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In Vivo and In Vitro Characterization of Murine T-Cell Clones Reactive to *Mycobacterium tuberculosis*

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Seventeen helper T-cell clones were derived by stimulating lymph node cells from sensitized C57BL/6 mice with *Mycobacterium tuberculosis* H37Ra, *M. tuberculosis* H37Rv, or purified protein derivative. Most clones cross-reacted with *Mycobacterium bovis* BCG, H37Ra, H37Rv, and purified protein derivative. However, four clones were able to differentiate H37Rv from H37Ra, or BCG from H37Ra and H37Rv. In addition, four other T-cell clones recognized recombinant antigens of 19 and 65 kilodaltons isolated from a genomic expression library of *M. tuberculosis* by using monoclonal antibodies. All clones were Ia restricted and had the Thy-1.2$^+$ Lyt-1$^+$ L3T4$^+$ Lyt-2$^-$ phenotype. On stimulation with antigen, all of the clones tested secreted interleukin-2 and gamma interferon but not B-cell stimulatory factor 1. All of the clones tested induced an antigen-specific delayed-type hypersensitivity response upon local cell transfer, although the magnitude of this response differed markedly among clones.

Tuberculosis remains a major health problem worldwide, with 7 million new cases of tuberculosis a year accounting for 400,000 deaths (7, 28). In 1984 in the United States, 22,255 cases of tuberculosis resulted in an estimated 2,000 deaths (3). The occurrence of tuberculosis among patients with acquired immunodeficiency syndrome (AIDS) (4), among the homeless (2), and among the elderly in nursing homes (24) have all contributed to a rising incidence of the disease in the United States, first noted in 1986. This highlights the need for an improved vaccine against tuberculosis, since vaccination with *Mycobacterium bovis* BCG is not always effective (1).

Studies with animal models clearly indicate that resistance to mycobacteria is a cell-mediated process that requires cooperation between thymus-dependent (T) lymphocytes and other cells to be effective (11, 12, 15, 19). Antibodies alone seem to contribute little to resistance (22), and activated macrophages appear to be the main effector cells (16, 27). Genetically determined primary resistance to infection with *M. bovis* BCG in mice appears to be expressed in the macrophage and may be controlled by a single gene (21). T cells regulate and maintain the cell-mediated immune response which follows primary exposure and forms the mechanism for acquired resistance. Whether cells capable of eliciting a delayed-type hypersensitivity response are the same as those providing a protective immune response remains an area of controversy.

We have established murine T-cell clones reactive to mycobacterial antigens in order to further investigate the mechanism by which these cells may mediate resistance to experimental tuberculosis infection and to define the antigens which induce protective T cells. T-cell clones which induce protective immunity could subsequently be used to select protective antigens produced by recombinant DNA technology as primary candidates for the development of an improved vaccine for tuberculosis.

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MATERIALS AND METHODS

Animals. C57BL/6, C3H.SW, BALB/c, B10.MBR, B10.A(4R), and B10.A(5R) mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). All of the mice used were between 5 and 12 weeks old.

Antigens. Purified protein derivative (PPD) of tuberculin was obtained from Connaught Laboratories, Ltd. (Willowdale, Ontario, Canada). *M. tuberculosis* H37Ra was obtained from Difco Laboratories (Detroit, Mich.) as a heat-killed, air-dried preparation and reconstituted to 1 mg (dry weight) per ml in phosphate-buffered saline (pH 7.2; 0.15 M). Stock cultures of *M. tuberculosis* H37Rv and *M. bovis* BCG Montreal were obtained from the American Type Culture Collection (Rockville, Md.). Antigen was made by treating log-phase cultures in 7H9 medium (Difco) containing 0.001% Tween 80 (Sigma Chemical Co., St. Louis, Mo.) with 2% paraformaldehyde for 2 h. After an extensive washing, BCG was reconstituted as 4×10^7 bacteria per ml in phosphate-buffered saline. H37Rv was further lyophilized and reconstituted in phosphate-buffered saline (1 mg [dry weight] per ml). Bovine serum albumin (BSA) was purchased from Sigma.

Recombinant mycobacterial antigens. Recombinant mycobacterial antigens isolated by using monoclonal antibodies from λgt11 recombinant DNA expression libraries of *M. tuberculosis* and *M. leprae* were tested as crude *Escherichia coli* lysates (29, 30). They were prepared by the method of Mustafa et al. (14). Briefly, single colonies of recombinant lysogens were incubated at 32°C in LB medium. Once cells had grown to a density at A_{600} of 0.5, they were transferred to incubators at 45°C and incubated for 20 min. Isopropyl-β-D-thiogalactopyranoside was added to 10 mM, and the cultures were incubated at 38°C for 1 h. After centrifugation, the cell pellet was frozen in liquid nitrogen and thawed, lysing the induced lysogen. Crude lysates were sterilized by filtration (0.22 μm pore size) and frozen at −70°C. The presence of mycobacterial antigens was confirmed by prob-

ing the crude lysates with the appropriate monoclonal antibodies.

Immunization. H37Ra and H37Rv were emulsified in incomplete Freund adjuvant and injected subcutaneously into all four footpads and at the base of the tail of each C57BL/6 mouse. All of the mice received 400 μg of these antigens.

Establishment of mycobacterium-specific T-cell clones. T-cell cultures were initiated and maintained as described by Kimoto and Fathman (10) and Kaufmann and Hahn (9) with modifications based on the methods of Sheppard et al. (23). Single-cell suspensions were prepared from inguinal, axillary, and periaortic lymph nodes harvested from mice immunized 14 days earlier. Cells were passed over a nylon wool column and incubated in 24-well trays (Costar, Cambridge, Mass.) with irradiated (2,500 rad) syngeneic spleen cells used as antigen-presenting cells (APC) and antigen in TCM (RPMI 1640 [GIBCO Laboratories, Grand Island, N.Y.] supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, 0.2 mM nonessential amino acids, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 100 IU of penicillin per ml, 100 μg of streptomycin per ml, 50 μg of gentamicin per ml, 0.05 mM 2-mercaptoethanol, and 10% fetal calf serum). Cells from animals immunized with H37Ra were stimulated with either PPD (10 μg/ml) or H37Ra (10 μg/ml). Cells from animals immunized with H37Rv were stimulated with H37Rv (10 μg/ml) only. After 4 to 6 days of culture in the presence of antigen, viable cells from the three cell lines were isolated on a Ficoll-sodium diatrizoate gradient (Histopaque; Sigma) and maintained for 8 to 10 days in the absence of APC and antigen at a density of 1×10^6 to 2×10^6 cells per ml. Resting cells were then suspended and cultured at a density of 1×10^5 to 2×10^5 cells per well with 5×10^6 APC per well and antigen in 24-well plates for 4 to 6 days, after which viable lymphocytes were isolated on Histopaque and maintained as described above for an additional 8 to 10 days. After three to six cycles of antigenic stimulation and rest, viable cells were cloned by limiting dilution at 1.0 to 0.1 cells per well in the presence of 5×10^5 APC, antigen (PPD, H37Ra, or H37Rv), and 10% interleukin-2 (IL-2; Electro-Nucleonics, Inc., Silver Spring, Md.) in 0.2 ml of TCM. After 10 to 14 days, positive wells were restimulated with APC, antigen, and 10% IL-2. After two cycles of stimulation in 96-well plates, positive wells were expanded into 24-well plates. IL-2 was omitted from the medium after one to two additional stimulations. Expanded clones were subsequently cycled between stimulation and rest, as described for the parent lines. Resting cells were routinely isolated on Histopaque gradients and extensively washed before use in experiments. For convenience, clones derived from parent lines stimulated with PPD, H37Ra, or H37Rv will be referred to as PPD, H37Ra, or H37Rv, clones and the corresponding antigens will be referred to as the primary antigens.

Proliferation assay. Cloned T cells (10^4) were cocultured with 5×10^5 irradiated, syngeneic spleen cells and antigen for 72 h in 0.2 ml of TCM. Cells were pulsed with 1 μCi of [^{3}H]thymidine (TdR; specific activity, 5 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) for 16 to 18 h before harvest on filter paper. TdR incorporation was measured by standard liquid scintillation counting methods. The response to an antigen was considered positive when there were more than 1,000 cpm over background counts and the proliferative response was at least twice that of the background. Dose responses were analyzed for each antigen to determine the optimum antigen concentration.

Lymphokine production. Cloned T cells (2×10^4) were incubated with or without antigen and 5×10^5 APC in 0.2 ml of TCM. Supernatants (100 μl) were harvested after 18 to 24 h and added at 50% (vol/vol) to 10^4 HT-2 cells for 24 h. [^{3}H]TdR (1 μCi) was added for the last 6 h, after which the cells were processed as described above. The presence of IL-2 or B-cell stimulatory factor 1 (BSF1) was determined by the method of Lichtman et al. (13). Briefly, HT-2 cells were incubated with supernatants from T-cell clones with or without either anti-BSF1 antibody (11B11, 1:1,000 dilution of ascites) or anti-IL-2 receptor (IL2R) antibody (7D4+ PC61, 1:100 dilution of ammonium sulfate-precipitated hybridoma culture supernatants), and the degree of inhibition of TdR incorporation was determined. Both monoclonal antibodies were known to specifically inhibit the response of HT-2 cells to either recombinant IL-2 (Genzyme, Boston, Mass.) or high-pressure-liquid-chromatography-purified BSF1 and recombinant BSF1. The presence of gamma interferon (IFN-γ) was assessed by a quantitative assay for the induction of Ia on resting macrophages (26). Peptone-induced peritoneal exudate cells from A/J mice were plated in microwells (5×10^4 cells per well). Nonadherent cells were removed, and the cultures were incubated for 24 h; antigen-stimulated T-cell culture supernatants (3 days old) or recombinant IFN-γ (Genentech, San Francisco, Calif.) were then added. After a 5-day incubation, wells were washed and incubated with monoclonal anti-I-A^k (1:10 dilution of 10.2.16 hybridoma supernatant) followed by ^{125}I-labeled sheep anti-mouse immunoglobulin G (IgG) (New England Nuclear Corp., Boston, Mass.). Cell-bound radioactivity per well was measured.

Surface phenotype. The surface phenotype of cloned T cells was determined by indirect immunofluorescence. Samples of cloned T cells were stained with monoclonal antibodies against Thy-1.2 (Miles Laboratories, Inc., Naperville, Ill.), Lyt-1.2 and Lyt-2.2 (Cedarlane, Westbury, N.Y.), and L3T4 (GK1.5) (5), followed by fluoresceinated goat anti-mouse IgG and IgM (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) for Lyt-1.2, Lyt-2.2, and Thy-1.2 and goat anti-rat antibody for GK1.5. The amount of background staining was determined by incubating the cells with fluoresceinated antibody alone. The cells were examined under a Leitz Orthoplan microscope.

DTH with T-cell clones. The local transfer of delayed-type hypersensitivity (DTH) reactions was done by injecting various numbers (10^4, 10^5, or 5×10^5) of T cells together with 20 μg of PPD in RPMI 1640 into one hind footpad (50 μl per footpad). The other footpad was injected with RPMI 1640 alone. Control mice received T cells without PPD. Additional control animals were treated with PPD alone. Footpad swelling was measured after 24 h, determined to be the time of maximal swelling, with a vernier caliper. Five days later, all of the footpads were rechallenged with antigen (20 μg) alone, and footpad swelling was measured 24 h later.

RESULTS

Establishment and antigen specificity of *M. tuberculosis*-reactive T-cell clones. A total of 17 cloned T-cell lines were obtained. Seven were derived from a PPD-driven parent line, and five each were from H37Ra- and H37Rv-stimulated lines. The in vitro proliferative response of these clones to four mycobacterial antigens is shown in Tables 1, 2, and 3. All of the clones were tested repeatedly over a range of antigen concentrations and consistently maintained the pattern of reactivity shown. No response was induced by the irrelevant antigen BSA. All PPD clones were broadly cross-

reactive to H37Ra, H37Rv, and BCG (Table 1; see Materials and Methods). In contrast, three of the five H37Ra clones either barely recognized H37Rv (clone F1) or not at all (clones G3 and F3), even though they all responded to PPD. Furthermore, clone G3 did not recognize BCG (Table 2). All five H37Rv clones cross-reacted with PPD and H37Ra. Of these, all but one (clone 3H10) recognized BCG (Table 3).

Surface phenotype of *M. tuberculosis*-reactive T-cell clones. All of the clones tested by immunofluorescence were found to be Thy-1.2$^+$ Lyt-1.2$^+$ L3T4$^+$ Lyt-2.2$^-$. In all cases, more than 95% of all cells reacted with monoclonal antibodies to the surface antigens.

***H-2* restriction of antigen-specific proliferation of *M. tuberculosis*-reactive T-cell clones.** The requirement for histocompatibility of nine T-cell clones and APC was examined in a standard proliferation assay. All of the clones tested responded to their primary antigen presented by APC from C57BL/6, C3H.SW, and B10.A(5R) mice (all *I-A^b*) and failed to proliferate when APC from BALB/c, B10.A(4R), or B10.MBR mice were used. A representative experiment with three clones is shown in Fig. 1. As anticipated, these experiments indicate that compatibility at the *I-A* sublocus of the *H-2* complex is sufficient and necessary for effective antigen presentation.

Lymphokine production. Recent data indicate that helper/inducer T cells fall into two distinct classes on the basis of lymphokine secretion profiles. One secretes BSF1 and the other secretes IL-2 and IFN-γ (13). Because of the potential biologic significance of this distinction, we evaluated the T-cell clones for the production of IL-2, BSF1, and IFN-γ. Supernatants from all nine clones tested could stimulate HT-2 cells. When monoclonal antibodies known to inhibit the response of HT-2 cells to recombinant IL-2 were added to the supernatants from antigen-stimulated T-cell clones, a marked reduction in proliferation was noted (Table 4). No attenuated HT-2 response was seen when anti-BSF1 antibodies were mixed with culture supernatants. The extent of [^{3}H]TdR incorporation by HT-2 cells incubated with supernatants from T cells cultured without antigen was less than 1,000 cpm. These findings indicate that all of the clones tested produce IL-2 and do not appear to produce detectable levels of BSF1. The controls for these experiments consisted of HT-2 cells incubated with recombinant human IL-2 (20 U/ml) or recombinant murine BSF1 (250 U/ml) with or without the anti-IL2R and anti-BSF1 monoclonal antibodies.

TABLE 1. Proliferative response of T-cell clones with PPD as primary antigen[a]

Clone	[^{3}H]TdR incorporation (cpm) by clone with antigen[b]:					
	None	PPD	H37Ra	H37Rv	BCG	BSA
G11	1,537	167,861	177,227	133,168	94,036	3,066
C11	323	6,672	14,654	3,347	2,869	339
D2	325	44,374	60,675	8,587	43,692	768
H10	468	61,293	64,047	44,319	48,435	293
C8	1,861	50,631	101,770	60,842	68,652	1,951
B8	758	65,432	60,985	51,864	68,480	1,338
D8	495	11,451	3,365	3,996	5,714	NT[c]

[a] Primary antigen is the antigen used to stimulate the parent line and clones derived from that line.

[b] Cloned T cells (10^4) were stimulated with PPD (10 μg/ml), H37Ra (20 μg/ml), H37Rv (10 μg/ml), BCG (10^6 organisms per well), or BSA (10 μg/ml) in the presence of 5×10^5 APC. Results are expressed as the arithmetic means of triplicate cultures. Standard deviations were generally less than 20% and are not shown. All responses were considered positive (see Materials and Methods) except with BSA and the clones without any antigen.

[c] NT, Not tested.

TABLE 2. Proliferative response of T-cell clones with H37Ra as primary antigen[a]

Clone	[^{3}H]TdR incorporation (cpm) by clone with antigen[b]:					
	None	H37Ra	PPD	H37Rv	BCG	BSA
C1	921	**104,121**	**88,270**	**36,317**	**42,236**	754
F1	414	**25,287**	**10,890**	1,411	**4,515**	583
F3	393	**2,080**	**1,481**	529	1,116	556
G3	914	**9,074**	**3,524**	908	965	716
H2	368	**9,250**	NT	6,024	NT	NT

[a] See Table 1, footnotes *a*, *b*, and *c*.

[b] Positive responses are indicated in boldface.

Consistently greater than 90% inhibition of [^{3}H]TdR incorporation was observed for each purified lymphokine in the presence of its blocking antibody (13).

The supernatants of clones G11, B8, and C1 were also tested for the presence of IFN-γ by a quantitative method to measure the induction of Ia on resting peritoneal adherent cells of A/J mice. All of the supernatants tested contained Ia-inducing activity. A representative experiment for two clones is shown in Table 5. Furthermore, the IFN-γ activity could be neutralized by the hamster monoclonal antibody specific for murine IFN-γ (H22.10.38.22) (Table 5) (D. S. Reynolds, W. H. Boom, and A. K. Abbas, J. Immunol., in press).

Response of T-cell clones to recombinant mycobacterial antigens. Sixteen clones were tested for their ability to recognize four recombinant antigens of *M. tuberculosis*. The recombinant antigens were isolated by monoclonal antibodies in a λgt11 expression library of genomic DNA of *M. tuberculosis*. These monoclonal antibodies recognize four distinct protein antigens of 71, 65, 19, and 14 kilodaltons (kDa) and are expressed in *E. coli* by λgt11 clones Y3272, Y3143, Y3147, and Y3247, respectively (7a, 29). We found that three T-cell clones (H10-PPD, 3H9-H37Rv, and 3F1-H37Rv) recognized the 19-kDa antigen (Y3147) and that one T-cell clone (G11-PPD) recognized the 65-kDa antigen (Y3143). None of the clones tested recognized the 71-kDa (Y3272) or the 14-kDa (Y3247) antigen. Representative experiments with all four positively reacting and two nonreactive clones (B8 and C1) are summarized in Table 6. Crude lysates of *E. coli* infected with λgt11 alone, without a mycobacterial DNA insert but containing the intact β-galactosidase molecule, gave no significant proliferative responses. Dose-response curves revealed that 5 to 10 μg of protein per ml of the crude lysates gave optimal proliferation (results not shown). Larger doses (20 to 50 μg) of the crude recombinant antigen preparations were found to be toxic for the T-cell clones. The presence of toxic or inhibitory factors may partially explain why the maximal response to the

TABLE 3. Proliferative response of T-cell clones with H37Rv as primary antigen[a]

Clone	[^{3}H]TdR incorporation (cpm) by clone with antigen[b]:					
	None	H37Rv	PPD	H37Ra	BCG	BSA
2B7	173	**6,945**	**2,779**	**26,410**	**5,097**	312
2B1	519	**17,360**	**4,319**	**19,833**	**9,092**	261
3H9	999	**109,016**	**160,777**	**157,682**	**72,782**	970
3F1	988	**40,145**	**39,765**	**43,990**	**25,518**	151
3H10	167	**16,458**	**19,598**	**25,417**	364	439

[a] See Table 1, footnotes *a*, *b*, and *c*.

[b] See Table 2, footnote *b*.

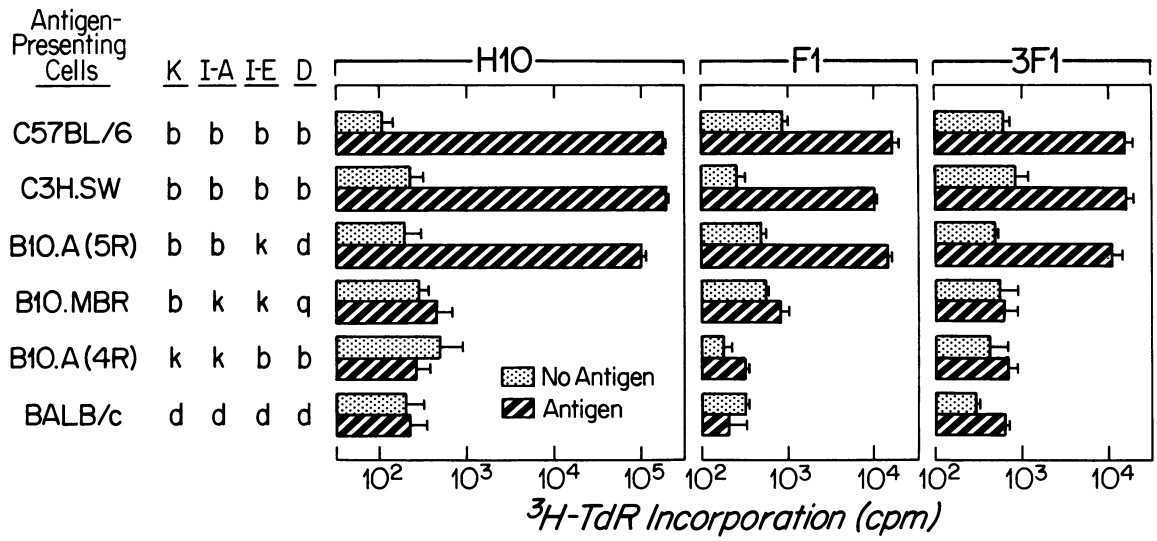

FIG. 1. *H-2* restriction of *M. tuberculosis*-reactive T-cell clones. T cells (10^4) were stimulated with and without antigen in the presence of 5×10^5 APC from various strains of mice in a standard proliferation assay. Clone H10 was stimulated with PPD, clone F1 with H37Ra, and clone 3F1 with H37Rv. Each bar represents the geometric mean and standard deviation of triplicate cultures. Haplotypes at *H-2* subloci K, I-A, I-E, and D (alleles *k*, *b*, *d*, and *q*) are indicated.

recombinant antigens was significantly lower than the maximal response to the primary antigen. With smaller doses (1 to 10 μg), nonspecific mitogenic effects were occasionally observed. Because the 65-kDa antigens of *M. tuberculosis* and *M. leprae* contain cross-reacting epitopes identified by monoclonal antibodies (7a), we tested the ability of clone G11 to proliferate in response to the 65-kDa *M. leprae* antigen. Clone G11 proliferated in response to the 65-kDa antigen of *M. leprae* (Table 7), although not as strongly as to the 65-kDa *M. tuberculosis* antigen. Negative controls in this experiment included λgt11 alone and the 71-kDa antigen of *M. tuberculosis*, Y3272, which was previously found to be nonreactive. These results indicate that the 65-kDa antigens of *M. tuberculosis* and *M. leprae* also share cross-reactive epitopes recognized by T cells.

Local transfer of DTH reactions. To determine the ability of the T-cell clones to mediate a specific local DTH response, various numbers of T cells (10^4, 10^5, and 5×10^5) were injected alone or together with 20 μg of PPD into a hind footpad of naive syngeneic C57BL/6 mice. The other footpad always received an equal volume of RPMI 1640. Footpad swelling was measured after 24 h, which was found to be the time of peak swelling. All nine of the clones tested elicited a DTH response, defined as a 10^{-1}-mm difference in swelling between experimental and control footpads. The clones could be differentiated by the strength of the peak response and the minimum number of cells necessary to elicit a response. Clones injected with BSA as an irrelevant antigen gave no DTH response (data not shown). The responses of five clones are shown in Table 8. The ability to confer

TABLE 4. IL-2 and BSF1 production by *M. tuberculosis*-reactive T-cell clones

Origin of lymphokine-containing supernatant	[^{3}H]TdR incorporation (cpm) of HT-2 cells in supernatant[a] with:		
	No antibody	Anti-BSF1	Anti-IL2R
Clone/stimulus			
G11/PPD	107,503	101,983	**31,075**
C1/H37Ra	67,121	64,518	**11,068**
F1/H37Ra	12,526	10,018	**1,601**
3F1/H37Rv	12,899	11,786	**1,465**
Controls (U/ml)			
rBSF1 (250)	25,650	**653**	19,109
rIL-2 (20)	105,643	95,901	**10,584**

[a] Supernatants were prepared by incubating 2×10^4 T cells with or without antigen in the presence of 5×10^5 APC. Supernatants were assayed with 10^4 HT-2 cells at 50% (vol/vol) in the presence or absence of anti-IL2R antibody (7D4 + PC61) or anti-BSF1 antibody (11B11). Controls used in these experiments were recombinant human IL-2 (rIL-2) and recombinant murine BSF1 (rBSF1). Results shown are the means of duplicate cultures. Boldface values are significantly different from results obtained when no antibody was added. Standard deviations were less than 10%.

TABLE 5. IFN-γ production by *M. tuberculosis*-reactive T-cell clones

Source (concn) of lymphokine-containing supernatant[a]	[^{125}I]anti-IgG bound/well (mean cpm)[b]
None	3,275
rIFN-γ (ng/ml)	
20	18,555
5	18,075
2	16,008
2 (+ 20 ng of anti-IFN-γ MAb per ml)	4,185
0.3	9,799
Clone G11 (%)	
10	19,450
1	9,781
0.1	5,455
Clone C1 (2.5%)	16,378

[a] Resting A/J adherent cells (10^4) were incubated for 5 days with either recombinant IFN-γ (rIFN-γ) or diluted supernatants from T-cell clones stimulated with antigen for 3 days. The anti-mouse IFN-γ monoclonal antibody (MAb) used was H22.10.38.22.

[b] Mean counts per minute of ^{125}I-labeled sheep anti-mouse IgG bound per well (described in Materials and Methods). Results are expressed as arithmetic means of duplicate cultures. Standard deviations were less than 10%.

TABLE 6. Proliferative response of T-cell clones to recombinant *M. tuberculosis* antigens[a]

Clone	[^{3}H]TdR incorporation (cpm)[b] with:						
			Lysates from *E. coli* containing:				
	No antigen	Primary antigen	λgt11 (no insert)	Y3143 (65 kDa)	Y3147 (19 kDa)	Y3247 (14 kDa)	Y3272 (71 kDa)
G11 (PPD)	1,914	**104,310**	1,375	**14,403**	1,310	1,755	1,669
H10 (PPD)	486	**36,967**	396	400	**2,656**	493	591
3H9 (H37Rv)	908	**107,188**	417	568	**3,286**	535	476
3F1 (H37Rv)	292	**16,699**	260	437	**1,268**	268	531
B8 (PPD)	903	**59,176**	1,122	1,503	1,346	1,442	1,211
C1 (H37Ra)	2,114	**120,190**	437	1,226	877	780	890

[a] T cells (10^4) were incubated in the presence of 5×10^5 APC with their primary antigen or crude lysates of *E. coli* (10 μg/ml) containing recombinant *M. tuberculosis* antigens of 65, 19, 14, or 71 kDa or λgt11 without inserts.

[b] Results are expressed as the arithmetic mean counts per minute of triplicate cultures. Standard deivations were generally less than 20% and are not shown. Boldface values are considered positive responses (see Materials and Methods).

immunological memory was measured by rechallenging all of the footpads with PPD 5 to 6 days after the initial transfer. The pattern on rechallenge was similar to the pattern observed on primary transfer. It is interesting that a DTH response developed in footpads previously injected with T cells alone on local challenge with antigen 5 to 6 days later, suggesting prolonged viability of the unstimulated T cells upon local transfer (data not shown).

DISCUSSION

The purpose of this study was to generate a panel of T-cell clones reactive to *M. tuberculosis* and to evaluate their in vitro and in vivo characteristics. By using both whole mycobacteria and PPD as primary antigens, we obtained 17 T-cell clones that were markedly heterogeneous in their response to four crude mycobacterial antigen preparations (PPD, BCG, H37Ra, and H37Rv). Although all of the clones reacted to various degrees to *M. tuberculosis* PPD, two clones (3H10 and G3) were able to differentiate BCG from H37Rv or H37Ra when whole bacteria were used as the antigen. In addition, three clones (F1, G3, and F3) proliferated strongly in response to H37Ra, but poorly or not at all in response to H37Rv. This may simply reflect the manner in which these antigens were prepared: heat-killed H37Ra versus paraformaldehyde-treated H37Rv and BCG. Lyophilization alone does not appear to change the antigenic constituents much, because when lyophilized paraformaldehyde-treated BCG was used, clone 3H10 was still unable to recognize BCG (data not shown). These findings, however, also raise the possibility that T cells recognize significant antigenic differences between H37Ra and H37Rv, as well as differences between H37Ra/H37Rv and BCG. However, Kaufmann and Fleisch (8) recently found that none of the L3T4$^+$ T-cell clones from H37Rv-immunized mice was able to distinguish among BCG, H37Rv, and H37Ra. All six of the clones examined in their study reacted to PPD. The antigen preparations used by these researchers were very similar to the ones used in the present study (8). In addition, of 31 monoclonal antibodies raised to various mycobacterial antigen preparations tested in a recent World Health Organization-sponsored workshop, six were specific for the *M. tuberculosis* complex. None, however, was able to differentiate BCG from *M. tuberculosis* or to differentiate among strains of *M. tuberculosis* (H. D. Engers and workshop participants, Letter, Infect. Immun. **51**:718–720, 1986).

The degree of heterogeneity in antigenic recognition of the T-cell clones is further confirmed by the response to four recombinant antigens. Of 17 clones, 4 (G11, H10, 3F1, and 3H9) recognize two recombinant protein antigens of 19 and 65 kDa, respectively. Furthermore, clone G11 also recognizes a 65-kDa protein antigen of *M. leprae*. From studies with monoclonal antibodies, it was found that many monoclonal antibodies were highly cross-reactive wtih the 65-kDa antigens of *M. tuberculosis* and *M. leprae* and that these antigens therefore shared many epitopes. It appears that these two molecules share T-cell epitopes as well. None of

TABLE 7. Proliferative response of clone G11 (PPD) to 65-kDa recombinant antigens of *M. tuberculosis* and *M. leprae*[a]

Stimulus	[^{3}H]TdR incorporation (cpm, mean ± SD)
None	706 ± 221
PPD	**169,828 ± 6,561**
λgt11	6,866 ± 1,939
Y3143	**60,548 ± 2,070**
Y3178	**27,107 ± 1,361**
Y3272	6,802 ± 215

[a] Crude lysates of *E. coli* containing antigens expressed by phages Y3143 (65 kDa; *M. tuberculosis*), Y3178 (65 kDa; *M. leprae*), and Y3272 (71 kDa; *M. tuberculosis*) were tested at a final concentration of 10 μg/ml. Results are expressed as the mean and standard deviation of triplicate cultures. Boldface values are significantly different from the corresponding controls.

TABLE 8. Footpad response to PPD upon local transfer of T-cell clones

Clone (no. of cells injected)	Footpad swelling (10^{-1} mm [mean ± SD])[a] after 24 h with antigen		Recall[b]
	None	PPD	
None	0.0 ± 0.0	0.4 ± 0.2	−
G11 (PPD)			
1×10^4	0.0 ± 0.0	0.6 ± 0.6	−
1×10^5	0.2 ± 0.3	0.9 ± 0.2	−
5×10^5	0.4 ± 0.2	1.4 ± 0.8	+
B8 (PPD)			
1×10^4	0.1 ± 0.2	1.5 ± 0.6	+
1×10^5	0.9 ± 0.2	3.1 ± 0.9	+
5×10^5	1.5 ± 0.7	5.0 ± 0.7	+
H10 (PPD)			
1×10^4	0.1 ± 0.2	0.5 ± 0.5	−
1×10^5	0.5 ± 0.0	0.8 ± 0.6	−
5×10^5	0.5 ± 0.4	1.6 ± 0.7	+
C1 (H37Ra)			
1×10^4	0.2 ± 0.3	0.6 ± 0.6	−
1×10^5	0.4 ± 0.2	1.8 ± 0.6	+
5×10^5	0.5 ± 0.4	3.2 ± 0.6	+
3F1 (H37Rv)			
1×10^4	0.1 ± 0.2	0.3 ± 0.3	−
1×10^5	0.0 ± 0.0	0.6 ± 0.9	−
5×10^5	0.6 ± 0.5	1.6 ± 0.8	+

[a] Footpad swelling measured as the difference between right and left where the right hind footpad received T cells with or without 20 μg of PPD and the left hind footpad always received RPMI 1640 alone. Values represent means and standard deviations of four to five mice per group.

[b] Recall is defined as $>10^{-1}$-mm difference between right and left hind footpads on rechallenge with 20 μg of PPD 5 to 6 days after cell transfer.

the other recombinant protein antigens of *M. leprae* and *M. tuberculosis* were found to be cross-reactive when evaluated with currently available monoclonal antibodies (7a). Our study represents the first report of murine T-cell clones which respond to some of the recombinant antigens isolated with monoclonal antibodies from expression libraries of *M. tuberculosis* and *M. leprae*.

The significance of the 65-kDa antigen of *M. tuberculosis* in human disease remains to be established. However, several lines of evidence suggest that it may be an important immunodominant antigen. First, antibodies to a 65-kDa antigen are present in the sera of patients with tuberculosis (25). From our studies and those of others, it is clear that a 65-kDa molecule is present in PPD (25). In addition, human helper T-cell clones isolated from patients with *M. tuberculosis* infections recognize the same recombinant 19- and 65-kDa antigens as the murine T-cell clones described here (17). Finally, a human T-cell clone derived from a patient with tuberculoid leprosy responds to a 64-kDa recombinant antigen of *M. bovis* BCG (6). These findings all suggest that the 65-kDa protein is a major mycobacterial antigen which is highly conserved across mycobacterial species and appears to carry many cross-reactive epitopes.

All of the cloned T cells tested carry the Thy-1^+ Lyt-1^+ L3T4^+ Lyt-2^- phenotype of helper T cells and proliferate only when antigen is presented by APC that are histocompatible at the *I-A* locus of the *H-2* complex. All of the clones tested appear to belong to the subset of helper T cells that secrete IL-2 and IFN-γ but not BSF1 (13). Consistent with this finding is the ability of the clones to mediate DTH in vivo, for which both IL-2 and IFN-γ, as macrophage-activating factors, may be important mediators. It is not yet definitively established whether the helper function for B cells correlates with profiles of lymphokine secretion. However, it is reasonable to postulate that IL-2- and IFN-γ-secreting helper T cells play a dominant role in cell-mediated immunity and that these are the lymphokines produced by the *M. tuberculosis*-reactive clones that we have generated.

All of the nine T-cell clones tested were able to transfer DTH locally. Clones could be differentiated on the basis of the degree of swelling observed in the footpads and the minimum number of cells necessary to elicit a DTH response. Pedrazzini and Louis (20) recently described a number of T-cell clones specific for PPD and BCG, some of which could not transfer DTH even when 10^6 cells were transferred. We did not observe this with the clones we developed. This may be the result of different selection pressures caused by different cloning strategies. Our clones were derived from long-term lines, whereas Pedrazzini and Louis (20) cloned their cells soon after isolating T cells from immunized mice.

We have thus demonstrated that despite similar abilities to produce lymphokine and transfer DTH, the helper/inducer T-cell clones recognize a diversity of mycobacterial antigens within the context of I-A histocompatible APC. It may be possible to differentiate clones by their capacity to inhibit the growth of *M. tuberculosis*. Studies to evaluate the ability of the *M. tuberculosis*-responsive T-cell clones described here to inhibit local growth of virulent *M. tuberculosis* are under way. T-cell clones may therefore be useful for the identification of potentially important antigens of *M. tuberculosis* obtained by recombinant DNA technology or by other means. A recent report by Orme (18) raises the possibility that different populations of protective T cells are generated during the course of *M. tuberculosis* infection in mice. These different populations may have different dominant antigenic specificities. This could make it more difficult to define protective antigens with T-cell clones derived from animals immunized with killed mycobacteria (18).

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