INFECTION AND IMMUNITY, June 1990, p. 1538–1544
0019-9567/90/061538-07$02.00/0
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Vol. 58, No. 6

Functional Analysis of *Histoplasma capsulatum*-Reactive T-Cell Hybridomas

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Activation of CD4$^+$ T cells is a crucial step in the elimination of *Histoplasma capsulatum* yeast cells from tissues. However, only a limited amount of information exists concerning the immunobiology of *H. capsulatum*-reactive T cells that are CD4$^+$. To facilitate the analysis of the functional activities of this T-cell subpopulation, we developed a panel of 10 murine T-cell hybridomas from splenocytes of immune C57BL/6 mice. All hybridomas reacted with monoclonal anti-CD4$^+$ antibody and released interleukin-2 after stimulation with histoplasmin. Within 3 weeks, the reactivity of hybridomas to histoplasmin declined dramatically, yet the cells responded vigorously to yeast-phase preparations that were enriched for cytosol, cell wall, or cell membrane. Of 10 hybridomas studied, only one recognized heterologous fungal antigens. Responsiveness to yeast-phase antigens was restricted by *I-A^b*. We mapped determinants in cytosol and cell wall or cell membrane by the technique of one-dimensional T-cell immunoblotting. The patterns of responses of hybridomas to cytosol were nearly uniform. All hybridomas responded to two immunodominant regions in cytosol with masses ranging from ≤18 to 26 kilodaltons (kDa) and 35 to 39 kDa. All hybridomas tested responded to determinants in the cell wall or cell membrane preparation with masses of 35 to 39 kDa. These hybridomas provide a useful tool for defining yeast-phase antigens that trigger T-cell activation.

Histoplasma capsulatum is a dimorphic fungal pathogen that produces a broad spectrum of disease. Most often, infection resolves without therapeutic intervention. The capacity of host defenses to limit the replication of this intracellular pathogen is chiefly dependent on a collaborative interaction between antigen-reactive T cells and macrophages (6, 7, 9, 14, 16). The precise sequence of events that results in growth restriction has not been defined. Accumulating evidence suggests that CD4$^+$ T cells, upon recognition of *H. capsulatum* antigens bound to class II major histocompatibility complex molecules, release lymphokines that activate the antifungal activity of macrophages (9, 18, 20). One of the principle lymphokines involved in arming murine macrophages is gamma interferon (19).

Although it is clear that T lymphocytes and, in particular, CD4$^+$ T cells play a central role in the elimination of *H. capsulatum* yeast cells (6, 7, 9), information about the biological properties of *H. capsulatum*-reactive CD4$^+$ T cells is incomplete. To gain a better understanding of the function of this T-cell subpopulation, we generated a panel of 10 T-cell hybridomas that respond to *H. capsulatum* antigens from splenocytes of C57BL/6 mice immunized with viable organisms. The data demonstrate that the T-cell hybridomas release interleukin-2 (IL-2) after stimulation with histoplasmin (HKC) or yeast-phase antigens. Only 1 of 10 hybridomas responds to heterologous fungal antigens. In addition, the recognition of yeast-phase antigens is restricted by *I-A^b*. T-cell immunoblotting was used to identify epitopes in yeast-phase antigens that stimulate T-cell hybridomas.

MATERIALS AND METHODS

Mice. Male C57BL/6, B10.A(4R), B10.A(5R), and B10.MBR mice were purchased from Jackson Laboratory, Bar Harbor, Maine.

Antigens. HKC was prepared as described previously (10). The protein content was 120 μg/ml. Yeast-phase preparations were isolated in the following manner. Strain G217B yeast cells were inoculated into Ham F-12 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) (17) and cultured for 36 h at 37°C in a gyratory shaker. Yeast cells were harvested and killed by incubation in phosphate-buffered saline containing thimerosal (1:10,000 dilution [wt/vol]) at room temperature for 1 h. Yeast cells were washed and suspended in phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, 5 μM leupeptin, and EDTA (0.2 mg/ml) at a dilution of 1 volume of packed cells per 2 volumes of buffer. Cells were disrupted in a bead beater (Biospec Products, Bartlesville, Okla.) at 4°C for 6 min with alternate 30-s cycles of homogenization followed by cooling. This treatment disrupted ≥99% of yeast cells. The homogenate was centrifuged at 450 × *g* for 5 min, and both supernatant and pellet were recovered. The supernatant was centrifuged again at 11,000 × *g* for 20 min at 4°C and decanted. The pellets that were isolated from the homogenate and supernatant were pooled and washed three times with phosphate-buffered saline. 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 overnight at 4°C. The soluble material was separated by centrifugation at 11,000 × *g* for 20 min and dialyzed against phosphate-buffered saline for 36 h to remove detergent. The supernatant, which is enriched for the cytosol of yeast cells, will be referred to as cytosol (Cyto). The solubilized particulate material, which is enriched for cell wall or cell membrane, will be referred to as cell wall/membrane (CW/M). The protein contents of Cyto and CW/M were 4.6 and 2.8 mg/ml, respectively, as determined by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.).

Coccidioidin and alkali-soluble, water-soluble cell wall

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extracts of *Blastomyces dermatitidis* (B-ASWS) and *Coccidioides immitis* (C-ASWS) were generously provided by Rebecca Cox, San Antonio State Chest Hospital, San Antonio, Tex. Cryptococcal antigen was a gift from Juneann Murphy, University of Oklahoma Health Sciences Center, Oklahoma City.

Immunization of mice and splenocyte preparation. Mice were injected subcutaneously with 10^6 viable yeast cells; 2 weeks later, they were inoculated intravenously with 10^6 viable yeast cells; and 3 weeks later, they were given 5×10^6 viable yeast cells intraperitoneally. Single-cell suspensions from spleens of mice were prepared as described previously (9).

Generation of T-cell hybridomas. Splenocytes from immunized mice were suspended at a concentration of 3×10^6/ml in RPMI 1640 containing 10% fetal bovine serum (FBS), 1% nonessential amino acids, 1% L-glutamine, 1% sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, and 10 μg of gentamicin per ml. One milliliter of cell suspension was dispensed into each well of a 24-well plate, and the cells were stimulated for 4 days with a 1:500 dilution of HKC. Lymphoblasts were harvested, washed, and fused with thymoma line BW5147.G.1.4 (referred to as BW5147) (splenocyte/thymoma ratio, 3:1) by use of 1 ml of 40% polyethylene glycol 1400-1600 containing 10% dimethyl sulfoxide. One hundred microliters of the cell mixture was plated at a density of 8×10^4 thymoma cells per well on Dulbecco modified Eagle medium containing hypoxanthine-aminopterin-thymidine, 10% FBS, and 10 μg of gentamicin per ml. One day prior to fusion, 6×10^3 syngeneic peritoneal cells in 0.1 ml of Dulbecco modified Eagle medium containing 10% FBS and gentamicin were dispensed into each well of a microdilution plate.

Actively growing hybridomas were expanded in hypoxanthine-aminopterin-thymidine-containing medium and tested for reactivity to HKC. All antigen-responsive hybridomas were grown in medium containing hypoxanthine and thymidine for 1 week and then maintained in Dulbecco modified Eagle medium containing 10% FBS and 10 μg of gentamicin per ml. Each hybridoma that responded to antigen was cloned by limiting dilution in 96-well plates; 0.3 of 1 hybridoma in 0.2 ml of Dulbecco modified Eagle medium containing 10% FBS, 10 μg of gentamicin per ml, and hypoxanthine-aminopterin-thymidine was cultured in the presence of 5×10^5 irradiated, syngeneic splenocytes. All actively growing cells were expanded and retested for antigenic reactivity.

Phenotypic analysis of hybridomas. Hybridomas (5×10^5) were incubated with 7.5 μg of either phycoerythrin-conjugated monoclonal anti-CD4 antibody or fluorescein-conjugated monoclonal anti-CD8 antibody (Becton Dickinson and Co., Mountain View, Calif.) for 30 min at 4°C and then washed. All cells were analyzed with an EPICS V apparatus (Coulter Electronics, Inc., Hialeah, Fla.).

SDS-PAGE of Cyto and CW/M and T-cell immunoblotting. Fifty micrograms of each preparation was electrophoresed in a 7.5 to 15% gradient polyacrylamide gel (11), and gels were silver stained (13).

One-dimensional immunoblotting studies were performed by the method of Abou-Zeid et al. (1). Cyto (960 μg) or CW/M (500 μg) was loaded into each well of a discontinuous 7.5 to 15% gradient polyacrylamide gel and electrophoresed at a constant current of 70 mA for 5 h. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were transferred to nitrocellulose (NC) (Bio-Rad Laboratories, Richmond, Calif.) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, 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 the transfer of high-M_r molecules. The NC was stained with Ponceau S, and lanes were cut. Each lane of NC-bound Cyto or CW/M was cut into 20 to 22 strips measuring 1 cm by 5 mm. Fractions were dissolved in dimethyl sulfoxide at room temperature for 1 h. The NC was repolymerized by adding 0.5 ml of 50 mM sodium carbonate-bicarbonate buffer (pH 9.6) while vortexing vigorously. The fractions were washed with RPMI 1640 and suspended in 1 ml of RPMI 1640.

Hybridoma stimulation assay. T-cell hybridomas were suspended in RPMI 1640 supplemented with 10% FBS, 1% nonessential amino acids, 1% L-glutamine, 1% sodium pyruvate, 5×10^{-5} M 2-mercaptoethanol, and 10 μg of gentamicin per ml. To each well of a microdilution plate were added 2×10^4 T cells in 0.1 ml of medium, 5×10^5 irradiated splenocytes in 0.1 ml of medium, and 50 μl of soluble antigen, NC-bound antigen, NC alone, or medium. Cultures were incubated overnight at 37°C in 5% CO_2. Subsequently, 100 μl of supernatant was removed and tested for IL-2 activity.

Assay of IL-2 activity. Test supernatants were added to 10^4 CTLL-2 cells in 0.1 ml of medium and cultured in triplicate at 37°C in 5% CO_2 for 28 h. Four hours before harvest, cells were pulsed with 1 μCi of [^{3}H]thymidine (specific activity, 6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.) and the uptake of radioactivity was measured with a liquid scintillation counter. As a positive control, CTLL-2 cells were incubated with supernatant obtained from rat splenocytes stimulated with concanavalin A in each experiment. Responses were considered positive when they were greater than or equal to threefold higher than the response of cells incubated with medium alone or with NC alone.

RESULTS

Development of hybridomas and phenotypic analysis. From 375 wells plated, 199 hybridomas (53.1%) were tested for the recognition of HKC, and 16 of 199 (8.0%) were antigen reactive. These hybridomas were cloned, and actively growing cells were tested for antigenic reactivity.

After cloning, 10 T-cell hybridomas were selected for further analysis. CD4 expression by the hybridomas was ≥88% for 8 of 10. CD4 expression by the remaining two was 80 and 83%, respectively. The mean percentage of hybridomas that were stained with anti-CD8 antibody never exceeded the percentage of BW5147 cells that were stained with anti-CD8 antibody (2.8%). Thus, hybridomas were $CD4^+$ $CD8^-$.

Antigenic reactivity of hybridomas. Hybridomas were stimulated with decreasing concentrations of HKC, and supernatants were tested for IL-2 activity. Each hybridoma responded to HKC in a dose-dependent manner (Table 1). A final HKC dilution of 1:100 (protein concentration per well, 1.2 μg/ml) induced optimal responses. The fusion partner, BW5147, failed to generate IL-2 activity when incubated with HKC.

Since splenocytes from immune mice had been stimulated with HKC prior to fusion, we sought to determine if the hybridomas recognized yeast-phase epitopes. Cells were stimulated with optimal dilutions of Cyto, CW/M, or HKC, and IL-2 release was measured. All hybridomas, but not BW5147, responded to each yeast-phase preparation (Table 2). Surprisingly, the reactivity of hybridomas to HKC was markedly diminished or lost (Table 2). The poor reactivity to this antigenic mixture was detected within 3 weeks of the

TABLE 1. Responses of T-cell hybridomas to HKC

Hybridoma	[^{3}H]thymidine incorporation by CTLL-2 cells (mean cpm ± SEM)[a] in response to[b]:			
	Medium	HKC (1:50)	HKC (1:100)	HKC (1:500)
1A1	165 ± 46	7,865 ± 1,301	9,037 ± 539	346 ± 63
2C4	295 ± 97	5,788 ± 333	10,728 ± 1,260	1,173 ± 206
3A41	76 ± 14	3,686 ± 394	7,699 ± 255	239 ± 81
2B3	80 ± 23	2,762 ± 268	7,930 ± 395	335 ± 88
2A2	131 ± 13	2,529 ± 134	7,665 ± 466	407 ± 56
1B4	345 ± 7	3,884 ± 377	8,557 ± 85	1,173 ± 206
3A42	170 ± 37	3,580 ± 344	8,018 ± 230	282 ± 73
3B1	127 ± 36	1,904 ± 120	6,367 ± 244	120 ± 21
3B2	155 ± 24	836 ± 104	3,437 ± 78	456 ± 166
1A4	180 ± 27	2,644 ± 141	7,108 ± 517	459 ± 120
BW5147	167 ± 21	103 ± 6	291 ± 72	243 ± 35

[a] Triplicate determinations. One representative experiment of at least two is shown.
[b] Numbers in parentheses after HKC indicate the final dilution of HKC. The final protein concentrations of HKC per well were 2.4 μg/l for the 1:50 dilution, 1.2 μg/ml for the 1:100 dilution, and 0.24 μg/ml for the 1:500 dilution.

TABLE 2. Responses of T-cell hybridomas to Cyto, solubilized CW/M, and HKC

Hybridoma	[^{3}H]thymidine incorporation by CTLL-2 cells (mean cpm ± SEM)[a] in response to[b]:			
	Medium	Cyto	CW/M	HKC
1A1	270 ± 87	16,453 ± 1,442	13,314 ± 408	2,571 ± 248
2C4	943 ± 50	18,748 ± 1,349	12,921 ± 63	3,064 ± 320
3A41	99 ± 7	9,082 ± 313	3,595 ± 22	933 ± 5
2B3	438 ± 23	12,654 ± 1,545	10,259 ± 60	1,310 ± 179
2A2	223 ± 54	9,548 ± 654	4,972 ± 236	364 ± 41
1B4	557 ± 64	10,735 ± 1,122	6,652 ± 849	1,261 ± 86
3A42	465 ± 29	9,639 ± 518	6,501 ± 317	1,726 ± 341
3B1	531 ± 68	4,804 ± 7	3,344 ± 65	669 ± 69
3B2	647 ± 65	21,219 ± 1,783	16,590 ± 264	4,264 ± 851
1A4	503 ± 106	12,074 ± 1,185	7,251 ± 796	1,451 ± 371
BW5147	201 ± 69	217 ± 86	270 ± 61	183 ± 19

[a] Triplicate determinations. One representative experiment of at least two is shown.
[b] Final dilutions and final protein concentrations of antigens per well were, respectively, 1:500 and 9.2 μg/ml for Cyto, 1:500 and 5.6 μg/ml for CW/M, and 1:100 and 1.2 μg/ml for HKC.

results shown in Table 1. Following this observation, cryopreserved hybridomas were retrieved and tested for reactivity to HKC as well as the yeast-phase preparations. The hybridomas retained vigorous responsiveness to the yeast-phase preparations, but reactivity to HKC either remained diminished or was nonexistent. For example, 2C4, which had been recovered from freezing, proliferated vigorously against Cyto (21,126 ± 699 cpm) but only weakly against HKC (2,656 ± 161 cpm; unstimulated cells, 242 ± 54 cpm). The yeast-phase preparations, therefore, contained determinants recognized by T cells. Moreover, there was an irreversible decrement or loss of reactivity to HKC.

Responses of hybridomas to heterologous fungal antigens. T-cell hybridomas were incubated with a battery of heterologous fungal antigens or Cyto, and IL-2 production was measured. Only one hybridoma, 1A1, responded to heterologous fungal antigens (Table 3). It reacted vigorously to C-ASWS and very weakly to B-ASWS. None of the hybridomas recognized cryptococcal antigen. With the exception of 1A1, the hybridomas demonstrated specificity for *H. capsulatum* antigens.

Major histocompatibility complex restriction of antigen presentation. T-cell hybridomas were cultured with irradiated splenocytes from C57BL/6 mice and from congenic B10.MBR, B10.A(4R), and B10.A(5R) mice in the presence or absence of Cyto. Data for 5 of 10 hybridomas are shown in Table 4. T cells recognized Cyto if they were incubated with antigen-presenting cells from C57BL/6 or B10.A(5R) mice but not B10.MBR or B10.A(4R) mice. Hence, antigen recognition requires compatibility at the *I-A* locus.

SDS-PAGE profiles of Cyto and CW/M. Cyto and CW/M were separated by electrophoresis with a 7.5 to 15% gradient gel, and gels were silver stained. Separation of Cyto by electrophoresis produced a complex pattern with bands ranging from <14 kilodaltons (kDa) to approximately 200 kDa (Fig. 1). Numerous silver-staining bands ranging from <14 to 200 kDa also were apparent in CW/M (A. Gomez, J. Rhodes, and G. Deepe, submitted for publication). Western blot (immunoblot) analysis did not demonstrate M or H antigens in Cyto or CW/M (data not shown).

T-cell immunoblotting analyses of Cyto and CW/M. Cyto and CW/M were separated by one-dimensional SDS-PAGE and transferred to NC, and fractions of NC-bound proteins were assayed for reactivity. All hybridomas recognized determinants in Cyto with masses ranging between ≤18 and 39 kDa. Results for 6 of 10 hybridomas are shown in Fig. 2. In addition, two immunodominant regions were present. One region had masses of ≤18 to 26 kDa, and the other had masses of 35 to 39 kDa.

The reactivity of the same six hybridomas to CW/M

TABLE 3. Responses of T-cell hybridomas to homologous and heterologous fungal antigens

Hybridoma	[^{3}H]thymidine incorporation by CTLL-2 cells (mean cpm ± SEM)[a] in response to[b]:					
	Medium	C-ASWS	Coccidioidin	B-ASWS	Cryptococcal antigen	Cyto
1A1	456 ± 33	23,648 ± 993	523 ± 137	1,265 ± 259	367 ± 54	21,346 ± 617
2C4	326 ± 65	396 ± 32	294 ± 35	383 ± 76	383 ± 76	16,984 ± 1,008
3A41	337 ± 37	349 ± 22	431 ± 108	307 ± 4	379 ± 42	19,134 ± 854
2B3	332 ± 15	519 ± 74	346 ± 10	382 ± 25	309 ± 27	19,760 ± 592
2A2	501 ± 89	413 ± 20	319 ± 25	489 ± 66	405 ± 23	21,005 ± 816
1B4	353 ± 40	313 ± 92	288 ± 13	287 ± 13	366 ± 38	19,730 ± 2,016
3A42	571 ± 85	448 ± 71	333 ± 26	454 ± 76	497 ± 105	18,420 ± 2,147
3B1	225 ± 11	237 ± 19	228 ± 15	252 ± 52	271 ± 37	14,305 ± 632
3B2	226 ± 5	197 ± 5	273 ± 38	223 ± 24	240 ± 24	13,768 ± 630
1A4	343 ± 65	244 ± 30	271 ± 12	469 ± 17	348 ± 58	20,632 ± 550

[a] Triplicate determinations. One representative experiment of at least two is shown.
[b] Optimal dilutions or concentrations of antigens were 50 μg/ml for C-ASWS, coccidioidin, and B-ASWS; 62 μg/ml for cryptococcal antigen; and 9.2 μg/ml for Cyto.

TABLE 4. Restriction of responses of T-cell hybridomas to Cyto by *I-A*[b]

Source of antigen-presenting cells	*H-2*[a]					Cyto[b]	[^{3}H]thymidine incorporation by CTLL-2 cells (mean cpm ± SEM)[c] in the presence of:				
	K	$A_\beta A_\alpha$	E_β	E_α	*D*		1A1	2C4	3A41	2A2	1A4
C57BL/6	*b*	*b*	(*b*)	*b*	*b*	−	223 ± 38	1,055 ± 117	391 ± 78	570 ± 7	250 ± 75
						+	23,025 ± 1,959	13,116 ± 1,607	21,195 ± 780	10,306 ± 429	6,915 ± 500
B10.MBR	*b*	*k*	*k*	*k*	*q*	−	123 ± 15	1,001 ± 191	80 ± 4	566 ± 34	182 ± 7
						+	120 ± 6	551 ± 40	97 ± 15	456 ± 31	65 ± 5
B10.A(4R)	*k*	*k*	(*k*)	*b*	*k*	−	155 ± 20	1,056 ± 11	264 ± 83	730 ± 129	71 ± 13
						+	136 ± 24	672 ± 26	200 ± 15	598 ± 4	58 ± 5
B10.A(5R)	*b*	*b*	*b*	*k*	*d*	−	234 ± 34	748 ± 9	208 ± 26	715 ± 12	121 ± 21
						+	11,947 ± 1,556	10,598 ± 808	10,803 ± 707	8,681 ± 196	4,908 ± 530

[a] Parentheses enclose silent alleles.
[b] Final protein concentration per well, 9.2 μg/ml.
[c] Triplicate determinations.

separated by electrophoresis is illustrated in Fig. 3. Only a single immunodominant region with masses of 35 to 39 kDa was apparent.

Fractions of NC-bound Cyto and CW/M do not depress responses to soluble antigen. Experiments were performed to determine if the fractions of NC-bound Cyto or CW/M contained molecules that would depress the responsiveness to soluble antigen. Hybridoma 2C4 was coincubated with 50 μl of NC-bound Cyto, NC-bound CW/M, or NC alone and an optimal dilution of soluble Cyto or CW/M. As a control, this hybridoma was incubated with soluble antigen alone. After 24 h, 100 μl of supernatant was removed from each well and tested for IL-2 activity. The mean response (± standard error of the mean) of 2C4 to soluble Cyto (14,223 ± 1,180 cpm; unstimulated cells, 2,168 ± 176 cpm) was not strikingly different from the proliferative activity of cells coincubated with soluble Cyto and individual fractions of NC-bound Cyto (range, 11,141 ± 690 to 20,180 ± 2,352 cpm). Similarly, the response of 2C4 to soluble CW/M (12,254 ± 586 cpm; unstimulated cells, same as above) did not differ substantially from that of cells coincubated with soluble CW/M and NC-bound CW/M (range, 12,301 ± 82 to 18,453 ± 958 cpm). Thus, none of the fractions contained inhibitors of the responsiveness of 2C4.

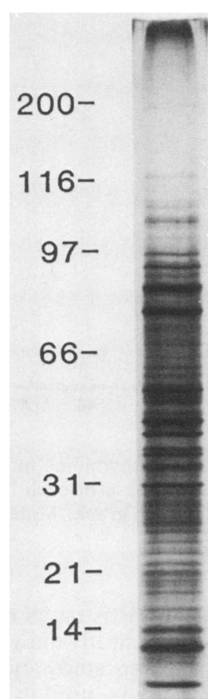

FIG. 1. Electrophoretic appearance of Cyto. The gel was silver stained. Molecular mass markers (in kilodaltons) are shown on the left.

Fractions that are not stimulatory do not contain inhibitors of IL-2 activity. We explored the possibility that the nonantigenic fractions stimulated the release of molecules that inhibited IL-2 activity or were toxic for CTLL-2 cells. Hybridoma 2C4 was incubated with 50 μl of fractions with masses of >39 kDa from NC-bound Cyto or fractions with masses of <35 and >39 kDa from NC-bound CW/M; 100 μl of supernatant was removed from each well 24 h later. CTLL-2 cells were cocultured with a suboptimal dilution (1:64) or IL-2 prepared from concanavalin A-stimulated rat splenocytes and test supernatants. The proliferative activity of CTLL-2 cells against a 1:64 dilution of IL-2 (18,040 ± 831 cpm) was considerably greater than that of cells cultured in medium (360 ± 60 cpm). The response of CTLL-2 cells to the admixture of IL-2 and supernatants from nonstimulatory fractions (range, 14,709 ± 1,659 to 21,977 ± 133 cpm) was similar to that of cells to IL-2 alone. The results indicate that supernatants from 2C4 that had been incubated with nonantigenic fractions did not contain inhibitors of IL-2 activity or molecules toxic for CTLL-2 cells.

DISCUSSION

In the field of mycology, others have reported the isolation of T-cell hybridomas from mice injected with cryptococcal antigen. These cells have been used as a source of factors that either suppress delayed-type hypersensitivity responses of immune mice to a cryptococcal filtrate (8) or inhibit phagocytosis by a subpopulation of murine macrophages (3). In the present investigation, the technique of immortalizing T cells by fusion with the thymoma line BW5147 was used for a different purpose. The objective was to produce a panel of $CD4^+$ T-cell hybridomas to identify antigens reactive with *H. capsulatum*-specific T cells. Thus, the findings described here represent one of very few reports of T-cell hybridomas in medical mycology and extend the range of usefulness of these cells.

For the past few years, this laboratory has analyzed the immunological properties of *H. capsulatum*-reactive T-cell clones generated from spleens of immune C57BL/6 mice (6, 7, 9). The development of T-cell hybridomas offers some advantages over T-cell clones. Hybridomas are autonomously replicating and do not require antigen-presenting cells, growth factors, or antigen for propagation. Moreover,

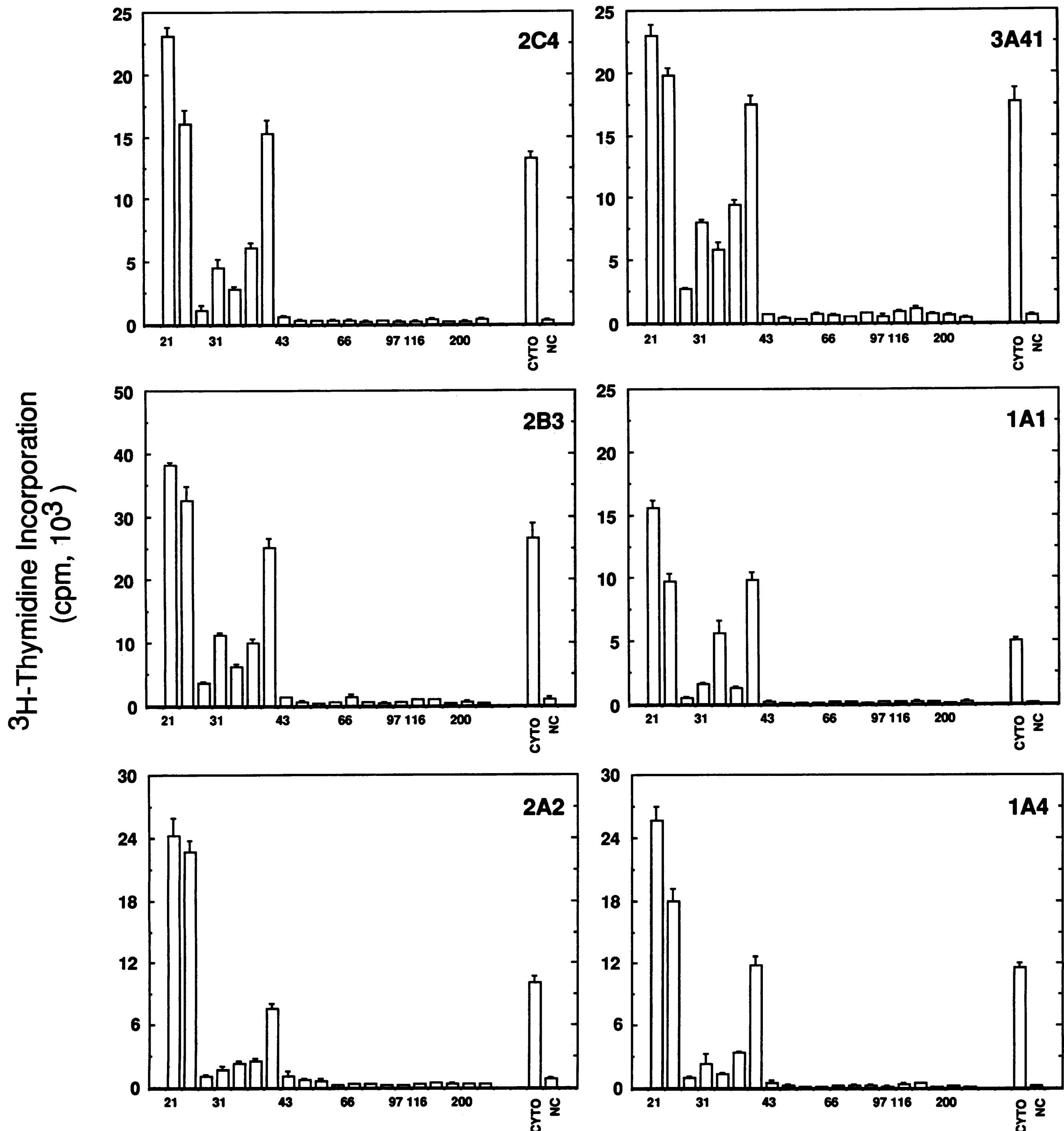

FIG. 2. T-cell immunoblotting analysis of responses of hybridomas to Cyto. Values represent [^{3}H]thymidine incorporation by CTLL-2 cells. The data are the means ± standard errors of the means of triplicate determinations. The response of each hybridoma to 9.2 μg of unfractionated Cyto bound to NC (CYTO) and to NC alone (NC) is depicted on the right side of each graph. Molecular mass markers (in kilodaltons) are shown on the abscissa. One representative experiment of two is illustrated.

experiments designed to examine antigenic reactivity can be performed more frequently with these cells than with T-cell clones. The reason is that after stimulation with antigen, T-cell clones usually require at least 7 days before they respond optimally to antigen again (15). By contrast, samples of hybridomas can be removed daily and tested for antigenic reactivity. We also have observed that the recovery of cryopreserved hybrids is much more successful than is that of T-cell clones. The disadvantages of hybridomas are that they cannot be used to study cytokine regulation of T-cell growth, nor can they be used in vivo as viable cells.

When the results of the current study are compared with results obtained with $CD4^+$ T-cell clones, several points emerge. Both types of antigen-reactive T cells were gener-

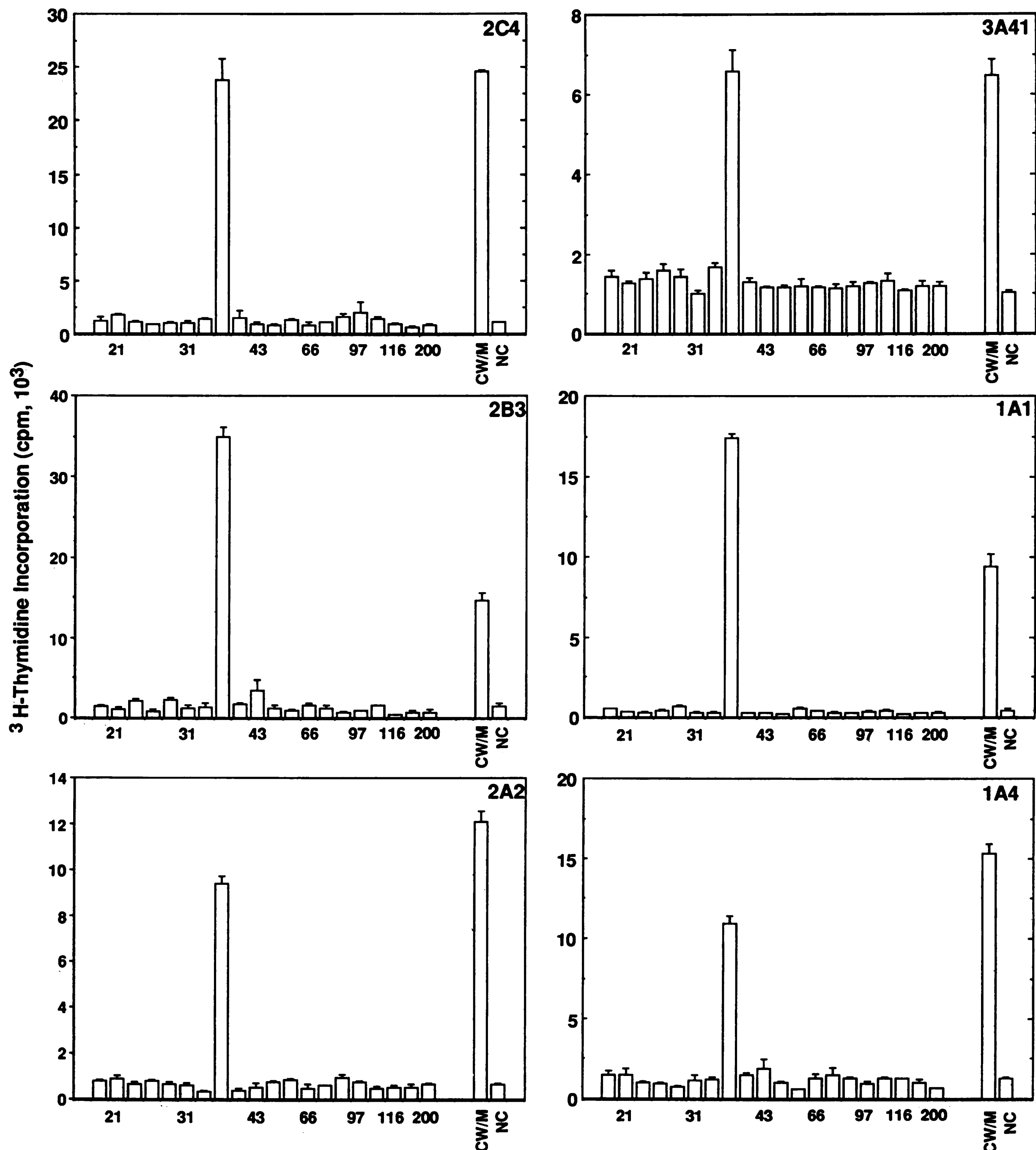

FIG. 3. Pattern of responses of hybridomas to electrophoresed CW/M. Values represent [^{3}H]thymidine incorporation by CTLL-2 cells. The data are the means ± standard errors of the means of triplicate determinations. The response of each hybridoma to 7.0 μg of unfractionated CW/M bound to NC (CW/M) and to NC alone (NC) is shown on the right side of each graph. Molecular mass markers (in kilodaltons) are shown on the abscissa. One representative experiment of two is illustrated.

ated from polyclonal populations that had been stimulated with HKC. However, the T-cell clones have been responsive to this antigenic preparation over the years, whereas the reactivity of hybridomas to HKC was greatly diminished or was lost (4, 8) within weeks of isolation. Although hybridomas were cryopreserved while they were responsive to HKC, the reactivity of cells retrieved from freezing remained irreversibly diminished. The explanation of this is not known.

In studies of antigenic specificity, only 1 of 10 hybridomas released IL-2 after incubation with heterologous fungal antigens. This finding contrasts with previous data in which T-cell lines and 6 of 12 clones responded to C-ASWS or B-ASWS (7). Also, extensive cross-reactivity among antigens from *H. capsulatum*, *B. dermatitidis*, and *C. immitis* has been demonstrated in cellular immune assays (4, 5). The low frequency of cross-reactive hybridomas, therefore, was unexpected. Furthermore, we used two additional approaches to isolate hybridomas that represented the repertoire of antigen-reactive T cells from immune mice. First, the

hybridomas were isolated from spleens of mice repeatedly inoculated with viable yeast cells to expose splenic T cells to a large number of putative antigens. Second, splenic T cells were stimulated with HKC in vitro only once. This step was taken to minimize selecting T cells that would only respond to a limited number of antigens in HKC. Because the majority of hybridomas are not cross-reactive, they can be used to define T-cell epitopes that are unique to *H. capsulatum*.

Much attention has focused on identifying and purifying antigens from pathogenic microbes. The technique of T-cell immunoblotting has simplified the detection of T-cell determinants from a complex mixture of antigens prepared from microbes (2, 12). The profiles of responses of individual hybridomas to electrophoretically separated Cyto or CW/M were fairly uniform. All hybridomas recognized two immunodominant regions in Cyto with masses of ≤18 to 26 kDa and of 35 to 39 kDa. Hybridomas responded to a single fraction in CW/M with a mass of 35 to 39 kDa.

CW/M and Cyto appeared to contain a common antigenic region. Since CW/M is not prepared in such a manner as to contain only membrane particles, it is possible that this molecule or molecules of 35 to 39 kDa are present only in Cyto and that they are copurified with CW/M. Alternatively, the determinants may exist separately in both fractions of *H. capsulatum*. The finding that antigens in Cyto and CW/M have similar masses does not imply that the amino acid sequences of the determinants are homologous. Additional studies will be required to determine if the antigenic molecules of 35 to 39 kDa in Cyto and CW/M share identity at the amino acid level.

The profile of responses of hybridomas to CW/M differs markedly from that observed with T-cell clones (Gomez et al., submitted). Five of six clones and a T-cell line respond to two immunodominant domains in CW/M with masses of 53 to 82 kDa. The difference in the pattern of responses between hybridomas and clones to CW/M indicates that there are numerous T-cell epitopes in this preparation from strain G217B yeast cells. Taken together, the heterogeneity of the responsiveness of T cells to CW/M raises concerns about which of the many determinants produce a protective response. In this regard, construction of a vaccine or an immunotherapeutic agent for histoplasmosis may remain elusive until protective epitopes can be distinguished from antigens that simply engage the T-cell receptor and do not confer protection.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-23017 from the National Institute of Allergy and Infectious Diseases. G. Deepe, Jr., is a recipient of Research Career Development award K04 AI-00856 from the National Institute of Allergy and Infectious Diseases.

We thank Angela Birch Smith for excellent secretarial assistance.

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