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Cryptococcal Capsular Polysaccharide-Induced Modulation of Murine Immune Responses

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Cryptococcus neoformans, an opportunistic fungal pathogen, often causes serious and life-threatening infections in immunocompromised hosts as well as in normal individuals. In the present study, purified cryptococcal capsular polysaccharide antigen was examined for its effect on several parameters of immune response and its ability to induce immune response to itself. Injection of the antigen into mice resulted in a dose-related specific antibody response which was detected at the individual antibody plaque-forming-cell level by a hemolytic assay in gel. Relatively low doses of cryptococci induced a maximal response, whereas higher doses resulted in a markedly depressed response. The antibody response to the cryptococcal capsular polysaccharide antigen appeared to be T cell independent and regulated by suppressor T cells, since mice injected with antilymphocyte serum or antithymocyte serum showed specific antibody responses to the antigen that were higher than those of untreated mice. It also markedly affected the in vitro mixed-lymphocyte reaction when added to cultures of mouse spleen cells being challenged in vitro with mitomycin C-treated allogeneic cells. The lower doses stimulated the response, whereas higher doses suppressed it. The macrophage response to yeast cells but not opsonized sheep erythrocytes was also modulated by the cryptococcal antigen.

It has been widely accepted that suppressed immune responses may be a prerequisite for clinical infection with opportunistic yeasts such as *Cryptococcus neoformans* (7, 8, 13). Infections by this yeast may be quite serious and life threatening, especially in immunocompromised hosts. However, local or systemic infections by this organism may occur with little or no evidence of preexisting immune derangements (4, 14). Thus, it was of interest to determine whether cryptococcal capsular polysaccharide, reputed to constitute a defense mechanism whereby the yeast avoids or blunts host cell phagocytosis, could affect immune responses per se. Previous studies in this laboratory have shown that a purified cryptococcal capsular polysaccharide antigen (CCPA) can markedly modulate the mitogenic as well as the primary in vivo and in vitro hemolytic antibody plaque responses of mouse spleen cells (I. C. Lee, J. F. Breen, F. R. Vogel, and H. Friedman, submitted for publication). In the present study, the specific antibody response to CCPA was assessed by an indirect plaque assay, as were the effects of antilymphocyte serum (ALS) and antithymocyte serum (ATS) on this immune parameter. Additionally, the possible immunomodulatory capacity of CCPA on the allogeneic response was investigated. The ability of this microbial capsular polysaccharide to affect phagocytosis of opsonized sheep erythrocytes (SRBCs) and unrelated yeasts (*Saccharomyces cerevisiae*) was also determined.

MATERIALS AND METHODS

Experimental animals. BALB/c and C57BL/6 female mice about 4 to 5 weeks of age were obtained from Jackson Laboratories, Bar Harbor, Maine. They were housed in groups of 10 in plastic mouse cages under pathogen-free conditions. The animals were maintained on water and Purina mouse pellets ad libitum.

Yeast culture and CCPA preparation. *C. neoformans* strain 371, a large capsule-forming variant of serotype A, B2550, was originally obtained from K. J. Kwon-Chung, National Institutes of Health, Bethesda, Md. The preparation of CCPA was performed exactly as described by Cherniak et al. (5). After preparation and purification, CCPA was dissolved in pyrogen-free saline at a concentration of 1 mg/ml as a stock solution, filter sterilized, and stored at −70°C until used. A control medium was prepared by the exact method used for preparing the polysaccharide except that no cryptococcal organisms were used. This control was included in all immunological assays.

CCPA-specific hemolytic plaque assay. To assay specific antibody responses to CCPA, BALB/c mice were injected intraperitoneally (i.p.) with doses of polysaccharide ranging from 0.01 to 100 μg contained in 0.5 ml

of buffered saline. After 5 days, mice were sacrificed by cervical dislocation, and spleen cell suspensions were prepared by "teasing" in medium 199 (GIBCO Laboratories, Grand Island, N.Y.) with forceps and serial passage through wire mesh screens and glass wool. The resulting spleen cell suspensions were diluted 1:5 with chilled medium 199 in 0.5-ml portions mixed with 50 μl each of guinea pig complement and SRBCs that were previously sensitized with CCPA as described by Baker et al. for pneumococcal polysaccharide (1). Samples of this mixture were assayed for hemolytic plaque-forming cells (PFCs) by using a modified PFC assay with the modified Cunningham chamber technique (6). Results obtained were expressed as CCPA-specific PFCs per spleen.

Antisera. Selected groups of mice being assayed for CCPA-specific PFCs also were treated in vivo with either rabbit anti-mouse ALS or ATS obtained from Microbiological Associates, Walkersville, Md. Undiluted antisera were administered as a single 0.3-ml i.p. injection concomitant with CCPA treatment. CCPA-specific PFCs were assayed on day 5 as described above.

Mixed-lymphocyte responses. Stimulator C57BL/6 spleen cells were prepared by incubating the cells at a concentration of 10^7 nucleated leukocytes per ml with 50 μg of mitomycin C (Sigma Chemical Co., St. Louis, Mo.) in RPMI 1640 medium (GIBCO) for 30 min at 37°C. The cells were then washed three times in complete medium and adjusted to a concentration of 2.5×10^6 cells per ml. The mitomycin C-treated cells (100-μl quantities) were mixed with equivalent volumes of responder BALB/c spleen cells at the same concentration in the round-bottom wells of microtiter plates (Nunc, Denmark). The cultures were treated with control extraction medium or CCPA equivalent to 1, 10, or 100 μg/ml and incubated for 72 h at 37°C. The plates were then pulsed with 0.5 μCi of [^{3}H]thymidine (New England Nuclear Corp., Boston, Mass.) and harvested after 18 h with a multiple automated sample harvester. The counts incorporated were assessed by standard liquid scintillation radiometry, and stimulation indexes were calculated by a standard formula.

SRBC phagocytosis. Peritoneal exudate (PE) cells were induced in BALB/c mice by i.p. injection of 1.0 ml of 5% dextran (Sigma). After 72 h, the PE cells were harvested by washing the peritoneum with 3 ml of RPMI 1640 medium and adjusted to a concentration of 8.0×10^5 nucleated cells per ml, and 100-μl samples were placed into 16-mm wells of Linbro plates (Linbro, Cambden, Conn.). After 1 h of incubation at 37°C, nonadherent cells were removed by washing with RPMI 1640 medium, and the adherent cells were refed with 2 ml of complete medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 μg of streptomycin and penicillin (GIBCO) per ml. The control extraction medium or CCPA, equivalent to 1, 10, or 100 μg/ml, was added to each well in 200-μl volumes, and the cells were incubated for 24 h at 37°C in 5% CO_2–air. The cultures were then washed twice with RPMI 1640 medium and opsonized, and chromium 51-labeled SRBCs were added. For preparation of the test SRBCs, 1 ml of a 5% suspension in Tris-phosphate buffer was mixed with 50 μCi of $Na_2{}^{51}CrO_4$ (New England Nuclear) and purified immunoglobulin G sheep hemolysin (Cappel Laboratories, Cockeysville, Md.) at a 1:800 dilution. SRBCs were then incubated at 37°C with continuous agitation for 30 min and washed three times in complete medium. After the final washing, they were resuspended in 25 ml of complete medium, and 1-ml aliquots were added to PE cell cultures. Phagocytosis was allowed to occur for 1 h at 37°C, and then nonphagocytized SRBCs were removed by two washes with RPMI 1640 medium. Adherent, noningested SRBCs were lysed by the addition of 1.0 ml of Tris-NH_4Cl buffer for 10 min, followed by two additional washes to remove residual radioactivity. The counts per minute of the phagocytized SRBCs were determined after treating the cultures with 600 μl of sodium dodecyl sulfate for 10 min. The supernatants were harvested, and 500-μl samples were counted by standard liquid scintillation radiometry.

Yeast cell phagocytosis. Normal BALB/c mice were sacrificed by cervical dislocation, and resident peritoneal cells were obtained by lavage with 3 ml of RPMI 1640 medium–10% fetal calf serum with 12-mm glass cover slips in Linbro 24-well plates. After 1 h, nonadherent cells were washed off as described above, and adherent cells were incubated further for 24 h in the presence of control extraction medium or CCPA. At this time, the adherent cells were washed twice with phosphate-buffered saline, and 1 ml of yeast suspension was added to each well. The yeast cells were prepared with heat-killed, commercially obtained bakers' yeast (*S. cerevisiae*). The yeast suspension was adjusted to 5.0×10^6 cells per ml in Dulbecco minimal essential medium (GIBCO) with 20% fetal calf serum before addition to the cover slip cultures. After 15 min of incubation at 37°C, the cover slips were washed twice with phosphate-buffered saline and fixed by dipping in fetal calf serum and air drying. The cover slips were stained with Camco Quik Stain (Scientific Products, Ocala, Fla.), and the degree of phagocytosis was assessed microscopically on the basis of two criteria: (i) the percentage of macrophages observed to ingest any number of yeast cells and (ii) percentage of macrophages seen containing five or more yeast cells.

RESULTS

CCPA-specific PFCs. BALB/c mice injected with graded doses of CCPA developed different numbers of specific-antibody-forming cells to the polysaccharide (Table 1). There were essentially no background PFCs to the polysaccharide in nonimmunized mice, since spleen cells from normal animals demonstrated fewer than 10 CCPA-specific PFCs per spleen. In contrast, when mice were injected with graded doses of CCPA, a marked increase in PFCs specific to the polysaccharide occurred. The 1.0-μg dose administered i.p. resulted in the highest PFC response, with the spleens of these mice developing approximately 10,000 specific PFCs per organ 5 days after immunization. This response was more than halved when the 10.0-μg concentration was used and decreased markedly at the 50- and 100-μg doses to approximately 1/30 of the maximum response. This dose response paralleled that reported for pneumococcal and other bacterial polysaccharides, where relatively high

TABLE 1. Specific antibody responses of mice to graded doses of CCPA

CCPA dose (μg)[a]	No. of animals per group	PFCs per spleen ± SD[b]
0	15	<10
0.1	5	$(4.8 \pm 3.6) \times 10^3$
1.0	15	$(9.6 \pm 5.3) \times 10^3$
10.0	16	$(4.1 \pm 3.7) \times 10^3$
50.0	5	$(2.2 \pm 2.5) \times 10^2$
100.0	10	$(3.1 \pm 2.5) \times 10^2$

[a] Groups of BALB/c mice were injected i.p. with the indicated doses of CCPA in 0.5 ml of phosphate-buffered saline.

[b] Average number of anti-CCPA PFCs per spleen 5 days after immunization.

doses resulted in less than optimum antibody responses.

Effects of ALS and ATS. It was of interest to determine whether CCPA could stimulate immunity in the absence of functional T lymphocytes since it is known that immune responses to bacterial polysaccharides, such as those obtained from pneumococci, are T-cell independent and are enhanced by the removal of suppressor T lymphocytes via treatment of challenged mice with anti-T-cell serum (2, 3). Mice treated with CCPA plus either ALS or ATS at the time of immunization had an approximately sixfold increase in the number of antibody-forming cells directed against the yeast antigen (Table 2). These animals were markedly immunosuppressed and showed longer allograft survival times after challenge with allogeneic skin grafts (data not shown), indicating that the antisera affected T-lymphocyte function. These results suggest that CCPA is a T-independent antigen and that suppressor T cells may influence the magnitude of the immune response as observed in the situation with pneumococcal polysaccharide.

Mixed-lymphocyte responses. The effects of CCPA on the mixed-lymphocyte response of BALB/c splenocytes against C57BL/6 alloantigens were examined since this response reflects the graft- and tumor-rejecting capacities of a host. The 1.0- and 10.0-μg doses of CCPA enhanced the allogeneic response by 45 and 64%, respectively, whereas the 100-μg concentration elicited a 30% decrease in this immune parameter (Table 3). It was also noted that cultivation of responder cells alone with the two lower CCPA doses resulted in increases of less than 20% in basal thymidine incorporation (data not shown).

TABLE 2. Effect of ALS and ATS on CCPA-specific PFC response

Animal group[a]	PFCs per spleen ± SD[b]
CCPA alone	$(5.2 \pm 1.1) \times 10^3$
CCPA plus ALS	$(3.0 \pm 1.1) \times 10^4$[c]
CCPA plus ATS	$(3.1 \pm 1.3) \times 10^4$[c]

[a] Groups of five BALB/c mice injected i.p. with 1.0 μg of CCPA in 0.5 ml of phosphate-buffered saline; antiserum-treated mice were given 0.3 ml of the indicated serum at the same time.

[b] Average number of anti-CCPA PFCs per spleen 5 days after immunization.

[c] Significant at $P < 0.05$.

TABLE 3. Effect of CCPA on mixed-lymphocyte responses of mouse spleen cell cultures

CCPA concn per culture (μg/ml)[a]	Mixed-lymphocyte response (% of control)[b]
0 (control)	100
1.0	145[c]
10.0	164[c]
100.0	70[c]

[a] Responder BALB/c and mitomycin C-treated stimulation C57BL/6 spleen cells (2.5×10^6/ml) mixed at a 1:1 ratio and graded doses of CCPA added at time zero.

[b] Average responses of five to six cultures pulsed with [^{3}H]thymidine 72 h after culture initiation. The results represent three replicate experiments.

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

Macrophage phagocytic activity. Another important parameter of the function of lymphocytes involves phagocytic uptake of antigen and particles by macrophages. Thus, it was of interest to determine whether CCPA affects phagocytic activity of murine peritoneal macrophages in vitro. Although 1.0 μg of CCPA had a slight but not significant effect on the uptake of SRBCs by PE cells, 10- and 100-μg doses had no detectable effect (Table 4). In contrast, the higher doses of CCPA markedly stimulated the phago-

TABLE 4. Effect of CCPA on phagocytic activity of murine peritoneal macrophages in vitro

CCPA per culture (μg/ml)[a]	% of control phagocytosis	
	SRBCs[b]	Yeast cells[c]
0 (control)	100	100
1.0	128	97
10.0	98	225[d]
100.0	97	214[d]

[a] Indicated dose of CCPA added to cultures of normal or mouse PE cells.

[b] BALB/c PE cells incubated with ^{51}Cr-labeled SRBCs for 1 h at 37°C.

[c] Normal BALB/c resident peritoneal cells incubated with heat-killed yeast cells for 15 min at 37°C. Data compare the number of macrophages ingesting five or more yeast cells.

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

cytic activity of peritoneal resident macrophages, as detected by increases in the percentage of phagocytic cells ingesting five or more yeast cells. The percentage of macrophages ingesting any number of yeast cells was not affected at any of the CCPA doses tested.

DISCUSSION

A number of studies have been reported concerning the immunochemistry of bacterial and fungal polysaccharides. The immune response to these polysaccharides is important in the host-parasite relationship. Polysaccharide antigens from bacteria such as *Streptococcus pneumoniae* (1) and from fungi such as cryptococci (9, 12) have been shown to induce a state of specific immunological nonresponsiveness or paralysis. The initial portions of this study concerning the dose response of CCPA as measured by a specific PFC response suggests a peak response after low-dose immunization, i.e., 1.0 μg, and suppression of this primary response after high-dose immunization, i.e., 50 and 100 μg. The significant increase in specific PFCs seen after ALS or ATS administration suggests a possible role for suppressor T cells in regulating the immune response to CCPA. Baker et al. (2) have demonstrated a similar effect of ALS on the PFC response to type 3 pneumococcal polysaccharide.

Experiments are in progress to further define the role of both helper and suppressor T cells in the immune response to CCPA. In contrast to others, who have shown that immunization with CCPA is capable of suppressing a secondary response to this polysaccharide (9, 12), we have demonstrated that T suppressor cells may have a role in immunoregulation of the primary response.

In addition to our findings regarding the specific PFC response to CCPA, it was of interest to note that the polysaccharide also modulates antigenically unrelated specific and nonspecific immune responses. Previous studies in this laboratory have shown that low doses of CCPA enhance the primary hemolytic antibody response to SRBCs, whereas larger doses (100 μg) depress this response. Since the immunoglobulin M antibody plaque response is known to be T helper cell dependent, the possibility that low CCPA concentrations nonspecifically enhance helper function must be considered. Furthermore, it is possible that transition from low to high CCPA doses represents a triggering threshold of helper versus suppressor activity. Thus, CCPA may serve as a probe for further delineating the intricacies of the immunoregulation during infection by yeast cells. The possibility that cryptococci or their products affect induction of suppressor macrophage populations should also be considered as well as the effects of CCPA on secondary immunoglobulin G antibody responses dependent upon helper T cells.

Studies based on the effect of CCPA on the mixed-lymphocyte responses were also of interest as this immune parameter is considered to be an in vitro correlate of delayed hypersensitivity. As observed in the present study, the relatively low doses of CCPA resulted in marked enhancement of this response, whereas the higher dose was suppressive. It is noteworthy that transplant recipients on any immunosuppressive regimen are frequent victims of cryptococcal and other opportunistic infections. If the data obtained in the murine model can be translated to the human system, then low doses of CCPA in situ may accelerate the allograft rejection, whereas higher doses may further suppress an already depressed immune system, thus possibly increasing the risk of other opportunistic infections.

Results from a number of laboratories have suggested that cryptococcal polysaccharide may modify the phagocytic activity of macrophages against intact yeast cells. For example, Kozel and Mastroianni (10) reported that cryptococcal polysaccharide inhibits both the attachment and the ingestion of non-encapsulated cryptococci by murine peritoneal macrophages. Additional studies by McGaw and Kozel (11) indicated that this polysaccharide can mask bound opsonins on cryptococci, thereby blocking agglutination of opsonized organisms by anti-immunoglobulin G serum. Although the exact mechanism for this inhibition is unknown, it would seem to be unrelated to the negative surface charge present in the capsular polysaccharide (9). The results of the present study indicate that a small dose of CCPA, i.e., 1.0 μg, induces a modest enhancement of opsonized SRBC phagocytosis, whereas the higher doses have little or no effect. Additionally, results of this study indicate that higher doses of CCPA actually enhance the ingestion of nonopsonized yeast cells. It is possible that *S. cerevisiae* and cryptococci may share a cross-reacting antigen, although the 24-h in vitro incubation period with CCPA would not appear to be adequate for primary sensitization. Further studies currently under way in this laboratory are involved with additional parameters of immune responses affected by CCPA as well as with attempts to delineate in more detail the target cell or cells for CCPA-induced immunomodulation. It appears likely that natural capsular constituents on an opportunistic microorganism such as cryptococci may modulate a variety of immune parameters by enhancing or suppressing selected cell classes dependent upon concentration. Additional studies in vivo with the polysaccharide and other cryptococcal antigens should provide information as to the rele-

vance of such immunomodulation to the infectious process per se.

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