amount of HIA ml/injection[b]	total amount (mg)
1	7	no HIA	—	—	
2	7	2 wk before beginning of IS	9 wk	0.4	17.6
3	7	same with IS	7 wk	0.4	13.7
4	7	2 wk after beginning of IS	5 wk	0.4	9.8
5	7	4 wk after beginning of IS	3 wk	0.4	5.9
6	7	6 wk after beginning of IS	1 wk	0.4	2.0
7	9	6 wk after beginning of IS	3 wk	0.4	5.9
8	7	same with IS	7 wk	0.2	6.8
9	7	same with IS	7 wk	0.6	20.5

[a]Each group initially consisted of 15 rats at the beginning. [b]Twice a week.

fication.

Statistical analysis

The counts of cystic forms were summarized by groups as arithmetical mean ± standard deviation. For comparison, ANOVA and Wilcoxon scores were applied, and probability values below 0.05 were considered significant.

RESULTS

Crude antigen and HIA

The crude antigen used for immunization was separated into several protein bands through SDS-PAGE and their antigenicity was screened by immunoblotting. The profile of protein bands and their antigenic bands was as described in a previous report (Hong *et al.*, 1995). The HIA used for this experiment reacted mainly to the 40-55, 92, 116, and 200 kDa bands (Fig. 1).

Survival of rats and infection by *P. carinii*

In each group, 2 to 12 of the original 15 rats survived the experiment and were included in the statistics. The rats that died during the experiment or showed pyogenic lung infection were excluded. All rats examined showed the cystic forms of *P. carinii* (Table 2).

Counts of *P. carinii* cystic forms by duration

Table 2 summarizes the counts of cystic forms of *P. carinii* by group. In group 1 rats,

which received steroid only, the count was 20.5-76.5 (mean 52.5 ± 19.3), while in group 2, which received HIA 0.4 ml for nine weeks (total protein content 17.6 mg), this was 1-126 (mean 54.1 ± 47.7). In group 3, in which HIA 0.4 ml was administered for seven weeks (total protein content 13.7 mg), the count decreased to 6-21 (mean 13.5 ± 10.6), which was significantly different to that of group 1 (p=0.0183). In groups 4 and 5, the mean count was 29.9 ± 32.9 (p=0.0407) and 31.6 ± 26.7 (p=0.0382) respectively, both were significantly lower than in group 1. In groups 6 and 7, the
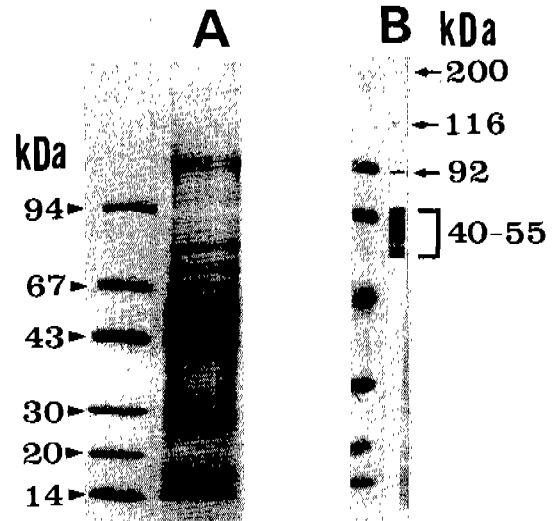

Fig. 1. A. SDS-PAGE analysis of crude antigen of rat *P. carinii*. **B.** Immunoblot of *P. carinii* crude antigen to hyperimmune IgG antibody (HIA).

Table 2. Number of cystic forms of *P. carinii* in 20 fields of oil immersion lens magnification by group

Group	No. of rats examined	No. of cystic forms		Statistical p values[a]	
		Range	Mean ± SD	ANOVA	Wilcoxon score
1	12	20.5-76.5	52.5 ± 19.3		
2	7	1.0-126.0	54.1 ± 47.7	0.9219	0.8990
3	2	6.0-21.0	13.5 ± 10.6[b]	0.0183	0.0547
4	10	2.0-102.0	29.9 ± 32.9[b]	0.0578	0.0407
5	12	1.0-72.5	31.6 ± 26.7[b]	0.0382	0.0566
6	7	4.0-80.0	35.8 ± 24.3	0.1149	0.1502
7	7	10.0-93.0	34.5 ± 27.3	0.1082	0.0570
8	6	8.5-59.6	28.2 ± 1737[b]	0.0191	0.0275
9	9	12.5-85.0	49.8 ± 26.5	0.7848	0.9150

[a]Non-significant for whole group, p=0.2451 by ANOVA and p=0.1867 by Wilcoxon scores; p-values of the count for each group are compared with that for group 1. [b]Statistically significant.
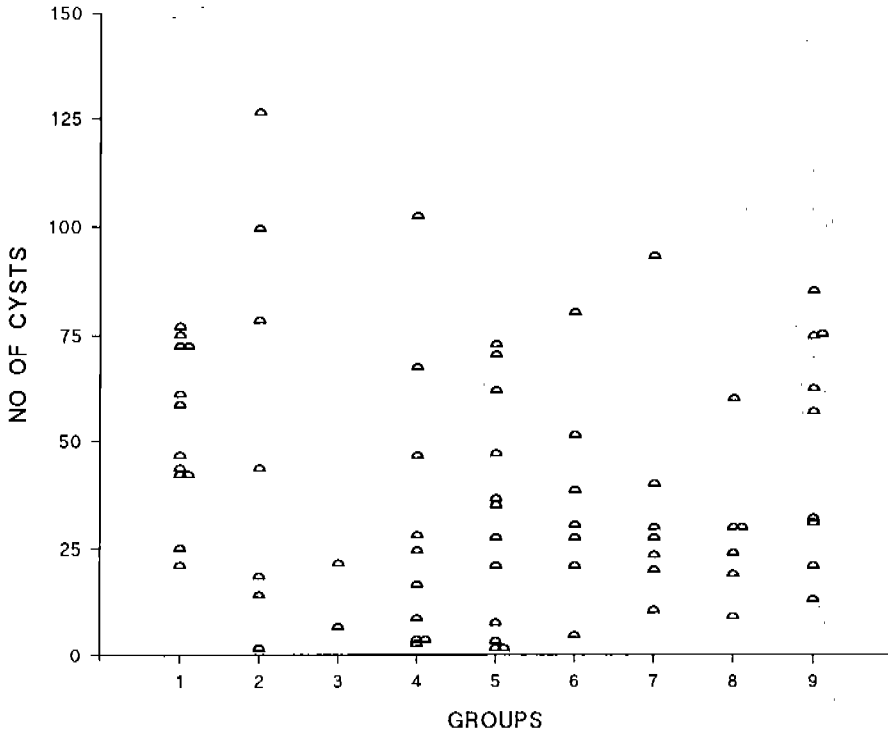
Fig. 2. Number of *P. carinii* cystic forms on 20 fields of immersion oil magnification of the rat lung impression smears by group.

mean count was 35.8 ± 24.3 and 34.5 ± 27.3 respectively, not significantly different from that of group 1. The counts of cystic forms are shown by group in Fig. 2.

Counts of *P. carinii* cystic forms by amount of HIA

In groups 8 and 9, each rat received HIA 0.2 ml (0.488 mg) and 0.6 ml (1.464 mg) respectively for seven weeks. They were infected by more cystic forms of *P. carinii* than those of group 3; the count was 28.2 ± 27.3 for group 8 and 49.8 ± 26.5 for group 9. The count difference between group 8 and group 1 was significant (p=0.0191), but that bewteen group 9 and group 1 was not (p=0.7848) (Table 2 & Fig. 2).

DISCUSSION

Humoral immunity is related with the development of *P. carinii* pneumonia, though cellular immunity plays the primary role (Gigliotti and Hughes, 1988; Peglow *et al.*, 1990; Lundgren *et al.*, 1992). Passive transfer of the monoclonal antibody or the antibody mixed with sensitized lymphocytes has been recognized as effective in the treatment of *P. carinii* infection in scid mice (Roths and Sidman, 1993). In normal control rats, neonates naturally have specific IgG antibody in their serum but it disappears within a month, reappearing 6 months from birth (Hong *et al.*, 1995). In immunosuppressed rats, the numbers of the organism increased according to decreases in serum IgG antibody (Hong *et al.*, 1995). Furuta *et al.* (1985), on the other hand, concluded that immune serum had no therapeutic effect on the infected mice, but their conclusion appears unconvincing. Since the serum was injected twice on the day of infection and again on the third day and the effect was evaluated 2-3 weeks after infection in that study, the amount of injected serum was too little and the experiment was too short for any concrete conclusion to be drawn.

The serum IgG antibody is known to be induced by the major surface antigen (MSG) on the outer membrane of *P. carinii* (Kovacs *et al.*, 1988; Blumenfeld *et al.*, 1990; Peglow *et al.*, 1990). The MSG consists of the 40-45, 50-55, 66, 92, 116, and 200 kDa bands, but the relation among them is still unknown. The HIA, isolated from immunized rabbit serum and used in the present study, reacted strongly to the 40-55, 92, 116, and 200 kDa antigen bands (Fig. 1); these must be MSG, and the HIA was confirmed to contain the antibody to them.

The present HIA reduced the counts of *P. carinii* cystic forms in rats of group 3, 4, 5 and 8, but not in groups 2, 6, 7 or 9. The effect found in groups 3 and 8 was prophylactic, while that in groups 4 and 5 was therapeutic. The rats in groups 6 and 7 first received HIA 6 weeks after immunosuppression, at which time *P. carinii* infection had already progressed, and they received HIA for 1 and 3 weeks respectively but not effective. The result showed that in rats, the heterogeneous HIA used in this study was effective only at the beginning or early stage of infection. Even during the early stage, however, rats must be injected with HIA at least 3 weeks.

In group 2 rats, which received HIA for nine weeks, counts of the organism did not decrease to any significant extent though injection of these rats started two weeks before the beginning of steroid injection. This means that in normal hosts HIA has no prophylactic effect on *P. carinii* infection. In addition, there was no effect on group 9 rats which received most HIA (total 20.5 mg), while the cystic form count fell in rats of groups 3, 4, 5 and 8 which received less HIA. This result suggested that there was no direct correlation between the total amount of HIA and the count of *P. carinii* cystic forms. Instead, a dose of 0.976 mg HIA was found to be optimal when injected biweekly for 5 to 7 weeks.

The present finding, on the other hand, can be interpreted as that the present HIA has no prophylactic or therapeutic effects, based on the fact that only two rats of group 3 survived for seven weeks. Though the count of cystic forms in the two rats showed the most significant reduction, the two rats were too few

for a definite conclusion. In fact, selective survival of the less infected rats is possible. Furthermore, because group 2 and 3 rats received HIA for more than seven weeks, the prophylactic effect in group 2 rats should have been the same as that in group 3 rats. This incongruent result may be an outcome of marked variation between individual rats.

Nontheless, the present HIA is effective because statistical significance was also found in groups 4 and 5. Because it reacted to major MSG antigenic bands, this HIA is theoretically more effective than monoclonal antibodies (Roths and Sidman, 1993).

Since HIA is a heterogeneous foreign protein which may induce serum sickness, its practical application may be difficult. It was necessary to set a control group which received non-immunized rabbit serum IgG antibody for exclusion of foreign serum effect. This effect should be evaluated in the next study. However, when an effective heterogeneous HIA is produced, it may be experimentally very useful and also provide basic information for the development of a vaccine against *P. carinii* infection.

In conclusion, the serum IgG antibody produced from hyperimmune rabbits, which reacts to MSG antigen bands, is partially effective for prophylaxis and the treatment of *P. carinii* infection in rats, especially during the early stage of infection. The mechanism of the protective role of the present IgG antibody is an interesting subject for further studies.

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=초록=

폐포자충증에 대한 이종항혈청 내 IgG 항체의 예방 및 치료효과

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폐포자충증에 대한 이종항혈청의 예방 및 치료효과를 검증하고자 이 연구를 수행하였다. 흰쥐 (위스타)를 15마리씩 나누어 9개 실험군을 설정하고, 스테로이드 주사로 면역억제시켜 폐포자충의 감염을 유발하였다. 흰쥐 유래의 폐포자충 조항원을 토끼에 투여하여 얻은 면역혈청 중에서 IgG 항체만을 분리하여 실험에 사용하였다. 각 실험군에 따라 면역억제 시작 2주전부터 억제 후 6주후 사이에 면역항체 주입을 시작하였고, 주 2회씩 7-9주 동안 주입하였다. 대부분의 실험군에서는 면역항체를 일회에 0.4 ml (단백질량 0.976 mg)씩 주입하였고, 용량에 따른 예방 및 치료효과를 비교하기 위하여 0.2 ml (단백질량 0.488 mg)와 0.6 ml (단백질량 1.464 mg) 주입 실험군을 설정하였다. 그 결과 실험군별로 실험기간인 7-9주간 살아남고 폐에 세균감염이 없는 흰쥐 2-12마리에서 결과를 얻었다. 폐도말검사 결과 모든 개체에서 폐포자충이 확인되었고, 실험군에 따라 포낭형 수의 평균이 최저 13.5 (3군)에서 최고 52.5 (1군) 사이에 있었다. 통계학적인 검증 결과 기준이 되는 1군에 비하여 유의한 병원체의 감소가 확인된 실험군은 0.4 ml의 항체를 주 2회씩 7주간 주입한 3군, 5주간 주입한 4군, 3주간 주입한 5군과, 0.2 ml를 7주간 주입한 8군이었다. 면역항체를 0.4 ml씩 9주간 주입한 2군과 항체를 0.6 ml씩 7주간 주입한 9군은 차이가 유의하지 않았다. 이 항체는 폐포자충의 표면에 있는 주항원 당단백질인 MSG (major surface glycoprotein) 중에서 40-55, 92, 116, 200 kDa 분획과 주로 반응하였다. 이들 항원 분획이 숙주의 방어기전과 관련된 항체를 유발하는 항원이며, 이 항원자극에 의하여 형성된 이종항체가 흰쥐의 폐포자충 감염초기에 수동면역의 효과가 있어 예방이나 치료에 활용될 수도 있음을 확인하였다.