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Murine and human B cell epitope mapping of the *Mycobacterium tuberculosis* 10-kD heat shock protein using overlapping peptides

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SUMMARY

The human immune response to the 10-kD *M. tuberculosis* protein was studied by a competition ELISA using monoclonal antibody (MoAb) SA-12. Twenty-five per cent of the sera from 20 patients with tuberculosis and none from 21 control subjects inhibited binding of SA-12 to the 10-kD antigen. To characterize the antigenic parts of the 10-kD antigen, overlapping decapeptides according to the amino acid sequence of the 10-kD protein were synthesized. In total, 91 sequential decapeptides, with an overlap of nine amino acids, were tested in ELISA with MoAb SA-12, human and murine sera (PEP scan). SA-12 recognized the amino acid sequence WDEDGEK (amino acid 50–56). All human sera, from patients with tuberculosis as well as from control subjects, gave almost identical undulating patterns of reactivity with the decapeptides. No relationship was found between the ability of the patients' sera to inhibit binding of MoAb SA-12 and the binding of these sera to the decapeptides comprising the epitope recognized by SA-12 in the PEP scan. Apparently, antibodies in patients' sera against the 10-kD protein are predominantly directed against discontinuous epitopes and, consequently, the continuous epitopes as presented in the PEP scan are not suitable to discriminate between patients with tuberculosis and control subjects. In the PEP scan, sera from BALB/c mice, both non-immunized and immunized with either live *M. tuberculosis* or the 10-kD protein gave similar patterns of reactivity, albeit different from the patterns obtained with the human sera. However, after immunization of the mice, clearly increased levels of antibodies to primary structures of the 10-kD protein were observed.

Keywords B cell epitopes *Mycobacterium tuberculosis* heat shock protein PEP scan

INTRODUCTION

The development of a serological test for tuberculosis has been hampered by the limited specificity and sensitivity of the available reagents (Ivanyi, Bothamley & Jackett, 1988). Although many constituents of the tubercle bacillus have been isolated and studied, virtually all of the proteins isolated from *M. tuberculosis* appear to possess cross-reactive parts that limit their potential as diagnostic antigens (Jackett *et al.*, 1988; Chan *et al.*, 1990). Characterization of epitopes that are specific for *M. tuberculosis* may lead to the synthesis and use of these epitopes in a serological test. By combining several peptides, specificity and sensitivity of a serodiagnostic assay could be optimalized.

An antigen with an apparent molecular weight of 10 kD has been isolated with the monoclonal antibody (MoAb) SA-12 from the culture supernatant of *M. bovis* BCG (Minden *et al.*, 1984). This antigen is known as MPB57 or BCG-a (Minden *et al.*, 1984; Yamaguchi *et al.*, 1988). The identical 10-kD antigen from *M. tuberculosis* has been valuable in the development of a serological test for tuberculosis (Verbon *et al.*, 1990). In Western blot 40% of patients, and in ELISA 50% of patients with tuberculosis and none of the control subjects had antibodies against the 10-kD antigen (Coates *et al.*, 1989; Verbon *et al.*, 1990). Gene analysis of the 10-kD antigen of *M. bovis* BCG and *M. tuberculosis* revealed identical nucleotide sequences encoding a protein of 99 amino acid residues, with a molecular mass of 10·7 kD (Yamaguchi *et al.*, 1988; Baird, Hall & Coates, 1989). This protein is 44% homologous with the GroES heat shock protein of *Escherichia coli* (Baird, Hall & Coates, 1988). Heat shock proteins are known to be highly conserved molecules in both prokaryotic and eukaryotic cells, which are induced at elevated temperature or other stress conditions (Lindquist, 1986). Another mycobacterial heat shock protein, the 65-kD antigen, which is a homologue of the *E. coli* GroEL protein

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(Young *et al.*, 1988), possesses cross-reactive as well as *M. tuberculosis* specific epitopes (Coates *et al.*, 1981). By analogy, the 10-kD antigen of *M. tuberculosis* is likely to contain both cross-reactive and specific epitopes.

Antigenic determinants, or epitopes, are the minimal segments of antigens that are recognized by antibodies or T cells. Epitopes have been classified as continuous or discontinuous (Atassi, 1975; Benjamin *et al.*, 1984). Discontinuous epitopes consist of amino acids from different parts of the polypeptides, brought together by the folding of the protein in its native structure. The continuous, or sequential epitopes are composed of a linear stretch of amino acids. The PEP scan has been a major advance enabling the identity of linear B cell epitopes to be established rapidly (Geysen, Meloen & Barteling, 1984; Geysen *et al.*, 1987).

The purpose of this study was to identify human and murine B cell epitopes of the 10-kD antigen of *M. tuberculosis*. Identification of *M. tuberculosis*-specific B cell epitopes on the 10-kD antigen would be a step towards accurate serodiagnosis of tuberculosis using synthetic peptides of these regions as reagents. A duplicate series of overlapping decapeptides was synthesized, based on the amino acid sequence of the 10-kD antigen, to mimic all possible continuous epitopes. These peptides were tested with human and murine sera and the MoAb SA-12.

MATERIALS AND METHODS

Peptides

Overlapping decapeptides were synthesized in duplicate and tested using the Epitope Scanning Kit (Cambridge Research Biochemicals, Cambridge, UK), according to the manufacturer's instructions. This is a modification of the technique of multiple peptide synthesis on polyethylene rods as described originally by Geysen *et al.* (1987). The decapeptides were synthesized according to the 100 amino acid sequence of the 10-kD antigen of *M. tuberculosis*, i.e. including the N-terminal methionine (Baird *et al.*, 1988; Yamaguchi *et al.*, 1988; Shinnick *et al.*, 1989). In total, 91 sequential decapeptides, with a nine amino acid overlap, were synthesized. In this paper amino acids are indicated by the single letter code, defined by the IUPAC–IUB Commission on Biochemical Nomenclature (1968).

ELISA

To block non-specific binding, the peptides still coupled to their solid-phase supports were incubated with 0·1% polyvinylpyrrolidone (PVP) in phosphate-buffered saline (PBS) with 0·05% Tween 20 (PBST), pH 7·4, for 1 h at room temperature in a polystyrene microtitre plate (Greiner, Nürtingen, Germany). New ELISA plates were used for subsequent incubation with MoAb or serum. The sera and MoAb SA-12 were diluted 1/100 and 1/5000, respectively, in PBST with 0·1% PVP, and 175 μl/well were added. After incubation for 1 h at room temperature the pins to which the peptides were attached were washed with PBST four times for 10 min. The appropriate horseradish-peroxidase-labelled conjugate (Pasteur Institute, Paris, France) was diluted 1/1000 in PBST with 0·1% PVP; 175 μl were added to each well and incubated for 1 h at room temperature. After washing the peptides (attached to the pins) with PBST five times for 10 min, 150 μl substrate solution, prepared by mixing equal volumes of tetramethylbenzidine solution (1·2 mg/ml in methanol) and 0·03% H_2O_2 in 50 mM sodium citrate, 0·1 M K_2HPO_4, pH 5·0, were added to each well and incubated for 20 min at room temperature in the dark. All incubation and washing steps were carried out with shaking.

The ELISA results were regarded as significant when the absorbance was 3 s.d. > mean value of the lowest 25% of signals (Geysen *et al.*, 1987). Absorbances were plotted at the position in the amino acid sequence of the N terminal amino acid of the peptides.

Competition ELISA

The competition assay was performed in an ELISA using peroxidase-labelled antibodies, as described by Wilkins & Ivanyi (1990). Briefly, polystyrene microtitre plates (Greiner) were incubated overnight at 4°C with 50 μl/well of a solution containing 20 μg/ml *M. tuberculosis* sonicate in PBS. Non-specific binding was blocked with 200 μl/well of 1% (w/v) dried milk in PBST and incubated for 1 h at 37°C. The liquid was then tipped off and the plates patted dry. Four five-fold dilutions of human sera (1/5–1/625; 25 μl/well) were added to duplicate wells and incubated at 37°C for 1 h. Twenty-five microlitres of a $1/10^6$ dilution of MoAb SA-12 were added per well. This dilution of MoAb gave 70% of maximum binding in wells without competing serum. The plates were incubated for 1 h at 37°C. The plates were washed four times with PBST and patted dry. Sheep anti-mouse conjugate labelled with horseradish peroxidase diluted 1/1000 in PBST with 1% milk was added (50 μl/well) and incubated for 1 h at 37°C. Tetramethylbenzidine substrate solution, as described above, was added and the absorbance was read in a Titertek Multiscan spectrophotometer at 405 nm after incubating for 40 min at room temperature.

SDS–PAGE

This was done as described by Laemmli (1970), on 13% (w/v) slab gels.

Western blot analysis

Antigens separated by SDS–PAGE were transferred to nitrocellulose as described previously (Kolk *et al.*, 1989).

Hydrophilicity

The value was determined according to the method of Hopp & Woods (1981).

Human sera

Sera were kindly provided by Dr J. E. Landheer, St Antonius Hospital, Nieuwegein, The Netherlands. Sera were obtained from 20 patients with a positive culture of *M. tuberculosis* who had received anti-tuberculous treatment for less than 2 months. Sixteen patients had pulmonary tuberculosis (code A) and four had extrapulmonary tuberculosis (code B). The extrapulmonary tuberculosis was located in cervical lymph nodes, urogenital system, abdomen or hip joint.

Control sera were obtained from 21 subjects: four BCG-vaccinated individuals (code C); five skin-test-positive persons without a history of BCG vaccination or previous tuberculosis (code D); eight skin-test-negative individuals (code E); two persons with a *M. avium* infection, diagnosed by a positive culture from sputum or blood (code F); and two patients with leprosy (code G, lepromatous leprosy and borderline lepromatous leprosy).

Table 1. Results of sera from 20 patients with tuberculosis and 21 control subjects in competition ELISA with MoAb SA-12

Serum	Inhibition (%)*
Patients with tuberculosis	
A1	67
A2	67
A3	30
A4	59
A5	41
A6–A16, B1–B4	<26
Control subjects	
C1–C4, D1–D5, E1–E8, F1, F2, G1, G2	<26

A, patients with pulmonary tuberculosis; B, with extrapulmonary tuberculosis; C, control subjects BCG vaccinated; D, skin-test-positive persons; E, skin-test-negative individuals; F, persons with *M. avium* infection; G, leprosy patients.

* Sera were tested at a dilution 1/5 and the percentage inhibition of the binding of MoAb SA-12 to *M. tuberculosis* sonicate is given.

Murine sera

Blood samples were taken from BALB/c mice before and after immunization. Eight-week-old BALB/c mice were immunized intraperitoneally either with 5×10^7 live *M. tuberculosis* or with the 10-kD protein isolated from nitrocellulose. For this, 7·5 mg of *M. tuberculosis* culture supernatant were separated by SDS-PAGE and subsequently blotted onto nitrocellulose membranes. Nitrocellulose containing the 10-kD band was cut out and sonified in PBS. The immunization procedure was repeated 2 weeks after the first immunization and a blood sample was taken a week later.

Monoclonal antibody

The MoAb SA-12 against the 10-kD protein of *M. bovis* BCG is of limited specificity (Engers *et al.*, 1986) and was kindly supplied by Dr P. Minden, Scripps Clinic, La Jolla, CA, and Dr P. Kelleher, The Woodlands, TX.

RESULTS

Competition ELISA with MoAb SA-12

A competition ELISA was used to evaluate the ability of human sera to inhibit binding of MoAb SA-12 to *M. tuberculosis* sonicate. None of the sera from control subjects gave any inhibition, while sera from five of the 20 patients with tuberculosis, inhibited binding of MoAb SA-12 to *M. tuberculosis* sonicate by 30% or more at a dilution of 1/5 (Table 1). This implies that 25% or more of these patients' sera contained antibodies against the 10-kD antigen.

Murine SA-12 epitope mapping

Overlapping decapeptides of the 10-kD antigen were used in a PEP scan to identify the epitope of SA-12 (Fig. 1). MoAb SA-12 reacted with decapeptides 48–51, covering the amino acid sequence 48–60 of the *M. tuberculosis* 10-kD antigen. The epitope recognized by MoAb SA-12 could be deduced from the

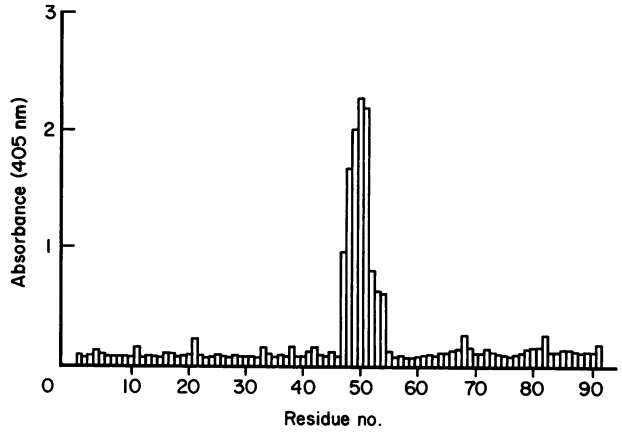

Fig. 1. PEP scan of decapeptides of the 10-kD antigen of *M. tuberculosis* with MoAb SA-12. Each peptide is indicated by the residue number of its N terminal amino acid on the 10-kD protein. The absorbance at 405 nm determined for each peptide in ELISA is shown.

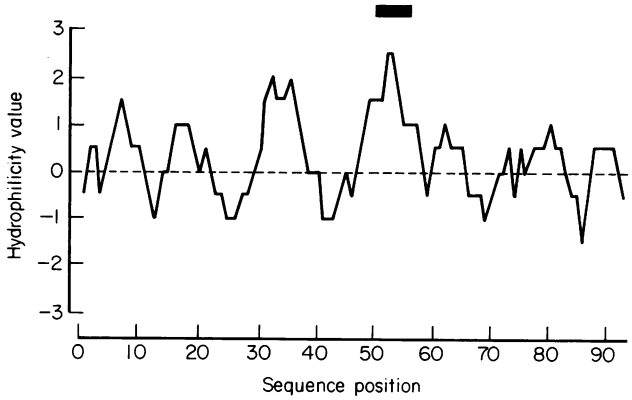

Fig. 2. Hydrophilicity profile of the 10-kD antigen of *M. tuberculosis*. Sequence position indicates the amino acid residue number of the N terminus from the 10-kD protein. The hydrophilicity profile, calculated according to Hopp & Woods (1981), is smoothed by taking a seven-point moving window average. The position of the SA-12 epitope is indicated by a solid bar.

Table 2. Hydrophilicity values and homology of the SA-12 epitope and corresponding regions on the *Escherichia coli* GroES and *Coxiella burnetti* HptA proteins

Protein	Epitope	Hydrophilicity value
GroES	ILENGEV * **	0·01
M. tuberculosis 10-kD	WDEDGEK **	1·37
HptA	PLDNGEV	0·07

* Amino acid homology in the corresponding regions.

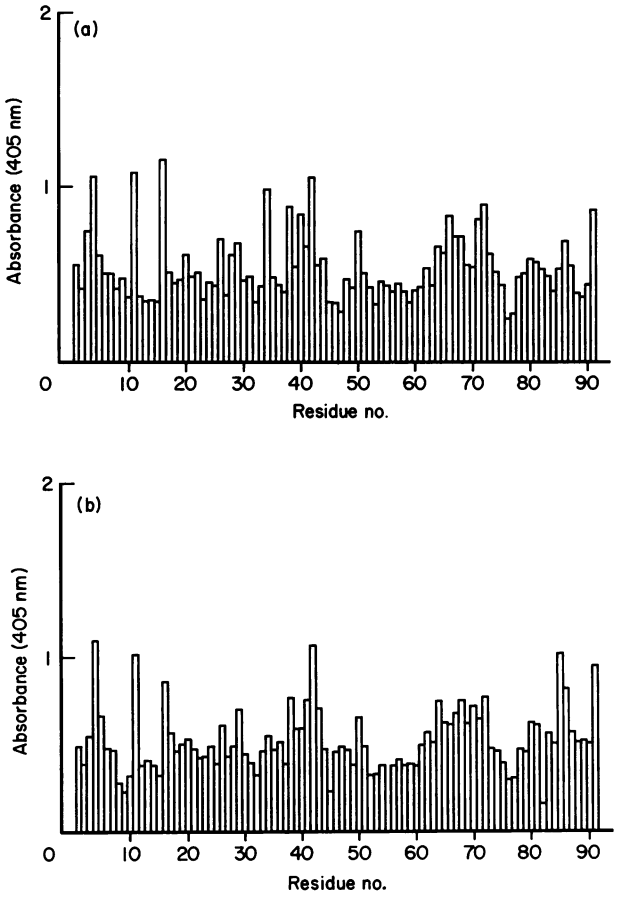

Fig. 3. PEP scans of decapeptides of the 10-kD antigen of *M. tuberculosis*. Each peptide is indicated by the residue number of its N terminal amino acid on the 10-kD protein. The absorbance at 405 nm determined for each peptide in ELISA is shown. (a) Representative example of serum from a patient with tuberculosis, positive in the competition ELISA with MoAb SA-12; (b) representative example of serum from a control subject, negative in competition ELISA with MoAb SA-12.

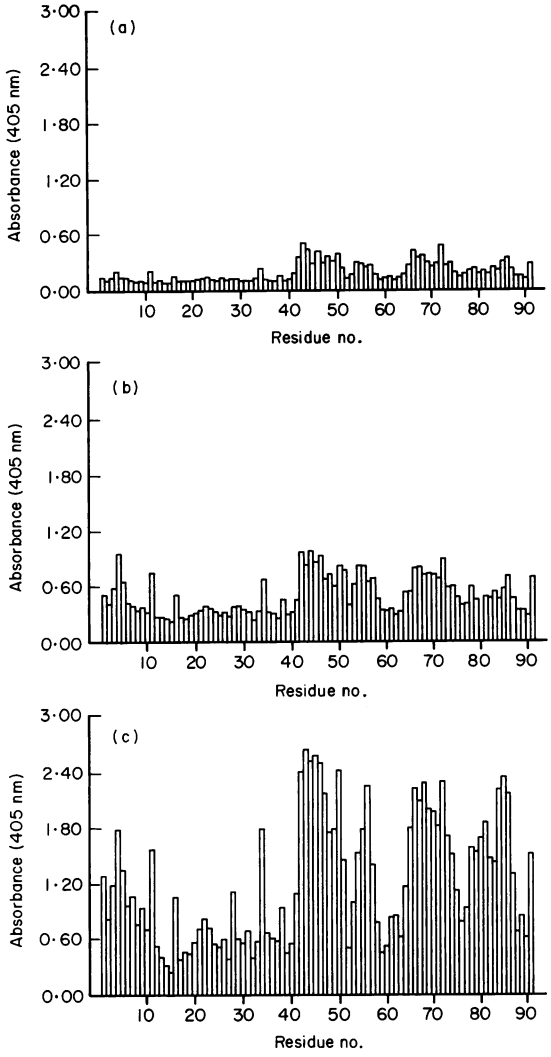

Fig. 4. PEP scans of decapeptides of the 10-kD antigen of *M. tuberculosis*. Each peptide is indicated by the residue number of its N terminal amino acid on the 10-kD protein. The absorbance at 405 nm determined for each peptide in ELISA is shown. (a) Serum of a non-immunized BALB/c mouse; (b) serum of a BALB/c mouse immunized with live *M. tuberculosis*; (c) serum of a BALB/c mouse immunized with the 10-kD antigen.

common sequence within these peptides: WDEDGEK (residues 50–56). The decapeptides 47, 52, 53 and 54 gave lower signals, suggesting that these peptides contained only part of the deduced epitope. Consistent with the antigenic determinant prediction by hydrophilicity analysis (Hopp & Woods, 1981) the epitope of SA-12 is positioned within the region of greatest local hydrophilicity (Fig. 2). The amino acid sequence WDEDGEK shared a three amino acids homology with the corresponding amino acid sequence of *E. coli* GroES protein and a two amino acids homology with the *Coxiella burnetti* HptA protein (Baird *et al.*, 1988) (Table 2). The hydrophilicity value of the corresponding heptapeptide sequences is by far the highest for the *M. tuberculosis* 10-kD antigen (Table 2).

B cell epitope mapping with human sera

Sera from nine patients with tuberculosis (A1–A8, B1) and from five control subjects (D1, E1–E4) were tested in the PEP scan to characterize the human B cell epitopes on the 10-kD antigen. Representative examples are shown in Fig. 3. All sera tested gave comparable undulating patterns of reactivity with the peptides. Similar patterns of reactivity were found in the duplicate PEP scan. When using the cut-off value, no epitopes were found that discriminated between patients with tuberculosis and control subjects. Additionally similar antibody levels were found with sera from both patients with tuberculosis and control subjects as measured by the absorbance in the PEP scan. Both IgM and IgG antibodies gave undulating patterns in the PEP scan, in patients with tuberculosis as well as in control subjects (data not shown). However, the undulating pattern was more often predominated by IgM antibodies. A control PEP scan done with goat anti-human IgG (heavy and light chain) conjugate alone revealed that the pattern was not caused by reactivity of the conjugate antibodies with the peptides (data not shown). Thus, the absorbances measured in the PEP scan were based on the reactivity of antibodies in the human sera with the peptides. Although the competition ELISA revealed the pres-

ence of antibodies to the 10-kD protein in at least five patients' sera, no increased levels of antibodies binding to peptides comprising the SA-12 epitope could be detected (Table 1 and Fig. 3).

B cell epitope mapping with murine sera

In order to investigate further the unexpected results obtained in the PEP scan with the human sera, three BALB/c mouse sera were tested in the PEP scan. One control mouse was not immunized, one mouse was immunized with live *M. tuberculosis* and one with the 10-kD antigen isolated from nitrocellulose. In the PEP scan all mouse sera, even that from the non-immunized mouse, showed a comparable undulating pattern with the overlapping decapeptides (Fig. 4). A gradual increase in detectable antibody level was found, represented as low peaks in the non-immunized mouse, higher peaks in the mouse immunized with live *M. tuberculosis* and clearly the most pronounced peaks in the mouse immunized with the 10-kD antigen (Fig. 4). Reactivity above the defined cut-off values was found with decapeptides 3–7, 41–51, 53–57, 64–75 and 78–87. Decapeptides 3–7 and 53–57 have the respective amino acid sequences KPLEDK and KRIPLD in common and probably represent single murine B cell epitopes. All other peaks consisted of 10 or more sequential decapeptides, indicating that overlapping epitopes were recognized by the polyclonal murine sera as a result of different B cell responses. The epitope WDEDGEK (decapeptide 48–51), recognized by MoAb SA-12 is situated within one of these regions of overlapping epitopes, i.e. decapeptides 41–51 (Fig. 4).

DISCUSSION

To map human and murine B cell epitopes, we performed a PEP scan of the amino acid sequence of the 10-kD protein of *M. tuberculosis* (Baird *et al.*, 1988). In the PEP scan, parts of the protein are mainly presented in their primary structures. Thus, whereas the PEP scan procedure is very useful to characterize continuous epitopes, it is not suitable to map discontinuous epitopes (Geysen *et al.*, 1987).

MoAb SA-12 appeared to react with the heptapeptide WDEDGEK (residues 50–56). According to the prediction of antigenic determinants by Hopp & Woods (1981), this epitope is located within the region of highest local hydrophilicity of the 10-kD protein. The corresponding regions on the *E. coli* GroES and the *C. burnetti* HptA proteins showed low hydrophilicity values, suggesting that these parts are less antigenic. Consistent with the prediction of Hopp & Woods (1981), the epitope of SA-12 was situated in a 'turn' region of the secondary structure of the 10-kD protein (Chou & Fasman, 1978; Rose, 1978). Such 'turn' regions are also considered to be antigenic (Novotny, Handschumacher & Bruccoleri, 1987).

In a competition ELISA, 25% of the sera of tuberculosis patients inhibited the binding of MoAb SA-12 to the 10-kD protein of *M. tuberculosis*, indicating the presence of antibodies to this antigen in sera from these patients. Surprisingly, all human sera, from control subjects as well as from patients, gave similar patterns of reactivity and approximately equal reactivities with the peptides in the PEP scan. Sera from patients that inhibited binding of MoAb SA-12 showed no increase in reactivity with decapeptides representing the epitope recognized by SA-12. One explanation for this may be that the continuous epitope of the MoAb is part of a discontinuous epitope recognized by the human sera. Additionally, binding of the MoAb might be inhibited through steric hindrance caused by antibodies in patients' sera. A similar discrepancy between inhibition of binding of MoAbs by human sera and the absence of binding of these sera to peptides comprising the corresponding epitopes defined by these MoAbs has been reported for the 65-kD and 36-kD *M. leprae* proteins (Meeker *et al.*, 1989; Klatser *et al.*, 1991).

Previously we have found that 35% of sera from patients with tuberculosis reacted with the 10-kD protein in Western blot; we supposed that these sera recognized continuous epitopes (Verbon *et al.*, 1990). However, a similar pattern of reactivity in the PEP scan was found with sera from patients with tuberculosis irrespective of the results with the 10-kD antigen in Western blot. Probably, the putative binding of antibodies to continuous epitopes is masked by high background reactivity found in the PEP scan. MoAbs also showed a marked reactivity with peptides that contained only part of the epitope (Fig. 1, peptides 47, 52–54; Klatser *et al.*, 1991). In our opinion, the PEP scan background reactivity found with human sera is due to the binding of polyclonal antibodies, directed against discontinuous epitopes, to peptides containing parts of these discontinuous epitopes (Berzofsky, 1985). Although binding of antibodies with continuous peptides constituting a part of their discontinuous epitope is generally considered to be weak (van Regenmortel, 1988), antibodies may bind more strongly to peptides covalently bound to polyethylene pins. This possibility is supported by the lower background reactivity of human sera with non-covalently bound peptides (unpublished results).

We were interested to see whether the phenomenon of the undulating pattern seen with human sera was also present in mice. Murine sera also showed an undulating pattern with the overlapping decapeptides which was, however, different from that found with human sera (Figs 3 and 4). Others also found that humans seem to recognize different epitopes than mice (Meeker *et al.*, 1989; Hartskeerl *et al.*, 1990). Increased exposure of mice to the 10-kD antigen, such as occurred after immunization with live *M. tuberculosis* and even more so after immunization with the 10-kD antigen itself, led to higher antibody levels against the same continuous epitopes that were recognized by the non-immunized mouse. This indicates that contact with the immunogen enhances a pre-existent antibody response (Burnett, 1959).

The fact that in human sera IgM antibodies more often predominated the undulating pattern might suggest the presence of polyreactive antibodies. These polyreactive antibodies or 'natural antibodies', mostly of the IgM type, are present in the sera from healthy humans, rodents and some fish species of very divergent phylogenetic order (Avrameas *et al.*, 1988). These polyreactive antibodies have the capacity to bind to multiple structurally unrelated antigens, including both foreign and self-antigens (Schwartz, 1988). Their polyreactivity is directed to epitopes on highly conserved molecules (Schwartz, 1988). These polyreactive antibodies may also serve as precursors for antibodies to exogenous antigens (Guilbert *et al.*, 1985). Our finding that MoAb SA-12 recognizes an epitope which is located in a broad area of B cell response even in the non-immunized mouse supports the assumption that natural antibodies are involved in the response to the 10-kD protein.

The PEP scan did not reveal distinct human B cell epitopes.

This may be due to the high background pattern caused by polyclonal antibodies which might partly consist of natural antibodies. The antibodies detectable in competition ELISA in sera from patients with tuberculosis are probably directed mainly against discontinuous epitopes. Since neighbouring residues may alter the antigenic conformation of a peptide (Kabsch & Sander, 1984; Wilson *et al.*, 1985; Berzofsky, 1985), additional residues in a longer peptide may contribute to a structure which more closely resembles the conformation of the epitope in its natural state. Studies are under way to investigate whether longer peptides and the purified recombinant 10-kD protein are more useful for the development of a serological test for tuberculosis.

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