PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 169, 427–431 (1982)

Altered Responsiveness of Murine Lymphoid Cells Treated with Cryptococcal Capsular Polysaccharide (41370)

IRA LEE, FRED VOGEL, JOHN BREEN, AND HERMAN FRIEDMAN

Department of Medical Microbiology and Immunology, Pathology, and Section of Infectious Diseases, Department of Internal Medicine, University of South Florida College of Medicine, James Haley Veterans Administration Hospital, and Tampa General Hospital, Tampa, Florida 33612

Abstract. Purified capsular polysaccharide derived from *Cryptococcal neoformans* was studied in terms of effects on immune responsiveness of mouse spleen cells to sheep red blood cells *in vivo* and *in vitro*. Injection of mice with small doses of polysaccharide enhanced the primary IgM antibody response, whereas larger doses suppressed the response. The polysaccharide also modulated the *in vitro* immune response of normal mouse spleen cells immunized with sheep erythrocytes. The blastogenic responsiveness of murine spleen cells to Con A and pokeweed mitogens were markedly suppressed by the highest dose of cryptococcal polysaccharide tested, i.e., 100 μg/ml. These results demonstrate that the purified polysaccharides of an opportunistic fungus can significantly modulate both antibody and mitogenic responses in the murine system, and suggest that this material may have a wider role in affecting host immune response mechanisms.

Cryptococcus neoformans causes severe infections, especially in hosts with underlying depression of immune responsiveness (1–3). While cryptococcal capsular polysaccharide (CCPA) has been shown to modulate the phagocytosis of cryptococcal organisms (4, 5), no knowledge has been available as to whether this microbial product can affect immune responses to unrelated antigens or mitogens. Thus, it was of interest to determine whether the cryptococcal capsular polysaccharide, reputed to constitute a defense mechanism whereby the yeast avoids host cell phagocytosis, affects various parameters of immune responsiveness. For this purpose, murine splenocytes were treated either *in vivo* or *in vitro* with CCPA, and their responsiveness to mitogens and sheep erythrocytes assessed.

Experimental Methods. *Animals.* BALB/c mice, 18–20 g each, were obtained from Jackson Laboratories, Bar Harbor, Maine, and housed in groups of 6–10 in plastic mouse cages under pathogen-free conditions. The animals were fed Purina mouse pellets and water *ad libitum*.

Cryptococcal antigen. Cryptococcus neoformans NIH B551a was grown in 1-liter flasks containing 300 ml of broth having the following composition in grams per liter of deionized water supplemented with trace elements: glucose, 40; urea, 1.29; KH_2PO_4, 1.36; $MgSO_4 \cdot 7H_2O$, 0.3; yeast extract, 0.2; thiamine–HCl, 2×10^{-3} *M* (Difco, Detroit, Mich.). CCPA was prepared from culture supernates as previously described (6). In brief, cultures were treated with sodium ethylmercurithiosalicylate, cells removed by centrifugation, and the supernates concentrated by vacuum evaporation. The polysaccharide was precipitated with cold absolute alcohol, redissolved, and reprecipitated with cetyltrimethylammonium bromide (Cetavlon, Eastman, Rochester, N.Y.). Following a second reprecipitation step with absolute alcohol, the CCPA was lyophilized. Stocks (1 mg/ml) of the polysaccharide were prepared in pyrogen-free saline, and stored at −70° until used. Control medium was prepared by the same extraction procedure without cryptococcal organisms being present, and included in all the immunologic assays described.

Antigen. Sheep erythrocytes (SRBC) were obtained from Lee Laboratories, Grayson, Georgia, in Alsever's solution. The SRBC were washed several times in cold PBS until clear of hemolysis and ad-

0037-9727/82/040427-05$01.00/0

justed to either 0.05% (v/v) or 10% (v/v) suspensions for *in vitro* or *in vivo* hemolytic plaque assays, respectively.

In vivo immunization. BALB/c mice were injected intraperitoneally on Day 0 with 0.5 ml of a 10% SRBC suspension, followed immediately by graded doses of CCPA contained in 1.0 ml PBS. Spleen cells were harvested on Day 4 to determine the IgM hemolytic antibody plaque response.

Spleen cell cultures. Experimental animals were sacrificed by cervical dislocation and spleen cells obtained by teasing individual spleens in RPMI 1640 medium (Gibco, Grand Island, N.Y.). Cell suspensions were washed twice in cold RPMI 1640 medium and adjusted to a concentration appropriate for the immunologic assays described below.

In vivo hemolytic plaque response. Splenocytes were obtained from mice primed *in vivo* with SRBC plus CCPA and nucleated cells adjusted to 2.5×10^6/ml in RPMI 1640. One hundred-microliter aliquots of these cells were assayed for their primary IgM hemolytic plaque response in a hemolysis-in-gel plaque assay as described by Yamada and Yamada (7).

In vitro hemolytic plaque assays. Spleen cells obtained from normal BALB/c mice were adjusted to 1.0×10^7/ml in BME medium (Gibco) with 10% fetal calf serum (Reheis, Kankakee, Ill.). One-milliliter aliquots were dispensed in triplicate into the wells of Linbro 24 wells plates (Oxnard, Calif.). To each well were added 200 μl of 0.05% SRBC, graded concentrations of CCPA contained in 200 μl PBS, and an additional 1 ml complete BME medium. After 5 days of culture at 37° in an atmosphere of 5% CO_2, the spleen cells were harvested, adjusted to a concentration of $1.0-2.0 \times 10^6$ cells/ml, and 100-μl aliquots assayed for IgM plaque responses as above.

Mitogens. Conconavalin A (Con A) and pokeweed mitogen (PWM) were obtained from Miles Laboratories, Elkart, Indiana, and Sigma Chemicals, St. Louis, Missouri, respectively. The mitogens were suspended at a concentration of 1 mg/ml in sterile PBS, these stock solutions filter sterilized and stored at −70° until used.

Blastogenic assays. Normal BALB/c spleen cells were adjusted to a concentration of 2.5×10^6 cells/ml in RPMI 1640 medium with 10% fetal calf serum. Portions of cells at this concentration were treated either with Con A or PWM equivalent to 1.0 and 2.5 μg/ml, respectively. Two hundred-microliter aliquots were then immediately dispensed into triplicate wells of Nunc 96-well flat-bottomed microliter plates (Gibco), and graded amounts of CCPA or control medium added in 20-μl amounts. After 72 hr of incubation at 37° in an atmosphere of 5% CO_2 all wells were pulsed with 0.5 μCi [^{3}H]thymidine (New England Nuclear Company, Boston, Mass.) and harvested 18 hr later with a multiple automated cell harvester. Counts incorporated were assessed by liquid scintillation counting and stimulation indices obtained by standard formula.

Experimental Results. Injection of mice with graded concentrations of purified CCPA markedly altered the PFC responsiveness of their spleen cells in a dose-dependent manner, as seen in Table I. Low doses of CCPA (0.5 and 10.0 μg/animal) gave moderate enhancement of this primary IgM antibody response, while the 1000 μg dose of polysaccharide markedly inhibited it.

The incubation of murine splenocytes from nontreated mice with CCPA during the induction phase of the *in vitro* antibody response also resulted in a dose-dependent alteration of this parameter, as shown in Table II. A concentration of 0.01 μg CCPA had a slight stimulating effect on the PFC response, while 0.1 μg resulted in approximately a 60% decrease in the antibody response. This depression then increased in a dose-dependent manner to a maximum effect with 100 μg CCPA/ml.

In order to determine if the polysaccharide had an effect on lymphocyte blastogenic responsiveness, mouse spleen cell cultures were treated with 1−100 μg/ml CCPA in conjunction with a nonspecific plant mitogen. As is evidenced in Table III, the responsiveness to Con A was unaffected at the 1- or 10-μg doses. However, 100 μg suppressed the response by about 60%. Similar results were observed with

TABLE I. ANTIBODY RESPONSE OF MICE TO SHEEP ERYTHROCYTES AFTER INJECTION WITH CRYPTOCOCCAL POLYSACCHARIDE

Polysaccharide concentration injected (μg/mouse ip)[a]	Antibody response to SRBC		Significant at $P \leq 0.05$[c]
	PFC/10^6 spleen cells[b]	Percentage of control	
None (control)	900 ± 68	100	—
0.5	1200 ± 130	135	*
1.0	1110 ± 95	125	NS
10.0	1410 ± 150	155	*
100.0	1150 ± 112	130	NS
1000.0	530 ± 43	60	*

[a] Mice injected with indicated concentration of CCPA at the same time as ip immunization with 0.5 ml of 10% SRBC.
[b] Average anti-RBC response ± SD of four to six mice per group 4 days after immunization.
[c] Determined by Student's t test.
* Significant at $P \leq 0.05$ as determined by Student's t test.

PWM as a mitogen. The CCPA had no direct mitogenic effect on the spleen cell cultures per se (unpublished observations).

Discussion. There have been many studies concerning the immunochemistry of bacterial and fungal polysaccharides. It is likely that immune responses to these microbial products may be important in the host–parasite relationship. Furthermore, polysaccharide antigens from bacteria such as pneumococci have been extensively studied in terms of induction of specific immunologic nonresponsiveness or immune paralysis (8, 9). In these studies, administration of polysaccharide to rodents alters the subsequent responsiveness of the animal to challenge with the same but not with unrelated polysaccharide antigens. Our *in vivo* PFC data indicate that simultaneous injection of CCPA with SRBC may alter the antibody response to this T-cell-dependent antigen. Lower doses of the polysaccharide enhanced the PFC response, while the high dose inhibited this response. The suppressive effect of CCPA was also noted in the *in vitro* hemolytic plaque response. Since cryptococcal polysaccharide is reputed to have antiphago-

TABLE II. EFFECT OF CRYPTOCOCCAL CAPSULAR POLYSACCHARIDE ON THE *IN VITRO* ANTIBODY RESPONSE OF MOUSE SPLEEN CELL CULTURES

Polysaccharide concentration (μg/ml)[a]	Antibody response to SRBC[b]		Significant at $P \leq 0.05$[d]
	PFC per 10^6 spleen cells ($\bar{x}$ ± SD)	Percentage of control response[c]	
None (control)	1380 ± 524	100	—
0.01	1650 ± 350	121	NS
0.1	609 ± 40	40	*
1.0	692 ± 40	46	*
10.0	530 ± 99	34	*
100.0	287 ± 115	15	*

[a] Cultures of 5 × 10^6 spleen cells from normal BALB/c mice immunized *in vitro* with 0.2 ml 0.05% SRBC and treated with indicated concentrations of CCPA.
[b] Antibody PFC response for three to six cultures per group 5 days after *in vitro* immunization.
[c] Corrected for background PFC counts.
[d] Determined by Student's t test.
* Significant at $P \leq 0.05$ as determined by Student's t test.

TABLE III. EFFECT OF CRYPTOCOCCAL POLYSACCHARIDE ON SPLEEN CELL BLASTOGENESIS TO PLANT MITOGENS

Polysaccharide concentration (μg/ml)[a]	Blastogenic responsiveness[b]			
	Con A		PWM	
	Stimulation indices	Percentage of control	Stimulation indices	Percentage of control
None (control)	58.1	100	29.1	100
1.0	58.7	101	29.7	102
10.0	54.0	93	28.5	98
100.0	22.1	38*	10.2	35*

[a] Concentration of cryptococcal polysaccharide added to cultures of 5×10^5 normal BALB/c spleen cells. Data shown represents six replicates in two experiments.

[b] Indicated mitogen added to cultures of spleen cells treated with CCPA; responses are expressed as stimulation indices and percentage of control responses 72 hr after culture initiation and 18 hr after pulse with [^{3}H]-thymidine.

* Significant at $P \leq 0.05$ as determined by Student's t test.

cytic properties, the possibility must be addressed that the depressed PFC responses may be due to coating of antigen by CCPA, resulting in decreased ingestion by macrophages involved in antigen presentation. Unpublished observations from our laboratory indicate that addition of CCPA to *in vitro* cultures of peritoneal exudate macrophages does not alter their phagocytosis of sheep erythrocytes in either opsonized or nonopsonized states. This argues against the hypothesis of antigen masking by CCPA, although it is possible that subsequent intracellular antigen processing may be affected.

The possibility that CCPA has direct toxic effects on immunocytes must also be considered. *In vitro* cultivation of murine splenocytes with 100 μg/ml of CCPA results in a modest (10–15%) decrease in viability at Day 1, but viability is comparable to that of controls by Day 5 *in vitro*, as determined by trypan blue viability counts. Such modest losses in viability cannot directly account for the large decreases in the *in vitro* and *in vivo* PFC responses. Furthermore, no differences in spleen size or total splenic lymphocyte numbers was noted in mice receiving CCPA during the *in vivo* PFC studies. Conceivably, the overall balance and function of splenic subpopulations (i.e., T helper, T suppressor, B cells, and macrophages) may be affected. Thus, the enhancement or suppression of antibody formation could be due to effects on cells other than antibody-forming B cells, such as regulatory T cells or macrophages. It should also be noted that the PFC assays only examined the primary antibody responses. The possible effects of CCPA on secondary responses, characterized by the IgG class of antibodies, have not been studied, but it would be of value to do so.

The nonspecific responsiveness of T lymphocytes to the mitogens Con A and PWM was not enhanced when 1- to 10-μg doses were used. However, 100 μg of CCPA significantly suppressed the blastogenic response, suggesting that T cells involved in recognition of mitogens and cell transformation could be inhibited. It seems unlikely that CCPA binds the mitogens directly, as preabsorption of CCPA with Con A–Sepharose does not ablate its ability to suppress mitogenic responses. Further studies in progress examine other immune parameters that may be modulated by CCPA, as well as target cells and mechanisms affected by this capsular product. Additional experimentation with other cryptococcal antigens may provide further information as to their relative importance to the cryptococcal infectious process per se.

1. Schimpff SC, Bennett JE. Abnormalities in cell-mediated immunity in patients with Cryptococcus

neoformans infection. J Allergy Clin Immunol 55:430–441, 1975.
2. Speckard A, Butler WT, Andriole V, Utz JP. Improved prognosis of cryptococcal meningitis with amphotericin B therapy. Ann Intern Med 58:66–83, 1963.
3. Butler WT, Alling DW, Spickard A, Utz, JP. Diagnostic and prognostic value of clinical and laboratory findings in cryptococcal meningitis. New Engl J Med 270:59–67, 1964.
4. Kozel TR, Mastroianni RP. Inhibition of phagocytosis by cryptococcal polysaccharide: Dissociation of the attachment and ingestion phases of phagocytosis. Infect Immun 14:62–67, 1976.
5. McGaw TG, Kozel TR. Opsonization of Cryptococcus neoformans by human immunoglobulin G: Masking of immunoglobulin G by cryptococcal polysaccharide. Infect Immun 25:262–267, 1979.
6. Cherniak R, Reiss E, Slodki ME, Plattner RD, Blumer SO. Structural and antigenic activity of the capsular polysaccharide of Cryptococcus neoformans serotype A. Mol Immunol 17:1025–1032, 1980.
7. Yamada H, Yamada A. Antibody formation against 2,4-dinitrophenyl-hapten at the cellular level. J Immunol 103:357–363, 1969.
8. Baker PJ, Barth RF, Stashak PW, Amsbaugh DF. Enhancement of the antibody response to type III pneumococcal polysaccharide in mice treated with antilymphocyte serum. J Immunol 104:1313–1315, 1970.
9. Baker PJ, Stashak PW, Amsbaugh DF, Prescott B. Regulation of the antibody response to type III pneumococcal polysaccharide II. Mode of action of thymic-derived supressor cells. J Immunol 112:404–409, 1974.

Received May 18, 1981. P.S.E.B.M. 1982, Vol. 169.