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Antigenicity and Immunogenicity of an Extract from the Cell Wall and Cell Membrane of *Histoplasma capsulatum* Yeast Cells

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In order to identify T-cell antigens from *Histoplasma capsulatum* yeast cells, we prepared a detergent extract of the cell wall and cell membrane of yeast-phase *H. capsulatum* G217B and analyzed its antigenicity and immunogenicity. Mice injected with viable *H. capsulatum* yeast cells or with 500 or 1,000 μg of the extract mounted a delayed-type hypersensitivity response to solubilized cell wall and cell membrane. Vaccination with this antigenic preparation conferred a protective immune response in mice that were challenged intravenously with *H. capsulatum* yeast cells. The extract induced in vitro proliferation by splenocytes from mice injected with either viable yeast cells or the soluble cell wall and cell membrane preparation. We also examined the profile of in vitro responses by a murine T-cell line and by cloned T cells to soluble cell wall and cell membrane by employing the technique of T-cell immunoblotting. Two prominent regions that stimulated the T-cell line and cloned T cells were identified. Fractions encompassing an area between 53 and 64 kDa caused proliferation by a T-cell line and five of six clones. Antigens recognized by the T-cell line and by three of six clones were contained in another area that extended from 69 to 82 kDa. The data demonstrate that this soluble extract from cell wall and cell membrane contains antigens recognized by T cells and mediates protective immunity. Moreover, T-cell immunoblotting provides a useful technique for mapping immunoreactive molecules from *H. capsulatum* yeast cells.

T lymphocytes, and in particular the $CD4^+$ subset, play a critical role in limiting the replication of *Histoplasma capsulatum* yeasts within macrophages (2, 4, 6, 11, 20, 23). It is believed that upon recognition of *H. capsulatum* antigens, $CD4^+$ T cells are stimulated to release lymphokines, among them gamma interferon (6). In turn, this mediator induces murine macrophages to inhibit intracellular proliferation of yeasts (25, 26).

Although yeast cells are the parasitic form of *H. capsulatum*, little is known about the identity of antigenic determinants from this phase that activate T cells or confer protection. Previous work has demonstrated that injection of ethylenediamine extracts of *H. capsulatum* yeast cells or cell walls into naive mice mediates a protective immune response (10). In another study, cell wall glycoproteins or soluble cytoplasmic material from *H. capsulatum* yeast cells elicited a delayed-type hypersensitivity (DTH) reaction in immunized guinea pigs and production of migration inhibition factor by peritoneal exudate cells from immune guinea pigs (8).

In this study, we have initiated a series of experiments to analyze the T-cell antigens from *H. capsulatum* yeast cells. A detergent extract of cell walls and cell membranes from *H. capsulatum* yeast cells (CW/M) was prepared and tested for its antigenicity and immunogenicity. The results demonstrated that this preparation induced a vigorous cell-mediated immune response both in vivo and in vitro. In addition, to identify the molecular masses of antigens in this detergent extract, we examined the blastogenic responses of a *H. capsulatum*-reactive T-cell line and T-cell clones to electrophoretically separated CW/M by employing the method of T-cell immunoblotting (1).

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MATERIALS AND METHODS

Mice. Male C57BL/6 mice were obtained from the Jackson Laboratory, Bar Harbor, Maine.

Injection of mice with *H. capsulatum* yeast cells. Preparation and inoculation of *H. capsulatum* G217B yeast cells have been described previously (3). Briefly, yeast cells were harvested after they were cultured for 36 h in brain heart infusion broth at a gyratory speed of 200 rpm and washed three times in a balanced salt solution. To immunize mice with viable *H. capsulatum* yeast cells, animals were injected subcutaneously with 10^6 yeast cells followed by an intravenous injection of 6×10^5 yeast cells 2 weeks later. After 3 weeks, mice received an intraperitoneal injection of 5×10^6 yeast cells.

In some experiments, mice were injected intravenously with 6×10^5 yeast cells. This is a sublethal inoculum that produces less than 5% mortality in mice 30 days postinoculation. In other studies, mice were injected intravenously with 4×10^6 yeast cells. This number of yeast cells causes 100% mortality in mice within 15 days.

Preparation of CW/M. To prepare CW/M, strain G217B yeast cells were inoculated into Ham's F-12 liquid medium supplemented with cystine (8.4 μg/liter), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) (6 g/liter), glutamic acid (1 g/liter), and glucose (18.2 g/liter) and were grown at 37°C in a shaking incubator for 48 h (24). Yeast cells were harvested and killed by incubation in phosphate-buffered saline (PBS), pH 7.2, containing thimerosal (1:10,000, wt/vol) at room temperature for 1 h. Yeast cells were washed and resuspended in PBS containing 1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, and 5×10^{-4} M disodium EDTA, at a concentration of 1 volume of packed cells to 2 volumes of buffer. Cells were disrupted in a bead beater (Biospec Products, Bartlesville, Okla.) at 4°C for 6 min by using alternating 30-s cycles of homogenization and cooling. This treatment disrupted ≥99% of the yeast cells.

The homogenate was centrifuged at 450 × *g* for 5 min; supernatant and pellet were recovered. The supernatant was centrifuged at 11,000 x *g* for 20 min at 4°C and decanted. The pellets that were isolated from both the homogenate and the supernatant were pooled and washed three times with PBS. The particulate material was boiled in 125 mM Tris, pH 6.9, containing 6 M urea, 20 mM 2-mercaptoethanol, and 1% (vol/vol) Tween 20 for 5 min. Solubilization in this buffer was continued at 4°C overnight. The soluble material was separated by centrifugation at 11,000 × *g* for 20 min and dialyzed against PBS for 36 h to remove detergent. The protein concentration of CW/M was 2.8 mg/ml, as determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.).

In vivo injection of mice with CW/M. CW/M was emulsified with an equal volume of complete or incomplete Freund's adjuvant (Difco Laboratories, Detroit, Mich.). Initially, mice were injected intraperitoneally with one-half of the total dose of CW/M emulsified in complete Freund's adjuvant; 7 to 10 days later, mice were injected intraperitoneally with the remaining half of the dose of CW/M admixed in incomplete Freund's adjuvant. In experiments presented below, mice were injected with a total of either 500 or 1,000 μg of CW/M.

Induction and measurement of DTH to CW/M. Groups of mice that were immunized with viable *H. capsulatum* yeast cells or injected with CW/M in Freund's adjuvant were challenged intradermally with 1 μg of CW/M in a volume of 0.05 ml. Footpad swelling was measured 24 h later with a digital micrometer. The DTH response was expressed as the percent increase in footpad size from that measured immediately before antigen challenge (4). As a control, DTH was measured in age-matched littermates that had been injected with an equal volume of buffer suspended in Freund's adjuvant.

Organ culture of *H. capsulatum*. Spleens from groups of five mice each were removed aseptically and homogenized individually in 10 ml of sterile saline by a Teflon tissue grinder. Homogenates were diluted serially, and 0.1 ml of each dilution was plated in triplicate onto brain heart infusion agar (2% agar [wt/vol]) containing 1% (wt/vol) dextrose, 0.01% (wt/vol) cysteine hydrochloride, 10 μg of gentamicin per ml, and 5% (vol/vol) defibrinated sheep erythrocytes. Cultures were incubated in a closed cabinet at 30°C for 7 to 10 days, and the number of mycelial colonies was counted (11).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of CW/M and T-cell immunoblotting. Fifty micrograms of CW/M was electrophoresed in a 7.5 to 15% gradient polyacrylamide gel (14), and the gels were silver stained (21). One-dimensional immunoblotting studies were performed by a method similar to that of Abou-Zeid et al. (1). Briefly, 500 μg of CW/M was loaded into each well of a discontinuous 7.5 to 15% gradient SDS-polyacrylamide gel and electrophoresed at 70-mA constant current for 5 h. Separated proteins were transferred to nitrocellulose (NC) (Bio-Rad Laboratories, Richmond, Calif.) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, and 0.5% SDS) for 16 h at 30 V and then for 1 h at 60 V. SDS was added to the buffer to enhance transfer of high-M_r molecules. NC was stained with Ponceau S, and lanes were cut. Each lane of NC-bound CW/M was cut into strips (1 cm × 5 mm), which were dissolved with 0.5 ml of dimethylsulfoxide at room temperature for 1 h. NC was repolymerized by adding 0.5 ml of 50 mM sodium carbonate–bicarbonate buffer, pH 9.6, while vortexing vigorously. Fractions were washed with RPMI 1640 and resuspended in 1 ml of RPMI 1640 containing 10% fetal bovine serum and 10 μg of gentamicin per ml.

Splenocyte preparation. Spleen cells from normal mice and mice immunized with yeast cells or injected with CW/M were prepared as described previously (7). Briefly, spleens were teased apart between two ground glass slides, and the cell suspension was washed three times in a balanced salt solution.

Proliferation assay. For the proliferation assay, resting cells from a murine T-cell line or from T-cell clones (6) were suspended in RPMI 1640 supplemented with 10% fetal bovine serum and 10 μg of gentamicin per ml. To each well of a 96-well microtiter plate were added 2×10^4 T cells in 0.1 ml, 5×10^5 irradiated splenocytes in 0.1 ml, and 50 μl of soluble CW/M (protein concentration in a well, 5.6 μg/ml) or 50 μl of NC-bound fraction. In proliferation assays of splenocytes, 4×10^5 cells in 0.2 ml of RPMI 1640 containing 10% fetal bovine serum and 10 μg of gentamicin per ml were added to each well. Cultures of the T-cell line and cloned T cells were incubated for 72 h at 37°C in 5% CO_2, whereas cultures of splenocytes were incubated for 144 h; 16 h before cell harvest, 0.5 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear, Boston Mass.) was added to each well. Cells were collected on glass fiber filters with a semiautomated harvester (MASH II; M. A. Bioproducts, Walkersville, Md.), and uptake of radioactivity was measured by liquid scintillation. Proliferative responses by cells exposed to soluble or NC-bound CW/M were considered positive when they were greater than three times that of cells incubated with plain NC (in assays of NC-bound antigen) or with medium.

An ovalbumin-reactive T-cell clone, 1S6, was isolated from splenocytes of C57BL/6 mice that had been immunized with antigen. This clone was propagated by biweekly stimulation with ovalbumin, 125 μg/ml, and fresh irradiated splenocytes from syngeneic mice.

Statistics. The Wilcoxon rank sum test was used for comparison of two groups. Fischer's exact test was used for comparison of two proportions.

RESULTS

Electrophoretic analysis of CW/M. CW/M was separated by SDS-PAGE under reducing conditions by using a 7.5 to 15% gradient gel which was silver stained. Separation of CW/M by electrophoresis produced a complex pattern with numerous bands ranging in molecular mass from 14 to >200 kDa (Fig. 1).

Mice immunized with viable *H. capsulatum* yeast cells or injected with CW/M mount a DTH response to CW/M. Mice were injected with viable yeast cells or with CW/M and footpad tested with 1 μg of CW/M 2 to 3 weeks after the last injection of the immunogen. Mice immunized with viable yeast cells mounted a significant DTH response to CW/M compared with nonimmune normal mice (Table 1). Moreover, mice injected with 500 or 1,000 μg of CW/M in Freund's adjuvant exhibited a DTH response that was significantly greater than that of animals administered an equal volume of buffer (Table 1).

Injection of mice with CW/M confers protective immunity. Mice were injected with a total of 500 or 1,000 μg of CW/M admixed with Freund's adjuvant. As a control, equal numbers of animals were given an identical volume of buffer in Freund's adjuvant. Two to 3 weeks after the last inoculation, groups of mice were challenged intravenously with a sublethal inoculum of *H. capsulatum* yeast cells. The number of

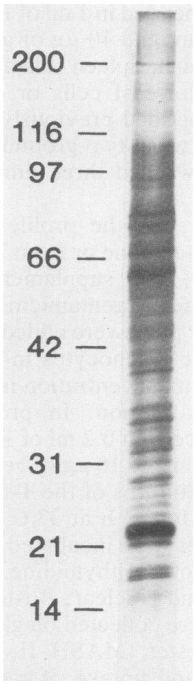

FIG. 1. SDS-PAGE profile of CW/M by using a 7.5 to 15% gradient gel. Gel was stained with silver. M_r markers (10^3) are displayed to the left of the gel.

H. capsulatum CFU in the spleens of mice was quantitated at 1 week of infection. Injection of 500 or 1,000 μg of CW/M significantly decreased the number of CFU in spleens of mice compared with that of infected controls (Table 2).

Subsequently, we sought to determine whether immunization with CW/M could protect mice against a lethal inoculum of *H. capsulatum* yeast cells. Groups of 10 mice each were injected with a total of 1,000 μg of CW/M in Freund's adjuvant or with buffer in Freund's adjuvant. This quantity of CW/M was selected because its protective efficacy in the aforementioned experiments was superior to that of the 500-μg injection. Two weeks after the last injection of CW/M, mice were injected intravenously with 4×10^6 yeast cells. Infected controls appeared ill (ruffled fur and huddling) by day 5. Three of 10 infected controls died on day 7. Four additional mice died on day 8, and the remaining three died on day 9. By contrast, only 1 of 10 immunized animals died during the 28-day observation period. This mouse became ill on day 13 and died on day 14. Therefore, CW/M significantly ($P < 0.00012$) protected mice against a lethal challenge with *H. capsulatum* yeast cells.

TABLE 1. DTH response of mice immunized with *H. capsulatum* yeast cells or CW/M to CW/M

Group	Immunization[a]	% Increase in footpad thickness (mean ± SEM)[b] for expt:	
		1	2
A	None	5 ± 1	2 ± 2
	Viable *H. capsulatum*	19 ± 3	19 ± 1
B	Buffer	3 ± 1	4 ± 1
	500 μg of CW/M	10 ± 2	13 ± 2
C	Buffer	1 ± 1	2 ± 1
	1,000 μg of CW/M	12 ± 1	12 ± 2

[a] Buffer and CW/M were suspended in Freund's adjuvant. One-half of the total dose of CW/M was admixed with complete Freund's adjuvant and injected intraperitoneally; 7 to 10 days later, the remaining amount of CW/M or buffer was admixed with incomplete Freund's adjuvant and injected intraperitoneally into mice.

[b] Percent increase = [(thickness 24 h after injection − thickness before injection) ÷ thickness before injection] × 100. Mean ± standard error of the mean (SEM) is for groups of five mice each. One microgram of CW/M was injected into the footpad of each mouse. Differences in percent increase between control and immunized mice were significant ($P = 0.01$) for all groups in both experiments.

TABLE 2. *H. capsulatum* burden in the spleens of mice immunized with CW/M

Immunization[a]	Mean CFU/spleen ± SEM (10^5) in expt[b]:	
	1	2
Buffer	29.0 ± 2.0	14.1 ± 0.4
500 μg of CW/M	9.5 ± 0.2[c]	7.3 ± 0.2[d]
Buffer	13.6 ± 0.1	23.9 ± 4.3
1,000 μg of CW/M	1.1 ± 0.2[c]	4.1 ± 0.8[c]

[a] See footnote *a*, Table 1.

[b] Mean ± SEM of groups of five mice each challenged intravenously with 6×10^5 yeast cells. Spleens were cultured at 1 week of infection.

[c] Differences in mean CFU per spleen between control and immunized mice were significant ($P = 0.01$).

[d] Differences in mean CFU per spleen between control and immunized mice were significant ($P < 0.05$).

Splenocytes from mice injected with viable yeast cells or with CW/M proliferate in vitro in response to CW/M. Splenocytes from mice immunized with viable *H. capsulatum* yeast cells responded vigorously to CW/M in vitro (Table 3). Likewise, spleen cells from mice injected with 500 to 1,000 μg of CW/M in Freund's adjuvant reacted in vitro to CW/M. On the other hand, the responses by spleen cells from normal controls or from mice injected with buffer admixed in Freund's adjuvant were not significantly different ($P > 0.05$) than the response by unstimulated cells (Table 3).

T-cell immunoblotting analysis of CW/M. Additional studies were performed to determine the molecular masses of antigens in CW/M that stimulate in vitro proliferation of *H. capsulatum*-reactive T cells. To accomplish this objective, we analyzed the responses of a murine T-cell line that is $CD4^+$ and of a panel of murine $CD4^+$ T-cell clones to electrophoretically separated CW/M. Since the T-cell line and cloned T cells were originally generated with histoplasmin (6), we initially determined whether these cells recognized CW/M. The T-cell line, JC1, and six T-cell clones responded vigorously to CW/M (Table 4). Thus, this extract contained determinants recognized by the T-cell line and all cloned T-cells.

An important concern was the reproducibility of results obtained by T-cell immunoblotting among individual gels. To address this issue, the proliferative activity of the T-cell line, JC1, was assessed to batches of CW/M that were electrophoresed on at least three separate occasions. The profiles of three individual fractionations are shown in Fig. 2. JC1 proliferated in response to several fractions with molecular masses ranging from 60 to 105 (Fig. 2A), from 48 to 124 (Fig. 2B), and from 43 to 87 (Fig. 2C) kDa. In addition, reactivity of JC1 was most pronounced to two fractions that extended from 55 to 73 (Fig. 2A), 62 to 72 (Fig. 2B), and 53 to 70 (Fig.

TABLE 3. In vitro proliferative response to CW/M by mouse splenocytes

Group	Immunization[a]	In vitro stimulus[b]	[^{3}H]thymidine incorporation (mean cpm ± SEM) in expt[c]: 1	2
A	None	Medium	339 ± 62	308 ± 40
		CW/M	492 ± 144	499 ± 104
	Viable *H. capsulatum*	Medium	445 ± 96	1,735 ± 108
		CW/M	6,790 ± 875	15,967 ± 85
B	Buffer	Medium	586 ± 105	1,436 ± 462
		CW/M	917 ± 93	1,423 ± 24
	500 μg of CW/M	Medium	586 ± 186	1,352 ± 274
		CW/M	6,294 ± 361	22,241 ± 1,266
	1,000 μg of CW/M	Medium	765 ± 175	937 ± 104
		CW/M	11,461 ± 867	24,769 ± 4,237

[a] See footnote *a*, Table 1.
[b] Final concentration of CW/M was 5.6 μg/ml.
[c] Mean ± SEM of duplicate determinations. Two of six experiments are shown.

TABLE 4. Proliferative response by *H. capsulatum*-reactive T cells to CW/M

Line or clone	[^{3}H]thymidine incorporation (mean cpm ± SEM)[a] of cells incubated in: Medium	CW/M
JC1	3,714 ± 504	34,829 ± 2,805
2.3H3	1,129 ± 203	36,401 ± 1,679
1.3G6	914 ± 50	17,001 ± 861
2.3E10	616 ± 220	23,502 ± 1,569
1.1C3	1,807 ± 158	48,602 ± 1,984
1.0C311	1,128 ± 76	23,765 ± 1,092
1.1E1	512 ± 134	4,653 ± 1,096

[a] Mean ± SEM of triplicate determinations. The data represent one representative experiment of at least two. Final protein concentration of CW/M in a microtiter well was 5.6 μg/ml.

2C) kDa. Thus, each T-cell immunoblot generated a highly reproducible pattern of responses by JC1.

T-cell clones were tested for reactivity to electrophoretically separated CW/M that was bound to NC. Figure 3 depicts a representative experiment for each clone. Four clones, 2.3H3, 1.3G6, 1.1C3, and 1.1E1, responded to molecules contained in a region that spanned from 53 to 64 kDa. Clones 2.3H3 and 1.3G6 also recognized antigens in fractions that extended to 82 kDa, and 1.1C3 reacted to antigens with molecular masses ranging from 72 to 81 kDa. Proliferative activity of 2.3E10 was to fractions encompassing 22 to 27 and 34 to 38 kDa. 1.0C311 recognized fractions that ranged from 38 to 42 and from 62 to 69 kDa. The response patterns by T-cell clones and the T-cell line to CW/M clearly indicated that there exist at least two prominent antigenic regions; one extended from 53 to 64 kDa, and the other spanned from 69 to approximately 82 kDa.

To determine if fractions from the electrophoresed CW/M were mitogenic, the ovalbumin-specific clone, 1S6, was incubated with NC-bound antigen and proliferation was measured. None of the fractions from CW/M caused a blastogenic response (Fig. 4).

DISCUSSION

H. capsulatum is an important pathogen of humans, especially since the advent of the epidemic of AIDS (16). It is generally acknowledged that successful resolution of infection depends in large measure on a complex interplay between T cells and macrophages. One of the critical interactions involved in collaboration between these two cell populations is the recognition of *H. capsulatum* yeast antigens by T cells. Yet, in the past decade, there has been little attention given to identifying the T-cell antigens from *H. capsulatum* yeast cells despite the fact that this form represents the tissue phase of the fungus. In fact, much of the previous work concerning T-cell responses of *Histoplasma* antigens has concentrated on histoplasmin, a product of the mycelial phase (6, 12, 13).

In this study, we have analyzed the antigenicity and immunogenicity of a detergent extract from a preparation of *H. capsulatum* yeast cells that is enriched for cell walls and cell membranes. Since T cells, and in particular the CD4$^+$ subpopulation, are a critical component of host defense mechanisms against this pathogen, experiments focused on the in vivo and in vitro T-cell-mediated responses to this extract. The results demonstrated that mice immunized with viable yeast cells mounted a DTH response to CW/M and that splenocytes from these animals recognized CW/M in

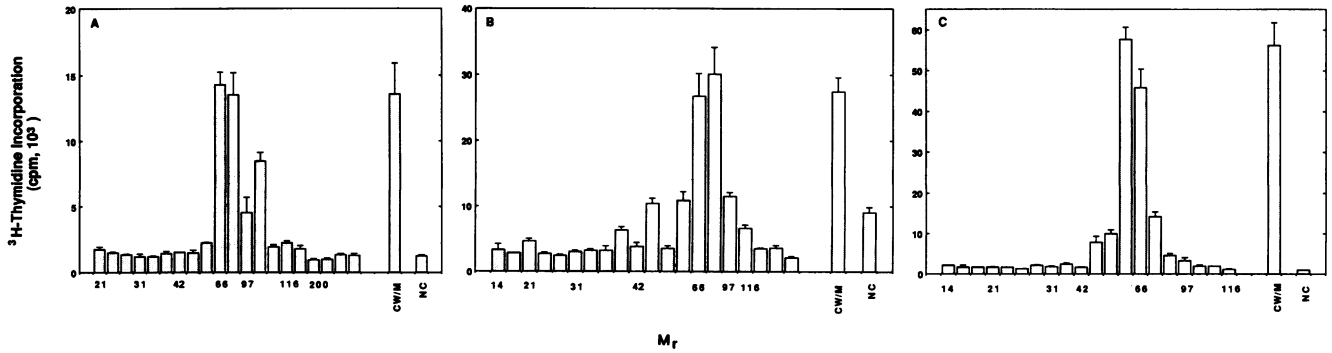

FIG. 2. Proliferative responses by JC1 to electrophoretically separated CW/M. Results from three individual gels with two different batches of CW/M are shown. Values represent the mean ± SEM of triplicate determinations for each fraction. The proliferative responses by JC1 to 7 μg of CW/M bound to NC and to NC alone are shown on the right of each graph. M_r markers (10^3) are displayed on the abscissa.

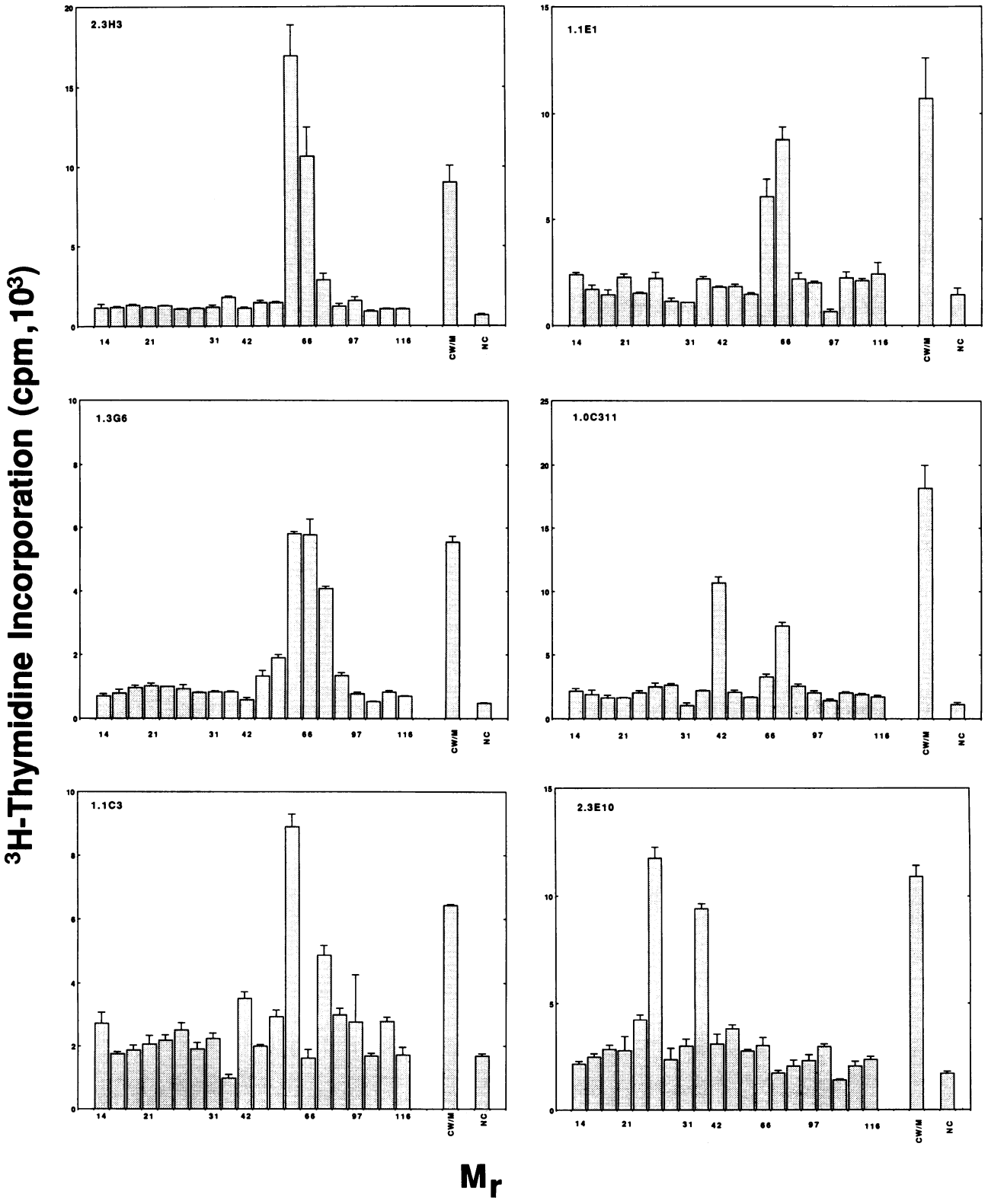

FIG. 3. Profile of responses by cloned T cells to electrophoresed CW/M. Values represent the mean ± SEM of triplicate determinations. The blastogenic response by each clone to 7 μg of CW/M that is bound to NC and to NC alone is shown on the right of each graph. M_r markers (10^3) are depicted on the abscissa. One representative experiment of two or more is shown.

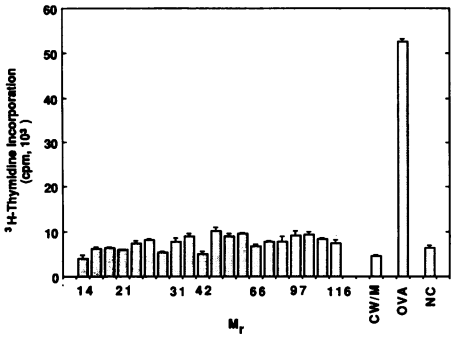

FIG. 4. Analysis of response by an ovalbumin-reactive clone, 1S6, to electrophoresed CW/M. Values represent mean ± SEM of triplicate determinations. The blastogenic response to 7 μg of unfractionated CW/M bound to NC, to ovalbumin (OVA) (125 μg/ml), and to NC alone are shown on the right of the graph. M_r markers (10^3) are displayed on the abscissa.

vitro. Injection of CW/M mixed in Freund's adjuvant into mice induced cutaneous DTH reactivity and in vitro proliferative responses by spleen cells to this antigenic preparation. Furthermore, CW/M did confer protective immunity against a sublethal as well as a lethal inoculum of *H. capsulatum* yeast cells. Thus, CW/M not only contains molecules recognized by T cells but also possesses antigens that can stimulate a protective immune response. It remains to be determined, however, whether the antigens that trigger T-cell proliferation can also mediate protective immunity.

There is limited information concerning the identity of immunogens from *H. capsulatum* yeast cells. The original studies demonstrated that both live and heat-killed yeast cells mediate protective immunity (19). Subsequently, Garcia and Howard (10) reported that an ethylenediamine extract from intact yeast cells or from cell walls enhanced survival in mice challenged intravenously with an inoculum of *H. capsulatum* yeast cells two times the 50% lethal dose. In addition, a ribosomal-protein complex from *H. capsulatum* yeast cells confers protection in mice against a lethal challenge with yeast cells (9). The finding that an antigenic preparation enriched in cell walls and cell membranes, CW/M, contains protective antigens extends the list of known immunogens isolated from *H. capsulatum*.

By SDS-PAGE analysis and silver staining, CW/M was a highly heterogeneous admixture of proteins that ranged in molecular mass from 14 to 200 kDa. To facilitate the identify of the T-cell antigens contained within this complex pool, we employed the method of T-cell immunoblotting (1). In other studies, this technique has provided a simple yet powerful tool with which to identify T-cell antigens that were present in heterogeneous mixtures of mycobacterial (15) and leishmanial (17, 18) antigens. We examined responses to electrophoretically separated CW/M by an oligoclonal T-cell line and monoclonal populations of T cells. This strategy was utilized because the immune system is organized in a clonal fashion.

The profiles of responses by an *H. capsulatum*-reactive T-cell line and T-cell clones were quite homogeneous. An area of CW/M extending from 53 to 64 kDa contained antigens recognized by the T-cell line and by five of six clones. Moreover, the T-cell line and three clones reacted with molecules present in a region extending from 69 to 82 kDa. However, the profiles of responses to CW/M by the T-cell line and cloned T cells differed from that of T-cell hybridomas isolated from spleens of immune C57BL/6 mice. Six hybridomas reacted to an antigen or antigens in CW/M that ranged in molecular mass from 35 to 39 kDa (5). Taken together, the findings indicate that there are diverse T-cell antigens in CW/M.

All clones responded to more than a single fraction (1 cm × 5 mm) of NC-bound antigen. For some clones, the stimulatory fractions were adjacent to one another. Several considerations may explain this finding. First, these regions of electrophoresed CW/M may contain individual antigens that have identical or similar T-cell epitopes. Second, adjacent fractions of NC-bound CW/M may have included a single protein band that had been partitioned into two sections. Alternatively, we cannot exclude the possibility that some of the antigens in the two fractions are degradation products of a single antigen, although efforts were made to minimize degradation by including several proteinase inhibitors during preparation of CW/M. In order to know which one of the above possibilities is correct, the amino acid sequences of stimulatory antigens will have to be deduced.

Our panel of *H. capsulatum*-reactive cells responded to yeast antigens, although they originally had been generated with histoplasmin. CW/M has been examined by microimmunodiffusion and Western immunoblot analysis for the presence of the M and H antigens, which are prominent antigens in histoplasmin. In numerous experiments, we have failed to identify either of these two glycoproteins in CW/M (10a). Since $CD4^+$ T cells recognize only small peptides in association with class II major histocompatibility complex molecules (22), it is conceivable that processing of histoplasmin or CW/M by antigen-presenting cells generates peptides with similar if not identical amino acid sequences.

In conclusion, we have generated a detergent extract from the cell wall and cell membrane of *H. capsulatum* yeast cells. This preparation contains antigens that induce both cutaneous DTH reactivity and in vitro proliferation of murine splenocytes and murine T-cell clones that are $CD4^+$. In addition, CW/M mediated a protective immune response in mice. The identification of a restricted number of T-cell antigens by T-cell immunoblotting provides a foundation for the isolation of individual antigens from the complex pool of proteins that is present in CW/M. Studies are underway to purify T-cell antigens and to examine their immunobiological activity, including their immunogenic potential.

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