

# Identification of T cell stimulatory epitopes from the 18 kDa protein of *Mycobacterium leprae*

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## Abstract

We have used different mouse strains to examine *in vivo* and *in vitro* responses to the 18 kDa protein of *Mycobacterium leprae*, which appears to be strongly immunogenic in both mice and humans. B and T cell stimulatory epitopes recognised by different strains of mice have been mapped using overlapping peptides that span the entire 18 kDa protein. Previous work established that immunization of mice with the 18 kDa protein results in specific antibody production to common B cell epitopes and immunization of mice with peptides containing these B cell epitopes resulted in the induction of specific IgG to only a limited subset of epitopes in each strain. Now we report that T cells purified from mice immunized with peptides that stimulate antibody production, proliferate *in vitro* when rechallenged. The proliferating T cells produce levels of IL-2 and IFN- $\gamma$ , that indicate antigen-specific T helper type 1 cells are present in significant numbers. Thus, a comparison of *in vivo* and *in vitro* data suggests that T cells bearing the phenotype associated with potentially protective cell-mediated responses can be primed *in vivo* by epitopes on small peptides. Since T cells from both strains of mice are capable of responding to the immunogenic synthetic peptides *in vitro*, but give different responses to the same peptides *in vivo*, factors other than epitope structure appear to influence T cell subset activation. This may have important implications for diseases such as leprosy where a polarized T cell response appears to develop and for the development of synthetic subunit vaccines.

## Introduction

A current approach to the problem of developing new generation vaccines is to identify dominant epitopes contained within major protein antigens of the infectious agent (1). This method has been particularly informative for research into leprosy, a chronic infectious disease caused by the obligate intracellular parasite *Mycobacterium leprae* (2), since viable laboratory models do not exist (3). The availability of genomic libraries of *M. leprae* DNA (4), and of a range of mAbs that bind antigens from lysates of whole *M. leprae* (5) has enabled the cloning of a number of genes that encode protein antigens defined by the immune response to *M. leprae* (6,7).

In this paper we discuss T cell responses to defined antigenic epitopes of the 18 kDa protein of *M. leprae*, which appears to

be a significant factor in human immune responses to *M. leprae* infection. Crude lysates of recombinant expression vectors producing this protein have been shown to stimulate human T cell clones specific for *M. leprae* (8). Lymphocytes from a large proportion of leprosy contacts also proliferate in response to an 18 kDa fusion protein (9) and some *M. leprae*-specific human T cell clones respond to a synthetic peptide duplicating part of the amino acid sequence of the 18 kDa protein (10). We have previously used antibody responses to synthetic peptides spanning the 18 kDa protein to identify B cell epitopes (11,12). We reasoned that if a peptide contained both B and T cell epitopes, immunization of mice with such a peptide should elicit antibody responses and provide an *in vivo* assay for the activation

of T cell help. T helper ( $T_H$ ) cell epitopes identified by this method in B10.BR mice and BALB/cJ mice showed considerable strain-specific variation (12).

T cell proliferation and cytokine ELISA assays have been used to examine the phenotype of responding T cells from both strains of mice to the 18 kDa protein or to peptides. T cell populations purified from mice immunized with 18 kDa protein or peptides recognise and respond *in vitro* to epitopes on many of the 20-mer peptides and these responding T cells appear to exhibit a predominantly  $T_H1$  phenotype. In particular, T cells from both strains of mice respond *in vitro* to epitopes located on peptides 1–20, 16–35, 31–50, and 61–80, which we have previously shown each contains an epitope that only stimulates T cell antibody help in B10.BR mice (12).

It is now well established that the class of  $T_H$  cell which is preferentially activated can determine whether protective immunity develops in response to immunization with a defined antigen (13,14). It has also been shown that the same antigen can induce a dominant  $T_H1$  response under some circumstances, but a  $T_H2$  dominated response under others (15). Furthermore, there is increasing evidence that antigen-specific expansion of T cell subsets may mediate the development of pathology in leprosy (16). The data presented in this paper also show that the ability to prime for a proliferative response *in vitro* is not sufficient to predict immunogenicity *in vivo*. This means that knowledge of the phenotype of responding T cells is as important as the identity of T cell stimulatory epitopes when considering the construction of synthetic subunit vaccines.

## Methods

### Animals

All strains of mice were obtained as breeding pairs from the Jackson Laboratory (Bar Harbor, ME) and maintained as inbred strains in the Department of Molecular Medicine at Auckland University, New Zealand. Mice were used in experiments at 6–10 weeks of age.  $F_1$  hybrid mice were bred at Auckland University.

### Preparation of antigen

The recombinant plasmid pML-3, which contains the gene for the 18 kDa protein of *M. leprae*, was used to transform the *Escherichia coli* strain DH5 $\alpha$  (6). Cells were grown to late log phase, harvested by centrifugation, and the 18 kDa protein extracted and purified as detailed elsewhere (6,7). The purified 18 kDa protein was checked by polyacrylamide gel electrophoresis and was consistently of greater than 95% purity.

Ten overlapping peptides which spanned the entire 18 kDa protein were synthesized by solid phase techniques (17) and then purified by reverse phase high performance liquid chromatography. These were residues 1–20, 16–35, 31–50, 46–65, 61–80, 76–95, 91–110, 106–125, 121–140, and 136–148.

Lyophilized, irradiated *M. leprae*, derived from infected armadillos, was supplied by Dr P. Brennan (Colorado State University, Fort Collins, CO) and *M. tuberculosis* from the National Tuberculosis Reference Laboratory (Greenlane Hospital, Auckland, New Zealand). All mycobacteria were stored in dry form under sterile conditions at 4°C until used as an antigen for immunization or in assays.

### Immunization

Antigens were prepared by dissolving lyophilized antigen in PBS, (150 mM NaCl, 2 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2) or in 0.1% acetic acid, if insoluble in PBS. The protocols used to stimulate antibody production and to detect responses by ELISA have been described previously (12).

To obtain populations of primed T cells, immunizations were given in 100  $\mu$ l of 50% Freund's incomplete adjuvant emulsified with 50% PBS. A volume of 25  $\mu$ l of this emulsion was injected into each of the hind footpads of the mouse and 50  $\mu$ l was injected at the base of the tail. Draining (popliteal and para-aortic) lymph nodes were removed 7–14 days after final immunization and restimulated *in vitro*. Control animals received the same volume of saline emulsified with Freund's incomplete adjuvant.

### T cell proliferation assays

Mice were sacrificed 7–14 days after immunization and the popliteal and para-aortic lymph nodes removed into BSS plus 10% FCS on ice. Adherent fat was removed from the lymph nodes and single lymph node cell suspensions were prepared by forcing the lymph nodes through a fine mesh stainless steel sieve. T cell populations derived from the lymph nodes were enriched by passage over nylon wool, using a modification of the technique of Julius *et al.* (18). Briefly, the eluted cells were subjected to two cycles of column purification. The recovery of T cells was typically 15–20% from lymph node, and the eluted cells contained less than 1% B220<sup>+</sup> and Mac-1<sup>+</sup> cells as determined by FACS analysis. Purified T cells did not proliferate significantly in the presence of the immunizing antigen unless exogenous antigen presenting cells (APCs) were added to the culture (data not shown). The T cells were resuspended in complete Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, including 2 mM glutamine, 100 U/ml each of penicillin 6-potassium and streptomycin sulfate, 10% FCS, and 50  $\mu$ M 2-mercaptoethanol) plus 5 ng/ml IL-1 at a concentration of  $2 \times 10^6$  cells/ml. Aliquots (100  $\mu$ l) of T cell suspension were dispensed into 96-well flat-bottomed plates containing 100  $\mu$ l of medium with antigen titrated over the range of 0–100  $\mu$ g/ml and  $10^4$  bone marrow-derived macrophages as a source of APCs. Cultures were incubated at 37°C in 10% CO<sub>2</sub> for 4 days, before the addition of 10  $\mu$ l of [<sup>3</sup>H]thymidine (New England Nuclear, Boston, MA) containing 0.25  $\mu$ Ci, to each well, and a further 8 h incubation. Plates were then harvested onto glass-fibre filter paper (Whatman, Maidstone, UK) and proliferation was assessed by [<sup>3</sup>H]thymidine incorporation, determined in a scintillation counter (Beckman model LS 8000, Fullerton, CA).

### Phenotype of responding cells

CD4<sup>+</sup> or CD8<sup>+</sup> cells were removed from cell populations by complement-mediated lysis, using either the anti-CD4 monoclonal antibody GK1.5 (20) or the anti-CD8 monoclonal antibody Lyt 2.2 (21) (diluted 1:50 in DMEM/10% FCS) for 30 min on ice. Cells were washed once and resuspended with the anti- $\alpha$  chain mAb MAR-18.5 (22) (diluted 1:100 in DMEM/10% FCS) of 30 minutes on ice and rewashed. Cells were then resuspended in DMEM, 0.5% FCS, 3.3% fresh rabbit complement, and incubated at 37°C for 30 min. Cells were washed twice, viable cell numbers determined, before use in proliferation assays.

Culture supernatants were removed at varying times from

antigen presentation assays and tested for the presence of the cytokines IL-2, IL-3, IL-4, and IFN- $\gamma$  by ELISA as previously described (19).

#### Establishment of T cell lines

Long-term T cell lines were established from single cell lymph node suspensions prepared from mice immunized with the 18 kDa protein as detailed above. Briefly, cells were cultured for 4 days with 10  $\mu\text{g/ml}$  18 kDa protein at  $5 \times 10^6$  cells/ml in 1.5 ml in 24-well culture plates. Blasts were recovered from the interface of a Histopaque 1083 (Sigma, St Louis, MO) gradient centrifuged at 300  $g$  for 15 min. Blasts were washed twice with medium, and cultured with  $1 \times 10^6$  irradiated syngeneic spleen cells and 2  $\mu\text{g/ml}$  18 kDa protein in 1.5 ml of complete medium. T cell lines were maintained by weekly stimulation with  $10^5$  T cells,  $1 \times 10^6$  irradiated syngeneic spleen cells, 2 ng/ml IL-2, and 2  $\mu\text{g/ml}$  18 kDa protein in 1.5 ml of complete medium.

#### Analysis of T cell line cytokines by the polymerase chain reaction (PCR)

T cell lines were harvested after 10 days stimulation with antigen and thoroughly washed. Cells were incubated with concanavalin A (5  $\mu\text{g/ml}$ ) and PMA (50 ng/ml) at  $5 \times 10^5$  cells/ml in 2 ml in 24-well plates. Cells were harvested after a further 6 h and total RNA prepared by guanidinium thiocyanate lysis, purification on a CsCl gradient, and phenol-chloroform extraction as previously described (23). The recovered RNA was resuspended at 1  $\mu\text{g}/13 \mu\text{l}$  in diethyl pyrocarbonate treated distilled, deionized water. cDNA synthesis was performed using 1  $\mu\text{g}$  of RNA in a total volume of 20  $\mu\text{l}$  containing 10 mM Tris-HCl, 8 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin, 500  $\mu\text{M}$  each dNTP, 1 mM dithiothreitol, and 20 U AMV reverse transcriptase (Promega, Madison, WI). The mixture was incubated at room temperature for 10 min, then 50 min at 45°C. The reaction was stopped at 90°C for 5 min and quenched on ice. RNase H was added (5 U), the mix incubated for a further 20 minutes at 37°C, and then diluted to 100  $\mu\text{l}$ . PCR reactions were performed on serial dilutions of cDNA (from 10 ng/ml) in a final volume of 25  $\mu\text{l}$  containing 10 mM Tris-HCl, 2 mM MgCl<sub>2</sub>, 50 mM KCl, 0.01% gelatin, 200  $\mu\text{M}$  each dNTP, 1  $\mu\text{M}$  each primer, 1 mM dithiothreitol, and 1 U AMV Taq polymerase (Perkin-Elmer Cetus, Norwalk, CT). PCR reactions were run for 60 cycles using a solid block thermal cycler 480 (Perkin-Elmer Cetus). Then, 5  $\mu\text{l}$  of each PCR reaction was electrophoresed through a 1.5% agarose gel and visualized with ethidium bromide to determine the size of the amplification products. Negative control including *E. coli* tRNA for the cDNA synthesis and no cDNA or no enzyme for the PCR reactions. Positive controls for PCR reactions were plasmids containing the appropriate gene. Each pair of primers spanned at least one intron, allowing mRNA to be distinguished from any contaminating genomic DNA, and were synthesized in the Department of Molecular Medicine.

## Results

#### Murine proliferative responses to the 18 kDa protein

To determine whether *in vitro* T cell proliferative responses were subject to a similar pattern of MHC restriction as previously shown for *in vivo* IgG responses to the 18 kDa protein, three strains of

mice, C57BL/10J, BALB/cJ, and B10.BR, were immunized with 18 kDa protein and after 7 days the draining lymph nodes were removed and T cells enriched as described in Methods. The recovered T cells were cultured with activated macrophages, as a source of APCs and 18 kDa protein as antigen. The proliferative responses described in Fig. 1(A) show that T cells derived from C57BL/10J mice immunized with the 18 kDa protein respond less vigorously than those from BALB/cJ or B10.BR mice. Thus, as previously observed for IgG responses to the 18 kDa protein (12), in an *in vitro* T cell proliferative assay, B10.BR and BALB/cJ are also high responder strains, while C57BL/10J is a low responder (24).

Pretreatment of purified T cells with anti-CD4 antibody and complement completely abrogated proliferation to the 18 kDa protein, while identical treatment using anti-CD8 antibody had little effect (Fig. 1B) showing that in all cases, responding T cells were of the CD4<sup>+</sup> phenotype. Inclusion of the mAb M5/114 (anti I-A<sup>d</sup>, anti I-E<sup>d,k</sup>) at 50  $\mu\text{g/ml}$  in *in vitro* restimulations of BALB/cJ T cells completely abolished proliferative responses, while inclusion of similar levels of MKD6 (anti-I-A<sup>d</sup>) or the mAb 10-3.6 (anti I-A<sup>k</sup>) had no effect. Proliferation of T cells from B10.BR mice was inhibited by mAb M5/114, but not MKD6 or 10.36, indicating that in both strains the proliferative response to all the peptides examined was I-E restricted (data not shown).

Mice were also immunized with either 18 kDa protein, *M. leprae*, *M. tuberculosis*, or ovalbumin and then rechallenged

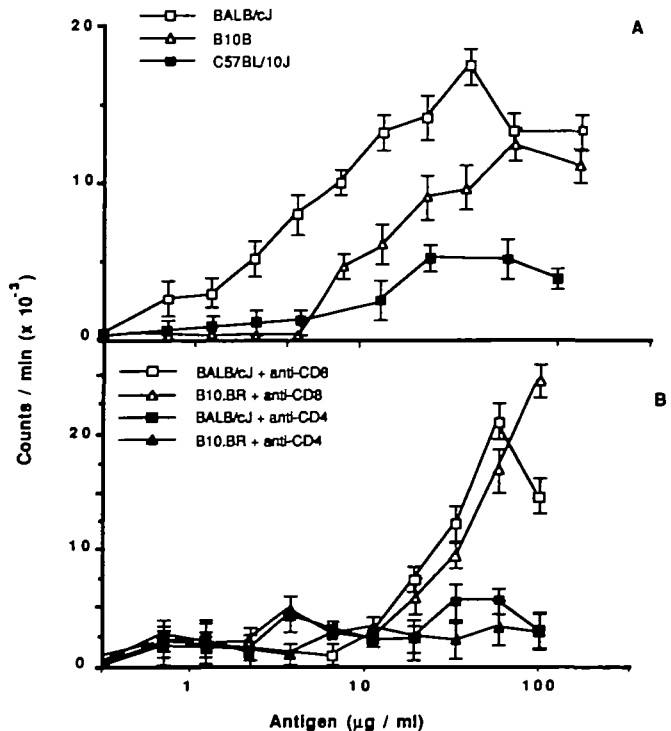
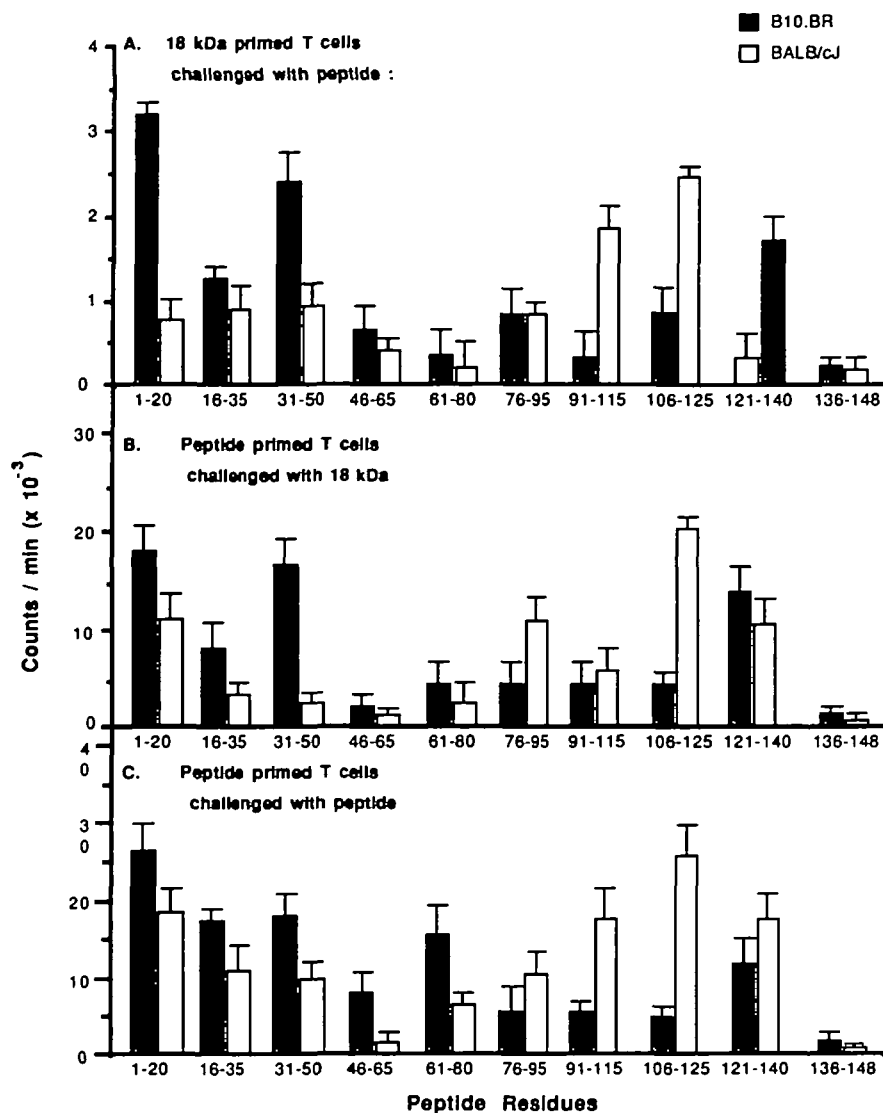


Fig. 1. (A) *In vitro* proliferative responses to the 18 kDa protein with T cells from 18 kDa protein-immunized BALB/cJ, B10.BR, and C57BL/10J mice, and syngeneic APCs as described in Methods. Each point represents the arithmetic mean of triplicate cultures. (B) Effect on T cell proliferation to 18 kDa protein of treatment of 18 kDa protein-reactive T cells with anti-CD4 and anti-CD8 mAbs. Each point represents the arithmetic mean of triplicate cultures.



**Fig. 2.** (A) *In vitro* proliferative responses of T cells from BALB/cJ and B10.BR mice immunized with the 18 kDa protein, cultured with 20-mer peptides (at 25  $\mu$ g/ml) and syngeneic APCs as described in Methods. Each point represents the arithmetic mean of triplicate cultures. Average background (T cells plus antigen without APCs) was 400 c.p.m. or below; proliferation in culture with 10  $\mu$ g/ml 18 kDa protein was ~25,000 c.p.m., in both strains. (B) *In vitro* proliferative responses of T cells from BALB/cJ and B10.BR mice immunized with individual 20-mer peptides, cultured with the 18 kDa protein (at 10  $\mu$ g/ml) and syngeneic APCs as described in Methods. Average background (T cells plus antigen without APCs) was 1000 c.p.m. or below, in both strains. Each point represents the arithmetic mean of triplicate cultures. (C) *In vitro* proliferative responses of T cells from BALB/cJ and B10.BR mice immunized with individual 20-mer peptides, cultured with the immunizing 20-mer peptide (at 25  $\mu$ g/ml) and syngeneic APCs as described in Methods. Average background (T cells plus antigen without APCs) was 900 c.p.m. or below, in both strains. Each point represents the arithmetic mean of triplicate cultures.

with each of these antigens *in vitro* (data not shown). T cells from mice immunized with the 18 kDa protein responded to rechallenge with the 18 kDa protein, *M. leprae*, or *M. tuberculosis* in culture. However, *M. leprae*-primed T cells did not proliferate significantly when restimulated in culture with 18 kDa protein. T cells from ovalbumin-immunized mice only responded when cultured with ovalbumin. Thus, the 18 kDa protein will stimulate T cells reactive with both *M. leprae* and *M. tuberculosis*, although responses to this protein constituted only a fraction of the total response to *M. leprae* immunization. The ability of the 18 kDa protein to stimulate T cells that responded to *M. tuberculosis* was interesting,

in that it suggested that there exist cross-reactive epitopes as yet undefined. The inability of BCG vaccination to consistently prevent the development of leprosy (25) implies that such crossreactive epitopes are not necessarily protective.

#### Mapping of T cell epitopes on the 18 kDa protein using 20-mer peptides

Groups of BALB/cJ and B10.BR mice were immunized with either the 18 kDa protein or 20-mer peptides. After 7 days the draining lymph nodes were removed, T cells purified, and cultured with exogenous macrophages and either 18 kDa protein or one of



**Table 1.** Cytokines produced by a secondary response *in vitro* with purified APCs

Antigen	BALB/cJ		B10.BR	
	IL-4 (ng/ml)	IFN- $\gamma$ (ng/ml)	IL-4 (ng/ml)	IFN- $\gamma$ (ng/ml)
1-20	<0.5	93.9 ( $\pm$ 20.1)	2.6 ( $\pm$ 0.9)	248.2 ( $\pm$ 61.0)
16-35	<0.5	<0.5	<0.5	94.9 ( $\pm$ 22.4)
31-50	<0.5	<0.5	<0.5	226.1 ( $\pm$ 48.5)
46-65	<0.5	<0.5	<0.5	<0.5
61-80	<0.5	<0.5	<0.5	42.0 ( $\pm$ 13.5)
76-95	<0.5	130.3 ( $\pm$ 30.8)	<0.5	41.9 ( $\pm$ 12.8)
91-110	<0.5	56.8 ( $\pm$ 10.6)	<0.5	42.8 ( $\pm$ 13.7)
106-125	<0.5	302.5 ( $\pm$ 60.2)	<0.5	41.9 ( $\pm$ 14.2)
121-140	<0.5	123.9 ( $\pm$ 21.8)	<0.5	184.2 ( $\pm$ 38.4)
136-148	<0.5	<0.5	<0.5	<0.5
18 kDa	1.9 ( $\pm$ 0.7)	284.7 ( $\pm$ 31.6)	1.6 ( $\pm$ 0.8)	261.9 ( $\pm$ 58.2)

Supernatants were taken at 60 h from the culture, fresh medium added and proliferation assayed by  $^3\text{H}$  incorporation as described in Methods. Supernatants were quantified against known recombinant IL-4 or IFN- $\gamma$  standards. In all cases, a high level of IFN- $\gamma$  in the supernatant correlated with increased levels of IL-2 and IL-3 (data not shown), which is indicative that the cytokines were produced by actively proliferating  $T_{\text{h}}1$  cells in both strains. Values have had background (response to a non-18 kDa peptide) subtracted. Samples where cytokine was not significantly above background are represented by '<0.5'. Values represent the means of triplicate cultures.

**Table 2.** Cytokines produced by T cell lines *in vitro*

T cell line	Strain	Peptide	IL-2	IL-3	IL-4	IFN- $\gamma$	Phenotype
1 BC, 5 BC	BALB/cJ	46-65	+	+	-	+	$T_{\text{h}}1$
2 BC	BALB/cJ	106-125	+	+	+	+	$T_{\text{h}}0$ or mixed $T_{\text{h}}1/T_{\text{h}}2$
3 BC, 4 BC	BALB/cJ	106-125	+	+	-	+	$T_{\text{h}}1$
1 BR, 5 BR	B10.BR	31/50	+	+	-	+	$T_{\text{h}}1$
3 BR, 4 BR	B10.BR	1-20	+	+	-	+	$T_{\text{h}}1$

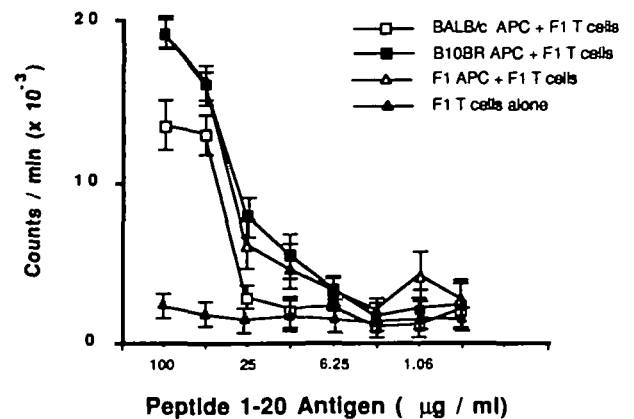
Cells were taken after 6 h concanavalin A and PMA stimulation, and RNA prepared as described in Methods. PCR products were quantified against  $\beta$ -actin and plasmids containing the gene in question after 60 cycles of PCR.

an insignificant response from unfractionated cells (Table 1). Immunization of both BALB/cJ and B10.BR mice induced significant levels of antibody reactive with this peptide (12). It is possible that this antibody response was largely supported by  $T_{\text{h}}2$  cells which often proliferate poorly *in vitro* (unpublished data) allowing  $T_{\text{h}}1$  cells to outgrow and dominate the long-term cultures, while not being obvious in short-term restimulations.

#### Response to peptide 1-20 in BALB/cJ mice

The data presented in this and a previous paper suggest an apparent paradox. Both B10.BR and BALB/cJ mice have B cells that recognise an epitope, or epitopes, on peptide 1-20, as immunization with intact 18 kDa protein led to the production of high titres of IgG that binds to this peptide (12). Immunization of both strains of mice with peptide 1-20 leads to the induction of antibody only in B10.BR, not BALB/cJ mice (12). These findings imply that only B10.BR, not BALB/cJ mice, have T cells capable of providing cognate help for B cell responses to epitopes located within peptides 1-20. However, the data presented in Fig. 2 and Table 1 show that purified T cells from BALB/cJ mice immunized with either 18 kDa protein or peptide 1-20 recognise and respond to an epitope or epitopes contained within peptide 1-20.

To investigate whether the differences in the responses shown by these mouse strains were due to different affinities of the peptide for MHC, bone marrow-derived APCs from B10.BR and



**Fig. 4.** Proliferative responses of T cells from  $F_1$  (BALB/cJ  $\times$  B10.BR) mice immunized with peptide 1-20 cultured with 20-mer peptides at 25  $\mu\text{g/ml}$  and  $F_1$  or parental APCs as described in the Methods section. Average background (T cells plus antigen without APCs) was 800 c.p.m. or below, in both strains. Each point represents the arithmetic mean of triplicate cultures.

BALB/cJ mice were used to present peptide 1-20 to T cells from  $F_1$  (BALB/cJ  $\times$  B10.BR) mice, which had been immunized with this peptide. It was found (Fig. 4) that BALB/cJ- or B10.BR-derived macrophages, pulsed with peptide 1-20 before

**Table 3.** Antibody isotypes produced *in vivo* (midpoint titres)

Antigen	BALB/cJ		B10.BR	
	IgG1	IgG2a	IgG1	IgG2a
1 – 20	1:100	undetectable	1:1030	1:100
91 – 115	1:1150	1:10	1:145	undetectable
18 kDa	1:1030	1:108	1:1150	1:143

IgG midpoint titres to the indicated antigens from BALB/cJ and B10.BR mice immunized multiple times with peptides 1 – 20, 91 – 115, and 18 kDa protein peptide. 91 – 115 was used rather than the 20-mer 91 – 110 since the former was a more potent stimulator of antibody cross-reactive with the whole 18 kDa protein (12). Results represent the mean of specific antibody in sera titrated from five individuals of each strain. The isotype specific ELISAs used antibodies for IgG1 (G1-6.5) and IgG2a (R12-4) from Pharmingen (San Diego, CA). Immunization with these peptides stimulated antibody production with a low proportion of IgG2a, a pattern associated with  $T_H2$  dominated responses (24). In contrast, the ratio of IgG1 and IgG2a is more even, when mice are immunized with the 18 kDa protein. Together with the results shown in Table 1, this suggests that both  $T_H1$  and  $T_H2$  cells may be activated.

irradiation, and then washed to remove free antigen, stimulated  $F_1$  T cells with similar efficiency, demonstrating that BALB/cJ-derived APCs process and present this peptide at a similar efficiency to those derived from B10.BR mice.

The strong proliferative response observed to *in vitro* rechallenge with peptide 1 – 20 by T cells derived from BALB/cJ mice shows that cells can be effectively primed by this peptide *in vivo*, but these cells do not provide help for effective antibody production (Table 3). This appears to be due to selective activation of  $T_H1$  cells *in vivo* (manuscript submitted).

## Discussion

The aim of this work has been to map regions within the 18 kDa protein that contain T cell epitopes, using an *in vitro* T cell proliferative assay and to compare these with T cell epitopes previously mapped by their ability to provide help for B cell responses (12). This approach may give insight into the factors governing activation of pathogenic or protective responses to the leprosy bacillus, given that the 18 kDa protein appears to be an important antigen in the response to *M. leprae* (8 – 10). In the mouse footpad model, immunization with *M. habana*, which unlike the majority of mycobacteria shares the 18 kDa protein, induced some protection against challenge with *M. leprae* (31), suggesting that this protein may contain epitopes with potential for protective immunization.

The specific epitopes which dominate an immune response may have a major impact on the progress of disease (27), due to the action of antigen-specific suppressor cells (28) or as a result of the imbalance of subsets of  $T_H$  cells (29). It has been postulated that the terminal poles of leprosy, which are characterized by either strong cell-mediated immunity or T cell unresponsiveness, may be caused by an imbalance in  $T_H1$  and  $T_H2$  cells, analogous to similar states studied in other disease models (30). Characterization of the epitopes recognised by T cells and the nature of the T cell response to dominant epitopes is therefore of great importance.

Our results indicate that while different strains of mice see similar B cell epitopes on the 18 kDa protein, they see different,

but overlapping, sets of T cell epitopes. This implies a degree of promiscuity in TCR – antigen – MHC interactions that could enable the construction of composite synthetic immunogens, recognised by divergent, or outbred populations. Moreover, the epitopes which evoked the strongest responses (as assessed by proliferation) in different mouse strains, are the same ones which provided the most potent T cell help for antibody production, (12) suggesting that the same epitopes are seen *in vivo* and *in vitro*. The cytokines produced *in vitro* (Tables 1 and 2) show that  $T_H1$  cells are effectively primed by these peptides, although analysis of the antibody isotypes suggest that  $T_H2$  responses are a significant part of the *in vivo* response (Table 3).

A striking feature of the responses described here is the difference between BALB/cJ and B10.BR mice with regard to their responses to peptide as compared with the whole 18 kDa protein. When T cells from BALB/cJ mice immunized and challenged *in vitro* with individual peptides were assayed for proliferation, responses were seen against those peptides which had stimulated a response with 18 kDa-primed T cells but, in addition, responses to peptides 1 – 20, 76 – 95, and 121 – 140 became apparent (Fig. 2). This indicates that BALB/cJ mice possess T cells reactive with epitopes contained on these peptides, but when immunized with the 18 kDa protein, these T cells are not effectively primed. In contrast, B10.BR mice respond effectively to the same peptides whether immunized with peptide or whole protein. Given that the APCs from BALB/cJ or B10.BR mice appear to present peptide 1 – 20 to  $F_1$  T cells with no significant difference in efficiency (Fig. 4), it seems unlikely that either antigen – MHC binding, or the reaction of the TCR and the antigen – MHC complex is in some way defective in BALB/cJ mice. The epitopes so far defined remain unchanged across a wide range of antigen concentrations, suggesting that T cell activation in these strains is not influenced by minor variations in the concentration of antigen. This leaves two likely explanations for these data. It is possible that variation in avidity of antigen-specific receptors in these two strains could lead to preferential uptake of antigen by different classes of APCs. Results obtained in our laboratory suggest that the nature of the response can be manipulated by influencing the type of APCs which presents to a mixed T cell population (manuscript submitted) with subsequent bias in the development of T cell subsets. Equally, it may be that variation in H-2 I-E molecules could lead to activation in one strain of specific suppressor cells, as suggested for *M. leprae*-specific HLA-DQ restricted human suppressor clones (28). Alternatively, this result may reflect real differences in the nature of the cellular populations of these mice, perhaps developed during the selection process. The development of systems in which antigenic epitopes have been described allows these questions to be further addressed, since at last we are able to analyse exactly what the effects of different immunization strategies are on defined T cell populations *in vivo*. This must help our understanding of the molecular processes involved in T cell recognition and activation.

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**Abbreviations**

APC	antigen presenting cell
DMEM	Dulbecco's modified Eagle's medium
PCR	polymerase chain reaction
T <sub>h</sub>	T helper cell

**References**

- Young, D., Lathigra, R., Hendrix, R., Sweetser, D., and Young, R. A. 1988. Stress proteins are immune targets in leprosy and tuberculosis. *Proc. Natl Acad. Sci. USA* 85:4267
- Fine, F. P. E. M. and Rodrigues, L. C. 1990. Modern vaccines Mycobacterial diseases. *The Lancet* 335:1016.
- Johnston, P. A. S. 1987. The search for animal models of leprosy. *Int. J. Lep.* 55:535.
- Young, R. A., Mehra, V., Sweetser, D., Buchanan, T. M., Clark-Curtiss, J., Davis, R. W., and Bloom, B. R. 1985. Genes for the major protein antigens of *Mycobacterium leprae*. *Nature* 316:450
- Engers, H. D., Abe, M., Bloom, B. R., Mehra, V., Britton, W., Buchanan, T. M., Khanolkar, S. K., Young, D. B., Closs, O., Gillis, T., Harboe, M., Ivanyi, J., Kolk, A. H. J., and Shepard, C. C. 1985. Workshop: results of a World Health Organisation-sponsored workshop on monoclonal antibodies to *Mycobacterium leprae*. *Infect. Immun.* 48:603.
- Booth, R. J., Harris, D. P., Love, J. M., and Watson, J. D. 1988. Antigenic proteins of *Mycobacterium leprae*. complete sequence of the gene for the 18 kDa protein. *J. Immunol.* 140:597.
- Booth, R. J., Grandison, P. M., Prestidge, R. L., and Watson, J. D. 1988. The use of a 'universal' yeast expression vector to produce an antigenic protein of *Mycobacterium leprae*. *Immunol. Lett.* 19:65.
- Mustafa, A. S., Gill, K. H., Nerland, A., Britton, W. J., Mehra, V., Bloom, B. R., Young, R. A., and Godal, T. 1986. Human T cell clones recognise a major *Mycobacterium leprae* protein expressed in *E. coli*. *Nature* 319:63
- Dockrell, H. M., Stoker, N. G., Lee, S. P., Jackson, M., Grant, K. A., Jouy, N. F., Lucas, R., Hasan, S. B., Hussain, R., and McAdam, K. P. W. J. 1989. T cell recognition of the 18 kDa protein of *Mycobacterium leprae*. *Infect. Immun.* 57:1979.
- Oftung, F., Shinnick, T. M., Mustafa, A. S., Lundin, K. E. A., Godal, T., and Nerland, A. H. 1990. Heterogeneity among human T cell clones recognising an HLA-DR4, Dw4-restricted epitope from the 18 kDa antigen of *Mycobacterium leprae* defined by synthetic peptides. *J. Immunol.* 144:1478.
- Doherty, T. M., Booth, R. J., Love, S. G., Gibson, J. D., Harding, D. R. K., and Watson, J. D. 1989. Characterisation of an antibody-binding epitope from the 18 kDa protein of *Mycobacterium leprae*. *J. Immunol.* 142:1691.
- Doherty, T. M., Bäckström, B. T., Love, S. G., Harding, D. R. K., and Watson, J. D. 1991. Immune responses to the 18 kDa protein of *Mycobacterium leprae*. Similar B cell epitopes but different T cell epitopes seen by inbred strains of mice. *J. Immunol.* 146:1934.
- Scott, P., Natovitz, P., Coffman, R. L., Pearce, E., and Sher, A. 1988. Immunoregulation of cutaneous leishmaniasis. T cell lines that transfer protective immunity or exacerbation belong to different T helper subsets and respond to distinct parasite antigens. *J. Exp. Med.* 168:1675.
- Haanen, J. B. A. G., de Waal Malefijt, R., Res, P. C. M., Kraakma, E. M., Ottenhoff, T. H. M., de Vries, R. R. P., and Spits, H. 1991. Selection of human T helper type 1-like T cell subset by mycobacteria. *J. Exp. Med.* 174:583.
- Liew, F. Y., Singleton, A., Cillari, E., and Howard, J. G. 1985. Prophylactic immunization against experimental leishmaniasis. V. Mechanism of the antiprotective blocking effect induced by subcutaneous immunization against *Leishmania major* infection. *J. Immunol.* 135:2101.
- Bloom, B. R., Salgame, P., and Diamond, B. 1992. Revisiting and revising suppressor T cells. *Immunol. Today* 13:131.
- Merrifield, R. B. 1969. Solid phase peptide synthesis. *Adv. Enzymol.* 32:221.
- Julius, M. H., Simpson, E., and Herzenberg, L. A. 1973. A rapid method for the isolation of functional thymus-derived murine lymphocytes. *Eur. J. Immunol.* 3:645.
- Cherwinski, H. M., Schumacher, J. H., Brown, K. D., and Mosmann, T. R. 1987. Two types of mouse helper T cell clone. III. Further differences in lymphokine synthesis between T<sub>H</sub>1 and T<sub>H</sub>2 clones revealed by RNA hybridization, functionally monospecific bioassays and monoclonal antibodies. *J. Exp. Med.* 166:1229.
- Dialynas, D. P., Wilde, D. B., Marrack, P., Pierres, A., Wall, K. A., Havran, W., Ollen, G., Loken, M. R., Pierres, M., and Kappler, J. 1983. Characterisation of the murine antigenic determinant, designated L3T4a, recognised by monoclonal antibody GK1.5: expression of L3T4a by functional T cell clones appears to correlate primarily with class II MHC antigen-reactivity. *Immunol. Rev.* 74:29.
- Bluestone, J. A., McKenzie, I. F., Melvold, R. W., Ozata, K., Sandrin, M. S., Sharrow, S. O. and Sachs, D. H. 1984. Serological analysis of H-2 mutations using monoclonal antibodies. *J. Immunogenet.* 11:197.
- Lanier, L. L., Gutman, G. A., Lewis, D. E., Griswold, S. T., and Warner, N. L. 1982. Monoclonal antibodies against the immunoglobulin kappa chains. *Hybridoma* 1:125.
- Chomczynski, P. and Sacchi, N. 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
- Harris, D. P., Bäckström, B. T., Booth, R. J., Harding, D. R. K., and Watson, J. D. 1989. The mapping of epitopes of the 18 kDa protein of *Mycobacterium leprae* recognised by murine T cells in a proliferation assay. *J. Immunol.* 143:1866.
- Bagshawe, A., Scott, G. C., Russell, D. A., Wigley, S. C., Merianos, A., and Berry, G. 1989. BCG vaccination in Karimui, Papua New Guinea, 1963-1979. *Bull. WHO.* 67:389.
- Coffman, R. L., Seymour, B. W. P., Lebman, D. A., Hiraki, D. D., Christiansen, J. A., Shrader, B., Cherwinski, H. M., Savelkoul, H. F. J., Finkelman, F. D., Bond, M. W., and Mosmann, T. R. 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol. Rev.* 102:1.
- Liew, F. Y., Millot, S. M., and Schmidt, J. A. 1990. A repetitive peptide of leishmania can activate T helper type 2 cells and enhance disease progression. *J. Exp. Med.* 172:1359.
- Salgame, P., Convit, J., and Bloom, B. R. 1991. Immunological suppression by human CD8<sup>+</sup> T cells is receptor dependent and HLA-DQ restricted. *Proc. Natl Acad. Sci. USA* 88:2598.
- Mosmann, T. R. and Coffman, R. L. 1989. Heterogeneity of cytokine secretion patterns and functions of helper T cells. *Adv. Immunol.* 46:111.
- Mason, D. 1990. Genetic variation in the stress response: susceptibility to experimental allergic encephalomyelitis and implications for human inflammatory disease. *Immunol. Today* 12:57.
- Lamb, F. I., Singh, N. P., and Coulston, M. J. 1990. The specific 18 kDa protein of *Mycobacterium leprae* is present in *Mycobacterium habana* and functions as a heat shock protein. *J. Immunol.* 144:1922.
- Margalit, H., Spouge, J. L., Cornette, J. L., Cease, K. B., DeLisi, C., and Berzofsky, J. A. 1987. Prediction of immunodominant helper T cell antigenic sites from the primary sequence. *J. Immunol.* 138:2213.