Antigen 85 (Ag85) complex proteins are major secretory products of Mycobacterium tuberculosis and induce strong cellular and humoral immune responses in infected experimental animals and human beings. We have previously shown that nanogram doses of these 30- to 32-kDa fibronectin-binding proteins inhibit local expression of delayed hypersensitivity by a T-cell fibronectin-dependent mechanism. Circulating levels of Ag85 might be expected to be elevated in patients with active tuberculosis and possibly to play a role in systemic anergy in these patients. To test this hypothesis, Ag85 was measured in serum and urine by a monoclonal antibody-based dot immunobinding assay in 56 patients and controls with known skin test reactivity. Median serum Ag85 levels were 50- to 150-fold higher in patients with active tuberculosis than in patients with active M. avium-intracellulare disease or other nontuberculous pulmonary disease or in healthy controls (P < 0.001). The median and range of serum Ag85 in patients with active tuberculosis was not significantly different between skin test-positive and -negative subjects. Patients with active M. avium disease could be distinguished from those with disease due to M. tuberculosis by monoclonal anti-Ag85 antibodies of appropriate specificities. No increases in urinary Ag85 were detected in any patient, regardless of the Ag85 level in serum. Chromatographic analysis and immunoprecipitation studies of serum revealed that Ag85 existed in the serum of these patients complexed to either fibronectin or immunoglobulin G (IgG). Uncomplexed circulating Ag85 was demonstrable in serum from fewer than 20% of patients with active tuberculosis. In patients with active tuberculosis, Ag85 is therefore likely to circulate primarily as complexes with plasma fibronectin and IgG rather than in unbound form. The existence of Ag85 complexes with plasma proteins would account for its lack of urinary clearance.

Tuberculosis is a global public health problem. A third of the world’s population is estimated to be infected with Mycobacterium tuberculosis, and tuberculosis is the most common cause of death of adults from infectious disease throughout the world (25). The recent increase in tuberculosis incidence in the United States resulting in part from the human immunodeficiency virus (HIV) epidemic has further focused interest on immunity to this disease (5).

Only actively dividing mycobacteria efficiently generate protective cell-mediated immunity to M. tuberculosis (30). Much recent research has therefore been focused on this organism’s secreted proteins. Proteins of the antigen 85 complex (Ag85A, Ag85B, and Ag85C) are major secretory proteins of actively replicating M. tuberculosis (40). They share high sequence homology at the nucleotide and protein level both with each other and with Ag85 from other mycobacterial species (41). This high degree of homology results in a particular Ag85 protein containing common epitopes found in many Ag85, in addition to unique species- and subtype-specific epitopes (11, 34). Ag85 complex proteins are mycolyltransferases (3). As such, they play an essential role in the final stages of mycobacterial cell wall synthesis, since inhibitors of this activity inhibit both the transfer and the deposition of mycolates into the mycobacterial cell wall and cell growth (3). The function of Ag85 complex proteins in mycobacterial physiology and pathogenesis of tuberculosis is otherwise incompletely understood (13, 17, 33).

Ag85 complex proteins induce delayed hypersensitivity, protective immune responses, and specific antibodies in infected mice and guinea pigs (2, 9, 15, 18–20, 27). They also induce readily elicitable cellular immune responses in cultured peripheral blood mononuclear cells of most healthy purified protein derivative of tuberculin (PPD)-positive people and a few patients with clinically active tuberculosis (16, 22). While levels of anti-Ag85 antibodies are often low in healthy PPD-positive subjects, they increase in patients with active tuberculosis (16, 38). Similar patterns of response are exhibited by healthy lepromin-positive subjects and patients with lepromatous leprosy (26).

Ag85 proteins bind to plasma and cellular fibronectins (1, 13), high-molecular-weight glycoproteins found in plasma and tissues that play important roles in cell motility and adhesion, development, phagocytic function, wound healing, and inflammation (23). Although microgram doses of Ag85 elicit delayed hypersensitivity reactions in sensitized guinea pigs, nanogram doses of these proteins inhibit local in vivo expression of delayed hypersensitivity by binding to and inactivating a specialized T-cell fibronectin produced after antigenic stimulation (13). This latter activity led us to hypothesize that patients with active tuberculosis might have high levels of circulating Ag85 proteins that could possibly play a role in the systemic anergy these patients often exhibit.

To examine this hypothesis, we measured Ag85 concentrations in serum and urine from patients and controls with known...
PPD skin test reactivity. We found serum Ag85 to be significantly increased in patients with active tuberculosis independent of skin test status. Ag85 in these patients circulates primarily as complexes with immunoglobulin G (IgG) and plasma fibronectin.

MATERIALS AND METHODS

Study population. The study population consisted of 56 patients and healthy controls at Metropolitan Hospital Center (New York, N.Y.). It included white (1 female, 4 males), black (10 females, 15 males), and Hispanic (11 females, 15 males) individuals. Samples included blood, postmortem tissues, and/or biopsy specimens.

Active tuberculosis (13), inactive tuberculosis (history of previously treated tuberculosis) (6), bacteriologically confirmed active M. avium-intracellulare disease (5), nontuberculous lung disease (20), and no disease (healthy controls) (12). Thirteen patients were HIV positive (eight males, five females). Of the patients with tuberculosis, one had pleural tuberculosis, one had tuberculous lymphadenitis, four had disseminated tuberculosis (pulmonary and either bone marrow or lymph node involvement) and seven had pulmonary tuberculosis (cavitory in one patient, associated with pleural effusion in another). Seven patients with tuberculosis, one with HIV disease, and one with nontuberculous lung disease refused their placement. Serum and urine samples were coded, and the code was refused until all assay measurements had been completed.

Purified proteins and antibodies. Ag85 complex proteins were purified from concentrated culture filtrates of M. hovis bacillus Calmette-Guérin (BCG) (9). Purified Ag85 complex contained 90% Ag85A, 6% Ag85B, and 4% Ag85C as judged by Western blotting against monoclonal anti-BCG Ag85 complex antibodies (11) and was stored in sterile aliquots at −80°C. For use as an antigen standard in dot blotting, the initial preparation of Ag85 used was arbitrarily assigned an immunoreactive Ag85 content of 1 mg/mL. Use of subsequent preparations was determined by parallel-line analysis of dot blots of initial and subsequent materials (24). Aliquots from a single standard preparation were used for these experiments; unitage was constant between the different aliquots of this preparation. Purified human plasma fibronectin was purchased (New York Blood Center, New York, N.Y.). Purified rabbit anti-fibronectin was a gift from Mary Haak-Frendscho, Promega Corp. (Madison, Wis.). It recognized only a 220- to 250-kDa dimer in human plasma and did not react with purified Ag85. It recognizes an N-terminal sequence spanning amino acids 261 to 280 in the C-terminal region of Ag85A (1). Ag85 in PBS (pH 7.2) for 24°C; they were then immunoprecipitated in a specific Ag85 epitope by Kruskal-Wallis one-way analysis of variance by ranks and a Dunn multiple comparison test.

RESULTS

Circulating Ag85 in patients with active tuberculosis. Mean levels of circulating Ag85 proteins measured with clone 240 anti-Ag85 were 50- to 150-fold higher in patients with active tuberculosis than in patients with active M. avium-intracellulare disease or other nontuberculous pulmonary disease or in healthy controls (P < 0.001, Dunn multiple comparison test) (Fig. 1). Median increases in Ag85 in patients with active tuberculosis were independent of PPD skin reactivity, acid-fast bacilli smear positivity, or stage of disease. There was also no significant relationship between circulating Ag85 levels and skin test reactivity in patients without tuberculosis. All 13 patients with active tuberculosis had serum Ag85 levels of >30 μg/mL. Only 5 of 43 patients without active tuberculosis (three with treated tuberculosis, one with PPD-positive and HIV-positive nontuberculous pulmonary disease, and one with sarcoidosis) had serum Ag85 levels above this level (Fig. 1A).

Immunoreactivity was strongly dependent on the antibody used. Even though clone 240 anti-BCG Ag85 showed weak reactivity with M. avium Ag85 in a previous study (11), it detected serum Ag85 in none of the five patients with active M. avium-intracellulare disease (Fig. 1A). When the remaining serum aliquots from eight patients with active tuberculosis and three patients with active M. avium-intracellulare disease were reassayed with an anti-BCG Ag85 monoclonal antibody (clone 17/4) that was strongly cross-reactive with M. avium Ag85 in...
vivo (11), elevated levels of serum Ag85 were detected in both groups of patients (data not shown). The difference in median serum Ag85 levels detected by clones 240 and 17/4 in sera from patients with *M. avium* disease was significant (*P* < 0.01, Dunn’s multiple comparison test), while the difference in median Ag85 levels detected by these two antibodies in patients with *M. tuberculosis* disease was not (*P* > 0.05).

Urinary levels of Ag85 were uniformly lower than serum
levels in all patients (Fig. 1B). They did not correlate with serum Ag85 concentration, patient diagnosis, or skin test reactivity.

Physical nature of circulating Ag85 in patients with active tuberculosis. Proteins of the Ag85 complex are small enough (30 to 32 kDa) to be cleared from the circulation by the kidney. The lack of urinary Ag85 in patients with active tuberculosis in the face of high levels of serum Ag85 suggested that Ag85 circulated as complexes with plasma proteins. To confirm this hypothesis, sera from six patients with active tuberculosis, two patients with treated inactive tuberculosis, three patients with nontuberculous pulmonary disease, and six healthy controls were fractionated by gel filtration chromatography, and the Ag85 content of each fraction was determined by immunoassay with clone 240 anti-Ag85. Sera from all patients with tuberculosis contained immunoreactive Ag85 associated with 440- to 580-kDa and 200-kDa materials, regardless of skin test reactivity. Figure 2A shows the results obtained with one such serum. These M_r values correspond to complexes of Ag85 with plasma fibronectin and IgG, respectively. In one of six patients, Ag85 was also reproducibly demonstrable in the 30-kDa region corresponding to unbound (“free”) Ag85 (data not shown). The presence of free Ag85 could not be related to an unusually high concentration of Ag85, as this particular serum contained only 210 μU of Ag85 per ml, which is slightly less than the median level of 300 μU/ml for this group. No Ag85 immunoreactivity was present in any serum fraction from healthy controls (Fig. 2) or from patients with treated inactive tuberculosis (not shown) or nontuberculous pulmonary disease (not shown).

Removal of IgG from the serum of tuberculosis patients with insolubilized protein A caused the disappearance of the 200-kDa but not the 440- to 580-kDa peak of Ag85 immunoreactivity on gel filtration chromatography of the residual supernatant (Fig. 2B). This remaining high M_r peak of Ag85 coeluted with immunoreactive fibronectin (Fig. 2B). IgG immune complexes precipitated by protein A contained large amounts of immunoreactive material with M_r values identical to those for purified Ag85 complex (Fig. 3A, lane 6) only in patients with active tuberculosis regardless of skin test status (Fig. 3A, lanes 1, 2). No immunoreactive Ag85 was present in the IgG immune complexes from other patient groups or from healthy controls (Fig. 3A, lanes 3 to 5).

The complete removal of IgG from each protein A-immunoprecipitated supernatant was confirmed by Western blotting; no residual IgG heavy and light chains were observed in any sample after protein A immunoprecipitation (data not shown). Subsequent immunoprecipitation of these IgG-depleted supernatants with anti-plasma fibronectin produced (i) supernatants in which the previously seen 440- to 580-kDa peak of immunoreactive Ag85 on gel filtration chromatography was absent (Fig. 2C) and (ii) immunoprecipitates containing large amounts of immunoreactive material with M_r values identical to those for purified Ag85 complex (Fig. 3B, lane 8) in all patients with active tuberculosis regardless of their PPD skin reactivity (Fig. 3B1, lanes 1 and 2). Fibronectin immunoprecipitates from all patients who did not have active tuberculosis contained either no immunoreactive material with M_r values identical to those for Ag85 complex (Fig. 3B1, lanes 3 to 5) or, in the case of 2 of 6 healthy control subjects (one PPD positive, one PPD negative), small amounts of immunoreactive material with M_r values identical to those for Ag85. Figure 3B1, lane 6, shows a fibronectin immunoprecipitate from a healthy, PPD-negative subject.

Ag85 could also be demonstrated in Ag85-fibronectin complexes generated in vitro (Fig. 3B1, lane 7). All fibronectin immunoprecipitates contained large amounts of immunoreactive fibronectin (Fig. 3B2, lanes 1 to 7). No Ag85 or fibronectin was precipitated by normal rabbit serum (Fig. 3B1, lanes 1 to 7) or if anti-fibronectin antibodies were omitted from the reaction mixture (data not shown).

Complexes of Ag85 with IgG and fibronectin were stable to repeated freeze-thaw cycles. IgG-Ag85 complexes could be demonstrated by immunoprecipitation even after three freeze-thaw cycles, and fibronectin-Ag85 complexes were demonstrable by immunoprecipitation even after six freeze-thaw cycles (data not shown). Thus, Ag85 proteins circulate in patients with tuberculosis in relatively stable complexes with IgG and fibronectin.

DISCUSSION
The pathophysiology of Ag85 proteins in patients with tuberculosis is complex. Median serum Ag85 levels were significantly higher in patients with active tuberculosis than in patients with active M. avium disease or other pulmonary diseases or with no disease, regardless of skin test reactivity or stage of disease. Although nanogram doses of Ag85 were previously shown to inhibit the local expression of delayed hypersensitivity in vivo (13), much higher serum levels in patients with active tuberculosis bore no relationship to PPD responsiveness. Moreover, high levels of Ag85 antigenemia in tuberculosis patients were not associated with appreciable Ag85 antigenuria. Ag85 is a small enough protein to expect that it would be readily cleared by the kidney from the circulation, much as other small secreted mycobacterial proteins are (36). The discovery that circulating Ag85 in patients with active tuberculosis existed primarily as complexes with fibronectin and IgG provided a reasonable explanation for its low renal clearance. Ag85 antigenuria in the presence of Ag85 complexes could of course occur if rapid dissociation of the circulating complexes led to the release of free antigen at some point in time (37). The relative stability of the complexes to freeze-thaw cycles, as well as their low dissociation constants (likely to be in the range of 10^{-7} to >10^{-10} M for Ag85-IgG by analogy with other polyclonal antibodies generated in response to repeated antigenic stimulation [27] and 10^{-7} M for Ag85-fibronectin [28]) makes rapid complex dissociation in the circulation appear unlikely.

It has been recognized for some time that mycobacterial growth products are detectable in tissues and tissue fluids of patients with active infections (6, 10, 29, 32, 35, 36, 42). Analysis of the pathophysiological interaction of growth products such as Ag85 with the host has previously focused on analyzing immune responses (16, 22, 26, 38) or the composition of circulating immune complexes (31). The availability of an immunoassay able to detect both unbound and complexed Ag85 has permitted the examination of other aspects of Ag85 pathophysiology. This ability to detect complexed Ag85 by immunoassay confirms an earlier report of detection of mycobacterial antigenemia by immunoassay in the presence of circulating immune complexes (35).

Large amounts of Ag85 proteins were demonstrable in plasma protein complexes only in sera from patients with active tuberculosis. No Ag85 was present in plasma fibronectin-Ag85 and IgG immune complexes in sera from patients with inactive tuberculosis or other pulmonary diseases. While no Ag85 was present in IgG immune complexes in sera from healthy controls, small amounts of Ag85 proteins were present in fibronectin-Ag85 complexes in sera from two of six healthy controls. The reason(s) for the presence of immunoreactive 32-kDa material associated with fibronectin detected by immunoprecipitation in the sera from normal individuals may reflect dif-
ferences in sensitivity between dot blotting and immunoprecipitation. They are, however, also consistent with the low levels of circulating Ag85 detected by dot blot in individuals not suffering from active tuberculosis. Incomplete removal of human IgG immune complexes from these samples is highly unlikely, since complete IgG removal was confirmed on each sample by Western blotting. Clone 240 anti-Ag85 is variably cross-reactive with Ag85 from many nonpathogenic mycobac-

FIG. 2. Chromatographic characterization of Ag85 in serum from a PPD-positive patient with active tuberculosis (●) and a PPD-positive healthy control (▲). (A) Ag85 determined by dot immunobinding and densitometry in serum from a PPD-positive patient with active tuberculosis. Ag85 was detected in association with 440- and 200-kDa materials. (B) The supernatant from protein A immunoprecipitation of serum from the PPD-positive patient shown in panel A no longer contains 200-kDa Ag85 but still contains 440-kDa Ag85. Ag85 coelutes with plasma fibronectin, as determined by dot immunobinding and densitometry, in patient serum (●) but not in the control serum (▲). (C) Ag85 is no longer present in the IgG-depleted supernatant shown in panel B following further immunoprecipitation with anti-human plasma fibronectin. Ag85 was not demonstrable in fractionated sera or supernatant from any healthy control either before or after the immunoprecipitations. The molecular sizes and the elution time of protein standards (in kilodaltons) and the units of plasma fibronectin (plasma FN) used to calibrate the column are indicated. See Materials and Methods for more details.
teria (11), and the low levels of immunoreactive Ag85 bound to plasma fibronectin and measured in sera from patients not diagnosed as having active tuberculosis could reflect this cross-reactivity, which perhaps results from transient or chronic colonization by nonpathogenic mycobacteria. The occurrence of immunoreactive Ag85 in healthy controls could even be a result of a normally silent initial infection with *M. tuberculosis* itself. Ag85 bound to fibronectin might be detectable for an extended period of time after it was generated, since such complexes, unlike IgG immune complexes, are not known to be preferentially removed from the circulation.

The Ag85 complex of *M. tuberculosis* consists of two bands seen in SDS-PAGE, one at 32 kDa containing Ag85A and Ag85C and the other at 30 kDa containing Ag85B (11, 14). Because clone 240 anti-Ag85 reacts equally well with Ag85A and Ag85B (11) and can recognize a 30- to 32-kDa doublet on Western blots of the purified Ag85 complex standard, the single band in Western blots of immunoprecipitates or purified Ag85 complex proteins is not simply due to lack of antibody specificity. While it could be indicative of large amounts of Ag85B not being present in the circulation of patients with active tuberculosis, it could also merely reflect assay variability that leads to a loss of resolution as a result of the particular doses of Ag85 complex proteins applied to the SDS-PAGE gel.

The increased circulating levels of Ag85 displayed by patients with active tuberculosis were similar in PPD-positive and PPD-negative (anergic) patients, and there was no significant difference in median serum levels of Ag85 between these two patient groups. Ag85 inhibits local expression of delayed hypersensitivity by binding to T-cell fibronectin (13), and increased levels of this secretory product might be expected to modify local host effector response at tissue sites of mycobacterial growth (32). However, elevated circulating levels of Ag85 had no apparent effect on systemic reactivity to PPD. This lack of effect of Ag85 on systemic expression of delayed-type hypersensitivity in these patients might reflect inhibition of its activity after formation of complexes with plasma proteins. Studies to confirm this hypothesis are currently in progress in our laboratory.

Although the numbers of tuberculosis patients examined were small, increases in serum Ag85 appeared to be independent of the stage of tuberculosis disease or the presence of acid-fast bacilli on sputum smears. Monoclonal anti-Ag85 antibodies of appropriate specificity distinguished patients with active tuberculosis from those with active *M. avium* disease. Elevated levels of serum Ag85 were also detected in a single patient with sarcoidosis, a disease of unknown etiology which has been suggested to be due to mycobacterial infection (4). These results suggest that measurement of circulating Ag85 might

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**FIG. 3.** Immunoprecipitation characterization of Ag85 in sera from patients with active tuberculosis and from controls. (A) Western blot analysis (SDS–10% PAGE gel) of protein A immunoprecipitates of serum from a PPD-positive patient with active tuberculosis (lane 1), a PPD-negative patient with active tuberculosis (lane 2), a PPD-positive patient with treated tuberculosis (lane 3), a PPD-positive patient with nontuberculous lung disease (lane 4), and a PPD-positive healthy control (lane 5). Lane 6 contains 10 μL of immunoreactive purified *M. bovis* BCG Ag85 complex proteins. Samples separated on SDS-PAGE, electroblotted to nitrocellulose, and developed with mouse clone 240 anti-Ag85 and ECL. Ag85 is present in circulating immune complexes only in patients with active tuberculosis. (B) Western blot analysis (SDS–10% PAGE gel) of rabbit anti-plasma fibronectin (anti-FN) or normal rabbit serum (NRS) immunoprecipitates of serum previously immunoprecipitated with protein A from a PPD-positive patient with active tuberculosis (lane 1), a PPD-negative patient with active tuberculosis (lane 2), a PPD-positive patient with treated tuberculosis (lane 3), a PPD-positive patient with nontuberculous lung disease (lane 4), a PPD-positive healthy control (lane 5), and a PPD-negative healthy control (lane 6) or of a mixture of purified human plasma fibronectin and purified *M. bovis* BCG Ag85 complex proteins (lane 7). Double arrows indicate the positions of the 32- and 30-kDa components of the Ag85 complex. Lane 8 contains 4.5 μL of immunoreactive purified *M. bovis* BCG Ag85 complex proteins. After removal of IgG, sera were incubated with monospecific rabbit anti-FN or NRS and then immunoprecipitated with protein A/G. The immunoprecipitates were separated by SDS-PAGE, electroblotted to nitrocellulose, and developed with either mouse clone 240 anti-Ag85 or rabbit anti-FN and ECL. See Materials and Methods for more details.
be developed into a diagnostic test for active mycobacterial infection that was independent of host immune response. Studies on larger numbers of patients with different stages of tuberculosis to examine this possibility are currently in progress.

In sum, median levels of circulating Ag85 are significantly higher in patients with active tuberculosis than in patients with other diseases or healthy controls. No increases in urinary Ag85 were detected in any patient, regardless of the serum Ag85 level. In patients with active tuberculosis, Ag85 circulates primarily as complexes with immunoglobulins and plasma fibrinogen rather than in unbound form. The existence of Ag85 complexes with plasma proteins accounts for its lack of urinary clearance.

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REFERENCES


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ERRATUM

Pathophysiology of Antigen 85 in Patients with Active Tuberculosis: Antigen 85 Circulates as Complexes with Fibronectin and Immunoglobulin G

STUART I. BENTLEY-HIBBERT, XIN QUAN, THOMAS NEWMAN, KRIS HUYGEN, AND HENRY P. GODFREY

Departments of Pathology and Medicine, New York Medical College, Valhalla, New York 10595, and Pasteur Institute of Brussels, B-1180 Brussels, Belgium

Volume 67, no. 2, p. 581–588. Page 583: Figure 1A should appear as shown below.