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Epidermal Growth Factor-Binding Protein in *Mycobacterium avium* and *Mycobacterium tuberculosis*: a Possible Role in the Mechanism of Infection

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Epidermal growth factor (EGF) is a potent mitogen for a variety of eukaryotic cells. EGF is found in a number of tissues and is prevalent in necrotic tissues and granulomata. The biological effect of EGF on mammalian cells is initiated by the binding to a specific receptor. Both *Mycobacterium avium* and *Mycobacterium tuberculosis* cause lung infections and localized or disseminated disease in both patients without AIDS and those with AIDS. Histopathologic studies show necrosis in the lung, liver, and splenic tissues of patients with disseminated mycobacterial infection. In the course of experiments to examine the effect of growth factors on macrophages, it was observed that *M. avium* and *M. tuberculosis* but not *Mycobacterium smegmatis* cultured in the presence of 5, 50, or 500 ng of EGF per ml grew significantly faster than mycobacteria cultured in the absence of EGF. ^{125}I-EGF was found to bind to *M. avium* and *M. tuberculosis*, and the binding was competitively inhibited by unlabeled EGF. A receptor for EGF was identified on mycobacteria. Incubation of mycobacteria with EGF prior to infection of macrophage monolayers resulted in faster bacterial growth within macrophages compared with that of mycobacteria not incubated with EGF. EGF-binding protein was cloned and expressed in *Escherichia coli*, and subsequently the protein was purified and the N-terminal amino acids were sequenced. These results suggest that EGF is a growth factor for pathogenic mycobacteria in granulomatous tissues and within macrophages and might enhance growth rates of both intracellular and extracellular mycobacteria in the site of infection.

Organisms of the *Mycobacterium avium* complex and *Mycobacterium tuberculosis* are important human pathogens that cause lung infection in immunocompetent hosts and disseminated infection in specific groups of immunocompromised patients (14, 18, 26). Mycobacteria are facultative intracellular microorganisms that preferentially infect macrophages (10). The host response to mycobacteria is associated with necrosis with or without granuloma formation (10). Areas both of caseation necrosis and granulomatous inflammation are rich in cytokines and growth factors, such as epidermal growth factor (EGF), fibroblast growth factor, and transforming growth factor α (13, 33). Granulomatous lesions are encountered chiefly in the lung, liver, and spleen of patients with disseminated disease (9). Both granuloma and liquefied caseous tissue are excellent growth media for mycobacteria, with extracellular bacteria reaching high numbers (9). In these lesions, mycobacteria are likely to be exposed to mammalian growth factors such as EGF (33). In an attempt to determine whether growth factors had any influence on the ability of human macrophages to inhibit growth of intracellular *M. avium*, it was our finding that as a control for the assay, *M. avium* grew significantly faster in the presence of recombinant human EGF than in the absence of it. On the basis of this observation, we sought to investigate the effect of EGF on the growth of extracellular as well as intracellular *M. avium* and *M. tuberculosis.*

MATERIALS AND METHODS

Bacteria. *M. avium* 101 (serovar 1), 100 (serovar 8), and 109 (serovar 4) were used for the assays. They were isolated from the blood of patients with AIDS, and their ability to cause disseminated infection in mice has been established in a number of laboratories. *M. tuberculosis* H37Rv and *Mycobacterium smegmatis* 11727 were purchased from the American Type Culture Collection (Rockville, Md.). Mycobacteria were cultured in Middlebrook 7H10 agar with oleic acid, albumin, dextrose, and catalase (OADC), and isolated colonies were suspended in Middlebrook 7H9 broth with OADC and cultured to the mid-log phase. The bacteria were then washed and subsequently resuspended in Hanks' buffered salt solution (HBSS) containing 0.1% Tween. The bacterial suspension was sonicated for 5 s (output, 20 W/min) to disperse clumps, vortex agitated, and left standing for 5 min. The top 1 ml of the suspension was removed, and the concentration of the bacteria was adjusted to 10^6 organisms per ml with the McFarland turbidity standard (5, 12). A sample of the final suspension was stained with Ziehl-Neelsen stain to examine the existence of bacterial clumps. The bacterial sample was also stained by the live-dead assay (Molecular Probe, Portland, Oreg.) to establish the percentage of viable organisms. An average of 70 to 75% of the organisms were found to be viable. A sample of the bacterial inoculum was plated onto Middlebrook 7H10 agar to confirm the number of bacteria in the inoculum.

A beta-hemolytic streptococcus (strain 4A, a clinical isolate) was cultured on blood agar. For the assays, the bacteria were scraped from the plates and washed before use.

Reagents. Recombinant human EGF was purchased from R&D Systems, Minneapolis, Minn.; recombinant human interleukin-1β (IL-1β) was also purchased from R&D Systems and had an activity of 2.1×10^5 U/mg of protein. Recombinant IL-6 was purchased from Genzyme (Cambridge, Mass.) and had an activity of 3.5×10^6 U/mg of protein. ^{125}I-EGF was purchased from Boehringer Mannheim (Indianapolis, Ind.) and had a specific activity of 45 μCi/μg. Mouse anti-human EGF and anti-human EGF receptor monoclonal antibodies, i.e., immunoglobulins G, were purchased from R&D Systems.

Growth of mycobacteria in the presence of EGF. To examine the effect of different concentrations of EGF on mycobacterial growth, we incubated *M. avium*, *M. tuberculosis*, and *M. smegmatis* with increasing concentrations of EGF (5, 50, and 500 ng/ml) in Middlebrook 7H9 broth plus OADC and compared the growth with that of bacteria grown in the absence of EGF. Recombinant human EGF was added at days 0 and 3. The culture was maintained under static conditions with the medium agitated by inversion of the tubes three times a day. At days 2 and 5, samples were obtained from the tubes, serially diluted, and plated onto Middlebrook 7H10 agar plates.

Human monocyte-derived macrophages. Cultures of human macrophages were established as reported previously (5). Briefly, monocyte-derived macrophages were cultured in 24-well plates containing 5×10^5 macrophages per well with RPMI 1640 plus 5% fetal bovine serum and 2 mM L-glutamine. Monolayers

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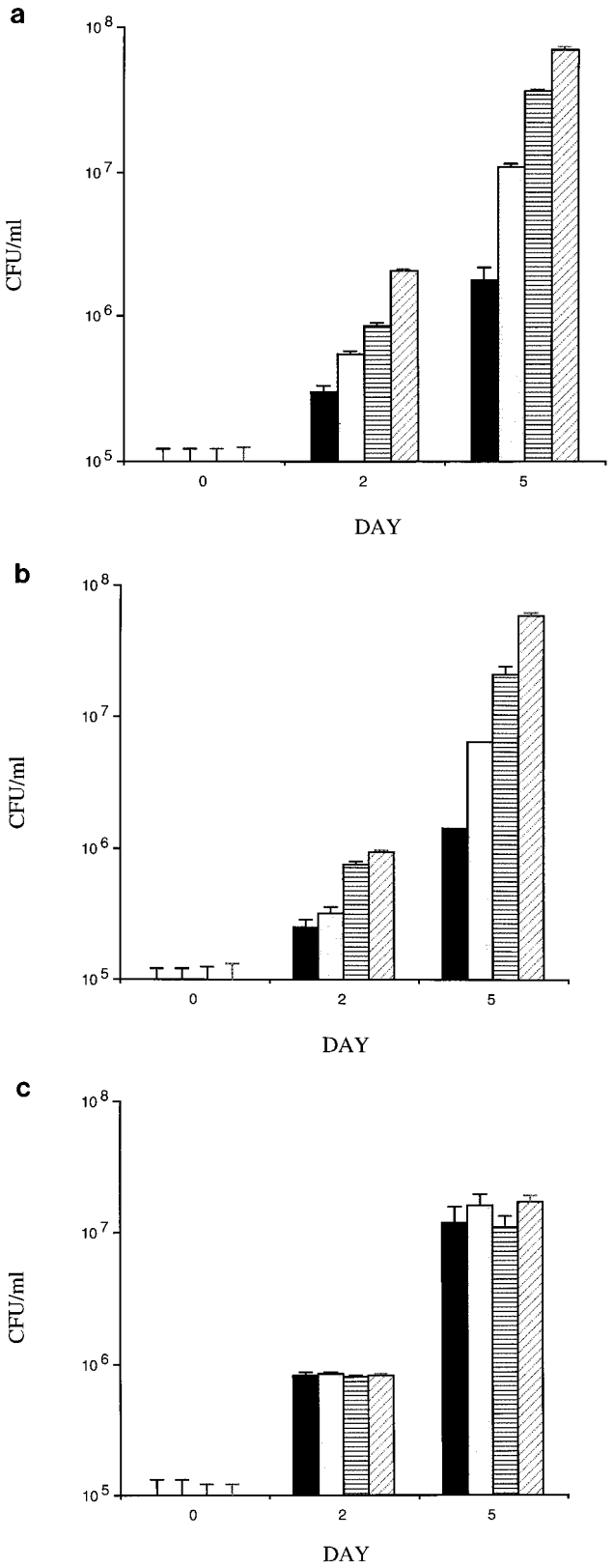

FIG. 1. Growth of *M. avium* 101 (a), *M. smegmatis* 11727 (b), and *M. tuberculosis* H37Rv (c) in Middlebrook 7H9 broth in the presence of EGF. Bacteria (10^5) were grown to the mid-logarithmic phase in Middlebrook 7H10 agar, harvested, washed twice, and cultured in Middlebrook 7H9 broth both in the absence (■; control) and presence of 5 (□), 50 (▤), or 500 (▨) ng of recombinant EGF per ml. EGF was replenished after 3 days. After 2 and 5 days, the bacteria were spun down, washed, serially diluted, and plated onto Middlebrook 7H10 agar to quantitate the viable organisms. Plates were maintained at 37°C for 10 days. The differences in growth rates were determined by comparing mycobacteria incubated with and without EGF. The data represent the means ± standard deviations of four different experiments. $P < 0.05$ for the comparisons between EGF-treated groups and the control.

were cultured for 7 days before infection. The number of viable cells in each monolayer was monitored by counting the cells every other day as described previously (5). No preferential detachment was seen in the different experimental and control groups.

Infection and quantitation of intracellular bacteria. Macrophage monolayers were infected with mycobacteria (10^6 bacteria per 10^5 cells) for 4 h. Monolayers were then washed three times with HBSS to remove extracellular bacteria. To quantitate the number of viable intracellular bacteria at time zero, three monolayers per experiment were lysed as described previously (5). Control with mycobacterial cell-free wells confirmed that the washing procedure removed at least 99.99% of the extracellular bacteria. Four days after infection, macrophage monolayers were lysed with sterile water and viable bacteria were subjected to the procedure described previously (5). The number of cells in the monolayer and the number of cells that detached from the monolayer were counted daily. We did not observe any differential detachment between the untreated control and experimental groups.

Binding of ^{125}I-EGF. Mycobacteria were exposed to ^{125}I-EGF (1 μg/ml) for 2 h at 4°C (maximal binding). Nonspecific binding was assessed by measuring the binding of ^{125}I-EGF in the presence of a 200-fold excess of unlabeled EGF. Bacteria were incubated in the presence of ^{125}I-EGF for 3 h and then washed three times with HBSS to remove unbound ^{125}I-EGF, and the radioactivity of the sample was determined with a Beckman gamma counter. Nonspecific binding of ^{125}I-EGF to the plastic tubes was ruled out by running control groups in which ^{125}I-EGF was added to tubes not containing bacteria and subsequently removed, and the tube was washed three times with HBSS.

Screening of *M. avium* genomic DNA library. An *M. avium* genomic library was constructed in the plasmid pMV261 as described previously (4) and inserted into *E. coli* HB101 by electroporation. Three hundred strains of *E. coli* HB101 transformed with *M. avium* DNA were screened by an immunodot assay as described previously (4), and the three positive clones were then examined in sodium dodecyl sulfate (SDS)–10% polyacrylamide gels. Gels for Western blotting (immunoblotting) were equilibrated, and separated proteins were transferred to nitrocellulose filters by methods described previously (6). The filters were then incubated with recombinant human EGF for 1 h and subsequently washed three times. EGF antibody was added for approximately for 1 h at room temperature; this was followed by washing and incubation with peroxidase–goat anti-mouse immunoglobulin G (List Biologicals, Campbell, Calif.) for another hour. Antigen-antibody complexes on washed filters were developed with 4-chloronaphthol, hydrogen peroxide, and phosphate-buffered saline (PBS).

Controls of bacteria without EGF were run in parallel.

PAGE and protein blotting. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out with a 10% (wt/vol) polyacrylamide separating gel and a 5% stacking gel by the method of Laemmli (21) as modified by us (6).

For studies of EGF binding, nitrocellulose membranes (containing the *M. avium* EGF-binding protein) were blocked for 1 h at room temperature with 1% gelatin (Sigma). The blots were then incubated for 1 h at room temperature with recombinant human EGF and, following washing, incubated another hour with mouse anti-human EGF antibody (1:1,000 dilution of a 10-μg/ml solution). The blots were then incubated with peroxidase–goat anti-mouse antibody immunoglobulin G and subsequently developed with a mixture of 4-chloronaphthol, hydrogen peroxide, and PBS.

Purification of the recombinant *M. avium* EGF receptor protein. *E. coli* transformed with a shuttle plasmid (pMV261 containing *M. avium* DNA) and overexpressing *M. avium* EGF-binding protein was lysed by sonication (power output, 20 W/s) for 5 min. The resulting material was centrifuged at 10,000 × *g* for 40 min, and the pellet was subsequently resolved in SDS–10% PAGE. The band corresponding to the recombinant *M. avium* protein was excised from the gel and processed by methodology described previously (3).

***M. avium* amino acid sequencing.** EGF-binding protein was extracted from *E. coli* and purified as described previously (3). The extracted protein was then subjected to SDS-PAGE and subsequently electrotransferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corp., Bedford, Mass.) by using 10 mM 2(*N*-morpholino)ethanesulfonic acid (pH 6.0)–20% methanol as the transfer buffer. The protein band was stained with Coomassie brilliant blue. The ~35,000-M_r protein band was then excised. Microsequencing by automated Edman chemistry was performed with an Applied Biosystems model 470A gas-phase sequencer (University of California, San Francisco, Biomolecular Resource Center).

Statistical analysis. Each experiment was repeated at least three times. All data in each experiment were obtained on duplicate wells, and the means ±

TABLE 1. Binding of ^{125}I-EGF to mycobacteria

EGF concn in medium (ng/ml)	^{125}I-EGF binding (cpm/min)[a]		
	M. avium	*M. tuberculosis*	*M. smegmatis*
None	6,316 ± 732[b]	5,712 ± 670[b]	664 ± 78
5	5,010 ± 261[b]	4,871 ± 419[b]	632 ± 64
50	3,976 ± 331[b]	3,247 ± 286[b]	654 ± 67
200	2,269 ± 210[b]	2,051 ± 204[b]	617 ± 51
500	1,443 ± 278[b]	1,106 ± 217[b]	632 ± 43

[a] By using ^{125}I-EGF with a specific activity of 45 μCi/μg, 10^6 bacteria were incubated with an increasing concentration of recombinant EGF, and the binding of ^{125}I-EGF to mycobacteria was examined by incubating bacteria at 4°C for 3 h (saturation occurred between 2 and 3 h). After the period of incubation, the bacteria were washed twice in HBSS and the amount of ^{125}I-EGF associated with mycobacteria was quantitated with a gamma counter. Background, defined as the nonspecific binding in counts per minute bound to the bacteria in the presence of a 200-fold excess of cold EGF, was less than 150 cpm/min and was subtracted from the results. The numbers are means ± standard deviations of three different experiments.

[b] $P < 0.05$ compared with the binding of ^{125}I-EGF to *M. smegmatis*.

standard deviations were calculated. Significance of the observation at identical time points was tested by the Student *t* test.

RESULTS

Bacterial suspensions were incubated in the presence or absence of EGF at three different concentrations. Boiled, inactivated EGF at 500 ng/ml was used as a negative control. After 2 and 5 days, the bacterial suspension was serially diluted and plated onto Middlebrook 7H10 agar. As shown in Fig. 1, culture in the presence of EGF was associated with a significant increase in CFU of *M. avium* and *M. tuberculosis* but not *M. smegmatis* after 2 and 5 days. The increase in growth of *M. avium* and *M. tuberculosis* was dependent on the concentration of EGF.

To test the specificity of EGF-mediated growth stimulation, *M. avium* and *M. tuberculosis* were cultured in Middlebrook 7H9 broth for 2 and 5 days in the presence of, per milliliter, 10, 10^2, and 10^3 U of IL-1 or IL-6, two cytokines that have been shown to be growth factors for microorganisms (28). No difference was seen in the CFU per milliliter values when mycobacterial growth in the presence of IL-6 or IL-1 was compared with that of the untreated control (data not shown).

To stimulate growth of mycobacteria in broth, EGF should be able to bind to a specific receptor on the mycobacterial cell wall. To determine whether mycobacteria would bind EGF, ^{125}I-EGF was incubated with *M. avium*, *M. tuberculosis*, or *M. smegmatis* in the presence of increasing concentrations of cold EGF at 4°C for 3 h (determined in preliminary assays to be the time required for maximal binding). As shown in Table 1, ^{125}I-EGF was able to bind to *M. avium* and *M. tuberculosis* but not *M. smegmatis*, and the binding was competitively inhibited by increasing concentrations of cold EGF.

Figure 2 shows the kinetics of binding of ^{125}I-EGF to *M. avium*. *M. avium* had a K_d of 2.0×10^{-10} M and an average of 450 ± 60 receptors per cell. To identify the EGF receptor on mycobacteria, a sonicated preparation of *M. avium* was resolved in SDS–12% PAGE under reduced conditions and then transferred to nitrocellulose paper by the Western blot technique. The nitrocellulose membrane was then incubated with recombinant EGF (Sigma Chemical Co., St. Louis, Mo.) for 1 h at 23°C, washed, incubated with mouse anti-human EGF antibody for an additional hour at 37°C, and subsequently incubated with a peroxidase–goat anti-mouse antibody and developed. As shown in Fig. 3, a band of approximately 35 to 37 kDa that was suggestive of EGF receptor on *M. avium* was observed.

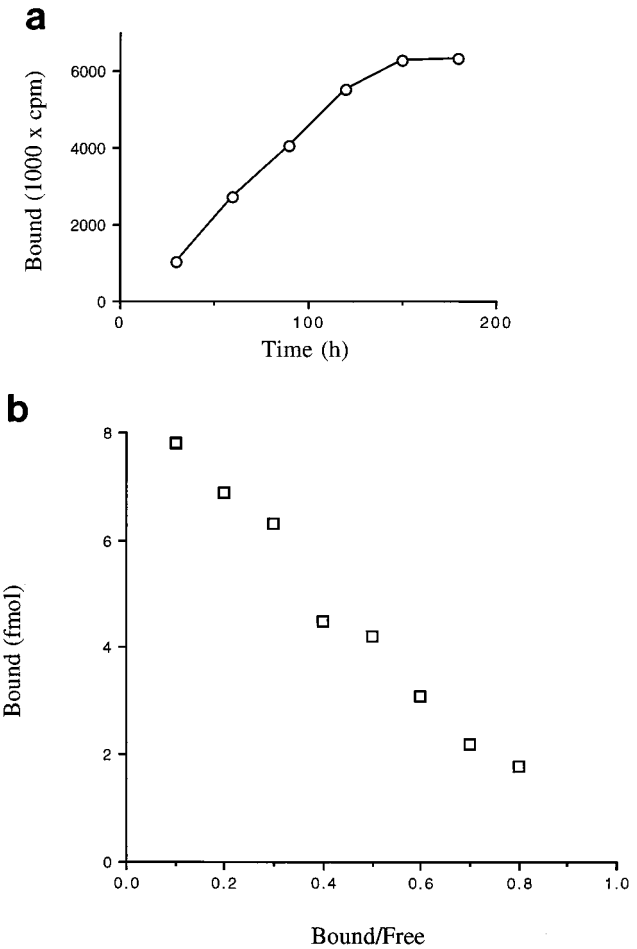

FIG. 2. (a) Kinetics of binding of ^{125}I-EGF to *M. avium*; (b) Scatchard plot analysis of the binding of ^{125}I-EGF to receptors on *M. avium*. Cells were incubated with increasing concentrations of ^{125}I-EGF at 4°C for 3 h.

Because mycobacteria are capable of intracellular growth (10), we investigated the effect of EGF on the growth of *M.*

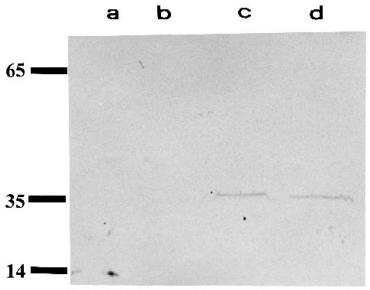

FIG. 3. Binding of anti-EGF antibody to *M. avium*. *M. avium* strains were prepared and resolved in 12.5% SDS as described previously (3). Proteins were then transferred to a nitrocellulose membrane by a previously described technique (3). The membrane was then treated with bovine albumin (1%) for 30 min, washed, and incubated with EGF for 1 h at room temperature. After washing, the nitrocellulose membrane was probed with anti-EGF antibody (Sigma) for 1 h, washed, and treated with peroxidase–goat-anti-mouse antibody. The membrane was then developed with 4-chloronaphthol and hydrogen peroxide. One band, of approximately 35 to 37 kDa, could be identified. Lanes: a, *M. avium* 101, without incubation with EGF (antibody steps were carried out as for the other lanes); b, *M. smegmatis*; C, *M. avium* 109; D, *M. avium* 101.

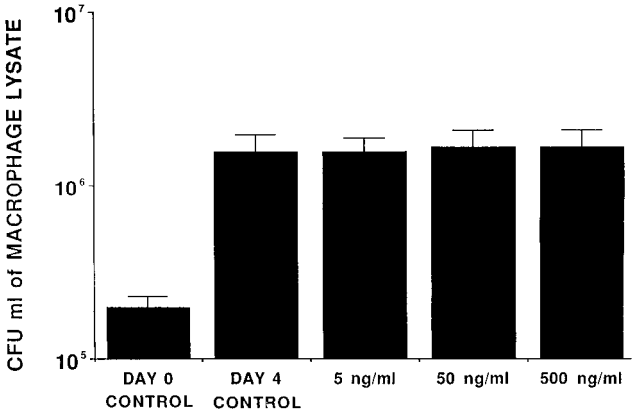

FIG. 4. Effect of EGF on intracellular growth of *M. avium*. *M. avium* (10^6 organisms) was used to infect a macrophage monolayer (10^5 macrophages). After 4 h, the extracellular bacteria were removed by washing (three times with HBSS). Monolayers were then treated with recombinant EGF (5, 50, or 500 ng/ml) for 4 days. Supernatant and EGF were replenished daily. After 4 days, the macrophages were lysed and the viable intracellular bacteria were quantitated as described previously (3). The numbers represent the means ± standard deviations of three different experiments. The numbers on the left indicate molecular weight markers (10^3).

avium within human macrophages. Infected macrophage monolayers (ratio, 10 bacteria/1 cell) were treated daily with EGF at 5, 50, or 500 ng/ml for 4 days. EGF had no effect on macrophage viability. After 4 days, the monolayers were lysed and the number of viable *M. avium* bacteria was determined. As shown in Fig. 4, treatment of macrophage monolayers with EGF had no influence on the growth of intracellular *M. avium.*

We then attempted to determine whether exposure of *M. avium* to EGF prior to phagocytosis had any effect on intracellular growth. *M. avium* cultured in Middlebrook 7H9 broth was treated with 5, 50, or 500 ng of EGF per ml for 2 days. Bacteria were then washed to remove EGF, and the inoculum was adjusted to 10^6 bacteria per ml. Bacteria were then used to infect human macrophages (ratio, 10 bacteria/1 cell). Whereas prior exposure to EGF did not influence phagocytosis by macrophages (data not shown), it resulted in an increase in the number of intracellular CFU when compared with *M. avium* that was not exposed to EGF (Fig. 5).

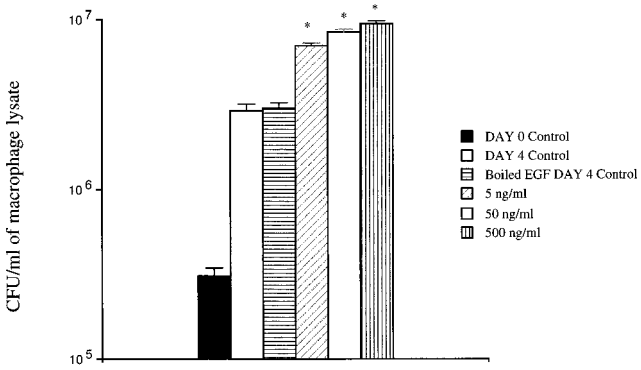

FIG. 5. Effect of incubation with *M. avium* with recombinant EGF prior to infection of macrophages on the intracellular growth of the organism. *M. avium* (10^7 bacteria) was incubated with recombinant EGF (5, 50, or 500 ng/ml) in Middlebrook 7H9 broth for 24 h at 37°C. The bacteria were washed, and the bacterial concentration was adjusted to 10^6 bacteria per ml. *M. avium* was then used to infect macrophage monolayers (10^5 cells) for 4 h. After this period, the monolayers were washed with HBSS to remove extracellular bacteria. Infected monolayers were incubated at 37°C for 4 days and then lysed to quantitate the viable intracellular bacteria. The numbers represent the means ± standard deviations of three different experiments. *, $P < 0.05$ for the comparisons between 5, 50, and 500 ng of EGF per ml and the control.

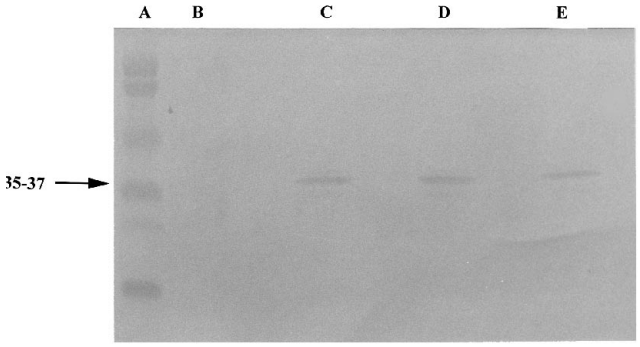

FIG. 6. Expression of recombinant EGF-binding protein of *M. avium* on *E. coli* HB101. Lanes: A, molecular weight markers; B, *E. coli* HB101; C, *E. coli* HB101 3 containing MAC DNA in plasmid pMV261; D, *E. coli* HB101 2 containing MAC DNA in plasmid pMV261; E, *E. coli* HB101 18 containing MAC DNA in plasmid pMV261. The numbers on the left indicate the molecular weight range (10^3) of the band indicated.

A genomic library of *M. avium* was created in pMV261 and transduced into *E. coli* HB101 (4). By screening transformed *E. coli* with mouse anti-human EGF antibody after incubation of the bacterium with human EGF, we were able to identify a protein of approximately 35 kDa that was not observed in the parent *E. coli* HB101 (Fig. 6). The 35-kDa protein was subsequently purified as described previously, and the N-terminal amino acids were sequenced. Figure 7 shows 20 amino acids of the *M. avium* EGF receptor. Search of the database showed that the recombinant *M. avium* 35-kDa protein has significant similarity to the streptococcal glyceraldehyde-3-phosphate dehydrogenase (GAPDH) that has been shown to correspond to a plasmin receptor protein in the bacterium (23). Anti-EGF receptor antibody was determined to bind to streptococci by Western blot (data not shown). The N-terminal amino acid of the mycobacterial GAPDH was shown to have 65% homology with the streptococcal GAPDH. Purified *M. avium* EGF receptor recombinant protein was subsequently shown to bind EGF in an enzyme-linked immunosorbent assay (data not shown).

DISCUSSION

Our data suggest that EGF produced in vivo during the process of tissue repair supports the growth of *M. avium* and *M. tuberculosis*. Furthermore, both organisms appear to have a receptor for EGF. The presence of receptors for cytokines on bacteria has been described previously. IL-1 binds to virulent *E. coli* but not avirulent *E. coli*, resulting in enhanced growth of the bacterium (28). IL-2 binds to *Candida albicans* (31), although the presence of high-affinity receptor on the fungus could not be demonstrated, suggesting that IL-2 binds nonspe-

^{1}AQGIRGREVFAINDIAAKNM^{20}	*M. avium*
^{1}IQNIEGVEVTAINDLADPNM^{20}	streptococci
^{1}RAAFLKNTVDVVSVNDPFIDLE^{20}	*S. mansoni*

FIG. 7. N-terminal amino acid sequence of EGF-binding protein of *M. avium* in comparison with N-terminal amino acid sequences of other similar proteins in the database (GenBank). The *Schistosoma mansoni* data are from reference 15.

cifically to the mannose-rich fungus. In addition, Denis and colleagues reported that both IL-2 and granulocyte-macrophage colony-stimulating factor stimulate growth of virulent *E. coli* (11). Most recently, high-affinity receptors for tumor necrosis factor alpha were described for *Shigella flexneri* (24). It was shown that *S. flexneri*-tumor necrosis factor alpha complex are taken up by macrophages with more efficiency than the bacterium itself. A similar mechanism that is capable of binding plasminogen, resulting in an enhanced ability of the microorganism to penetrate endothelial cell monolayers, was described for *Borrellia burgdorferi*. Furthermore, a number of pathogenic bacteria have receptors for different plasma proteins and extracellular matrix proteins, such as plasminogen, fibronectin, collagen, and laminin (17, 22, 32). A fibronectin receptor has been described for *M. tuberculosis*, *M. avium*, and *Mycobacterium leprae* (1, 29, 30). A previous study by Hide et al. (16) showed that antibodies to EGF receptor from mammalian cells cross-react with *Trypanosoma brucei* and recognize a 135-kDa protein on the parasite surface. EGF was shown to act in vitro as a growth factor for its procyclic form in both the absence and presence of serum.

The nature and function of the EGF receptor on mycobacteria are unclear, but the ability of recombinant EGF to stimulate bacterial growth indicates that the protein is most likely expressed on the bacterial surface. In addition, the ability of unlabeled EGF to compete with ^{125}I-EGF suggests that the binding is specific. The protein was cloned and overexpressed in *E. coli*. The protein sequence of the N-terminal amino acid of the purified mycobacterial EGF receptor protein showed that it is a GADPH with significant homology to the group A streptococcal GAPDH (23). In streptococci, GAPDH was shown to bind plasmin (23), fibronectin lysozyme, and cytoskeletal proteins (27). In eukaryotes, glycolytic enzymes, of which GAPDH is a key one, are found to be associated with membranes and subcellular cytoskeletal structures (2). Recently, proteins with a similar structure were found to be membrane bound in both eukaryotes and prokaryotes and involved in a variety of functions independent from its catalytic property (8, 15).

The presence of GAPDH on the surface of pathogenic mycobacteria may indicate a mechanism that rapidly connects the extracellular milieu with the intracellular trafficking machinery, sending signals that probably facilitate the adaptation of the bacteria to challenges encountered in the constantly changing extracellular environment. In fact, this possibility is supported by observations that GAPDH has been shown to have alternative functions besides glycolytic activity, such as bundling of microtubules, protein kinase activity, and uracil DNA glycosylase activity (19, 20, 25).

The implications of an EGF receptor on mycobacteria are presently unknown. However, it is plausible to hypothesize that EGF might have a role in the extracellular growth of *M. avium* and *M. tuberculosis* in the host, primarily in both granulomatous and necrotic tissues.

Further studies are needed to determine the regulation of expression of the receptor in mycobacteria and the function of the mycobacterial EGF receptor in vivo.

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