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Function and Antigen Recognition Pattern of L3T4$^+$ T-Cell Clones from *Mycobacterium tuberculosis*-Immune Mice

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T-cell clones were established from *Mycobacterium tuberculosis*-immunized mice. These clones had the phenotype Thy-1$^+$ L3T4$^+$ Lyt-2$^-$ and were restricted by the *H-2I-A* locus. After antigen stimulation, the T-cell clones secreted interleukin-2 and gamma interferon. Factors produced by these T-cell clones activated normal bone marrow macrophages for antimycobacterial activity in vitro. Furthermore, the T-cell clones could adoptively confer delayed-type hypersensitivity on normal recipient mice. These findings indicate that the T-cell clones expressed relevant functions of antimycobacterial immunity. The antigen reactivity of the T-cell clones to different mycobacterial species ranged from broad cross-reactivity to stringent specificity, and none of the clones distinguished between *M. tuberculosis* and *M. bovis*. Thus, *M. tuberculosis*-immune helper/inducer T cells of identical phenotype, genetic restriction, and function varied in their antigen specificity. T-cell clones of the type described will facilitate functional characterization of mycobacterial antigens on the T-cell level.

Tuberculosis is a chronic bacterial infection which affects more than 50 million people worldwide (2). The etiological agent, *Mycobacterium tuberculosis*, is an intracellular bacterial pathogen, and acquired resistance is mediated by specific T lymphocytes and expressed by activated mononuclear phagocytes (12). Since the failure of *M. bovis* BCG vaccination against tuberculosis in a well-controlled trial, it has been emphasized that efforts should be undertaken to develop novel control measures (2, 31). As a first step towards this goal, characterization of T cells reactive against *M. tuberculosis* is required. We have therefore established T-cell clones from mice immunized with *M. tuberculosis* and characterized their phenotype, genetic restriction, function, and antigen specificity. The clones were class II-restricted, Thy-1$^+$ L3T4$^+$ Lyt-2$^-$, and expressed a homogeneous functional pattern but varied in their specificity for different mycobacterial species. These findings suggest that cross-reactive as well as specific T-cell clones are involved in the cellular immune response to *M. tuberculosis*.

MATERIALS AND METHODS

Mice. C57BL/6, B10.A (4R), B10.A (5R), and B10.MBR mice raised under specific-pathogen-free conditions in our own breeding facilities were used at 8 to 12 weeks of age.

Bacteria and bacterial antigens. *M. bovis* strain BCG Phipps was kindly provided by R. North, Trudeau Institute, Saranac Lake, N.Y., and *M. tuberculosis* H37Rv by J. K. Seydel, Forschungsinstitut Borstel, Bostel, Federal Republic of Germany (FRG). The other mycobacterial strains were kindly provided by W. Brehmer, Robert-Koch-Institut, Berlin, FRG. Mycobacterial species were grown in Dubos broth (Difco Laboratories) supplemented with bovine serum albumin and Tween 80 at 37°C with shaking. Cultures were centrifuged and suspended in phosphate-buffered saline, and the number of CFU was determined by plating 1:10 dilutions on Middlebrook-Dubos agar (Difco). Washed mycobacteria were heat-killed (60°C, 60 min) and ultrasonicated three times at 60 W for 3 min each with a Branson Sonifier B12 with microtip. Purified protein derivative (PPD) was obtained from the Statens Serum Institute, and lyophilized killed *M. tuberculosis* H37Ra was from Difco Laboratories.

Establishment of T-cell clones. C57BL/6 mice were immunized with 100 μl of killed *M. tuberculosis* H37Ra in incomplete Freund adjuvant (10 mg/ml) subcutaneously (s.c.) into the base of the tail. After 8 days, draining lymph nodes were removed. T cells were enriched by passage over nylon wool columns, and afterward 5×10^2 cells were seeded per 0.2 ml of medium in round-bottomed microculture plates. Cultures were done in the presence of 2×10^5 irradiated spleen cells as accessory cells (AC) and 5 μg of killed *M. tuberculosis* H37Ra cells as antigen in 0.2 ml of Iscove modified Dulbecco medium (IMDM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, and antibiotics (Gibco) at 37°C under 7% CO_2. After 4 days, cultures were fed with 2×10^5 AC, antigen, and 10% crude interleukin-2 (IL-2). After another 7 days of culture, wells with growing cells were identified visually, and the cells were expanded. The lines were recloned in the presence of AC, antigen, and IL-2, and clones were expanded in Costar 3506 trays as described above.

IL-2 preparation. Lewis rat spleen cells (5×10^6/ml) were cultured in the presence of 5 μg of concanavalin A per ml, and after 24 h the supernatants were collected. Supernatants were supplemented with 20 mg of α-methylmannoside per ml and used in crude form.

Phenotype analysis. Cells were treated with anti-Thy-1.2 (Olac), anti-L3T4 (GK1.5 [6]) or anti-Lyt-2.2 (HO2.2 [11]) monoclonal antibodies and afterwards with fluorescein isothiocyanate-coupled goat anti-mouse or anti-rat immunoglobulin antiserum (Dianova) as described previously (18). After being washed, the cells were analyzed with an Ortho Cytofluorograph 50 H (Ortho Diagnostic Systems).

Proliferation assay. T cells were purified over Urovison-Ficoll (density, 1.077), and then graded numbers of T cells cultured with 2×10^5 irradiated (3,300 rads) spleen cells as AC and mycobacterial antigens per 0.2 ml of medium in round-bottomed microculture plates. After 3 days, cultures were pulsed with 1 μCi of [^{3}H]thymidine ([^{3}H]TdR; Radiochemical Center, Amersham, England), and 18 h later [^{3}H]TdR incorporation was determined.

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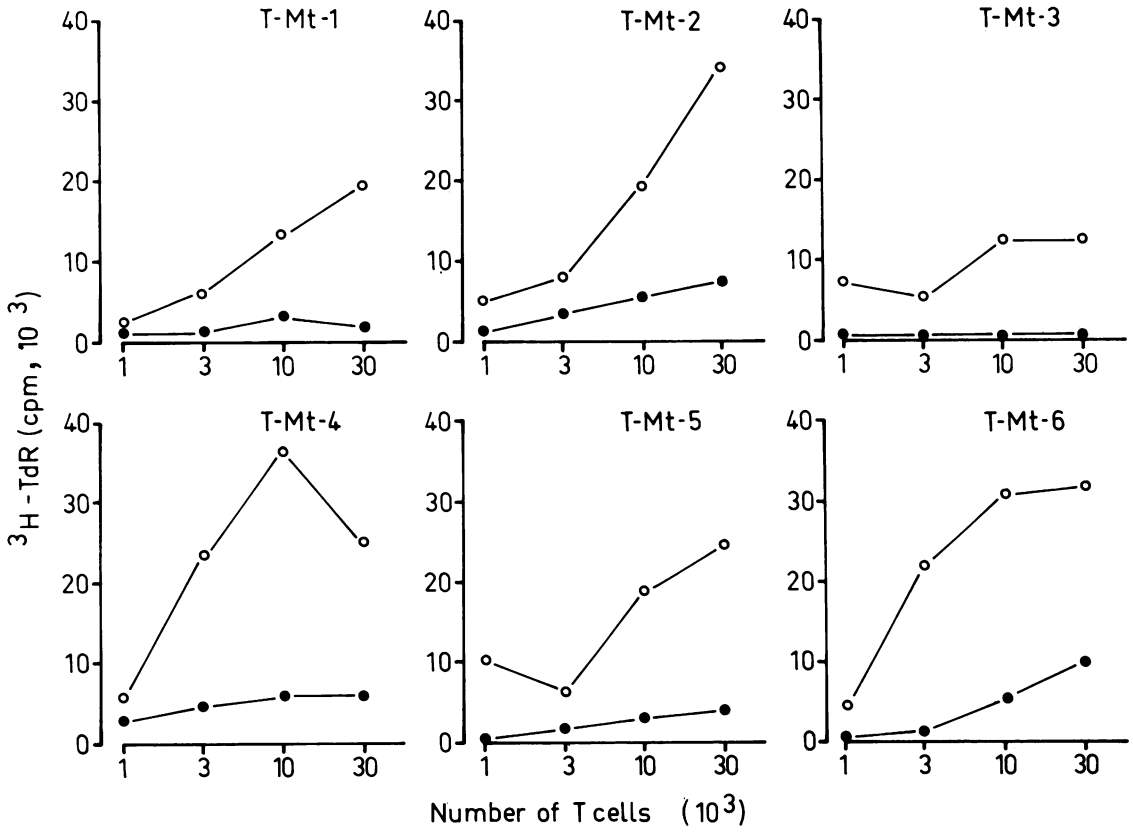

FIG. 1. Graded numbers of cloned T cells were cultured in the presence (○) or absence (●) of irradiated spleen cells and *M. tuberculosis*, and after 4 days proliferative responses were determined by [^{3}H]TdR incorporation.

IL-2 and IFN-γ assay. After purification over Urovison-Ficoll, 10^4 T cells were cultured with 2×10^5 AC and 5 μg of PPD per 0.2 ml of medium, and after 24 h the supernatants were removed. IL-2 activities were determined on an IL-2-dependent cell line as described previously (15). For determination of gamma interferon (IFN-γ), supernatants were added to 5×10^3 L929 cells as described previously (17). After 24 h, 10^3 vesicular stomatitis virus were added, and inhibition of cytopathic effect was determined visually. Some cultures were done in the presence of a specific rabbit anti-IFN-γ antiserum (final dilution, 1:50).

Activation of macrophages for antimycobacterial activity. Bone marrow cells were cultured in serum-free IMDM as described previously (10) and used at day 9 of culture. Activation of macrophages for antimycobacterial activity was done by the method of Rook and Rainbow (29) with modifications. Bone marrow cells (5×10^4/0.2 ml of medium) together with supernatants from T-cell cultures were incubated overnight in flat-bottomed plates (Nunc), and after being washed, 2×10^6 viable *M. bovis* BCG organisms were added. After 4 days, cultures were washed, macrophages were lysed with saponin (0.1%), and mycobacteria were pulsed with 1 μCi of [^{3}H]uracil for 8 h. Incorporation of [^{3}H]uracil was taken as an indicator of mycobacterial growth. Cultures were performed in IMDM without antibiotics or serum.

Adoptive DTH. To determine adoptive delayed-type hypersensitivity (DTH), T cells were mixed with PPD, and 50 μl containing 10^5 T cells and 5 μg of PPD was injected s.c. into one hind footpad. Footpad swelling was measured 24 h later with a dial gauge caliper as described previously (19).

RESULTS

Establishment of different *M. tuberculosis*-reactive T-cell clones. To establish different T-cell clones reactive to *M. tuberculosis*, T cells from *M. tuberculosis*-immune mice were seeded at limiting cell numbers (5×10^2 cells per well) without prior bulk cultivation. Of 192 wells, 18 showed growth, and 8 selected lines were cloned. Each of the six clones described here was derived from a different cell line. The six clones had the phenotype Thy-1$^+$ L3T4$^+$ Lyt-2$^-$ as revealed by analysis with the fluorescence-activated cell sorter. Graded numbers of cloned T cells were stimulated by syngeneic AC and *M. tuberculosis* as antigen, and proliferative responses were determined. Stimulation of the T-cell clones depended on the presence of AC and antigen, and hence the clones were *M. tuberculosis* reactive (Fig. 1). Similar results were obtained when PPD was used as the antigen (data not shown). In previous experiments we have titrated these two antigens and found increasing antigen-specific responses with up to 2.5 to 5 μg of PPD and mitogenic effects at >10 μg of PPD per 0.2 ml of medium. With killed *M. tuberculosis* H37Ra, optimal antigen-specific proliferation in the absence of polyclonal responses was observed at 5 to 10 μg/0.2 ml.

***H-2* restriction of *M. tuberculosis*-reactive T-cell clones.** For determination of the *H-2* restriction of the *M. tuberculosis*-reactive T-cell clones, T cells were cultured with AC from different congenic mice in the presence of *M. tuberculosis* antigen. Proliferation was only observed in the presence of AC from C57BL/6 and B.10A(5R) mice, but not in the presence of AC from B10.A(4R) and B10.MBR mice (Table

TABLE 1. *H-2* restriciton of *M. tuberculosis*-reactive T-cell clones[a]

Mouse strain	Source of AC: *H-2* complex K	I-A	I-E	D	[^{3}H]TdR uptake (cpm) with clone: None	T-Mt-1	T-Mt-2	T-Mt-3	T-Mt-4	T-Mt-5	T-Mt-6
C57BL/6	*b*	*b*	*b*	*b*	83	36,199	41,346	25,890	9,241	12,883	37,962
B10.A(4R)	*k*	*k*	*b*	*b*	94	1,242	1,375	1,244	819	884	1,066
B10.A(5R)	*b*	*b*	*k*	*d*	88	48,958	37,761	42,860	10,602	10,018	39,750
B10.MBR	*b*	*k*	*k*	*q*	61	1,928	1,025	1,747	1,409	1,516	1,557
None					52	776	854	1,190	997	968	411

[a] Cloned T cells (10^4) were cultured with irradiated spleen cells from different mouse strains and killed *M. tuberculosis*. After 4 days, proliferative responses were determined by [^{3}H]TdR uptake. Values are means of triplicate cultures; the standard deviation was <10%.

1). Thus, the T-cell clones recognized mycobacterial antigens in association with class II molecules.

Lymphokine secretion by *M. tuberculosis*-reactive T-cell clones. Cloned T cells were stimulated by syngeneic AC and PPD. After 24 h, supernatants were collected, and the IL-2 and IFN activities in these supernatants were determined. The culture supernatants of the six T-cell clones contained IL-2 and IFN (Table 2). The IFN activity was completely inhibited by addition of a specific anti-IFN-γ antiserum to the cultures (data not shown). We therefore conclude that the *M. tuberculosis*-reactive T-cell clones, after stimulation by antigen plus AC, produced IL-2 and IFN-γ.

In vitro stimulation of antimycobacterial mechanisms in normal bone marrow macrophages. It is generally accepted that acquired resistance against intracellular bacteria is expressed by macrophages which are activated by T-cell-derived lymphokines (12). To investigate whether the lymphokines produced by *M. tuberculosis*-reactive T-cell clones could activate antimycobacterial mechanisms, we used an in vitro technique in which replication of live *M. bovis* BCG organisms within macrophages is measured by [^{3}H]uracil uptake. As a source of macrophages we used in vitro-cultured bone marrow macrophages, because we found only small variations with this cell type, whereas peritoneal macrophages markedly varied in different experiments. Cloned T cells were stimulated by syngeneic AC and PPD, and after 24 h supernatants were collected. Bone marrow macrophages were cultured with these supernatants for 24 h, and then live *M. bovis* BCG organisms were added. The inhibition of [^{3}H]uracil uptake by *M. bovis* BCG was taken as an indicator of the antimycobacterial effects exerted by activated macrophages. Macrophages activated by supernatants from antigen-plus-AC-stimulated T cells markedly reduced [^{3}H]uracil uptake by *M. bovis* BCG (Table 3). Hence, the T-cell clones were capable of activating macrophages for antimycobacterial activity in vitro.

Adoptive DTH transfer by *M. tuberculosis*-reactive T-cell clones. DTH is an accepted in vivo parameter of cell-mediated immunity against intracellular bacteria (12). We therefore wanted to assess whether the *M. tuberculosis*-reactive T-cell clones could confer DTH against PPD on normal recipient mice. T cells were mixed with PPD and injected locally (s.c.) into one hind footpad, and 24 h later footpad swelling was determined. In the presence of PPD, the six T-cell clones induced significant footpad swelling (Table 4), showing that they were capable of transferring DTH.

Antigen reactivity pattern. Killed and ultrasonicated organisms of different mycobacterial species (equivalent to 10^6 CFU) were cultured with cloned T cells and AC. In previous studies with heterogeneous T-cell populations, it had been found that the mycobacterial preparations and concentrations used induced optimal responses. The reactivity pattern of the clones ranged from broad to stringent antigen specificity (Table 5). Thus, clone T-Mt-4 recognized a determinant shared by all mycobacterial species tested and hence showed broad cross-reactivity; clone T-Mt-6 recognized a determinant shared by *M. tuberculosis*, *M. bovis*, and *M. ulcerans*, but not by *M. smegmatis*, *M. avium*, *M. scrofulaceum*, *M. intracellulare*, or *M. vaccae* and hence showed intermediate cross-reactivity; clone T-Mt-3 recognized a determinant shared only by *M. tuberculosis* and *M. bovis* but not by the other mycobacterial species and hence showed stringent specificity. These findings suggest that (i) at least some of the T-cell clones recognized distinct determinants of *M. tuberculosis*, (ii) that the panel of T-cell clones used, *M. tuberculosis* and *M. bovis* were not distinguishable,

TABLE 2. Lymphokine production by *M. tuberculosis*-reactive T-cell clones[a]

T-cell clone	Lymphokine activity: IL-2 ([^{3}H]TdR uptake [cpm])	IFN-γ (titer)
T-Mt-1	7,360	27
T-Mt-2	34,015	27
T-Mt-3	9,572	9
T-Mt-4	26,923	81
T-Mt-5	15,128	27
T-Mt-6	18,459	9
None	76	<3

[a] Cloned T cells were cultured with irradiated spleen cells and PPD, and 24 h later supernatants were collected. IL-2 and IFN activities were determined as described in Materials and Methods. In the absence of either AC or antigen, IL-2 activities were <2,000 cpm and IFN were titers <3. Values are means of triplicate cultures; the standard deviation was <10%.

TABLE 3. Stimulation of antimycobacterial activity in normal bone marrow macrophags by *M. tuberculosis*-reactive T-cell clones[a]

T-cell clone	[^{3}H]uracil uptake (cpm): With PPD and AC	Without PPD and AC	% Inhibition of [^{3}H]uracil uptake
None	5,053		
T-Mt-1	562	4,076	86
T-Mt-2	836	4,143	80
T-Mt-3	111	3,658	97
T-Mt-4	77	3,132	98
T-Mt-5	83	3,124	97
T-Mt-6	127	2,537	95

[a] Cloned T cells were cultured in the presence or absence of irradiated spleen cells and PPD. After 24 h, supernatants were removed and their capacity to activate antimycobacterial effects in normal bone marrow macrophages was evaluated as described in Materials and Methods. Values are means of triplicate cultures; the standard deviation was <30%.

TABLE 4. Adoptive DTH conferred by *M. tuberculosis*-reactive T-cell clones[a]

Clone	Footpad swelling (mm [10^{-1}])	
	With antigen	Without antigen
T-Mt-1	7.0	1.0
T-Mt-2	3.5	1.0
T-Mt-3	4.0	1.5
T-Mt-4	5.5	1.0
T-Mt-5	4.0	1.5
T-Mt-6	3.5	1.5
None	0.5	

[a] Cloned T cells were mixed with PPD or medium and injected (s.c.) into one hind footpad. Footpad swelling was determined as described in Materials and Methods. Values are medians for four animals.

and (iii) at least one of the determinants was unique for *M. tuberculosis* and *M. bovis*, whereas the others were shared with atypical mycobacteria.

DISCUSSION

The aim of the present study was to establish *M. tuberculosis*-reactive T-cell clones, to characterize them functionally and phenotypically, and to use them for analysis of antigenic relatedness between mycobacterial species on the T-cell level. The clones had the phenotype Thy-1^+ L3T4^+ Lyt-2^- and were class II restricted. After stimulation with antigen plus syngeneic AC, they produced the lymphokines IL-2 and IFN-γ. Importantly, the T-cell clones conferred DTH reactions on normal recipient mice and rendered normal bone marrow macrophages capable of limiting mycobacterial growth in vitro. Thus, the *M. tuberculosis*-reactive T-cell clones were of the helper/inducer type, as judged by the criteria of function, phenotype, and genetic restriction. Murine T-cell clones of helper type have also been generated from mice immunized with *M. bovis* (28) and *M. leprae* (14).

Acquired resistance against intracellular bacterial pathogens depends on antigen-specific T cells, which activate macrophages for increased bacteriocidal or bacteriostatic activity, and macrophage activation is mediated by lymphokines produced by antigen-stimulated T cells (12). Furthermore, studies by Mackaness (21) have provided evidence that acquired resistance and DTH are closely related activities of the immune response against intracellular bacteria.

The *M. tuberculosis*-reactive T-cell clones produced the lymphokines IL-2 and IFN-γ, which are known to be important mediators of T-cell immunity. Interestingly, these clones also stimulated bone marrow macrophages to inhibit the growth of *M. bovis* in vitro. While our data suggest that the T-cell clones were capable of activating antimycobacterial defense mechanisms, they do not allow us to decide whether the increased antimycobacterial activity was mediated by IFN-γ or by another lymphokine produced by the T-cell clones. Although in several studies IFN-γ has been found to exert potent macrophage activation (24), experiments in the human system indicate that factors derived from *M. tuberculosis*-reactive T cells which are distinct from IFN-γ can activate macrophages (1), and results on the effects of IFN-γ-activated human blood monocytes on mycobacterial growth are controversial (7, 30). Furthermore, our data do not allow us to decide whether mycobacteriostatic or mycobactericidal effects were induced in T-cell lymphokine-activated bone marrow macrophages because the [^{3}H]uracil uptake assay only measures bacterial RNA synthesis. In any case, both mechanisms could contribute to mycobacterial restriction in the host. Currently we are studying the effects of recombinant IFN-γ on growth inhibition of *M. tuberculosis* and *M. bovis* by murine bone marrow macrophages to elucidate these questions (I. Flesch and S. H. E. Kaufmann, manuscript in preparation).

The *M. tuberculosis*-reactive T-cell clones were capable of mediating DTH reactions after local transfer. We used local T-cell transfers because we (13) and others (5) have found that after systemic transfer, cloned T cells are trapped in the lung and thereby prevented from mediating their biological functions in vivo.

Together our findings show that *M. tuberculosis*-reactive T cells of the helper type alone are capable of activating macrophage functions in vitro and in vivo which are relevant to antituberculous immunity and furthermore that recognition of a single mycobacterial epitope by T cells is sufficient to induce these functions. This notion, of course, does not exclude the involvement of other lymphocytes (notably class I-restricted Lyt-2^+ T cells) in acquired antituberculous resistance. Indeed, it has been shown that Lyt-2^+ T cells are required for adoptive protection of mice against subsequent infection with the intracellular bacteria *Listeria monocytogenes* and *M. tuberculosis* (20, 25), and studies performed in the *L. monocytogenes* system point to a role of both class II-restricted L3T4^+ and class I-restricted Lyt-2^+ T cells in cellular antibacterial immunity (4, 9, 16–20, 37). We have

TABLE 5. Reactivity pattern towards different mycobacterial species of *M. tuberculosis*-reactive T-cell clones[a]

Bacterial antigen	[^{3}H]TdR uptake (cpm) of clone						
	None	T-Mt-1	T-Mt-2	T-Mt-3	T-Mt-4	T-Mt-5	T-Mt-6
M. tuberculosis Rv	95	15,117	9,483	27,698	26,166	9,140	12,563
M. bovis	109	16,648	15,088	19,960	14,770	10,612	4,990
M. smegmatis	128	12,609	435	358	8,124	7,402	819
M. avium	300	14,850	1,216	404	12,608	1,474	1,315
M. scrofulaceum	295	454	17,127	1,253	9,141	938	998
M. intracellulare	116	9,704	8,572	987	10,575	115	750
M. ulcerans	85	27,968	31,975	352	26,960	17,483	18,200
M. vaccae	106	281	383	848	9,208	803	586
PPD	78	16,315	12,014	18,130	19,765	7,876	9,553
L. monocytogenes	105	241	412	296	183	589	414
None	64	127	154	344	798	155	206

[a] Cloned T cells (10^4) were cultured with irradiated spleen cells (2×10^5) and with killed and ultransonicated organisms of different mycobacterial species (equivalent to 10^6 CFU), with PPD (5 μg), or with killed *L. monocytogenes* organisms (10^7 CFU). After 4 days, proliferative responses were determined by [^{3}H]TdR uptake. Values are means of triplicate cultures, the standard deviation was <10%.

recently established Lyt-2+ T-cell clones with reactivity to *L. monocytogenes* (18), *M. tuberculosis*, and *M. leprae* (unpublished data). The availability of L3T4+ and Lyt-2+ T-cell clones specific for antigens of intracellular bacteria will allow us to further analyze the contribution of both subsets to antibacterial immunity on the single-cell level.

We have used the *M. tuberculosis*-reactive T-cell clones as tools for functional characterization of the antigenic relatedness between different mycobacterial species on the T-cell level. In earlier studies, cross-reactivity between different mycobacterial species has been observed on the level of heterogeneous T-cell populations (3, 22). Our study shows that on the clonal level, marked heterogeneity exists in the antigen reactivity pattern of single T cells, which ranges from broad cross-reactivity to stringent specificity. However, even the most stringent T-cell clone, T-Mt-3, failed to distinguish between *M. tuberculosis* and *M. bovis*. A high degree of DNA homology between *M. tuberculosis* and *M. bovis* has been found, showing that they are closely related species or even belong to the same species (33). In addition, it appears that *M. tuberculosis* and *M. bovis* cannot be distinguished with monoclonal antibodies (8).

On the other hand, the T-cell clones showed a heterogeneous recognition pattern towards atypical mycobacteria despite their homogeneity with respect to function, phenotype, and genetic restriction. T-cell clones with broad cross-reactivity, such as T-Mt-4, may be relevant for the cross-protection between *M. tuberculosis* and atypical mycobacteria as observed recently in the murine system (26).

Since the recent failure of *M. bovis* BCG vaccination to protect against subsequent infection with *M. tuberculosis*, there has been much speculation about the possibility that *M. tuberculosis* expresses unique epitopes with particular relevance to protection (2, 27, 31–33). Our study does not lend support to this hypothesis because none of the clones distinguished between *M. tuberculosis* and *M. bovis*, and even clones recognizing different epitopes expressed a similar functional and phenotypic pattern.

Recently, recombinant mycobacterial proteins have been cloned and expressed in *Escherichia coli* (34–36). The availability of a panel of T-cell clones with well-characterized function and antigen reactivity patterns will make it possible to analyze the antigenicity of these proteins on the T-cell level and will allow the association of distinct mycobacterial antigens with defined T-cell functions. This approach may facilitate the identification of immunodominant antigens of *M. tuberculosis* for T cells, with potential value for the diagnosis and therapy of tuberculosis.

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