The Korean Journal of Parasitology
Vol. 35, No. 1, 31-38, March 1997

Skin ulcer and immunoblot patterns by inoculation sites in BALB/c mice infected with *Leishmania major*

Mejeong LEE, Jong-Kook LEE, Seong-Beom KIM,
Soon-Hyung LEE and Sung-Tae HONG*

Department of Parasitology and Institute of Endemic Diseases,
Seoul National University College of Medicine, Seoul 110-799, Korea

Abstract: The skin ulcer in *Leishmania major* infection is known to be variable according to the inoculation sites even in a susceptible host. The present study traced the immunoblot patterns by the site of inoculation and duration of infection in BALB/c mice. *L. major* were subcutaneously inoculated on the nose, footpad, and back of the mice, in a dose of 3×10^6 promastigotes. Sera of the mice were collected every 10 days after inoculation. SDS-PAGE separated soluble protein bands of the promastigotes and immunoblot was carried out with the infection sera. The skin ulcer first appeared on the nose at 15 days, and on the footpad at 17 days after inoculation. The ulcer on the back appeared after 90 days. In the mice with ulcer on the nose or footpad, serum IgG antibody reacted to 202, 139, 98, 83, 81, 67, 65, 62, 59, 54, 52, 42, 26, and 23 kDa bands at 20 days after inoculation. In mice inoculated on the back, however, the immunoblot showed visible reactions with 202, 83, 81, 74, 67, 65, 62, 59, 54, 52, 20 and 17 kDa bands at 90 days after inoculation. The present result showed that the antigenic protein bands of *L. major* promastigotes were differed by the inoculation site and duration of infection. Since the skin ulcer and the serum antibodies to antigenic bands between 67-52 kDa appeared simultaneously, it is suggested that the serum IgG antibodies may play a role in formation of the skin ulcer in BALB/c mice.

Key words: *Leishmania major*, BALB/c mice, antigenic bands, serum IgG antibody, nose, footpad, back

INTRODUCTION

Leishmania major is an obligate intracellular parasite of family Trypanosomatidae, which replicates in macrophages, but epidermal Langerhans cells are also a potential host cell for initiation of the infection (Moll, 1993; Axelrod *et al.*, 1994). It induces ulceration and granuloma formation of the skin in humans or rodents.

The clinical manifestation of *Leishmania* infection is known to vary by genetic background of the host, dose of infection, and strain of the parasite. Skin infection of mice with *L. major* usually develops as either of two divergent patterns. Resistant mice, such as C57BL/6, respond immunologically by activation of gamma interferon (IFN-*r*) producing $CD4^+$ helper T (Th1) cells. On the other hand, susceptible mice of BALB/c strain respond by proliferation of interleukin-4 (IL-4)

• Received 17 February 1997, accepted after revision 25 February 1997.

• This study was supported by a Clinical Research grant (#088) from the Seoul National University Hospital (1995).

*Corresponding author

producing Th2 cells. The preferential development of Th1 or Th2 cells, determines different outcomes of the disease (Solbach & Laskay, 1995; Kemp *et al.*, 1996). Because Th1 cells predominate in C57BL/6 mice, cellular immunity makes the host resistant to the infection. However, as Th2 cells predominate in BALB/c mice, humoral immunity makes the host susceptible.

Kirkpatrick *et al.* (1987) found site-related variations in development of the skin lesion in different strains of mice as well as in the golden hamster. Nabors & Farrell (1994) observed that SWR mice are capable of controlling primary infections on the footpad even though they are extremely susceptible to infection on the rump. According to the site of inoculation in SWR mice, patterns of IFN-r and IL-4 production are different. The previous studies have shown that development of the skin lesion depends on species of the host and the cellular immunologic status. Most of the people with ulcerative cutaneous leishmaniasis develop strong delayed hypersensitive reaction to *Leishmania* antigens and low antibody titers, while those with non-ulcerating forms show the reversed reaction (Gutierrez, 1990).

However, any role of antibodies in leishmaniasis is not known. The present study chronologically monitored production of serum antibodies in experimental *L. major* infections by 3 inoculation sites.

MATERIALS AND METHODS

The parasite and the host

The strain of *Leishmania major* used in this study was shared from the parasitology laboratory of the University of Toronto, Canada. The promastigotes were maintained at 24°C by passage in NNN medium or minimum essential medium (GIBCO Laboratories, Grand Island, N.Y.) containing 10% fetal bovine serum (GIBCO). Five-week-old male BALB/c mice were purchased from the Myung Jin Co., Ltd., Seoul and used for this experiment.

Inoculation of *L. major* and collection of sera

Mice were subcutaneously inoculated on the nose, hind footpad, and back with 3×10^6 stationary-phase promastigotes of *L. major*. Each group included 5 mice. Their sera were collected every 10 days between 10 and 100 days after inoculation.

Antigen preparation

Promastigotes of cultivated *L. major* were washed and disrupted for 20 seconds by sonication at maximum capacity (sonicator, Heat Systems-Ultrasonics, Inc., N.Y.). They were then centrifuged at 12,000 rpm for 60 min, and the supernatant was stored at -70°C until required for use as the crude antigen. The protein fractions were separated in gels of 12.5% SDS-PAGE, and antigenic bands were screened by immunoblot with the collected sera (Tsang *et al.*, 1983). The primary antibody was diluted 1:100 and the secondary antibody, peroxidase-conjugated goat anti-mouse IgG, was diluted 1:1000 (Cappel, U.S.A.). The substrate, 0.6% chloronaphthol, 0.2% diaminobenzidine and 0.02% H_2O_2/PBS, was applied for color development.

Measurement of skin temperature

The skin temperature was measured on the nose, footpad and back with a wire digital thermometer.

RESULTS

Ulceration at inoculation sites with *L. major*

The skin ulcer appeared first on the nose 15 days, on the footpad 17 days, and on the back 90 days after inoculation (Fig. 1 & Table 1). The mice infected on the nose developed the more severe ulcer and tissue destruction than those infected on the footpad, and all of the nose infected mice died 40-50 days after inoculation.

Protein fractions of *L. major* on SDS-PAGE

Crude extract of *L. major* promastigotes was separated in gels of 12.5% SDS-PAGE. More

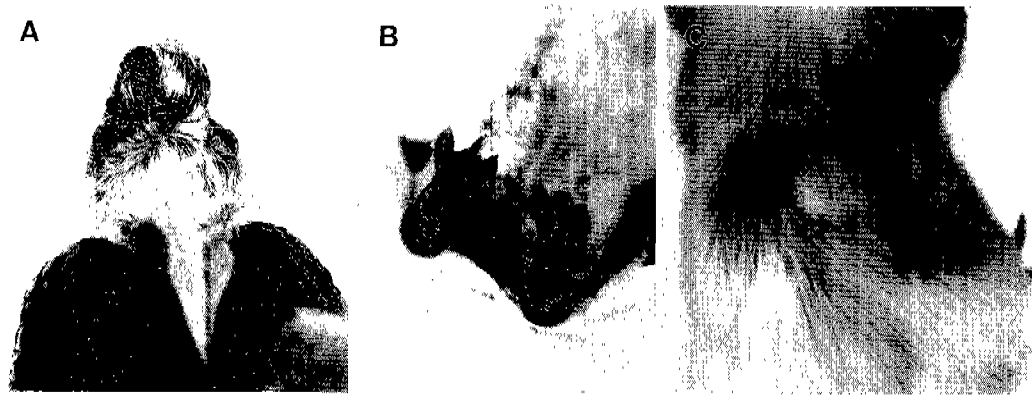

Fig. 1. A. Ulceration on the nose of a BALB/c mouse at 20 days after inoculation of *L. major* promastigotes. **B.** Ulceration on the footpad of a BALB/c mouse at 20 days after inoculation of *L. major* promastigotes. **C.** Skin lesion on the back of a BALB/c mouse at 90 days after inoculation of *L. major* promastigotes.

Table 1. Number of mice with ulceration at the inoculation site of *L. major* by duration of infection

Days after inoculation	No. of mice with skin lesion at		
	nose	footpad	back
0	0/5	0/5	0/5
15	1/5	0/5	0/5
18	5/5	1/5	0/5
23	—	5/5	0/5
85	—	—	2/5
90	—	—	4/4

than fifteen bands from 29 kDa to 120 kDa were recognized (Fig. 2).

Anti-*L. major* IgG antibody in mouse sera of 3 inoculation sites

In the mice which were inoculated on their nose, anti-*L. major* IgG antibody faintly reacted to the 202, 139, 98, 83, 81, 52, 42, 26 and 23 kDa bands 10 days after inoculation, and strongly to the 202, 139, 98, 83, 81, 67, 65, 62, 59, 54, 52, 42, 26, and 23 kDa bands at 20 days (Fig. 3). In the mice inoculated on the footpad, the immunoblot response to *L. major* antigen was the same with that of the nose inoculated mice. Compared to these, even 80 days after inoculation, the immunoblot showed weak reactions to 202, 83, 81 and 65 kDa bands in mice inoculated on the back (Fig. 4). At 90 days after inoculation, the

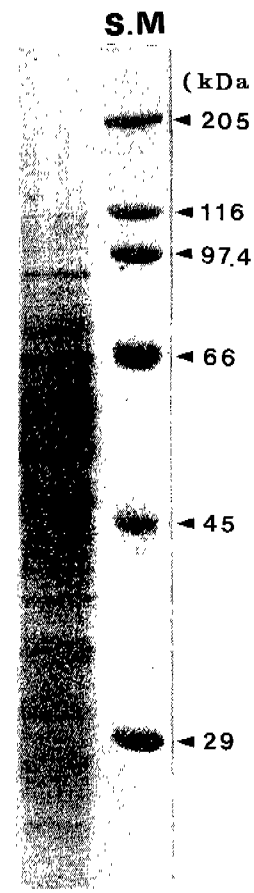

Fig. 2. Crude extract of *L. major* promastigotes in 12.5% SDS-PAGE.

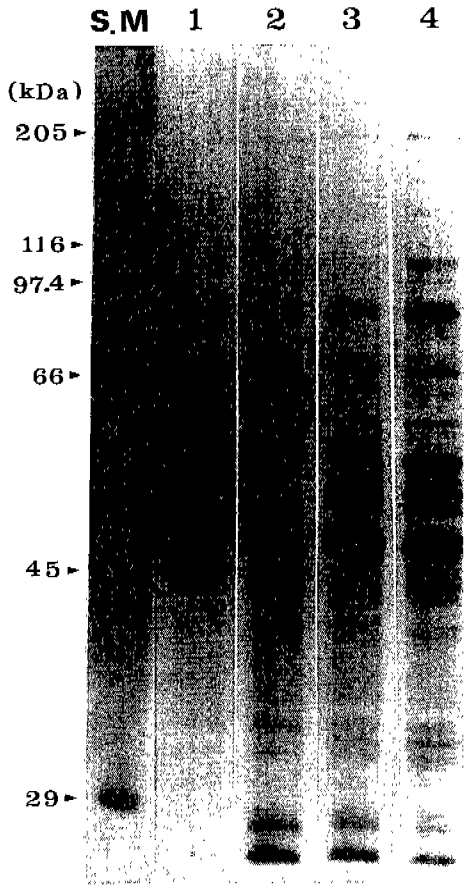

Fig. 3. Immunoblot patterns of *L. major* crude antigen with sera of mice with nasal ulceration. Lane 1, 10 days after inoculation; 2, 20 days; 3, 30 days; 4, 40 days after inoculation.

antibodies to antigenic bands of 202, 83, 81, 74, 67, 65, 62, 59, 54, 52, 20 and 17 kDa appeared distinctively in serum of mice with the back ulcer. Main antigenic bands were between 71 and 53 kDa (Fig. 5).

Measurement of skin temperature

The surface temperature was 29°C on the nose, 28°C on the footpad, and 34°C on the back.

DISCUSSION

In the present study, the skin ulcer developed differently by the inoculation sites in *L. major* infected BALB/c mice. The skin ulcer appeared first on the 15th day in the nose-inoculated mice, and on the 17th-18th day in the footpad-inoculated mice. Compared with the nose- or footpad-inoculated groups, the ulcer appeared after 90 days in the back-inoculated mice. Furthermore, all of the nose-inoculated mice were suffered from more severe ulcer than mice of other groups and died 40 to 50 days after infection due to visceral dissemination. The different clinical manifestation may be an outcome of the different balance between offensive force of *L. major* and host defence response to the infection. The offensive force may be determined by degree of proliferation and spread of *L. major*, and by immune reaction. The present finding suggested that the nose and the footpad are the better habitat for *L. major* proliferation than the back in BALB/c mice.

There must be several factors partially related with growth of *L. major* in the skin and also with subsequent clinical manifestations. The factors are immune response, local differences in skin temperature, differences in the degree of lymphatic microvasculature and lymphatic drainage, differences in densities of epidermal dendritic cells (Langerhans cells), pH, nutrients, and serum components (Nabors & Farrell, 1994, Zilberstein & Shapira, 1994).

The most important factor regulating the clinical manifestations of leishmaniasis is the immune response. Many studies have suggested that inbred mice generally develop either of two divergent patterns of immune response to infection of *L. major*. The response is known to be decided by preferential development of Th1 or Th2 cells at the initial phase of infection. Since Th2 cells are derived mainly in *L. major* infected BALB/c mice, the mice are susceptible (Solbach & Laskay, 1995). Although cytokine response and T cell subset population were not studied in the present study, the mice with skin ulcer showed activities of serum IgG antibodies. This finding conforms with that humoral immune response is insufficient to protect the mice, and also cellular immunity is more important than the humoral response in *L. major* infection.

It is also plausible that the difference of the skin ulceration at the inoculation sites is

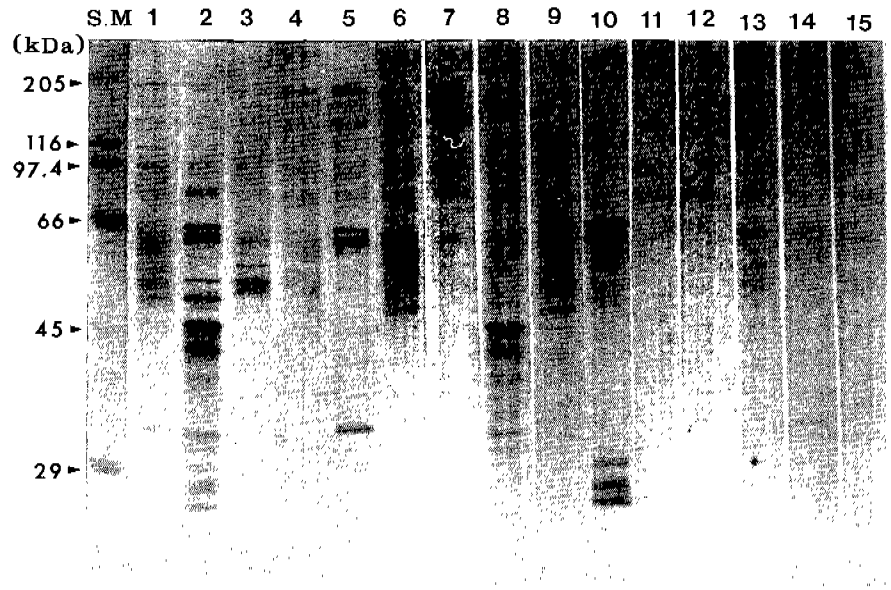

Fig. 4. Immunoblot patterns of *L. major* crude antigen with sera collected at 40 days after inoculation. Lanes 1-5: sera from mice with nasal ulcer; lanes 6-10: sera from mice with pedal ulcer; lanes 11-15: sera from mice with dorsal inoculation.

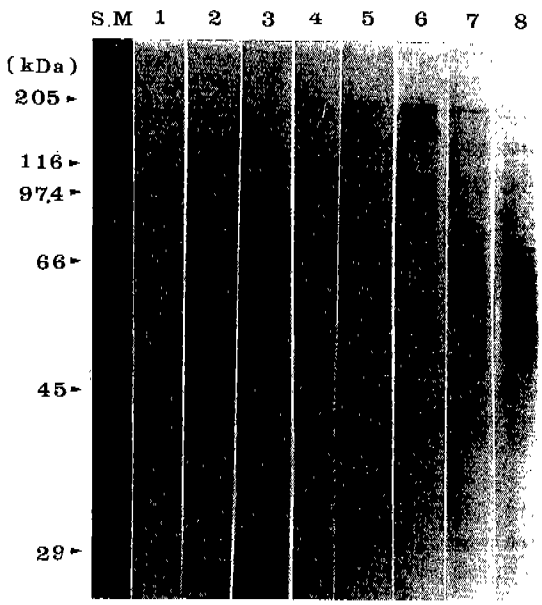

Fig. 5. Immunoblot patterns of *L. major* crude antigen with sera of mice with dorsal ulceration. Lanes 1-6, intervals of 10 days after inoculation; 7, 90 days; 8, 100 days.

regulated by the different immune responses. On this point of view, Nabors & Farrell (1994) mentioned that tail-inoculated SWR mice were more susceptible than footpad-inoculated mice. This phenomenon might suggest that different immune response is induced by the inoculation sites in a mouse. In data of Nabors & Farrell (1994), BALB/c mice were extremely susceptible to *L. major* which were inoculated on the footpad and the tail. It should be studied further whether nose-inoculation and back-inoculation show different immune responses in BALB/c mice.

All of the *Leishmania* inoculated mice showed no or very faint immunoblot response before the skin ulcer developed. In mice inoculated on the nose or footpad, serum IgG antibody reacted faintly to 202, 139, 98, 83, 81, 52, 42, 26 and 23 kDa bands at 10 days after inoculation, but it reacted strongly to these bands and additionally to 67, 65, 62, 59, and 54 kDa bands at 20 days. Contrary to this, the serum IgG antibody of mice inoculated on the back reacted only to 4 protein bands of 202, 83, 81, and 65 kDa from 10 to 80 days. However at 90 days, the reaction became stronger to the bands and additionally to 74, 67, 59, 54, and 52 kDa bands. The reaction to 74-52 kDa bands corresponded to the clinical development of ulcers. In this connection, Nabors & Farrell

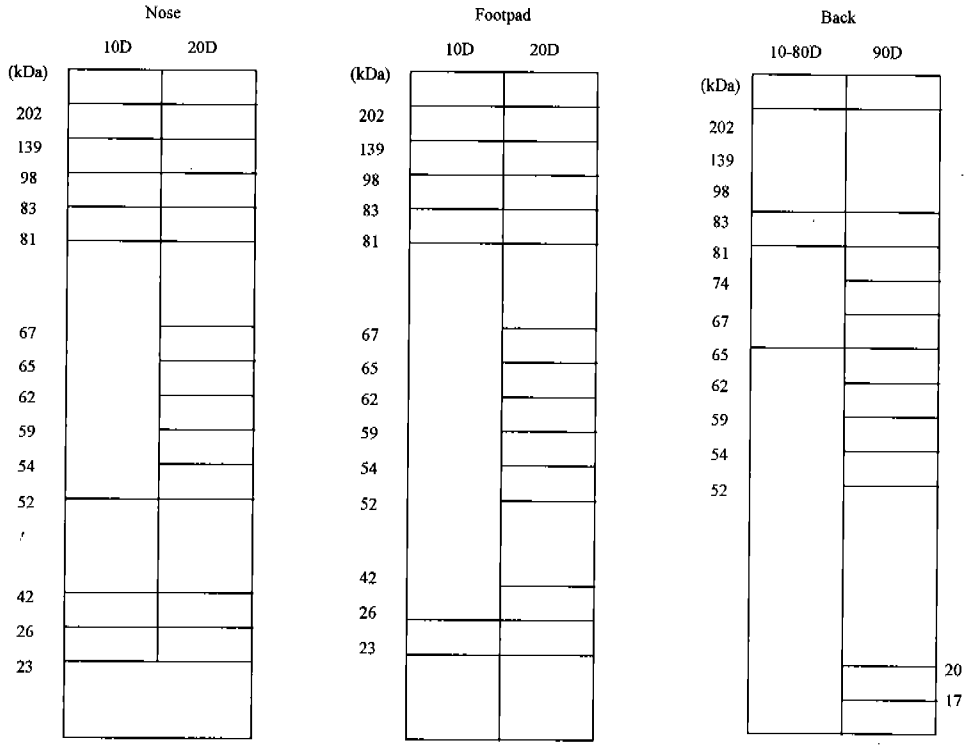

Fig. 6. Summary of immunoblot patterns of IgG antibody to crude antigen of *L. major* by inoculation sites.

(1994) observed that production of IL-4 was significantly increased in BALB/c mice 10 days after footpad inoculation. The increased IL-4 stimulated active production of antibodies.

Production of IL-4 by lymph node cells of BALB/c mice increased continuously by 10 days after inoculation but began to decrease at 8 weeks. During the period, production of IFN-*r* was found to decrease in mice infected at either site (Nabors & Farrell, 1994). The amount of IgE antibodies in serum is known to be high in chronically infected BALB/c mice regardless of the site of infection. In our study, the antibodies increased to be detectable in the serum at 15 to 20 days after inoculation, and the skin ulcer appeared also at 15 to 20 days after inoculation on the nose or footpad. Especially at the time of ulceration, antibodies to 67-52 kDa bands newly appeared. From these findings it is suggested that the serum antibodies are also important in formation of the skin ulcer.

The skin surface temperature was 28-29°C on the nose and footpad while it was 34°C on the back. Although Leon *et al.* (1995) found no correlation between *in vitro* infectivity and temperature, the temperature might influence the initial proliferation at the inoculation sites. Biegel *et al.* (1983) found that *L. mexicana* was grown well in cultured macrophages at 34°C, but its growth was restricted at 37.5°C. The combination of both low pH and high temperature induces transformation of the promastigote to the amastigote in all *Leishmania* species (Zilberstein & Shapira, 1994). In addition to the above factors, density of Langerhans cells may be involved to the different development of ulcer although distribution of the cell is not well defined by the site.

In conclusion, the present findings revealed that serum IgG antibodies to antigen of *L. major* promastigotes appeared in different patterns between nose- or footpad-inoculated mice and back-inoculated mice. The serum IgG antibodies to the antigenic bands increased significantly when the skin ulcer

appeared at the site of inoculation. This finding suggests that the IgG antibodies to the antigenic bands especially to the bands between 67 and 52 kDa should play a role in the pathogenesis of the skin ulcer.

REFERENCES

Axelrod O, Klaus S, Frankenburg S (1994) Antigen presentation by epidermal Langerhans cells in experimental cutaneous leishmaniasis. *Parasite Immunol* **16:** 593-598.

Biegel D, Topper G, Rabinovitch M (1983) *Leishmania mexicana*: Temperature sensitivity of isolated amastigotes and of amastigotes infecting macrophages in culture. *Exp Parasitol* **56:** 289-297.

Gutierrez Y (1990) Diagnostic pathology of parasitic infections with clinical correlations. p20-54, Lea & Febiger, Philadelphia.

Kemp M, Theander TG, Kharazmi A (1996) The contrasting roles of CD4+ T cells in intracellular infections in humans: leishmaniasis as an example. *Immunol Today* **17**(1): 13-16.

Kirkpatrick CE, Nolan TJ, Farrell JP (1987) Rate of *Leishmania*-induced skin-lesion development in rodents depends on the site of inoculation. *Parasitology* **94:** 451-465.

Leon LL, Soares MJ, Temporal RM (1995) Effects of temperature on promastigotes of several species of *Leishmania*. *J Euk Microbiol* **42**(3): 219-223.

Moll H (1993) Epidermal Langerhans cells are critical for immunoregulation of cutaneous leishmaniasis. *Immunol Today* **14**(8): 383-386.

Nabors GS, Farrell JP (1994) Site-specific immunity to *Leishmania major* in SWR mice: the site of infection influences susceptibility and expression of the antileishmanial immune response. *Infect Immun* **62**(9): 3655-3662.

Solbach W, Laskay T (1995) *Leishmania major* infection: the overture. *Parasitol Today* **11**(10): 394-397.

Tsang VCM, Perralta JM, Simons AR (1983) Enzyme-linked immunoelectrotransfer blot techniques (EITB) for studying specificities of antigen and antibodies separated by gel electrophoresis. *Methods Enzymol* **92:** 377-391.

Zilberstein D, Shapira M (1994) The role of pH and temperature in the development of *Leishmania* parasites. *Ann Rev Microbiol* **48:** 449-470.

=초록=

BALB/c 마우스에서 큰리슈만편모충의 감염부위에 따른 궤양형성과 혈청 면역반응

이미정, 이종국, 김성범, 이순형, 홍성태

서울대학교 의과대학 기생충학교실 및 의학연구원 감염병연구소

BALB/c 마우스에서 감염부위와 감염기간에 따른 숙주 체액 면역반응의 변화를 알아보고자 하였다. 배양한 큰리슈만편모충의 전편모형(promastigote)을 BALB/c 마우스의 코, 등, 발바닥 부위로 나누어 각각 3 × 10^6마리씩 피하 감염 후 10-100일 동안에 궤양의 형성과정을 관찰하고 채혈하여 SDS-PAGE와 면역이적법을 시행하여 각 부위별로 나타나는 항체 반응을 관찰하였다. 외관상으로는 감염 15일부터 코에 감염시킨 마우스에서 먼저 궤양이 형성되기 시작하였고, 코에 궤양이 나타난 후 2-3일 뒤에 발에서 궤양이 형성되었으며 등에서는 감염시킨 후 90일이 되어서야 궤양이 관찰되었다. 감염후 20일에 실시한 면역이적법에 의하면 코 감염군에서는 202, 139, 98, 83, 81, 67, 65, 62, 59, 54, 52, 42, 26, 23 kDa의 항원성 분획이 관찰되었고 발 감염군에서의 항원 분획양상도 코 감염군과 같았으나 등감염군에서는 202, 83, 81, 65 kDa의 희미한 항원성 분획이 관찰되었다. 그러나 감염 후 90일이 경과한 등 감염군에서는 202, 83, 81, 74, 67, 65, 62, 59, 54, 52, 20, 17 kDa의 항원 분획이 관찰되었다. 이상의 결과로부터 감염부위와 감염기간에 따라 큰리슈만편모충에 대한 혈청반응이 항원 분획에 따라 다르게 나타남을 관찰하였다. 이 차이는 세 감염부위의 온도차에 의한 결과일 가능성도 있으나 다른 부위에 감염될 경우 한 숙주 내에서도 다른 면역반응이 유발되어 나타날 수도 있다고 추측하였다. 특히 궤양 형성 시기와 혈청내 67-52 kDa 분획에 대한 항체 출현 시기가 일치하는 것으로 보아 궤양 형성에 이 항체가 관여할 가능성이 있음을 시사한다.

〔기생충학잡지 35(1): 31-38, 1997년 3월〕