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Analysis of a Genomic DNA Expression Library of *Mycobacterium tuberculosis* Using Tuberculosis Patient Sera: Evidence for Modulation of Host Immune Response

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DNA obtained from a human sputum isolate of *Mycobacterium tuberculosis*, NTI-64719, which showed extensive dissemination in the guinea pig model resulting in a high score for virulence was used to construct an expression library in the lambda ZAP vector. The size of DNA inserts in the library ranged from 1 to 3 kb, and recombinants represented 60% of the total plaques obtained. When probed with pooled serum from chronically infected tuberculosis patients, the library yielded 176 recombinants with a range of signal intensities. Among these, 93 recombinants were classified into 12 groups on the basis of DNA hybridization experiments. The polypeptides synthesized by the recombinants were predominantly LacZ fusion proteins. Serum obtained from patients who were clinically diagnosed to be in the early phase of *M. tuberculosis* infection was used to probe the 176 recombinants obtained. Interestingly, some recombinants that gave very strong signals in the original screen did not react with early-phase serum; conversely, others whose signals were extremely weak in the original screen gave very intense signals with serum from recently infected patients. This indicates the differential nature of either the expression of these antigens or the immune response elicited by them as a function of disease progression.

There has been, for the past several years, an ongoing search to identify *Mycobacterium tuberculosis* antigens capable of conferring protective immunity against tuberculosis as well as antigens with potential for use as specific diagnostic reagents. These efforts have received a boost owing to the resurgence of tuberculosis over the last 5 to 10 years. The application of hybridoma and recombinant DNA technologies to the study of *M. tuberculosis* resulted in major advances in the identification and characterization of several *M. tuberculosis* antigens and their genes (11, 32). Most of these studies used recombinant expression libraries of *M. tuberculosis* genomic DNA as a source of *M. tuberculosis* genes (39) and monoclonal or polyclonal antibodies (6, 7, 12, 38) as reagents to identify recombinants expressing immunodominant mycobacterial antigens. One may question the relevance of studying B-cell-reactive antigens from a pathogen such as *M. tuberculosis*, resistance to which is predominantly mediated by T cells. However, several of these antigens were subsequently shown to elicit T-cell responses in tuberculosis patients and/or *Mycobacterium bovis* BCG-vaccinated healthy individuals (9, 20, 23, 37). Nevertheless, no single antigen has shown promise for use as a candidate vaccine, although hsp65 has been reported to contribute towards protective immunity against tuberculosis in the mouse model (33).

During the course of an active infection, *M. tuberculosis* modulates the immune response of the host. This is evident from reports documenting loss of skin test reactivity in advanced stages of disease with a concomitant rise in antibody titers (2, 30). This immunologic deficit reverts to normal with clinical improvement on chemotherapy, suggesting that the high load of bacterial antigens was mediating suppression of T-cell function. Moreover, the ability of *M. tuberculosis* to alter those functions of a macrophage that play a key role in eliciting a protective immune response has also been elegantly documented (26, 34, 36). The identity of *M. tuberculosis* antigens responsible for modulating host immune responses is, however, not known.

Since the landmark observations of Mackaness (18) it has been recognized that lymphoid cells are required for protection against *M. tuberculosis*. Nevertheless, human T-cell lines reactive to *M. tuberculosis* sonicates respond weakly to live bacteria (27). In the mouse model, only live *M. tuberculosis*, but not dead organisms, could protect against a challenge dose of virulent *M. tuberculosis* and could adoptively transfer protection through T cells (24). However, delayed-type hypersensitivity responses were shown to be efficiently induced by killed bacilli. These observations have led to the commonly held belief that live mycobacteria elaborate certain specific antigens, missing in killed preparations, that play an important role in eliciting protective immunity. Most of the currently well-characterized antigens of *M. tuberculosis* were identified with antibodies raised against antigen preparations obtained from killed organisms. The paradoxical immune-suppressing phenomenon observed during active *M. tuberculosis* infection as opposed to the immunogenicity and adjuvanticity of killed preparations suggests that the antigens which suppress host immune responses are also produced only by actively growing *M. tuberculosis*. One may reasonably surmise that an extremely successful pathogen such as *M. tuberculosis* would turn on the expression of a battery of genes that would be advantageous for surviving within the host, against the onslaught of a complex immune system. In fact, host-specific gene expression has been demonstrated in other pathogenic bacteria, such as *Salmonella typhimurium* (19) and *Borrelia burgdorferi* (4). Presumably, the activation of such differentially expressed genes is brought about in response to environmental cues unique to the host environment.

The clinical picture in tuberculosis infections is indicative of selective suppression of cell-mediated immune responses of the host with simultaneous enhancement of antibody titers. The inability of antibodies to confer protection against tuber-

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culosis notwithstanding, they may recognize B-cell epitopes in the antigens relevant for protection and for altering host immune response, thus serving as valuable reagents. We therefore screened a genomic DNA expression library of *M. tuberculosis* with pooled serum obtained from patients with full-blown tuberculosis in an attempt to identify the above-mentioned categories of antigens. The library was constructed with DNA from a highly virulent field isolate of *M. tuberculosis* obtained from the sputum of an infected patient. We describe in this report identification of several *M. tuberculosis* proteins reactive to sera from tuberculosis patients. Among these we have found proteins that show differential reactivity to pooled serum obtained from recently infected as opposed to chronically diseased tuberculosis patients. This is suggestive of alterations either in the expression of these proteins or in the host immune response to these proteins as the disease progresses from the establishment to the maintenance phase.

MATERIALS AND METHODS

Mycobacterial strains. *M. tuberculosis* strains were isolated from sputum of patients with pulmonary tuberculosis by culturing on Lowenstein-Jensen egg-based solid medium (16). Isolate NTI-64719 used for the experiments reported here was identified to be *M. tuberculosis* at the National Tuberculosis Institute, Bangalore, India, on the basis of the following criteria set out in the Centers for Disease Control manual (15): (i) rough, corded, and nonpigmented colonies; (ii) growth at 37°C but not at 25 or 45°C; (iii) low growth rate; (iv) negative for arylsulfatase and catalase activities; (v) lack of growth on MacConkey agar; (vi) highly positive for niacin production and nitrate reduction; and (vii) resistance to thiophen-2-carboxylic acid hydrazide. In addition, the identity of the isolate was confirmed by the following tests developed at the National Tuberculosis Institute in Bangalore (22): (i) inability to grow in the presence of *para*-nitrobenzoic acid and thiosemicarbazone and (ii) lack of growth in synthetic N medium. Individual colonies obtained were suspended in gel saline (0.5% gelatin, 0.9% sodium chloride) at a concentration of $\sim 10^8$ cells per ml and stored frozen at -70°C in aliquots.

Determination of virulence. The virulence of each isolate was tested in the guinea pig animal model. Bacilli (10^7 in 0.5 ml of gel saline) from each isolate were injected intramuscularly into the thigh muscle of duplicate guinea pigs. Animals were sacrificed on 42nd day, and the extent of dissemination of the bacilli in the spleen, liver, and lung was used as a measure of virulence (21).

Construction of a genomic expression library of *M. tuberculosis*. Aliquots (20 ml) of Lowenstein-Jensen medium were dispensed into milk dilution bottles and solidified in the horizontal position. *M. tuberculosis* NTI-64719 was seeded on the surface of the solid medium and grown for 1 week. Then 2 ml of 2.0 M glycine was added to each bottle, and growth was continued for 5 days. Bacilli were harvested by scraping the surface cultures gently and washing the surface with lysis buffer (25 mM Tris [pH 8.0], 10 mM EDTA, 50 mM glucose). Washed bacilli were resuspended in lysis buffer (2 ml/g [wet weight]); lysozyme and Tween 80 were added at final concentrations of 5 mg/ml and 0.2%, respectively; and the mixture was incubated in a shaker at 37°C for 14 h. Sodium dodecyl sulfate (SDS) was then added to a concentration of 1%, and the suspension was held at 50°C for 15 min, at which time lysis resulting in an increase in viscosity was observed. The sample was diluted to 5 ml with lysis buffer and extracted with phenol twice followed by chloroform. DNA was precipitated from 0.1 M sodium acetate, pH 5.0, with 0.6 volume of isopropanol.

DNA was randomly sheared by sonication in a Heat Systems-Ultrasonics, Inc., model W-380 sonifier with a microtip. Fragments ranging in size from 1 to 3 kb were recovered from low-melting-point agarose gels following electrophoresis. The ends of the fragments were made blunt with a combination of Klenow enzyme and T4 DNA polymerase and methylated with *Eco*RI methylase. *Eco*RI linkers were added, and the fragments were ligated to lambda ZAP II arms (Stratagene, La Jolla, Calif.) and packaged in vitro with Gigapack extracts according to the manufacturer's instructions. A test sample of the library was plated on *Escherichia coli* XLI-Blue in the presence of 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 25 μl of 40-mg/ml 5-bromo-4-chloro-3-indolyl-β-D-thiogalactoside (X-Gal). The library was amplified on lawns of *E. coli* XLI-Blue, and the lysates obtained, which had a titer of 10^{10} PFU/ml, were stored in aliquots at -70°C.

Tuberculosis patient serum. Patients were divided into two categories: one group consisted of chronically infected individuals, suffering from disease for more than a year and with bacilli in their sputum; the second group consisted of individuals reporting symptoms of tuberculosis for the first time and who were clinically judged to be in very early stages of disease. Pooled serum was absorbed serially against whole *E. coli* XLI-Blue cells, XLI-Blue sonicates, and a lambda ZAP–blue-plaque lysate of XLI-Blue cells. Serum samples were stored in the presence of 0.05% sodium azide.

Screening of the lambda ZAP::*M. tuberculosis* library. Immunological screening of the library was carried out using a pool of 13 serum samples from chronically infected patients at a dilution of 1:50. The library was plated at a density of 2×10^4 plaques in 150-mm-diameter petri dishes. Plaques were transferred to 10 mM IPTG-impregnated nitrocellulose membranes, blocked, treated with serum in blocking buffer overnight at 4°C, and washed and incubated with protein A-horseradish peroxidase (Amersham) at a dilution of 1:3,000 for 1.5 h. Plaques were visualized with diaminobenzidine hydrochloride as a substrate. Rescreening of the positive plaques using serum from recently infected patients used a pool of 18 serum samples at a dilution of 1:50.

Five monoclonal antibodies (listed in Table 1) were obtained from the World Health Organization (WHO)-World Bank repository. Monoclonal antibodies 2F8-3 and 2CI-5 (3) were kind gifts of Kris Huygen of the Institute Pasteur du Brabant in Belgium, and A-30 (1) was a kind gift of Rama Mukherjee, National Institute of Immunology, New Delhi, India. These monoclonal antibodies were used to screen the recombinants obtained by screening with tuberculosis patient sera.

DNA analysis. Individual plaques were picked from the top agar and suspended in 1.0 ml of SM buffer (50 mM Tris [pH 7.5], 0.1 M sodium chloride, 0.2% magnesium sulfate, 2.5% gelatin) at 4°C overnight. Fifty microliters of the eluted phage particles was used to infect 4-ml cultures of XLI-Blue. Lambda DNA isolated from the lysates was digested with *Eco*RI to release the inserts. DNA electrophoresed in 0.8% agarose gels was transferred to nylon membranes and hybridized with total *M. tuberculosis* $H_{37}R_v$ DNA labelled with ^{32}P by nick translation in a solution consisting of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 15× Denhardt's solution, and 0.1% sodium lauryl sulfate for 14 h at 65°C. The membranes were washed thrice with 0.3× SSC containing 0.1% SDS at 65°C for 30 min, dried, and exposed to Kodak X-Omat X-ray film. In other experiments, insert DNA fragments were purified from low-melting-point agarose gels and labelled with ^{32}P by nick translation. Recombinant plaques were patched on a lawn of *E. coli* XLI-Blue cells and incubated at 37°C for 6 to 8 h. Plaques were transferred to nylon membranes (NEN-DuPont), cross-linked by UV irradiation, and hybridized to labelled insert DNA fragments for 14 h. Membranes were washed thrice in 0.3× SSC at 65°C and once in 0.1× SSC at 65°C and exposed to Kodak X-Omat X-ray film.

All recombinant DNA techniques and screening of the lambda ZAP library were carried out according to the methods of Sambrook et al. (28). Restriction and modification enzymes were from Boehringer Mannheim GmbH.

Protein analysis. The inserts were recovered as single-stranded filamentous phage by excision using Exassist helper phage according to the manufacturer's instructions (Stratagene) and introduced into *E. coli* SOLR. Cultures were grown to an A_{600} of 0.4 and induced for 3 h with 10 mM IPTG. Cells were harvested and lysed with 10 mM sodium phosphate buffer (pH 8.0) containing 6 M urea, 0.1% sodium lauryl sulfate, and 0.1% β-mercaptoethanol. Proteins were analyzed on SDS–10% polyacrylamide gels by the Laemmli discontinuous buffer system (17). Gels were stained with 0.25% Coomassie brilliant blue R250 in 40:10:50 methanol-acetic acid-water. For Western blot (immunoblot) analysis, proteins electrophoresed on SDS-polyacrylamide gels were transferred to nitrocellulose membranes with a Novablot semidry transfer unit (Pharmacia LKB) at 100 mA for 1 h. Membranes were blocked in phosphate-buffered saline (PBS) containing 0.25% gelatin and 0.25% bovine serum albumin for 2 h. Pooled patient serum was then used at a dilution of 1:200 in blocking buffer overnight at 4°C. Membranes were washed in PBS containing 0.02% Tween 20, treated with a 1:3,000 dilution of protein A-horseradish peroxidase conjugate (Amersham) for 1 h, washed, and developed with diaminobenzidine hydrochloride.

RESULTS

Construction of the lambda ZAP II::*M. tuberculosis* library. A mixture of DNA fragments with completely random end points was generated from the genomic DNA of *M. tuberculosis* NTI-64719 by sonication. Sonicated DNA ends were repaired with a combination of Klenow fragment of *E. coli* DNA polymerase and T4 DNA polymerase, because of the ill-defined nature of ends generated by this physical shearing procedure. After fractionating on 0.8% agarose gels, fragments ranging in size from 1 to 3 kb were recovered and used to construct the library. This size range was deemed optimal to ensure that all open reading frames will be obtained as LacZ fusion proteins. Larger sizes were avoided because of the potential to lose genes that are not fused to the *lacZ* open reading frame and whose expression in the recombinant may depend on a subset of mycobacterial promoters incapable of functioning in *E. coli*. Ligation of lambda ZAP II arms to mycobacterial DNA fragments was carried out at a vector/insert ratio of 10:1. We obtained 10^6 plaques per μg of vector DNA, of which less than 2% were blue when plated on X-Gal and IPTG. Analysis of 12 random white plaques from the library revealed the presence

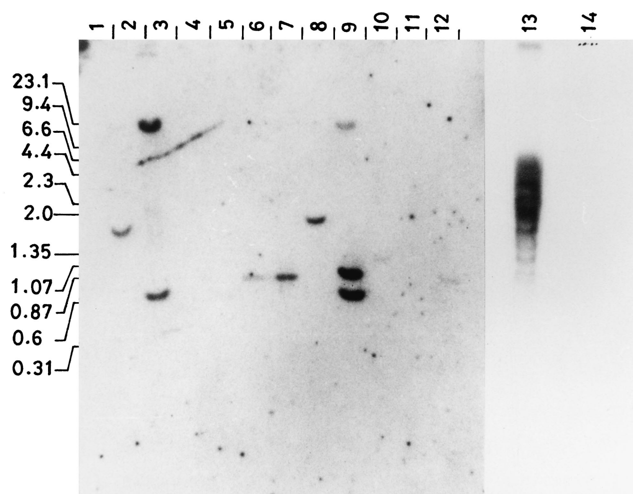

FIG. 1. Southern blot analysis of recombinants from the lambda ZAP II::*M. tuberculosis* library. DNA obtained from 12 white plaques was digested with *Eco*RI, Southern blotted, and probed with labelled *M. tuberculosis* $H_{37}R_v$ genomic DNA. Lanes 13 and 14 contain 2 μg each of *Bam*HI-digested genomic DNA from *M. tuberculosis* NTI-64719 and *E. coli* DH10B, respectively. Sizes are given on the left in kilobase pairs.

of inserts in 7, ranging in size from 600 bp to 2.0 kb. All inserts hybridized specifically to labelled total genomic DNA from *M. tuberculosis* $H_{37}R_v$ (Fig. 1). The specificity of the probe is evident from hybridization to total genomic DNA only from *M. tuberculosis* (lane 13) but not from *E. coli* (lane 14). The signal in lane 13 had a restricted and high size range, since the *Bam*HI digestion of the *M. tuberculosis* DNA had not proceeded to completion; the signal, however, accurately reflected the ethidium staining pattern of the sample. In addition, it is to be noted that the genomic DNA samples shown in lanes 13 and 14 were electrophoresed on a separate agarose gel; consequently, the size markers shown on the left of Fig. 1 apply only to lanes 1 to 12.

Screening of the lambda ZAP II::*M. tuberculosis* library with tuberculosis patient serum. Plaques (4×10^5) were screened with pooled serum from chronically infected tuberculosis patients. A total of 176 recombinants gave positive signals. The plaques were subjected to two rounds of purification. Figure 2 shows 50 representative plaques patched on a lawn of *E. coli* XLI-Blue and probed with the pooled patient serum initially used to screen the library. As can be seen, the recombinants exhibited a wide range of signal intensities, ranging from very strong to barely above the background level obtained with blue plaques (arrows). We found the detection method using protein A conjugated to horseradish peroxidase superior to that using goat anti-human immunoglobulin conjugate because of better specificity and sensitivity. Twelve randomly selected plaques out of the 176 signals were picked and analyzed for the presence of insert DNA. As can be seen in Fig. 3A, all 12 carried inserts ranging in size from 1 to 3 kb that hybridized to *M. tuberculosis* genomic DNA (Fig. 3B). The strong signal intensity in clone 85 is likely due to multiple copies of this fragment in the *M. tuberculosis* genome.

In order to identify recombinants that carried the same or overlapping segments of DNA, we purified insert DNA from 12 clones and hybridized the labelled insert DNA to all 176 recombinant plaques. A total of 93 recombinants fell into the 12 groups represented by these 12 inserts. One of these insert DNA segments was represented only once in the panel of 176 serum-reactive clones (clone 73), while two others (clones 61 and 88) were represented 14 and 17 times, respectively. The majority of inserts were represented by two to four recombinants from among the 176 serum-reactive clones. This number is to be expected in view of the number of plaques originally screened, which was 10-fold in excess of the number required to represent the complete genome.

We screened all 176 recombinants with seven monoclonal antibodies that recognize well-characterized antigens of *M. tuberculosis* (Table 1). Only two of these, TB68 and TBC-1, reactive to a 16-kDa protein and the 38-kDa antigen 5 of *M. tuberculosis*, respectively, reacted with 2 clones each of the 176 (Fig. 4). Surprisingly, the 65-kDa heat shock protein and the 19-kDa antigen of *M. tuberculosis*, which have been reported earlier to elicit humoral immune responses in tuberculosis patients (13, 35), were not represented in the panel of 176 recombinants that we had obtained. It is likely that antibodies recognizing the highly conserved heat shock proteins were removed from the serum during preabsorption with *E. coli* lysates.

Analysis of recombinant proteins. In order to study the proteins encoded by these recombinants in detail, we recovered the insert DNA as pBluescript plasmids by excision from the lambda ZAP genome using Exassist helper phage. Coinfection of *E. coli* XLI-Blue cells with the lambda recombinant and helper phage causes excision and release of the phagemid carrying the recombinant DNA as filamentous phage. Reinfection of *E. coli* SOLR cells with the filamentous phage resulted in their propagation as plasmids. Induction of such cultures with IPTG gave levels of recombinant proteins far in excess of that obtained during growth of the lambda recombinants. Figure 5A shows a Coomassie-stained SDS-polyacrylamide gel of 10 recombinants in SOLR, induced with IPTG for 3 h. The presence of multimers of recombinant proteins in several lanes is due to the absence of sulfhydryl reagents in the SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer. Figure 5B shows a Western blot analysis of the same recombinants using pooled patient sera. We observed that SDS denaturation weakens the reactivity of some recombinant proteins to patient serum while the reactivity of other recombinants is enhanced by the same treatment. For example, recombinant 61 in Fig. 5B reacts very weakly with pooled serum from tuberculosis patients on Western blots, in contrast to a strong reactivity of plaque lifts. Reactivity of one of the recombinant proteins

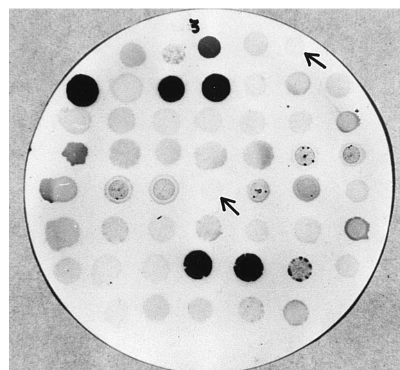

FIG. 2. Analysis of lambda ZAP II::*M. tuberculosis* recombinants using pooled tuberculosis patient serum. Fifty representative recombinants identified in the original screen were plaque purified and patched on a lawn of *E. coli* XLI-Blue. Plaques were transferred to a nitrocellulose membrane and probed with pooled tuberculosis patient serum as described in Materials and Methods.

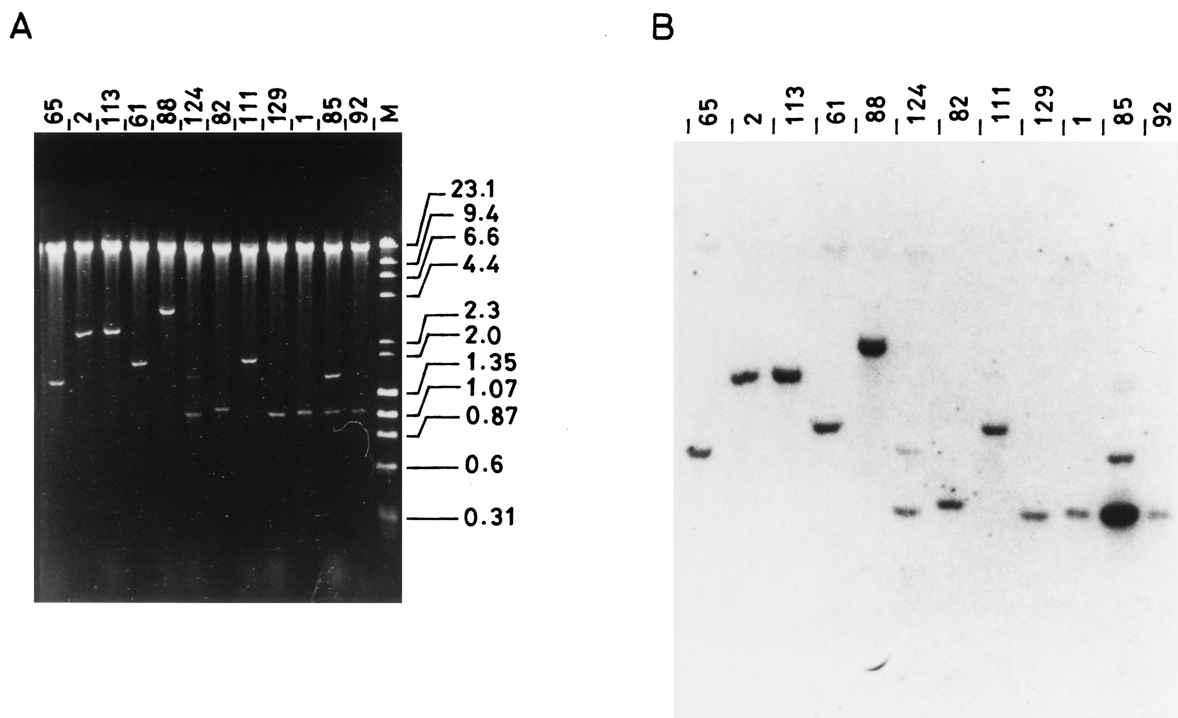

FIG. 3. Southern analysis of patient serum-reactive lambda ZAP II::*M. tuberculosis* recombinants. Twelve recombinants that gave positive signals when screened with patient serum were analyzed as described in the legend to Fig. 1. (A) Ethidium bromide-stained agarose gel of electrophoresed recombinant DNAs. Sizes are on the right in kilobase pairs. (B) Southern analysis of gel shown in panel A hybridized to total *M. tuberculosis* DNA.

(clone 113) to patient serum was lost on Western blotting of the SDS-polyacrylamide gel-SDS electrophoresed protein. Some recombinant proteins were judged to be products of transcripts arising from mycobacterial promoters by virtue of being independent of IPTG for expression.

Screening recombinant plaques with serum from patients with early-phase tuberculosis. The T-cell response to *M. tuberculosis* infection in mice has been reported to undergo a shift from Th1 to Th2 effector populations, as judged by their cytokine profiles in response to antigens of *M. tuberculosis* (25). In leprosy, where the shift from tuberculoid to lepromatous form as a measure of disease progression is clinically distinguishable, Th1 effector T cells have been reported to give way to Th2 T cells (14). However, in tuberculosis the clinical picture does not permit easy distinction of early from well-established phases of infection. We were therefore curious to find out if serum obtained from patients in early stages of tuberculosis would recognize all 176 recombinants identified by using serum from chronically infected patients. A screen of the 176 recombinants using pooled early-phase tuberculosis patient serum revealed interesting differences in the reactivity of antigens specified by some of these recombinants to the two serum pools. Panels A and C in Fig. 6 show reactivity of the recombinants to early-phase serum, while panels B and D show reactivity of the same recombinants to late-phase serum. Arrows point to some recombinants showing differential reactivity to the two serum pools. Panel B contains three recombinants with very strong reactivity to late-phase serum which do not react with early-phase serum. On the other hand, arrows in panels A and C point to a few recombinants whose reactivity to early-phase serum was much stronger than signals obtained with late-phase serum.

One of these differentially reacting recombinants (clone 75) that gave a very strong signal only with late-phase serum was analyzed further. Recombinant protein expressed from excised pBluescript plasmids were electrophoresed on SDS-polyacrylamide gels and immunoblotted onto the two serum pools. Lane 3 in Fig. 7A shows the recombinant protein expressed in clone 75, while lane 2 shows an equally abundant recombinant protein from clone 73 that showed no differential reactivity when plaques were screened. While the recombinant protein from clone 73 showed reactivity to pools of both early- and late-phase sera (lanes 1 in panels B and C, respectively), recombinant protein from clone 75 reacted only with a pool of late-phase sera (lanes 2, panels B and C). To rule out the possibility that this difference is due to variations in reactivities of individual serum samples, the recombinant proteins from clones 73 and 75 were analyzed by Western blotting to each of the 18 early-phase serum samples that was used to make the pool.

TABLE 1. Monoclonal antibodies used to screen serum-reactive recombinants from the lambda ZAP::*M. tuberculosis* library

Monoclonal antibody	Centers for Disease Control-WHO reference no.	*M. tuberculosis* antigen recognized (kDa)	Immunoglobulin subclass	Reference
TB68	IT 20	16	G1	WHO
TB78	IT 13	65 (hsp65)	G1	WHO
TB23[a]	IT 19	19	—[b]	WHO
TB71	IT 23	38 (Antigen 5)	G2b	WHO
TBC-1	IT 34	38 (Antigen 5)	M	WHO
2F8-3[a]	—	44	G2a	3
2C1-5[a]	—	44	G2a	3
A30	—	30	G1	1

[a] Cross-reactive with other mycobacteria.
[b] —, none.

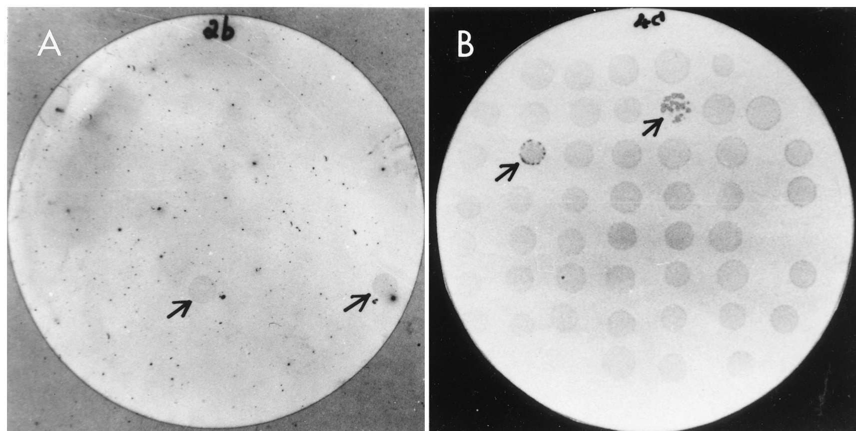

FIG. 4. Analysis of human serum-reactive recombinants with monoclonal antibodies. Recombinant plaques patched on lawns of *E. coli* XLI-Blue were transferred to nitrocellulose membranes and probed with individual monoclonal antibodies listed in Table 1. (A) Filter probed with TBC-1; (B) filter probed with TB68. See Table 1 for antigen specificities of the monoclonal antibodies. Arrows indicate reactive recombinants.

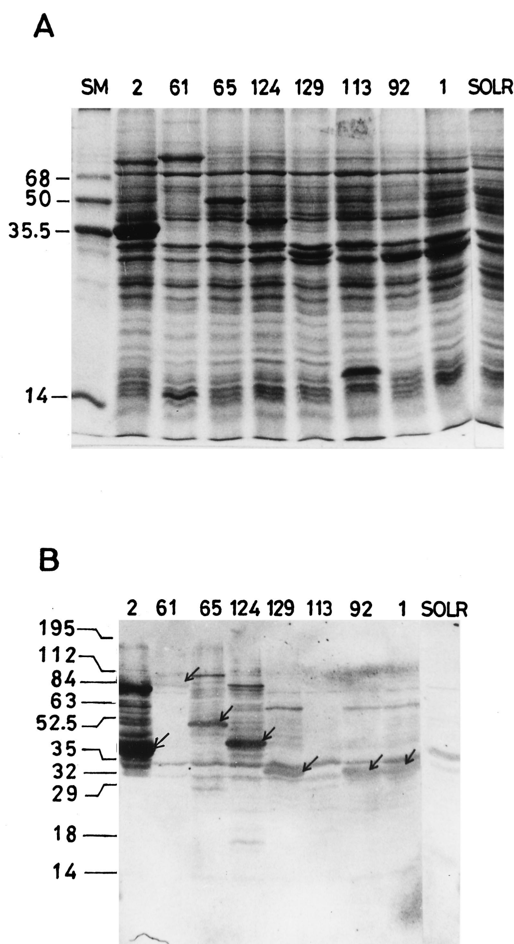

FIG. 5. Western blot analysis of recombinant clones. Inserts from 10 individual recombinants recovered as pBluescript plasmids were introduced into *E. coli* SOLR. IPTG (10 mM)-induced cell lysates were electrophoresed by SDS–10% PAGE, transferred to nitrocellulose membranes, and probed with pooled late-phase tuberculosis patient serum. Sizes are given on the left in kilodaltons.

None of the serum samples reacted with recombinant 75, while all reacted with recombinant 73, albeit with various intensities (data not shown). These findings demonstrate alterations in the host immune response to *M. tuberculosis* antigens during the course of an active infection, perhaps reflecting differences in expression patterns of these antigens.

DISCUSSION

Our studies were carried out with an expression library constructed using genomic DNA from a virulent human isolate of *M. tuberculosis*. Organisms obtained from the sputum and tested for virulence in the guinea pig model were expanded only once on Lowenstein-Jensen solid medium before DNA was isolated for construction of the library. We avoided strains such as $H_{37}R_v$ as a source of DNA since the presence of virulence-associated gene products may be called into question in such laboratory-adapted strains. The library was found to

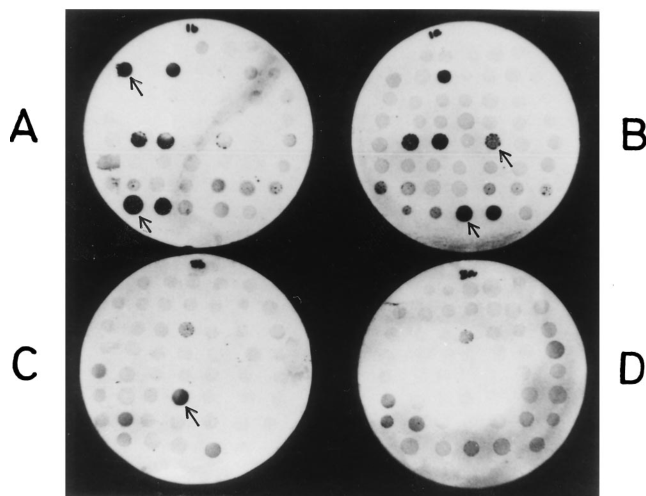

FIG. 6. Screening lambda ZAP II::*M. tuberculosis* recombinants with pooled early-phase tuberculosis serum. Recombinants obtained in the original screen were probed with 1:50-diluted pooled serum from patients in early and late stages of *M. tuberculosis* infection as described in Materials and Methods. (A and C) filters treated with early-phase sera; (B and D) filters treated with late-phase sera. Arrows indicate differentially reactive recombinants.

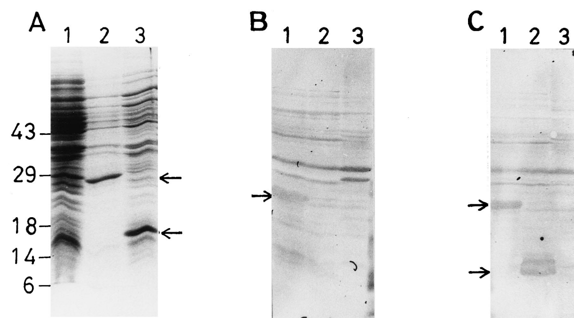

FIG. 7. Analysis of *E. coli* SOLR lysates of differentially reacting recombinants. (A) Coomassie-stained SDS–10% PAGE. Lanes: 1, control; 2, recombinant 73; 3, recombinant 75. (B and C) Western blot analysis using early- and late-phase pooled sera, respectively. Lanes: 1, recombinant 73; 2, recombinant 75; 3, control *E. coli* SOLR. Sizes are indicated on the left in kilodaltons. Arrows indicate the recombinant protein bands.

have a high proportion, namely, 60%, of recombinants. The size range of insert DNA of 1 to 3 kb selected for construction of the library appears to have been vindicated, judging from the large number of recombinants, namely, 176, identified in our screen.

We have identified several genes whose polypeptide products are reactive to serum from tuberculosis patients. We found that none of the patient serum-reactive clones corresponds to the immunodominant antigens such as hsp65 and 19-kDa antigen (9, 35). We could, however, identify among our recombinants clones expressing hsp10, the 16-kDa antigen, and antigen 5 (6). These observations are somewhat similar to those of Sathish et al. (29), who found that clones in a *Mycobacterium leprae* expression library identified on the basis of reactivity to leprosy patient sera did not correspond to the major heat shock or stress proteins of *M. leprae*. The failure to identify clones expressing the well-known heat shock proteins such as hsp65 could be due to the extensive preclearing of the patient serum with *E. coli* proteins which may have depleted all antibodies reactive to these highly conserved proteins. However, hsp10 of *M. tuberculosis* obviously displays unique epitopes reactive to antibodies in patient serum that are not present in the *E. coli* homolog. It is to be borne in mind that nonpolypeptide antigenic determinants cannot be identified by these approaches based on expression of mycobacterial genes in an *E. coli* background. For example, mycobacterial cell wall-derived lipids and carbohydrates have been shown to be very potent modifiers of the functions of different subsets of immune cells (5, 8, 10, 31, 40).

Our observations indicate that a set of antigens, in addition to the ones identified so far, are immunodominant in the host during an active infection. Preliminary nucleotide sequence analysis of some of the recombinants that we obtained revealed that they represent heretofore unreported genes of *M. tuberculosis*. Some of these antigens may be elaborated only by live, actively replicating *M. tuberculosis*. Previous observations (24, 27) strongly suggest that the T cells responding to this category of antigens are the major mediators of protective immunity. Nevertheless, the B-cell responses to these antigens can serve as a handle in establishing their identity in recombinant systems such as a genomic expression library where every potential open reading frame can be surveyed. However, the immunological relevance of antigens identified in our screen needs to be confirmed on the basis of the pattern of reactivity to T cells obtained from susceptible patients and resistant individuals. Indeed, one of the novel antigens that we identified stimulates lymphoproliferative responses in healthy contacts of tuberculosis patients.

The use of patient serum to recognize recombinant proteins bound to nitrocellulose permits identification of epitopes that would be seen on native proteins of *M. tuberculosis*. In fact, the recombinant protein synthesized by clone 113 has no reactivity to patient serum on denaturation by SDS and heat. All recombinant phages that we surveyed from among the 176 clones synthesized one and sometimes two recombinant proteins.

The identification of recombinant proteins with differential reactivity to serum from early and late phases of tuberculosis infection throws open the exciting possibility of studying tuberculosis antigens that modulate the host immune system. That such a phenomenon does take place has been consistently suggested by the observations on the clinical manifestations of tuberculosis (2, 30) as well as by the demonstration in the mouse model (25) of the presence of two distinct waves of T cells with different antigen reactivities and cytokine profiles. It is to be noted that antigens which may be strongly recognized in the early phase of infection but with no reactivity to serum from chronically infected patients would have been missed in our study. The number of such antigens is not likely to be high, since the early phase of tuberculosis infection is dominated by T-cell responses with low antibody levels. Such antigens can, however, be identified by screening the lambda ZAP library with early-phase serum. One may speculate that the tubercle bacillus would target the cells specifying innate immunity in the host during the early establishment phase of infection and cells and mediators specifying acquired immunity during the later maintenance phase of infection. This would logically be achieved through differential gene expression, and the immune cells of the host are bound to be valuable reagents in the search for such genes.

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