Characterization of Fibronectin-Binding Antigens Released by *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG

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Fibronectin (FN)-binding antigens are prominent components of short-term culture supernatants of *Mycobacterium tuberculosis*. In 3-day-old supernatants, a 30-kilodalton (kDa) protein was identified as the major FN-binding molecule. In 21-day-old supernatants, FN bound to a double protein band of 30 and 31 kDa, as well as to a group of antigens of larger molecular mass (57 to 60 kDa). FN-binding molecules in this size range, but not of 30 to 31 kDa, were also found in sonicates. We showed that the 31- and 30-kDa FN-binding bands correspond to components A and B of the BCG85 complex, previously shown to be abundant in culture supernatants of *Mycobacterium bovis* BCG. Thus, a polyclonal antibody to the BCG85 complex bound to the 30- and 31-kDa antigens and inhibited binding of FN to them on immunoblots of the culture filtrates. Similarly, FN bound to the purified components of the BCG85 complex, and this binding was blocked by the antibody. A monoclonal antibody, HYT27, also bound both to the BCG85 components A and B and to the 30- and 31-kDa FN-binding molecules of *M. tuberculosis*, but it did not block the binding of FN. Related molecules appear to be present on the surface of BCG and to mediate the binding of BCG to FN-coated plastic surfaces, since this binding could also be blocked by the polyclonal anti-BCG85 antibody and by the purified components of BCG85, particularly component A, but not by monoclonal antibody HYT27. The binding of these mycobacterial antigens to FN appears to be of very high affinity, and we suggest that this property of major secreted antigens of *M. tuberculosis* indicates an important role in mycobacterial disease and in the binding of BCG to tumor cells during immunotherapy of bladder cancer.

There has been increasing interest in the possibility that live mycobacteria secrete antigens which may be relevant to rapid recognition of bacilli by cells of the immune system. This possibility was highlighted by the fact that many T-cell clones which recognize sonicated mycobacteria fail to recognize live ones and that peak production of protective T cells in the infected animal occurs only when organisms are rapidly multiplying (14, 18). Since secreted antigens are likely to have important protective roles, we have used [35S]methionine-labeled supernatants of 4- to 7-day-old cultures to identify proteins which are secreted or released by living metabolizing organisms of *Mycobacterium tuberculosis* and *Mycobacterium bovis* BCG (2, 3). Western blot analysis showed that antigens defined in supernatants of 3- to 5-day-old cultures constituted a small subset of those seen in supernatants of organisms cultured for longer periods, when leakage of material from dead organisms becomes important (1, 2).

Some secreted antigens have the interesting property of binding to fibronectin (FN) (2). FN, which is a large glycoprotein commonly found in plasma and extracellular matrix, has many biological activities. It binds to a number of macromolecules, including collagen, gelatin, fibrin, and heparin, and participates in cell surface interactions between eucaryotic cells and microorganisms (9, 12, 22). FN has been observed to bind in a receptor-mediated manner to several bacteria and parasites including *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Treponema pallidum* (5, 13, 20, 21). It has been suggested that FN has a role as an

adherence factor important in the pathogenesis of infective endocarditis (6).

We recently demonstrated that mycobacteria bound to FN and that BCG culture supernatants inhibited attachment of organisms to FN-coated surfaces (15). Moreover, the attachment of BCG to the bladder wall was shown to be mediated by FN, suggesting that this interaction is necessary for the initiation of the antitumor activity in intravesical BCG therapy for murine bladder cancer (16).

The aims of this study were to define the FN-binding antigens which are secreted or released by *M. tuberculosis* and *M. bovis* BCG and to establish their relationship to antigens of similar molecular weight described in other publications. These were the BCG85 complex which is abundant in culture supernatants of BCG (25), antigens recognized by monoclonal antibodies of the HYT27 group in culture filtrates of *M. tuberculosis* (19, 27), and an antigen designated P32 purified from BCG supernatant (4).

We show here that these antigens are all related and that they also mediate the binding of whole organisms to FN-coated surfaces. These molecules must be present on the surface of the bacilli, as well as being secreted.

MATERIALS AND METHODS

Mycobacterial strains. The strains of *M. tuberculosis* used were a laboratory strain, H37Rv, and a clinical isolate. The British (Glaxo Pharmaceuticals, Ltd., Greenford, United Kingdom), Brazilian, and Canadian *M. bovis* BCG substrains were obtained commercially as lyophilized preparations.

Preparation of secreted proteins. [35S]methionine-labeled

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proteins in culture supernatants of mycobacteria were obtained as described previously (2, 3). In brief, bacteria were grown as suspensions in iron and carbon source-supplemented methionine-free Eagle minimum essential medium (Flow Laboratories, Inc., McLean, Va.) containing [^{35}S]methionine for 4 days in an atmosphere of 5% (vol/vol) CO_2. Cultures were then passed through filters (Millex GV; 0.22-μm pore size; Millipore Corp., Bedford, Mass.), and filtrates were stored at −70°C. Before use in binding assays, radiolabeled supernatants were filtered on PD-10 columns (Pharmacia, Uppsala, Sweden) to separate ^{35}S-labeled proteins from free [^{35}S]methionine.

Supernatant of a 3-day-old culture of *M. tuberculosis* was prepared by the same method but without labeled methionine and then concentrated 10-fold and desalted by gel filtration on a PD-10 column. The 21-day-old culture filtrate was prepared by growing *M. tuberculosis* as a pellicle on Sauton medium, and bacteria were then removed by sequential filtration through Whatman no. 1 paper and Millipore filters (0.2-μm pore size).

Other antigens. The BCG85 components were purified from 14-day-old culture filtrate of a Danish BCG strain as described in detail elsewhere (25) by combining ammonium sulfate precipitation with column chromatography. A 21-day-old culture filtrate of the Japanese BCG strain was kindly provided by S. Nagai, Osaka City University Medical School, Osaka, Japan. Sonicates of *M. tuberculosis* H37Rv and *M. bovis* BCG were prepared as described previously (17). The sonicate of armadillo-grown *Mycobacterium leprae* was provided by R. J. W. Rees, National Institute for Medical Research, London, United Kingdom. The recombinant form of the 65-kilodalton (kDa) protein of BCG was a gift from J. D. A. van Embden, National Institute of Public Health and Environmental Hygiene, Bilthoven, The Netherlands.

FN. Human FN was purified from plasma by affinity chromatography on gelatin-Sepharose as described by Vuento and Vaheri (23).

Production of antisera and monoclonal antibodies. Rabbit antiserum against the BCG85 complex of *M. bovis* BCG was produced as described previously (25). The monoclonal antibodies used were HBT7, HYT27, HYT29, and HYT2; they were produced and characterized as previously described (19). Antibodies to FN which were prepared by immunizing rabbits or goats with purified FN gave a monospecific response against human serum proteins in immunoelectrophoresis.

FN binding assays. Flat-bottomed Removawells were incubated with 100 μl of FN at 125 μg ml^{-1} or 10-fold serial dilutions in phosphate-buffered saline for 90 min at 37°C. Control wells were coated with human or bovine serum albumin. Wells were washed with phosphate-buffered saline supplemented with 0.1% (wt/vol) bovine serum albumin, and 50-μl aliquots of ^{35}S-labeled proteins (25,000 cpm) obtained from culture supernatants were added. Each replicate was assayed in duplicate. After 1 h of incubation at 37°C, the wells were washed and dried and bound isotope was quantitated by liquid scintillation spectroscopy. To measure mycobacterial adherence to FN, we labeled bacteria with [^{3}H]uracil as described previously (17), and 4 × 10^5 CFU were added to wells and incubated for 3 h at 37°C. Each replicate was assayed in quadruplicate.

Binding specificity of ^{35}S-labeled proteins from culture filtrates or ^{3}H-labeled BCG to FN was ascertained by incubating FN-coated wells with anti-FN antibodies for 1 h at 37°C. Normal rabbit or goat sera were used as controls.

Wells were washed and adherence of ^{35}S-labeled proteins or ^{3}H-labeled BCG was determined as described above. The monoclonal antibodies (HBT7, HYT27, HYT29, and HYT2) and anti-BCG85 antiserum were tested for inhibition of BCG attachment to FN-coated surfaces. ^{3}H-labeled BCG were incubated with appropriate antibody concentrations for 1 h at 37°C, washed with phosphate-buffered saline, and then assayed for binding to FN. The effects of purified BCG85A and BCG85B antigens on the attachment of ^{3}H-labeled BCG to FN was determined by adding each antigen (protein concentration ranging from 0.3 to 30 μg/ml) to wells coated with FN. The 65-kDa protein of BCG was used as a control at the same concentrations. After 1 h of incubation at 37°C, the wells were washed and ^{3}H-labeled BCG were added as described.

Western blotting (immunoblotting). Antigen samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a discontinuous buffer system (11) on slab gels of 12.5% acrylamide. A mixture of standard protein markers (MW-SDS-200; Sigma Chemical Co., St. Louis, Mo.) was used for the determination of molecular mass. Proteins were transferred onto nitrocellulose paper with a semi-dry electroblotter (Ancos, Olstykke, Denmark) (10). Proteins were localized by staining with Aurodye, a colloidal gold solution (Janssen Life Sciences Products, Olen, Belgium). Nitrocellulose membranes were incubated with antibodies as described previously (2). For the identification of FN-binding antigens, immunoblots were incubated with FN (50 μg ml^{-1}) for 2 h at 37°C and probed with peroxidase-conjugated rabbit anti-FN immunoglobulin at a 1:500 dilution (DAKO A246). The specificity of anti-BCG85 antibodies and HYT monoclonal antibodies toward the FN-binding proteins was checked by incubating immunoblots of culture filtrate and purified BCG85 antigens with anti-BCG85 antiserum or HYT monoclonal antibodies first and then with FN and anti-FN antibodies.

RESULTS

FN-binding activity of secreted proteins. [^{35}S]methionine-labeled proteins from culture supernatants of *M. tuberculosis* H37Rv and two BCG substrains were incubated with FN-coated surfaces. Binding of radiolabeled proteins is shown in Fig. 1. The radioactivity recovered from surfaces coated with bovine serum albumin (usually 50 to 85 cpm) was subtracted from values obtained from incubations with FN. Attachment of mycobacterial culture supernatants to FN reflected a dose-dependent binding, since increasing amounts of radioactive proteins bound to higher concentrations of FN (Fig. 1A). At the FN concentration (125 μg ml^{-1}) used throughout the study, similar amounts of radiolabeled proteins in mycobacterial culture supernatants were found to bind to FN. Moreover, pretreatment of FN-coated surfaces with anti-FN antiserum diminished attachment of labeled proteins by 50 to 70% (Fig. 1B). No inhibition was observed with normal serum.

Selective recognition of mycobacterial antigens by FN. Western blot analysis of 21-day-old culture filtrates and sonicates of *M. tuberculosis* and *M. bovis* BCG and of *M. leprae* sonicate was used to determine the binding specificity of FN. FN reacted with two sets of mycobacterial proteins (Fig. 2B, lanes 1 to 4). FN bound to one set of antigens in the range of 57, 58, and 60 kDa in both culture filtrate and sonicate. The other group of antigens recognized by FN was only present in culture filtrate and was a double protein band with molecular masses of 30 and 31 kDa. FN reacted poorly with *M. leprae* sonicate (Fig. 2B, lane 5).
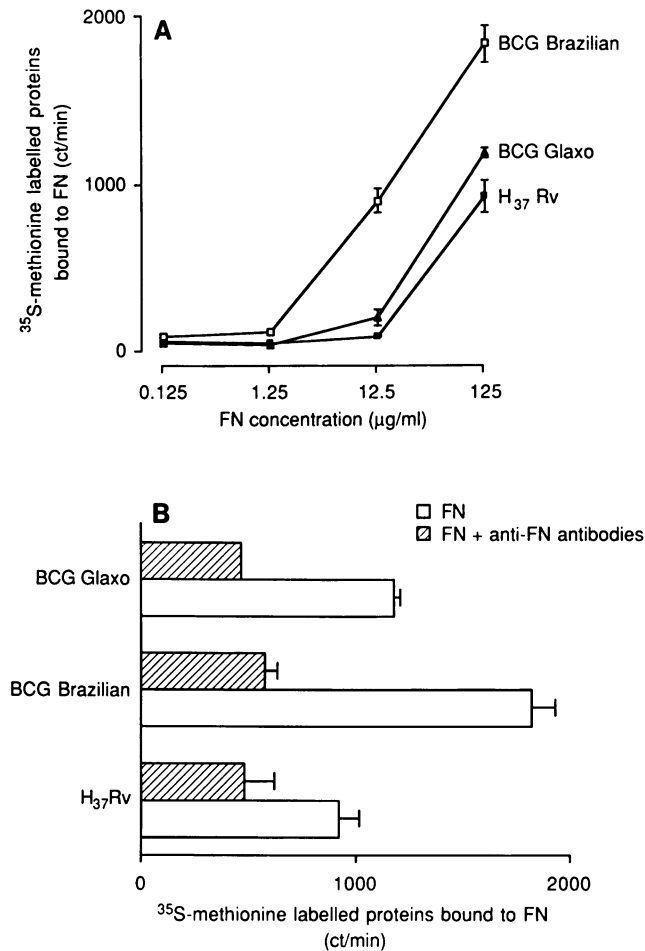
FIG. 1. Binding to FN-coated surface of [35S]methionine-labeled proteins in culture supernatants of *M. tuberculosis* H37Rv and two BCG substrains. (A) Effect of coating plastic surfaces with increasing concentrations of FN. (B) Inhibition of binding of [35S]methionine-labeled proteins to FN by goat anti-FN antibodies at a 1:50 dilution. Normal goat serum did not show any inhibiting capacity. The background obtained from bound radiolabeled proteins to surfaces coated with bovine serum albumin was subtracted from the values reported. Bars indicate standard error of the mean.

Secretion of FN-binding proteins by actively metabolizing mycobacteria was further investigated by comparing the FN activity patterns of supernatants of 3- and 21-day-old cultures of *M. tuberculosis*. Immunoblotting of the short-term supernatant with FN detected one major protein band at 30 kDa (Fig. 2C, lane 3).

To determine the chemical nature of the FN-binding molecules, we next tested the binding of FN on immunoblots treated with proteolytic enzymes or with periodate. Incubation with trypsin (50 μg ml^{-1}) for 1 h at 37°C destroyed the FN-binding properties of these molecules, suggesting the involvement of a polypeptide moiety in mycobacterial antigens. In contrast, pretreatment of immunoblots with 0.1 M sodium periodate for 2 h at 4°C in the dark did not prevent FN binding.

Immunological relationship between the 30- to 31-kDa FN-binding antigens, the BCG85 complex, and the HYT27 antigens. In previous reports, two antigenic components, BCG85A (31 kDa) and BCG85B (29 kDa), of the BCG85 complex of BCG were characterized and shown to be

partially identical (25). By crossed immunoelectrophoresis, these antigens were found in culture filtrates of both BCG and *M. tuberculosis* and reacted with monoclonal antibody HYT27, which recognized a double protein band at 32 and 33 kDa (24). On the basis of these findings, we looked for a possible relationship to the FN-bindng antigens by Western blot analysis. Protein staining and immunoblotting with polyclonal anti-BCG85 antiserum on blots of culture filtrate of *M. tuberculosis* and of purified BCG85A and BCG85B antigens demonstrated that the double protein band binding to FN corresponds to the two purified components of the BCG85 complex (Fig. 3A and B). The preparations of BCG85A and BCG85B gave additional protein bands which were recognized by the polyclonal rabbit antiserum, suggesting either that some contaminating components were present in the antigen preparation used for immunization or that, more probably, degradation products were present. Incubation with each of the four monoclonal antibodies showed that only HYT27 reacted as strongly as the polyclonal rabbit antiserum. Monoclonal antibody HYT27 bound to a double band at 30 and 31 kDa in the culture filtrate and to single bands in the purified preparations of BCG85A and BCG85B at 31 and 30 kDa, respectively (Fig. 3C).

We checked whether FN bound on immunoblots of purified BCG85A and BCG85B, and the binding patterns are compared in Fig. 3D. FN reacted with the 31-kDa band in BCG85A, while two bands at 30 and 25 kDa were seen in BCG85B. Since FN bound to antigens BCG85A and BCG85B, we next determined the ability of polyclonal rabbit anti-BCG85 antibody and of monoclonal antibody HYT27 to inhibit FN binding on immunoblots. Anti-BCG85 antibody considerably reduced FN binding both to antigens BCG85A and BCG85B, and to the 30- and 31-kDa double band in the culture filtrate (Fig. 3E). Treatment of immunoblots with preimmune rabbit serum or with monoclonal antibody HYT27 prior to incubation with FN did not affect the binding (data not shown).

Relationship between BCG85 complex and bacterial surface proteins that bind FN. Supernatants from BCG cultures were previously shown to inhibit the binding of BCG to FN-coated surfaces (15). Characterization of the FN-binding properties of mycobacteria prompted us to examine further the relationship between surface proteins and soluble proteins in supernatants of mycobacterial cultures which correspond to the BCG85 components. Anti-BCG85 antibodies inhibited BCG attachment to the FN surface to the same degree as anti-FN antibodies (Fig. 4A). Normal rabbit serum did not inhibit BCG binding. As expected, preincubation of 3H-labeled BCG with any of the monoclonal antibodies of the HYT27 group produced no inhibition of BCG attachment (data not shown). We next tested the ability of purified antigens BCG85A and BCG85B to inhibit BCG binding to FN. Both antigens inhibited the attachment of BCG bacilli to FN-coated surfaces (Fig. 4B). Antigen BCG85A was more effective than BCG85B at a concentration of 3 μg ml^{-1} and, in a separate experiment, produced complete inhibition of 3H-labeled BCG attachment to FN at 7 μg ml^{-1}. The 65-kDa protein of BCG was used as a control and did not affect the binding.

DISCUSSION

The secreted antigens of *M. tuberculosis* and BCG substrains were further studied by using FN, monospecific antisera, and monoclonal antibodies. Actively growing mycobacteria release antigens which bind to FN. The binding to
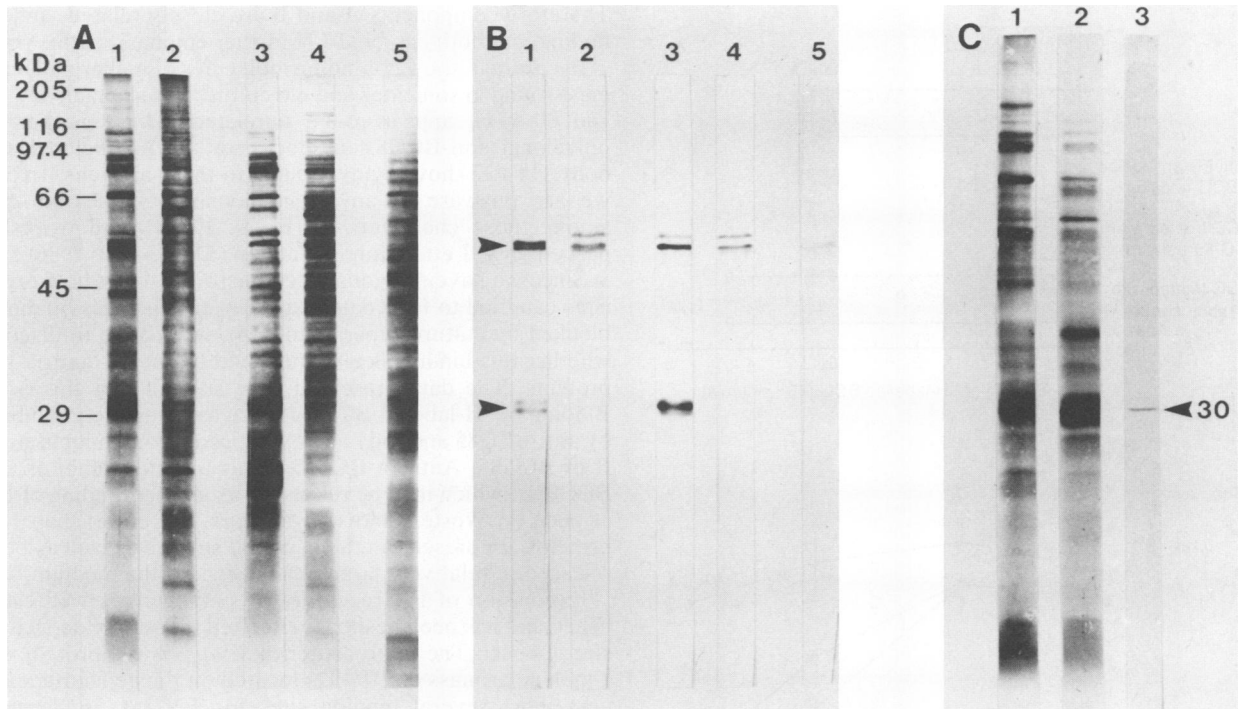
FIG. 2. Identification of FN-binding antigens on immunoblots of culture filtrates and sonicates of *M. tuberculosis*, *M. bovis* BCG, and *M. leprae*. (A) Aurodye-stained protein profiles of 21-day-old culture filtrate (lane 1) and sonicate (lane 2) of *M. tuberculosis*, 21-day-old culture filtrate (lane 3) and sonicate (lane 4) of *M. bovis* BCG, and *M. leprae* sonicate (lane 5). (B) Immunoblots probed with FN. Lanes 1 to 5 correspond to the antigen preparations stained in panel A. (C) Lanes 1 and 2 are 21- and 3-day-old culture supernatants of *M. tuberculosis* stained with Aurodye, and lane 3 is the immunoblot of lane 2 incubated with FN. The positions of molecular mass markers are shown.

FN could be inhibited by anti-FN antibodies. The FN-binding antigen in 3-day-old culture supernatants of *M. tuberculosis* was identified by Western blot analysis and shown to be a major protein band of 30 kDa. In 21-day-old culture supernatants, FN bound to a double protein band with molecular masses of 30 and 31 kDa and also to a group of antigens of higher molecular mass (57, 58, and 60 kDa). The latter, but not the 30- and 31-kDa components, were also
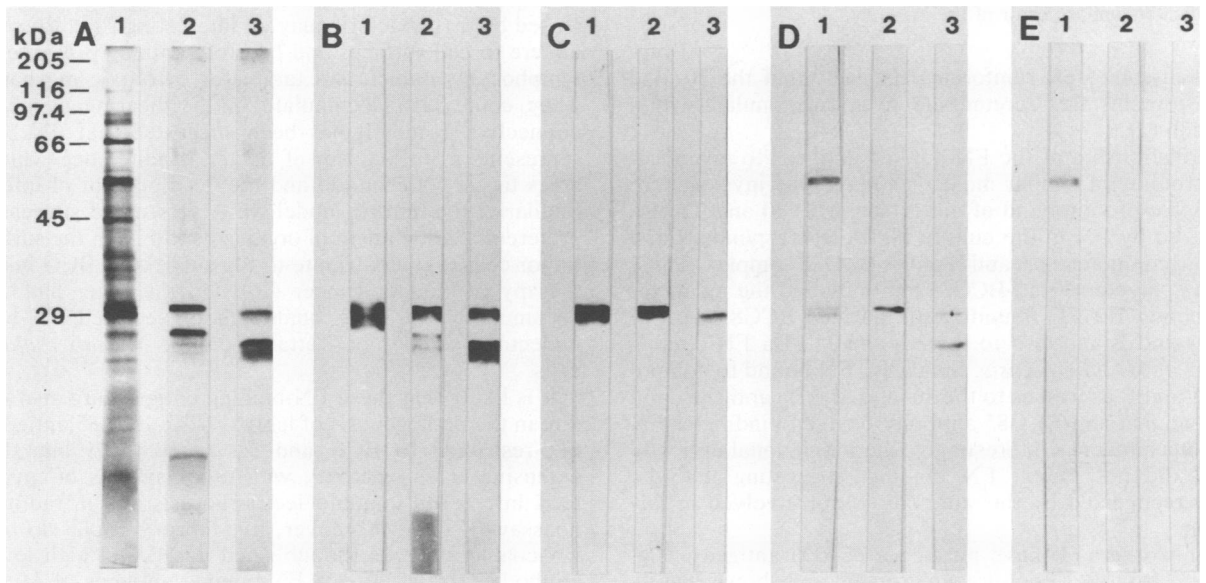

FIG. 3. Western blot analysis of culture supernatant of *M. tuberculosis* and purified BCG85A and BCG85B antigens of *M. bovis* BCG. Lanes 1, 2, and 3 in all panels are proteins in the culture supernatant of *M. tuberculosis*, BCG85A, and BCG85B, respectively. Reference lanes were stained with Aurodye (A). Immunoblots were incubated with polyclonal rabbit anti-BCG85 antiserum (B), monoclonal antibody HYT27 (C), FN (D), or anti-BCG85 antiserum first and then FN (E). The positions of molecular mass markers are shown.
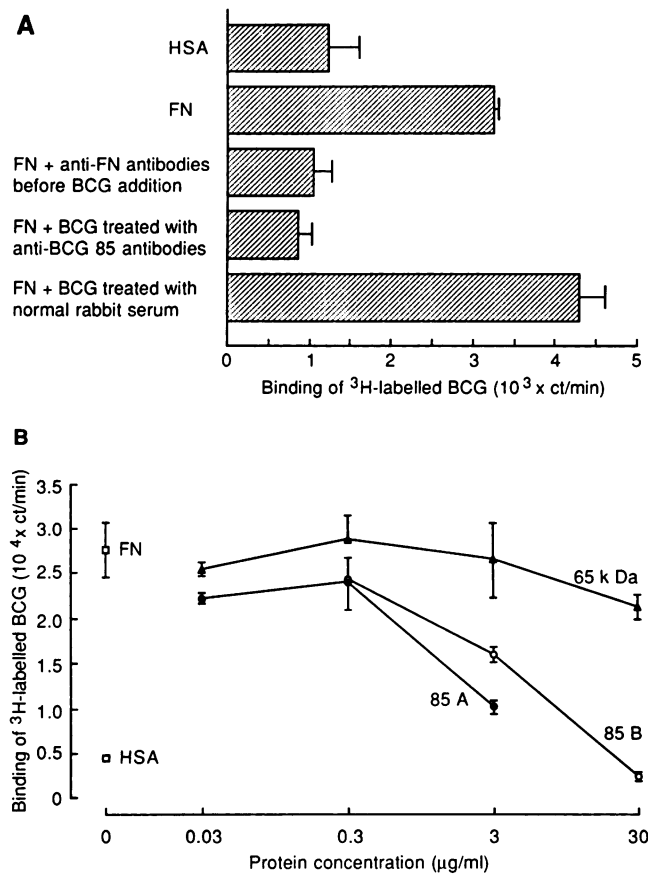
FIG. 4. Effects of anti-BCG85 antibody (A) and of purified antigens BCG85A and BCG85B (B) on the attachment of ^{3}H-labeled BCG to FN-coated surfaces. (A) BCG were pretreated with anti-FN antibodies, anti-BCG85 antibodies, or normal rabbit serum at a 1:50 dilution. (B) FN-coated wells were incubated with the indicated amount of inhibitor and then assayed for adherence of ^{3}H-labeled BCG. Control wells were coated with human serum albumin (HSA). Bars indicate standard error of the mean.

present in sonicates, reinforcing the view that the 30- and 31-kDa proteins are secreted and do not accumulate within the bacilli (2).

The relationship of the FN-binding antigens to mycobacterial proteins of similar molecular mass was investigated. The double protein band of molecular mass 30 and 31 kDa recognized by FN in the culture filtrate corresponds to the antigenic components A and B of the BCG85 complex. Thus, both the polyclonal anti-BCG85 antibody and the monoclonal antibody HYT27 bound to the purified BCG85 components A and B and also to the 30- and 31-kDa FN-binding proteins of M. tuberculosis. Similarly, FN bound to components A and B as well as to the 30- and 31-kDa antigens, and the polyclonal anti-BCG85 antibody blocked binding of FN to all four antigens. Interestingly, the monoclonal antibody HYT27 did not inhibit FN binding, suggesting that the epitope recognized by this antibody is not involved in this function.

Other workers (4) have purified a 32-kDa antigen designated P32 from BCG culture supernatant and shown that its NH$_2$-terminal amino acid sequence is identical to that of a protein, MPB59, isolated from the Japanese substrain. However P32 corresponds to component A of the BCG85 complex, whereas MPB59 corresponds to component B (26).

Therefore components A and B are closely related, and our finding that both can bind FN further emphasizes this point.

In contrast, the FN-binding molecules of higher molecular mass found in sonicates and old culture supernatants (57, 58, and 60 kDa) appear to be unrelated. Thus, neither the polyclonal anti-BCG85 antibody nor the monoclonal antibody HYT27 showed any binding to these antigens. In fact, we are unaware of any monoclonal antibodies to them among those characterized (H. D. Engers and workshop participants, Letter, Infect. Immun. 51:718–720, 1986).

Since we have previously demonstrated that whole organisms can bind to FN-coated surfaces and that this binding is blocked by culture supernatant (15), we wished to discover whether this binding is also mediated by the 30- and 31-kDa proteins. The data presented here suggest that this is so. Binding of ^{3}H-labeled BCG to FN was completely inhibited by anti-BCG85 antibody and by antigenic components A and B of BCG85. Antigen BCG85A was a better inhibitor than BCG85B, which may be related to its greater binding of FN, as seen on Western blots. It appears, therefore, that these proteins are present on the bacterial surface as well as being released in relatively large quantities into the medium.

Expression of FN receptors by pathogens is well established and has been best characterized for staphylococci and streptococci. The FN receptor of S. aureus is a protein with a molecular mass of 210 kDa located on the cell surface and containing several binding sites for FN (5). In group A streptococci, lipoteichoic acid has been identified as the major surface receptor (13), although a papain-sensitive protein binding to FN has also been reported (20). Gram-negative organisms, on the whole, do not bind to FN (6).

The interaction between FN and mycobacterial receptors appeared to be very strong. In a previous report, we showed that culture supernatants of BCG inhibited the attachment of BCG to FN-coated surfaces (15). Since the inhibitory activity of the supernatant could be removed by affinity chromatography on FN-Sepharose, we attempted to isolate the FN-binding antigens by using this technique. However, it proved very difficult to elute the bound proteins. This very strong binding suggests that the FN-binding antigens released by mycobacteria play an interesting role. They could adhere to cell surfaces and be preferentially presented to T lymphocytes or act as a target for cytotoxic mechanisms. They could also accumulate on FN-bearing elements of connective tissue. It has been suggested that FN binding represents a mechanism of bacterial adherence which enables tissue colonization and the development of infection. Similarly, the murine model we have studied suggests that preferential attachment of organisms to FN on the surface of tumor cells may contribute to the success of BCG immunotherapy of bladder cancer (16). On the other hand, local accumulation of large quantities of secreted FN-binding molecules could block attachment of M. tuberculosis to cells.

It is likely that these FN-binding antigens are also important in the pathogenesis of leprosy. The BCG85 antigens are not restricted to BCG and M. tuberculosis but showed extensive cross-reactivity with other species of mycobacteria in crossed immunoelectrophoresis and in radioimmunoassays (7, 26). Moreover, live leprosy bacilli do bind to FN-coated surfaces (unpublished data), and high levels of antibodies to the 30-kDa FN-binding antigens of M. tuberculosis have been detected in sera of lepromatous leprosy patients (E. Filley, C. Abou-Zeid, M. Harboe, and G. A. W. Rook, manuscript in preparation). Similarly, the corresponding antigen BCG85B was shown to induce a marked humoral

immune response in armadillos and monkeys during development of systemic mycobacterial infection after inoculation with *M. leprae* (26). These observations suggest that the 30-kDa protein is a secreted antigen of this organism too, but unstudied because it is essentially absent from sonicates of *M. leprae* and is depleted from the surface during the extraction procedures required to prepare this organism from armadillo tissues. The fact that potent antisera prepared against armadillo-grown *M. leprae* sonicate failed to show evidence of antibodies to these antigens is compatible with the evidence that they are secreted.

There is also direct evidence that these antigens evoke responses in human tuberculosis. In a recent study, tuberculin-positive volunteers showed significant lymphoproliferation and gamma interferon production to antigen P32, but no anti-P32 antibodies (8). In contrast, high levels of immunoglobulin G to P32 and low blastogenesis were observed in patients with poor general health and advanced tuberculous lesions.

In conclusion, this study resolved the confusion surrounding a series of reports of antigens in the 30- to 31-kDa range and showed that these are all concerned with the same antigens, which have in addition the relevant biological property of high affinity for FN. Work is now in progress to clone the genes since preliminary data suggest a role in both leprosy and tuberculosis.

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