

Immunological Unresponsiveness Induced by Cryptococcal Capsular Polysaccharide Assayed by the Hemolytic Plaque Technique

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Numerous studies have suggested that cryptococcal capsular polysaccharide could induce an immunological paralysis. To investigate this possibility, mice were given various concentrations of purified cryptococcal polysaccharide and then 14 days later were challenge-immunized with the same material in Freund's incomplete adjuvant. Anticryptococcal agglutinin titers were determined at various periods after polysaccharide treatment and after challenge immunization. At the same periods the hemolytic plaque technique was used to determine the number of spleen cells capable of producing antibody against cryptococcal polysaccharide. The data indicated that there was a transitory immune response which preceded tolerance induction. In animals given the largest doses of polysaccharide, "in vivo" neutralization was responsible for low serum agglutinin titers during the transitory response. The capsular polysaccharide was considered to have induced immunological unresponsiveness at the highest concentration, because challenge immunization did not stimulate an increase in the number of plaque-forming cells (PFC). A sixfold increase in numbers of PFC was found in animals injected initially with the lowest concentration of polysaccharide. These results support the idea that tolerance was due to terminal differentiation without proliferation of the immunocompetent cells. The central failure of the immune mechanism which was apparent in the paralyzed mice was temporary under the conditions of this experiment.

Effects of *Cryptococcus neoformans* capsular polysaccharide on host defense mechanisms are not well understood. One effect appears to be the induction of immunological paralysis if the antigen is given in sufficient quantities. This idea was first suggested by Gadebusch in 1958 (7). Abrahams and Gilleran (1) found a narrow range between an amount of antigen that elicited effective resistance and a larger amount that caused a marked reduction in protection. They felt that the increased amount of polysaccharide present in the larger doses was the cause of the "paralyzing" effect. Other studies have shown that high concentrations of polysaccharide prevent rabbits from building high antibody titers in response to killed-cell vaccine (Cozad et al., *Bacteriol. Proc.*, p. 81, 1963) or in response to purified polysaccharide in Freund's adjuvant (Cozad et al., *Bacteriol. Proc.*, p. 122, 1970). These findings indicate induction of immunological unresponsiveness; however, one cannot rule out the possibilities that the results were due to a neutralization or "masking" of antibody by persisting polysaccharide. This study was de-

signed to determine effects of the polysaccharide at the cellular level, thereby attempting to establish more specifically the mechanism involved in the apparent immunological paralysis. By employing the hemolytic plaque technique, the numbers of antibody-producing cells were detected at various periods after treatment with polysaccharide and after challenge immunization of animals which had been pretreated with polysaccharide. Serum agglutinin titers were determined at the same time periods.

MATERIALS AND METHODS

Animals. Ten-week-old male CBA/J inbred mice (Jackson Laboratory, Bar Harbor, Maine) were given, intraperitoneally (ip), one of the following: 0.5, 5, 50, or 500 μ g of cryptococcal polysaccharide or saline. Fourteen days later, the mice were challenge-immunized with 0.1 μ g of polysaccharide in incomplete Freund's adjuvant. Six mice from each polysaccharide dosage group and saline control group were exsanguinated on days 4, 14, 18, 21, and 28 after initial treatment. Spleens were used for enumeration of plaque-forming cells (PFC); sera were titered by the microagglutination test.

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, La. It was obtained in 1961 from Lorraine Friedman and has been maintained in our laboratory since that time on Sabouraud 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*.

Isolation and purification of polysaccharide. Crude polysaccharide was prepared by growing *C. neoformans* on neopeptone-dextrose dialysate broth (5) at 30 C for 3 days, and by employing the precipitation methods of Evans and Theriault (6). Purification techniques of Gadebusch (7) were used. The dried polysaccharide was stored in a desiccator at room temperature. For use, the appropriate quantity of polysaccharide was dissolved in sterile physiological saline (0.85% NaCl).

The polysaccharide preparation gave strongly positive anthrone, Bial, and Dische tests. By using the Lowry assay for protein, and crystalline bovine serum as a standard, the protein content was 1.7% of the total dry weight of the polysaccharide. There was no absorption maximum at 260 nm using a Beckman DB-G spectrophotometer.

Preparation of polysaccharide-incomplete Freund's adjuvant emulsion. For challenge immunization an incomplete Freund's adjuvant-polysaccharide emulsion was prepared by continuous grinding and dropwise addition of one volume of polysaccharide solution (2.5 μ g of polysaccharide/ml) into a mortar containing one volume of a mixture of five parts Marcol 52 (Humble Oil and Refining Co.) and one part Arlacel A (Hill Top Research, Inc., Miami, 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 ip with 0.1 ml of the emulsion. This amount contained 0.125 μ g of polysaccharide.

Preparation of antigen for sensitization of sheep red blood cells (sRBC). After growing *C. neoformans* 184 in neopeptone dialysate broth for 2 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 fluid was adjusted to pH 7.0 by the addition of 0.1 N NaOH. The cell-free extract was then dialyzed against four changes of pH 7.0 physiological saline and stored at -20 C until used. This antigen, designated HA antigen, was used for sensitization of sheep erythrocytes.

One milliliter of the HA antigen had 560 μ g of sugar by the anthrone test and 750 μ g of protein by the biuret method of analysis. There was no absorption maximum at 260 nm.

Sensitization of sRBC. A 0.2-ml amount of washed, packed sRBC was suspended in 10 ml of 0.01 M phosphate-buffered saline (PBS 0.15 M NaCl), pH 7.2, and 0.2 ml of undiluted HA antigen was added

to the cell suspension. The mixture was incubated at 37 C for 45 min, and then the sensitized cells (sRBC-HA) were washed three times in PBS. Sheep RBC treated in the same manner but with 0.2 ml of saline substituted for HA antigen were prepared and are referred to as "sham-sensitized" sRBC. Sensitized erythrocytes and sham-sensitized erythrocytes were used in microhemolysis tests and hemolytic plaque assays.

Microhemolysis test. This test was employed to determine if sRBC-HA would lyse in the presence of specific antiserum and complement, a prerequisite for using these sensitized cells in the hemolytic plaque technique. Heat-inactivated, sRBC-absorbed, anti-cryptococcal serum was diluted twofold in barbital sodium-HCl-buffered saline, pH 7.5 (VBS) (10) using Microtiter, U-shaped bottom, disposable, nonflexible plates (Cooke Engineering Co.). To each of the serum dilutions was added 0.025 ml of a 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 resuspend all cells and were incubated an additional hour at 37 C with occasional shaking during the first 30 min. After overnight storage at 4 C, the test results were recorded. The microhemolysis titer was defined as the highest serum dilution in which there was any visible red color in the supernatant fluid.

The specificity of the hemolytic reaction was confirmed by demonstrating that hemolysis could be inhibited by the addition of 5 μ g of cryptococcal polysaccharide, by absorbing the antiserum with cryptococcal cells, and by using sham-sensitized sRBC.

Hemolytic plaque technique. The procedure used to detect and enumerate plaque-forming mouse spleen cells specific for cryptococcal antigen was primarily that of Jerne, Nordin, and Henry (9). 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 medium (MEM, Schwarz BioResearch, Orangeburg, N.Y.). A 0.5-ml amount 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 of DEAE dextran per ml. After 15 min at room temperature, the plates were incubated at 37 C for 1 hr. Each plate was flooded with 1.5 ml of a 1:5 dilution of guinea pig complement in VBS, and the plates were again incubated for 30 min at 37 C. After 2 hr at room temperature the excess complement was removed and the plates were refrigerated at 4 C overnight. Reading of the plates was facilitated by staining with benzidine as described by Jerne et al. (9).

Three plates per spleen were prepared and plaques were counted. Total nucleated cell counts were made for each spleen cell suspension so that results could be expressed as PFC per 10^6 nucleated cells. Similar curves were obtained whether the data were expressed

in terms of PFC/10⁶ spleen cells or in terms of PFC/spleen; therefore, the latter terms were arbitrarily chosen to be used in this study.

Microagglutination test. The Microtiter technique using lucite V-shaped bottom Microtiter plates (Cooke Engineering Co.) was employed for determining agglutinin titers. PBS (0.01 M, pH 7.2) was used as the diluting fluid. All solutions used in Microtiter tests were made with distilled, deionized water. The microagglutination test was performed by making serial twofold 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. The Formalin-killed cells were prepared by the procedure of Neill, Abrahams, and Kapros (12). They were diluted to give 60% transmission using 0.5 by 4 inch (ca. 1.3 by 10.2 cm) tubes in a Spectronic 20 (Bausch & Lomb) spectrophotometer set at 500 nm. The Microtiter plates were sealed and incubated for 2.5 hr, 18 inches (ca. 46 cm) from a 60 w bulb in a goose-neck lamp. The increased temperature due to the lamp facilitated reading of titers because end points appeared sharper. 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.

RESULTS

Average numbers of PFC in polysaccharide- and saline-treated mice are shown in Fig. 1. Each point represents the mean number of PFC/spleen for six mice. The two horizontal black lines denote the range in numbers of PFC/spleen in mice treated with saline only. Fourteen animals were used to derive background counts, and those counts ranged from 32 to 66 PFC/spleen with a mean of 43.4. This is in accordance with the background range of 40 to 60 PFC/normal CBA mouse spleen reported by Aisenberg and Wilkes (2).

Four days after initial polysaccharide treatment, all dosage groups showed an increase in numbers of PFC. By day 14 the PFC levels were back to normal. At this time the animals were given a challenge-immunizing dose of 0.1 μg of polysaccharide in Freund's incomplete adjuvant. Four days after challenge, mice pretreated with 0.5 μg of polysaccharide showed a dramatic increase in numbers of PFC compared to animals which had been given the 500-μg dose of polysaccharide. In fact, at 4 days after challenge immunization, the animals' abilities to respond to challenge with an increase in numbers of PFC were inversely related to the concentration of polysaccharide initially given. By day 28 of the experiment, or 14 days after challenge, the animals pretreated with the two highest doses of polysaccharide began to show an increase in numbers of PFC, and there was no significant difference between any of the groups at that time period.

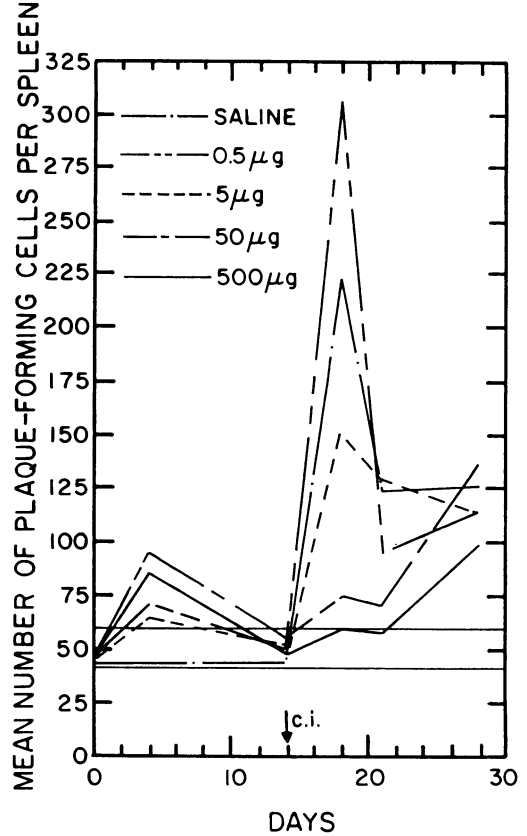


FIG. 1. Mean numbers of plaque-forming cells (PFC) in CBA/J mice treated with various amounts of cryptococcal polysaccharide on day 0. Each point represents mean number of six mice. Horizontal black lines denote background range of PFC in saline treated mice. Arrow indicates challenge immunization with 0.125 μg of polysaccharide in incomplete Freund's adjuvant.

Plaque size was quite uniform throughout all of the plates.

Figure 2 shows the mean log₂ microagglutinin titers of the same animals represented in the hemolytic plaque assay. After initial polysaccharide treatment the mean antibody titer was highest in the low-dosage group. As polysaccharide dosage was increased there was a decrease in mean titers. After challenge immunization (ci) all polysaccharide-treated animals showed an increase in titers; however, the peak titer after ci for each dosage group did not exceed the peak titer for that group after initial polysaccharide treatment. The saline controls responded to ci with essentially the same peak titers noted for animals showing a primary response to a single injection of 0.5 μg of poly-

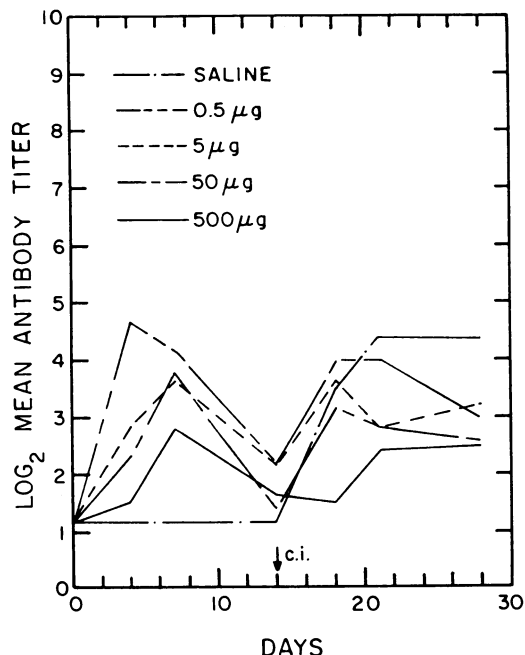


FIG. 2. Mean \log_2 agglutinin titers of CBA/J mice treated with various amounts of cryptococcal polysaccharide on day 0. Each point denotes mean \log_2 titer of six mice. Arrow indicates challenge immunization with $0.125 \mu\text{g}$ of polysaccharide in incomplete Freund's adjuvant.

saccharide. Throughout the experiment the mean titers for the $500\text{-}\mu\text{g}$ dosage group were significantly lower than titers for the $0.5\text{-}\mu\text{g}$ dosage group. The animals treated with 5 and $50 \mu\text{g}$ of polysaccharide had mean titers which ranged between the extreme titers of the high- and low-dosage groups.

Effects of treatment with increasing concentrations of polysaccharide are best shown by comparisons of numbers of PFC and agglutinin titers at various time periods (Fig. 3). There was no significant difference in numbers of PFC among dosage groups at 4 days after polysaccharide injection; however, agglutinin titers decreased rapidly as polysaccharide concentration increased (Fig. 3A). At 4 and 7 days after ci, both numbers of PFC and agglutinin titers showed descending curves when plotted against concentration of polysaccharide, but the curves did not parallel (Fig. 3B and C). By 14 days after ci, numbers of PFC were almost the same for all dosage groups, and the agglutinin titers showed a close parallel (Fig. 3D).

DISCUSSION

All four concentrations of cryptococcal capsular polysaccharide were capable of stimulating

a primary immunological response in mice as indicated by the increase in anticryptococcal antibody-producing cells and the production of agglutinating antibodies (Fig. 1 and 2). The initial response was a transient one which peaked and had disappeared by 14 days after polysaccharide injection in every case except in animals given 0.5 or $5 \mu\text{g}$ of polysaccharide (whole yeast cell agglutination test). Development of a transient immunological response in the form of protective immunity prior to the establishment of paralysis has been reported with pneumococcal polysaccharide (11, 14).

At 4 days after polysaccharide injection, all concentrations of polysaccharide had stimulated a similar increase in numbers of PFC, and all plaques appeared to be uniform in size indicating that the amount of antibody produced by each was approximately the same. It would be reasonable to expect agglutinin titers to be approximately the same in all groups, but the agglutinin titers did not parallel the numbers of PFC during the initial response (Fig. 3A). An explanation for these divergent results could be that "in vivo" neutralization was occurring in animals given the higher concentrations of antigen.

One might argue that the passive hemolytic plaque assay system and the whole yeast cell agglutination test were not detecting antibodies directed toward the same antigenic components, and this was the reason for the divergent results. As Allen and Friedman (3) suggested in their work with immuno-plaque assays using *Escherichia coli* and *E. coli* lipopolysaccharide, it is plausible that only major antigenic component(s) of the polysaccharide preparation could sensitize the red blood cell, whereas the whole microbial cells were mosaics of major and minor antigenic components. If this were the case, one would expect the hemolytic plaque technique to detect only a portion of that population of antibody-forming cells producing antibody detected by the whole-cell agglutination test. In Fig. 3 the numbers of PFC and agglutinin titers have been compared at the four different time periods. In every case except one, when anticryptococcal antibody-forming cells were found in the mice there were also agglutinating antibodies. The exception was found 4 days after polysaccharide treatment in the group of animals which had received the $500\text{-}\mu\text{g}$ concentration (Fig. 3A). These data support the idea that the agglutination test was detecting antibody directed against several antigenic components, whereas the plaques were due to antibody directed against a more limited number of antigenic component(s) which coated red blood cells. The two tests were not detecting totally different populations of

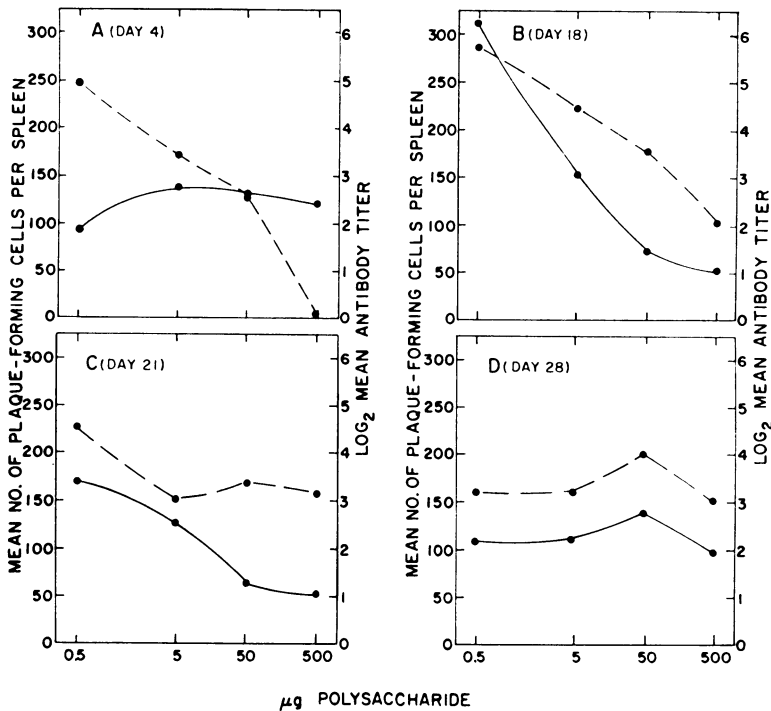


FIG. 3. Comparison of mean numbers of plaque-forming cells (PFC) and mean \log_2 titers in CBA/J mice treated with various amounts of cryptococcal polysaccharide. Solid line denotes PFC; broken line denotes \log_2 titers. Days indicate time after initial polysaccharide treatment. Animals represented in B, C, and D had been also subjected to challenge immunization with 0.125 μg of polysaccharide in incomplete Freund's adjuvant on day 14.

antibodies but rather one test was detecting only a portion of the population, and the other test detected the "whole" population. The exception noted in Fig. 3A can be explained again by "in vivo" neutralization of antibody by an excess of polysaccharide.

After challenge immunization an effect totally different from "in vivo" neutralization was seen. A state of immunological unresponsiveness had been established in mice which had initially received 500 or 50 μg of polysaccharide. The immuno-plaque assay data showed that the apparent unresponsiveness was due to an absence or to very low numbers of antibody-producing cells (Fig. 1). These results support Sterzl's hypothesis of exhaustive differentiation without proliferation of competent cells as a result of stimulation with high concentrations of antigen (16).

Baker et al. (4), Howard (8), and Siskind (13) seem to agree that in immunological paralysis induced by pneumococcal polysaccharide there are two mechanisms involved, one being neutralization of antibody and the other a suppression of antibody synthesis. The results of this study suggest that both mechanisms are also

involved in paralysis induced by cryptococcal polysaccharide. Cryptococcal polysaccharide is a weaker immunogen than pneumococcus polysaccharide, as indicated by the relatively low antibody titers and numbers of PFC. Even though the responses were low in comparison to responses to other antigens, there was a statistically significant difference in numbers of PFC in the high-dosage group and the low-dosage group on day 18 (Fig. 1).

Tolerant mice began to show an increase in numbers of PFC 14 days after the challenge immunization. This recovery of responsiveness could be due to the replenishment of the population of immunocompetent cells. The recovery may have been accelerated by the fact that the *ci* dose of polysaccharide was emulsified with incomplete Freund's adjuvant. It is possible that water-in-oil emulsions might be able to call out young, immunologically capable cells which are then stimulated to differentiate by the residual antigen (15). Further studies need to be done to substantiate these suggestions.

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