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Immunoblot analysis for serum antibodies to *Pneumocystis carinii* by age and intensity of infection in rats

Sung-Tae HONG[1]*, Mejeong LEE[1], Min SEO[1], Dong Hyun CHOO[2], Hyung Ro MOON[2] and Soon-Hyung LEE[1]

Department of Parasitology and Institute of Endemic Disease[1], Department of Pediatrics[2], Seoul National University College of Medicine, Seoul 110-799, Korea

Abstract: The present study aims to observe changing patterns of serum antibody to *Pneumocystis carinii* in normal rats of different ages and in immunosuppressed rats. The serum IgG antibody was observed by immunoblotting with crude antigen of *P. carinii* which were purified from the lungs of infected rats. The crude antigens separated in SDS-PAGE resolved more than 20 protein bands from 20 to 200 kDa. Of them, 40-45, 50-55, 116 and 200 kDa bands were major antigens of *P. carinii*. Most of the normal rats of up to 4 weeks had the antibodies reacting the 4 bands, but none of 8-week-old rats revealed the specific antibody. After the rats grew for 40 weeks, all were found to have the antibody in their serum. Same pattern of serum antibody level by age was found in ELISA. When immunosuppressed rats became heavily infected, the antibody in their serum decreased distinctively. The present results suggest that antibodies in normal newborn rats are transferred from their mother and lowered up to 8 weeks. Thereafter, the levels of the antibodies begin to increase by natural exposure to *P. carinii*. It was also confirmed that the intensity of *P. carinii* infection is inversely related with levels of serum antibodies.

Key words: *Pneumocystis carinii*, rats, antigenic bands, IgG antibody, immunoblot, ELISA, age, intensity of infection

INTRODUCTION

Pneumocystis carinii is a complex group of pathogenic protists commonly found in the lungs of humans and mammals throughout the world. *P. carinii* is very important because it causes fatal pneumonia in immunocompromised hosts, such as the patients with cancer chemotherapy, prolonged steroid therapy, organ transplantation, malnutrition, and AIDS. Definite diagnosis of the pneumonia is demonstrating the organism in bronchoalveolar lavage or in the lung tissue. Serodiagnosis, which is one of major diagnostic methods of infectious diseases, is regarded meaningless in *P. carinii* pneumonia (Maddison *et al.*, 1982; Peglow *et al.*, 1990). However, serological findings are still useful in immunobiological and epidemiological characterization of *Pneumocystis* or its infection (Hong, 1991; Smulian *et al.*, 1993; Moon *et al.*, 1995).

Previous serological studies revealed that normal healthy persons over 2 to 4 years have antibodies to *P. carinii* in their serum. In Korea, infants began to reveal the serum antibodies one year after birth (Hong, 1991; Moon *et al.*,

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* Corresponding author

1995). Since the organism is transmitted by air, its infection occurs in neonatal period. Although the rat is the best experimental model, the pattern of chronological infection after birth is still unknown.

When the host loses immunity, both of cellular and humoral, overt pneumonia develops (Pifer *et al.*, 1987; Walzer *et al.*, 1989). Therefore the level of the serum antibodies is expected to be reduced in clinical cases, but the findings were controversial in human studies (Maddison *et al.*, 1982; Peglow *et al.*, 1990). The inconsistency of serum antibody level in patients may be interpreted to be caused by uneven intensity of infection. In rats, as the immunity is suppressed more, the intensity of infection becomes severer. In this sense, experimental rat is a good model for observation of relation between the antibody level and intensity of infection.

The present study has two purposes. The first is monitoring the anti-*P. carinii* antibody in serum of healthy rats of different ages. The second is observation of serum antibody response according to the intensity of infection. We analyzed immunoblotting patterns and ELISA of the subjected rats.

MATERIALS AND METHODS

1. Collection of sera from healthy and immunosuppressed rats

Wistar rats were supplied from the animal laboratory of Seoul National University College of Medicine. They were kept in a conventional animal quarter with regular commercial diet and tap water. As summarized in Table 1, the sera were collected from healthy rats from 5 days to 40 weeks after birth. Their sera were stored at -70°C until used.

2. Infection of *P. carinii* in rats

Wistar rats from the same vendor were kept in a conventional animal room and weekly injected with 10 mg/kg methyl-prednisolone (Depomedrol®, Korea Upjohn Ltd., Seoul). Commercial diet and tap water mixed with ampicillin 1 mg/ml were supplied. Their sera were collected from 1 to 12 weeks after beginning of the injection (Table 1).

Table 1. The rats used for the present experiment

Groups of rats	No. of rats
Normal healthy rats	
5 days	10
2 weeks	10
4 weeks	19
6 weeks	18
8 weeks	10
10 weeks	15
13 weeks	50
40 weeks	10
Immunosuppressed rats	
1 weeks	5
2 weeks	19
3 weeks	13
4 weeks	9
5 weeks	7
6 weeks	10
7 weeks	19
8 weeks	9
9 weeks	21
12 weeks	9

3. Determination of intensity of *P. carinii* infection

The lungs of the immunosuppressed rats were isolated after ether anesthesia and bleeding. The lungs were smeared by impression on slide glasses and were stained in Diff-Quik solution (Fisher Scientific, U.S.A.). The smear specimens were microscopically screened and the intensity of *P. carinii* infection was graded by counting the number of cysts in 20 high power fields; no cyst was as 0, 1 to 9 cysts as I, 10 to 19 cysts as II, 20 to 99 as III, 100 or more as IV (Hong *et al.*, 1994).

4. Preparation of *P. carinii* crude antigen

P. carinii were purified from the lung homogenate as described previously (Hong, 1991). Briefly, the lungs of infected rats were minced and homogenized in a Stomacher blender (Seward Medical, U.K.). After erythrocytes were lysed in 0.85% ammonium chloride solution, the cell pellet was washed 3 times in phosphate buffered saline. The cellular pellet was filtered through membranes of 10 μm pore. The purified organisms were

homogenized finally by sonication for 30 seconds. Supernatant after centrifugation for 60 minutes at 12,000 rpm was stored at -70°C and regarded as crude antigen.

5. SDS-PAGE and immunoblotting

The crude antigen preparations were electrophoresed in 0.1% SDS and 12.5% polyacrylamide gels. Each well of the gel was loaded with 30 μg protein. The protein bands in the gel were transferred onto nitrocellulose membrane (Nytran®, Hoefer Scientific Instruments, U.S.A.) in an electrical chamber. The protein bands on the nitrocellulose membrane were then reacted with serum specimens (diluted 1:100). The serum antibody ligated to antigenic protein bands was reacted with peroxidase-conjugated goat antibody to rat IgG (Cappel Co., U.S.A.). The substrate for color development was 4-chloro-1-naphthol and diaminobenzidine (Sigma Co., U.S.A.).

6. ELISA

The ELISA procedure was carried out as described in Hong (1991). The crude antigen was diluted 1:400 in carbonate buffer (pH 9.6), serum 1:20, and the conjugate (peroxidase conjugated anti-rat IgG, Cappel Co., U.S.A.) 1:4,000. The substrate was O-phenylenediamine 4 mg/10 ml. The absorbance was recorded at 490 nm with an ELISA reader (Dynatech Inc., U.S.A.).

RESULTS

1. Protein bands of *P. carinii* crude antigen separated by SDS-PAGE

Proteins in the crude antigen of *P. carinii* were separated into about 20 bands between 20 to 205 kDa (Fig. 1). The crude antigens of different batches of rat *P. carinii* from showed same patterns of bands (data not shown).

2. Anti-*P. carinii* IgG antibody in serum of normal rats of different ages

The serum specimens of normal rats included IgG antibodies which reacted with numerous protein bands of *P. carinii* (Fig. 2). Among the bands, 40-45, 50-55, 116 and 200 kDa bands were antigenic (Moon *et al.*, 1995). These 4 bands appeared in all of newborns of 5

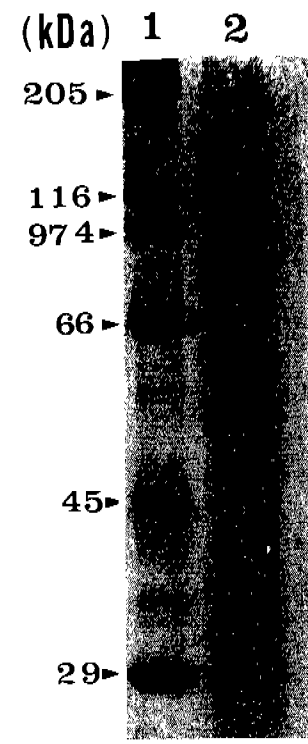

Fig. 1. SDS-PAGE findings of crude antigen of *P. carinii*, Coomassie brilliant blue R-250 stained protein bands in 12.5% gel. Lane 1, size marker; lane 2, crude antigen of *P. carinii* used in this experiment, W13-32.

days or fully grown adults of age 40 weeks, but not in 8 weeks old rats (Fig. 2 & Table 2).

The ELISA with sera of normal rats showed the same tendency of the absorbances changed by different age groups, 0.380 in 5 days group to 0.425 in 40 weeks group (Table 3).

3. Anti-*P. carinii* IgG antibody in serum of immunosuppressed rats

The immunosuppressed rats in a batch showed some variations of the immunoblotting patterns. This result might be understood by individual variation of the immunosuppression. This difference was also recognized by different intensity of *P. carinii* infection. However, when the reaction was rearranged by the intensity of *P. carinii* infection, the sera from more heavily infected rats showed much lower antibody reactions than those from less infected rats (Fig. 3 & Table 4). The present finding revealed that the rats failed to produce

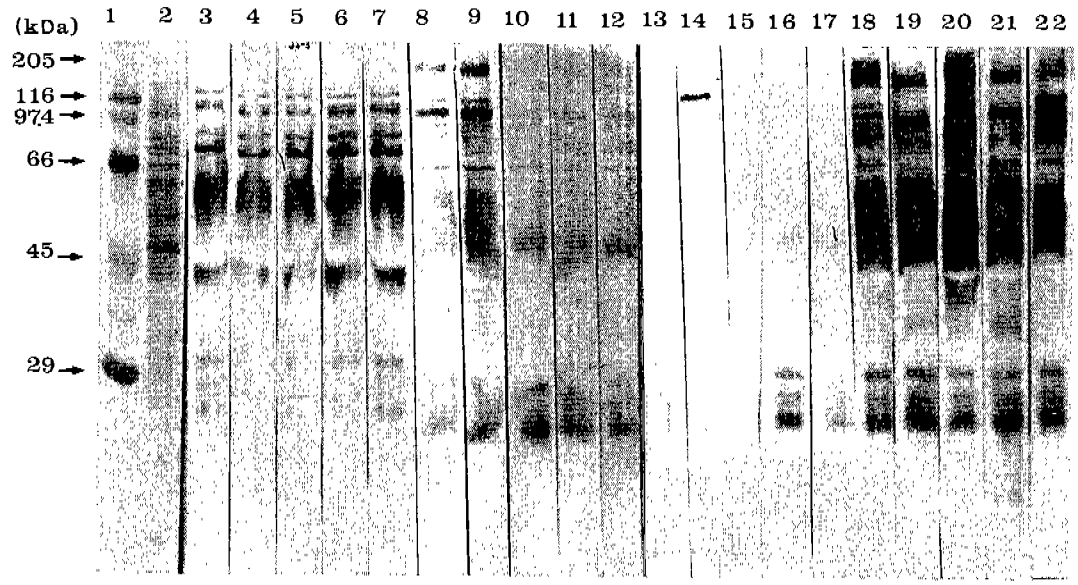

Fig. 2. Immunoblotting pattern between W13-32 *P. carinii* antigen and sera of normal rats of different ages. Lanes 1, size marker; 2, amido black 10B stained protein bands; 3-7, 2 week old rats; 8-12, 4 week old rats; 13-17, 10 week old rats; 18-22, 40 week old rats.

Table 2. Anti-*P. carinii* IgG antibody in serum of normal rats by immunoblotting

Age of rats	No. of rats exam	No. of rats reacted to antigenic bands (kDa)					Positive rate (%)
		40-45	50-55	116	200	None	
5 days	10	5	7	0	0	3	70
2 weeks	10	10	10	10	10	0	100
4 weeks	10	5	6	10	8	0	100
6 weeks	10	0	0	5	4	5	50
8 weeks	10	0	0	0	0	0	0
10 weeks	10	0	5	3	3	5	50
13 weeks	10	1	1	3	2	7	30
40 weeks	10	10	10	10	10	0	100

Table 3. Anti-*P. carinii* IgG antibody levels in serum by ELISA in normal rats

Age	No. of rats	Absorbances	
		Range	Mean
5 days	7	0.327-0.499	0.380
2 weeks	10	0.399-0.586	0.455
4 weeks	19	0.028-0.204	0.124
6 weeks	18	0 -0.150	0.098
10 weeks	15	0.051-0.161	0.122
13 weeks	48	0.031-0.507	0.156
40 weeks	8	0.225-0.639	0.425

anti-*P. carinii* antibody as they were suppressed of their immunity. The intensity of *P. carinii* infection also increased by progress of immunosuppression (Fig. 3). As presented in Fig. 4, the serum antibody still remained in most of rats after 2 weeks of immunosuppression. However, according to the age of rats, the antibody reactions after 2 weeks of immunosuppression were found different. The immunoblotting pattern was basically same as found in normal rats of the same age group, however, the patterns of individual rat serum varied greatly in adult rats of age 40 weeks (Fig. 4).

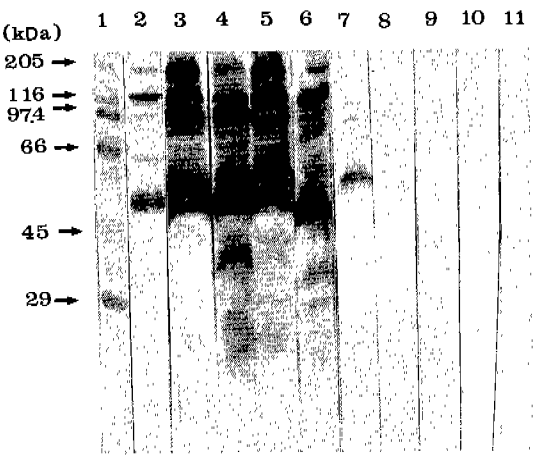

Fig. 3. Immunoblotting pattern between W13-32 *P. carinii* antigen and sera of immunosuppressed rats. Lanes 1, size marker; 2-6, sera of rats in intensity of infection I; 7-9, sera of rats in intensity of infection II; 10, serum of a rat in intensity of infection III; 11, serum of a rat in intensity of infection IV.

Antibody test by ELISA showed similar changing pattern of antibodies by intensity of infection (Table 5 & Fig. 5).

DISCUSSION

The immunoblot and antibody test by ELISA of the present experiment show that normal healthy albino rats have anti-*P. carinii* IgG antibodies in their serum when they are born. Serum levels of the antibody decrease as they grow up to 8 weeks but increase again until 40 weeks old. Another finding is that the serum antibody is inversely correlated with the intensity of *P. carinii* infection. These conclusions were proved by the findings that all of the normal rats of 5 days, 2, 4 and 40 weeks after birth showed anti-*P. carinii* antibodies in their serum but 8 week old rats showed no antibodies. Only a small proportion of the rats of age groups, 6, 10 and 13 weeks after birth, were observed to have the serum antibody. When the rats were suppressed of their immunity, they showed less antibodies even in lightly infected rats. As *P. carinii* infection progressed to intensity III and IV, the antibody rate was 52.3% and 0%.

Several previous data recorded very similar immunoblotting patterns of rat *P. carinii*. Sera of 6, 10 and 21 week old normal rats were found to include low amount of the antibody to rat *P. carinii* antigen by ELISA and no antibody was detected to the major antigenic band of 110 kDa by immunoblotting (Graves *et al.*, 1986). However the serum antibody increased in dilution titer by ELISA and became positive by immunoblotting as the rats grew to adults of 36 to 64 weeks old. The result of Graves *et*

Table 4. Anti-*P. carinii* IgG antibody patterns analyzed by intensity of infection

Intensity of infection[a]	No. rats examined	No. of rats reacted to antigenic bands (kDa)					Positive rate (%)
		40-45	50-55	116	200	None	
0	37	0	10	17	17	20	45.9
I	13	0	4	5	4	7	46.2
II	19	0	8	15	12	3	84.2
III	21	0	3	11	7	10	52.3
IV	10	0	0	0	0	10	0

[a]Intensity of *P. carinii* infection was graded by the number of cysts in 20 high power microscopic fields by impression smear of the lungs. 0, no cysts; I, less than 10 cysts; II, 10 to 19 cysts; III, 20 to 99 cysts; IV, more than 100 cysts.

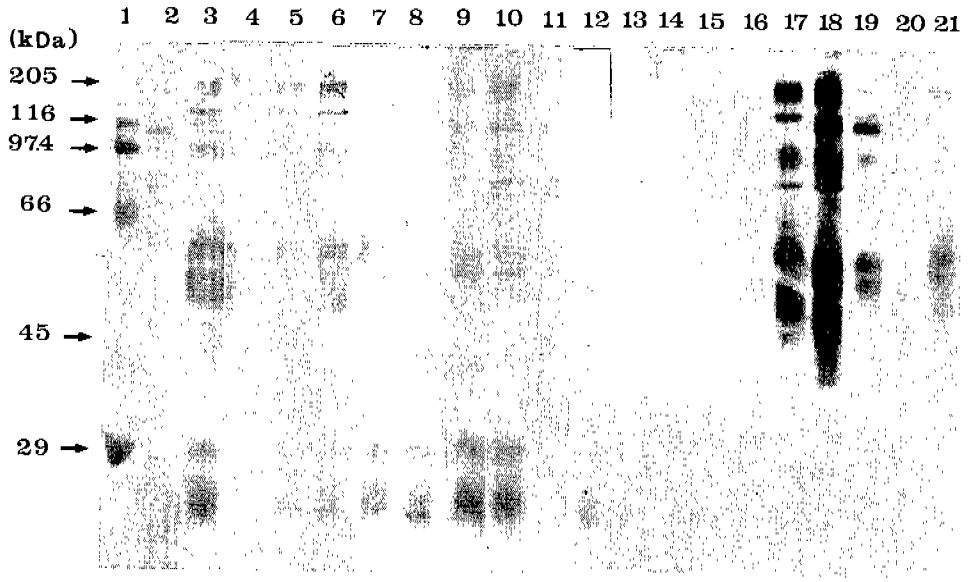

Fig. 4. Immunoblotting pattern between W13-32 *P. carinii* antigen and sera of rats of 2-week immunosuppression. Lanes 1, size marker; 2, amido black 10B stained protein bands; 3-6, sera of 2 week old rats; 7-11, sera of 5 week old rats; 12-16, 8 week old rats; 17-21, sera of 40 week old rats.

Table 5. Anti-*P. carinii* IgG antibody levels by ELISA in serum of immunosuppressed rats

Duration of immunosuppr	No. of rats	Absorbances	
		Range	Mean
5 weeks	7	0.020-0.567	0.241
6 weeks	7	0.097-0.624	0.322
9 weeks	21	0 -0.429	0.196
12 weeks	9	0.091-0.530	0.249

al. (1986) showed that the rats became infected by *P. carinii* before 36 to 64 weeks after birth. The present experiment also reveals that the rats are infected by *P. carinii* 40 weeks after birth. The newborn rats have the antibody transferred from their mother, but the antibody decreases gradually after birth up to 8 weeks. In the animal room of this experiment, the newborn rats were not infected during the first 8 weeks. The serum antibody after 8 weeks must have been produced from their own immune system. In natural environment, newborn rats are exposed just after birth to *P. carinii* because this protist is regarded as ubiquitous. However, the rats may be protected by the maternal antibodies in early life after birth. As the rats are infected and exposed to the antigen afterwards, they begin to produce the antibody. The process takes maximum 40 weeks in albino rats in our laboratory.

Walzer *et al.* (1987) confirmed that IgM and IgG antibodies were not detectable in immunosuppressed rats by IFA and Western blot but the antibody levels rose 18 weeks after withdrawal of immunosuppression. The rebound production of the antibody was found very strong. Their result revealed that the antibodies recognized 45, 50, 116 and 200 kDa bands of crude antigen of rat *P. carinii*. The present immunoblot result showed most of the immunosuppressed rats failed to produce the antibody even though they had the organisms in their lungs. The lower the antibody levels in

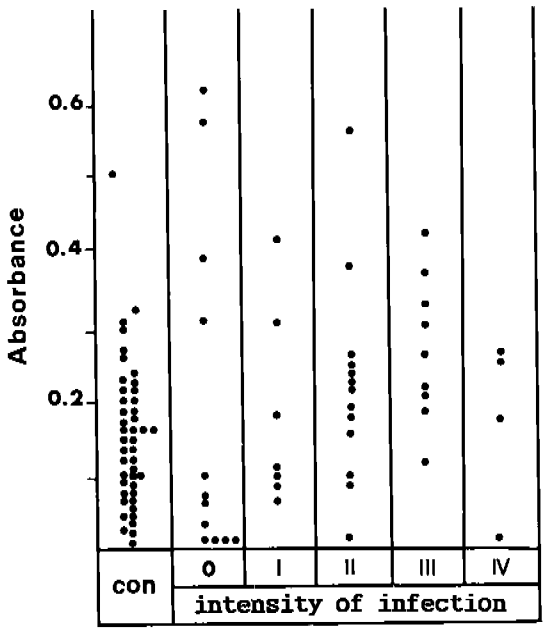

Fig. 5. Anti-*P. carinii* antibody levels measured by ELISA according to intensity of infection in immunosuppressed rats. Con, sera of control rats of 40 weeks age; 0-IV, sera of immunosuppressed rats by intensity of infection; 0, no cysts in 20 immersion oil fields; I, less than 10 cysts; II, 10 to 19 cysts; III, 20 to 99 cysts; IV, more than 100 cysts.

the serum, the severer the infection became. The present finding and result of Walzer *et al.* (1987) suggest that humoral immunity is important for prevention of pneumocystosis although cellular immunity has been regarded as the principle defence mechanism in pneumocystosis (Walzer *et al.*, 1989). The reduced concentration of IgG antibodies was also noticed in HIV-infected humans (Burns *et al.*, 1990).

In the present results, the 4 bands, 40-45, 50-55, 116 and 200 kDa, were recognized as antigens of *P. carinii* of Korean rats. These bands cross-reacted with serum specimens of normal Koreans (Moon *et al.*, 1995). Graves *et al.* (1986) found that 55-60 and 110-116 kDa bands were common antigens of *P. carinii* from rats and humans. Walzer *et al.* (1987) observed that 45, 50 and 116 kDa bands were major antigens. Bauer *et al.* (1993) recognized 40, 45 and 120 kDa bands from rat *P. carinii*. The antigenic bands of those 3 papers and the present experiment may be regarded as same. The size difference of the 4 bands is very slight and can be interpreted as a variation made by the preparation of antigen and SDS-PAGE system. Therefore, we confirm that *P. carinii* from laboratory rats in Korea is very similar in its antigenicity with those in other countries. Of course there were some minor bands in the present experiment and other records. Since the rats of the present study were not bred in the pathogen free quarter, they might be infected with other pathogens. Therefore the sera from normal or immunosuppressed rats might include antibodies reacting with antigenic bands of other microorganisms. This should be evaluated further.

The role of the 4 different antigen bands is not clearly defined. The normal rats produced the antibodies to the 50 kDa band slightly earlier and stronger (Walzer *et al.*, 1987). In the present data, the reaction with 50-55 kDa appeared stronger in rats of 10 weeks after birth. On the contrary, the band 40-45 kDa disappeared first in immunosuppressed rats. However, any speculation about the difference of these bands is impetuous. Only if the immunoblotting pattern is consistent in every *P. carinii*-infected rat, the pattern will indicate the infection. Further studies can answer it.

The present results confirmed that newborn rats have the antibody to *P. carinii* of maternal origin and the rats become infected 40 weeks after birth. When the rats lose their immunity, they fail to produce enough antibodies for protection and thus *P. carinii* can multiply.

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=초록=

흰쥐의 연령과 감염과정에 따른 폐포자충에 대한 항체형성 양상

홍성태[1], 이미정[1], 서 민[1], 주동현[2], 문형로[2], 이순형[1]

서울대학교 의과대학 기생충학교실 및 풍토병연구소[1], 소아과학교실[2]

정상 흰쥐의 출생 후 연령과 면역억제된 흰쥐의 감염강도에 따른 혈청 내 폐포자충(*Pneumocystis carinii*)에 대한 항체 형성을 관찰하고자 이 연구를 수행하였다. 폐포자충을 발현시키고 순수분리하여 수용성 항원을 만들고 이를 이용하여 전기영동과 면역이적법(Immunoblot)과 효소면역법(ELISA)을 시행하여 항체반응을 관찰하였다. SDS-PAGE에 의하여 분리된 항원의 단백질 분획은 20-200 kDa의 범위에서 20개 이상이 관찰되었다. 이들 분획 중에서 인체 양성표준 혈청에 116 kDa 분획이 강하게, 45-55 kDa 및 100 kDa 분획이 약하게 반응하였고, 흰쥐 양성표준 혈청과는 40-45 kDa 분획과 강하게 그리고 50-55 kDa, 116, 200 kDa 분획과 약하게 반응하였다. 연령 별로 이 네 분획과의 반응을 보면, 출생 5일-6주까지 양성률 50-100%이고 8주에는 0%가 되었고, 10주 이후에는 증가하여 40주에 100%가 되었다. 폐포자충의 감염량이 증가하여 감염강도가 IV인 흰쥐에서는 면역이적으로 측정할만큼 항체를 가진 개체가 없었다. 이러한 정상과 면역억제된 흰쥐의 혈청반응 유형은 효소면역법에서도 확인할 수 있었다. 네 항원 분획 중 40-45 kDa에 대한 혈청반응이 각 군에서 낮게 관찰되었으나 그 생물학적 의의는 아직 평가하기 어렵다. 이 결과는 흰쥐가 출생하면 모체에서 유래한 혈청 내 항폐포자충 항체를 8주가 경과하면서 모두 소실하고 그 이후에 10주부터 40주에 이르기까지 자연감염에 의해 항체를 서서히 형성하는 것을 의미한다. 면역 억제에 의하여 흰쥐가 항체를 효과적으로 생산하지 못하게 되면 혈청 내 항체량에 반비례하여 폐포자충의 감염량이 늘어난다. 이는 항체에 의해 매개되는 체액면역이 폐포자충의 감염과 직접 관련이 있다는 증거가 된다.

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