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Development and Characterization of *Histoplasma capsulatum*-Reactive Murine T-Cell Lines and Clones

GEORGE S. DEEPE, JR.,[1]* JAMES G. SMITH,[1] GERALD SONNENFELD,[2] DAVID DENMAN,[3] AND WARD E. BULLOCK[1]

Department of Medicine[1] and Department of Radiology,[3] University of Cincinnati College of Medicine, Cincinnati, Ohio 45267, and Department of Microbiology and Immunology, University of Louisville School of Medicine, Louisville, Kentucky 40292[2]

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Experimental studies have suggested that antigen-specific T lymphocytes are important mediators of resistance to infection with the pathogenic fungus *Histoplasma capsulatum*. To gain a better understanding of the role of T lymphocytes, we developed murine T-cell lines and clones that recognized *Histoplasma* antigens. These T cells were of the helper/inducer phenotype (Thy-1.2$^+$ Lyt-1$^+$ L3T4$^+$ Lyt-2$^-$) and exerted multiple immunological functions. T-cell lines and 12 clones proliferated vigorously in response to histoplasmin; the T-cell lines and 6 clones also were reactive with heterologous fungal antigens prepared from either *Blastomyces dermatitidis* or *Coccidioides immitis*. Recognition of antigen by T cells was *H-2* restricted; in the absence of antigen, four clones demonstrated alloreactivity. All T-cell clones conferred local delayed-type hypersensitivity responses when injected with antigen into footpads of mice. Ten of 12 T-cell clones released interleukin-2 after stimulation with antigen, and all clones tested secreted interferon. Moreover, culture supernatants from antigen-stimulated clones armed peritoneal macrophages to inhibit intracellular growth of *H. capsulatum* yeast cells. All clones assayed exerted nonspecific help. Thus, development of T-cell clones should facilitate analysis of the regulatory properties of *Histoplasma*-specific T cells.

Human infection with the pathogenic fungus *Histoplasma capsulatum* produces a wide spectrum of disease ranging from a mild respiratory illness to a progressive disseminated form. Most commonly, the infection is self-limiting and is detected by the presence of a delayed-type hypersensitivity (DTH) response to *Histoplasma* antigens. An effective host response that controls infection with *H. capsulatum* appears to require the activation of cell-mediated immune responses. Several experimental studies support this concept. First, mice treated with antilymphocyte serum or athymic mice are highly susceptible to infection with *H. capsulatum* (1, 37). Second, antigen-stimulated T lymphocytes from mice immunized with *H. capsulatum* secrete a factor, presumably gamma interferon (IFN-γ), that arms macrophages (Mϕ) to restrict intracellular growth of yeast-phase organisms (38). Third, T lymphocytes from immunized mice can transfer to naive mice protection against a lethal challenge with *H. capsulatum*, whereas B lymphocytes do not (35).Thus, cellular immunity and, in particular, T lymphocytes play important roles in host defenses against invasion to this fungus; however, the precise mechanisms by which these cells contribute to host resistance remain poorly understood.

An important advance in cellular immunology has been the development of monoclonal populations of antigen-specific T cells that can be propagated in vitro for prolonged periods. Studies of cloned T-cell lines (TCL) have greatly enhanced our knowledge about the biological properties of T cells. To this end, we have produced TCL and cloned T cells that are *Histoplasma* reactive from spleens of C57BL/6 mice immunized with viable *H. capsulatum* yeast cells. The cloned T cells proliferate in response to histoplasmin and, in some cases, to heterologous fungal antigens. In addition, these cells mediate local DTH responses when injected with antigen into footpads of mice, they release regulatory lymphokines after stimulation with antigen, and they exert helper activity.

* Corresponding author.

MATERIALS AND METHODS

Mice and preparation of *H. capsulatum*. Male C57BL/6, B10.A(4R), B10.A(5R), B10.MBR, BALB/c, BDP/J, C3H/HeJ, SJL/J, and SWR/J mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. *H. capsulatum* yeast cells, strain G217B, were prepared as described previously (12).

Antigens and reagents. Sheep erythrocytes (SRBC) were purchased from Colorado Serum Co., Denver. Histoplasmin (HKC-43) was a gift from Norman Goodman, University of Kentucky, Lexington. It was prepared from seven mycelial strains of *H. capsulatum* grown in Smith asparagine medium. Before use, HKC-43 was dialyzed against phosphate-buffered saline, pH 7.2, for 24 h. Alkali-soluble, water-soluble cell wall extracts of mycelium-phase *Blastomyces dermatitidis* (B-ASWS) and *Coccidioides immitis* (C-ASWS) were provided generously by Rebecca Cox, San Antonio State Chest Hospital, San Antonio, Tex. Cryptococcal antigen (Cnef) was a gift of Juneann Murphy, Norman, Okla. Purified protein derivative was obtained from Lederle Laboratories, Pearl River, N.Y. Rabbit antibody to mouse interferon-α/β was purchased from Lee Biomolecular, San Diego, Calif.

IL-2-enriched supernatants. A preparation of interleukin-2 (IL-2) was produced by stimulating Lewis rat splenocytes with 5 μg of concanavalin A per ml for 48 h. The supernatants were harvested, and 20 mg of α-methyl-mannoside per ml was added. This preparation was filter sterilized and stored at −70°C.

Generation of TCL. TCL were developed after the method of Kimoto and Fathman (20). C57BL/6 mice were injected

subcutaneously with 10^6 viable yeast cells. Two weeks later, spleens were removed and teased apart between two ground-glass slides in Hanks balanced salt solution (HBSS). After removal of tissue debris by sedimentation, cells were washed twice and suspended in RPMI 1640 containing 10% heat-inactivated 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 (complete medium) at 3×10^6 cells per ml. One milliliter of cell suspension was dispensed into each well of a 24-well plate (Becton Dickinson Labware, Oxnard, Calif.), and the cells were stimulated for 4 days with a 1:500 final dilution of HKC-43. Subsequently, the cells were harvested and centrifuged over Lympholyte-M (Accurate Chemical and Scientific Corp., Westbury, N.Y.) for 20 min at 400 × *g*. Lymphoblasts were removed, washed once, and suspended in complete medium. To each well of a 24-well plate were added 1×10^5 to 2×10^5 viable cells and 2×10^6 irradiated (1,500 R) syngeneic splenocytes in complete medium without HKC-43. After 10 days of "rest," 1×10^5 to 2×10^5 viable cells were recultured with 2×10^6 fresh, irradiated splenocytes and a 1:500 dilution of HKC-43 for 4 days. T cells were propagated by alternating cycles of stimulation for 4 days and rest for 10 days.

Cloning of T cells. T-cell clones were derived by limiting dilution in 96-well plates (Microtest II; Becton Dickinson Labware); 0.3, 1, or 3 resting T cells in 0.1 ml of complete medium were cultured in the presence of 5×10^5 irradiated syngeneic splenocytes, 1:500 dilution of HKC-43, and 10% (vol/vol) IL-2-containing supernatants. After 14 days, wells exhibiting growth were transferred to 24-well plates with the addition of 2×10^6 irradiated splenocytes, HKC-43, and 10% IL-2-enriched supernatants. The clones were thus expanded in 24-well plates by restimulating 1×10^5 to 2×10^5 cells per well every 7 to 10 days with 2×10^6 irradiated splenocytes, HKC-43, and 5% IL-2-containing supernatants.

Proliferation assay. Resting T cells were suspended in RPMI 1640 supplemented with 10% FBS and 10 μg of gentamicin per ml. To each well of a 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 HKC-43 or a heterologous antigen. The cells were incubated for 72 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 culture. 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 a liquid scintillation counter.

Phenotypic analysis of T cells. Antigen-stimulated T cells were centrifuged over Lympholyte-M, washed, and suspended in RPMI 1640 containing 0.1% sodium azide and 1% FBS (staining medium). To 5×10^5 cells in 0.2 ml was added one of the following monoclonal antibodies: 7.5 μg of fluorescein-conjugated anti-Thy-1.2, anti-Lyt-1, or anti-Lyt-2 (Becton Dickinson, Mountain View, Calif.). Supernatant from hybridoma GK1.5 (0.2 ml) was added to a sample of cells to label the L3T4 surface antigen (13). Fluorescein-conjugated goat anti-rat immunoglobulin G (Kirkegaard and Perry, Gaithersburg, Md.) was used as a secondary antibody at a 1:40 dilution. Cell incubations were carried out at 4°C for 30 min and were followed by two washes in staining medium. All cells were suspended in 0.5 ml of staining medium prior to analyses with a FACS 440 (Becton Dickinson).

Measurement of DTH responses. Resting T cells were suspended at 10^7 cells per ml in undiluted HKC-43 or HBSS; 50 μl of cells suspended in HKC-43 was inoculated intradermally into the right hind footpad, whereas cells in HBSS were injected into the left hind footpad. A separate group of mice were injected with 50 μl of HKC-43 alone. Footpad thickness was measured 24 h later with a digital micrometer. The DTH response was expressed as the percent increase in footpad size over that measured immediately before challenge with cells.

Generation of lymphokine-containing supernatants. Supernatants were prepared in 24-well plates by incubating 10^5 resting T cells with 2×10^6 irradiated syngeneic splenocytes in the presence or absence of HKC-43 at a final volume of 1 ml per well. At various times after culture initiation, supernatants were harvested, centrifuged, and stored at −70°C until assayed.

Assay of IL-2 activity. To twofold dilutions of test supernatants were added 4×10^3 CTLL-2 cells, and triplicates were cultured at 37°C in 5% CO_2 for 28 h. Four hours before harvest, cells were pulsed with 0.5 μCi of [^{3}H]thymidine.

Assay of IFN activity. Antiviral activity was determined by a plaque reduction assay, using the Indiana strain of bovine vesicular stomatitis virus and murine L-929 cells (30). The IFN titer in supernatants was designated as equivalent to the reciprocal of the highest dilution of the sample that reduced virus plaques by 50%. In this assay system, one IFN unit was equal to 0.88 NIH G-002-904-511 reference unit.

Assay for Mϕ-activating factor(s). Inhibition of intracellular growth of *H. capsulatum* was assayed by the method of Wu-Hsieh and Howard (38); peritoneal Mϕ were harvested in RPMI 1640 medium containing 10 U of heparin per ml from mice inoculated intraperitoneally 3 days previously with 2 ml of Difco Proteose Peptone (Difco Laboratories, Detroit, Mich.). Mϕ were washed and then suspended in RPMI 1640 containing 10% FBS and 0.1% gentamicin to a final concentration of 10^6 cells per ml; 1-ml samples were placed on 25-mm round glass cover slips in 35-mm plastic dishes and incubated for 2 h at 37°C. The dishes were then washed extensively, and peritoneal Mϕ were incubated with medium, a 1:2 dilution of culture supernatant from resting T-cell clones, or a 1:2 dilution of culture supernatant from antigen-stimulated T-cell clones for 3 h. Subsequently, the Mϕ monolayers were inoculated with 2×10^5 *H. capsulatum* yeast cells. Extracellular yeasts were removed after 1 h, and cover slips from each group were removed and stained with Diff-Quik (Dade Diagnostics Inc., Aquada, P.R.). The remaining monolayers were reincubated with medium or supernatants for an additional 15 h before staining. The number of yeast cells within 100 infected Mϕ was counted in a blinded fashion, and the mean number of yeast cells per infected Mϕ was calculated.

To characterize partially the Mϕ-activating factor(s), supernatants were dialyzed against a glycine-HCl acid buffer, pH 2, for 24 h followed by dialysis against RPMI, pH 7, for an additional 24 h. As a control, supernatants were dialyzed against RPMI, pH 7, for 48 h. Heat stability of the factor was assessed by incubating supernatants for 30 min at 56°C. The capacity of rabbit anti-mouse IFN-α/β to alter the regulatory effect of supernatants was tested by adding 750 U of anti-IFN-α/β or, as a control, an equal volume of rabbit serum to each milliliter of a 1:4 dilution of test supernatants.

Assay for helper activity. Resting T cells (10^4) were cocultured in 48-well plates (Costar, Cambridge, Mass.) with a preparation of B cells plus accessory cells (3×10^6 per well) in the presence of 3.5×10^6 SRBC per well and with or without HKC-43. The B cells plus accessory cells were prepared by depletion of T cells from splenocyte suspensions. Splenocytes were suspended in RPMI 1640 containing

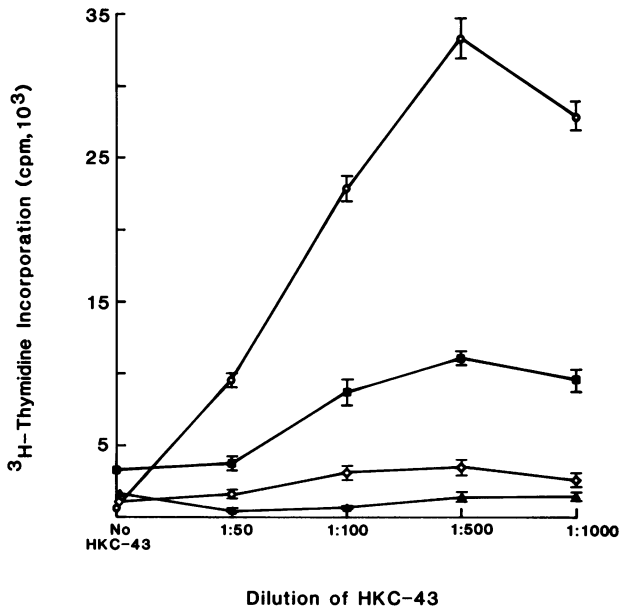

FIG. 1. Proliferative response by unfractionated splenocytes from normal (▲) and *H. capsulatum*-inoculated (◇) mice and by JC1 (○) and JC2 (■) cells to HKC-43. Splenocytes (4×10^5) were cultured for 5 days with or without HKC. T cells (2×10^4) were cultured with 5×10^5 irradiated syngeneic splenocytes in the presence or absence of HKC-43 for 3 days. Data are expressed as mean ± standard error of triplicate determinations. Representative experiment of three experiments is depicted.

5% FBS at a concentration of 10^7 cells per ml and incubated in a 1:5 dilution of monoclonal anti-Thy-1.2 antibody (HO-13-4-9) (22) at 4°C for 30 min followed by a 30-min incubation at 37°C with a 1:10 dilution of Low-Tox M rabbit complement (Accurate Chemical and Scientific Corp.). Quadruplicate cocultures were incubated at 37°C in 5% CO_2 for 4 days. The number of plaque-forming cells to SRBC was quantitated by the method of Cunningham and Szenberg (11).

Statistics. The Wilcoxon rank sum test was used for statistical analysis.

RESULTS

Proliferative response by splenocytes and TCL from *Histoplasma*-inoculated mice. C57BL/6 mice were injected subcutaneously with 10^6 viable yeasts; 2 weeks later, spleen cells from immunized and control mice were tested for their responsiveness to HKC-43 in vitro. Spleen cells from mice inoculated with *H. capsulatum* yeasts mounted a significant response ($P < 0.05$) to HKC-43 versus control mice (Fig. 1). A 1:500 dilution of HKC-43 induced optimal proliferation of spleen cells.

Two TCL, JC1 and JC2, were developed from spleen cells of immunized mice as described in Materials and Methods. After six cycles of stimulation and rest, JC1 and JC2 were assessed for reactivity to HKC-43. JC1 responded vigorously to HKC-43 in a dose-dependent manner (Fig. 1). JC2 also proliferated in response to antigen, although the proliferative activity of JC2 was less than that of JC1. As with unfractionated splenocytes from *H. capsulatum*-immunized mice, JC1 and JC2 responded maximally to a 1:500 dilution of HKC-43.

Cloning of TCL. JC1 and JC2 were cloned by limiting dilution in 96-well microtiter plates. The percentage of growing cells in plates seeded with 3, 1, and 0.3 cells per well was as follows: JC1—18% of 3 cells per well, 9% of 1 cell per well, and 4% of 0.3 cell per well; JC2—10% of 3 cells per well, 5% of 1 cell per well, and 3% of 0.3 cell per well. Because of the low clonal efficiency, all actively growing wells were likely to be true clones (>95% probability of clonality by Poisson statistics). Twelve clones that subsequently survived the expansion process were subjected to further analysis.

Phenotypic characterization of TCL and cloned T cells. After eight cycles of stimulation and rest, JC1 and JC2 were ≥95% Thy-1.2$^+$ Lyt-1$^+$ L3T4$^+$ Lyt-2$^-$. Surface immuno-

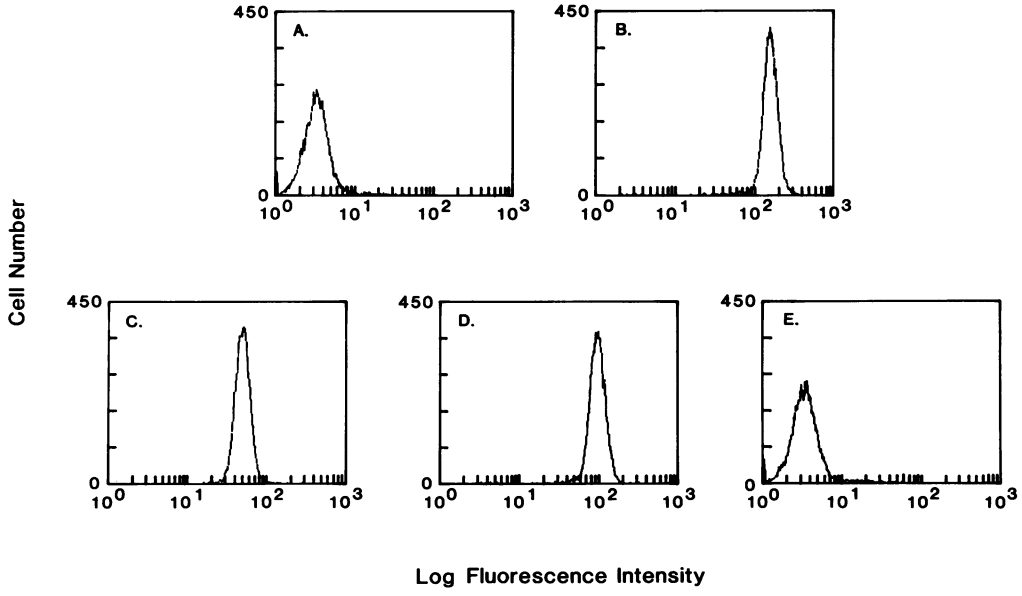

FIG. 2. Immunofluorescence profile of clone 1.3G6. Panels illustrate cells that were unstained (background fluorescence) (A) or stained with anti-Thy-1.2 (B), anti-Lyt-1 (C), anti-L3T4 (D), or anti-Lyt-2 (E), as described in Materials and Methods. Immunofluorescence was analyzed by a FACS 440.

TABLE 1. Proliferative response by TCL and T-cell clones to HKC-43 and heterologous antigens

TCL or clone	Proliferative response (mean cpm ± SEM) to [a]:					
	Medium	HKC-43	B-ASWS	C-ASWS	Cnef	PPD
JC1	320 ± 14	33,439 ± 1,255	18,537 ± 995	4,835 ± 742	209 ± 9	618 ± 58
JC2	3,963 ± 143	16,251 ± 272	10,430 ± 720	8,385 ± 602	3,085 ± 12	4,530 ± 383
1.03C11	942 ± 50	29,648 ± 665	795 ± 167	734 ± 167	1,146 ± 86	1,398 ± 93
1.1C3	721 ± 71	17,632 ± 14	731 ± 14	1,939 ± 432	582 ± 158	704 ± 113
1.1D5	778 ± 38	23,396 ± 3,308	571 ± 79	6,504 ± 535	1,432 ± 256	1,207 ± 7
1.3G4	494 ± 70	30,882 ± 1,347	1,198 ± 131	10,423 ± 1,504	955 ± 116	1,275 ± 266
1.3G6	835 ± 245	26,974 ± 2,720	1,791 ± 282	13,848 ± 551	751 ± 103	1,492 ± 68
2.3D3	2,196 ± 777	33,243 ± 1,372	2,390 ± 247	2,732 ± 267	3,699 ± 10	3,073 ± 818
2.3E10	2,626 ± 8	38,865 ± 439	2,240 ± 269	1,710 ± 187	3,319 ± 411	2,403 ± 468
2.3H3	426 ± 88	12,592 ± 1,102	279 ± 58	996 ± 26	666 ± 61	654 ± 28

[a] Mean of triplicate determinations. Optimal dilution or concentration of antigens: HKC-43, 1:500 for JC1 and JC2; HKC-43, 1:100 for clones; B-ASWS and C-ASWS, 40 μg/ml; Cnef, 1:100; purified protein derivative (PPD), 25 μg/ml.

globulin was not detected on cells. All cloned T cells were >98% Thy-1.2$^+$ Lyt-1$^+$ L3T4$^+$ Lyt-2$^-$. The fluorescence intensity of cloned T cells stained with fluoresceinated anti-Lyt-2 did not differ from unstained cells. A representative fluorescence profile of one clone is illustrated in Fig. 2. Thus, *Histoplasma*-reactive cloned T cells were of the helper/inducer phenotype.

Antigen specificity of TCL and cloned T cells. The proliferative response by the TCL and 12 clones to HKC-43 and to a battery of heterologous antigens was examined to determine the antigen specificity of these T cells. Several points emerged from these studies. First, in contrast to the parent lines, all cloned T cells responded optimally to a 1:100 dilution of HKC-43 rather than to a 1:500 dilution (data not shown). Second, both B-ASWS and C-ASWS induced proliferation of the TCL, although the response to these antigens was much less than that of the response to HKC-43 (Table 1). Third, the proliferative activity of cloned T cells to heterologous fungal antigens varied widely. Of the eight clones presented in Table 1, four (1.03C11, 2.3H3, 2.3E10, and 2.3D3) were responsive only to HKC-43. The remaining four clones demonstrated cross-reactivity to B-ASWS or C-ASWS. 1.1C3 and 1.1D5 responded only to C-ASWS; 1.3G4 and 1.3G6 responded to both B-ASWS, albeit weakly, and C-ASWS. Of the four additional clones not depicted in Table 1, two recognized only HKC-43, and two responded to both B-ASWS and C-ASWS. Clone 1.1E1 was the only 1 of 12 in which the response induced by a heterologous fungal antigen, in this case B-ASWS (17,417 ± 713 cpm), was equal to that of HKC-43 (17,364 ± 1,244 cpm; medium control, 867 ± 43 cpm). Lastly, neither the TCL nor the cloned T cells responded to Cnef or purified protein derivative.

Major histocompatibility complex: restriction of antigen presentation and alloreactivity of cloned T cells. To determine whether allogeneic accessory cells could present antigen to cloned T cells, irradiated splenocytes (as a source of accessory cells) from several inbred strains of mice were cultured with T cells in the presence or absence of HKC-43. TCL and cloned T cells responded significantly ($P < 0.05$) to HKC-43 only in the presence of syngeneic splenocytes. The data for four representative clones are presented in Table 2. In the absence of HKC-43, these same four clones mounted a moderate to strong proliferative response (as judged by a stimulation index of ≥10) to one or more allogeneic stimulators (Table 2). Thus, although presentation of HKC-43 to T cells was *H-2* restricted, 4 of 12 clones were dual reactive; that is, they responded not only to nominal antigen in the presence of syngeneic splenocytes, but also to alloantigens.

In parallel studies, cloned T cells were cultured with irradiated splenocytes from the congenic mice B10.MBR, B10.A(4R), and B10.A(5R) in the presence or absence of HKC-43 (Table 3). T cells responded to HKC-43 when

TABLE 2. Proliferative response by cloned T cells to HKC-43 in the presence of syngeneic or allogeneic accessory cells

Source of accessory cells[a]	*H-2* haplotype	HKC-43[b]	[^{3}H]thymidine incorporation (cpm ± SEM)[c] by:			
			1.1E1	1.3D1	2.3D3	2.3H3
C57BL/6	*b*	−	499 ± 82	801 ± 56	370 ± 61	564 ± 33
		+	12,388 ± 11	22,259 ± 1,449	24,167 ± 1,416	30,576 ± 877
BALB/cJ	*d*	−	1,538 ± 208	3,341 ± 252	199 ± 24	**10,092 ± 1,615**[d]
		+	836 ± 123	3,305 ± 261	129 ± 25	18,155 ± 1,217
C3H/HeJ	*k*	−	**20,545 ± 1,526**	1,848 ± 245	409 ± 78	4,236 ± 645
		+	10,341 ± 835	2,217 ± 81	215 ± 46	7,019 ± 581
BDP/J	*p*	−	1,961 ± 73	**27,362 ± 3,300**	**14,033 ± 1,235**	1,063 ± 466
		+	971 ± 227	17,832 ± 826	7,237 ± 797	650 ± 21
SWR/J	*q*	−	3,288 ± 380	2,748 ± 1,318	1,148 ± 297	**9,264 ± 612**
		+	848 ± 71	3,410 ± 50	485 ± 59	5,426 ± 1,136
SJL/J	*s*	−	668 ± 213	3,535 ± 448	1,829 ± 418	**6,988 ± 106**
		+	738 ± 90	3,333 ± 273	859 ± 214	2,306 ± 10

[a] 5×10^5 irradiated splenocytes from each strain of mice cultured with 2×10^4 cloned T cells per well.
[b] Final dilution of HKC-43 was 1:100.
[c] Mean of triplicate determinations.
[d] Boldface values are positive responses to allogeneic accessory cells as judged by stimulation index (cpm of T cells with allogeneic accessory cells/cpm of T cells with syngeneic accessory cells) of ≥10.

TABLE 3. *H-2* region restriction of antigen presentation to cloned T cells

Source of accessory cells[a]	*H-2*					HKC-43[b]	[^{3}H]thymidine incorporation (mean ± SEM)[c] by:					
	K	$A_\beta A_\alpha$	E_β	E_α	D		1.1C3	1.1D5	1.3G6	2.3D3	2.3E10	2.3H3
C57BL/6	*b*	*b*	(*b*)[d]	*b*	*b*	−	2,325 ± 47	193 ± 13	968 ± 225	594 ± 298	771 ± 153	758 ± 87
						+	37,972 ± 2,504	7,773 ± 636	47,499 ± 5,488	19,510 ± 1,206	53,744 ± 3,914	13,663 ± 879
B10.MBR	*b*	*k*	*k*	*k*	*q*	−	1,969 ± 203	205 ± 25	2,400 ± 518	264 ± 54	1,507 ± 345	8,058 ± 2,007
						+	1,338 ± 202	73 ± 9	7,105 ± 252	338 ± 27	886 ± 169	9,917 ± 359
B10.A(4R)	*k*	*k*	(*k*)[d]	*b*	*k*	−	1,587 ± 414	245 ± 32	2,279 ± 340	387 ± 120	2,510 ± 681	916 ± 141
						+	1,070 ± 299	131 ± 13	1,932 ± 375	371 ± 128	899 ± 238	395 ± 50
B10.A(5R)	*b*	*b*	*b*	*k*	*d*	−	1,262 ± 68	242 ± 43	662 ± 146	196 ± 14	802 ± 114	1,146 ± 175
						+	29,523 ± 3,710	14,909 ± 1,266	48,329 ± 4,906	24,641 ± 4,470	32,742 ± 1,438	20,447 ± 1,156

[a] 5×10^5 irradiated splenocytes cocultured with 2×10^4 cloned T cells.
[b] Final dilution of HKC-43 was 1:100.
[c] Mean of triplicate determinations.
[d] Parentheses indicate silent allele.

incubated with accessory cells from C57BL/6 or B10.A(5R) mice but not B10.MBR or B10.A(4R) mice. Therefore, antigen presentation requires compatibility at the I-A locus.

Mediation of local DTH by cloned T cells. Cloned T cells were injected with or without HKC-43 into footpads of C57BL/6 mice, and footpad swelling was measured 24 h later. All cloned T cells assayed conferred local DTH reactivity when injected with HKC-43 (Table 4). In addition, the percent increase (mean ± standard error) in footpad swelling to HKC-43 alone (7 ± 1%) was similar to that of T cells in HBSS. Histopathologic examination of footpads from mice inoculated with T cells suspended in HKC-43 revealed a heavy infiltration of both polymorphonuclear leukocytes and monocytes in a ratio of 2:1. Furthermore, there was mild perivascular invasion by both polymorphonuclear leukocytes and monocytes. This histological picture resembles the DTH reaction to polysaccharides in mice (27).

IL-2 production. TCL and cloned T cells were incubated with or without HKC-43, and supernatants from these cultures were assayed for IL-2 activity. The kinetics of IL-2 production by JC1 and JC2 are illustrated in Fig. 3A. At 6 h, IL-2 activity was detectable in culture supernatants of the antigen-stimulated TCL; peak IL-2 activity was measured at 24 h. Thereafter, little or no IL-2 was contained in culture supernatants. Unstimulated JC1 and JC2 did not secrete measurable levels of IL-2 (Fig. 3A). Ten of 12 clones produced measurable amounts of IL-2 in response to HKC-43; results from 8 clones that secreted IL-2 are presented in Table 5. Production of this lymphokine was variable. Culture supernatant from antigen-stimulated 1.03C11 increased [^{3}H]thymidine incorporation by CTLL-2 cells only 6-fold above medium control, whereas supernatant from antigen-stimulated 1.3G4 enhanced this incorporation by 85-fold. That 2 of the 12 clones failed to release detectable levels of IL-2, yet could proliferate in response to HKC-43, suggests that these T cells utilized all of this essential T-cell growth factor as rapidly as they generated it.

TABLE 4. Cloned T cells mediate local DTH responses to HKC-43

Cloned T cells[a]	% Increase in footpad thickness (mean ± SEM)[b]	
	HBSS challenge	HKC-43 challenge
1.03C11	8 ± 1	33 ± 3[c]
1.1C3	9 ± 1	33 ± 2
1.1D5	8 ± 1	38 ± 4
1.3G4	11 ± 1	53 ± 3
1.3G6	11 ± 2	48 ± 3
2.3D3	11 ± 2	52 ± 5
2.3E10	8 ± 1	36 ± 4
2.3H3	11 ± 2	45 ± 3

[a] C57BL/6 mice were injected with 5×10^5 cloned T cells suspended in either HKC-43 or HBSS into right and left footpads, respectively. Footpad swelling was measured 24 h later.
[b] Mean increase from groups of five mice.
[c] Change in footpad thickness is significantly greater ($P < 0.01$) than that of T cells suspended in HBSS.

IFN production. The kinetics of IFN generation by JC1 and JC2 after HKC-43 stimulation differed from that of IL-2 production by these cells. IFN production by JC1 was maximal at 48 h and increased gradually thereafter (Fig. 3B), whereas IFN generation by JC2 peaked at 48 h and then declined. Interestingly, IFN activity in culture supernatants from unstimulated JC2 cells was similar to that of supernatants from antigen-stimulated JC2 cells at or beyond 24 h of culture. Data from 8 of 12 clones are presented in Table 5. Supernatants from all unstimulated clones except 1.03C11 contained detectable IFN activity. Stimulation of clones with HKC-43 enhanced IFN generation by 3- to 27-fold.

Effect of supernatants from antigen-stimulated T-cell clones on intracellular growth of *H. capsulatum*. Additional experiments were conducted to determine if lymphokine-containing supernatants from clones could stimulate peritoneal Mϕ to inhibit intracellular growth of yeast-phase *H. capsulatum*. Supernatants from 48-h cultures of unstimulated or antigen-stimulated T-cell clones were harvested and tested at a 1:2 dilution. Supernatants from all antigen-stimulated clones sharply reduced intracellular growth of yeasts. The results for two clones are shown in Table 6.

Supernatants from antigen-stimulated T-cell clones were studied further to characterize partially the factor(s) responsible for arming Mϕ. The activity of the factor(s) was abrogated by acid treatment but was unaffected by heat (Table 6). Addition of 750 U of rabbit anti-mouse IFN-α/β to each milliliter of culture supernatant did not alter the activity of supernatants. Thus, the biological characteristics of this factor(s) are similar to that of IFN-γ (16).

TCL and cloned T cells mediate nonspecific help. Since cloned T cells were of the helper/inducer phenotype, we examined whether these T cells could exert helper activity. T cells were cultured with a preparation of syngeneic B cells plus accessory cells in the presence or absence of HKC-43. All cultures were immunized with SRBC, and the number of plaque-forming cells to SRBC was measured 4 days later. Seven clones were assessed for their capacity to function in the helper mode, and the results with five clones are presented in Fig. 4. Coculture of cloned T cells with B cells in

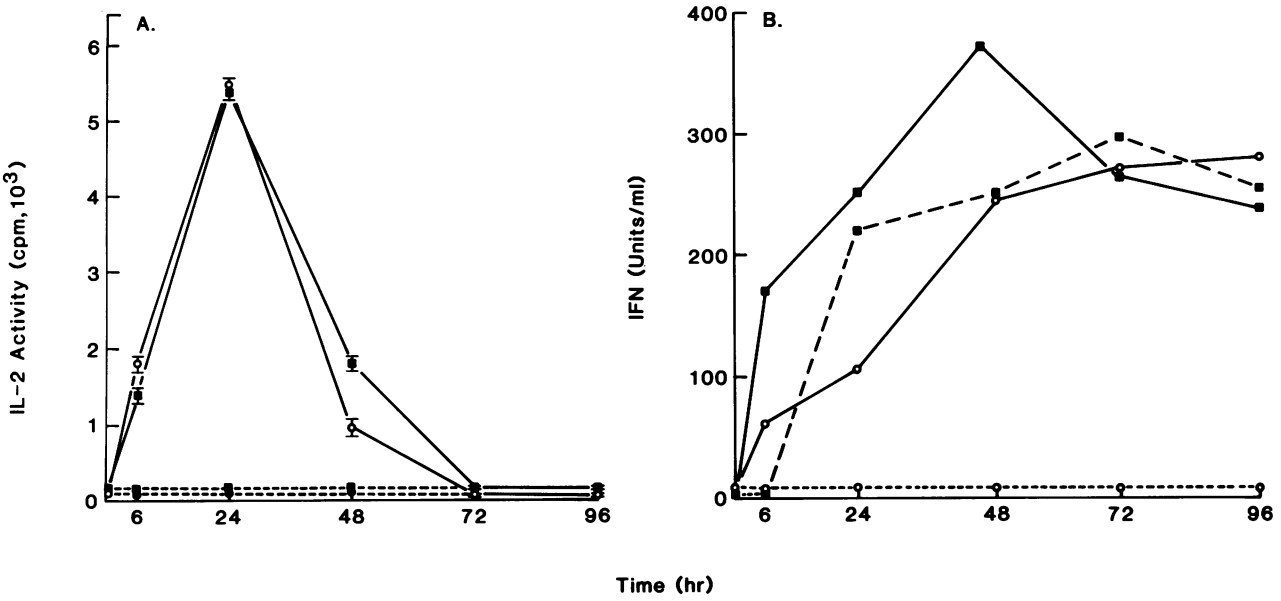

FIG. 3. Kinetics of lymphokine production by JC1 (○) and JC2 (■). IL-2 activity (A) and IFN levels (B) in culture supernatants from T cells stimulated with a 1:500 dilution of HKC-43 (——) or in supernatants from unstimulated T cells (- - -). IL-2 activity was measured in a 1:4 dilution of test supernatants and was assayed by [^{3}H]thymidine incorporation by CTLL-2 cells. Data are expressed as mean ± standard error of triplicate determinations. Response by CTLL-2 cells cultured in a 1:4 dilution of IL-2-enriched supernatants was 12,905 ± 352 cpm. IFN levels in supernatants of irradiated splenocytes incubated with HKC-43 1:500 was <30 U/ml. Representative experiment of three experiments is shown.

the presence of HKC-43 markedly enhanced plaque-forming cell responses to SRBC. Thus, as measured in this helper assay, T cells could nonspecifically stimulate secretion of immunoglobulin by B cells.

DISCUSSION

In the field of infectious diseases, murine and human cloned T cells have been produced that are reactive with antigens prepared from diverse pathogenic microorganisms including bacteria (18, 24), parasites (21, 18), and viruses (8, 15). In the present investigation, we have used the T-cell-cloning methodology to isolate cloned T cells that recognize *Histoplasma* antigens. Our findings, therefore, extend to fungi the range of antigen-specific cloned T cells that are reactive with antigens derived from pathogens. *Histoplasma*-reactive TCL and cloned T cells were characterized phenotypically as Thy-1.2$^+$ Lyt-1$^+$ L3T4$^+$, that is, of the helper/inducer phenotype. Functional analyses of these T cells revealed that they recognized *Histoplasma* antigens both in vitro and in vivo. In addition, cloned T cells secreted regulatory lymphokines in response to stimulation with HKC-43 and mediated helper activity. Thus, the generation of cloned T cells provides a useful tool for elucidating the regulatory mechanisms involved in host resistance to *H. capsulatum*.

TABLE 5. IL-2 and IFN production by cloned T cells

Cloned T cells	IL-2 activity (cpm ± SEM)[a]		IFN (U/ml)[b]	
	Medium	HKC-43[c]	Medium	HKC-43[c]
1.03C11	107 ± 17	676 ± 85	<30	166
1.1C3	27 ± 2	2,057 ± 207	96	265
1.1D5	80 ± 42	3,115 ± 432	88	700
1.3G4	49 ± 13	4,179 ± 322	100	283
1.3G6	75 ± 12	2,160 ± 771	122	1,980
2.3D3	81 ± 40	1,053 ± 87	81	1,389
2.3E10	123 ± 51	6,777 ± 364	85	728
2.3H3	267 ± 64	2,844 ± 528	81	2,230

[a] Mean by CTLL-2 cells cultured in triplicate. Supernatants were harvested 24 h after culture initiation and assayed at a final dilution of 1:4. Response by CTLL-2 cells cultured in a 1:4 dilution of IL-2-enriched supernatants ranged from 12,216 ± 410 to 14,776 ± 913 cpm. The response of CTLL-2 cells cultured in medium alone was <125 cpm.

[b] Supernatants were harvested 48 h after culture initiation and then assayed.

[c] Final dilution of HKC-43 was 1:100.

It is clear from the present study that HKC-43 contains an immunodominant epitope that is recognized by antigen-specific T cells. Six T-cell clones exhibited a very restricted pattern of antigen recognition in which they responded only to HKC-43. The highly specific nature of these cloned TCL provides evidence that, for T-cell recognition, *H. capsulatum* contains a unique epitope(s) that is not shared with antigens prepared from *B. dermatitidis* or *C. immitis*.

Others have demonstrated extensive cross-reactivity among antigens from *H. capsulatum*, *B. dermatitidis*, and *C. immitis* as assessed by DTH reactivity, serological assays, or lymphocyte transformation assays (9, 10). Therefore, it is of interest that the TCL and six clones also proliferated in response to heterologous fungal antigens derived from *B. dermatitidis*, or *C. immitis* or both. Although the response to these antigen preparations was considerably less than that to HKC-43, except for one clone, the cross-reactive nature of the TCL and some clones further supports the assertion that fungal antigens have shared epitopes.

To date, an antigen or antigens that are common to *H. capsulatum*, *B. dermatitidis*, and *C. immitis* have not been identified definitively, although it has been proposed that galactomannan may be a surface antigen present on both *H. capsulatum* and *B. dermatitidis* (4). Several additional moieties from mycelium- or yeast-phase *H. capsulatum* have been shown to be immunoreactive in various immunological assays. These substances include glycolipids (5), polysac-

TABLE 6. Partial characterization of lymphokine that arms Mϕ to inhibit intracellular growth of *H. capsulatum*

Expt	Supernatant prepn[a]		Treatment of supernatant	Mean no. of yeasts/infected Mϕ[b]	
	Clone	HKC-43[c]		0 h	16 h
1	Medium			1.99 ± 0.17	5.33 ± 0.08
	1.3G6	−		2.11 ± 0.07	6.05 ± 0.07
		+	None	2.19 ± 0.05	2.26 ± 0.12
		+	Dialysis, pH 2	2.15 ± 0.10	5.23 ± 0.16
		+	Dialysis, pH 7	2.29 ± 0.06	2.32 ± 0.09
		+	56°C, 30 min	2.16 ± 0.17	2.30 ± 0.18
2	Medium			2.00 ± 0.08	5.86 ± 0.09
	2.3H3	−		2.19 ± 0.06	5.39 ± 0.01
		+	None	2.21 ± 0.11	2.41 ± 0.02
		+	Dialysis, pH 2	2.02 ± 0.11	5.69 ± 0.22
		+	Dialysis, pH 7	2.31 ± 0.11	2.69 ± 0.08
		+	56°C, 30 min	2.10 ± 0.17	2.47 ± 0.28
3	Medium			2.18 ± 0.03	5.85 ± 0.18
	1.3G6	+	None	2.35 ± 0.09	2.50 ± 0.04
		+	Anti-IFN-α/β[d]	2.28 ± 0.09	2.44 ± 0.02
		+	Rabbit serum[d]	2.23 ± 0.10	2.35 ± 0.10
	2.3H3	+	None	2.38 ± 0.04	2.67 ± 0.18
		+	Anti-IFN-α/β[d]	2.58 ± 0.06	2.55 ± 0.14
		+	Rabbit serum[d]	2.35 ± 0.13	2.39 ± 0.11

[a] Supernatants assayed at a 1:2 dilution.
[b] Mean of triplicate determinations.
[c] Final dilution HKC-43 was 1:100.
[d] 750 U of anti-IFN-α/β or, as control, rabbit serum added to each milliliter of 1:4-diluted supernatant.

charides or a polysaccharide-protein complex (34), a protein, M antigen (7), and ribosomal protein (35). Furthermore, Sprouse (31) has isolated a glycoprotein from HKC-43 that mediates DTH reactivity in experimental animals. Therefore, from a practical standpoint, cloned T cells will facilitate the identification of shared and unique epitopes on the antigen molecule(s) from *H. capsulatum*.

Class II major histocompatibility complex molecules on the surfaces of antigen-presenting cells regulate, in part, antigen recognition by T cells, and the proliferation of T cells in response to antigen requires compatibility at the *H-2* I-A or I-E locus (6). Similarly, recognition of HKC-43 by cloned T cells was restricted to identity at the *H-2* I-A locus on accessory cells; irradiated splenocytes from a variety of inbred strains of mice with different *H-2* haplotypes failed to present HKC-43 to T cells.

Four of 12 clones (33%) responded vigorously to irradiated allogeneic splenocytes from at least one inbred strain of mice

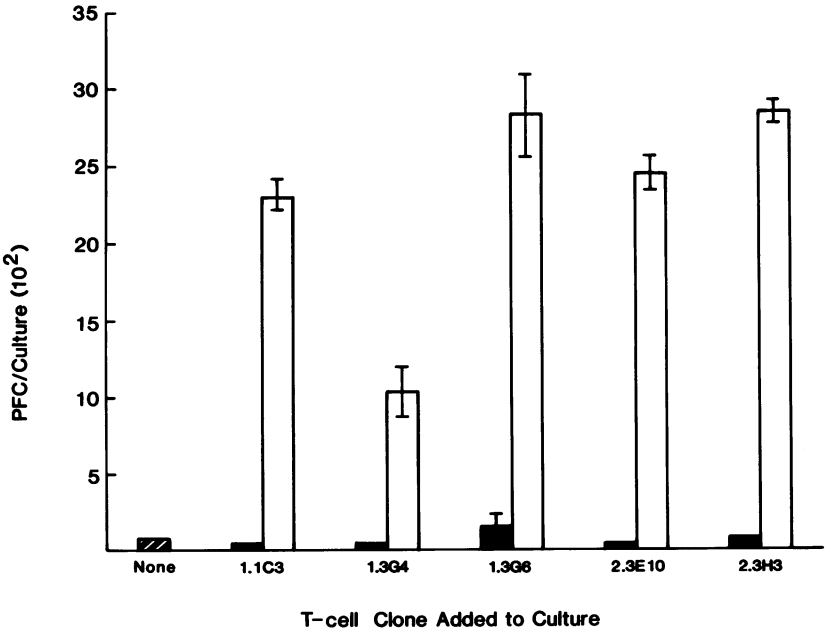

FIG. 4. Cloned T cells mediate nonspecific helper activity. Resting T cells (10^4) were cultured with 3×10^6 B cells in the presence of 3.5×10^6 SRBC and with (□) or without (■) a 1:100 dilution of HKC-43. The number of plaque-forming cells (PFC) per culture to SRBC was assessed 4 days later. Data are expressed as mean ± standard error of quadriplicate determinations.

in the absence of HKC-43. Thus, these clones were dual reactive in that they responded to nominal antigen in the context of *H-2* I-A compatibility and to alloantigens on the surface of accessory cells. Others also have isolated T-cell clones that are dual specific; in the absence of antigen, these alloreactive clones recognize allogeneic class I or class II major histocompatibility complex-encoded molecules of Mls antigens, which are non-*H-2* alloantigens (17, 32, 36). In studies that have examined large numbers of T-cell clones for responsiveness both to nominal antigen in the context of self-major histocompatibility complex and to alloantigens alone, the percentage of alloreactive clones has ranged from 18 to 61% (23; reviewed in reference 3). The frequency of alloreactive T-cell clones among *Histoplasma*-specific clones is in accord with the previous investigations, but the true frequency may be greater than reported because only five allogeneic stimulators were used.

To conclude that a single T cell expresses dual reactivity requires that the population being analyzed be monoclonal. It is conceivable that if the T-cell population is polyclonal, one T-cell subpopulation may react to alloantigen and another may react to HKC-43. This seems unlikely since the probability of clonality of *Histoplasma*-specific T-cell clones was >95%. These results confirm the finding that a single T-cell population may recognize both nominal antigen and alloantigen.

H. capsulatum-reactive TCL and most cloned T cells produced measurable levels of IL-2 in response to HKC-43, and they also elaborated IFN following stimulation with antigen. In the absence of antigen, JC2 and all but one clone also secreted detectable levels of IFN. Generation of multiple lymphokines by monoclonal populations of T cells has been described previously (21, 25). Indeed, Prystowsky et al. (25) detected 10 different lymphokine activities in culture supernatants from a single clone stimulated with antigen. In this study, supernatants from cloned T cells were assayed directly for two lymphokines. However, it is probable that clones secreted several factors since they exerted helper activity in vitro. In this regard, antibody formation has been shown to require the collaboration of several lymphokines including IL-2, B-cell growth factor, T-cell replacing factor, and colony-stimulating factor (14).

Supernatants from antigen-stimulated clones contained a factor(s) capable of arming Mϕ to restrict intracellular growth of *H. capsulatum*, and this lymphokine is similar to a previously described factor produced by splenic T cells from mice immunized with *H. capsulatum* (38). Partial characterization of the factor secreted by clones that mediates this activity revealed it to be acid labile, but heat stable. Addition of anti-IFN-α/β antiserum to culture supernatants did not alter the capacity of the factor to arm Mϕ. Thus, the factor(s) in supernatants shares some characteristics with IFN-γ, although reports have differed concerning the heat stability of IFN-γ. Some have reported that murine IFN-γ is heat stable, whereas others have found it to be heat labile (26, 33). Nevertheless, the acid lability of this lymphokine in conjunction with the failure of anti-IFN-α/β to block its effect strongly suggests that IFN-γ is at least partially responsible for stimulating Mϕ to restrict fungal growth. The recent finding that Mϕ-activating factors distinct from IFN-γ are produced by human T-cell clones also raises the possibility that IFN-γ is not the only factor in culture supernatants that can stimulate Mϕ to exert anti-*Histoplasma* activity (2).

The release of soluble mediators by cloned T cells thus may represent an important mechanism by which these cells contribute to acquired resistance to infection with *H. capsulatum*. Secretion of IL-2 in response to antigen may lead to cellular activation and expansion of antigen-reactive cells (29). Generation of IFN, especially IFN-γ, may enhance the antifungal activity of Mϕ and may augment antigen recognition by T cells by increasing Ia antigens on the surface of accessory cells (33). The availability of T-cell clones that recognize *Histoplasma* antigens will facilitate the confirmation or refutation of these hypotheses. In addition, isolation of T-cell clones will provide the foundation for identification of a receptor for *H. capsulatum* on T cells and for the creation of anti-idiotypic antibodies to the T-cell receptor that may be useful in disease prevention.

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