LIM, THUANG SENG

RESPONSES IN MICE FOLLOWING INTRANASAL OR INTRAPERITONEAL INFECTION WITH CRYPTOCOCCUS NEOFORMANS

The University of Oklahoma PH.D. 1979

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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

RESPONSES IN MICE FOLLOWING INTRANASAL OR INTRAPERITONEAL INFECTION WITH *CRYPTOCOCCUS NEOFORMANS*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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degree of

DOCTOR OF PHILOSOPHY

BY

THUANG SENG LIM

Norman, Oklahoma

1979

RESPONSES IN MICE FOLLOWING INTRANASAL OR INTRAPERITONEAL INFECTION WITH CRYPTOCOCCUS NEOFORMANS

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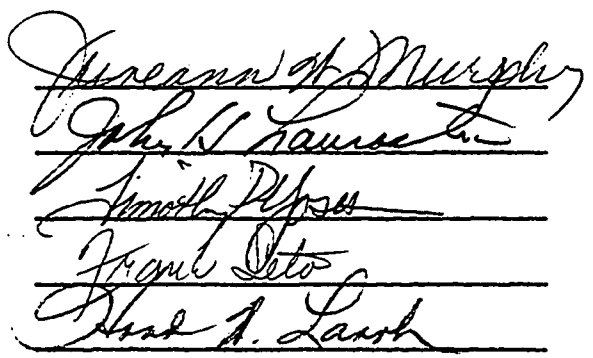

DISSERTATION COMMITTEE

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DEDICATION

This work is dedicated to the memory of my late father, Lim Peng Chor. His undying love, hard work and self-sacrify provided me the opportunity to attain a higher education.

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ABSTRACT

Inbred CBA/J mice were used in developing a defined *in vivo* mouse model for studying host-parasite relationship in cryptococcal infections. Mice were infected either intranasally (IN) or intraperitoneally (IP) with 10^3 viable *C. neoformans* cells and organism growth profiles in the lungs, spleens, livers and brains of the infected animals were determined over a 92 day study period. Humoral and delayed type hypersensitivity (DTH) responses were also determined in these mice at weekly intervals throughout the time period. Intranasally infected mice developed strong DTH responses to a cryptococcal culture filtrate (CneF) antigen, and there was a good correlation between acquisition of DTH and the reduction of *C. neoformans* in the infected tissues. Intraperitoneally infected mice showed a slower rate of reduction in numbers of *C. neoformans* in the tissues and had a somewhat suppressed DTH response to CneF antigen. Anticryptococcal antibody was not detected in either IN or IP infected mice, but cryptococcal polysaccharide antigen titers were high in both groups. The transfer of sensitized spleen cells from IN infected mice

to syngeneic naive recipient mice resulted in transfer of the DTH response to cryptococcal antigen to recipients.

Splenic enriched T-cells and sera were obtained from inbred CBA/J mice injected 7 or 35 days earlier with either 10^3 viable C. neoformans or sterile physiological saline. The transfer of day 7 sensitized enriched T-cells or normal enriched T-cells did not transfer immunity to C. neoformans or DTH to CneF antigen to the recipients. However, day 35 sensitized enriched T-cells when transferred to recipient mice were able to confer immunity as indicated by the reduction in numbers of C. neoformans cells in the tissues and they also transferred the DTH response to the CneF antigen. Sera from either sensitized or normal mice was unable to transfer immunity to recipient animals. These results suggested that there was a time requirement for development of the immune response in the donor mice and that T-cells were crucial in the host defense against a cryptococcal infection. Culturing of day 35 C. neoformans sensitized T-cells in the presence of homologous antigen (CneF) but not in the presence of heterologous antigen (PPD or DNFB) induced migration inhibition factor (MIF) production, thus indicating that lymphocytes from C. neoformans injected mice were specifically sensitized to CneF antigen.

RESPONSES IN MICE FOLLOWING INTRANASAL OR INTRAPERITONEAL INFECTION WITH *CRYPTOCOCCUS NEOFORMANS*

CHAPTER I

INTRODUCTION

Over the years, the pathogenesis of *Cryptococcus neoformans* has been widely studied and has been the subject of several reviews (1,2,27,31). Evidence developed through studies on various aspects of host-parasite relationships of this infection has suggested that both humoral (12,14,15, 18) and cell mediated (9,13,19,20,30,33) immune (CMI) responses are important in defense against a cryptococcal infection. However, the exact role of humoral antibody in protection against cryptococcosis is still uncertain. More evidence has been generated supporting CMI as the important factor in host defense against a cryptococcal infection.

It has been known for some time that cryptococcosis occurs more frequently in patient with defective CMI (10,32 35,36); however, cryptococcosis also has been known to occur in patient who did not suffer from any apparent defect in

CMI. In view of the wide distribution of this organism in nature, and the potentially frequent encounter with the etiological agent without development of actual disease, we were impelled to developed a defined animal model that would allow systematic examination of the pathogenesis of C. neoformans and the host responses to this organism.

Cryptococcal infections in humans most often involves the lung parenchyma with the respiratory tract being considered the primary portal of entry (8,25). However, occasionally the organisms disseminate to the brain and other organs despite the presence of natural anticryptococcal factors in serum and saliva (24). Pathological examination of chronically infected tissues from cryptococcal patients reveals tissue lesions consisting of granulomas containing macrophages, lymphocytes, epitheloid and giant cells (25). Although the granulomatous lesions are often widely spread in murine cryptococcosis, similar lesions as those seen in human infections are present in the brains, lungs, spleens and livers of cryptococcus infected mice (5). Because of the similarity between human and murine cryptococcosis, we have chosen to use the mouse to develop a laboratory animal model for studying this disease. Furthermore, there is already available a great bank of knowledge on immunological functions and immunogenetics related to delayed-type

hypersensitivity (DTH) and CMI phenomena in various strains of mice which can provide guidelines for using the murine model in a systematic and relatively in depth study on the acquired host defense mechanisms in cryptococcosis. The easy handling and rapid reproduction of mice also are advantages which will enable us to use more animals for the study which in turn will provide for a more accurate assessment of the host-etiological agent relationships in this infection.

Our primary goal in this study was to define the cryptococcosis-murine model using two routes of infection in one inbred strain of mice. The parameters defined were: (a) C. neoformans growth profiles in various organs (i.e. lungs, spleens, livers and brains). (b) DTH responses, (c) cryptococcal antigen levels, and (d) antibody titers in serum. All parameters were followed over a relatively long time period (92 days) post infection.

In addition, experiments were conducted to demonstrate that the DTH response could be passively transferred to normal syngeneic recipients with spleen cells from sensitized animals but not with serum from sensitized mice, or serum or spleen cells from control animals.

MATERIALS AND METHODS

Mice. Inbred 8 to 12 weeks old CBA/J mice of both sexes bred in the University of Oklahoma animal facilities were used in this study.

Organism. Cryptococcus neoformans 184A as described by Murphy and Cozad (29) was employed throughout this work.

Infection of mice. CBA/J mice were given 1.0×10^3 viable C. neoformans cells intranasally (IN) in two 5 ul physiological saline droplets or 1.0×10^3 cells intraperitoneally (IP) in 0.5 ml of physiological saline (SPS). Control animals were inoculated with equivalent volumes of SPS either intranasally or intraperitoneally.

Antigen preparation. The cryptococcal culture filtrate (CneF) antigen used for footpad testing was prepared according to the procedure described by Cauley and Murphy (9).

Determination of DTH profile. On days 1, 3, 7 and weekly intervals thereafter until 92 days post-infection, 10 mice (5 males and 5 females) from each group were selected randomly

for footpad testing. The right and left hind footpads of these mice were measured before (R_0 and L_0) and 24 hours after (R_{24} and L_{24}) intradermal injection of 0.03 ml antigen in the right footpad and 0.03 ml SPS in the left. The net increase of footpad swelling was calculated by the following equation:

$$(Right_{24} - Right_0) - (Left_{24} - Left_0) = \text{Net footpad increase.}$$

A net footpad swelling of 0.3 mm or more was considered to be a positive response.

Method of footpad measurement. Mouse footpads were measured by using an instrument designed and constructed in our laboratory. This instrument consisted of a substage 1000 watt light source, a convex lens and a 3 inches diameter hole. Directly above and clamped tightly over the hole was a transparent 7½ cm ruler with a 1 cm thick plastic block glued on the 1 cm mark of the ruler. The ruler was marked off in mm. Above the ruler, was a lens system and an adjustable mirror which projected the light coming from the base onto a screen. A board with a scale calibrated in 0.005 mm was placed on the wall 20 feet away from the projector. To use the apparatus, a mouse was put into a 50 ml syringe with a hole cut near the end of the syringe so that the foot could be pulled out and placed against the plastic block on the ruler. The thickness of the footpad was read by measuring

the image projected on the calibrated board on the wall. Three people were involved in measuring the footpads, a reader, a projectionist to place the footpads against the block and a third person who handed mice to the projectionist and who recorded the results. This system eliminated any possibilities for biased reading since the reader and the projectionist did not know from what group each mouse came.

Organism growth profile. At 3 hours after mice received either intranasal or intraperitoneal infection with C. neoformans, and at 2, 4, 8 days and weekly intervals thereafter for 92 days, 5 male mice from each group were exanguinated, and serum samples were collected. The mice were autopsied, and spleens, livers, lungs and brains were collected for determination of the numbers of viable C. neoformans using the procedure previously described by Cauley and Murphy (9).

Serological tests. The whole yeast cell microagglutination test as described by Murphy and Cozad (29) was employed to measure the anticryptococcal antibody response in both the IN and IP infected mice. Rabbit anticryptococcal antiserum with a titer of 1:640 was used as a positive control while normal rabbit serum served as a negative control. The level of cryptococcal antigen in the sera of these mice was assayed using the latex cryptococcal antigen test described by Bloomfield

et al (4). A 25 ug per ml solution of capsular polysaccharide isolated from cultures of C. neoformans and a normal rabbit serum were used as positive and negative controls respectively, in the latex agglutination test.

Preparation of lymphoid cells. Mice were given intranasally either 1.0×10^3 viable C. neoformans in 0.01 ml SPS or the same volume of SPS. On days 7, 28, 35 and 44 post-infection, sensitized mice and saline control mice were sacrificed by exsanguination, serum was collected and stored at -20 C until needed. Spleens from these mice provided the lymphoid cells for transfer. Donor cell pools were prepared by teasing the spleen with sterile forceps over a 120 mesh stainless steel screen into a petri dish containing 2 ml of Dulbecco's phosphate buffered saline (DPBS). Red blood cells present among the dissociated lymphoid cells were lysed using ammonium chloride tris buffered solution (6). Cells were washed once with DPBS and again with SPS. The viability of lymphocytes was determined by trypan blue exclusion test and the final cell suspension was adjusted to about 5×10^8 viable lymphocytes per ml of SPS.

Transfer of lymphocytes. At various time intervals, sensitized and normal lymphoid cells were obtained from donor mice and were injected intravenously into designated groups of recipient

mice. Eight recipients were used at each time period; 5 mice were given approximately 1.0×10^8 sensitized lymphocytes, and 3 animals received approximately the same number of normal lymphocytes. Delayed hypersensitivity responses of the recipient mice were checked by footpad testing 20 hours after the mice received lymphoid cell transfers. On the days lymphocytes were transferred, 5 C. neoformans infected and 3 SPS inoculated donor mice were footpad tested to determine the state of sensitization of these mice.

Transfer of serum. Sera obtained from donor mice given 10^3 viable C. neoformans 7 or 35 days earlier were injected intravenously in 0.5 ml volumes into recipient mice. Delayed type hypersensitivity responses were determined by footpad testing 20 hours following serum transfer.

Statistical analyses. The means and standard error of the means programmed on a Hewlett-Packard calculator model 9810A were used in analyses of data.

RESULTS

Quantitation of viable C. neoformans in tissues and the DTH profile. Three hours after intranasal inoculation with 10^3 viable C. neoformans cells, a few cryptococci were isolated from the lungs of only one mouse (Fig.1 and Table 1). All other organs cultured remained sterile for the first 8 days of the experiment. By day 15 after infection, one of the five mice autopsied had cryptococci in all organs cultured; however, two of five (40%) had positive lung cultures. The first peak in numbers of cryptococci cultured (Fig.1 and 2A) as well as number of culturally positive mice came at 22 days post infection (Table 1). Eighty percent of the mice had cryptococci in the lungs by this time. Following this maxima the number of colony forming units (CFU) in the lungs declined rapidly, and by day 43 C. neoformans was not cultured from the lungs of a single animal. However, one week later 80% of the mice had low numbers of cryptococci in organs other than lungs. Lungs remained free of organisms from day 43 through 57. On day 50 post intranasal infection, another peak in CFU of C. neoformans and in percentage of mice with positive cultures was observed. The number of CFU making the second peak was approximately 2 log units

lower than that of the first peak, indicating that the mice were maintaining C. neoformans populations at low levels. Again by day 64 and continuing through day 92, C. neoformans was cultured from lungs of the infected animals. However, only 20% of the inoculated population had positive cultures during this segment of the experiment.

Although only a few IN infected animals showed detectable levels of C. neoformans in the lungs during the first week after infection, an experiment performed to determine the site of cryptococci localization following intranasal inoculation revealed about 70% of the infecting dose was in the head region, excluding the brain, during the first and second weeks after IN infection.

The organism growth profiles in the intraperitoneally infected mice showed different patterns from those of the intranasally infected mice. Three hours following IP infection, 3 of 5 mice showed positive cryptococci cultures, with one mouse having cryptococci in spleen and liver and the other 2 mice having cryptococci in either the spleen or the liver (Fig.3 and Table 2). By day 3, 100% of the mice had cryptococci in livers and spleens but not in other organs. Like the IN infected mice, organism counts in the IP infected mice also peaked at 22 days post infection (Fig. 4A) with 100% of mice having cryptococci in the livers, spleens and lungs.

After day 22, mean CFU of C. neoformans began to decline but was followed by a second peak on day 43 and a third peak on day 71, respectively. The second and third peaks in mean numbers of CFU were one log lower than the first peak. After the third peak, the mean CFU continued to decline through day 92. This cyclic pattern of organism growth was also apparent in IN infected animals, whether the infecting dose was given by droplets, as in this study, or in aerosol form (Bates, R.A. and J.W. Murphy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, F26, p.89). Also, from day 29 through 85, the percentages of IP infected mice having positive cultures fluctuated between 80 to 100% except on day 36 when only 60% of the mice were culturally positive for cryptococci.

Following the intranasal infection with C. neoformans, positive DTH responses to CneF antigen were not detected until day 22, when 20% of the mice tested developed positive DTH footpad responses (Fig. 2B). By day 29, 100% of the mice had developed positive DTH to CneF antigen. The intensity of this response reached a maximal level on day 43 and remained at a relatively high level throughout the 92 day study period. However, the DTH responses to CneF antigen in the IP infected mice appeared to be somewhat suppressed (Fig. 4B) when compared to that of the IN infected mice (Fig. 2B). Although DTH first appeared in 20% of the IP mice at day 22 and peaked at day

43 as in the IN group, the overall mean increases in footpad thickness were significantly lower than those of the IN mice. The percentage of IP infected mice showing positive DTH responses was also substantially lower than in the IN group, with 40% of the mice having positive responses at any given time throughout the experiment (Table 2). The 5 female mice used in DTH profile determinations at each time period were not used in organism growth profile studies. However, DTH measurements from the female mice following IN or IP infection were not significantly different from the footpad measurements of the male mice.

The IN and IP infected mice that survived throughout the study period were autopsied at 183 days post infection, and no cryptococci were cultured from any of the four organs assayed.

Serological data. The level of cryptococcal polysaccharide (CP) antigen in the sera of IN and IP infected mice are shown in Figure 5. Cryptococcal polysaccharide antigen was detected in IP mice as early as 1 day post infection; however, in the IN mice, CP antigen was not demonstrated until day 7. The level of CP antigen in sera of IN and IP mice remained relatively constant up to day 14, and each group of mice had a mean $\log_2$ titer of about 9.0 at that time. After that period,

levels of CP antigen increased rapidly in the IP infected mice to a peak mean $\log_2$ titer of 12.5 by day 36 post infection; whereas, the IN infected mice showed a peak mean $\log_2$ titer of 11.5 on day 43. The increase of CP antigen levels in IN and IP mice came after the numbers of C. neoformans had reached maximal levels and had started to decline. Following the peak increase in CP antigen titers, the levels of CP antigen in the sera began to decrease rapidly; and by day 92, both the IN and IP mice had mean $\log_2$ titers of 8.0.

Anticryptococcal antibodies in sera of IN and IP infected mice were not detectable despite carefully repeated attempts to demonstrate the presence of agglutinating antibody.

Transfer of DTH. Following intranasal inoculation with 10^3 C. neoformans, DTH responses were not detected in these donor mice on day 7. However, on day 28, 35 and 44 post infection positive DTH responses to CneF antigen were observed (Fig. 6). This was in complete agreement with the DTH profile shown in Figure 3B. The recipient mice that received spleen cells obtained from donor mice infected 7, 28, 35 or 44 days previously, showed a progressive increase in sensitivity to CneF antigen (Fig. 6). The recipients of day 7 sensitized spleen cells as well as all recipients of normal spleen cells from saline control mice did not have positive DTH responses to CneF antigen. There were only 40% of the day 28 sensitized

spleen cells recipients with positive footpad responses; whereas, all recipients of day 35 or 44 sensitized spleen cells had positive DTH responses to CneF antigen. Delayed hypersensitivity responses were negative in all mice that received transfers of serum obtained from donor mice immunized with either C. neoformans or saline at 7 or 35 days earlier.

Figure 1. Organism growth profile on lungs, brains, livers and spleens of mice infected intranasally with 10^3 viable C. neoformans.

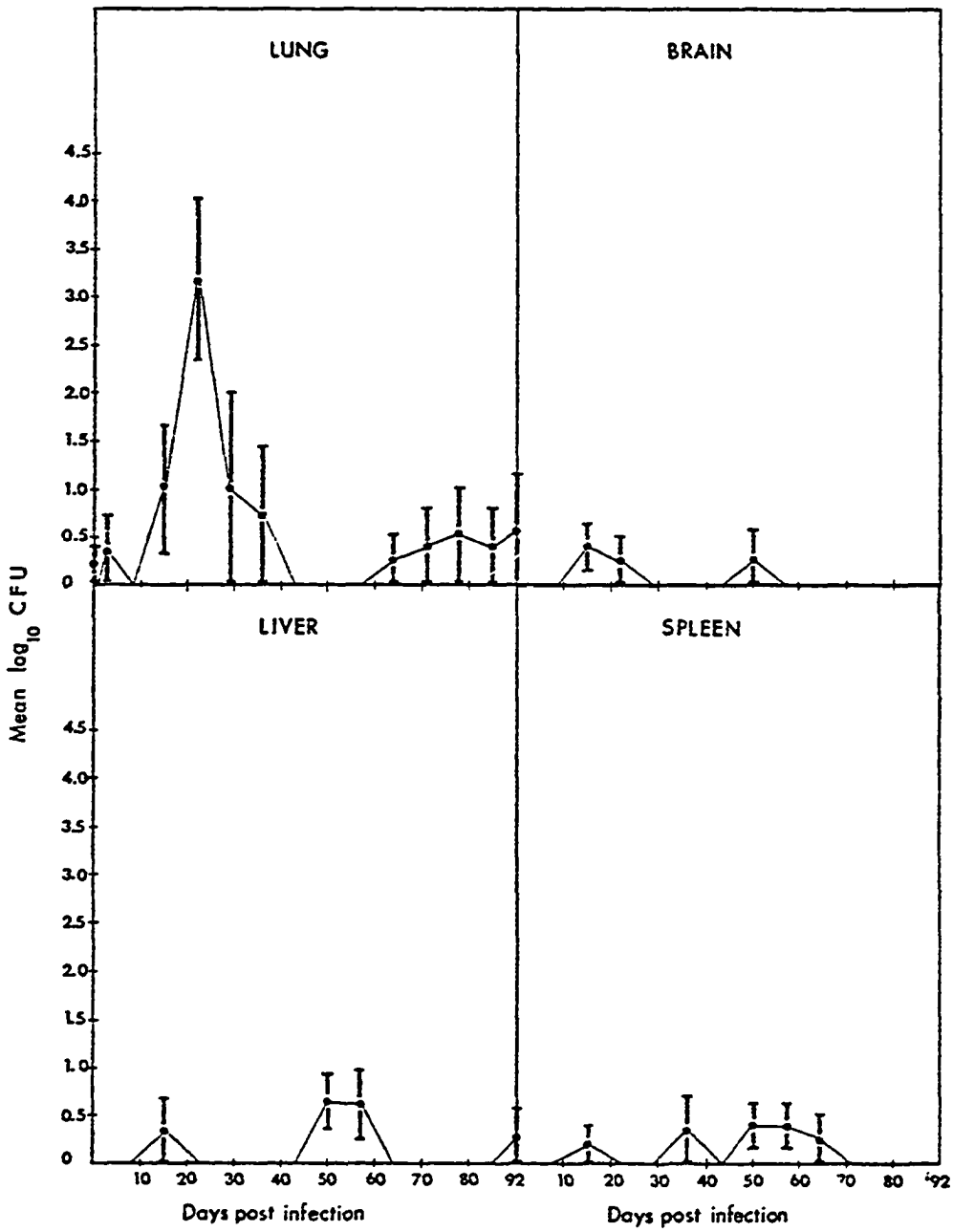
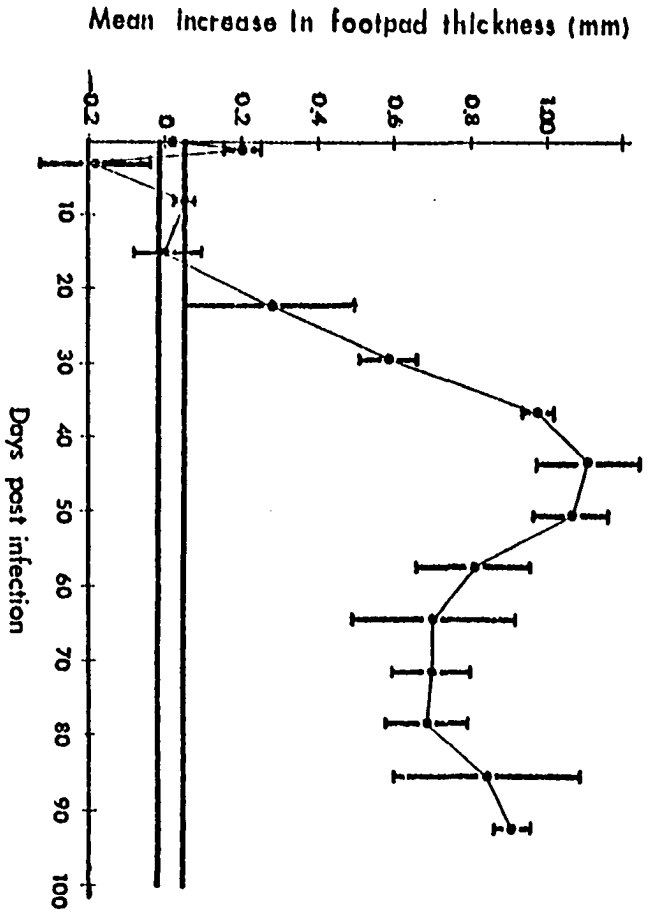

LUNG
BRAIN
LIVER
SPLEEN
Mean log10 CFU
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0
10 20 30 40 50 60 70 80 92
Days post infection

Figure 2A. Mean colony forming units (CFU) of mice infected intranasally with 10^3 viable C. neoformans.

Figure 2B. Footpads delayed hypersensitivity responses to cryptococcal cultured filtrate antigen in intranasally infected mice. Horizontal solid lines (———) indicate the range of delayed hypersensitivity responses in saline control mice.


Mean Increase In footpad thickness (mm)
-0.2
0
0.2
0.4
0.6
0.8
1.0
Days post infection
10
20
30
40
50
60
70
80
90
100

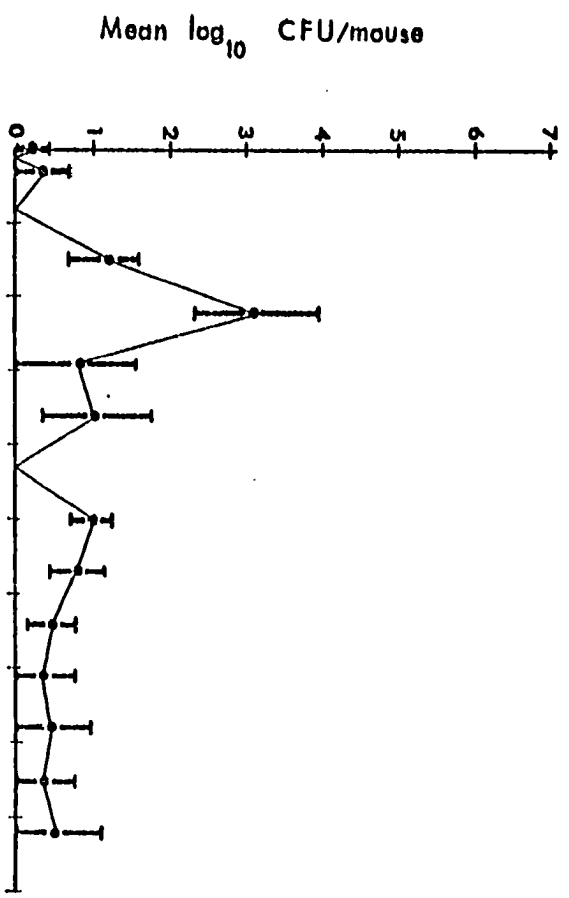

Mean log10 CFU/mouse
0
1
2
3
4
5
6
7

Table 1. Comparison of the percentage of intranasally infected mice with positive cryptococci culture and the percentage of those showing positive DTH responses.

INDIVIDUAL ORGANS	* PERCENT SHOWING POSITIVE CRYPTOCOCCAL CULTURES ON DAY :															
	0	1	3	8	15	22	29	36	43	50	57	64	71	78	85	92
LUNG	20	0	20	0	40	80	20	20	0	0	0	20	20	20	20	20
LIVER	0	0	0	0	20	0	0	0	0	60	40	0	0	0	0	20
SPLEEN	0	0	0	0	20	0	0	20	0	40	40	20	0	0	0	0
BRAIN	0	0	0	0	20	20	0	0	0	20	0	0	0	0	0	0
% TOTAL MICE WITH POSITIVE CULTURES	20	0	20	0	40	80	20	40	0	80	60	40	20	20	20	20
ALL MICE	** PERCENT OF MICE WITH A POSITIVE DTH RESPONSE															
	0	0	0	0	0	20	100	100	100	100	100	80	100	100	100	100

** 10 mice (5 male & 5 female) were used at each test period.
* 5 male mice were used on each autopsy period.

Figure 3. Organism growth profile on lungs, brains, livers and spleens of mice infected intraperitoneally with 10^3 viable C. neoformans.

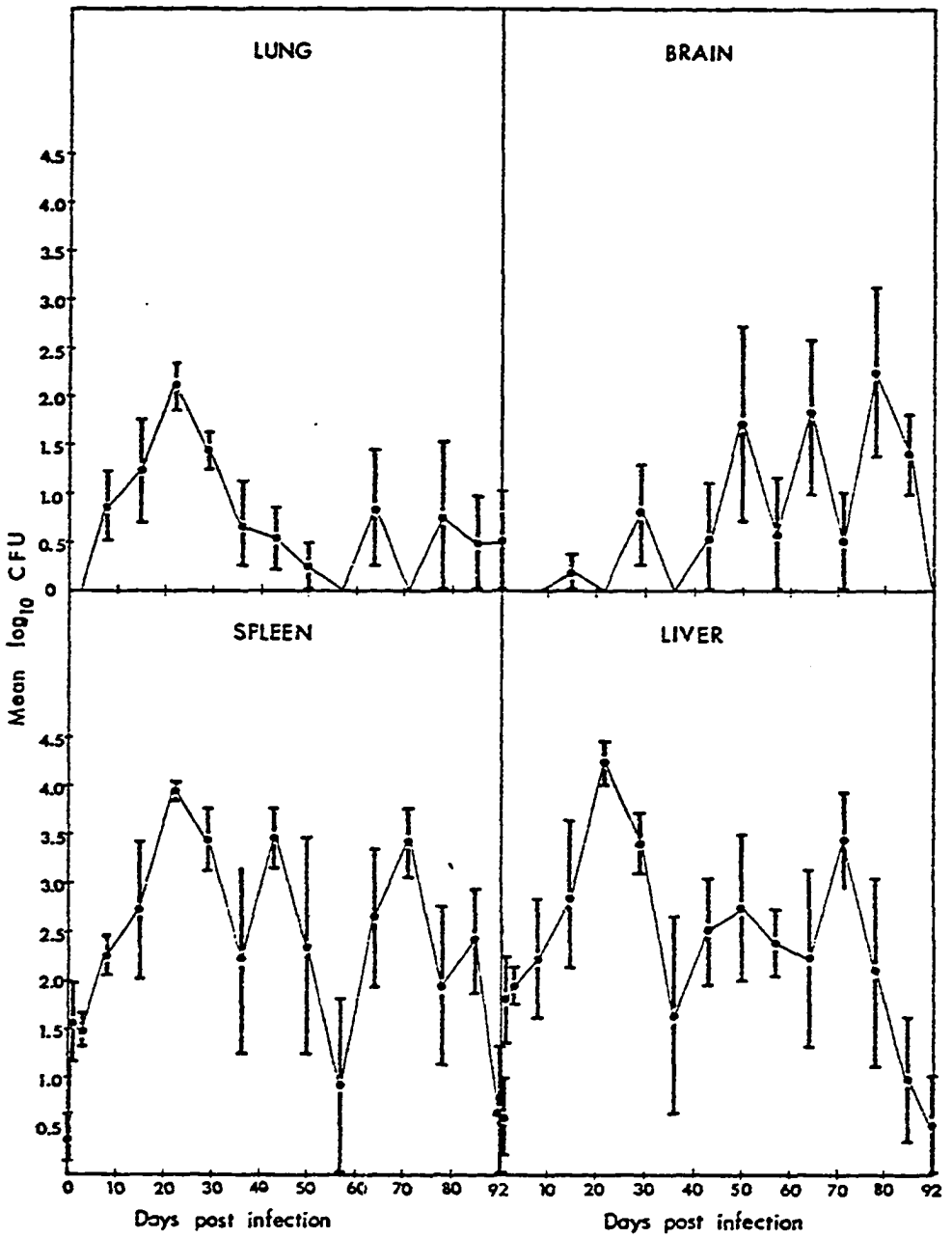

LUNG
BRAIN
SPLEEN
LIVER
Mean log10 CFU
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
0
10
20
30
40
50
60
70
80
92
Days post infection
Days post infection

Figure 4A. Mean colony forming units (CFU) of mice infected intraperitoneally with 10^3 viable C. neoformans.

Figure 4B. Footpads delayed hypersensitivity responses to cryptococcal cultured filtrate antigen in intraperitoneally infected mice. Horizontal solid lines (——) indicate the range of delayed hypersensitivity responses in saline control mice.

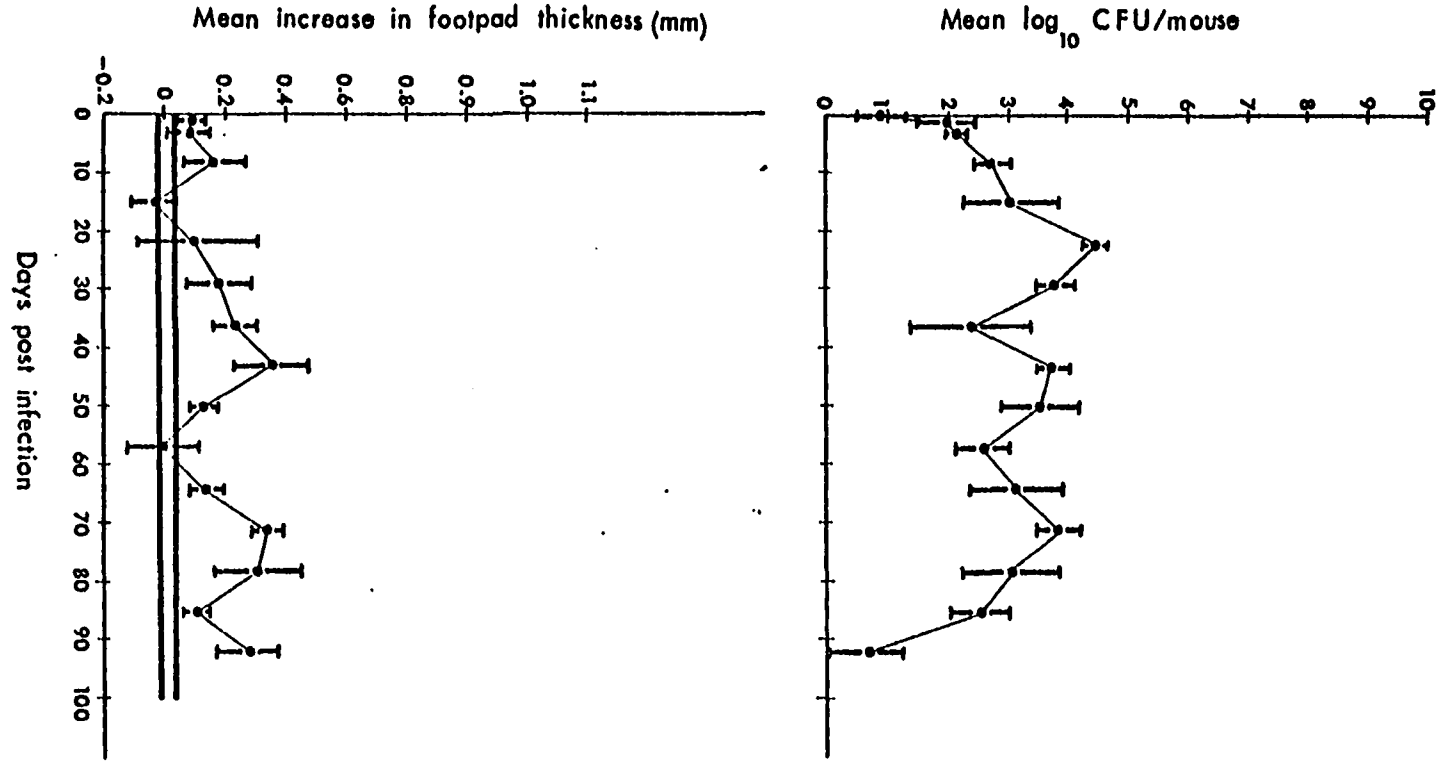

Mean increase in footpad thickness (mm)
-0.2
0
0.2
0.4
0.6
0.8
0.9
1.0
1.1
Days post infection
0
10
20
30
40
50
60
70
80
90
100
Mean log10 CFU/mouse
0
1
2
3
4
5
6
7
8
9
10

Table 2. Comparison of the percentage of intraperitoneally infected mice with positive cryptococci culture and the percentage of those showing positive DTH responses.

INDIVIDUAL ORGANS	* PERCENT SHOWING POSITIVE CRYPTOCOCCAL CULTURES ON DAY :															
	0	1	3	8	15	22	29	36	43	50	57	64	71	78	85	92
LUNGS	0	0	0	60	60	100	100	40	40	20	0	40	0	40	20	20
LIVER	40	80	100	80	80	100	100	40	100	80	80	60	100	80	40	0
SPLEEN	40	80	100	100	80	100	100	60	100	60	20	80	100	80	80	20
BRAIN	0	0	0	0	20	0	40	0	20	40	20	60	20	80	20	0
% TOTAL MICE WITH POSITIVE CULTURES	60	80	100	100	80	100	100	60	100	80	80	80	100	100	80	20
ALL MICE	** PERCENT OF MICE WITH A POSITIVE DTH RESPONSE															
	0	0	0	0	0	20	40	40	40	0	20	0	40	40	0	40

** 10 mice (5 males & 5 females) were used at each test period.
* 5 male mice were used on each autopsy period.

Figure 5. Cryptococcal polysaccharide antigens in serum of IN (●) and IP (▲) infected mice.

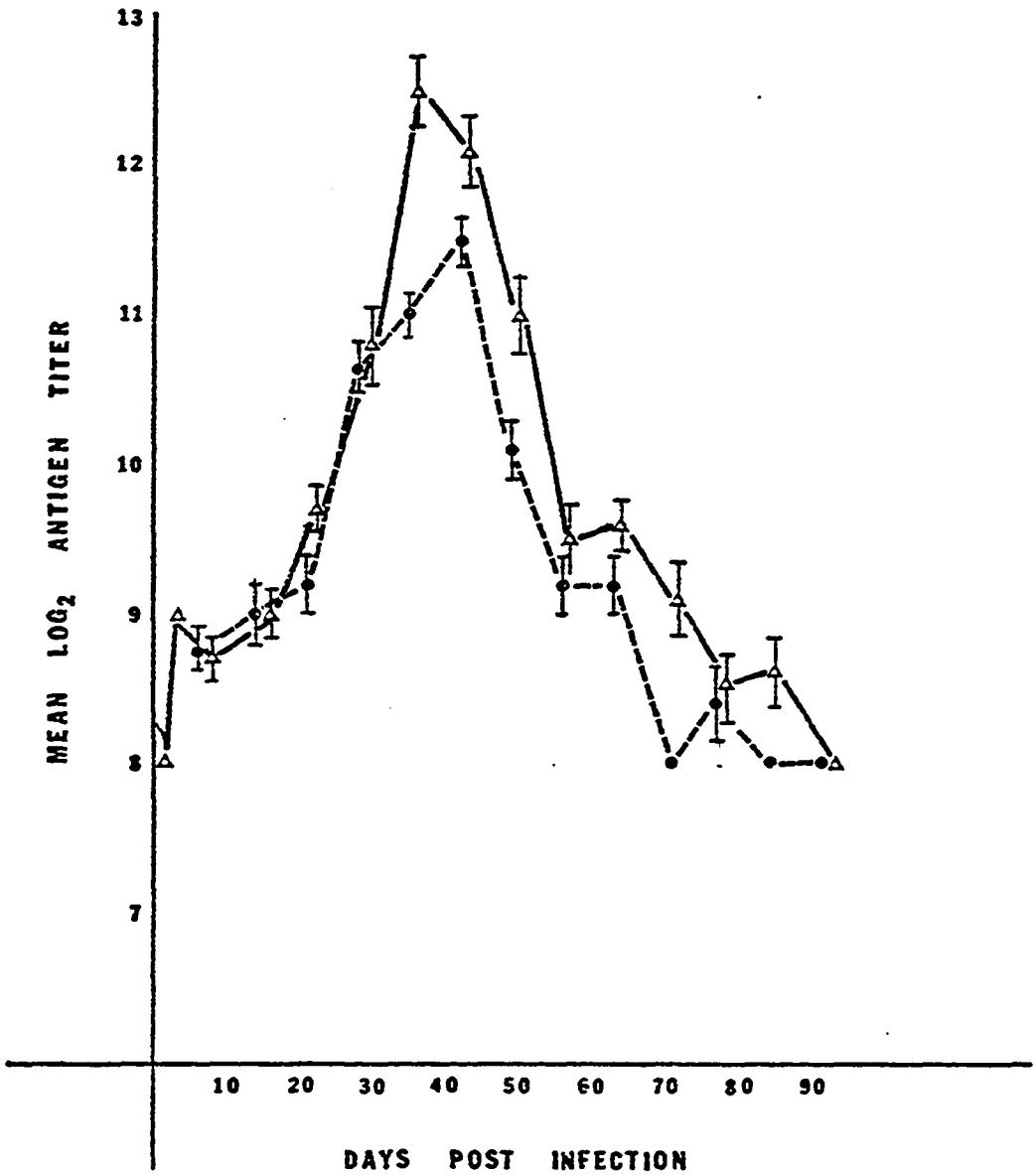
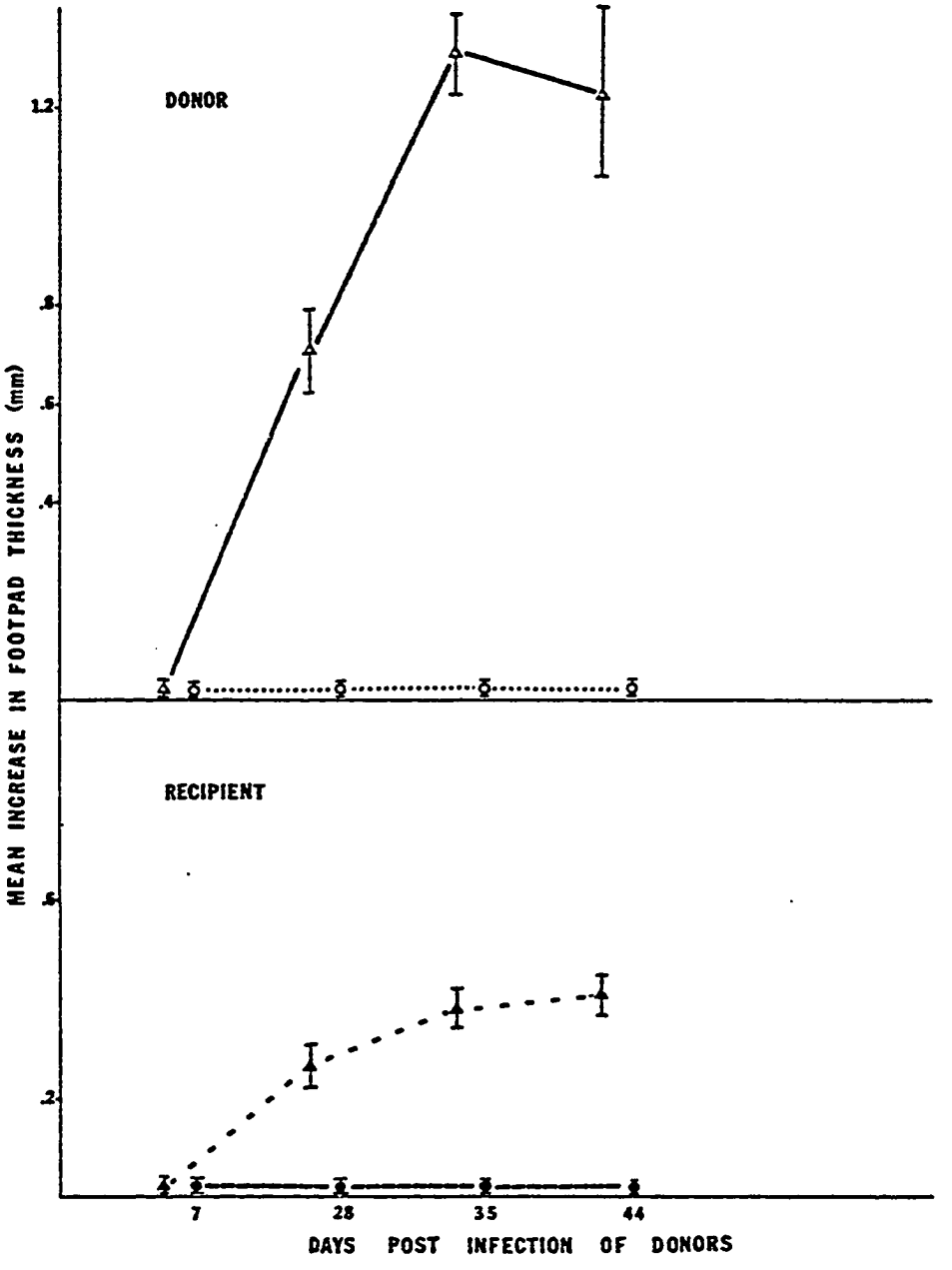

13
12
11
10
9
8
7
MEAN LOG2 ANTIGEN TITER
10
20
30
40
50
60
70
80
90
DAYS POST INFECTION

Figure 6. Footpads delayed hypersensitivity responses of donor and recipient mice.


DONOR
RECIPIENT
1.2
.8
.6
.4
.2
MEAN INCREASE IN FOOTPAD THICKNESS (mm)
7
28
35
44
DAYS POST INFECTION OF DONORS

DISCUSSION

Although various routes such as intracerebral, intravenous, intraperitoneal and subcutaneous have been used in the past to study the pathogenesis of experimental murine cryptococcal infections, no one has actually followed the course of the disease for a long period of time and simultaneously examined many different parameters of host responses. In this study, we have demonstrated that mice infected intranasally developed a greater degree of delayed hypersensitivity to cryptococcal cultured filtrate antigen than mice infected with the same number of C. neoformans through the intraperitoneal route.

As DTH in the IN infected mice increased in intensity, as measured by the increase in footpad thickness, it was paralleled by a rapid decrease in the number of C. neoformans cells in the animals. Furthermore, the intensity of the DTH responses in those mice stayed fairly high following the peak response, and the numbers of C. neoformans cells remained low after the initial decrease. Thus in the IN infected mice, there was an association between the acquisition of DTH response and the reduction of C. neoformans in tissues.

Similar associations between DTH and immune protection against C. neoformans have also been demonstrated by others (19,21, 30). Dykstra and Friedman (16) demonstrated that subcutaneous vaccination with viable C. neoformans conferred protection against a subsequent IV challenge with 10^2 viable cryptococci but only if the challenge was given at least 3 weeks (21 days) after vaccination. A similar time requirement for immunity to show a visible effect was observed also in this study. The IN and IP infected mice did not begin to show a decrease in the number of cryptococci until after 22 days post-infection (Fig. 2A & 4A), at which time the DTH response was showing the most rapid increase in intensity.

In the IP infected mice, the DTH response to CneF antigen was significantly lower than in the IN infected group, and the numbers of cryptococci were much higher in the IP infected mice than in the IN infected animals. The low DTH responses and the high percentage of animals with C. neoformans in tissues in the IP infected group could have been due to the route of infection. Hay and Reiss (23) recently showed that mice infected intravenously with 10^3 viable C. neoformans developed weaker CMI responses than mice infected subcutaneously. Exactly what causes the depressed DTH responses following IP infection is not yet known, but one possibility could be that presentation of antigen by the IP or IV routes preferentially induces suppressor cells or suppressor factors. In fact, in

suppressor cell and suppressor factor studies, many workers used the IP or IV routes of injecting the antigen to preferentially induce suppression over hypersensitivity (11,22,28,34). Another possibility for the observed low DTH responses in IP infected mice could be that the IP route of infection is not efficient in stimulating the appropriate population of lymphocytes responsible for the expression of DTH response.

Only a few IN infected animals showed detectable numbers of organisms in the lung during the first two weeks following infection. However, approximately 70% of the infecting dose of C. neoformans could be cultured from regions of the head excluding the brain during the same time period. Also, based on the fact that 100% of the IN treated animals developed strong DTH responses to CneF antigen by 28 day post infection, we assume that the IN treated mice were indeed infected with C. neoformans even though the infection could not be always detected by the cultural methods employed in these experiments. Another important feature observed from the organism growth profiles of IN and IP infected mice was the phenomenon of cyclic growth patterns. The IN and IP infected mice showed an eventual decline of C. necformans in various organ tissues. These animals achieved the reduction of organisms through a fluctuation between increase and decrease in number of cryptococci in the tissue and with each sucessive increase in number of C. neoformans being less than the previous increase.

Similar cyclic patterns of C. neoformans growth also have been observed in Balb/c mice infected through the aerosol route using a Henderson apparatus (Bates, R.A. and J.W. Murphy, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, F26, p.89).

The profiles of CP antigen detected in the sera of IN and IP infected mice from day 1 to day 35 were similar to results obtained by Cauley and Murphy (9). The increase in CP antigen levels following increase in number of cryptococci in these animals confirmed the idea that number of cryptococci in tissues is reflected by level of CP antigen in the sera. Besides possibly reflecting the number of C. neoformans in the tissues, the effect level of CP antigen has on host's immune response have not been defined in our model. Several studies (3,7,17,26) have indicated CP antigen could inhibit the ability of macrophages to phagocytize C. neoformans, however, results of this study did not indicate that CP caused detrimental effects on the ability of the animals to eliminate the infection. Murphy and Cozad (29) have shown that 500 ug of cryptococcal polysaccharide injected into CBA/J mice induced immunological paralysis. The highest mean CP antigen levels detected in sera of IP and IN mice in this study were 500 ug and 300 ug, respectively, which were levels capable of inducing immunological paralysis. Since no agglutinating antibodies were detected in either the IP or IN infected mice throughout the 92 day study period, it is possible that immune paralysis

was induced by the polysaccharide antigen or the antibodies produced were neutralized by the high levels of CP antigen. Circulating CP antigen did not appear to affect the CMI responses since the animals developed substantial DTH reactions which were presumably responsible for reducing the numbers of cryptococci. Finally, the ability of spleen cells, but not sera from DTH positive donor mice, to transfer DTH to syngeneic naive recipients confirmed that the DTH responses detected in this study were indeed cell-mediated.

In summary, this study provides information on cellular and humoral immune responses of mice with disseminated cryptococcosis over a prolonged period of time. It also presents a detailed account of organism growth profiles in various organs as a consequence of 2 different routes of inoculations. In addition, it provides two very well defined murine cryptococcosis models for further in depth studies on the role of T-lymphocytes and their products (e.g., lymphokines), the relationship of DTH to immune protection, and immune suppression in cryptococcal infection.

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CELL TRANSFER OF IMMUNITY TO CRYPTOCOCCOSIS

CHAPTER II

INTRODUCTION

Immunological studies on the host defense against a cryptococcal infection have provided a substantial amount of indirect evidence (1-3,11,14,15) indicating the importance of cell-mediated immunity (CMI) in the protection against this disease. However, direct evidence to support this concept has been lacking. In humans, cryptococcosis has frequently been found to occur in patients with defective CMI (5,32,33). Studies in mice have demonstrated also an increased susceptibility to cryptococcosis in immunologically deficient animals (nude mice) when compared to normal mice (7,16). Data suggest that deficiencies as in nude mice result in increased susceptibility and inability to mount an effective immune response. However, no data is yet available to show directly that cryptococcal sensitized T-lymphocytes are responsible for immune protection.

There are numerous studies indicating T-lymphocytes

participate in many immunological functions (6,8-10,20,25,31). A deficiency of T-cells would affect not only cell-mediated antimicrobial activity but also helper cell function in the production of antibodies. Furthermore, T-cell regulation of immune responses also would be affected by a T-cell deficiency. Therefore, by demonstrating that immunity can be transferred to syngeneic animals with sensitized T-lymphocytes but not with serum from sensitized animals or T-cells from control animals, then the role of CMI in protective immunity against cryptococcosis will be established. In an earlier study we demonstrated that mice infected intranasally acquired DTH responsiveness and immunity to C. neoformans at the same time, and that the sensitized lymphocytes were capable of transferring DTH to naive recipient mice (T.S. Lim, Ph.D. Dissertation., U. of Oklahoma, Norman. 1979). Therefore, the objective of this study is to attempt to provide a direct evidence that cryptococcal sensitized T-lymphocytes play a definite role in elimination of the cryptococcal cells from the infected host.

MATERIALS AND METHODS

Mice. CBA/J mice were bred in the animal facilities of the University of Oklahoma using breeding stock purchased from Jackson Laboratory, Bar Harbor, Maine. Inbred mice of both sexes, ages 8 to 12 weeks were used in the study.

Organism. Cryptococcus neoformans 184A as described by Murphy and Cozad (26) was employed throughout this study.

Immunization of mice. Following ether anesthesization, mice were immunized intranasally with 1.6×10^3 viable yeast cells suspended in two 5 ul saline droplets using a 50 ul Hamilton glass syringe attached to a 27 gauge needle. Control mice were similarly given 10 ul of saline intranasally.

Collection and enrichment of T-lymphocytes. Lymphoid cells from immunized and saline control mice were harvested according to procedures described previously (T.S. Lim, Ph.D. Dissertation., U. of Oklahoma, Norman. 1979.). The nylon wool method described by Julius et al (19) was used for obtaining lymphocyte populations enriched for T-cells.

Determination of homogeneity of enriched T-lymphocytes suspension. The amount of T-cell homogeneity in enriched T-lymphocyte suspensions was determined by treating the non-enriched and enriched population of T-cell with AKR/J anti-CBA/J antiserum (anti-Thy 1.2) plus absorbed guinea pig complement (GPC) and assaying for cell viability using the trypan blue dye exclusion assay. Also direct and indirect fluorescein labeled antibody techniques were performed which labeled the B-cells and B and T-cells, respectively. Therefore, by subtracting the number of cells stained directly (B-cells) from those stained indirectly (T and B-cells) with fluorescein labeled reagents, the number of T-lymphocytes in the enriched T-cell populations were obtained.

The direct fluorescent antibody staining of lymphocytes using fluorescein conjugated goat anti-mouse IgG (GAMIG) (Cappel Lab., Inc. Cochranville, Pa.) was done by adding 1.0×10^6 enriched T-lymphocyte in 0.5 ml of phosphate buffered saline (PBS) containing fetal calf serum (FCS) into a 1.5 ml conical polypropylene micro-test tube. Using PBS with 5% FCS, a 1/8 dilution of conjugated GAMIG was made and 0.05 ml of this dilute antiserum was added to the cell suspensions. The tubes were mixed well, incubated at room temperature for 30 minutes, then centrifuged at 230 x g for 5 minutes. The supernatants were carefully aspirated and discarded. The cells were washed once with 0.5 ml of

PBS containing 5% FCS to remove any unbound fluorescein labeled antibody. Afterwhich, each cell pellect was resuspended with 0.01 ml of glycerin in PBS (pH 7.2-7.6). The entire cell suspension in each micro-test tube was transferred to a microscope slide and covered with a 22 x 22 mm cover slip. The number of lymphocytes which showed peripheral fluorescence were counted at 400X using a Leitz Wetzlar Ortholux fluorescent microscope.

The indirect fluorescent antibody staining of lymphocytes was identical to those described for the direct fluorescent antibody staining except that lymphocyte suspensions were first incubated with 1:4 dilution of anti-Thy 1.2 mouse serum for 30 minutes at room temperature. This permitted the binding of T-lymphocytes by anti-Thy 1.2 antibody. After incubation, the cells were washed, centrifuged, and the supernatant was discarded. Fluorescein labeled GAMIG was then added to the cells as described earlier so as to label T and B cells.

DTH response in donor mice. On 7 and 35 days post IN administration of C. neoformans, mice were randomly selected and footpad tested for DTH responsiveness to cryptococcal cultured filtrate antigen (CneF) according to procedure described by Cauley and Murphy (7).

Transfer of enriched T-lymphocytes. On days 7 and 35 following intranasal administration of 1.6×10^3 viable C. neoformans

cells or saline, mice were put under deep ether anesthesia. Blood was obtained through cardiac puncture, and sera were collected, pooled and stored at -20C until needed. Spleens were removed from these donor mice and used for obtaining enriched T-lymphocyte populations.

Recipient mice were divided into 4 groups and were given intravenously 1.8×10^4 viable C. neoformans cells. Two hours later, group 1 mice were intravenously (IV) injected with 0.2 ml saline while group 2 mice received approximately 1.0×10^8 sensitized enriched T-lymphocytes in 0.2 ml saline suspension. Group 3 mice were injected IV with 1.0×10^8 sensitized enriched T-lymphocytes which had been treated with anti-Thy 1.2 antiserum plus complement to lyse the T-cells and group 4 mice were given 1.0×10^8 enriched T-lymphocytes obtained from normal saline treated mice.

Approximately 20 hours after lymphocytes transfer, 5 mice from each group were selected randomly and footpad tested to determine DTH responsiveness to CneF antigen.

Serum transfer. Immune and normal sera obtained earlier from sensitized and non-sensitized mice were used for transfer to recipient mice. Two groups of mice were intravenously injected with 1.0×10^4 viable C. neoformans cells, 2 hours prior to the injection of serum. One group received intravenously 0.5 ml of serum from non-sensitized mice; whereas the other group received 0.5 ml of serum from sensitized

mice. The same volume of the appropriate serum was administered again on days 3, 6 and 10 post challenge to those mice which had not been sacrified during the previous autopsy period. Twenty hours after the first serum transfer, 5 mice from each group were footpad tested with CneF antigen.

Viability counts for C. neoformans. One day after the injection of lymphocytes or serum, 3 mice from each group were sacrified, and organs were collected. The numbers of viable yeast cells in the spleens, livers, lungs and brains of each mouse were determined according to procedure described by Cauley and Murphy (7). Similarly autopsies were done on days 3, 7, 10 and 14 post passive transfer of lymphocytes and on days 7 and 14 post serum transfer.

Macrophage migration inhibition test. The macrophage migration inhibition test was used to demonstrate the specificity of sensitized lymphocytes for C. neoformans. Mice were either sensitized with 10^3 viable C. neoformans intranasally, with 0.5% 2,4-dinitro-1-fluorobenzene (DNFB) (Sigma Chemical Co.) according to procedure described by Phanuphak et al (30) or with 0.5 ml of complete Freund's adjuvant (CFA) (Difco) subcutaneously on the back and on the left and right side of the abdominal. One week after receiving 0.5 ml of CFA, mice were given another 0.1 ml of CFA intraperitoneally. Mice that

received only sterile saline were used as controls.

Thirty-five days after C. neoformans immunization, 4 days after the last skin painting with DNFB or 7 days after the last injection of CFA, 4 mice from each group were sacrificed. Spleens from these mice were removed and enriched populations of T-cells were obtained. The enriched T-cells were adjusted to a concentration of about 5.0×10^6 cells/ml in tissue culture Medium 199 containing 27 mM sodium bicarbonate supplemented with 5% FCS and containing 50 units of sodium penicillin G and 50 ug of streptomycin per ml. Enriched T-lymphocytes from C. neoformans sensitized mice were cultured in eight 16 x 125 mm plastic tissue culture tubes (Falcon Plastic) by adding 2.0 ml of the enriched T-cell suspension into each tube. A 0.1 ml volume of predetermined optimal dose of CneF antigen having a concentration of 100 ug of protein was added to 2 of the 8 tubes. Another two tubes were added with 0.1 ml of a 0.05% DNFB in saline. Finally, to the remaining 4 tubes of T-cell suspensions, 2 tubes were added with 0.1 ml of 25 ug/ml of purified protein derivative (PPD) (Parke Davis & Co., Detroit, Mi.) and the other 2 tubes were added with 0.1 ml sterile saline. The final volume in each tube was adjusted to 2.5 ml by adding o.1 ml of 3.0×10^4 cells/ml of light mineral oil stimulated peritoneal exudate cells and more tissue culture Medium 199. Similar procedure was also carried out with enriched T-cells from CFA and DNFB sensitized mice. The tubes were allowed to incubate for 48

hours at 37C under 5% CO_2 -95% air. Supernatants were collected, and migration inhibition factor (MIF) activity was determined according to procedure described by Harrington and Stastny (18) using light mineral oil stimulated macrophages from normal mice.

Statistical analyses. The mean, standard error and unpaired t-tests programmed on a Hewlett packard model 9810A calculator were used in analyses of the data.

RESULTS

DTH responses. Footpad responses of donor mice and lymphoid cell recipient mice are shown in Figure 1. Donor mice immunized intranasally with viable C. neoformans 7 days earlier showed negative DTH responses to CneF antigen; however, by day 35 post infection, donor mice demonstrated strong DTH responses to CneF antigen. Recipient mice receiving either sensitized, non-sensitized, or anti-Thy 1.2 antibody treated sensitized enriched T-cells from day 7 donor mice did not have positive responses to CneF antigen when footpad tested 20 hours after cell transfer. However, mice receiving sensitized enriched T-cells from day 35 donors showed positive DTH responses; whereas the other groups of mice receiving day 35 non-sensitized or anti-Thy 1.2 treated day 35 sensitized T-cells did not develop DTH responses to CneF antigen.

Effect of cell transfer. On days 1, 3, 7, 10 and 14 following intravenous challenge with 1.0×10^4 viable C. neoformans and transfer of sensitized, non-sensitized, anti-Thy 1.2 antibody treated sensitized lymphocytes or saline injection, 3 mice from each of the 4 groups were autopsied to determine

the C. neoformans cell numbers in various organ tissues. There were no significant differences (throughout the autopsy period) in the number of C. neoformans CFU among the infected organ tissues of the 3 groups of days 7 donor cells recipients and the group 1 challenge-control that received C. neoformans and saline. By day 14, the number of mean C. neoformans CFU in group 1 challenge-control, group 2 of the days 7 sensitized T-cells recipients, group 3 of the anti-Thy 1.2 treated sensitized T-cells recipients and in group 4 of the normal T-cells recipients were 1.717×10^7, 1.565×10^7, 1.781×10^7 and 1.776×10^7 respectively. Although there were no significant differences on day 1 in the mean numbers of C. neoformans CFU among the 3 groups of day 35 donor cells recipients and the group 1 challenge-control that received only C. neoformans and saline, by day 3, group 2 mice that received sensitized enriched T-cells began to show a slower increase in organism counts with a mean CFU per mouse of about 4.0×10^3; whereas, the other 3 groups showed a greater increase in C. neoformans cells with an average of 3.0×10^4 CFU per mouse. On day 7, 10 and 14 recipient mice in groups 1, 3 and 4 showed a rapid increase in mean C. neoformans CFU, but group 2 recipient mice continued to have a slower rate of increase in C. neoformans cells in tissues. By day 14, the mean CFU for group 2 mice was 1.06×10^5, and the mean CFU for group 1, 3 and 4 were 4.39×10^6, 4.70×10^6 and

4.62×10^6 respectively. The differences in mean CFU between group 2 recipient mice and the other 3 groups of recipient mice beginning from day 3 through day 14 were highly significant as indicated by the unpaired student's t-test ($P < 0.005$).

Effects of serum transfer. Two hours after intravenous challenge with approximately 1.0×10^4 viable C. neoformans, mice were each given 0.5 ml of pooled serum obtained earlier from immunized or saline control donor mice. On day 1, 7 and 14 after challenge and serum injection, three mice from each group were randomly picked for autopsy and determination of numbers of C. neoformans cells in tissues. On day 3, day 6, and again on day 10, those mice that had not been autopsied, were given another 0.5 ml of "immune" donor serum or saline control donor serum. No differences were observed in the organism growth profiles obtained from mice receiving day 7 sensitized donor serum or day 7 saline control mice serum (Figure 3). Similar observation was also found between mice that were given day 35 immunized donor serum and mice that received day 35 saline control donor serum (Figure 4). In both cases, C. neoformans continued to increase throughout the study even though the recipient mice were being given "immune" serum injections. The mean numbers of CFU were not significantly different between the test and the control groups of mice at any time interval.

Migration inhibition test. The degree of T-lymphocyte specificity in mice immunized with C. neoformans was determined by using 2 unrelated antigens, PPD and DNFB in a migration inhibition assay. Results of this study are presented in Table 1. Sensitized lymphocytes obtained from C. neoformans, CFA and DNFB immunized mice did not produced MIF when cultured in the presence of heterologous antigen. However, when homologous antigen was added to the lymphocyte cultures, MIF was produced as measured by the assay. In other words, PPD and DNFB did not stimulate C. neoformans sensitized lymphocytes to produce MIF; however, the CneF antigen did stimulate a factor which inhibited migration of macrophages, thus showing specificity of C. neoformans sensitized T-cells to CneF antigen ($P < 0.025$).

Figure 1. Footpads delayed hypersensitivity responses of donor mice and their lymphoid cell recipients to Cnef antigen. Group 1 received 1.0×10^4 viable C. neoformans. Group 2 received 1.0×10^4 viable C. neoformans + 1.0×10^8 day 7 or day 35 enriched T-cells from sensitized donor mice. Group 3 received 1.0×10^4 viable C. neoformans + 1.0×10^8 anti-theta and guinea pig complement treated day 7 or day 35 sensitized enriched T-cells. Group 4 received 1.0×10^4 viable C. neoformans + 1.0×10^8 normal enriched T-cells. I indicates immunized mice, C indicates saline control mice, IS indicates mice received serum from immunized donor mice and NS indicates mice received normal serum from saline control donor.

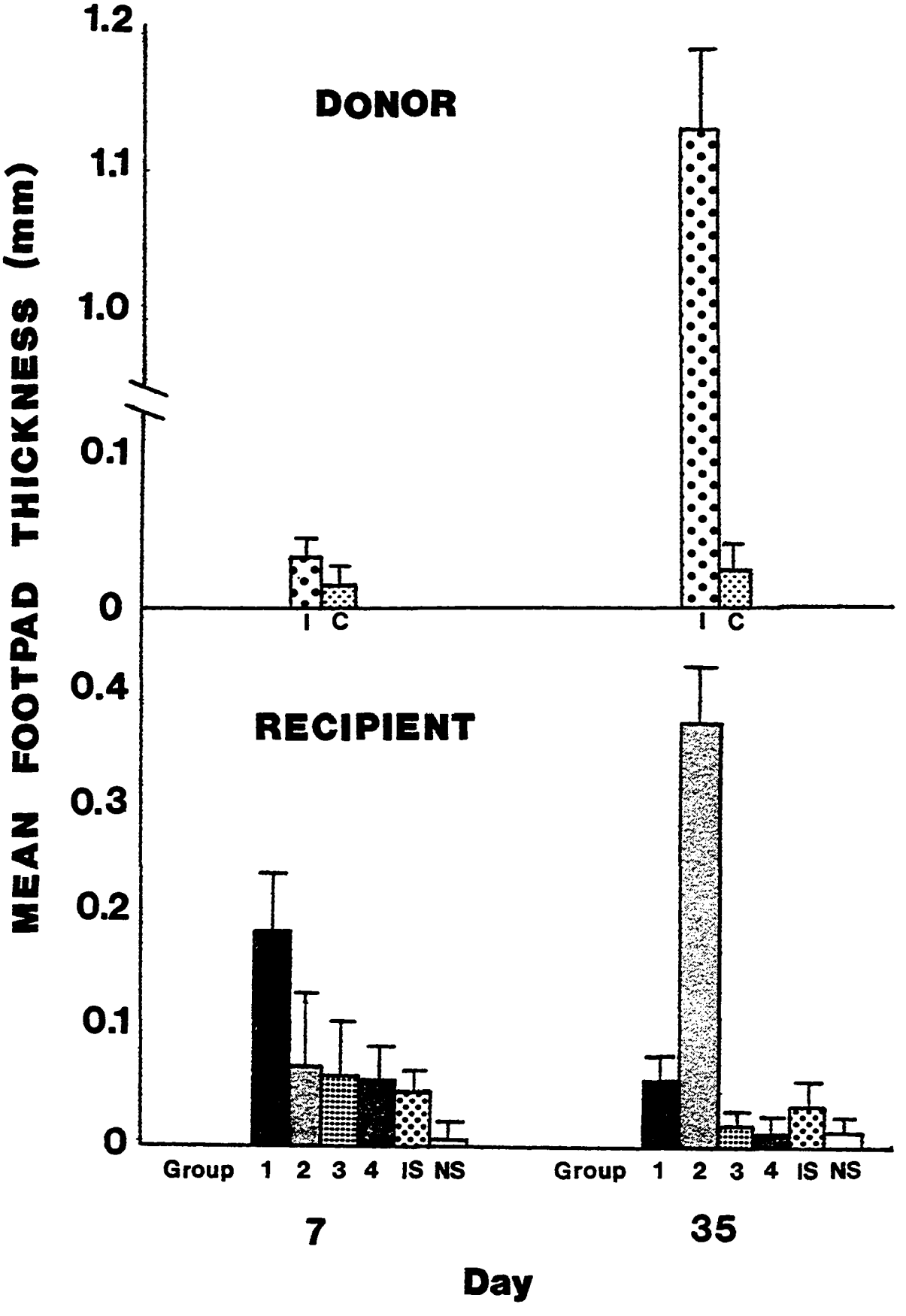
MEAN FOOTPAD THICKNESS (mm)
1.2
1.1
1.0
0.1
0
DONOR
I C
I C
0.4
0.3
0.2
0.1
0
RECIPIENT
Group 1 2 3 4 IS NS
Group 1 2 3 4 IS NS
7
35
Day

Figure 2. Organism growth profile of day 7 and day 35 recipient mice. Group 1 received IV 1.0 x 10^4 viable C. neoformans (▲--▲), group 2 received 1.0 x 10^4 viable C. neoformans + 1.0 x 10^8 day 7 or day 35 enriched T-cell from sensitized donor (o---o), group 3 received 1.0 x 10^4 viable C. neoformans + 1.0 x 10^8 anti-theta plus guinea pig complement treated day 7 or day 35 sensitized splenic T-cells (●--●) and group 4 received 1.0 x 10^4 viable C. neoformans + 1.0 x 10^8 normal splenic T-cells from day 7 or day 35 saline control donor (△—△).

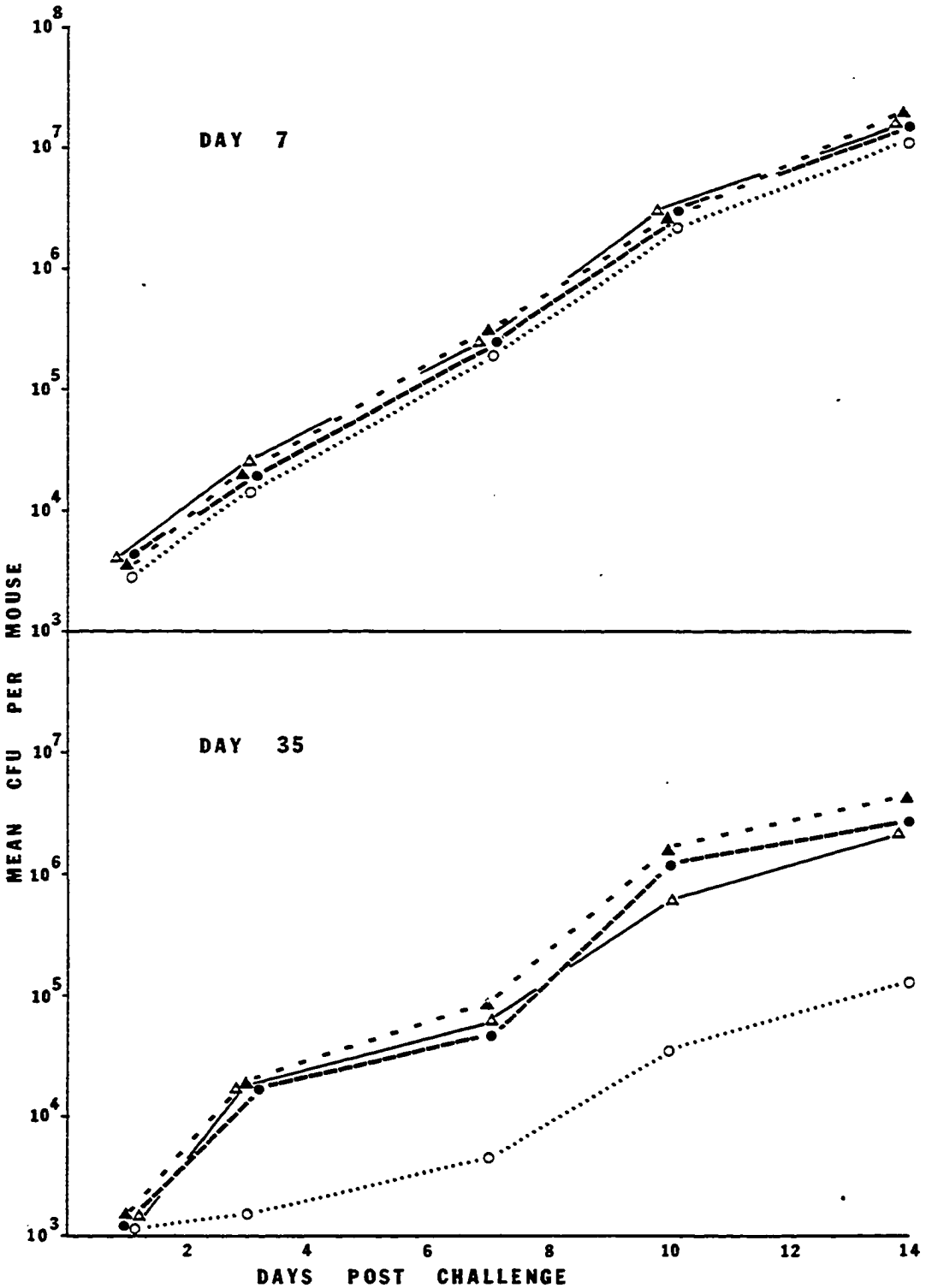
DAY 7
DAY 35
MEAN CFU PER MOUSE
10^8
10^7
10^6
10^5
10^4
10^3
2
4
6
8
10
12
14
DAYS POST CHALLENGE

Figure 3. Organism growth profile of mice receiving serum from donor mice inoculated intranasally 7 days earlier with 10^3 C. neoformans (□--□) or with sterile saline (○—○). Arrow indicates time when serum was injected.

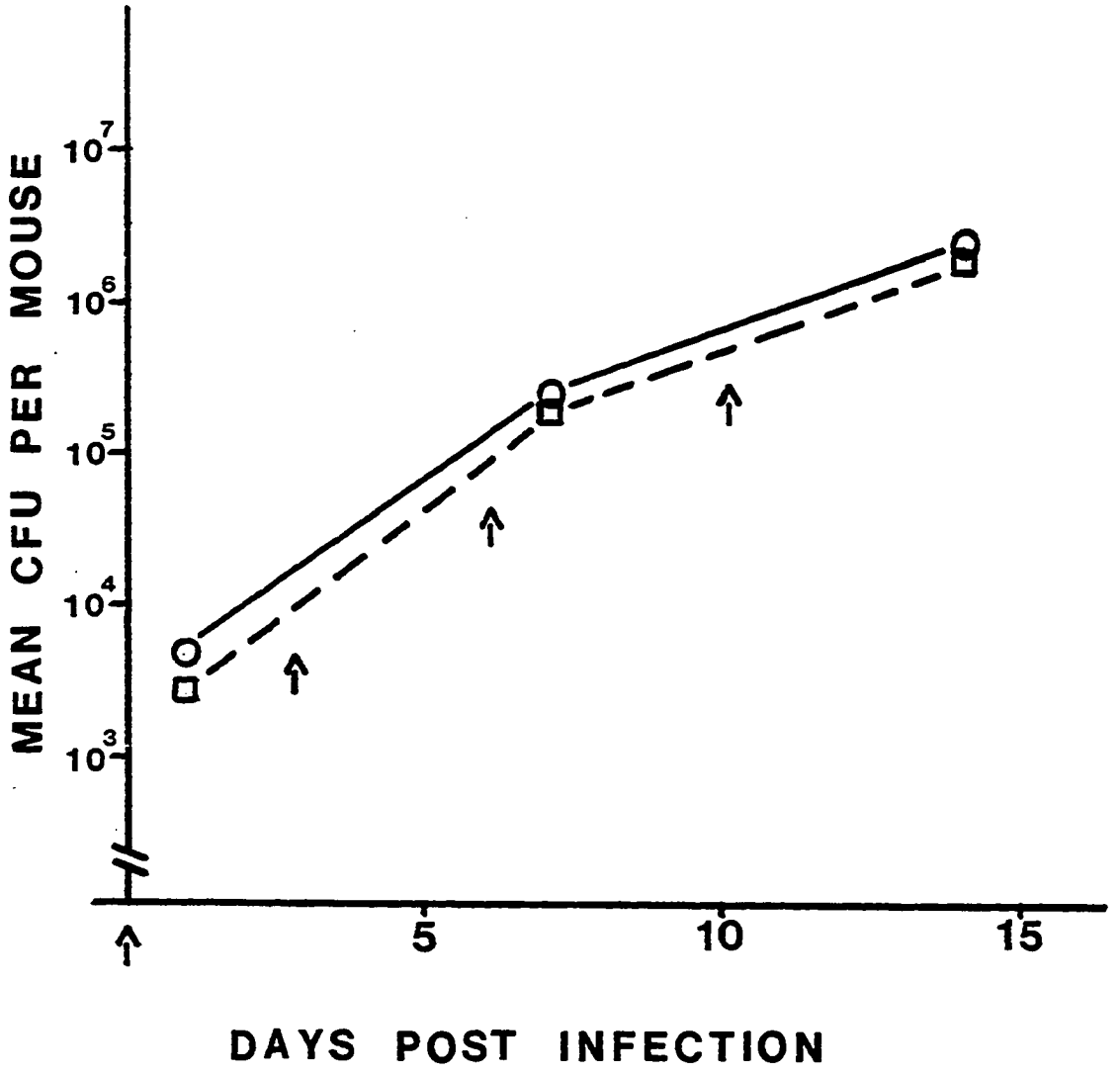
MEAN CFU PER MOUSE
10^7
10^6
10^5
10^4
10^3
5
10
15
DAYS POST INFECTION

Figure 4. Organism growth profile of mice receiving serum from donor mice inoculated intranasally 35 days earlier with 10^3 C. neoformans (□--□) or with sterile saline (o—o). Arrow indicates time when serum was injected.

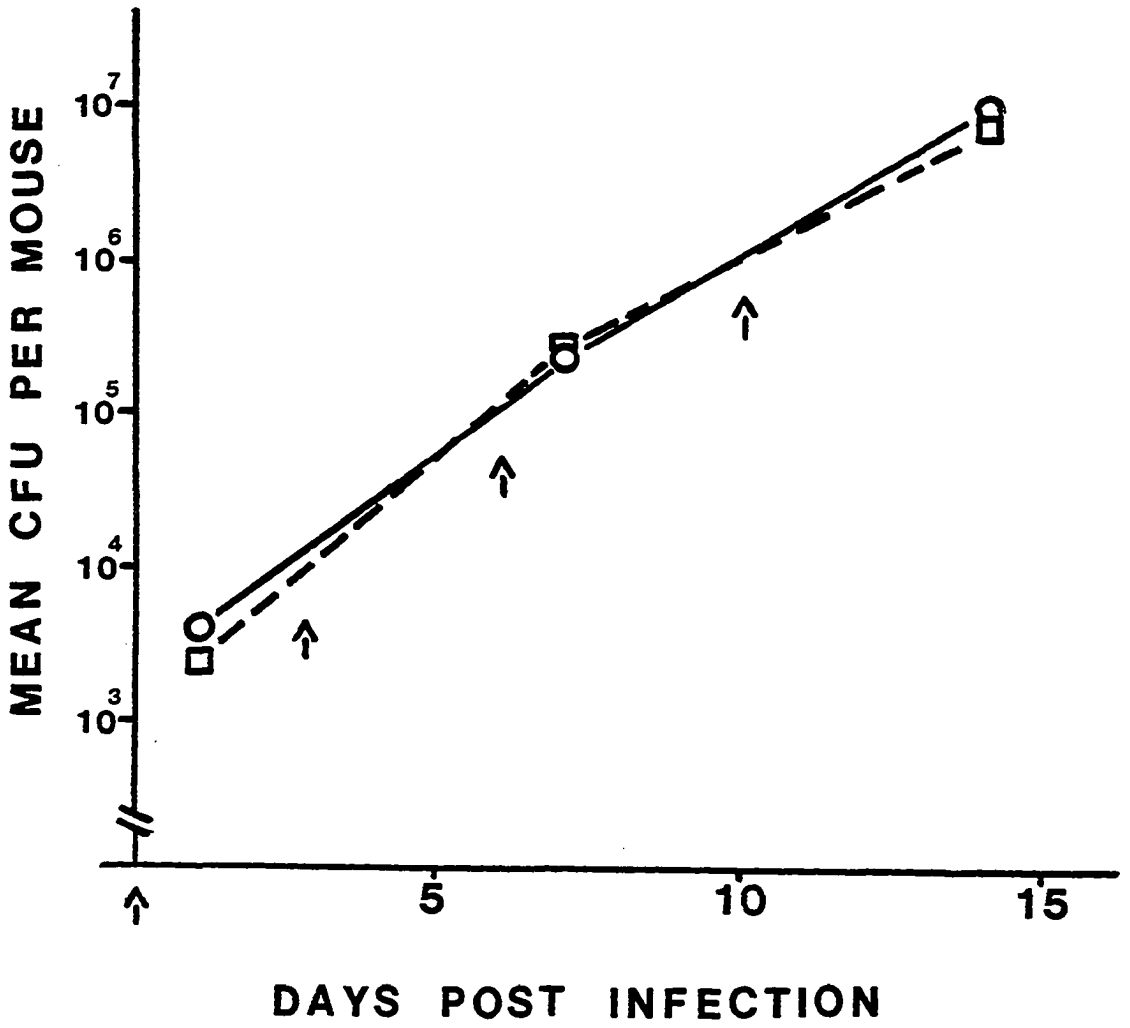
MEAN CFU PER MOUSE
10^7
10^6
10^5
10^4
10^3
5
10
15
DAYS POST INFECTION

Table 1. Specificity of MIF production by C. neoformans, CFA and DNFB sensitized lymphocytes upon stimulation with antigen.

NUMBER OF IMMUNE MICE	IMMUNIZING ANTIGEN	MIGRATION DISTANCE				MIGRATION RATIO**		
		ANTIGEN IN ASSAY MEDIUM						
		CneF	DNFB	PPD	SALINE	CneF:SAL	DNFB:SAL	PPD:SAL
4	Cryptococcus neoformans	21.50 17.00 (19.25)	40.25 37.75 (39.00)	37.00 39.25 (38.125)	42.00 45.00 (43.50)	0.4425	0.8965	0.8764
4	DNFB	46.00 39.00 (42.50)	28.75 24.25 (26.50)	41.25 38.00 (39.625)	46.00 50.75 (48.375)	0.8785	0.5478	0.8191
4	CFA	38.75 41.25 (40.00)	34.50 39.25 (36.875)	24.25 21.00 (22.625)	40.25 43.25 (41.75)	0.9580	0.8832	0.5419
4	SALINE	44.25 47.25 (45.75)	43.00 45.50 (44.25	42.75 50.00 (46.375)	45.75 47.50 (46.625)	0.9812	0.9490	0.9946

** The migration ratio was calculated by dividing the migration distance of PEC assayed in CneF, DNFB or CFA medium by distance in saline medium. () The mean PEC migration distance of a duplicated MIF assay in media containing either homologous or heterologous antigen.

DISCUSSION

In this study of experimental cryptococcosis, we have shown that adoptive immunity to Cryptococcus neoformans can be conferred upon syngeneic naive recipients by live T-lymphocytes obtained from mice actively immunized with viable C. neoformans. However, the ability of these lymphoid cells to confer immunity appeared to be time related. Dykstra and Friedman (12) pointed out in their study that immunity to C. neoformans was observed only if the mice were challenged with 10^2 viable C. neoformans at least 3 weeks after they received subcutaneous immunization. In our study enriched splenic T-cells isolated from mice immunized intranasally 7 days earlier with 10^3 viable C. neoformans failed to confer immunity to recipient mice challenged intravenously with approximately 10^4 viable C. neoformans. Organism growth profiles on this group of recipient mice and 2 other groups of recipients which were given either anti-Thy 1.2 treated sensitized enriched T-cells or normal enriched T-cells did not show any significant differences from the control group which was intravenously given 10^4 viable C. neoformans only. On the other

hand, when enriched splenic T-cells were obtained from donor mice immunized 35 days earlier with 10^3 viable C. neoformans and transferred to naive recipients, immunity was conferred on the recipient mice. However, if the lymphoid cells were first treated with anti-Thy 1.2 antibody plus complement to eliminate the T-lymphocytes before transfer to the recipient mice, no immunity was conferred on the recipients. These results indicated that sensitized T-cells were responsible for controlling the numbers of C. neoformans cells. Similar role for T-cells was also observed in murine coccidioidomycosis (4). Furthermore, the inability of serum from C. neoformans immunized donor mice to confer immunity on recipient mice further confirmed the role of T-cells in the host defense against a cryptococcal infection. This absence of a protective role on the immune serum in disseminated cryptococcosis was in contrast to the role of humoral antibody in systemic candidiasis where antibody appeared to play an important role in the host defense (29).

It is also of interest to note that in murine cryptococcosis there was only a 2 to 3 days latent period between cell transfer and the expression of immunity in the recipient mice. This was in contrast to the adoptive immunity to tuberculosis studies where the latent period was more than 7 days (22,23). It has also been shown that immunity to tuberculosis required both the small and large lymphocytes (24) and that

lymphocytes which confer immunity may differ from cells that transfer delayed hypersensitivity (13,21). Whether such features will be seen also in the immune responses of murine cryptococcosis are not known. In our opinion, we have undoubtedly demonstrated the importance of T-lymphocyte in host defense against a cryptococcal infection and that the DTH response was associated with immunity to cryptococcosis in donor (T.S. Lim, Ph.D. Dissertation., U. of Oklahoma, Norman. 1979.) and recipient mice. Furthermore, in our murine cryptococcosis study, the MIF assay performed using our cryptococcal cultured filtrate antigen (7,27) and two other unrelated antigens, PPD and DNFB, demonstrated that T-lymphocytes from C. neoformans immunized mice were highly specific for CneF antigen.

Our study demonstrated that the enriched population of splenic T-cells obtained from C. neoformans sensitized mice were able to transfer delayed hypersensitivity and anti-cryptococcal immunity as well as showing specificity to CneF antigen in MIF production. Nevertheless, we are not certain if the population of T-cells responsible for any of these three responses indeed belong to the same subpopulation or to different subpopulations of T-cells. Studies on other infectious disease, such as listeriosis, have failed to demonstrate any parallel between the degree of hypersensitivity and resistance to infection (17,28). Therefore, in

murine cryptococcosis, further study is needed to resolve this question of direct association between DTH and cryptococcal immunity.

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