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Mapping of T cell epitopes using recombinant antigens and synthetic peptides

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Two complementary approaches were used to determine the epitope specificity of clonal and polyclonal human T lymphocytes reactive with the 65-kd antigen of *Mycobacterium leprae*. A recombinant DNA sublibrary constructed from portions of the 65-kd gene was used to map T cell determinants within amino acid sequences 101-146 and 409-526. Independently, potential T cell epitopes within the protein were predicted based on an empirical analysis of specific patterns in the amino acid sequence. Of six peptides that were predicted and subsequently synthesised, two (112-132 and 437-459) were shown to contain human T cell epitopes. This corroborated and refined the results obtained using the recombinant DNA sublibrary. Both of these regions are identical in *M. leprae* and *M. tuberculosis* and are distinct from the known B cell epitopes of the 65-kd protein. This combination of recombinant DNA technology and peptide chemistry may prove valuable in analysis of the cellular immune response to infectious agents.

Key words: epitopes/mycobacteria/peptides/recombinant DNA/T cells

Introduction

Identification of antigenic determinants within protein molecules is of theoretical importance in understanding the fundamental interactions involved in immune responses and of potential practical value in the design of 'subunit' vaccines and specific diagnostic reagents. Information has accumulated recently on the architecture of both antibody binding sites and T cell epitopes for a number of structurally well-defined proteins such as lysozyme and myoglobulin (Benjamin *et al.*, 1984) and considerable interest has been shown in the use of synthetic peptides for modulation of antibody responses to infectious agents (Lerner, 1984). Several predictive methods have been described for the identification of potential B cell epitopes (Hopp and Woods, 1981; Tainer *et al.*, 1984; Westhof *et al.*, 1984; Barlow *et al.*, 1986). Because an effective immune response to many pathogens, such as intracellular parasites, is dependent on T cell activation (Hahn and Kaufman, 1981), it is also necessary to develop strategies to identify T cell determinants. The observation that T cell determinants generally consist of short linear peptide sequences has stimulated the development of predictive theories for T cell epitopes that are based on analysis of amino acid sequence (DeLisi and Berzofsky, 1985; Rothbard, 1986).

Pathogenic mycobacteria are the cause of widespread chronic diseases, particularly in developing countries, with an estimated 30 million individuals suffering from tuberculosis and a further 10-15 million individuals with leprosy (Bloom and Godal, 1983). The outcome of mycobacterial infection is thought to be determined by the interaction of mycobacterial antigens with T cells (Mackaness, 1964) and recent studies have identified some of the mycobacterial proteins which are involved in recognition by human T cells (Emmrich *et al.*, 1986; Mustafa *et al.*, 1986; Ottenhoff *et al.*, 1986; Young *et al.*, 1986; Lamb and Young, 1987; Oftung *et al.*, 1987).

The gene for a major mycobacterial antigen, the 65-kd protein, has been cloned from *Mycobacterium tuberculosis* (Young *et al.*, 1985a; Husson and Young, 1987) and from *M. leprae* (Young *et al.*, 1985b). Sequence data shows that this antigen is highly conserved in the two mycobacteria (Mehra *et al.*, 1986; Shinnick, 1987). The 65-kd protein is recognised by a high proportion of the many different murine monoclonal antibodies raised against mycobacterial extracts (Engers *et al.*, 1985,1986), suggesting that the antigen is important in the B cell response to mycobacteria. A recombinant DNA expression method has been used to map the epitopes recognised by six of these monoclonal antibodies (Mehra *et al.*, 1986).

The 65-kd antigen is also involved in the T cell response to infection, and T cell clones from patients with tuberculosis or leprosy have been shown to proliferate in response to the 65-kd protein (Emmrich *et al.*, 1986; Lamb *et al.*, 1986; Oftung *et al.*, 1987). However, the precise location of the T cell determinants within the molecule has not yet been established.

In this paper we have exploited two new techniques to map human T cell epitopes within the 65-kd protein, one based on recombinant DNA expression (Mehra *et al.*, 1986) and the other based on the synthesis of peptides whose sequences are predicted to constitute T cell determinants (Rothbard, 1986).

Results

Expression of antigens by recombinant DNA clones

Construction of a set of recombinant DNA clones containing overlapping fragments of the structural gene of a protein can be used to determine the location of antigenic sites within the molecule. Screening of such a sublibrary with monoclonal antibodies has been used to identify six B cell epitopes in the 65-kd protein (Mehra *et al.*, 1986) and we have used an analogous approach to locate T cell determinants. Figure 1 illustrates the regions of the gene covered by each of the recombinant DNA clones.

Expression of antigens in lysogenic strains derived from the phage clones listed in Figure 1 was analysed by Western blotting as shown in Figure 2. The phage clone which was used for construction of the sublibrary (Y3178) contains a DNA insert coding for the entire 65-kd protein of *M. leprae* but with an orientation reversed with respect to the *lacZ* gene (Mehra *et al.*, 1986). The antigen expressed by this clone has a mol. wt of ~65 kd and is clearly not fused to β-galactosidase (lane 8). An analogous

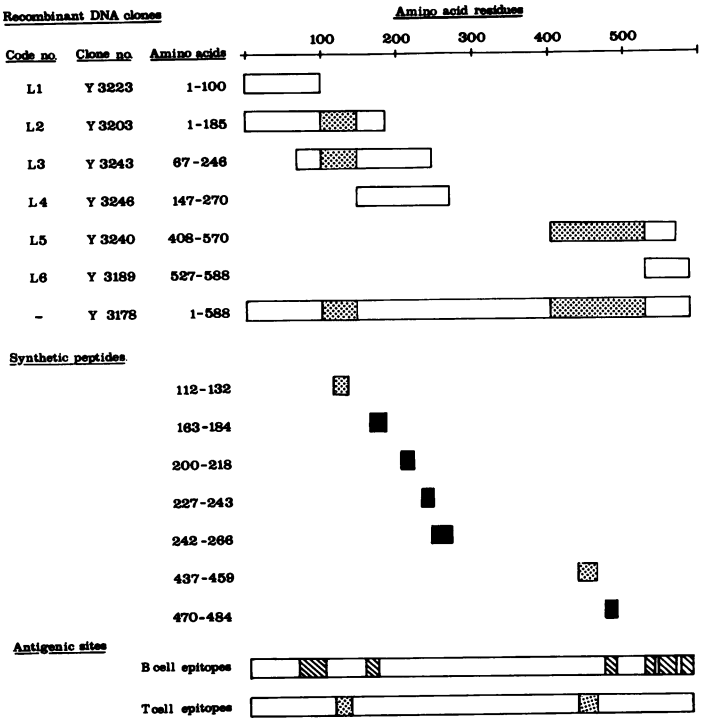

Fig. 1. Map showing areas of the 65-kd protein covered by DNA sublibrary clones and synthetic peptides. The amino acid residues of the 65-kd protein (1−588, based on proposed *M. leprae* coding sequence) are shown on the scale along the top. The upper portion of the diagram shows the areas of the gene covered by the DNA sublibrary clones, while the lower portion shows the synthetic peptides. The stippled areas correspond to the regions recognised by T cells.

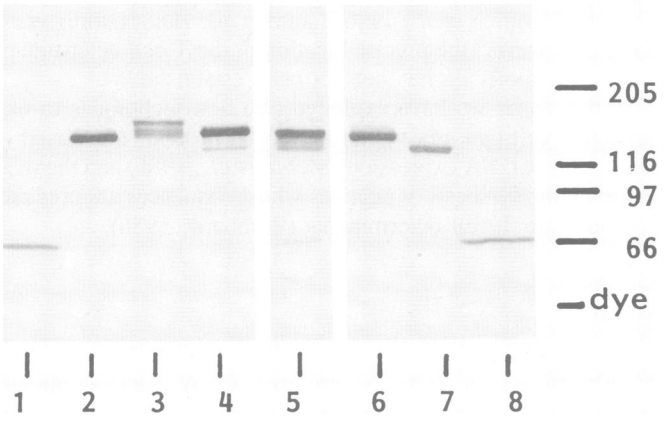

Fig. 2. Expression of mycobacterial antigen by recombinant DNA clones. Lysogens prepared in *E. coli* Y1089 using the λgt11 phages shown in Figure 1 were grown and induced as described in the text. Western blots were prepared and developed with monoclonal antibodies C1.1 (**lanes 1−4**), IIH9 (**lane 5**) and IIC8 (**lanes 6−8**). **Lane 1** contained lysogen from clone Y3150; **lane 2**, Y3223; **lane 3**, Y3203; **lane 4**, Y3243; **lane 5**, Y3246; **lane 6**, Y3240; **lane 7**, Y3189; **lane 8**, Y3178. On the right-hand side of the figure is shown the migration position of standard protein markers with mol. wts (in kd) as indicated.

situation exists for clone Y3150 which expresses the 65-kd protein of *M. tuberculosis* (lane 1). In contrast, all of the sublibrary clones used in this study have insert DNA oriented with the *lacZ* gene (Mehra *et al.*, 1986) and Western blot analysis demonstrates that in each case the mycobacterial antigenic determinant is expressed as a high mol. wt fusion protein linked to β-galactosidase (Figure 2, lanes 2−7).

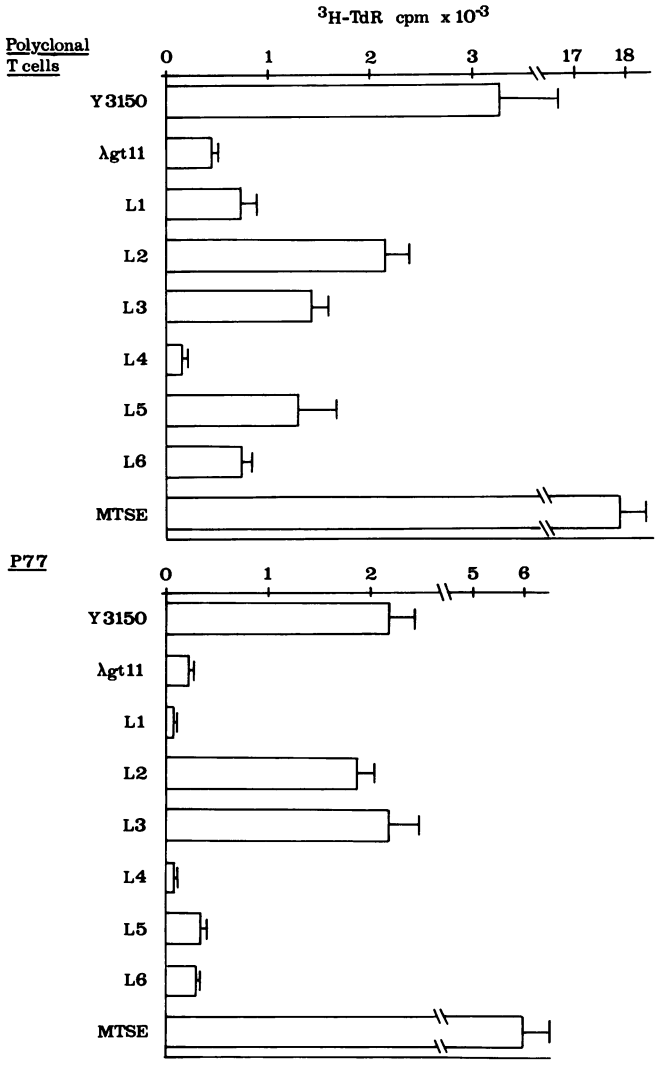

Fig. 3. T cell recognition of antigens expressed by recombinant DNA clones. Unfractionated ascitic lymphocytes were stimulated with extracts from Y3150, λgt11 (control lysogen with no insert DNA), L1, L2, L3, L4, L5, L6 (all at 30 μg protein/ml) and MTSE (1 μg protein/ml). Proliferation as correlated with [^{3}H]TdR incorporation was determined at day 6. The results are expressed as mean c.p.m. ± SEM of triplicate cultures. The response of T cells to medium alone was 575 ± 10 c.p.m. ± SEM. T cells of clone P77 were stimulated with the same antigens as above in the presence of autologous irradiated PBMC as a source of APC. Proliferation was determined at 72 h. The control response of P77 to APC in the absence of antigen was 325 ± 4 c.p.m. ± SEM.

After ultrasonication, the fusion protein from each clone was found to be present in approximately equal amounts in the soluble and insoluble fractions. Addition of ammonium sulphate to the soluble fraction (final concentration, 50% saturation at 0−4°C) resulted in complete precipitation of fusion proteins as judged by Western blot analysis. Previous experiments (data not shown) have indicated that *E. coli* extracts have a non-specific inhibitory effect on T cell proliferation assays which can be substantially decreased by this simple ammonium sulphate fractionation step.

T cell recognition of fusion proteins from 65-kd sublibrary

Products expressed by the intact 65-kd gene (Y3150) and the sublibrary clones L2 (residues 1-185), L3 (67−246) and L5

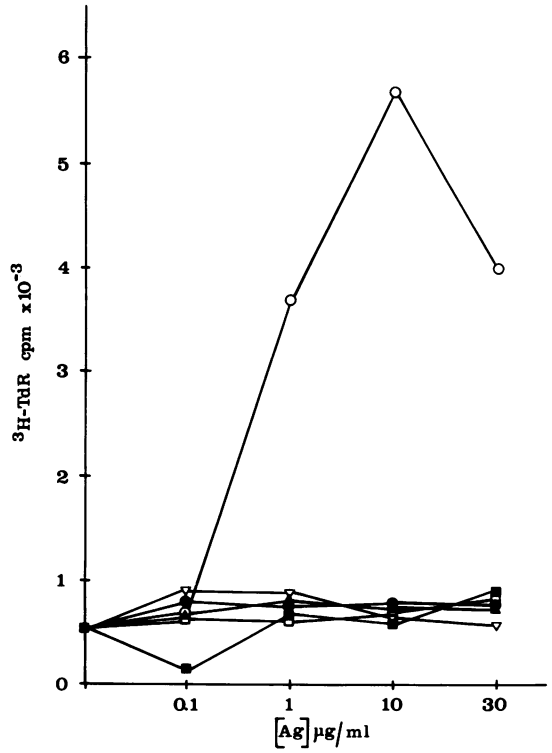

Fig. 4. Response of T cell clone P77 to synthetic peptides of 65-kd antigen. T cells of clone P77 were stimulated with peptides 112–132 (○), 163–184 (▽), 200–218 (□), 227–243 (▲), 242–266 (●), 437–459 (■) in the presence of autologous irradiated EBV-B cells. Proliferation was determined at 72 h.

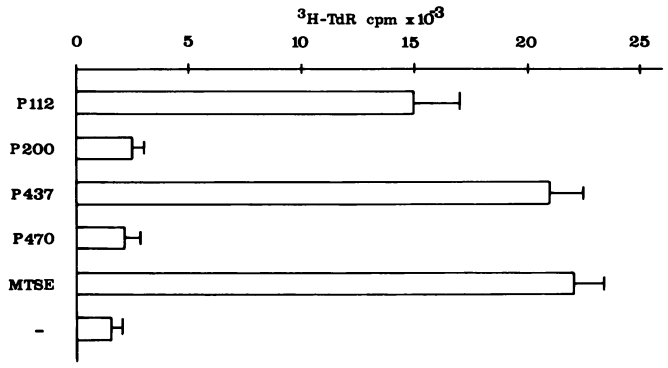

Fig. 5. Polyclonal T cell response to synthetic peptides of 65-kd antigen. Unfractionated ascitic lymphocytes were stimulated with peptides 112–132, 200–218, 437–459, 470–485, MTSE or alone in medium. Proliferation was determined at day 6. The results show the maximum proliferation of a dose–response curve for each antigen.

(408–570) induced a proliferative response in the polyclonal T cells of the ascitic effusion as compared to the control of a lysogen prepared using λgt11 without insert DNA (Figure 3). The response to L1, L4 and L6 was <2-fold higher than that of the background control of the λgt11 lysogen. This suggested that at the polyclonal level at least two regions of the 65-kd protein (residues 101–146 and 409–526) contained T cell epitopes (Figure 1). The response of the ascitic T cells to MTSE served as a positive control (Figure 3).

The pattern of recognition of the 65-kd sublibrary fusion proteins by T cells of clone P77, isolated from the ascitic effusion and previously reported to be specific for the 65-kd antigen (Lamb

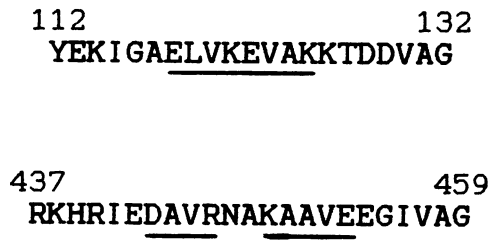

Fig. 6. Amino acid sequence of residues 112–132 and 437–459 of the 65-kd protein. Amino acids are represented by the single letter code. The underlined residues represent the characteristic patterns associated with the presence of predicted T cell epitopes (Rothbard, 1986).

et al., 1986), was also determined. Clone P77 proliferated in response to L2 and L3 but not to the adjacent sequences L1 and L4. In contrast to the response observed with ascitic T cell population, clone P77 was not stimulated by extracts from the recombinant clones covering the carboxyl terminus of the protein (L5 and L6) (Figure 3). The cloned T cells responded to the intact 65-kd protein (Y3150) and to MTSE but not to the control extract from a lysogen prepared from λgt11 without insert DNA. This suggests that the epitope recognised by P77 is located within residues 101–146.

Mapping of T cell epitopes with synthetic peptides

A high proportion of the peptides which have been reported to function as T cell determinants contain a distinctive pattern within their amino acid sequence (Rothbard, 1986). This pattern consists of a charged, or a glycine residue, followed by two or three consecutive hydrophobic residues, and terminating with a polar amino acid. The 65-kd antigen was analysed for the presence of such sequences and six peptides containing patterns similar to those in previously defined epitopes were identified and synthesised. Figure 4 lists the six peptides along with a control peptide (470–484) which contains a known B cell epitope (Mehra *et al.*, 1986) but lacks the characteristic T cell pattern.

Each peptide was tested with both the T cell clone and with the polyclonal population using the proliferation assay. T cells of clone P77 proliferated in a dose-dependent manner when stimulated with peptide 112–132 in the presence of antigen presenting cells, but not with the control peptides 163–184, 200–218, 227–243, 242–266 and 437–459 all from the 65-kd protein (Figure 4). The proliferation was equal to that elicited by preparations containing the intact protein or any of the fragments produced by the sublibrary clones, thus allowing us to conclude that this peptide did indeed contain the epitope for clone P77.

When the response of the uncloned T cells to the peptides was analysed, the same peptide (112–132) was found to be stimulatory and there was also a substantial response to the peptide 437–459 (Figure 5). By means of six peptides we were able to identify two distinct regions of the 65-kd protein that were stimulatory for the ascitic T cells of the donor selected in this study. These two peptides are located within the same two regions of the protein which were identified as immunostimulatory by the recombinant sublibrary approach. The amino acid sequences of the two stimulatory peptides are shown in Figure 6, with the residues corresponding to the predictive patterns underlined.

Discussion

The approach used here for mapping of epitopes recognised by T cells using a λgt11 sublibrary prepared from the gene coding

for the 65-kd antigen is analogous to that previously used to identify monoclonal antibody binding sites on the same protein (Mehra *et al.*, 1986). Although recognition of antigens in lysates from *Escherichia coli* by T cell clones has been reported (Lamb *et al.*, 1986; Mustafa *et al.*, 1986) we have been able to extend this technique to identify regions within a molecule which stimulate proliferation of both clonal and uncloned T cells. It is important to note that both 'free' antigens and antigens expressed as β galactosidase fusion proteins were recognised in these assays, although it is conceivable that a fusion point very close to the T cell epitope could cause a 'false negative' result if it leads to an alteration in antigen processing mechanisms. The polyclonal T cell population used in this study was obtained from an ascitic effusion and is highly enriched in T cells reactive with mycobacteria. Proliferation of polyclonal T cell preparations in response to *E. coli* lysates is quite variable; only preparations with strong responses to specific antigen and low background responses to *E. coli* itself will be suitable for this type of analysis.

The second strategy we have used to identify T cell epitopes is to test the ability of peptide antigens directly to stimulate T cells. Rothbard (1986) has identified a common pattern in the amino acid sequence of peptides containing known T cell epitopes and it is possible that the presence of such a pattern can be used to identify novel T cell epitopes. As a prototype, we have used the 65-kd antigen to test the possibility of using this approach to predict previously undefined T cell epitopes in a relatively large open reading frame. Because the known T cell epitopes are still quite small in number, the power of such an empirical prediction must, however, remain limited at the present time. Nonetheless we were able to identify two stimulatory epitopes in a polyclonal population by synthesising six peptides containing predicted T cell epitopes without knowledge of the results obtained using the recombinant DNA sublibrary. The two stimulatory peptide sequences fell within the regions of the protein identified by sublibrary mapping.

Both sequences 112–132 and 437–459 of the 65-kd protein are identical in *M. leprae* and *M. tuberculosis* (Mehra *et al.*, 1986; Shinnick, 1987) and this is consistent with the cross-reactivity observed at the clonal level for T cells specific for this protein (Emmrich *et al.*, 1986; Lamb *et al.*, 1986). Using mouse monoclonal antibodies, both species-specific and cross-reactive B cell epitopes have been identified on the 65-kd antigen (Mehra *et al.*, 1986) and it is interesting to note that none of these B cell epitopes overlap with the two T cell epitopes described here. Previous analysis of other protein antigens also suggests that T and B cell epitopes within a molecule need not overlap (Lamb and Green, 1983; Benjamin *et al.*, 1984). Whether T cells binding to the cross-reactive epitopes are able to function as helper cells to stimulate production of antibodies to adjacent specific or cross-reactive B cell epitopes remains to be investigated.

Whether the two regions of the 65-kd protein within which we have identified T cell epitopes are immunodominant at the population level or reflect the repertoire of restriction elements that this individual is able to use requires further investigation. The latter has been found to be true for the human cytotoxic T cell response to influenza virus where residues 335–349 of nucleoprotein are recognised only in the context of HLA-B37 (McMichael *et al.*, 1986). Each of the peptides contains two sets of predictive patterns as shown by the underlined residues in Figure 6 (118–121/122–125 and 443–446/449–453). It may therefore be necessary to test the effect of amino acid substitution at particular residues in order to analyse the association of these peptides with HLA-encoded determinants.

The experimental observations reported here show that a recombinant DNA sublibrary can be used for initial localisation of T cell epitopes within a protein. Determination of the nucleotide sequence of sublibrary clones allows derivation of an amino acid sequence which can be analysed for identification of potential T cell epitopes. The complementary strategy of using predicted peptide antigens verified and refined the results obtained using the recombinant DNA sublibrary. The combination of the two techniques leads to a rapid identification of the immunologically important sites within protein antigens. This approach to analysis of T cell recognition of the 65-kd protein from mycobacteria may be generally applicable to the investigation of other infectious agents and may allow production of simple defined peptide antigens which can be used as probes for detailed analysis of cellular immune responses.

Materials and methods

Antigens

Mycobacterium tuberculosis H37RV was grown for 8 weeks as a surface pellicle on Sauton's medium. A soluble extract (MTSE) was prepared by disruption of cobalt-irradiated organisms using a Braun MSK cell disintegrator at 4000 r.p.m. for 2 min at 5–10°C. Bacterial debris was removed by centrifugation at 30 000 *g* for 1 h, and supernatant material filtered through a 0.45-micron filter. Protein concentrations in the soluble extracts were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Preparation of antigen from recombinant DNA clones

Preparation and characterisation of a λgt11 sublibrary from the DNA insert of clone Y3178 (*M. leprae* 65-kd antigen) has been described previously by Mehra (1986). The sublibrary clones used in the present study are shown in Figure 1 along with their relationship to the amino acid sequence of the 65-kd protein as deduced from the insert endpoint analysis (Mehra *et al.*, 1986). Other recombinant clones used in this study were Y3178 (*M. leprae* 65-kd antigen; Young *et al.*, 1985b) and Y3150 (*M. tuberculosis* 65-kd antigen; Young *et al.*, 1985a).

Lysogens were prepared from phage clones in *E. coli* Y1089 as described by Huynh *et al.* (1985). After growth in L-broth at 37°C to an absorbance of 0.5 at 600 nm, lysogens were induced by incubation for 20 min at 45°C, followed by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to 10 mM and further incubation at 38°C for 1 h. Cells were harvested by centrifugation, resuspended in one-tenth volume of phosphate-buffered saline (PBS), and lysed by sonic disruption using a Rinco B1070 ultrasonic probe at a maximum output for 20 s. Bacterial debris was removed by centrifugation at 10 000 *g* for 20 min, and an equal volume of saturated ammonium sulphate solution was added with vigorous mixing to the supernatant fractions. After 1 h at 0–4°C, precipitate material was collected by centrifuging at 10 000 *g* for 20 min and resuspended in PBS. The protein concentration in the 50% ammonium sulphate fractions was determined by the method of Lowry *et al.* (1951).

Expression of mycobacterial antigenic determinants in recombinant lysates was analysed by Western blotting using monoclonal antibodies Cl.1 (Mehra *et al.*, 1986), IIH9 and IIC8 (Gillis and Buchanan, 1982). Bacterial pellets and samples from the soluble and insoluble portions obtained after sonication of *E. coli* strains were mixed with sample buffer for SDS–polyacrylamide gel electrophoresis under reducing conditions (Laemmli, 1970) and incubated on a boiling water bath for 2 min. Samples (~5 μg of protein per lane) were applied to SDS–polyacrylamide gels (6% w/v, final acrylamide concentration) and subjected to electrophoresis at 15 mA per gel for 50 min using a mini-gel system supplied by Hoeffer Scientific Instruments (San Francisco, CA). Nitrocellulose blots were prepared from gels by electroblotting at 50 V for 1 h (Towbin *et al.*, 1979). Blots were washed with 0.2% Triton X-100 in PBS and non-specific binding was blocked by carrying out subsequent incubations in the presence of 5% dried milk powder in PBS–Triton. Develpment of Western blots with monoclonal antibodies and horseradish peroxidase conjugated secondary antibody was performed as described previously (Young *et al.*, 1986).

Synthetic peptides

The synthetic peptides were prepared using solid-phase methods on an Applied Biosystems 430 A peptide synthesiser as previously described (Townsend *et al.*, 1986). The particular regions of the protein that were synthesised were selected on the basis of the presence of a pattern of either four or five amino acids (charged/glycine followed by two or three hydrophobic residues and then a polar amino acid) (Rothbard, 1986). These two patterns have a high statistical correlation with known helper and cytotoxic T cell epitopes. The 65-kd protein contains 25 patterns of four amino acids and 12 patterns of five, which could be ordered by

analysing the amino acid sequence in comparison with previously defined T cell epitopes.

Preparation of lymphocytes

Mononuclear leucocytes reactive with *M. tuberculosis* were isolated from peripheral blood (PBMC) and the ascitic effusion of a patient by centrifugation on a discontinuous density gradient of Ficoll–Hypaque were resuspended in complete medium, RPMI 1640 supplemented with A+ serum, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin.

Isolation of human T lymphocyte clones

Human T cell clones reactive with *M. tuberculosis* were isolated as previously described (Lamb and Young, 1987). Briefly, ascitic lymphocytes (2.5×10^4/ml) were stimulated with MTSE (1 μg protein/ml) in 96-well, round-bottom microtitre plates (Nunc, Roskilde, Denmark) in complete medium. Lymphoblasts were enriched on Ficoll–Hypaque and cloned by limiting dilution (0.3 cells/well in Terasaki plates) in the presence of autologous irradiated (3000 rads) PBMC mixed (1:1) with ascitic lymphocytes (5×10^4/ml), MTSE (1 μg/ml) and 10% (v/v) interleukin-2 (IL-2; Lymphocult T, Biotest-Serum Institute GmbH, Frankfurt, FRG) (Lamb *et al.*, 1982). At day 7, growing clones were transferred to 96-well, flat-bottom microtitre plates and subsequently to 24-well plates. At each transfer the clones received filler cells, antigen and IL-2. The clones were expanded and maintained by the addition of fresh IL-2 every 3–4 days, and filler cells together with specific antigen every 7 days. Before use in proliferation assays the T cell clones were rested for 6–8 days after the last addition of filler cells.

Proliferation assays

Cloned T-lymphocytes (5×10^4/ml) were cultured with soluble antigen in the presence of autologous irradiated PBMC (1.25×10^5/ml) or EBV transformed B cells (5×10^4/ml) in a total volume of 200 μl in 96-well, round-bottom plates as previously described (Lamb and Young, 1987). After 72 h incubation [^{3}H]-methyl thymidine (1 μCi; [^{3}H]TdR; Amersham International, Amersham, UK) was added to the cultures for 8–16 h, and then harvested onto glass-fibre filters. Unfractionated lymphocyte cultures (1×10^6/ml) were pulsed with [^{3}H]TdR after 6 days incubation. Proliferation as correlated with [^{3}H]TdR incorporation was measured by liquid scintillation spectroscopy. The results are expressed as the mean counts per minute (c.p.m.) ± % error of the mean for triplicate cultures.

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