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A novel approach to the identification of T-cell epitopes in *Mycobacterium tuberculosis* using human T-lymphocyte clones

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SUMMARY

Current approaches to the analysis of antigens involved in the cellular immune response to mycobacterial infection rely on the initial identification and isolation of molecular components using monoclonal antibodies. In order to overcome the constraints of this approach, we have utilized a procedure involving T-cell recognition of antigens fractionated by polyacrylamide gel electrophoresis (SDS–PAGE) and added to proliferation assays after blotting onto nitrocellulose membranes. Analysis of human T-cell responses to *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG by this procedure revealed distinctive patterns of reactivity to different molecular weight components indicative of the selective recognition of immunodominant and species-specific determinants. Human T-cell clones were subsequently derived, and SDS-PAGE immunoblotting was used to identify the antigen recognized by each clone. Three epitopes defined by individual T-cell clones were identified on separate polypeptides with molecular weights 16,000–18,000 (clone P53), 18,000–20,000 (clone P57) and 52,000–55,000 (clone P35). This study demonstrates the potential application of T-cell cloning in conjunction with SDS-PAGE immunoblotting for the dissection and analysis of the cellular immune response to pathogenic agents during human infection.

INTRODUCTION

A considerable effort has been made over many years to identify molecular components of pathogenic mycobacteria that are involved in stimulation or suppression of the human immune response during infection (Brennan, 1984; Daniel, 1984). The potential rewards of such research are the development of novel diagnostic reagents and the production of vaccines effective in controlling diseases such as leprosy and tuberculosis (Arnon, 1984). The generation and characterization of mouse monoclonal antibodies directed towards mycobacterial antigens have represented a significant advance in this area of resarch (Engers 1985, 1986; Ivanyi, Morris & Keen, 1985) and, as a result of these studies, a limited number of protein antigens of *Mycobacterium tuberculosis* and *Mycobacterium leprae* have been identified. The independent generation of many monoclonal antibodies with overlapping specificities in separate laboratories suggests that these proteins represent immunodominant mycobacterial antigens with respect to the murine antibody response (Engers, 1985, 1986). Representative monoclonal antibodies from workshops organized by the World Health Organisation (Engers, 1985, 1986) have been used with considerable success to screen for expression of these major antigens in recombinant DNA libraries (Thole *et al.*, 1985; Young *et al.*, 1985a, b). Since protection from mycobacterial infection is a function of the cell-mediated immune system (Hahn & Kaufmann, 1981), several laboratories have initiated studies involving characterization of the human T-cell response to these antigens (Mustafa *et al.*, 1986; Ottenhoff *et al.*, 1986; Emmrich *et al.*, 1986; Young *et al.*, 1986).

While it seems likely that the monoclonal antibody-defined proteins are immunodominant for murine B cells (Engers, 1985, 1986; Ivanyi *et al.*, 1985), it is not clear that the same pattern of immunodominance will be reflected in the human T-cell response to mycobacteria. It is possible, for example, that antigens that cause marked T-cell proliferation during infection may stimulate little or no antibody formation following immunization with a mycobacterial extract. There is therefore a need to supplement the present monoclonal antibody-based approach to antigen identification with an approach that directly identifies antigens recognized by human T cells. The recent development of an SDS-PAGE assay for screening T-cell clones (Young & Lamb, 1986) provides the possibility of such an approach, and we describe here its application to the identification of antigens of *M. tuberculosis* involved in the human T-cell response.

Abbreviations: BCG, Bacillus calmette-guerin; [^{3}H]TdR, tritiated methyl thymidine; IL-2, interleukin-2; MBSE, *M. bovis* soluble extract; MTSE, *M. tuberculosis* soluble extract; SDS–PAGE: sodium dodecyl-sulphate–polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Antigens

Mycobacterium tuberculosis H37Rv and *Mycobacterium bovis* BCG were grown for 8 weeks as a surface pellicle on Sauton's

medium. Soluble extracts were prepared by disruption of cobalt-irradiated organisms using a Braun MSK cell disintegrator at 4000 r.p.m. for 2 min at 5–10°. Bacterial debris was removed by centrifugation at 30,000 *g* for 1 hr, and supernatant material was 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 standard.

Preparation of immunoblots

Dot blots and SDS–PAGE immunoblots were prepared as described previously (Young & Lamb, 1986). Samples of mycobacterial extracts for electrophoresis contained 20 μg of protein per lane. Polyacrylamide gels were run on a 'mini-gel' apparatus (Hoeffer Scientific Instruments, San Francisco, CA) to a total length of 2 cm (Table 2) or 4 cm (Figs 1 and 2), or on a full-sized gel apparatus (BioRad Instruments, Richmond, CA) to a total length of 15 cm (Fig. 2, insert). Proteins were transferred to nitrocellulose by electroblotting as described elsewhere (Young & Lamb, 1986).

Preparation of lymphocytes

Mononuclear leucocytes isolated from peripheral blood (PBMC) and the ascitic effusion of a patient reactive with *M. tuberculosis* by centrifugation on a discontinuous density gradient of Ficoll-Hypaque (Boyum, 1968) were resuspended in complete medium, RPMI-1640 supplemented with 10% pooled A+ serum, 2 mM L-glutamine and 100 IU/ml penicillin/streptomycin.

Isolation of human T-lymphocyte lines and clones

Ascitic lymphocytes ($2{\cdot}5 \times 10^5$/ml) were stimulated for 6 days with a soluble extract of *Mycobacterium tuberculosis* strain H37Rv (MTSE; 1 μg protein/ml) in 96-well round-bottomed microtitre plates (Nunc, Roskilde, Denmark) in complete medium. The activated lymphocytes were isolated on Ficoll–Hypaque and expanded as a long-term T-cell line (P anti-Rv) with autologous irradiated (3000 rads) PBMC mixed (1 : 1) with ascitic lymphocytes (5×10^5/ml) and MTSE (1 μg/ml) in the presence of 10% interleukin-2 (IL-2; lymphocult T, Biotest-Serum Institut GmbH, Frankfurt, FRG) or cloned by limiting dilution as previously described (Lamb *et al.*, 1982). T-cell clones reactive with mycobacterial antigens were isolated as follows. Ascitic T lymphoblasts were diluted [0·3 cells/well in Terasaki plates (Nunc)] in the presence of irradiated autologous PBMC mixed (1 : 1) with ascitic lymphocytes (5×10^5/ml), 10% IL-2 and MTSE (1 μg/ml). Growing clones at Day 7 were transferred to 96-well flat-bottomed microtitre plates and subsequently to 24-well plates then 25 cm^2 flasks. 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 6–8 days after the last addition of filler cells.

Proliferation assays

T-lymphocyte clones and long-term lines (5×10^4/ml) were cultured with soluble or nitrocellulose-bound antigen in the presence of autologous irradiated PBMC/ascitic lymphocytes (5×10^5ml) in 96-well flat-bottomed microtitre plates as previously described (Young & Lamb 1986). Briefly, after 72 hr incubation the cultures were resuspended and the nitrocellulose strips discarded prior to the addition of tritiated methyl thymidine (1 μCi; [^{3}H-]TdR; Amersham International, Amersham, Bucks) for 8–16 hr and then harvested onto glass fibre filters. The duration of unfractionated lymphocyte cultures (1×10^6/ml) was 5 days. 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.) ± % standard error of the mean for triplicate cultures.

RESULTS

T-cell proliferation in response to nitrocellulose-bound antigen

T lymphocytes obtained from ascitic effusions or from the peripheral blood of tuberculosis patients were found to proliferate in response to *M. tuberculosis* extract supplied either as soluble antigen or in the form of a solid-phase antigen bound to nitrocellulose. Table 1 shows a representative experiment demonstrating proliferation of a T-cell line derived from an ascitic effusin. As described previously for the clonal response to a viral antigen (Young & Lamb, 1986), a higher concentration of solid-phase antigen was required to stimulate an equivalent response to that obtained with soluble antigen. No proliferation was observed in the absence of accessory cells, and the nitrocellulose itself was not mitogenic (Table 1).

Differential pattern of reactivity on the polyclonal T-cell response to SDS–PAGE immunoblots of *M. tuberculosis* and *M. bovis*

In order to identify the molecular weight of the components stimulating the proliferative response of the polyclonal T-cell population, mycobacterial extracts were subjected to SDS-PAGE and, following transfer to nitrocellulose membrane, the different antigen factions were tested in proliferation assays. Figure 1 shows the response of ascitic T cells to *M. tuberculosis* and *M. bovis* BCG extracts examined in this manner. A distinct

Table 1. Activated T lymphocytes recognize antigens blotted on nitrocellulose

			Response ([^{3}H]TdR incorporation c.p.m. ± SEM)	
T cells	APC	Antigen	Nitrocellulose bound	Soluble
+ *	−	—	375 ± 14	
+ †	+	—	180 ± 9	286 ± 23
+	+	MTSE 0·1 μg/ml	5482 ± 10	24,197 ± 21
+	+	+ 1·0 μg/ml	21,445 ± 16	30,997 ± 35
+	+	+ 10 μg/ml	31,620 ± 1	55,381 ± 20
+ ‡	+	+ 1·0 μg/ml + nitro	—	22,478 ± 18
+	−	IL-2	—	36,474 ± 36

H37Rv-reactive T-cell line (P anti-Rv 5×10^4/ml) was stimulated with MTSE as soluble antigen or nitrocellulose-bound as dot-blots together with irradiated autologous ascitic lymphocytes and PBMC (5×10^5/ml) as a source of antigen-presenting cells (APC). Proliferation as correlated with [^{3}H]TdR incorporation was determined at 72 hr. The results are expressed as the mean c.p.m. ± SEM of triplicate cultures. Control responses of T cells to (*) medium, (†) APCs without antigen, and (‡) soluble antigen in the presence of nitrocellulose.

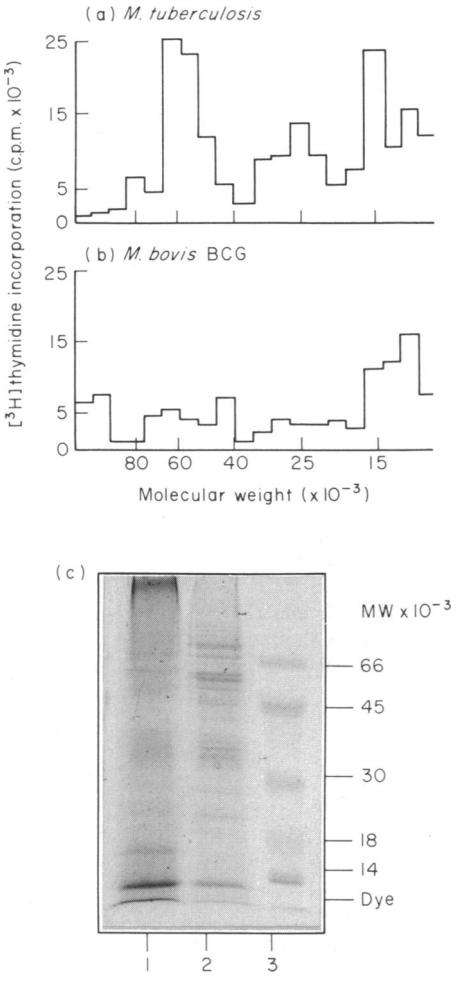

Figure 1. Differential pattern of reactivity of the polyclonal T-cell response to SDS-PAGE antigens on immunoblots of *M. tuberculosis* and *M. bovis* BCG. Unfractionated ascitic lymphocytes (1×10^6/ml) were cultured for 5 days with *M. tuberculosis* and *M. bovis* BCG on nitrocellulose blots (20 fractions) or as soluble antigen. Proliferation was determined as described in the legend to Table 1. The response to soluble *M. tuberculosis* and *M. bovis* BCG (1 μg protein/ml) was 112,411 ± 15 and 71,246 ± 9 c.p.m. ± SEM, respectively. Background proliferation of the T cells to nitrocellulose or to medium alone were 637 ± 14 and 587 ± 22 c.p.m. ± SEM. (a) *M. tuberculosis* and (b) *M. bovis* BCG extracts (20 μg protein per lane) were prepred and run on SDS–polyacrylamide gels, and proteins were blotted onto nitrocellulose; (c) A representative gel stained with Coomassie blue: Lane 1, *M. tuberculosis* extract; Lane 2, *M. bovis* BCG extract; Lane 3, molecular weight markers.

pattern of peaks and troughs in the proliferative response to *M. tuberculosis* extract was observed (Fig. 1a) with peaks particularly in the molecular weight regions of 51,000–66,000, 24,000–28,000, and 12,000–18,000. While the overall response of this T-cell population to *M. bovis* BCG extract at 1μg/ml (71,246 ± 15) was comparable to the *M. tuberculosis* response (112,411 ± 15), quantitative and qualitative differences in response to the two extracts were observed during analysis by SDS-PAGE immunoblotting (Fig. 1a and b). The higher molecular weight fractions from *M. bovis* BCG (51,000–66,000 and 24,000–28,000) stimulated a lower response than those from *M. tuberculosis*, although comparable proliferation in response to the low molecular weight material (< 20,000) was observed. Visual inspection of polyacrylamide gels stained for total protein (Fig. 1c) did not indicate a marked difference in the concentration of proteins in these molecular weight regions between the two mycobacterial extracts.

Analysis by T-cell cloning

In order to carry out a more detailed analysis of the T-cell response to the antigens of different molecular weights, a set of T-cell clones was established from the T-cell population shown in Fig. 1. Nine of the clones were selected for detailed analysis and these are listed in Table 2.

All of the clones proliferated in response to the *M. tuberculosis* extract, while several showed little or no response to *M. bovis* BCG. Seven strongly proliferative clones (> 10,000 c.p.m. to *M. tuberculosis*-soluble extract) showed a clearly positive response to nitrocellulose-bound antigen, while the remaining two clones (P2 and P56) showed only a weak proliferation to solid-phase antigen. All of the clones were tested for recognition of high, medium or low molecular weight fractions obtained from 2-cm mini-immunoblots, and positive results (proliferation > 1000 c.p.m.) were observed with five of the clones (Table 2). Clones P35, P53 and P57 were then analysed using 4-cm SDS-PAGE immunoblots divided into 20 fractions each (Fig. 2). Clone P35 responded to two adjacent fractions in the high molecular weight region (50,000–60,000 (Fig. 2) and subsequent more detailed analysis using 70-fraction immunoblots indicated a molecular weight of 52,000–55,000 for the antigen recognized by this clone (Fig. 2a, insert). The other two clones (P53 and P57) that both showed some proliferation in response to *M. bovis* BCG recognized antigens with molecular weight 16,000–18,000 (Fig. 2b) and 18,000–20,000 (Fig. 2c), respectively.

DISCUSSION

The results presented in this paper demonstrate a novel and important approach to the molecular analysis of the antigens of a microbial pathogen that interact with the human cellular immune system. It is possible to use SDS–PAGE immunoblotting to analyse the contribution of antigens of different molecular weight to the overall polyclonal T-cell response to a mycobacterial extract. The extension of this analysis to screen the polyclonal response of tuberculosis patients and normal individuals to extracts prepared from different mycobacteria represents a potential approach to the identification of species-specific antigens and of antigens that are recognized as immunodominant during infection. Since the concentration of protein in each fraction of the gel has not at this stage been assayed, it cannot be stated whether the immunodominance represented by the peaks is a reflection of the concentration of particular antigens or of the concentration of T cells with appropriate specificities. Visual inspection of gels stained for total protein does not demonstrate the occurrence of particularly high concentrations of protein in sections of the gel stimulating maximum T-cell proliferation, and immunodominance based on immune recognition rather than on relative antigen concentration would therefore appear more likely. Occurrence of antigens causing a suppression of T-cell proliferation in the

Table 2. Specificity pattern of MTSE-induced T-cell clones for immunoblotted *M. tuberculosis*

	Clone no.								
	P2	P35	P48	P53	P56	P57	P65	P77	P83
(A) *Soluble antigen*									
1. MTSE	7251	28,115	11,584	45,507	8998	19,053	13,772	12,020	25,851
2. MBSE	818	406	1804	14,902	279	13,466	895	805	3155
3. IL-2 response	36,798	83,042	64,290	29,105	39,019	88,258	37,818	61,611	51,867
4. APC control	283	273	121	113	95	176	155	127	346
5. Medium control	86	109	105	75	55	176	74	103	83
(B) *Nitrocellulose-bound antigen*									
1. MTSE	1387	9195	4108	19,175	1441	9388	2804	7069	7868
2. Immunoblot > 50,000	221	5778	739	2039	380	575	167	351	479
3. Immunoblot 35,000–50,000	345	399	2580	4944	276	586	298	238	464
4. Immunoblot 20,000–35,000	533	430	231	699	126	464	353	252	497
5. Immunoblot 20,000	431	568	1387	7553	356	2431	884	538	1226
6. Nitrocellulose control	451	352	214	137	128	159	252	237	457

Cloned T cells ($2{\cdot}5 \times 10^4$/ml) were stimulated with MTSE or *M. bovis* BCG soluble extract, MBSE (1 μg protein/ml) as a soluble antigen (A1, A2) or the same concentration of MTSE bound to nitrocellulose (B1). Each clone was also tested with four sections prepared from 2-cm MTSE immunoblots corresponding to the approximate molecular weight ranges listed for samples B2–B5. Results from the major reactive section are underlined for each clone. Controls included medium alone (A5), with antigen-presenting cells (A4) and with uncoated nitrocellulose membrane (B6). Proliferation assays were performed as described in the legend to Table 1.

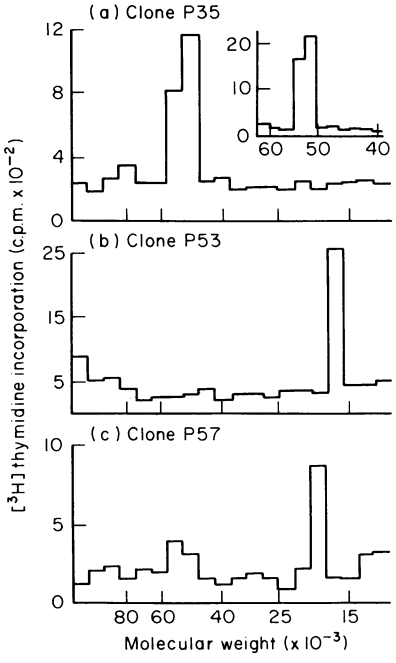

Figure 2. Identification of antigens recognized by *M. tuberculosis*-reactive T-cell clones using immunoblot analysis. T cells of clones P35, 53 and 57 ($2{\cdot}5 \times 10^4$/ml) were cultured with SDS–PAGE-separated immunoblots (20 fractions) together with irradiated PBMC/ascitic lymphocytes and assayed as described in the legend to Table 1. The specificity of clone P35 was analysed in more detail using the MW fractions (40,000–60,000) of a 15-cm (75 fractions) immunoblot [insert in (a)]. Results are shown as the mean of triplicate assays, the standard error for peak samples was less than 15% in each case.

areas of the gel corresponding to the troughs cannot be dismissed at this stage. It is also possible that the immunodominance apparent by this technique may reflect other properties of the proteins such as their degree of binding to nitrocellulose and the efficiency of processing and presenting particular antigens from the solid phase. While being aware of these reservations, analysis by SDS–PAGE immunoblotting does offer the potential of obtaining considerably more information than simple proliferation assays with unfractionated extracts, and this approach may be expected to lead to the identification of immunodominant antigens of important biological function of specificity in complex antigen mixtures.

In addition to the analysis of polyclonal responses, SDS-PAGE immunoblotting allows a new role for T-cell clones in the identification and analysis of mycobacterial antigens. Several authors have described the generation of human T-cell clones recognizing mycobacterial extracts (Mustafa *et al.*, 1986; Ottenhoff *et al.*, 1986; Emmrich *et al.*, 1986) and, in some cases, particular clones have been shown to respond to individual antigens identified using mouse monoclonal antibodies (Ottenhoff *et al.*, 1986), or by recombinant DNA techniques in association with the same antibodies (Mustafa *et al.*, 1986; Emmrich *et al.*, 1986). In this paper we have demonstrated a direct approach to the identification of the antigen specificity of human T-cell clones that is not dependent on an antibody-based analysis. Two of the T-cell clones described here (P53 and P57) recognize antigens in a molecular weight range 16,000–20,000 that overlaps with the 19,000 antigen recognized by known monoclonal antibodies (Engers, 1986) and further analysis to determine whether the T-cell antigen in either case is identical to the 19,000 protein will be of interest. A third clone (P35) recognizes an antigen in the molecular weight region 52,000–

55,000, which is well separated from the monoclonal antibody-defined proteins at 65,000 and 38,000 (Engers, 1986). This suggests that the T- and B-cell repertoires as regards their specificity for mycobacterial antigens need not be identical as has been reported for other antigens (Berzofsky, Richman & Kilon, 1979; Lamb & Green, 1983). It is interesting to note that this clone, which did not respond to *M. bovis* BCG, recognized an antigen in the molecular weight range that appeared to be relatively rich in *M. tuberculosis*-specific antigens. Of the 33 monoclonal antibodies analysed during the WHO workshop on antibodies to *M. tuberculosis*, none was found to discriminate completely between tubercle bacilli and *M. bovis* BCG (Engers, 1986). This study therefore not only demonstrates a novel approach to the analysis of antigen specificity of T-cell clones, but has also resulted in the identification of an *M. tuberculosis* antigen that is not represented in the previous set of monoclonal antibody-defined proteins. Not all of the T-cell clones investigated in these experiments recognized the SDS-PAGE immunoblots, and therefore this procedure may not be applicable for all T cell subsets or for those T cells with low-affinity receptors. Furthermore, where T-cell clones responded to more than one fraction of the four fraction (2-cm) immunoblots, this may result from inadequate separation of the determinants, or it may be that one determinant can exist on multiple molecular weight proteins as evidence by monoclonal antibodies recognizing the 65,000 'multiple band' antigen of mycobacteria (Ivanyi *et al.*, 1985). Therefore, further analysis of the T-cell clones described in this paper using extracts from different strains of mycobacteria and using *Escherichia coli* clones expressing recombinant mycobacterial DNA (Young *et al.*, 1985a, b) is currently in progress.

Thus, the experiments described here indicate two potentially important areas for the application of SDS-PAGE immunoblotting to further research on the molecular analysis of mycobacterial immunity. These are the analysis of immunodominant antigens by screening of polyclonal responses, and the identification of novel antigens and epitopes using T-cell clones. A panel of well-characterized human T-cell clones recognizing different mycobacterial antigens could prove useful in complementing the set of antigens previously defined by mouse monoclonal antibodies (Engers, 1985, 1986). Screening of recombinant DNA libraries (Thole *et al.*, 1985; Young *et al.*, 1985a, b) with such T-cell clones could provide an approach towards the isolation of the relevant antigens in quantities suitable for thorough immunological analysis.

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