Differential Immunogenicity of Novel Mycobacterium tuberculosis Antigens Derived from Live and Dead Bacilli

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Mouse serum raised against killed antigen preparations of Mycobacterium tuberculosis failed to recognize most of the recombinant antigens of M. tuberculosis that were originally identified by reactivity to tuberculin (TB) patient sera. Similar results were obtained with serum from guinea pigs immunized with live and killed mycobacteria. Antibodies raised against seven random TB patient serum-reactive antigens detected each of these antigens in the sonicate preparation. The nucleotide sequences of the genes for these seven antigens revealed that all represented hitherto unreported genes of M. tuberculosis. Our results suggest differential presentation to the host immune system of the same antigens derived from live and killed mycobacteria.

It is a well-established fact that generation and recall of protective immunity to Mycobacterium tuberculosis can be achieved only with live, actively replicating, not dead, mycobacteria (5, 6). Early experiments (7) proved that the subset of T cells responsible for eliciting good delayed-type hypersensitivity responses following immunization with live Mycobacterium bovis bacillus Calmette-Guérin (BCG) were different from the population responsible for conferring protective immunity. The phenotype and kinetics of emergence of these populations were subsequently analyzed (4). In another study (9), human T-cell lines reactive to sonicated antigens of M. tuberculosis showed little or no response to live organisms. These observations have led to the intriguing conclusion that living mycobacteria possibly elaborate antigens, perhaps secreted into the extracellular environment of the bacilli, the immune response to which leads to protection. Alternately, it is believed that the expression of certain antigens responsible for protective immunity may be turned on or upregulated in bacilli actively replicating in vivo, namely, within the host environment. Neither the molecular mediators nor the immunological mechanisms involved in this protection have been defined.

We had previously identified 176 recombinants representing approximately 40 unique genes of M. tuberculosis by screening a genomic DNA expression library for a field isolate of M. tuberculosis with pooled tuberculosis (TB) patient sera (1). In the present study, we report that only a minority of 26 of these antigens tested elicited antibodies in three strains of mice immunized with sonicated antigen preparations of M. tuberculosis. Antibodies raised against 7 of these recombinant proteins in mice, however, revealed the presence of each of these proteins in M. tuberculosis sonicates.

The present experiments used M. tuberculosis NTI-83949, isolated from the sputum of a patient with pulmonary TB as reported earlier (1) and grown in Middlebrook 7TH medium. TB patient serum samples were obtained from inmates of the Shantibai Devarao Shivaram Tuberculosis and Chest Diseases Hospital who had been suffering from the disease for a period of 1 to 10 months. The diagnosis of TB was based on the presence of acid-fast bacilli in sputum and/or radiological findings. To generate antisera to the recombinant proteins, 10 μg of the protein band excised from the Coomassie blue-stained sodium dodecyl sulfate (SDS)-polyacrylamide gel emulsified with incomplete Freund’s adjuvant was injected intraperitoneally into mice (BALB/c, C57 Black, and Swiss) twice at 15-day intervals and followed 20 days later by a third injection with antigen suspended in phosphate-buffered saline. Serum samples obtained from the three strains of mice were pooled for use in the studies reported here. Mice were immunized with sonicates of M. tuberculosis at 150 μg of protein per injection.

Mycobacterial protein extracts were prepared by sonication (total time of 15 min at a setting of 3 in a Branson model 450 Sonifier) of bacilli grown in Middlebrook 7TH broth, harvested in mid-log phase, and suspended in sterile water containing protease inhibitors. Approximately 100 μg of protein was electrophoresed per lane of an SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (BA85; Schleicher and Schuell), and probed with pooled mouse or human sera, followed by treatment with an appropriate horseradish peroxidase-conjugated secondary antibody. Western blot analysis of recombinant Escherichia coli SOLR lysates (1) utilized sufficient total protein containing 100 to 300 ng of the specific recombinant protein, as judged from Coomassie blue-stained gels. Recombinant pBlueScript plasmids were sequenced by using the T3 primer in an ABI Prism automated sequencing system using Taq DNA polymerase.

Differential reactivity of M. tuberculosis antigens to sera generated against live and killed bacilli. E. coli lysates obtained from TB patient serum-reactive recombinants were immunoblotted to pools of TB patient sera and pools of mouse sera at equivalent titers. Only 10 of 26 recombinant proteins tested showed reactivity to the pooled mouse sera. Figure 1 shows the results obtained with 19 representative recombinant proteins. Whereas all the recombinant proteins reacted well, as was expected with the pooled TB patient sera (Fig. 1A), only 5 of the 19 reacted to pooled mouse sera (Fig. 1B), although both sera showed good reactivity to total M. tuberculosis sonicates (Fig. 1, lanes TB). In addition, very low dilutions of mouse sera identified two more recombinant proteins, namely, 11 and 39 (data not shown). In clone 186, the size of the mouse serum-reactive protein was much larger than the one reactive to patient sera. This recombinant, whose insert size was approximately 3.5 kb, perhaps specified two different proteins, both of mycobacterial origin, with one being expressed from a mycobacterial promoter within the insert. Similar results were obtained when the reactivities to recombinant proteins of antisera

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obtained from guinea pigs infected with live *M. tuberculosis* were compared with those from guinea pigs immunized with sonicated antigen preparations of *M. tuberculosis* (B). Clone numbers are indicated above the lanes. Lanes TB, *M. tuberculosis* sonicate. Molecular sizes are given on the right in kilodaltons.

The absence of antibodies to most of the recombinant proteins in mouse sera generated against sonicated preparations of *M. tuberculosis* raised the possibility that these antigens were absent in bacteria grown in vitro. In order to address this issue, antisera against seven recombinant proteins, represented by clone numbers ARRMADB 2, 11, 14, 39, 73, 268, and 283 (five of which, as mentioned above, failed to elicit any antibodies in mice), were raised in mice. Figure 2A shows a Coomassie blue-stained SDS-polyacrylamide gel of *E. coli* lysates from the seven recombinants under study. The corresponding *M. tuberculosis* sonicate proteins identified by Western blotting with antisera raised against each of these recombinants are shown in Fig. 2B. The sizes of the proteins varied from 26 to 95 kDa (Table 1). Antibodies to recombinant 283 identified, in addition to a strong band of 38 kDa, a second, weaker band of 85 kDa. Evidently, all seven antigens are present in significant amounts in *M. tuberculosis* grown in vitro. The nucleotide sequences of these seven recombinant genes were then determined. When compared with all the entries in the database, all seven were found to be previously unreported, newly described genes of *M. tuberculosis*.

Multiple reports (3, 5, 7, 9) documenting the singular capability of live tubercle bacilli to elicit protective immunity against TB raised the interesting possibility that the host immune system during disease is privy to uniquely processed and presented antigens of live *M. tuberculosis* not obtained from a killed preparation of the organism. In these reports, T cells of

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FIG. 1. Differential reactivity of recombinant *M. tuberculosis* proteins to sera from mice and TB patients. *E. coli* lysates from recombinant clones were electrophoresed on SDS–12.5% polyacrylamide gels, transferred to nitrocellulose membranes, and probed with pooled antisera from TB patients (A) or pooled sera from three strains of mice immunized with sonicated antigen preparations of *M. tuberculosis* (B). Clone numbers are indicated above the lanes. Lanes TB, *M. tuberculosis* sonicate. Molecular sizes are given on the right in kilodaltons.

FIG. 2. (A) Western blot analysis of *M. tuberculosis* sonicates to antirecombinant sera. (B) *E. coli* lysates of the seven recombinants reported in the text (clones 2, 11, 14, 39, 73, 268, and 283) electrophoresed on SDS–12.5% polyacrylamide gels and stained with Coomassie blue. Antisera raised against each of the recombinant proteins shown in panel A were Western blotted to *M. tuberculosis* sonicates. Lanes correspond to the clone numbers indicated at the top of the lanes in panel A. Molecular sizes are given on the right in kilodaltons.
the host were shown to be capable of recognizing differences between live and dead mycobacteria. Our results show that humoral immune responses can also distinguish live from dead mycobacteria. Preliminary nucleotide sequence analysis pointed to the novel nature of the seven antigens studied in this report, which are currently being characterized (1a). The differential response observed in our experiments may be attributed to (i) the absence of these antigens in in vitro-grown bacilli or (ii) the poor immunogenicity of the sonicate proteins in the mouse. The former possibility does not seem to apply to the seven antigens discussed in this report, since they were present in in vitro-grown bacilli. The loss of specific epitopes as a result of sonication is a possibility to be considered. We, however, believe this to be unlikely, since both sera used in the experiments reported here are polyclonal in nature and would therefore have reacted to other epitopes on degraded proteins if any had been generated. Alternately, these antigens may be synthesized by the bacilli at much higher levels during in vivo growth, resulting in their enhanced immunogenicity. Indeed, in Salmonella typhimurium, genes identified by the use of the in vivo expression technology included several metabolic functions required for survival and growth under the nutrient-limiting conditions prevailing in the host (2). In addition, the presence of the other differentially reactive antigens in bacilli grown in vitro needs to be confirmed. The latter possibility also does not apply to the seven antigens studied, since the recombinant proteins were capable of eliciting very high levels of antibody responses in mice. This raises the possibility that there are differences in the presentation of the same antigens to the host immune system when these antigens are elaborated by living bacilli as opposed to being derived from killed bacilli. In pursuit of candidates that are dominant antigens during immunization with living mycobacteria, fractionated culture filtrate antigens of BCG were screened with guinea pig antisera, resulting in the identification of a proline-rich 45/47-kDa complex that reacted only to serum from guinea pigs immunized with live bacilli (8). Our results suggest that the host immune response would perhaps be a valuable tool for identifying both virulence-associated proteins of mycobacteria and protective antigens unique to living bacilli that are also immunogenic in the host.

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