Immune Response to *Cryptococcus neoformans* Soluble Polysaccharide: Immunological Unresponsiveness

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Mice injected with 100 to 800 μg of *Cryptococcus neoformans* soluble polysaccharide showed a reduced ability to produce antibody after a challenge immunization with polysaccharide emulsified in Freund incomplete adjuvant. These animals were considered immunologically unresponsive. Animals given an initial injection of 25 or 50 μg of polysaccharide responded to a challenge immunization in the same manner as control animals. Reversion of unresponsive mice to antibody production without further antigenic stimulation did not occur during a 12-week experimental period. These animals exhibited a partial response to challenge immunization 8 weeks after induction of unresponsiveness, and they were fully responsive to challenge immunization at 12 weeks. Animals given a single dose of 0.1, 0.4, or 1.6 μg of polysaccharide produced a marked anamnestic response after challenge immunization. Repeated injections of subimmunogenic doses of polysaccharide did not produce a marked anamnestic response and would induce unresponsiveness only when the cumulative dose reached 100 to 400 μg of polysaccharide, suggesting that injected cryptococcal polysaccharide might be sequestered in some manner until an amount of antigen sufficient for induction of unresponsiveness is accumulated. This possibility was confirmed by immunofluorescence studies that revealed a long-term deposition of polysaccharide in the tubular epithelial cells of the kidney.

The reported incidence of positive tests for humoral antibody in cryptococcal infections has varied from 45 to 80% (2, 12, 16). Although studies have shown no apparent relation between antibody titer and severity or type of infection (9, 12), Diamond and Bennett (5) reported in a study of prognostic factors in cryptococcal meningoencephalitis that a negative test for serum antibody correlated significantly with high risk of death from cryptococcal meningoencephalitis as well as risk of relapse after antifungal therapy. Unfortunately, the pattern of immune response to cryptococcal polysaccharide is erratic; in addition, when circulating antibody is found, titers are usually low, regardless of antibody assay technique. On occasion, humoral antibody has been detected early in the infection, throughout the infection, or concurrent with circulating polysaccharide. In many instances, detectable levels of antibody cannot be demonstrated. Conversion of antibody tests from negative to positive appears to be a rare occurrence (2).

Positive tests for antibody appear to be inversely related to the quantity of antigen in the patient. For example, 22 of 29 patients with cryptococcal meningoencephalitis and a negative India ink smear of cerebrospinal fluid had detectable antibody in their serum, whereas only 4 of 40 patients with positive India ink smears had positive tests for antibody (2). Conversely, the incidence of positive antibody tests in patients increases when the patients exhibit solitary lung lesions, no central nervous system involvement, or early central nervous system involvement (9, 10, 19). These observations of cryptococcosis in human beings as well as several studies of immune response to cryptococcal polysaccharide in animals (17) have led to speculation that antibody is neutralized by excess antigen or that some form of immunological unresponsiveness might result from supraoptimal doses of cryptococcal polysaccharide. Murphy and Cozad (18) demonstrated that immunological unresponsiveness can occur, and that unresponsiveness appears to be the result of a decline in the number of cells capable of producing antibody rather than neutralization of antibody by excess antigen. These findings suggest that immune unresponsiveness may play some role in interpreting the poor immune response observed in human cryptococcosis.

The role of antibody and immunological unresponsiveness in cryptococcosis is unknown; however, Diamond et al. (6) have suggested that
cryptococcal antibody is not directly protective. Instead, antibody production may be simply a concomitant of an active host response to infection. The recent description of an antibody-dependent cell-mediated mechanism for killing of Cryptococcus neoformans suggests a more active role for antibody in resolution of the disease (3, 4). Given the value of antibody tests as an indicator of patient prognosis and the potential of antibody-dependent cell-mediated killing as an effector mechanism in cryptococcosis, the concept of immune unresponsiveness assumes new importance. Unfortunately, immune unresponsiveness induced by C. neoformans capsular polysaccharide remains largely uncharacterized. The present study was undertaken to provide additional information as to how immune unresponsiveness is induced and maintained in experimental animals. The specific aims of this study were (i) to confirm the induction of immune responsiveness, (ii) to determine the effects of antigen dose and duration of exposure to antigen on the induction of immune unresponsiveness, and (iii) to determine whether recovery from paralysis can occur and how such a recovery might relate to circulating antigen.

**MATERIALS AND METHODS**

**Organism.** C. neoformans strain 613 was used throughout this study and has been described (15).

**Animals.** Eight- to ten-week-old female Swiss mice weighing 20 to 26 g were used. Food and water were supplied to the animals ad libitum.

**Soluble polysaccharide.** The method used to purify cryptococcal polysaccharide was a modification of that used by Evans and Theriault (7) and has been described in detail elsewhere (15).

**Immunization of mice.** Mice were immunized subcutaneously with 0.1 ml of polysaccharide emulsified in an equal volume of Freund incomplete adjuvant (BBL, Bioquest). All immunizations were done with an optimal immunizing dose of 20 μg of cryptococcal polysaccharide (17).

Mice were bled from the ophthalmic venous plexus with a Pasteur pipette. The serum was separated and inactivated at 56°C for 30 min, and each specimen was adsorbed with 0.1 ml of packed sheep erythrocytes for 2 h at room temperature.

**Passive hemagglutination.** Polysaccharide was coupled to sheep erythrocytes by a modification of the method used by Baker et al. (1) for pneumococcal polysaccharide. The sensitization procedure and the procedure for assay of serum antibody have been reported (16).

**Assay for antigen.** A microtiter complement fixation assay was used to measure cryptococcal polysaccharide. Immune rabbit serum prepared as previously described (15) and antigen were diluted in Veronal buffer, and 5 50% hemolytic complement units were added. The mixture was allowed to stand overnight at 5°C. A total of 25 μl of optimally sensitized sheep erythrocytes (3 × 10⁷/ml) was added, and lysis was recorded after 1 h of incubation at 37°C. The optimal dilution of immune serum was determined by block dilution of serum and polysaccharide. Fixation of complement was noted with as little as 0.2 μg of polysaccharide.

**Immunofluorescence studies.** Cryptococcal polysaccharide was identified in tissue sections by indirect immunofluorescence. Cryptococcal antisera was prepared in rabbits against whole cells of C. neoformans strain 613 (15). Fluorescein-conjugated immunoglobulin G fraction of goat anti-rabbit immunoglobulins (A, G, and M) was obtained from Chappel Laboratories, Inc. (lot no. 7500). The fluorescein conjugate contained 1.7 mg of antibody protein per ml. Unfixed frozen sections 4 to 5 μm thick were cut on a cryostat. Cryptococcal antisera was applied to slides containing the tissue sections and incubated for 30 min at 37°C in a humidified atmosphere. The slides were washed for 5 min in each of three changes of phosphate-buffered saline (pH 7.2), reincubated with fluorescein-labeled goat anti-rabbit immunoglobulins for 30 min at 37°C, and washed three times in phosphate-buffered saline. Normal rabbit serum was substituted for anti-cryptococcal serum as a negative control.

**Statistical analysis.** Statistical analysis of data was done by analysis of variance. Homogeneity of error was confirmed on all data by Bartlett’s test (20). All tests for significance were done at the P = 0.05 level.

**RESULTS**

Induction of immunological unresponsiveness. The experimental procedure used to demonstrate immune unresponsiveness to cryptococcal polysaccharide was similar to the protocol used by Selton et al. (8) with pneumococcal polysaccharide. Groups of mice were given intraperitoneal injections of saline. Control groups were injected intraperitoneally with saline. All mice were challenged 10 days later with a subcutaneous injection of 0.2 ml of 25 to 800 μg of polysaccharide in saline. A control group was injected intraperitoneally with saline. All mice were challenged 10 days later with a subcutaneous injection of 0.2 ml of 25 to 50 μg of cryptococcal polysaccharide emulsified in Freund incomplete adjuvant. All mice were bleed 17 days after challenge, and their serum was titrated by passive hemagglutination. Induction of immune paralysis by the initial injection of polysaccharide was indicated by an antibody titer lower than that of the control. The results are shown in Fig. 1. Mice given an initial dose of 25 and 50 μg of polysaccharide responded to the challenge immunization with about the same antibody titer as the control group. Unresponsiveness increased with dosage in animals receiving paralyzing doses of 100, 200, 400, and 800 μg of polysaccharide. These data confirm a report by Murphy and Cozad (18) on induction of immunological unresponsiveness by cryptococcal polysaccharide.

Duration of immunological unresponsiveness. Studies on cryptococcosis in human beings have shown that seroconversions from
of production was assessed by determining the number of mice showing unresponsiveness to 10 injections (0.2 ml) of polysaccharide. Serum was titrated with passive hemagglutination.

Groups of 40 mice each were given intraperitoneal injections (0.2 ml) of 100, 200, or 400 μg of cryptococcal polysaccharide in saline. A control group was given an initial injection of saline. At 1, 4, 8, and 12 weeks after the initial paralyzing injection, 10 mice from each group were bled to obtain a small sample of serum. The mice were challenged subcutaneously 2 days later with 20 μg of polysaccharide in Freund incomplete adjuvant. Serum specimens obtained 17 days later were titrated by passive hemagglutination along with serum obtained before challenge.

Sera taken from animals before the challenge immunization at 1-, 4-, 8-, and 12-week intervals were uniformly negative; therefore, a spontaneous recovery from unresponsiveness in the form of measurable antibody production did not appear to occur over that period of time.

Passive hemagglutination titers obtained from mice challenged 1, 4, 8, and 12 weeks after injection of the paralyzing dose are shown in Fig. 2. Mice given an initial injection of 100 μg of polysaccharide showed only limited response to the challenge inoculation given after 1 and 4 weeks. These data correlate well with results shown in Fig. 1. Mice treated with 200 and 400 μg of polysaccharide had geometric mean titers of less than 1:2 after challenge. Mice challenged 8 weeks after induction of unresponsiveness showed an increased response; however, antibody titers of animals given paralyzing doses of 200 and 400 μg of polysaccharide remained somewhat below control levels. Recovery from unresponsiveness appeared to be complete 12 weeks after the initial injection of polysaccharide. Examination of the 12-week data by analysis of variance showed no significant (P = 0.05) difference between the response to challenge by animals given 100, 200, or 400 μg of polysaccharide and the control group.

Current serological methods used in cryptococcosis utilize assays for both cryptococcal antibody and antigen. Therefore, it was of interest to relate the time of recovery from unresponsiveness shown in Fig. 2 to levels of polysaccharide in serum of mice. Groups of 40 mice each were injected intraperitoneally (0.2 ml) with 50, 100, 200, or 400 μg of cryptococcal polysaccharide in saline. Four or five animals from each group were bled at 1-h, 12-h, 1-day, 3-day, 7-day, and 14-day intervals, and levels of polysaccharide in serum were assayed by complement fixation. The results (Fig. 3) indicate that circulating polysaccharide was not detectable (assay sensitivity = 0.2 μg) 7 days after injection of 50, 100, and 200 μg of cryptococcal polysaccharide. Circulating polysaccharide was detected 7 but not 14 days after injection of 400 μg of polysaccharide. Thus, an interval of at least 8 weeks...
Fig. 3. Clearance of cryptococcal polysaccharide from serum of mice injected intraperitoneally with 50 (○), 100 (△), 200 (□), and 400 (●) μg of polysaccharide. Antigen was measured by complement fixation using immune rabbit serum.

elapsed between clearance of detectable circulating antigen and recovery from unresponsiveness.

Effects of long-term and low-dose exposure to cryptococcal polysaccharide. Cryptococcosis often follows a chronic course, and the patient may be exposed to cryptococcal polysaccharide for long periods of time. Presumably, the dose of polysaccharide to which the patient is exposed may vary considerably. An experiment was done to determine the effects of prolonged exposure to polysaccharide upon the total amount of polysaccharide necessary to induce immune unresponsiveness. Three groups of mice were divided into 8 lots of 10 animals each. Each lot of mice was given a total of 0.1, 0.4, 1.6, 6.4, 25, 100, or 400 μg of polysaccharide in saline or an injection of saline alone. The first group was given a single intraperitoneal injection of the specified amount of polysaccharide. The second group of mice received the specified amount of polysaccharide in a total of four intraperitoneal injections given every other day; i.e., an animal that was to receive 400 μg of polysaccharide was given injections of 100 μg on alternate days for four injections. Injections of polysaccharide for the third group of mice were divided into 16 doses given every other day. Control animals were given injections of saline. All mice were challenged 10 days after the last dose of polysaccharide with a subcutaneous injection (0.2 ml) of 20 μg of cryptococcal polysaccharide emulsified in Freund incomplete adjuvant. Blood was drawn 17 days later, and serum was titrated by passive hemagglutination.

The results (Fig. 4) showed that all animals given a single injection of 0.1, 0.4, and 1.6 μg of polysaccharide responded to the challenge immunization at levels considerably above the control group. Animals given 6.4 and 25 μg responded with titers similar to those of the control. Increasing degrees of immune unresponsiveness were noted in mice treated with 100 and 400 μg of polysaccharide.

Unlike the case in animals given a single injection, dividing polysaccharide doses of 0.1, 0.4, 1.6, and 6.4 μg into four injections produced a response to the challenge immunization that did not differ significantly (P = 0.05) from the response of the control group. The mean titer of animals given 25 μg of polysaccharide may have been slightly below the control response, whereas animals given 100 and 400 μg of polysaccharide were almost totally unresponsive.

Animals given polysaccharide in 16 injections responded to the challenge almost identically to animals given 4 injections. Total polysaccharide doses of 0.1, 0.4, 1.6, 6.4, and 25 μg did not significantly (P = 0.05) affect the production of humoral antibody. Animals given a total of 100 and 400 μg responded quite poorly.

The data in Figure 4 suggested that cryptococcal polysaccharide might be sequestered in some manner until an amount of polysaccharide sufficient for induction of paralysis is accumulated. Accordingly, an experiment was done to consider this possibility. Mice were injected intraperitoneally with 100 μg of polysaccharide, and they were sacrificed at varying time intervals over a 14-week period. Several organs were removed, and frozen sections were examined by immunofluorescence to determine the distribution of antigen in the body. A faint amount of nonspecific background fluorescence was observed when tissues from untreated mice were examined or when normal rabbit serum was substituted for anti-cryptococcal serum. Immunofluorescent examination of tissue from treated tissue 1 day and 8 days after injection of antigen showed a weak granular staining of the connec-
tive tissue in many organs including lung, liver, spleen, thymus, lymph node, and heart. Localization of antigen in these tissues diminished rapidly, and cryptococcal polysaccharide could not be found in these tissues 4 weeks after injection of polysaccharide. There appeared to be no localization of polysaccharide either on or in the macrophages of any organ studied. Localization of polysaccharide in the kidney was in marked contrast to any other tissue studied. Accumulation of the polysaccharide in the tubular epithelial cells was noted 24 h after injection of polysaccharide. The polysaccharide appeared as fine, intensely stained granules that appeared to be located within the tubular cells. The granules enlarged, reaching a maximum approximately 4 weeks after injection of polysaccharide (Fig. 5). After 4 weeks, the granules gradually subsided in both number and staining intensity; however, weak staining was still apparent in the tubular epithelial cells at the termination of the experiment 14 weeks after injection of polysaccharide. At no time did there appear to be any localization of polysaccharide in the glomerulus.

**DISCUSSION**

The ability of cryptococcal polysaccharide to induce immunological unresponsiveness was clearly demonstrated by Murphy and Cozad (18). Their results showed this unresponsiveness to be the result of a suppression of antibody synthesis rather than a neutralization of antibody by excess antigen. The present study confirmed the induction of immunological unresponsiveness by cryptococcal polysaccharide. Our use of circulating antibody as an indicator of immunological responsiveness rather than measurement of antibody production at a cellular level, however, makes it impossible to confirm the mechanism described by Murphy and Cozad (18).

Escape from immunological unresponsiveness in the form of reversion to production of antibody to cryptococcal polysaccharide without further antigenic stimulation was not seen during a 12-week observation period. This may explain the infrequency of reports of seroconversion from negative to positive antibody tests.

**Fig. 5.** Kidney from mouse injected intraperitoneally with 100 µg of cryptococcal polysaccharide and examined after 4 weeks. Frozen sections were incubated with normal rabbit serum (A) or anti-cryptococcal serum (B) and then incubated with fluorescein-labeled goat anti-rabbit immunoglobulins (fluorescence illumination, x312).
noted by Bindschadler and Bennett (2). Partial escape from unresponsiveness in the form of response to challenge immunization with polysaccharide was seen 8 weeks after induction of unresponsiveness, and recovery appeared complete after 12 weeks. Recovery from unresponsiveness occurred long after antigen was no longer detectable in serum. Recovery also correlated well with the loss of antigen that had localized in the various body organs. A very small amount of polysaccharide could be found in the kidney at the time of recovery, but presumably this amount of antigen was insufficient for maintenance of unresponsiveness.

Within limits of our experimental design, induction of "low-dose" unresponsiveness did not occur. The possibility of low-dose unresponsiveness at lower antigen doses cannot, however, be excluded. Rather than induction of a low-dose form of unresponsiveness, repeated injections of low doses of antigen induced unresponsiveness when the total antigen dose amounted to 100 to 400 μg, which was the same amount of polysaccharide necessary to induce unresponsiveness if given as a single injection. This result may have some importance in the interpretation of serological tests in cryptococcosis, since our data show that induction of unresponsiveness in the host appears to be dependent upon the total amount of polysaccharide to which the host is exposed, with duration of exposure being of little consequence.

Studies on the effects of multiple injections of antigen on induction of unresponsiveness suggested that cryptococcal polysaccharide might be sequestered in some manner until an amount of polysaccharide sufficient for induction of paralysis is accumulated. Immunofluorescent studies provided direct evidence for long-term deposition of cryptococcal polysaccharide in the tubular epithelial cells of the kidney. Significant long-term deposition of cryptococcal polysaccharide was not noted in other organs that were studied. The pattern of tissue localization of cryptococcal polysaccharide appears markedly different from the distribution of type II and III pneumococcal polysaccharide. Kaplan et al. (11) reported that the most constant and striking concentrations of pneumococcal polysaccharides were found in cells of the reticuloendothelial system, the capillary endothelium, and fibroblasts throughout the body. We observed no localization of cryptococcal polysaccharide in macrophages. These in vivo results also confirm earlier in vitro observations that cryptococcal polysaccharide does not bind to cultured macrophages (14). The distribution of cryptococcal polysaccharide in human cryptococcosis is not known and remains an area for further study.

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LITERATURE CITED

