THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

IMMUNOLOGICAL UNRESPONSIVENESS INDUCED BY CAPSULAR POLYSACCHARIDE OF *CRYPTOCOCCUS NEOFORMANS*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

JUNEANN W. MURPHY

Norman, Oklahoma

1969

IMMUNOLOGICAL UNRESPONSIVENESS INDUCED

BY CAPSULAR POLYSACCHARIDE OF

CRYPTOCOCCUS NEOFORMANS

APPROVED BY

Gary C. Cozad

R B Clark

Howard K. Lamb

Glenn H. Ulender

John A. Lancaster

DISSERTATION COMMITTEE

ACKNOWLEDGMENT

I greatly appreciate the guidance, suggestions, and stimulating discussions provided throughout this study by Dr. George C. Cozad.

For contributing helpful suggestions in the preparation of this manuscript and for serving on my dissertation committee, I wish to thank Drs. Howard W. Larsh, J. Bennett Clark, John H. Lancaster, and Simon H. Wender.

Also I am grateful to Dr. David M. Adamson, Suhaila Al-Naib, Larry Garrett, and Don Wiggins who assisted in this work.

Finally I wish to express a very special thank you to my husband for his cooperation, help, and encouragement and to my daughters for their sacrifices.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

IMMUNOLOGICAL UNRESPONSIVENESS INDUCED BY CAPSULAR POLYSACCHARIDE OF *CRYPTOCOCCUS NEOFORMANS*

CHAPTER I

INTRODUCTION

High levels of capsular polysaccharide of *Cryptococcus neoformans* have been demonstrated in the body fluids of patients with meningeal and disseminated cryptococcosis (5, 7, 8, 30). The polysaccharide levels decline during the recovery phase of the disease, and circulating antibodies appear. Transitory antibody titers have been reported by Gordon and Vedder (18) in some patients in early stages of the disease. Polysaccharide titers seem to reflect the severity of the disease state; the presence of antibodies indicates a favorable prognosis (18). A number of studies have been done with polysaccharide preparations isolated from *C. neoformans*; however they have not provided sufficient information to determine the exact role that the capsular polysaccharide plays in the disease process of cryptococcosis.

The antigenic characteristics of the capsular material of C. neoformans have been studied by various workers. Kligman (26) failed to induce precipitins in rabbits by injecting crude capsular polysaccharide of C. neoformans. Neither agglutinins nor precipitins could be demonstrated in rabbits given purified polysaccharide, intravenously (33). However, in a more recent study Al-Naib (4) has shown a transient agglutinin response in rabbits injected intraperitoneally with 5000 µg or 0.005 µg of purified polysaccharide. Pretreatment of rabbits with cryptococcal polysaccharide greatly decreased the agglutinin titers produced in response to formalin-killed C. neoformans cells (33). Unsuccessful attempts have also been made to demonstrate humoral antibodies in mice injected with 5, 25, or 100 µg of polysaccharide in complete Freund's adjuvant (20). When polysaccharide conjugated to bovine gamma globulin was used as the immunogen, agglutinins specific for the polysaccharide could be demonstrated (19).

Further information about the antigenic nature of the capsular polysaccharide of C. neoformans can be obtained from protection studies which have been done using the polysaccharide or immunogens containing polysaccharide. Gadebusch (16) showed that the subcutaneous administration of varying amounts of purified capsular polysaccharide, given 14 days before a virulent challenge dose of C. neoformans, failed to protect mice. In fact, the

polysaccharide at the highest concentrations made the animals more susceptible to challenge. Gadebusch therefore suggested that the high doses of polysaccharide had induced immunological "paralysis." Louria (28) demonstrated a similar effect when he immunized mice with heat killed, strongly encapsulated C. neoformans cells, and found that after lethal challenge, they died somewhat earlier than controls. Mice immunized with a weakly encapsulated strain were slightly protected against challenge. Abrahams and Gilleran (2) reported that immunization of mice with large doses of formalin-killed cells (6×10^8 cells/injection) induced a "paralyzing" effect which they suggested to be due to an increase in amount of capsular material present in the vaccine. Gadebusch (16) showed mice could be protected when injected with a polysaccharide-resin complex; however, Goren (19) failed to protect mice by immunizing with polysaccharide conjugated to bovine gamma globulin. Using Bordetella pertussis as an adjuvant with killed cryptococcal cells as a vaccine, Abrahams (1) demonstrated a 95 to 100% survival rate in mice after challenge.

Since the demonstration of antibodies or protection in animals after treatment with C. neoformans capsular polysaccharide or polysaccharide-containing-antigens has been somewhat inconsistent, it seems important at this time to attempt to explain this variation. All of the previously cited results indicate that the polysaccharide is capable

of stimulating an immunological unresponsive state when given in sufficient quantities and an immune state when given in very small quantities or with a material which increases its adjuvanticity. The effects that cryptococcal polysaccharide have on the immunological response in animals is reminiscent of those effects produced by pneumococcal polysaccharides. Felton (15) noted that mice treated with larger than immunizing amounts of pneumococcal polysaccharide could not survive subsequent challenge with the homologous organism, and he called this phenomenon immunological paralysis. Currently, it is generally agreed that the terms immunological tolerance, immunological paralysis, immunological unresponsiveness, and antigen overloading can be used interchangeably, since all of these phenomena represent aspects of the same basic biological mechanisms (10, 11, 27). An operational definition of tolerance used in a review by Leskowitz (27) is: "tolerance represents a state of specific immunologic nonreactivity to an antigenic stimulus which, in normal animals, would be followed by a recognizable and measurable response."

It seems to be agreed by most reviewers (11, 21, 27, 36) that the bulk of evidence supports "central" failure of the immunologic apparatus as the mechanism for immunological unresponsiveness. Dresser and Mitchison (11) explain "central" failure as an alteration of the properties of immunologically competent cells in such a way that a

restricted range of reactivity is deleted. Paralysis resulting from neutralization of antibody has been suggested from some studies using pneumococcal polysaccharide (11). This is the strongest evidence provided which supports a mechanism of paralysis other than "central" failure.

The possibility that cryptococcal polysaccharide can induce immunological unresponsiveness has been suggested by Gadebusch (16), Salvin (34), Abrahams and Gilleran (2), and Bennett and Hasenclever (6). Some workers have attributed the reduction of antibody titers (33), the failure to demonstrate antibodies (40), and the lack of protection (17) in animals after subjection to cryptococcal polysaccharide to be due to antibody neutralization by excess antigen. There is a lack of understanding of how the animal's immunological system responds to cryptococcal polysaccharide; therefore the purposes of this study were to determine whether or not cryptococcal polysaccharide could induce tolerance in accordance with Leskowitz's operational definition; and if so to gain further knowledge concerning the mechanism by which the state of immunological unresponsiveness was established.

CHAPTER II

MATERIALS AND METHODS

Experimental animals. Eight to 10 week old New Zealand white rabbits weighing 1.2-2.4 kg were used in studying the immunological responses to varying concentrations of capsular polysaccharide.

Male and female 6-8 week old Swiss white hybrid mice from a colony maintained in our laboratory were used in LD_{50} and infection studies. For hemclytic plaque studies, 10 week old male CBA/J inbred mice (Jackson Laboratory, Bar Harbor, Maine) were used.

Organism. Cryptococcus neoformans isolate 184 used throughout this work is a weakly encapsulated form which was originally isolated in 1958 from lungs of a patient at Charity Hospital, New Orleans, Louisiana. It was obtained in 1961 from Dr. Lorraine Friedman and has been maintained in our laboratory since that time on Sabouraud's glucose agar. Antiserum prepared against strain 184 formed precipitin bands on double diffusion agar plates with Type A polysaccharide but not with Types B or C; therefore 184 was considered to be a Type A C. neoformans.

Determination of LD_{50} and infection of mice. The organism was grown in flasks of neopeptone-dextrose dialysate broth (12) at 30 C with constant shaking for 3 days. Hemocytometer counts were made, and appropriate suspensions were prepared in sterile physiological saline (SPS). Spread plates were made to determine the number of viable cells. The 24 day LD_{50} was determined by injecting 100 mice with varying numbers of viable C. neoformans cells. Each mouse was injected with 0.1 ml of the appropriate cell concentration and the number of survival days was noted. Calculation of the LD_{50} was done according to the method of Reed and Muench (32).

Preparation of formalin-killed C. neoformans cells. After growing 48 hr at 37 C on slants of medium prepared with 20 g agar, 10 g neopeptone, 10 g glucose, 2 g yeast extract, and 5 g NaCl in 1 l distilled water, cells of C. neoformans were suspended in 0.85% NaCl containing 2% formalin and were stored for 24 hr at 25 C. The cells were washed once in SPS and heated for 30 min at 56 C. Following a second washing, the cells were suspended in SPS containing 0.5% formalin and were stored at 4 C. Before using the cells were washed three additional times with SPS. Cells prepared in this manner were used in immunization of rabbits and in microagglutination tests.

Isolation and purification of polysaccharide. Crude polysaccharide was prepared by growing C. neoformans on

neopeptone-dextrose dialysate broth (12) at 30 C for 3 days and by employing the methods of Evans and Theriault (13). Purification techniques of Gadebusch (16) were used. The dried polysaccharide was stored in a desiccator at room temperature. For use the appropriate quantity of polysaccharide was dissolved in SPS and centrifuged at 27,750 x g for 20 min. The upper 3/4 of the solution was used in preparing the desired concentrations of polysaccharide.

Preparation of polysaccharide-incomplete Freund's adjuvant emulsion. For challenge immunization of mice an incomplete Fruend's adjuvant-polysaccharide emulsion was prepared by continuous grinding and drop-wise adding of 1 volume of polysaccharide solution (2.5 µg polysaccharide/ml) into a mortar containing 1 volume of a mixture of 5 parts Marcole 52 (Humble Oil and Refining Co.) and 1 part Arlacel A (Hill Top Research, Inc., Miamiville, Ohio). After all the polysaccharide had been added and the mixture appeared white, further emulsification was carried out by passing the material through an 18 gauge needle until the emulsion formed discrete and stable drops on the surface of water. For challenge immunization each mouse was injected intraperitoneally (ip) with 0.1 ml of the emulsion. This amount contained 0.125 µg of polysaccharide.

Preparation of antigen for sensitization of sheep red blood cells (SRBC). After growing C. neoformans 184 in neopeptone dialysate broth for 3 days at 30 C, formalin was

added to give a 2% final concentration, and the culture was allowed to remain at room temperature for 18 hr. The cells were removed by centrifugation and the supernatant was brought to pH 7.0 using 0.1 N NaOH. The cell free extract was then dialysed against 4 changes of pH 7.0 saline and stored at -20 C until used. This antigen, referred to from here on as HA antigen, was used for sensitization of sheep erythrocytes as well as a skin test antigen.

Sensitization of SRBC. Two tenths ml of washed, packed SRBC was suspended in 10 ml of phosphate buffered saline pH 7.2 (PBS), and 0.2 ml of HA antigen was added to the cell suspension. The mixture was incubated at 37 C for 45 min, then the sensitized cells (SRBC-HA) were washed 3 times in PBS. Sheep erythrocytes sensitized in this manner were used in microhemagglutination tests, microhemolysis tests, and hemolytic plaque assays.

Preparation of antiserum. Five New Zealand white rabbits were hyperimmunized by giving each rabbit intravenously (iv) 1 ml containing 5×10^8 formalin-killed cryptococcal cells every other day for a total of 7 injections. After 7 days the rabbits were given another series of 7 injections of formalin-killed cells. Bleeding was done from the central ear artery 7 days after the last injection. Sera were collected and stored at -20 C in 1 ml volumes until used. All 5 rabbits were skin tested with 0.1 ml of HA antigen the day after they were bled.

Serological procedures.

1. Agar gel double diffusion plate. The agar gel diffusion methods of Ouchterlony as modified by Heiner (22) were employed. Instead of ordinary agar, purified agar (Difco) was used. Capsular polysaccharide at a concentration of 5000 µg/ml was placed in the center well, and six test sera were put into the surrounding wells. A positive control serum was included each time the test was run. All rabbit serum samples collected were checked for precipitins in this manner.

2. Microagglutination test. The Microtiter technique using lucite V-shaped bottom Microtiter plates (Cooke Engineering Co.) was employed for determining agglutinin titers in rabbit and mouse sera. For titrating rabbit serum 0.85% NaCl was used as the diluent, and for mouse serum PBS was used as the diluting fluid. All solutions used in Microtitre tests were made with distilled, deionized water. The microagglutination test was performed by making serial two-fold dilutions of serum so that each well in the Microtiter plate contained 0.025 ml of diluted serum. To each serum dilution was added 0.025 ml of a suspension of washed formalin-killed C. neoformans cells which had been diluted to give 60% transmission using 1/2" x 4" tubes in a Spectronic "20" (Bausch and Lomb) spectrophotometer set at 500 mµ. The Microtiter plates were sealed and incubated for 2.5 hr about 18" from a desk lamp before being read for

agglutination. The highest dilution of serum in which there was not a smooth white button of cells in the well apex was taken as the titer.

3. Microhemagglutination test. For the microhemagglutination test to detect antibodies against C. neoformans, disposable, non-flexible, "V" shaped bottom Microtiter plates were used. Serum was diluted as for microagglutination tests; however the diluent was 1% normal rabbit serum in saline in this procedure. All test sera and serum used in the diluent had been heat inactivated at 56 C for 30 min and had been adsorbed with 0.15 ml of packed SRBC per ml of serum for 30 min at room temperature. To each serum dilution was added 0.025 ml of a 0.5% suspension of SRBC-HA made up in 1% normal rabbit serum in saline. Plates were sealed and incubated in a 37 C incubator for 1 hr, followed by 18 hr at 4 C. The titer was considered to be the highest serum dilution in which there was not a smooth button formed in the well apex. Each time microhemagglutination tests were run a diluent control, a normal serum control, and a positive serum control were included.

4. Microhemolysis test. This test was employed to see if SRBC-HA would lyse in the presence of specific antiserum and complement, for this is a prerequisite for using the sensitized cells in the hemolytic plaque technique. Heat inactivated, SRBC adsorbed serum was diluted in twofold manner in veronal buffered saline pH 7.5 (VBS) in

Microtiter, "U" shaped bottom, disposable, non-flexible plates. To the serum dilutions was added 0.025 ml of 0.5% suspension of SRBC-HA in VBS. After the plates had incubated at 37 C for 1 hr, 0.025 ml of a 1:10 dilution of guinea pig complement in VBS was added to each well. The plates were gently shaken to make sure all cells were resuspended and then incubated an additional hour at 37 C with occasional shaking during the first 30 min of this incubation period. After an overnight storage at 4 C, the plates were read. The microhemolysis titer was the highest serum dilution in which there was hemolysis. The following controls were run:

VBS + SRBC-HA + Complement

Test Serum + SRBC-HA + VBS

Normal Serum + SRBC-HA + Complement

Positive Serum + SRBC-HA + Complement

To determine whether or not a serum sample was anti-complementary, the serum was added to a SRBC-hemolysis system before the final incubation with complement. If hemolysis was inhibited by the serum then it was considered to be anti-complementary.

5. Adsorption and inhibition procedures. To determine if the microhemagglutination and the microhemolysis tests were specific for antibodies against cryptococcal antigens the following procedures were employed:

a. Adsorption of antiserum with formalin-killed C. neoformans cells. To 0.2 ml of thrice washed, packed C. neoformans cells was added 1 ml of titered test antiserum. After thorough mixing the suspension was incubated at room temperature for 30 min, then centrifuged, and the serum was collected. The adsorbed serum was retitered by the microhemagglutination and microhemolysis techniques.

b. Inhibition of hemagglutination and hemolysis with purified polysaccharide. The antiserum was diluted as for microhemagglutination or microhemolysis tests; however before the addition of SRBC-HA, 0.025 ml of 200 µg/ml concentration of polysaccharide was added to each well. The plates were incubated for 1 hr at 37 C before the remainder of the respective test was performed.

Hemolytic plaque technique. The procedure used to detect and enumerate plaque forming cells in the mouse spleen which were specific for cryptococcal antigen was primarily that of Jerne, Nordin, and Henry (25). Spleen cell suspensions were made by pressing the freshly excised spleen through a stainless steel fine mesh sieve with 5 ml of cold Spinner base Minimal Eagle's Medium (MEM) (Schwarz Bio-Research, Orangeburg, N.Y.). Five-tenths ml of the spleen cell suspension and 0.1 ml of a 20% suspension of SRBC-HA were added to 2 ml of 0.75% agar in MEM containing 1 mg of diethylaminoethyl dextran (DEAE dextran) (Pharmacia, Uppsala, Sweden). The suspension was mixed and poured onto

a layer of 10 ml of 1.4% agar in MEM containing 1 mg/ml of DEAE dextran. After 15 min at room temperature the plates were incubated at 37 C for 1 hr. One and one half ml of a 1:5 dilution of guinea pig complement in veronal buffered saline was added to each plate, and the plates were again incubated at 37 C for 30 min. After 2 hr at room temperature the excess complement was removed from the plates, and they were refrigerated at 4 C overnight. Each plate was covered with 3 ml of a hemoglobin stain made by mixing 10 ml of 2% benzidine solution in glacial acetic acid with 0.5 ml of 30% H_2O_2 and 90 ml of distilled water. When the plate had turned blue the stain was decanted, and the plaques were enumerated under a stereoscope using the 0.75 X ocular. Three plates were done per spleen.

Experimental design. To determine what effects C. neoformans polysaccharide had on the immunological response in animals, all the animal studies were designed to initially treat the animals with varying concentrations of polysaccharide, followed 14 days later by challenging the immunological capacity of the animals. The challenge was by one of the following: formalin-killed C. neoformans cells, viable C. neoformans cells, or polysaccharide in incomplete Freund's adjuvant.

1. Immunological unresponsiveness in rabbits. Twenty-one rabbits were divided into 7 groups of 3 rabbits per group, so that the mean body weight of each group was

1.7 ± 0.1 kg. On day 0 each group of animals was given a different concentration of purified polysaccharide or saline ip, and 14 days later all rabbits were challenge immunized with a series of 7 iv injections, each of 6×10^7 formalin-killed cells, given every other day for 14 days. Seven days after the last challenge immunizing injection all rabbits were bled from the central ear artery, and sera were titered by means of the microagglutination test. Table 1 shows the treatment schedule in detail.

2. Immunological unresponsiveness in mice.

a. Challenge with viable C. neoformans cells. Sixty, 6-8 week old female Swiss white mice were used in this study. For each concentration of polysaccharide or saline, 10 mice were used. The polysaccharide or saline was given ip on day 0. All mice were challenged on day 14 with 1 LD_{50} of C. neoformans, iv. The number of days each animal survived after challenge was noted, and the mean life expectancy was determined by summing all survival days of all mice in the group and dividing by the number of mice in that group. The statistical test employed to determine if there was a significant difference in the mean life expectancies of test animals and controls was Student's t test. Polysaccharide concentrations used and further details are listed in Table 2.

b. Challenge with polysaccharide in incomplete Freund's adjuvant. In this study 90, ten week old, CBA/J

Table 1.--Schedule for polysaccharide treatment and challenge immunization of rabbits

Group No.	Weight of rabbit (kg)	Group averag weight (kg)	Polysaccharide given ip (μg/ml)	Challenge Immunization	
				No. cells given iv	Days given[a]
1	1.3 1.8 2.5	1.8	Saline	6×10^7	14,16,18, 20,22,24, and 26
2	1.3 1.6 2.4	1.7	0.005	6×10^7	14,16,18, 20,22,24, and 26
3	1.5 1.7 2.1	1.7	0.05	6×10^7	14,16,18, 20,22,24, and 26
4	1.5 1.7 1.9	1.7	5.0	6×10^7	14,16,18, 20,22,24, and 26
5	1.5 1.6 2.0	1.7	500	6×10^7	14,16,18, 20,22,24, and 26
6	1.5 1.5 2.3	1.8	5,000	6×10^7	14,16,18, 20,22,24, and 26
7	1.6 1.6 1.8	1.7	50,000[b]	6×10^7	14,16,18, 20,22,24, and 26

[a]Indicates day relative to injection of polysaccharide on day 0.

[b]This amount of polysaccharide was contained in 5 ml.

Table 2.--Schedule for polysaccharide treatment and challenge of mice with viable *C. neoformans*

Group No.	Number of mice per group	Polysaccharide given ip (μg/0.1ml)	Challenge Immunization	
			No. viable cells, iv	Day given[a]
10	10	500	1.9×10^5	14
12	10	5	1.9×10^5	14
14	10	0.05	1.9×10^5	14
16	10	0.0005	1.9×10^5	14
18	10	0.00005	1.9×10^5	14
20	10	saline	1.9×10^5	14

[a]Indicates day relative to injection of polysaccharide on day 0.

male mice were used. The mice were divided into 5 groups of 18 mice each. On day 0 the animals in each group were injected ip with one of the following concentrations of polysaccharide: 500 μg, 50 μg, 5 μg, 0.5 μg or saline. On days 4, 7, and 14, three mice from each group were bled by exsanguination. On day 14 all remaining mice in each group were injected ip with 0.1 ml. of polysaccharide in incomplete Freund's adjuvant. Then three mice from each group were bled on days 18, 21, and 28. Hemolytic plaque assays were done on the spleens of each animal bled on days 4, 18, 21, and 28. This complete experiment was repeated on 90 more CBA/J 10 week old male mice, to give a total of 6 mice per concentration group per bleeding time. Table 3 shows in detail the treatment and bleeding schedules.

Table 3.--Schedule for polysaccharide treatment, bleeding, hemolytic plaque assay and challenge immunization of mice

	Polysaccharide given ip (μg/0.1ml)	No. of mice bled for serum titration			Polysaccharide in ICF[a] ip (μg/0.1ml)	No. of mice bled for serum titration		
Day	0	4[b]	7	14	14	18[b]	21[b]	28[b]
	500	3	3	3	0.125	3	3	3
	50	3	3	3	0.125	3	3	3
	5	3	3	3	0.125	3	3	3
	0.5	3	3	3	0.125	3	3	3
	saline	3	3	3	0.125	3	3	3

[a]ICF = Incomplete Freund's Adjuvant

[b]Hemolytic plaque assay were done on all mice bled on these days.

CHAPTER III

RESULTS AND DISCUSSION

Evaluation of serological tests and antigen specificity. To use the hemolytic plaque assay technique to detect and enumerate plaque forming spleen cells which were producing antibodies specific for C. neoformans, a sensitizing antigen had to be produced and a method for coating sheep erythrocytes with the cryptococcal antigen had to be developed. After finding a good sensitizing antigen and devising a method for tagging the SRBC, it was then necessary to find serological procedures whereby one could test, (1) the efficiency of the sensitization process, (2) the specificity of the tagged cells, and (3) the ability of the sensitized cells to lyse in the presence of specific antiserum and complement.

Five rabbits were used to produce high titer cryptococcal antiserum for testing of the sensitized erythrocytes. Preliminary testing of sensitivity and specificity of the SRBC-HA for the cryptococcal system was done with rabbit anti-cryptococcal serum. Larger quantities of serum could be obtained from these animals, thereby making possible

repeated testing of the same sample. The hemagglutination and hemolysis tests were also performed on mouse serum with similar results as with rabbit serum; however titers were lower in mouse sera, and after adsorbing the sera with SRBC, the titers were always reduced 1 to 3 tubes. Table 4 shows a comparison of titers determined by using the microagglutination test, the microhemagglutination test, the microhemolysis test with sera not adsorbed with SRBC, and the microhemolysis test with sera adsorbed with SRBC, plus precipitin results from double diffusion plates. In all cases the hemagglutination titers were lower than the agglutination titers which indicated that the hemagglutination test was not as sensitive as was the agglutination test for rabbit anti-cryptococcal antibodies. The hemagglutination test did not appear to be less sensitive than the agglutination test in the mouse system; however due to an insufficient amount of mouse antiserum this could not be verified through repeated testing of the same serum sample.

In three of the five rabbits the hemolysis titers were higher than either the agglutination or the hemagglutination titers. This increase of titer was probably not due to the hemolysis test being more sensitive but rather that the hemolysis test demonstrated a different population of antibodies than did the agglutination or hemagglutination test. This conclusion was drawn because different immunoglobulin classes can be distinguished, in many instances, by

Table 4.--Comparison of results from various serological test procedures

Rabbit No.	Log_2 titer and (Reciprocal of titer)				
	Agglut-ination	Hemagglut-ination	Hemolysis Serum non adsorbed	Hemolysis Serum adsorbed[a]	Precip-itin
1286	5 (32)	4 (16)	8 (256)	8 (256)	Neg[b]
1296	9 (512)	8 (256)	7 (128)	7 (128)	Neg
1571	7 (128)	5 (32)	8 (256)	8 (256)	Pos[c]
1517	0 (Neg)	------	7 (128)	3 (8)	Neg
2394	5 (32)	4 (16)	5 (32)	0 (Neg)	Neg
NRS[d]	0 (Neg)	0 (Neg)	0 (Neg)	0 (Neg)	Neg

[a]Serum was adsorbed with SRBC

[b]Neg = negative

[c]Pos = positive with 2 precipitin bands

[d]NRS = normal rabbit serum

function, for example IgM readily fixes complement and has a high cytolytic or hemolytic efficiency while the other classes fix complement less readily and are weakly or not at all cytolytic (31).

By comparing the hemolysis titers before and after adsorption with sheep red blood cells, a determination could be made of how antibodies against SRBC effected the hemolysis titer results. If the anti-cryptococcal titer was high enough to mask the titer to sheep erythrocytes, then there was no change in titer after adsorption; however if the anti-cryptococcal titer was low then it was mask by anti-SRBC antibodies. Therefore adsorption of the serum was necessary to get an accurate anti-cryptococcal titer.

To confirm that the hemolysis and hemagglutination tests using the erythrocytes coated with HA antigen were specific for C. neoformans, two more tests were done. For the first, the antiserum was adsorbed with formalin-killed C. neoformans cells, then retitered by the two test systems. The results are shown in Table 5. In all but one case, the titers were negative after adsorption with C. neoformans cells. The second test was an inhibition procedure whereby capsular polysaccharide was allowed to incubate with antiserum dilutions before the sensitized erythrocytes were added. The polysaccharide completely blocked the hemagglutination or the hemolysis in each case (Table 5). If the hemolysis or hemagglutination had been due to some

Table 5.--Anti-cryptococcal titers before and after adsorption with C. neoformans cells and inhibition with polysaccharide

Serological test	Rabbit No.				
	1286	1296	1571	1517	2394
Hemagglutination					
Untreated serum	4 (16)[a]	8 (256)	5 (32)	------	4 (16)
Adsorbed serum[b]	0 (Neg)[c]	0 (Neg)	0 (Neg)	------	0 (Neg)
Poly. treated[d]	0 (Neg)	0 (Neg)	0 (Neg)	------	0 (Neg)
Hemolysis					
Untreated serum	8 (256)	7 (128)	8 (256)	3 (8)	0 (Neg)
Adsorbed serum[b]	3 (8)	0 (Neg)	0 (Neg)	0 (Neg)	0 (Neg)
Poly. treated[d]	0 (Neg)	0 (Neg)	0 (Neg)	0 (Neg)	0 (Neg)

[a] Log_2 titer (Reciprocal of titer)

[b] Serum was adsorbed with C. neoformans cells

[c] Neg = Negative

[d] Serum was treated with 200 µg of polysaccharide

antigen-antibody system other than cryptococcal, then neither the adsorption nor the inhibition procedures would have changed the titers of the antisera. The HA antigen was considered specific for C. neoformans antibodies.

To determine the effectiveness of the HA antigen for detecting delayed hypersensitivity (cell-bound antibody), the 5 hyperimmunized rabbits, 10 normal rabbits, and 10 guinea pigs which had histoplasmin positive skin tests were skin tested with HA antigen. The results are shown in Table 6. Only the 2 rabbits with the highest hemolysis titers (1/256) gave positive skin reactions. All normal rabbits and all histoplasmin positive guinea pigs were negative with HA antigen. From the very limited testing done in this study there is some evidence that the HA antigen might prove to be an effective skin testing antigen. There was no cross reactivity with Histoplasma infected animals, and untreated animals were uniformly negative. Whether the HA antigen was detecting all sensitized animals can not be answered from this study. Further work is necessary before an accurate evaluation can be made on the effectiveness of HA antigen as a skin testing agent.

Effects of cryptococcal polysaccharide on the immunological responsiveness of rabbits. Rabbits were injected, ip, with various concentrations of cryptococcal polysaccharide and 14 days later given a challenge immunization series of formalin-killed C. neoformans cells. Seven days

Table 6.--Delayed skin reactivity in rabbits using HA antigen as the skin testing material

Rabbit No.	24 hr Induration (mm)	24 hr Erythema (mm)
1286	10 x 10	15 x 18
1296	Negative	5 x 5
1571	13 x 10	18 x 13
1517	Negative	Negative
2394	Negative	12 x 12
Normal[a]	Negative	Negative

[a]Ten normal rabbits showed the same results

after the challenge immunization series, the animals were bled and agglutination titers were determined. The results of this experiment are shown in Figure 1. The rabbits treated initially with 50,000 μg of polysaccharide showed essentially no response to the challenge immunization (reciprocal titer of 1.26). Normal rabbit sera gave reciprocal titers of 1 to 2. Animals initially treated with saline (controls) responded to the challenge immunization with an average reciprocal titer of 12.6. These results fulfilled Leskowitz's definition of tolerance, for the group of animals given 50,000 μg of polysaccharide showed "a specific non-reactivity to the antigenic stimulus (challenge immunization) which in normal animals, would be followed by a recognizable and measurable response (reciprocal titer of 12.6)." If the lack of a significant titer in the group receiving 50,000 μg of polysaccharide had been due to a simple neutralization of antibodies by excess polysaccharide rather than immunological tolerance, then the curve of Figure 1 would not have been as it is but rather would have appeared as a line of continuous descent from 12.6, the reciprocal titer of the control animals, to the point 1.26, the reciprocal titer of the animals treated with the highest concentration of polysaccharide. The fact that the animals initially treated with 500 μg showed an enhanced immunological response to challenge immunization (reciprocal titer of 16) also was evidence against simple antibody neutralization. The

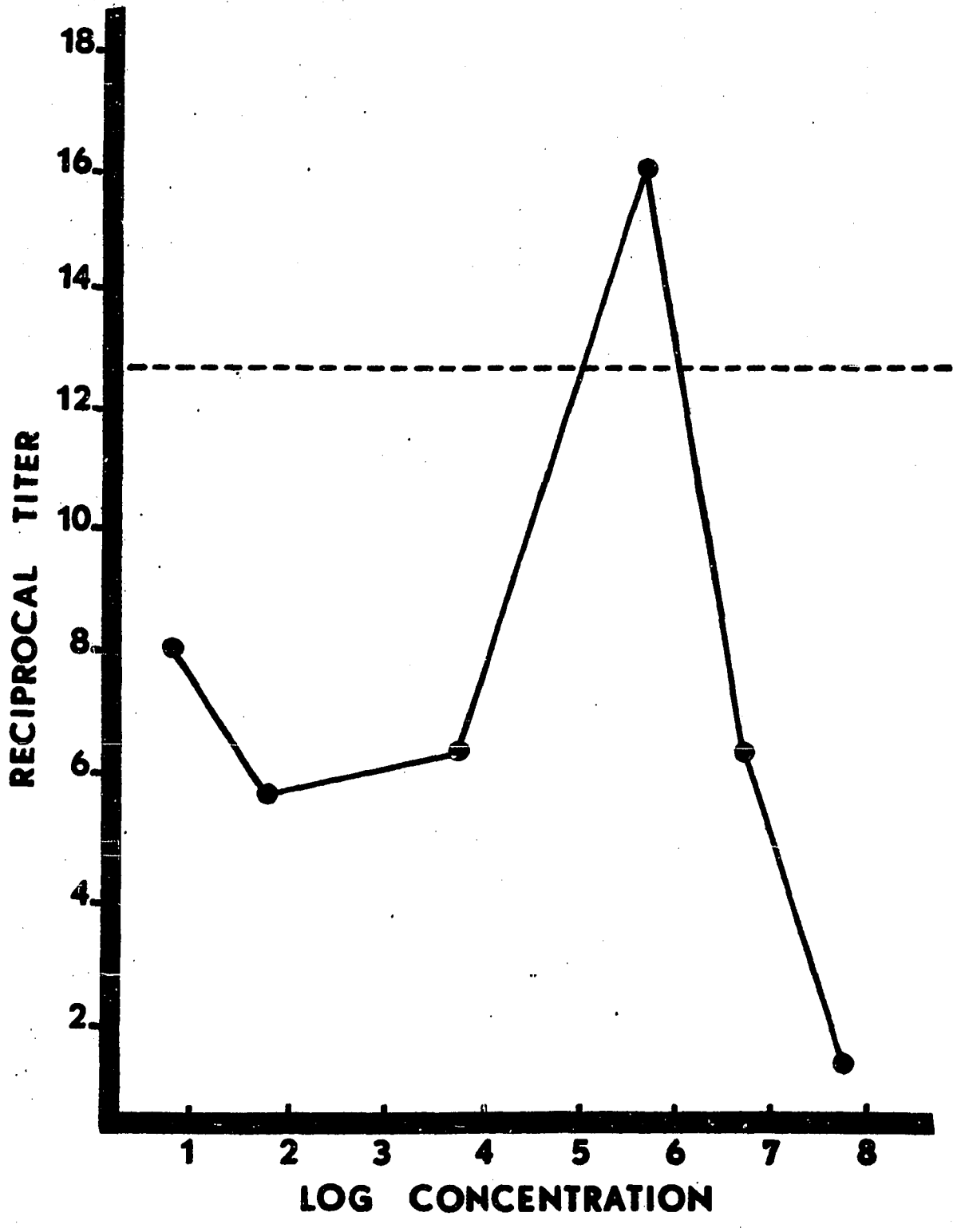

Figure 1. Effects of cryptococcal polysaccharide concentration (mμg) on agglutinin titers in rabbits after challenge immunization. Dashed line represents average titer of controls.

boosted agglutinin response indicated that 500 µg was an immunizing dose of cryptococcal polysaccharide.

The challenge immunization protocol for the rabbits consisted of 7 injections of 6×10^7 cryptococcal cells extended over a 14 day period and introduced a mosaic of antigens. For a more accurate determination of immunizing and paralyzing doses of polysaccharide, a challenge immunization procedure using a single injection containing only a small quantity of polysaccharide would have been much better.

Effects of pretreatment with polysaccharide on the mean life expectancy in mice challenged with C. neoformans. Mice, given various concentrations of polysaccharide ranging from 0.00005 µg to 500 µg, were challenged 14 days later with 1 LD_{50} of C. neoformans. The mean life expectancy (MLE) for each concentration group was determined and compared to MLE of the control (saline treated) group using Student's t test. The only group which showed a significant difference from controls was that group treated with 5 µg of polysaccharide (Figure 2). The MLE of that group was greater than the controls which indicated some protection stimulated by the 5 µg of polysaccharide. These results indicated that the immunizing dose for cryptococcal polysaccharide was similar to that found for pneumococcal and Klebsiella polysaccharides. Howard (23) reported 0.5 and 5 µg of pneumococcal Type III polysaccharide to be immunizing doses and 500 µg to

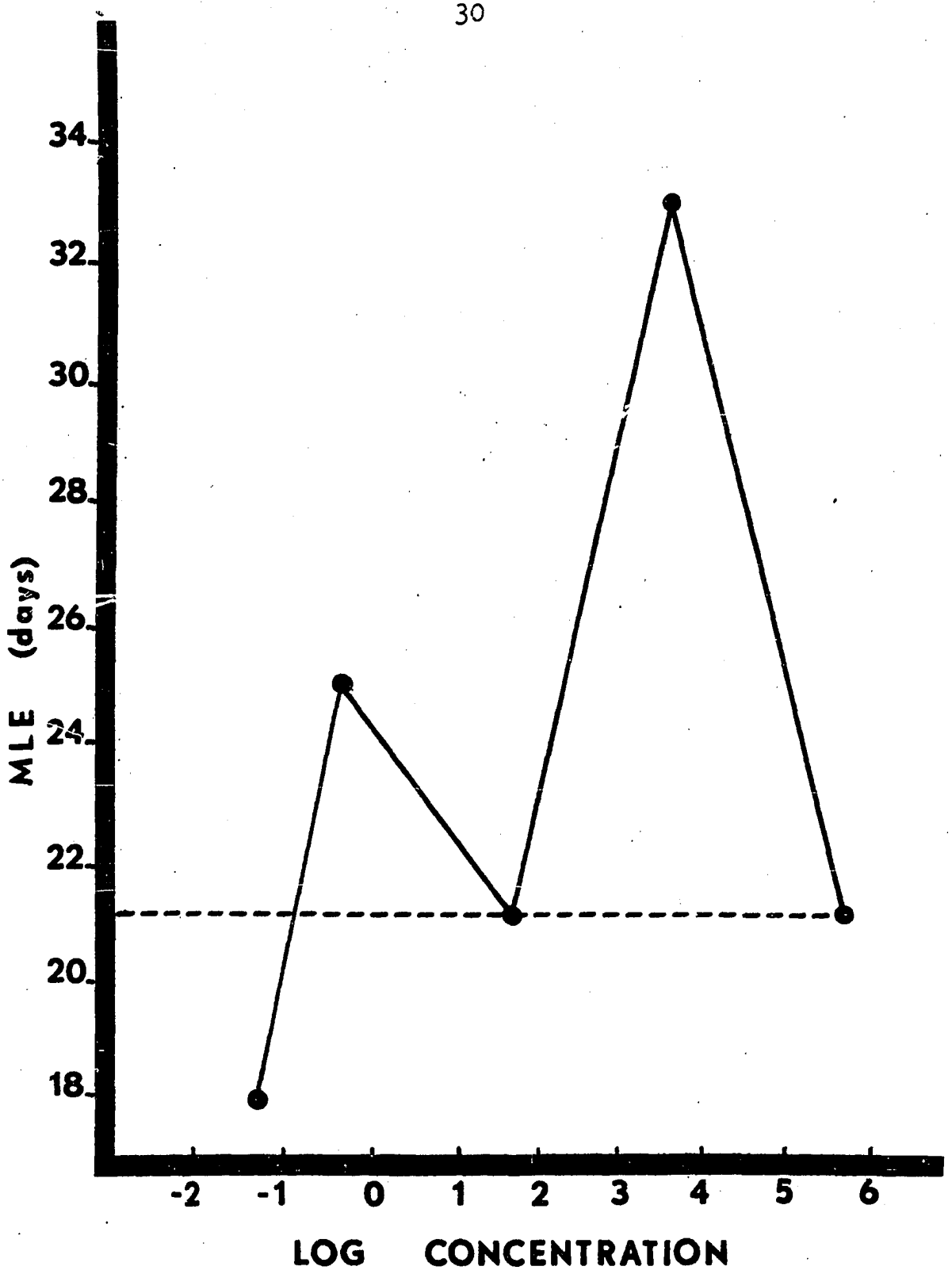

Figure 2. Effects of cryptococcal polysaccharide concentrations (mμg) on mean life expectancy (MLE) in mice after challenge with 1 LD_{50} of C. neoformans. Dashed line represents MLE of controls.

be a paralyzing dose in CBA mice. Wu and Tice (41) showed that 5 µg of Klebsiella polysaccharide would immunize mice, while 1000 µg paralyzed them. A 0.5 µg concentration of cryptococcal polysaccharide was not used in this experiment; however it was in a later study and did prove to be an immunizing dose in mice (Figure 5). Whether or not 500 µg of cryptococcal polysaccharide was a paralyzing dose for mice could not be determined from the MLE study. The animals in that group died at the same rate as those in the control group. One would expect animals treated with 500 µg of polysaccharide to die faster because their immunological responses had been paralyzed by the polysaccharide. The increase in death rate was not observed in this study possibly because the challenge dose of Cryptococcus was so great that the ability of an animal to produce antibodies was of no significance.

The extremely small doses of polysaccharide were used to demonstrate "low dose" paralysis with this antigen; however if "low dose" paralysis was produced, it was not demonstrated for the same reason as for "high dose" paralysis. Low-zone tolerance was first described by Mitchison (29) using bovine serum albumin. This phenomenon has not been reported with polysaccharides; however Siskind and Howard (35) tried without success to elicit a "low dose" zone of paralysis with pneumococcal polysaccharide.

Effects of treatment with polysaccharide on the number of plaque forming cells in mouse spleens and agglutinin titers before and after challenge immunization. This study was designed to determine whether or not antibody-producing cells were present in mouse spleens after the animals had been subjected to immunizing and paralyzing doses of cryptococcal polysaccharide. In addition, it was to demonstrate how the antibody-producing cell populations compared with antibody titers.

Responses of CBA/J mice injected, ip, with saline, 0.5, 5, 50, or 500 µg of cryptococcal polysaccharide were evaluated by means of hemolytic plaque assay technique using polysaccharide coated sheep erythrocytes and by means of agglutination tests. Four days following polysaccharide injection the number of plaque forming cells in the mouse spleens were determined, and sera were assayed for agglutinins. The results from the hemolytic plaque assays are in Figure 3. Agglutinin results are in Figure 4. Each point on the two graphs was an average of the results from 6 mice.

Animals treated with saline (controls) had 36 plaque forming cells (PFC) per spleen. Aisenberg and Wilkes (3) reported a background range of 40 to 60 PFC per normal CBA mouse spleen. Agglutinin titers of normal mouse sera or saline treated mouse sera were found to average 1/2.1 to 1/3.1.

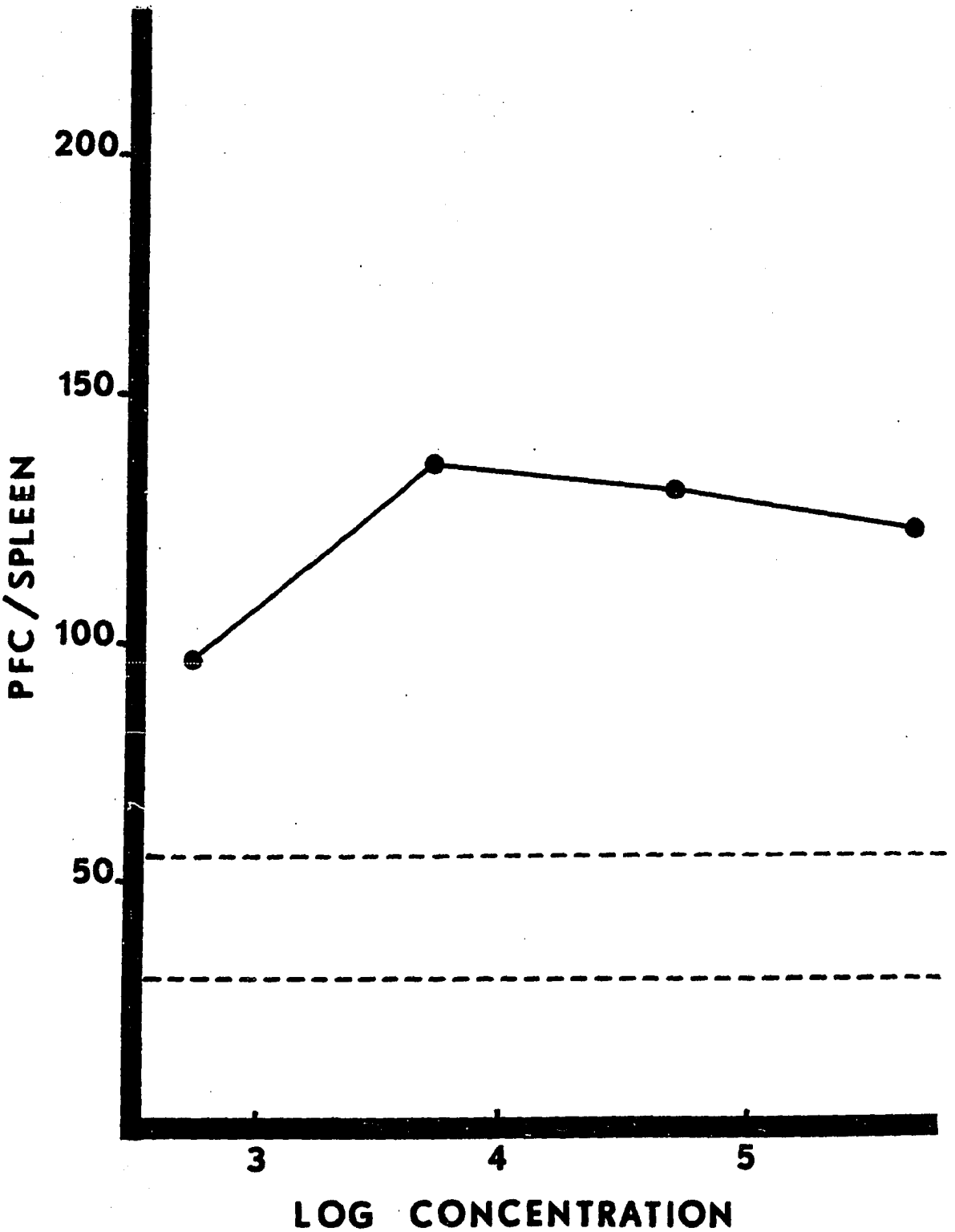

Figure 3. Effects of cryptococcal polysaccharide concentration (mμg) on numbers of plaque forming cells (PFC) in the mouse spleen, 4 days after polysaccharide injection. Area in dashed lines represents background levels of PFC in normal CBA mice.

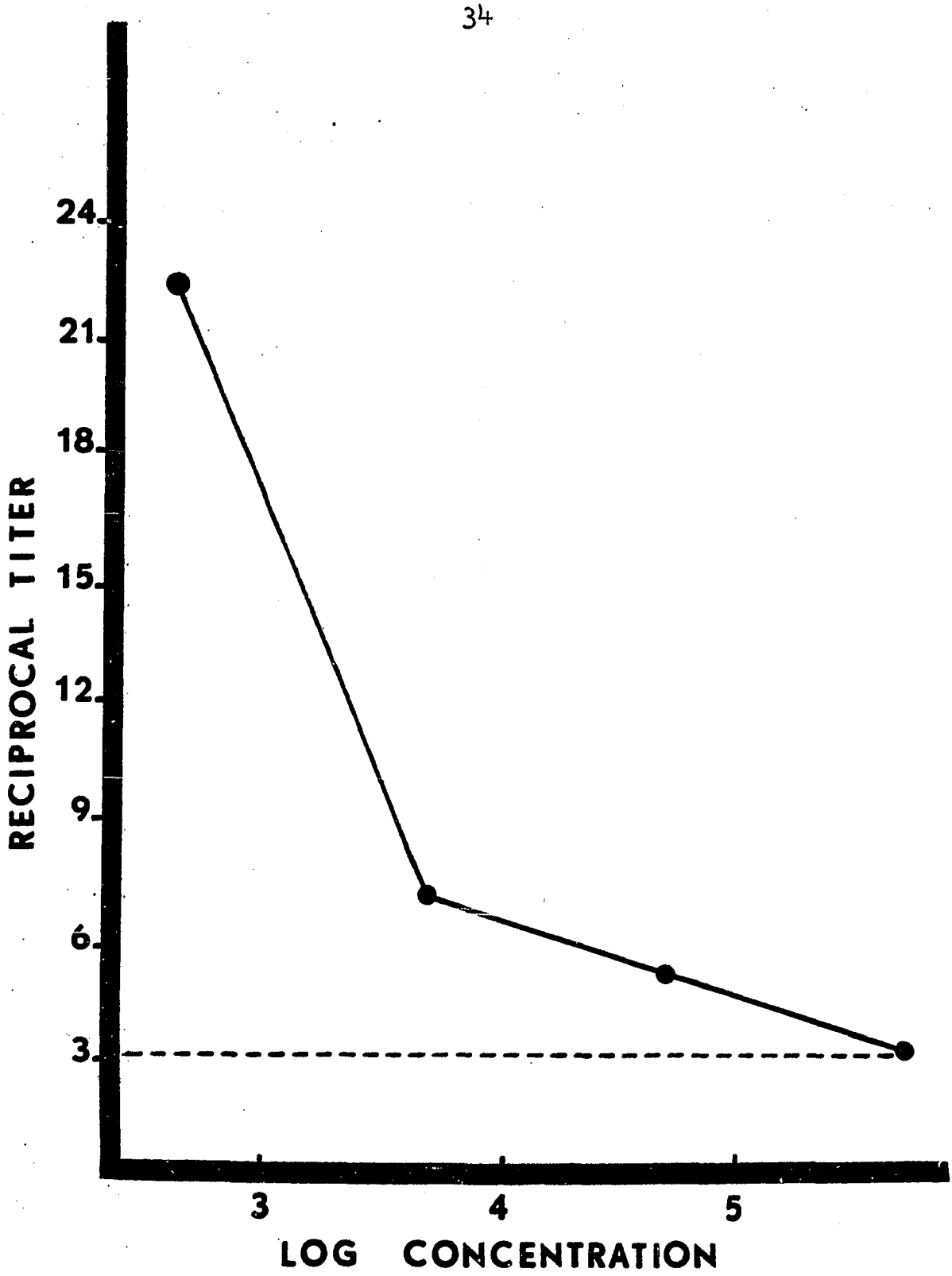

Figure 4. Effects of cryptococcal polysaccharide concentration (mμg) on agglutinin titers 4 days after polysaccharide injection. Area in dashed lines represents titer levels of normal CBA mice.

Specific antibody-forming cells were demonstrated in all animals treated with polysaccharide. The greater numbers of PFC were found in the groups treated with 5, 50, and 500 µg of polysaccharide as compared to the 0.5 µg group. However, the average agglutinin titers peaked in the group receiving 0.5 µg of polysaccharide and showed a continuous reduction with increasing concentration groups (Figure 4). The highest concentration group (500 µg) had an average titer which was not above background. They therefore were considered as negative.

To explain these results two assumptions were made: (1) that the antibody produced to one injection of polysaccharide was primarily macroglobulin and (2) that antibody titers rose in parallel with the number of antibody-producing cells. The former assumption was supported by the work of Al-Naib (4) and the fact that IgM is considered to be the first antibody to appear after antigen stimulation (14, 31). The latter assumption was supported by the findings that in the SRBC antigen-antibody system the number of PFC paralleled the 19S antibody titers (24, 25). Upon the basis of the two assumptions, the antibody titers in the 5, 50, and 500 µg groups should have greatly exceeded the titer of the 0.5 µg group, because the former groups had many more PFC in their spleens than did the 0.5 µg group. However this was not the case. The gradual decrease in titers with increasing concentration groups was probably due to

neutralization of antibody, the greatest number of antibodies being neutralized by 500 µg, less neutralized with 50 µg, and still less with 5 µg.

Agglutinin titers also were determined at 7 and 14 days after polysaccharide treatment. These results appear in Figure 5. The agglutinin titers peaked at 4 days in the 0.5 µg group and at 7 days in all other concentration groups, then the titers fell rapidly during the following 9 and 7 days, respectively. The rise and fall in titers shown in each concentration group followed the form of a typical primary immunological response.

The next study was designed to investigate what effect challenge immunization would have on agglutinin titers and the numbers of PFC in animals which had been given saline or one of the 4 concentrations of polysaccharide. The challenge immunization (c.i.) of 0.125 µg of cryptococcal polysaccharide in incomplete Freund's adjuvant was given 14 days after initial polysaccharide treatment. This time interval was chosen because Brooke (9) showed that an induction period was required for the production of tolerance to pneumococcal polysaccharide. Hemolytic plaque assays and agglutinin titers were determined 4 and 7 days following challenge immunization.

Four days following challenge immunization, the numbers of PFC were greatest in the 0.5 µg group, followed by the controls (Figure 6). Animals treated with 5 µg had

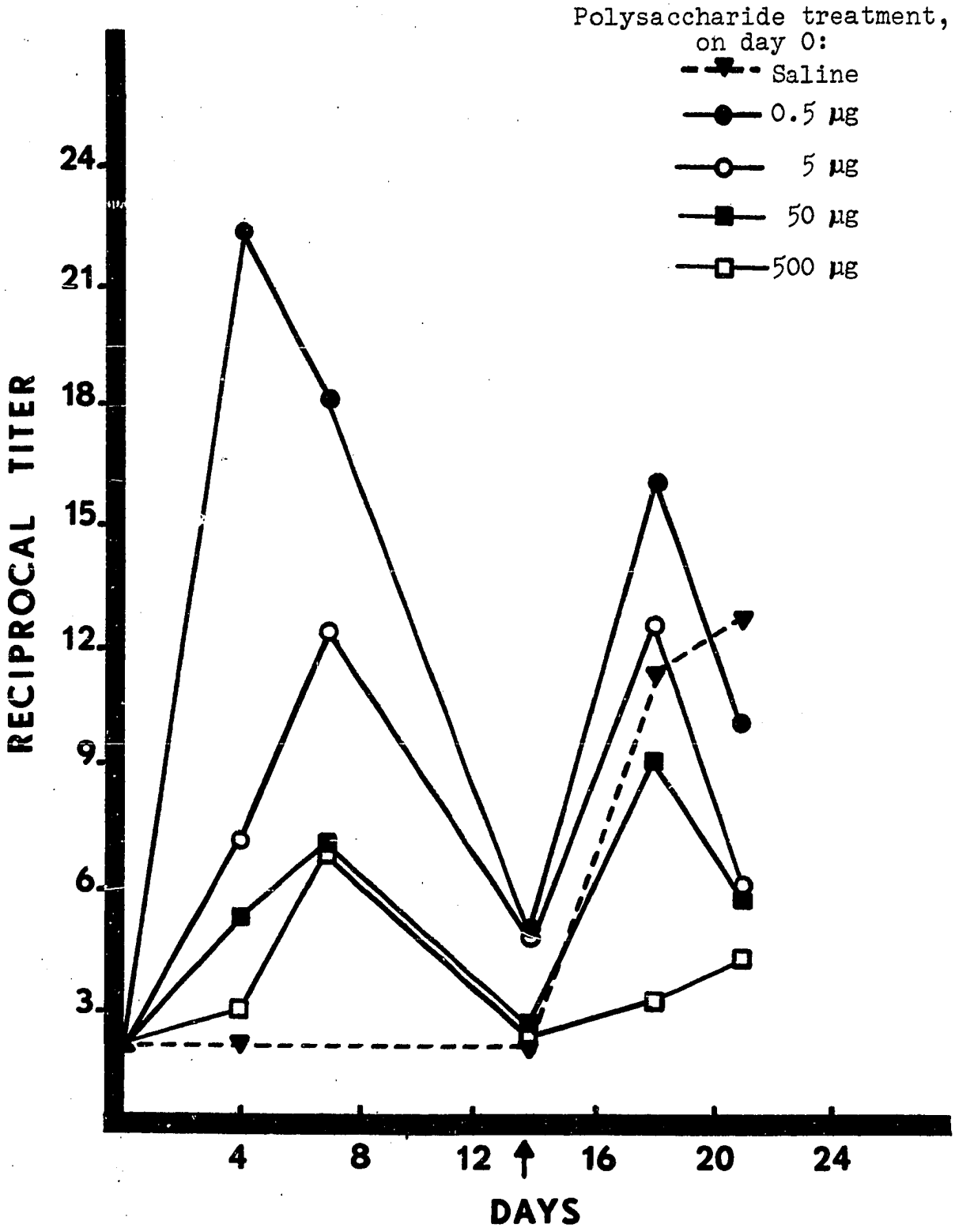

Figure 5. Agglutinin titers in mice treated with various concentrations of cryptococcal polysaccharide on day 0. ↑ indicates challenge immunization with 0.125 µg polysaccharide in incomplete Freund's adjuvant.

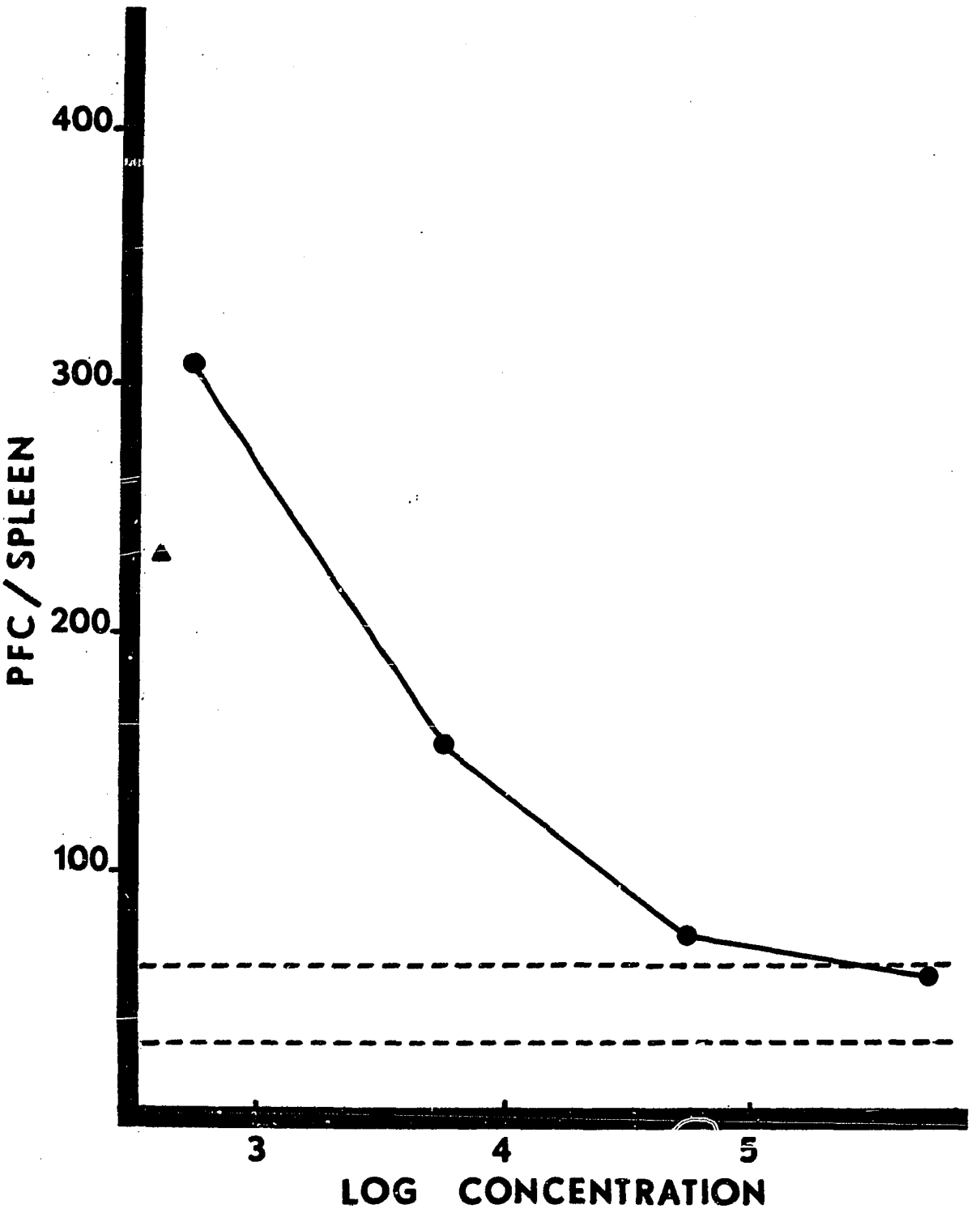

Figure 6. Average numbers of plaque forming cells (PFC) 4 days after challenge immunization in the spleens of animals previously treated with various concentrations of polysaccharide (mμg). Dashed lines enclose background PFC count in normal CBA mice. ▲ represents controls.

fewer PFC than did the control group. The number of PFC found in the group treated with 50 μg of polysaccharide was just above background which indicated only a slight response to challenge immunization. Animals treated with 500 μg of polysaccharide were unable to respond to the challenge immunization; thus demonstrating they were tolerant. Agglutinin titers were positive in all groups except the 500 μg treated one. The agglutination results are shown in Figure 5.

By 7 days after challenge immunization the number of PFC had dropped in all groups (Figure 7). However by 14 days after c.i., the numbers of PFC were increasing in the groups initially treated with 50 and 500 μg of polysaccharide. There was still a trend of decrease in PFC in the other 3 groups (Figure 7). The increase in PFC in the two high concentration groups can be explained better after the following discussion.

The data shown in Figure 7 indicated that all the doses of polysaccharide stimulated a production of specific antibody forming cells. A similar response to pneumococcal Type III polysaccharide has been noted independently in Sterzl's laboratory (38) and by Howard (23). By means of the hemolytic plaque technique using polysaccharide coated mouse erythrocytes, it was demonstrated in Sterzl's laboratory that the antibody-forming cells were present at 6 days after polysaccharide injection and disappeared subsequently

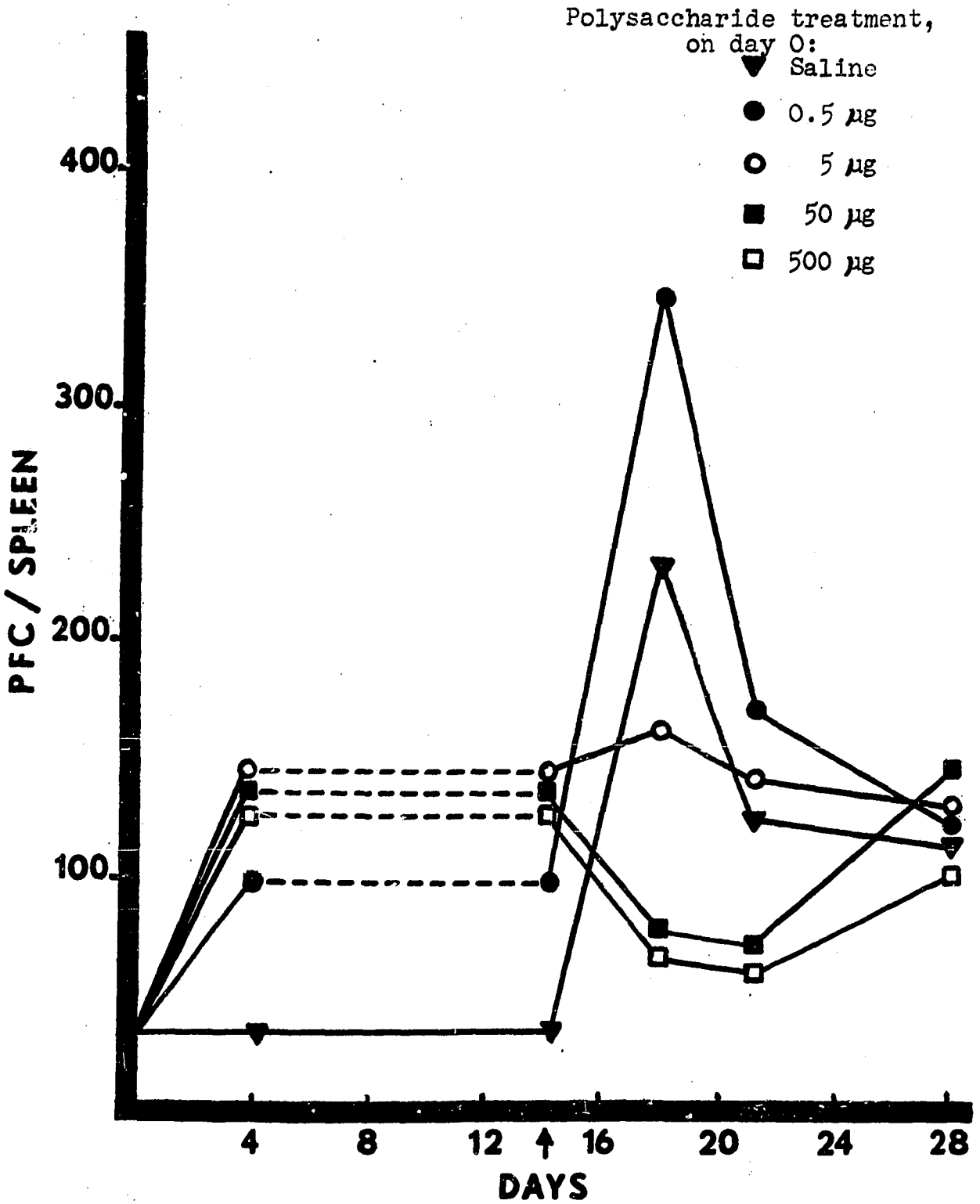

Figure 7. Average numbers of plaque forming cells (PFC) in CBA mice treated with various concentrations of polysaccharide on day 0. ↑ indicates challenge immunization with 0.125 µg polysaccharide in incomplete Freund's adjuvant.

as the state of paralysis was established. Howard using the Biozzi rosette technique noted an increase in rosette-forming cells after treatment with paralyzing doses of polysaccharide but no decrease over a period of 28 days.

After challenge immunization it was apparent that 500 µg was a paralyzing dose, and partial paralysis was induced with 5 and 50 µg. Five-tenth µg was considered an immunizing dose.

According to the "unitarian concept" of immunocompetent cell differentiation, if all stages of differentiation of immunocompetent cells occur in the presence of antigen then proliferation is restricted (37, 38, 39). When large concentrations of cryptococcal polysaccharide were injected subcutaneously, the polysaccharide could be detected in all reticulo-endothelial tissue 4 weeks later (16). Therefore when a paralyzing dose of cryptococcal polysaccharide was given, all susceptible immunocompetent cells were activated and differentiated without proliferation into antibody-forming cells. This group of cells was presumably what the 4 day hemolytic plaque assay was demonstrating. Sterzl (38) reported that antibody-producing cells have a half-life of 2-3 days. Considering this half-life and since the antibody-forming cells did not proliferate, the challenge immunization stimulated only a small number of immunocompetent cells. The results of the hemolytic plaque assays on days 18 and 21 reflected that very few immunocompetent cells differentiated

into antibody-forming cells in animals treated with 50 and 500 µg of polysaccharide. By day 28 the amount of polysaccharide remaining in the animal was probably in the immunizing dose range; therefore as new immunocompetent cells were produced they were stimulated to differentiate and proliferate. This would account for the increase in antibody forming cells noted at 28 days in the 50 and 500 µg groups.

CHAPTER IV

SUMMARY AND CONCLUSIONS

An antigen isolated from the culture filtrate of C. neoformans was effective in coating SRBC which could subsequently be used to detect specific anti-cryptococcal antibodies in microhemagglutination, microhemolysis, and hemolytic plaque assay techniques. The antigen also proved to be a potentially valuable skin testing antigen for cryptococcosis.

Immunological unresponsiveness was demonstrated in rabbits treated with 50,000 µg of cryptococcal polysaccharide. Five hundred µg appeared to be an immunizing dose, for rabbits given this dosage responded to challenge immunization with higher agglutinin titers than did animal injected with saline.

In mice, paralysis could be induced with 500 µg of C. neoformans polysaccharide, and an immune response could be elicited with 0.5 or 5 µg. Prior to establishment of paralysis there was a transient phase of increased numbers of antibody-forming cells in the spleen; however the antibody

produced by the cells could not be detected in the serum, presumably due to neutralization by excess antigen. Following induction of tolerance, challenge immunization did not elicit an increase in antibody-forming cells. This indicated a "central" failure of the immune mechanism. Spontaneous recovery from paralysis was suggested because there was an increase in antibody-forming cells 28 days after injection of the paralyzing dose of polysaccharide.

The results of this study indicate that in cryptococcosis, the circulating polysaccharide serves two functions: first, it neutralizes antibody produced during the tolerance induction period, and secondly it stimulates all susceptible immunocompetent cells to differentiate without proliferation. As long as polysaccharide levels remain sufficiently high in the body fluids of the patient, both activities are taking place. When polysaccharide levels are reduced, for example after treatment with amphotericin B, then the patient slowly gains control of the disease as the immunocompetent cells are replenished.

BIBLIOGRAPHY

1. Abrahams, I. 1966. Further studies on acquired resistance to murine cryptococcosis: Enhancing effect of Bordetella pertussis. J. Immunol. 96:525-529.

2. Abrahams, I. and T. G. Gilleran. 1960. Studies on actively acquired resistance to experimental cryptococcosis in mice. J. Immunol. 85:629-635.

3. Aisenberg, A. C. and B. Wilkes. 1967. Immunological tolerance induced by cyclophosphamide assayed by plaque spleen cell method. Nature 213:498-499.

4. Al-Naib, S. M. 1969. Immunological aspects of Cryptococcus neoformans. Masters Thesis. Univ. of Okla.

5. Anderson, K. and M. Beech. 1958. Serological tests for the early diagnosis of cryptococcal infection. Med. J. Aust. 2:601-602.

6. Bennett, J. E. and H. F. Hasenclever. 1965. Cryptococcus neoformans polysaccharide: Studies of serological properties and role in infection. J. Immunol. 94:916-920.

7. Bennett, J. E., H. F. Hasenclever, and B. S. Tynes. 1964. Detection of cryptococcal polysaccharide in serum and spinal fluid: Value in diagnosis and prognosis. Trans. Assn. Amer. Phys. 77:145-150.

8. Bloomfield, N., M. A. Gordon, and D. F. Elmendorf, Jr. 1963. Detection of Cryptococcus neoformans antigen in body fluids by latex particle agglutination. Proc. Soc. Exp. Biol. Med. 114:64-67.

9. Brooke, M. S. 1964. Breaking of immunological paralysis by injection of a specific depolymerase. Nature 204:1319-1320.

10. Chase, M. W. 1959. Immunologic tolerance. Ann. Rev. Microbiol. 13:349-376.

11. Dresser, D. W. and N. A. Mitchison. 1968. The mechanism of immunological paralysis. Advan. Immunol. 8:129-181.

12. Evans, E. E. and J. R. Kessel. 1951. The antigenic composition of Cryptococcus neoformans: II. Serologic studies with the capsular polysaccharide. J. Immunol. 67:109-114.

13. Evans, E. E. and R. J. Theriault. 1953. The antigenic composition of Cryptococcus neoformans: IV. The use of paper chromatography for following purification of the capsular polysaccharide. J. Bacteriol. 65:571-577.

14. Fahey, J. L. 1965. Antibodies and immunogloblins I. Structure and function. JAMA 194:71-74.

15. Felton, L. D. 1949. The significance of antigen in animal tissues. J. Immunol. 61:107-117.

16. Gadebusch, H. H. 1958. Active immunization against Cryptococcus neoformans. J. Infect. Dis. 102:219-226.

17. Gadebusch, H. H., P. A. Ward, and E. P. Frenkel. 1964. Natureal host resistance to infection with Cryptococcus neoformans. III. The effect of cryptococcal polysaccharide upon the physiology of the reticuloendothelial system of laboratory animals. J. Infect. Dis. 114:95-106.

18. Gordon, M. A. and D. K. Vedder. 1966. Serologic tests in diagnosis and prognosis of cryptococcosis. JAMA 197:961-967.

19. Goren, M. B. 1967. Experimental murine cryptococcosis: Effect of hyperimmunization to capsular polysaccharide. J. Immunol. 98:914-922.

20. Goren, M. B. and G. M. Middlebrook. 1967. Protein conjugates of polysaccharide from Cryptococcus neoformans. J. Immunol. 98:901-913.

21. Hasek, M., A. Lengerova and T. Hraba. 1961. Transplantation immunity and tolerance. Advan. Immunol. 1:1-66.

22. Heiner, D. C. 1958. Diagnosis of histoplasmosis using precipitin reactions in agar gel. Pediatrics 22:616-627.

23. Howard, J. G. 1968. Properties of antigens, p. 28-31. In M. Landy and W. Braun (ed.) Immunological tolerance. Academic Press, New York.

24. Jerne, N. K. and A. A. Nordin. 1963. Plaque formation in agar by single antibody-producing cells. Science. 140:405.

25. Jerne, N. K., A. A. Nordin and C. C. Henry. 1963. In Cell bound antibodies. p. 109. Wistar Institute Press, Philadelphia.

26. Kligman, A. M. 1947. Studies of the capsular substance of Torula histolytica and the immunologic properties of Torula cells. J. Immunol. 57:395-401.

27. Leskowitz, S. 1967. Tolerance. Ann. Rev. Microbiol. 21:157-180.

28. Louria, D. B. 1960. Specific and non-specific immunity in experimental cryptococcosis in mice. J. Exp. Med. 111:643-665.

29. Mitchison, N. A. 1965. Induction of paralysis in two zones of dosage. Proc. Roy. Soc. London, Ser. B 161:275-292.

30. Neill, J. M., J. Y. Suggs, and D. W. McCauley. 1951. Serologically reactive material in spinal fluid, blood, and urine from a human case of cryptococcosis (torulosis). Proc. Soc. Exptl. Biol. and Med. 77:775-778.

31. Pike, R. M. 1967. Antibody heterogeneity and serological reactions. Bacterol. Rev. 31:157-174.

32. Reed, L. J. and H. Muench. 1938. A simple method for estimating 50% end point. Am. J. Hyg. 27:493-496.

33. Richey, J. 1965. Immunological investigations of Cryptococcus neoformans. Masters Thesis. Univ. of Okla.

34. Salvin, S. B. 1959. Current concepts of diagnostic serology and skin hypersensitivity in the mycoses. Am. J. Med. 27:97-114.

35. Siskind, G. W. and J. G. Howard. 1966. Studies on the induction of immunological unresponsiveness to pneumococcal polysaccharide in mice. J. Exp. Med. 124:417-429.

36. Smith, R. T. 1961. Immunological tolerance of non-living antigens. Advan. Immunol. 1:67-129.

37. Sterzl, J. 1966. Immunological tolerance as the result of terminal differentiation of immunologically competent cells. Nature 209:416-417.

38. Sterzl, J. 1968. Properties of antigens, p. 27-28. In M. Landy and W. Braun (ed.) Immunological tolerance. Academic Press, New York.

39. Sterzl, J. and A. M. Silverstein. 1967. Developmental aspects of immunity. Advan. Immunol. 6:337-459.

40. Walter, J. E. and R. D. Jones. 1968. Serodiagnosis of clinical cryptococcosis. Am. Rev. Resp. Dis. 97:275-282.

41. Wu, W. G. and K. L. Trice. 1967. Influence of cellular factors on immune unresponsiveness induced by Klebsiella pneumoniae capsular antigen. J. Bact. 94:896-901.