

## Effects of Immunization with *Cryptococcus neoformans* Cells or Cryptococcal Culture Filtrate Antigen on Direct Anticryptococcal Activities of Murine T Lymphocytes

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**Immunizing CBA/J mice with intact *Cryptococcus neoformans* cells or with a cryptococcal culture filtrate antigen (CneF) induces an anticryptococcal delayed-type hypersensitivity response. Recently, it has been shown that two phenotypically different T-cell populations are responsible for delayed-type hypersensitivity reactivity in mice immunized with intact cryptococcal cells, whereas only one of those populations is present in mice immunized with soluble cryptococcal antigens in complete Freund's adjuvant (CFA). The purpose of this study was to determine if differences occur with regard to direct anticryptococcal activity between T-lymphocyte-enriched populations from mice immunized with intact viable or dead cryptococcal cells and similar cell populations from mice immunized with the soluble cryptococcal culture filtrate antigen, CneF, emulsified in CFA. The percentage of lymphocytes which form conjugates with *C. neoformans* and the percentage of cryptococcal growth inhibition *in vitro* are greater with T-lymphocyte-enriched populations from mice sublethally infected with *C. neoformans* or from mice immunized with intact heat-killed cryptococcal cells in the presence or absence of CFA than with lymphocyte populations from mice immunized with CneF-CFA. Enhanced anticryptococcal activity of T lymphocytes could be induced by immunizing mice with heat-killed *C. neoformans* cells of serotype A, B, C, or D as well as by immunizing with a similar preparation of an acapsular *C. neoformans* mutant but not by immunizing with CFA emulsified with CneF prepared from any one of the *C. neoformans* isolates. These data indicate that the soluble cryptococcal culture filtrate antigens do not induce the same array of functional T lymphocytes as whole cryptococcal cells.**

T lymphocytes are clearly important in protection against the yeast-like organism *Cryptococcus neoformans*, the etiological agent of cryptococcosis. This is evident from the fact that the individuals most susceptible to disseminated cryptococcal infections are those with T-cell deficiencies, especially individuals with AIDS (3, 6, 14, 27). The need for T lymphocytes in protection against *C. neoformans* also has been well documented with the murine model (8, 9, 15, 22). T-cell-deficient SCID and nude mouse strains are highly susceptible to fatal infections with *C. neoformans* (2, 5, 10). When immunologically competent mice are appropriately immunized with *C. neoformans* cells or with cryptococcal antigens, the animals develop a T-cell-dependent, anticryptococcal cell-mediated immune (CMI) response that is detectable by footpad testing for delayed-type hypersensitivity (DTH) (1, 13, 16, 18). In several studies, the development of anticryptococcal DTH reactivity has been associated with more effective clearance of the organism from infected tissues or with protection against subsequent *C. neoformans* infection (2, 4, 10, 15, 17, 21).

The anticryptococcal DTH response can be induced by giving a sublethal infecting dose of *C. neoformans*, by immunizing with heat-killed cryptococcal cells, or by immunizing with a soluble cryptococcal culture filtrate antigen (CneF) emulsified in complete Freund's adjuvant (CFA) (1, 4, 16, 18). Adoptive transfer studies have shown that CD4<sup>+</sup> T cells from CneF-CFA-immunized mice transfer the anticryptococcal DTH response to naive syngeneic mice (13, 18), whereas both CD4<sup>+</sup> and CD8<sup>+</sup> T cells from mice immunized with intact *C. neoformans* cells will transfer anticryptococcal DTH (18). These find-

ings suggest that immunization with intact *C. neoformans* cells induces a broader array of T-cell subsets with anticryptococcal DTH reactivity than can be induced with soluble cryptococcal antigens in CFA. We have previously shown that immunization of mice with intact heat-killed cryptococcal cells not only induces anticryptococcal DTH reactivity but also induces in the spleens and lymph nodes of the mice a population of T lymphocytes that will directly bind and inhibit *C. neoformans* growth *in vitro* (4, 25). The purpose of the study presented here was to determine whether immunization with the soluble cryptococcal antigen CneF in CFA, which induces a strong anticryptococcal DTH response in mice, will also induce a population of T cells that can directly bind to *C. neoformans* and inhibit cryptococcal growth.

### MATERIALS AND METHODS

**Mice.** Inbred, female CBA/J mice (*H-2<sup>k</sup>*) were purchased from Jackson Laboratory, Bar Harbor, Maine. The animals were maintained in the University of Oklahoma Health Science Center animal facility and used between 8 and 12 weeks of age.

**Organisms.** For all experiments unless otherwise indicated, *C. neoformans* serotype A isolate 184A was used for treatment of mice, for preparation of soluble culture filtrate antigen CneF, and for target cells in the conjugation and growth inhibition assays. In specified experiments, other isolates of *C. neoformans* were used. The isolates were serotype B isolate 435B, serotype C isolate 13C, and serotype D isolate 3501D (provided by J. Bennett and J. Kwon-Chung), as well as the serotype D acapsular isolate Cap67 (provided by E. Jacobson) (11). *C. neoformans* cultures were maintained on modified Sabouraud agar (MSAB) slants. Heat-killed *C. neoformans* cells for immunization were prepared by harvesting the blastoconidia in endotoxin-free sterile physiologic saline solution (SPSS) from the MSAB slants after 3 days of growth at room temperature and then incubating the cryptococci for 1 h at 60°C. After heat treatment, the sterility of each culture was demonstrated by the lack of growth on MSAB plates. CneF derived from each of the isolates listed above was produced as previously described (1).

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**Infections and immunizations with *C. neoformans* cells or with CneF.** To establish an infection, mice were given approximately  $5 \times 10^3$  viable *C. neoformans* cells intranasally as previously described (1, 16), and control mice were given an equal volume of SPSS by the same route. For immunization, three different protocols were used. One immunization protocol consisted of injecting each mouse with  $10^7$  heat-killed cryptococcal cells emulsified in CFA (1:1, vol/vol) subcutaneously at two sites on the lower abdomen (4). Preliminary experiments with increasing 10-fold increments in numbers of heat-killed *C. neoformans* ( $10^3$  to  $10^8$  cells) showed that  $10^7$  cryptococci with or without CFA induced the strongest DTH responses in CBA/J mice. Control mice for this group were similarly injected with an equal volume of SPSS emulsified in CFA (1:1, vol/vol). For the second immunization protocol, each mouse was injected subcutaneously with  $10^7$  heat-killed *C. neoformans* cells in SPSS at two sites on the lower abdomen, and control mice for this group received an equal volume (0.2 ml) of SPSS by the same route. The third immunization protocol entailed injecting each mouse with 0.1 ml of soluble CneF emulsified in CFA (1:1, vol/vol) at two sites on the lower abdomen. Control mice for this group received 0.1 ml of SPSS emulsified in CFA (1:1, vol/vol) injected subcutaneously at each of two sites on the lower abdomen.

**Detection of DTH reactivity.** The effectiveness of the infection and immunization procedures to induce an anticryptococcal CMI response was determined by footpad testing of the mice on day 34 after infection and on day 7 after the CneF-CFA or heat-killed *C. neoformans* treatment. The times after infection or immunization of the mice to be used in each protocol to elicit the anticryptococcal DTH reaction were selected on the basis of results from previous studies (16, 24). Five mice from each group were footpad tested by injecting 30  $\mu$ l of SPSS into one hind footpad and 30  $\mu$ l of CneF into the other hind footpad (1). The footpads were measured before injection and 24 h after injection to determine the amount of swelling, which is indicative of the level of anticryptococcal DTH reactivity (1).

**Preparation of effector and target cell populations.** Spleens from at least three mice were pooled, and single-cell suspensions were made. The spleen cells (SPC) were passed over nylon wool columns, and the nylon wool-nonadherent (NWN) cells were collected (12). These NWN-SPC populations were used as effector cells in the conjugation and cryptococcal growth inhibition assays. All effector cell populations were suspended in tissue culture media (TCM) consisting of RPMI 1640 with 10% fetal bovine serum. The NWN-SPC populations were depleted of B cells and macrophages (less than 1% of each) and were enriched for T cells (90 to 95% CD3<sup>+</sup> cells), as judged from flow cytometric analysis (25). To assess whether there were viable cryptococcal cells present in the spleens of infected mice, 1/10 of the total SPC suspension or the NWN-SPC pool was plated on MSAB plates and incubated for 3 days at room temperature before colony counts were made. No *C. neoformans* colonies grew on the MSAB plates, indicating that there were fewer than 10 viable cryptococci per spleen.

The target cells for the conjugation and growth inhibition assays were prepared by growing *C. neoformans* cells on MSAB slants for 1 day at room temperature, then transferring the cryptococcal cells to 10 ml of TCM, and growing the cells overnight at 37°C in 7% CO<sub>2</sub>. The cryptococcal cells grown in TCM were washed three times and then resuspended in fresh TCM before being used in the assays. Each target cell preparation was examined microscopically to ensure that the preparation was a single-cell suspension. In addition, the capsule width and particle size were measured on 200 cells of each of the four encapsulated isolates used in these studies after culturing of the cryptococci under the conditions used for preparation of target cells. The follow data were obtained on mean capsule width  $\pm$  1 standard deviation and particle size  $\pm$  1 standard deviation, respectively: isolate 184A,  $1.3 \pm 0.45 \mu$ m and  $6.5 \pm 1.19 \mu$ m; isolate 435B,  $2.2 \pm 2.6 \mu$ m and  $9.4 \pm 6.3 \mu$ m; isolate 13C,  $1.2 \pm 1.2 \mu$ m and  $6.9 \pm 2.3 \mu$ m; and isolate 3501D,  $2.5 \pm 1.4 \mu$ m and  $9.6 \pm 3.5 \mu$ m.

**Conjugation assay.** Conjugate formation between NWN-SPC effector cells and *C. neoformans* target cells was performed as previously described (7, 23, 26). Briefly,  $10^6$  NWN-SPC per 0.1 ml of TCM were mixed with  $5 \times 10^5$  viable *C. neoformans* cells per 0.1 ml of TCM (effector-to-target cell ratio of 2:1) in triplicate polystyrene Falcon tubes (12 by 75 mm; Becton Dickinson, San Jose, Calif.) and incubated for 2 h at 37°C in 7% CO<sub>2</sub>. After incubation, the conjugates were enumerated microscopically to determine the percentage of effector cells attached to *C. neoformans* cells. The number of effector cell-*C. neoformans* conjugates in a minimum of 200 effector cells was counted. In each sample, the percentage of effector cells bound to two or more cryptococcal cells was assessed and was consistently less than 1.

**Assay for growth inhibition of *C. neoformans*.** The growth inhibition assay was performed as previously described (25). Briefly,  $10^6$  NWN-SPC per 0.1 ml of TCM and  $5 \times 10^5$  viable *C. neoformans* cells per 0.1 ml of TCM were dispensed into quadruplicate wells of a flat-bottom 96-well microtiter plate (Limbro Scientific Co., Hamden, N.Y.). For controls,  $5 \times 10^5$  viable *C. neoformans* cells in 0.2 ml of TCM were added to quadruplicate wells. The assays were incubated at 37°C for 24 h in 7% CO<sub>2</sub>, after which time the cells in the wells were mixed and samples were removed, diluted, and plated on MSAB plates. The plates were incubated for 3 days at room temperature, and the CFU were counted. The percent growth inhibition was calculated as follows: [(number of CFU from control wells - number of CFU from test wells)/number of CFU from control wells]  $\times$  100.

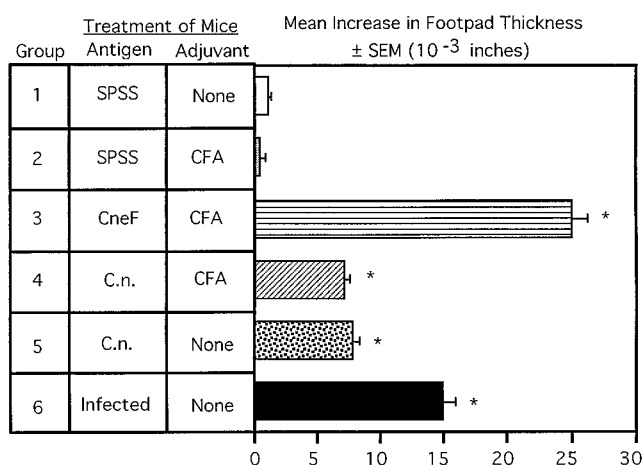


FIG. 1. DTH responses elicited by CneF in mice treated 8 days earlier with SPSS, SPSS-CFA, CneF-CFA, or heat-killed *C. neoformans* cells (C.n.) with or without CFA or infected intranasally 35 days earlier with viable *C. neoformans* cells. Data are representative of three experiments with five mice per group per experiment. Error bars represent SEM. \*, significant difference ( $P < 0.001$ ) compared with control group 1 or 2. One inch = 2.54 cm.

**Statistical analysis.** Calculations of means, standard errors of the means (SEMs), and unpaired Student's *t* test were used to analyze the data. In comparing two groups, a *P* value of  $\leq 0.05$  indicates a significant difference between the groups.

## RESULTS

**Detection of anticryptococcal DTH reactions in *C. neoformans*-treated, CneF-CFA-treated, and control mice.** To ensure that the mice treated with *C. neoformans* cells or with CneF-CFA developed an anticryptococcal CMI response, their levels of DTH reactivity were measured. When footpad tested with CneF, mice immunized with CneF-CFA, intact cryptococcal cells-CFA, or cryptococcal cells alone or mice infected with *C. neoformans* developed DTH responses which were significantly greater than reactions observed in control mice (Fig. 1; compare groups 3 to 6 with groups 1 and 2;  $P < 0.001$ ). Emulsifying cryptococcal cells in CFA before immunization did not significantly alter the level of DTH responsiveness of the mice (Fig. 1; compare group 4 with group 5). The DTH reactions in mice immunized with intact killed *C. neoformans* cells in the presence or absence of CFA were significantly less than the DTH responses of mice immunized with CneF-CFA (Fig. 1; compare group 4 or 5 with group 3;  $P \leq 0.002$ ). Furthermore, DTH responses of mice immunized with killed cryptococcal cells were also slightly but significantly less than the anticryptococcal DTH reactions assessed at day 35 after mice were given an intranasal dose of viable *C. neoformans* (Fig. 1; compare group 4 or 5 with group 6;  $P \leq 0.003$ ). In addition, the DTH reactions of the infected mice were significantly lower than the anticryptococcal DTH reactions of the mice immunized with soluble antigen in CFA (Fig. 1; compare group 6 with group 3;  $P = 0.002$ ).

**Direct interactions of NWN-SPC with *C. neoformans* cells.** Immunizing mice with cryptococcal cells increases, in comparison with controls, the percentage of T cells which bind to *C. neoformans* cells in vitro (25). To determine if treating mice with CneF-CFA also increases the ability of T lymphocytes to bind to *C. neoformans* cells, the percentages of conjugates formed with NWN-SPC from mice treated 8 days earlier with SPSS, SPSS-CFA, CneF-CFA, *C. neoformans*-CFA, or *C. neoformans* cells alone or infected 35 days earlier with viable *C.*

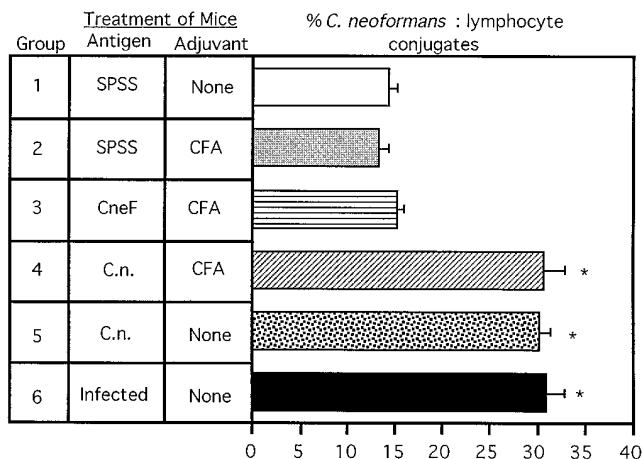


FIG. 2. Mean percentages of lymphocytes that formed conjugates with *C. neoformans* after a 2-h incubation. Lymphocytes were NWN-SPC isolated from mice treated 8 days earlier with SPSS, SPSS-CFA, CneF-CFA, or *C. neoformans* cells (C.n.) with or without CFA or infected intranasally 35 days earlier with viable *C. neoformans* cells. Three mice per group were used in each experiment, and data are representative of three experiments. Error bars represent SEM. \*, significant difference ( $P < 0.001$ ) compared with control group 1 or 2.

*neoformans* were assessed. NWN-SPC from mice treated with intact *C. neoformans* cells formed significantly greater numbers of conjugates with *C. neoformans* than did NWN-SPC from the respective control mice (Fig. 2; compare groups 4 to 6 with groups 1 and 2;  $P < 0.001$ ). Emulsification of *C. neoformans* cells in CFA prior to immunization did not significantly alter the percentage of NWN-SPC which bound cryptococcal cells (Fig. 2; compare group 4 with group 5). In contrast, the percentage of NWN-SPC from CneF-CFA-treated mice that bound cryptococci was similar to the percentage of control lymphocyte-*C. neoformans* conjugates (Fig. 2; compare group 3 with group 1 or 2). These data suggest that treating mice with CneF-CFA does not enhance the ability of the T cells to form conjugates with *C. neoformans* cells.

**Inhibition of *C. neoformans* growth by NWN-SPC.** Previously we have shown that binding of T lymphocytes to *C. neoformans* is prerequisite to inhibition of cryptococcal growth (25). Thus, lymphocyte populations which do not bind to cryptococcal cells would not be expected to mediate inhibition of *C. neoformans* growth. To determine if this prediction holds in the present study, *C. neoformans* growth inhibition assays were done with NWN-SPC effector cell populations from the same six groups of mice used in the conjugate assays. As anticipated, NWN-SPC from mice treated with CneF-CFA did not inhibit the growth of *C. neoformans* cells any better than did NWN-SPC from control mice (Fig. 3; compare group 3 with group 2). In contrast, NWN-SPC obtained 35 days after infection of mice with *C. neoformans* as well as similar SPC populations taken 8 days after immunization with heat-killed cryptocoeci were able to inhibit *C. neoformans* growth significantly better than NWN-SPC from control mice or NWN-SPC from mice treated 8 days earlier with CneF-CFA (Fig. 3; compare groups 4 to 6 with group 1, 2, or 3;  $P < 0.001$ ). As with conjugate formation, emulsifying intact cryptococcal cells in CFA before immunization did not significantly alter the ability of NWN-SPC to inhibit the growth of *C. neoformans* (Fig. 2; compare groups 4 and 5). In one experiment, we also assessed the abilities of NWN-lymph node cells from mice treated 8 days earlier with CneF-CFA or SPSS-CFA to inhibit the growth of *C. neoformans* and found the percent growth inhibition to be  $11.4 \pm 1.9$

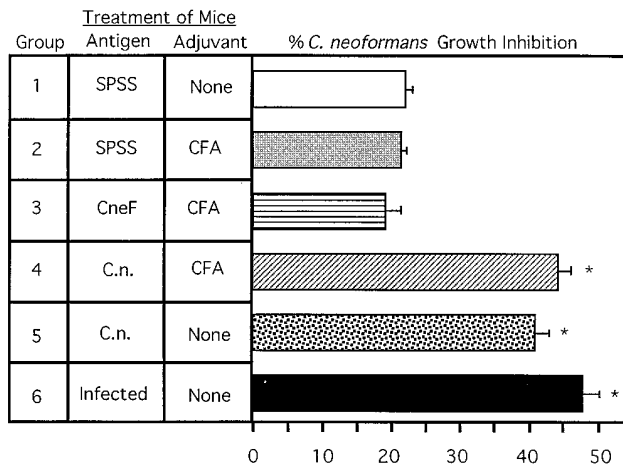


FIG. 3. Mean percent growth inhibition of *C. neoformans* mediated by NWN-SPC from mice that were treated 8 days earlier with SPSS, SPSS-CFA, CneF-CFA, or *C. neoformans* cells (C.n.) with or without CFA or infected intranasally 35 days earlier with viable *C. neoformans* cells. Three mice per group were used in each experiment. The data are representative of three experiments. Control wells contained  $(1.3 \pm 0.07) \times 10^6$  viable *C. neoformans* cells. Error bars represent SEM. \*, significant difference ( $P < 0.001$ ) compared with control group 1 or 2.

or  $9.7 \pm 0.8$ , respectively. Both results were at the lower end of the range of the percentages of cryptococcal growth inhibition obtained with NWN-lymph node cells from mice given saline 8 days previously but significantly below the percentage of cryptococcal growth inhibition with NWN-lymph node cells from mice immunized with *C. neoformans* cells ( $37.1\% \pm 1.0\%$  in this experiment).

**Kinetics of the anticryptococcal responses.** Although no increase in lymphocyte-mediated anticryptococcal activity was detected when lymphocytes were isolated 8 days after CneF-CFA treatment, it is possible that anticryptococcal activity of the lymphocytes is transient, with measurable levels occurring at a time other than 8 days after immunization. To assess this possibility, NWN-SPC were collected from CneF-CFA-treated mice on days 1, 4, 6, 8, 10, and 12 after immunization and assayed. Also, in separate groups of mice, anticryptococcal DTH responses were determined at each of the times. Anticryptococcal DTH reactivity was detectable by 4 days after immunization with CneF-CFA and continued to increase until day 6. From days 6 through 12, the level of DTH reactivity in the CneF-CFA-immunized mice remained constant (Fig. 4A). In contrast, DTH reactions of mice immunized with intact heat-killed *C. neoformans* were not detectable until 6 days after immunization; the DTH reactivity did not peak until 8 days and then remained constant for the remainder of the experimental period. The level of the DTH reactivity in the *C. neoformans*-immunized mice never reached the level of the DTH reactivity observed in CneF-CFA-immunized mice (Fig. 4A; CneF-CFA group compared with C.n. [*C. neoformans*-immunized] group on days 4 to 12;  $P \leq 0.005$ ). Control mice did not display anticryptococcal DTH responses at any time point tested (Fig. 4A).

The NWN-SPC obtained on day 8, 10, or 12 after immunization with heat-killed cryptocoeci displayed significantly increased abilities to bind and inhibit the growth of *C. neoformans* compared with NWN-SPC from control mice (Fig. 4B and C). In contrast, irrespective of the time that the effector cells were obtained after immunization, NWN-SPC from CneF-CFA-immunized mice had no greater ability to bind or

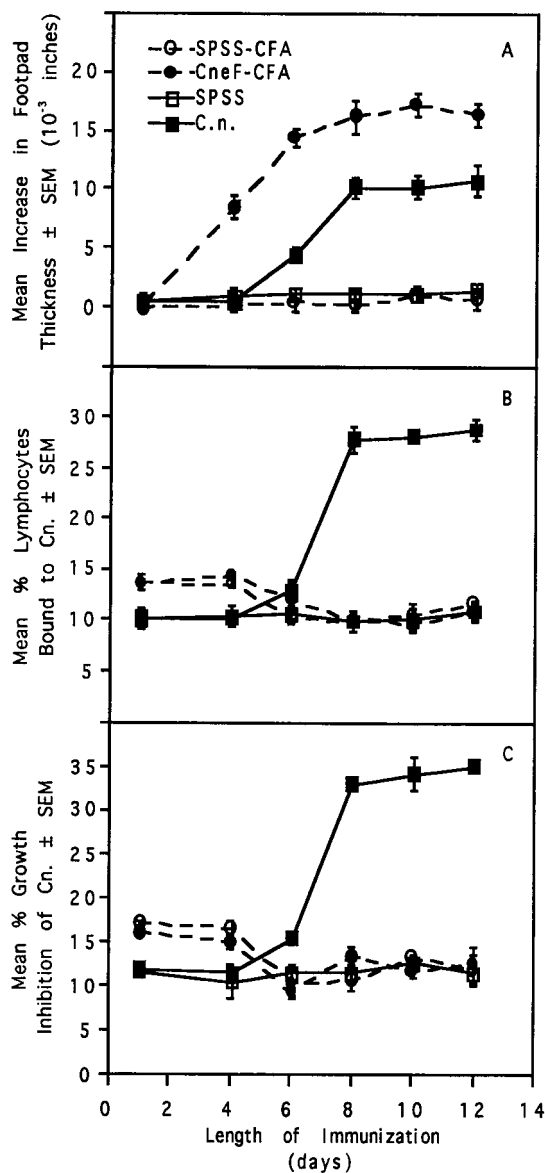


FIG. 4. Kinetics of DTH reactions, mean percent lymphocyte-*C. neoformans* conjugates, and mean percent lymphocyte-mediated growth inhibition of *C. neoformans* after immunization with *C. neoformans* cells (C.n.) or soluble antigen (CneF-CFA). (A) DTH responses elicited by CneF in mice treated 1, 4, 6, 8, 10, or 12 days earlier with SPSS-CFA, CneF-CFA, SPSS, or C.n. (B) Mean percent conjugates formed after a 2-h incubation of *C. neoformans* with NWN-SPC isolated from mice that were treated 1, 4, 6, 8, 10, or 12 days earlier with SPSS-CFA, CneF-CFA, SPSS, or C.n. (C) Mean percent growth inhibition of *C. neoformans* mediated by NWN-SPC from mice that were treated 1, 4, 6, 8, 10, or 12 days earlier with SPSS-CFA, CneF-CFA, SPSS, or C.n. Five mice per group were used in DTH experiments, and three mice per group were used in conjugation and growth inhibition experiments. The data are representative of three experiments. Error bars represent SEM.

inhibit the growth of *C. neoformans* than NWN-SPC from SPSS-CFA-treated control mice (Fig. 4B and C). The NWN-SPC from mice treated with SPSS-CFA or CneF-CFA displayed a slight but significant and reproducible increase in anticyptococcal activity over lymphocytes from SPSS- or *C. neoformans*-treated mice on days 1 and 4 after immunization (Fig. 4B and C;  $P \leq 0.05$ ). By day 6 after treatment, the direct anticyptococcal activity of lymphocytes from animals given CFA was back to control levels.

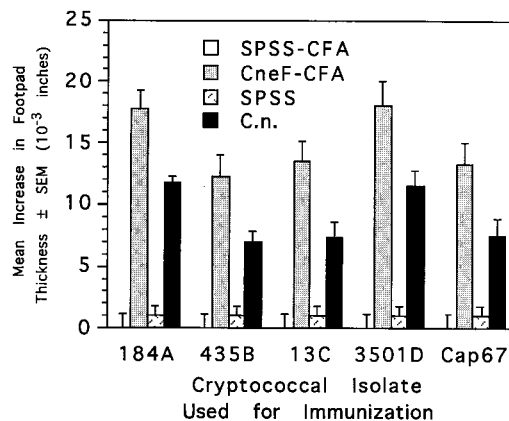


FIG. 5. DTH responses elicited by CneF-isolate 184A in mice treated 7 days earlier with SPSS-CFA; CneF-CFA from *C. neoformans* isolate 184A, 435B, 13C, 3501D, or Cap67; SPSS; or heat-killed *C. neoformans* cells (C.n.) of isolate 184A, 435B, 13C, 3501D, or Cap67. Data are representative of two experiments with five mice per group per experiment. Error bars represent SEM. \*, significant difference ( $P < 0.001$ ) compared with control group 1 or 2.

**Induction of T-cell-mediated anticyptococcal activity by various isolates of *C. neoformans* cells and their CneF.** From this investigation as well as from earlier studies, it is clear that intact *C. neoformans* isolate 184A cells can induce, in the lymph nodes and spleens of mice, T lymphocytes which have enhanced abilities to bind and inhibit *C. neoformans* cell growth compared with control T lymphocytes (4, 25). In this study, we tested the potential of immunization with heat-killed cryptococcal cells or culture filtrate antigen prepared from two *C. neoformans* var. *gattii* isolates (435B and 13C) and from one additional *C. neoformans* var. *neoformans* isolate (3501D) as well as one acapsular mutant (Cap67) to induce DTH reactivity and to induce T lymphocytes with direct anticyptococcal activity. All *C. neoformans* isolates used for immunization, whether injected as intact cells or as a soluble culture filtrate in CFA, induced anticyptococcal CMI responses detectable by footpad testing (Fig. 5). As with *C. neoformans* isolate 184A, mice immunized with CneF prepared from any one of the *C. neoformans* isolates and footpad tested with CneF prepared with 184A (CneF-184A) had stronger DTH responses than did mice immunized with the intact cryptococcal cells of the respective isolate and footpad tested with CneF-184A (Fig. 5). DTH responses of mice immunized with *C. neoformans* cells or with CneF of isolate 184A or 3501D were slightly but significantly greater than the DTH responses of mice immunized with isolate 435B or 13C (Fig. 5;  $P \leq 0.05$ ). Mice immunized with the acapsular mutant Cap67, which was derived from isolate 3501D, had significantly smaller footpad responses to CneF-184A than did mice immunized with 3501 (Fig. 5; when the immunogen was CneF-CFA,  $P = 0.03$ ; when the immunogen was intact cryptococcal cells,  $P = 0.01$ ).

The NWN-SPC from mice immunized with the intact cryptococcal cells, regardless of the *C. neoformans* isolate, displayed direct anticyptococcal activity, as determined by in vitro growth inhibition of isolate 184A cryptococcal cells, when compared with NWN-SPC from SPSS-injected mice (Fig. 6;  $P < 0.005$ ). In contrast, none of the NWN-SPC populations from the CneF-CFA-treated groups inhibited the growth of isolate 184A any better than the SPSS-CFA control NWN-SPC (Fig. 6). The anticyptococcal activity of lymphocytes isolated from

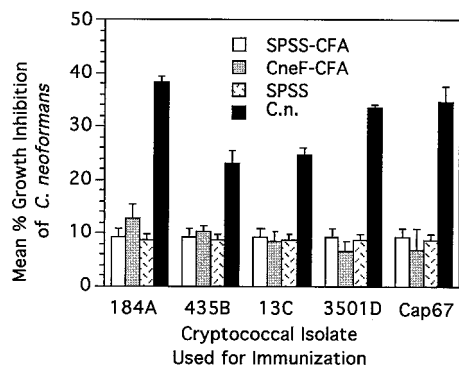


FIG. 6. Mean percent growth inhibition of *C. neoformans* isolate 184A mediated by NWN-SPC from mice that were treated 8 days earlier with SPSS-CFA; CneF-CFA from isolate 184A, 435B, 13C, 3501D, or Cap67; SPSS; or *C. neoformans* cells (C.n.) from isolate 184A, 435B, 13C, 3501D, or Cap67. Three mice per group were used in each experiment. The data are representative of two experiments. Error bars represent SEM.

mice treated with *C. neoformans* cells of isolate 184A or 3501D was slightly but significantly greater than that mediated by lymphocytes from mice immunized with *C. neoformans* isolate 435B or 13C cells (Fig. 6;  $P \leq 0.008$ ). Since it is possible that the lower reactivity of the NWN-SPC from groups of mice immunized with isolate 435B or 13C could have been due to the use of a heterologous isolate for assessment of the direct anticryptococcal activity, we also used as target cells the same isolate used for immunization. When the experiment was performed with target cells homologous to the cryptococcal isolate used for immunization, we found that lymphocytes from mice treated with cryptococcal cells but not with CneF from isolate 184A, 435B, 13C, or 3501D inhibited the growth of viable 184A, 435B, 13C, or 3501D cells, respectively, better than did lymphocytes from SPSS-treated control mice (Fig. 7;  $P < 0.005$ ). Immunization with the CneF from the same isolate as the target cells did not induce a significant increase in direct anticryptococcal activity in the lymphocytes over control levels (Fig. 7). As observed with the heterologous target cells (Fig. 6), when the target cells were homologous to the immunizing cells, lymphocytes from mice treated with intact cells of *C. neoformans* isolate 184A or 3501D displayed greater direct anticryp-

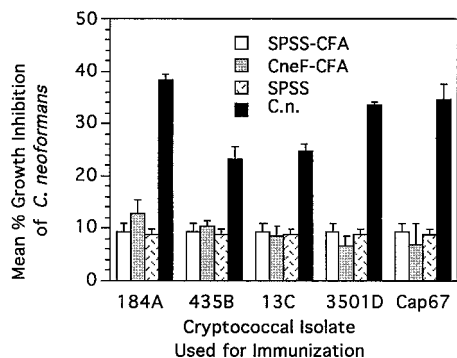


FIG. 7. Mean percent growth inhibition of *C. neoformans* mediated by NWN-SPC from mice that were treated 8 days earlier with SPSS-CFA; CneF-CFA from isolate 184A, 435B, 13C, 3501D, or Cap67; SPSS; or *C. neoformans* cells (C.n.) of the designated isolate. Target cells were of the same *C. neoformans* isolate as used for immunization. Three mice per group were used in each experiment. The data are representative of two experiments. Error bars represent SEM.

tococcal activities than lymphocytes from mice treated with *C. neoformans* isolate 435B or 13C (Fig. 7;  $P \leq 0.01$ ).

## DISCUSSION

The results presented here demonstrate that there are functional differences in lymphocytes from mice immunized with the soluble cryptococcal antigen CneF in CFA and in lymphocytes from mice immunized with intact *C. neoformans* cells. Although both immunogens, CneF-CFA and intact cryptococcal cells, induce anticryptococcal DTH responses, only immunization with intact *C. neoformans* cells up-regulates the direct anticryptococcal activity of murine lymphocytes. Lymphocytes with direct anticryptococcal activity are induced as effectively with viable cryptococcal cells as with heat-killed cryptococcal cells. Up-regulation of lymphocyte-mediated direct anticryptococcal activity by immunization with intact cryptococcal cells is not a unique feature of *C. neoformans* isolate 184A, a serotype A isolate. At least one isolate from each of the other three *C. neoformans* serotypes as well as the acapsular mutant strain Cap67, when injected subcutaneously into mice as intact organisms, are able to induce NWN-SPC with direct anticryptococcal activity. Furthermore, the direct anticryptococcal activity of the lymphocyte populations is not specific for the isolate which was used to induce the response, because all four of the isolates used as intact cells for immunization of mice induced NWN-SPC populations that had direct anticryptococcal activity against the homologous *C. neoformans* isolate as a target cell and against the heterologous isolate 184A. Presence or absence of the capsule on the cryptococcal cells does not influence the induction of the direct anticryptococcal activity. This is evident from the fact that intact cells of Cap67, the acapsular mutant which was derived from *C. neoformans* isolate 3501 (11), are able to induce direct anticryptococcal activity in the lymphocytes as effectively as the encapsulated parent strain 3501. Emulsification of the intact cryptococcal cells in CFA does not alter the level of direct anticryptococcal activity induced in the NWN-SPC. This is demonstrated by lack of difference in the direct anticryptococcal activity of NWN-SPC from mice given *C. neoformans* cells in the absence of CFA and of NWN-SPC from mice immunized with intact cryptococcal cells emulsified in CFA. Injection of mice with CFA mixed with SPSS or CneF does induce a slight increase in the direct anticryptococcal activity of NWN-SPC during the first 4 days after treatment, suggesting that CFA has an early effect on direct anticryptococcal activity, but the effect does not extend to 8 days after immunization, when the direct anticryptococcal activity of the NWN-SPC can be related to an active, acquired immune response induced by intact cryptococci. Together these data indicate that neither CFA nor early effects of CFA on the system alter the level of direct anticryptococcal activity observed at 8 days after immunization with intact cryptococcal cells.

Since we were unable to detect direct anticryptococcal activity in the NWN-SPC obtained 8 days after immunization of mice with CneF-CFA, we made two additional attempts to induce direct anticryptococcal activity in NWN-SPC populations by immunizing with soluble cryptococcal antigens. First, we performed a kinetic study of the direct anticryptococcal activity after immunization with CFA emulsified with CneF prepared from isolate 184A. If the time after immunization when the NWN-SPC were assessed for conjugate formation and growth inhibition of *C. neoformans* was not appropriate for detection of the activity, then the kinetic study should identify a time when the activity is present. However, by measuring the direct anticryptococcal activity of the NWN-SPC at 2-day in-

tervals through day 12 after immunization with CneF-CFA, no direct anticryptococcal activity was observed at any time. Our second attempt to induce direct anticryptococcal activity in NWN-SPC by immunization with soluble antigen was done by using four additional preparations of CneF, each made with a different isolate of *C. neoformans*. None of the CneF-CFA preparations induced the direct anticryptococcal response at 8 days after immunization, indicating that the CneF prepared with *C. neoformans* isolate 184A was not the only soluble antigen preparation incapable of inducing direct anticryptococcal activity. The lack of activity was not due to the animals being resistant to sensitization, because all of the immunized mice displayed solid CMI responses as detected by footpad swelling reactions to CneF. Furthermore, the CFA was not likely to be inhibiting the response because CFA had no measurable effects on the direct anticryptococcal activity induced by intact cryptococcal cells. All considered, it appears that soluble culture filtrate antigens are not sufficient to boost direct anticryptococcal activity of the T lymphocytes.

The three immunization protocols used, i.e., infection with viable cryptococci, immunization with heat-killed *C. neoformans* cells with or without CFA, and immunization with CneF-CFA, induced anticryptococcal CMI responses as indicated by the DTH reactions of the immunized mice. CneF-CFA induced the strongest DTH responses yet did not induce direct anticryptococcal activity in the splenic T lymphocytes or in lymph node T cells, for that matter. These results are in accord with the findings of Mody et al. (18), who showed that immunization of mice with intact *C. neoformans* cells induces two populations of T lymphocytes, CD4<sup>+</sup> and CD8<sup>+</sup> cells, which are capable of transferring anticryptococcal CMI reactivity to naive mice (18). In contrast, immunization with soluble cryptococcal antigen in CFA induces only CD4<sup>+</sup> T cells capable of transferring anticryptococcal DTH reactivity (18). Our results are similar in that immunization with intact cryptococcal cells induced two functional T-cell populations or one T-cell population with two functions, i.e., DTH reactivity and direct anticryptococcal activity. As in the model of Mody et al. (18), in our experiments soluble cryptococcal antigen in CFA did not induce both populations of lymphocytes or both T-cell functions. Mice immunized with soluble antigen displayed only one of the two functions, DTH reactivity.

The differences in phenotypes and functions of T cells induced by the intact organism and by soluble products of the cryptococci might be explained by examining the processes that occur during the induction stage of the responses. Little is known in this regard; however, from our current understanding of T-cell induction processes, one might speculate that in the case of *C. neoformans*, two different induction pathways may be possible. When CneF-CFA is the immunogen, one would expect that antigen-presenting cells (APC) would phagocytize the oil droplets containing the cryptococcal antigen, process the antigen, and present the appropriate portion of the antigen on the class II major histocompatibility complex molecules on the APC surface to T cells bearing the complementary T-cell receptor (20). The interaction of the APC-bearing antigen and the T cell would be expected to cause the T cells to differentiate and proliferate, resulting in mature Th1 cells capable of DTH reactivity upon restimulation with antigen (19). On the other hand, when intact *C. neoformans* cells are the immunogen, not only does the above pathway for induction of T cells occur, but since T lymphocytes can bind directly to cryptococci, one might predict that the direct T-cell interactions with the cryptococcal cells might induce another activation pathway in the lymphocytes. For example, conjugate formation between T cells and cryptococcal cells might induce increases or changes

in receptor expression in the T cells, might induce maturation of the T cells along with associated functions, or possibly could induce maturation and proliferation of the T cells. It is possible that this latter induction pathway is the process by which the CD8<sup>+</sup> DTH-responsive cells are induced in the model described by Mody et al. (18) and by which the T cells with direct binding and growth inhibitory activity for *C. neoformans* are induced in our model.

When intact *C. neoformans* cells were used as the immunogen, the levels of T-cell-mediated direct anticryptococcal activity induced by each of the various *C. neoformans* isolates mirrored the levels of DTH reactivity induced by the respective *C. neoformans* isolate. In general, immunization with the *C. neoformans* var. *neoformans* cells (isolates 184A and 3501D) stimulated higher DTH reactivity and induced higher direct anticryptococcal activity in lymphocyte populations than were induced by immunization with *C. neoformans* var. *gattii* cells (isolates 435B and 13C). This was true when a *C. neoformans* isolate heterologous or homologous to the immunizing *C. neoformans* isolate was used as the target cells for the binding and growth inhibition assays. The differences in results on direct anticryptococcal activity between T lymphocytes from the *C. neoformans* var. *neoformans* (184A and 3501D)-immunized mice and T lymphocytes from *C. neoformans* var. *gattii* (435B and 13C)-immunized mice cannot be attributed to variance in capsule size between the *C. neoformans* isolates, because isolates 184A and 13C have similar capsule widths (1.3 and 1.2  $\mu\text{m}$ , respectively) and isolates 435B and 3501D have similar capsule widths (2.2 and 2.5  $\mu\text{m}$ , respectively). These observations suggest, but do not establish, that isolates of *C. neoformans* var. *neoformans* are slightly more immunogenic than *C. neoformans* var. *gattii* isolates. Additional isolates of each variety must be evaluated for their abilities to induce T cells responsible for direct anticryptococcal activity and DTH reactivity to substantiate this speculation.

The results from this study represent important basic knowledge which is prerequisite to examining the potential of each of the immunization protocols for its effectiveness as a vaccine against *C. neoformans*. From the high level of DTH reactivity induced by the CneF-CFA, one might predict that the soluble antigen in an appropriate adjuvant might provide the greatest degree of protection. However, it could also be argued that the intact killed cryptococci, which induce a lower DTH response but also stimulate direct anticryptococcal activity in the T lymphocytes, might be as effective as the soluble antigen in protecting the host. Additional studies must be performed to resolve which type of immunization protocol offers the greatest amount of protection against a challenge infection with *C. neoformans*. The ability to induce an anticryptococcal CMI response with one isolate of *C. neoformans* and detect the response with a different isolate suggests that a single isolate of *C. neoformans* could be used for a vaccine which would be effective against other isolates of *C. neoformans* without regard to the serotype of the eliciting isolate.

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