Mycobacteria and Human Autoimmune Disease: Direct Evidence of Cross-Reactivity between Human Lactoferrin and the 65-Kilodalton Protein of Tubercle and Leprosy Bacilli

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We document here by Western immunoblotting and immunogold ultracytochemistry that polyclonal antibodies against human lactoferrin (Lf) bind to tubercle and leprosy bacilli. In situ immunogold labeling of *Mycobacterium leprae* (present in armadillo liver and in human skin) and of *Mycobacterium tuberculosis* indicated that receptors for anti-Lf antibodies were present both on the cytoplasm and on the envelope of the bacilli. We found by immunoblotting that the 65-kDa heat shock protein is the major component of *M. leprae* and *M. tuberculosis* that is responsible for the binding of the anti-Lf probe. Furthermore, we show that anti-Lf immunoglobulin G eluted from the nitrocellulose-transferred mycobacterial 65-kDa protein band did bind back to Lf. Ultracytochemistry of biopsy samples of human lepromas showed that dead or severely damaged *M. leprae* was strongly marked by the anti-Lf antibodies; a similar pattern of immunogold marking was observed on *M. leprae* when antibodies against the 65-kDa mycobacterial protein were used. Our results offer direct evidence that the 65-kDa protein of leprosy and tubercle bacilli is recognized with specificity by antibodies against the human protein Lf. The Lf-65-kDa protein antigenic cross-reactivity may contribute to the formation of autoantibodies and immune complexes as well as to other autoimmune events that are frequent in tuberculosis and leprosy. Our immunocytochemical data also suggest that the cross-reactivity may persist for some time after the death of mycobacteria in infected hosts.

Association between the immune response against mycobacterial infections and autoimmune disease has long been suspected, particularly for autoimmune arthritis (6, 51, 55). The first historical evidence for this association was reported by Poncet at the end of the 19th century (48). It was confirmed later (e.g., reference 32) and strengthened by the observation of arthritic episodes after *Mycobacterium bovis* BCG immunotherapy (66). Elevated levels of antimycobacterial antibodies in sera of patients with rheumatoid arthritis were reported (7, 68), as was the presence of reactive T cells in the synovium (25, 31). Numerous studies of the so-called adjuvant rat model of arthritis have concurred to demonstrate an etiologic role of mycobacteria in autoimmune arthritis (9, 15, 16, 19, 70, 71).

We have recently reported that two mycobacterial species (*M. smegmatis* and *M. avium*) have receptors for polyclonal antibodies raised against human lactoferrin (Lf) and that these receptors pertain to the 65-kDa protein of these cultivable mycobacteria (1). This Lf-65-kDa protein cross-reactivity, if extended to *M. tuberculosis* and *M. leprae*, would become a candidate factor for the etiology of the autoimmune phenomena of tuberculosis and leprosy (36). There are, however, some structural differences between the 65-kDa protein of *M. avium* and *M. smegmatis* and its homologs in *M. tuberculosis* and *M. leprae*. These differences are immunologically relevant, since they are known to lead to differences in epitope recognition (4, 40, 43, 76). Thus, before considering a role for the Lf-65-kDa protein

cross-reactivity in human tuberculosis and leprosy, it is necessary to directly determine whether anti-Lf antibodies bind with specificity to *M. leprae* and *M. tuberculosis*. We used two different methods to address this question: immunoblotting and high-resolution immunocytochemistry. We demonstrate here that anti-Lf sera do react with the 65-kDa protein of both tubercle and leprosy bacilli, and we document the subcellular distribution of the anti-Lf labeling on both mycobacterial species in situ. We found that remnants of dead, extensively degraded *M. leprae* bacilli present in human lepromas were strongly marked by the anti-Lf probe; this suggests that the Lf-65-kDa protein immune cross-reactivity may persist in the host well after *M. leprae* bacilli are dead.

MATERIALS AND METHODS

***M. tuberculosis*.** Strain H37Rv of *M. tuberculosis* was grown in Löwenstein-Jensen medium, washed several times by pelleting and resuspension in saline-0.04% Tween 80, and fixed in an aldehyde mixture (see below) to be processed further for electron microscopy. *M. tuberculosis* homogenates were a kind gift of Arend Kolk and Sjoukje Kuijper of the N. H. Swellengrebel Laboratory of Tropical Hygiene of the Royal Tropical Institute, Amsterdam, The Netherlands.

***M. leprae*.** Liver samples from one armadillo naturally infected with *M. leprae* (collected by one of us [M. T. Silva] in southern Louisiana with the collaboration of F. Portaels and G. Walsh) were aldehyde fixed to be processed later for immunocytochemistry. Skin biopsy samples of lepromas of two Portuguese patients with lepromatous leprosy (50),

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FIG. 1. Western blots of human Lf (lane 1; 1 µg of protein), whole-cell homogenate of *M. tuberculosis* (lane 2; 25 µg of protein), whole-cell homogenate of *M. leprae* (lane 3; 25 µg of protein), and the *E. coli*-derived 65-kDa *M. bovis* BCG recombinant protein (lane 4; 0.2 µg of protein) labeled with polyclonal antibodies against human Lf. The anti-Lf antibodies label the 65-kDa protein of both tubercle (lane 2) and leprosy (lane 3) bacilli. The reference molecular mass standards were bovine serum albumin (66 kDa) and egg albumin (45 kDa). Molecular masses in kilodaltons are marked on the left.

supplied by Poiarés Batista and M. Lurdes Ferreira (Department of Dermatology, Faculty of Medicine, University of Coimbra, Coimbra, Portugal) were used both for diagnosis and for immunocytochemistry. One of the patients was biopsied before the beginning of treatment, and the other was biopsied after 6 days of treatment with rifampin, clofazimine, and dapsone. Homogenates of isolated *M. leprae* bacilli were offered to us by Paul Klatser (N. H. Swellengrebel Laboratory of Tropical Hygiene, Royal Tropical Institute).

Antibodies. Two different polyclonal antibodies against human Lf were used: (i) an immunoglobulin G (IgG) fraction of rabbit serum produced by subcutaneous injection of Lf in incomplete Freund's adjuvant (27; purchased from Dakopatts A/S, Copenhagen, Denmark [catalog no. A186]) and (ii) an affinity-purified anti-Lf IgG fraction raised in rabbits (purchased from Sigma [catalog no. L 3262]). Polyclonal antibodies against the *Escherichia coli*-derived recombinant *M. bovis* BCG 65-kDa protein (62, 64, 65) were obtained from rabbits and affinity purified.

Immunoblotting (Western blotting). Protein extracts of *M. tuberculosis* and *M. leprae* were concentrated in acetone at -20°C , centrifuged, and dried. They were then resuspended and denatured in sample buffer, boiled at 100°C for 3 min, and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% polyacrylamide) at 20 mA for 1 to 2 h. Aliquots of *E. coli*-derived recombinant *M. bovis* BCG 65-kDa protein (62, 64, 65) were also used. The gels were electroblotted overnight onto a nitrocellulose membrane (67). The nitrocellulose blots were washed with phosphate-buffered saline (PBS) and blocked for 1 h in PBS with 10% fetal calf serum or 5% skim milk. They were incubated with the anti-Lf antibodies for 2 to 4 h, washed in two changes of PBS-0.1% Nonidet P-40 during 30 min, incubated again for 2 h with peroxidase-labeled anti-rabbit IgG (Amersham, London, England), washed in PBS-0.1%

FIG. 2. Western blot of Lf (lane 1, 2 µg of protein; lane 2, 4 µg of protein) labeled with IgG eluted from a nitrocellulose transfer of the recombinant 65-kDa mycobacterial protein that had been incubated with anti-Lf antibodies. This immunochemical result demonstrates that the anti-Lf IgGs that bind to the mycobacterial protein are the same antibodies that recognize Lf.

Nonidet P-40, and then washed for 10 min in PBS. The peroxidase reaction was initiated by 2.8 mM 4-chloro-1-naphthol (or 100 µg of diaminobenzidine per ml) and 0.015% hydrogen peroxide in PBS and stopped by washing the blots in water. Controls for anti-Lf labeling were done with (i) anti-Lf serum absorbed with an excess of Lf and (ii) a nonspecific serum. To elute the IgG molecules of anti-Lf sera that bound to the recombinant *M. bovis* 65-kDa protein, we used the affinity purification method of Olmsted (46), as modified and adapted to Western blotting by others (29, 60). Briefly, nitrocellulose transfers of Lf were incubated with the antibody fraction recovered from the transferred 65-kDa protein band. The binding of the eluted antibodies to Lf was detected in nitrocellulose transfers of the human iron-binding protein by the immunolabeling method (peroxidase reaction) described above.

Electron microscopy. The isolated mycobacteria and the fragments of *M. leprae*-infected armadillo liver and skin biopsy samples of patients with leprosy were fixed in 0.3 to 0.5% glutaraldehyde-2% formaldehyde in 0.1 M phosphate buffer, pH 7.4, for 2 h at 4°C (24). The fragments were rinsed in PBS with 1 mM glycyl-glycine to quench free aldehyde sites (2), dehydrated in graded ethanols, and embedded in LR White resin (The London Resin Co., Ltd., Woking, England). Isolated *M. tuberculosis* were preembedded in a aldehyde-cross-linked matrix of bovine serum albumin (BSA) (3, 42). Resin polymerization was achieved overnight at 60°C . Thin sections of the tissues were collected on Parlodion-coated grids and treated for immunogold cytochemistry.

Immunogold ultracytochemistry. The grids with tissue or mycobacterial sections were incubated in the following solutions: PBS; PBS with 1% BSA; anti-Lf or anti-65-kDa protein antibodies raised in rabbits (see "Antibodies" above) in 1:32 to 1:64 dilutions in PBS; PBS with 1% BSA; and 10-nm colloidal gold particles coated by goat anti-rabbit IgG (Sigma [catalog no. G 3766]) in a 1:20 dilution in PBS containing 1% BSA, 3% NaCl, 5% fetal calf serum, and 0.05% Tween 80. The grids were then rinsed in the colloidal gold vehicle, PBS, and water. The grids were stained with uranyl acetate and lead citrate and were viewed in a Siemens Elmiskop 1A electron microscope. Two control experiments

FIG. 3 AND 4. Immunogold labeling (black dots) of tubercle bacilli by anti-Lf antibodies viewed in low-magnification (Fig. 3) and high-magnification (Fig. 4) electron micrographs of thin-sectioned bacteria. The immunogold probe labels both the cytoplasm and envelope of *M. tuberculosis*; the cell wall shows the highest density of marking seen on the bacilli. Magnification: Fig. 3, $\times 83,000$; Fig. 4, $\times 152,000$. Bars, $0.1\ \mu\text{m}$.

FIG. 5. Electron micrograph of a thin section of liver tissue from a nine-banded armadillo naturally infected with *M. leprae*. The leprosy bacilli are contained in large vacuoles of the armadillo Kupffer cells and are labeled by the immunogold method (black dots) with anti-Lf antibodies. Notice that the cytoplasm of the macrophage (at the top of the figure) is not significantly marked by the anti-Lf antibodies. Magnification, $\times 68,000$. Bar, $0.3 \mu\text{m}$.

were performed to search for labeling artifacts. (i) Nonspecific binding of the gold conjugate was screened by omitting the incubation in anti-Lf or anti-65-kDa protein antibodies in the immunogold procedure described above. (ii) Nonspecific

binding of antibodies to the sections was studied with samples treated with preimmune rabbit sera or with rabbit sera not specific for Lf or the 65-kDa protein (rabbit anti-human IgG).

FIG. 6. Electron micrograph of a thin section of a human skin leproma from a patient with lepromatous leprosy. The immunogold labeling (black dots) of the *M. leprae* bacilli indicates labeling with anti-Lf antibodies. The gold marker binds to both the cytoplasm and the cell wall of *M. leprae*. Some of the gold labeling is seen outside and in the vicinity of the bacilli (arrowheads at the top of the figure). Magnification, $\times 120,500$. Bar, $0.1\ \mu\text{m}$.

RESULTS

Western blotting. The Western blotting method was used to investigate whether anti-Lf sera bound specifically to proteins of tubercle and leprosy bacilli separated by SDS-PAGE. We found that the anti-Lf IgG bound with specificity to a protein band with a molecular mass of about 65 kDa present in both *M. tuberculosis* and *M. leprae* homogenates (Fig. 1). Comparison of the positioning and labeling of the two mycobacterial homogenates with that of nitrocellulose transfers of the 65-kDa protein of *M. bovis* BCG confirmed that the band did correspond to the 65-kDa protein (Fig. 1). The binding of the anti-Lf sera to the 65-kDa proteins of *M. tuberculosis* and *M. leprae* was specific, since it was not detected in the control labeling experiments: preimmune rabbit serum, antisera not specific for Lf, and Lf-absorbed anti-Lf IgG did not react with tubercle or leprosy bacilli or with the *M. bovis* 65-kDa protein.

Because we used polyclonal anti-Lf antibodies, we decided to employ the affinity purification method of Olmsted

(46), as modified by others (29, 60), to search for direct evidence that the anti-Lf IgGs that bound to the recombinant 65-kDa *M. bovis* BCG protein were the same antibodies that labeled Lf. This method involved two steps: the anti-Lf IgG bound to the 65-kDa protein was first eluted from Western blot transfers of the mycobacterial protein and was then reused to label nitrocellulose transfers of Lf. These experiments showed that the anti-Lf antibodies that bound to the recombinant 65-kDa mycobacterial protein did have the capacity to bind to Lf with specificity (Fig. 2).

Immunogold ultracytochemistry. We performed immunogold staining to determine by electron microscopy the subcellular distribution of anti-Lf labeling on *M. tuberculosis* and *M. leprae* sections. Antigenic preservation of the tissues required the use of fixation protocols (i.e., a low concentration of glutaraldehyde and no osmium postfixation) that do not offer the best mycobacterial ultrastructure (compare examples in references 56 and 58). This requirement results in some loss of microanatomical information (e.g., the visu-

alization of membranes) but nonetheless offers the topographical distinction between the cytoplasm and envelope areas of mycobacteria.

Both the cytoplasm and the envelope of tubercle and leprosy bacilli were labeled by the anti-Lf antibodies. The labeling was represented by the numerous black dots (10 nm in diameter) of immunogold seen in the mycobacterial profiles (Fig. 3 through 8). Heavy anti-Lf immunogold labeling was found on *M. tuberculosis* (Fig. 3). In high-magnification electron micrographs, it was clear that the cell wall showed the highest density of labeling and that the cytoplasm also depicted significant marking by the immunogold probe (Fig. 4). In the liver of a naturally infected armadillo, *M. leprae* cells were also heavily labeled by the anti-Lf probe; here, the leprosy bacilli were located inside large vacuoles of Kupffer cells (Fig. 5). Similar labeling results were obtained with anti-Lf antibodies on *M. leprae* present in skin macrophages from a human leproma (Fig. 6). A few gold spheres were sometimes detected outside, but near, the mycobacterial profiles (Fig. 6). In summary, all *M. leprae* cells seen either in the skin of human patients or in armadillo liver showed positive marking by the anti-Lf IgGs. In addition, when examining skin leproma tissue from a patient undergoing pharmacological treatment (rifampin, clofazimine, and dapsone), we also found heavy anti-Lf marking on degraded *M. leprae* that showed the ultrastructural features of nonviable bacilli. Furthermore, the most conspicuously altered bacilli (i.e., *M. leprae* remnants made up just of cell wall debris) present in the patient's skin continued to show a high density of anti-Lf labeling (Fig. 7). The pattern of anti-Lf immunogold marking of this *M. leprae* cell-wall debris (Fig. 7) was comparable to that obtained when antibodies against the 65-kDa mycobacterial protein were used (Fig. 8).

DISCUSSION

We document here that antibodies raised against human Lf specifically bind to tubercle and leprosy bacilli. We also show that the 65-kDa protein is, as in the case of *M. smegmatis* and *M. avium* (1), the major mycobacterial component responsible for the binding of the anti-Lf antibodies. This work, therefore, offers direct demonstration of antigenic cross-reactivity between a human protein (Lf) and a molecule of the infectious agents of tuberculosis and leprosy.

Lf is an iron-binding protein stored in neutrophilic granulocytes and in exocrine cells associated with mucosal surfaces of the body and is present in small amounts in plasma (24, 38). Lf is an immunomodulator that acts on macrophages (12, 13, 21, 37, 45), the same cells that are parasitized by mycobacteria. Macrophages also carry surface receptors for Lf (8). On the other hand, the 65-kDa protein is a major antigen of pathogenic mycobacteria, leading to strong humoral and cellular immune responses (5, 23, 28, 34, 35, 47, 73, 75). This mycobacterial antigen has been implicated in experimental and human autoimmune arthritis (31, 51, 68, 69, 72) and in autoimmune insulin-dependent diabetes (22). In the new, comprehensive rethinking of the immune re-

sponse put forward by Cohen and Young, the dominant, conserved microbial antigens, such as the 65-kDa mycobacterial protein, are viewed as pivotal elements in immune recognition of microorganisms and in the induction of autoimmune disease (17, 18, 20).

The antigenic cross-reactivity between the *M. tuberculosis* and *M. leprae* 65-kDa protein and human Lf may contribute to the high levels of autoantibodies often reported to be present in sera of patients with tuberculosis and leprosy, particularly lepromatous leprosy (10, 26, 30, 61). Recently, other instances of cross-reactivity between mycobacterial antigens and human tissues have been reported (44, 49). The presence of Lf in plasma is evidence in favor of the formation of autoanti-Lf antibodies adding to the pool of circulatory antigen-antibody complexes found in human mycobacterial infections. The occurrence of high concentrations of circulating autoantibodies, particularly during *erythema nodosum leprosum* episodes of leprosy, is known to be quite harmful for patients with lepromatous leprosy, mainly because of the deposition of immune complexes in kidneys (30, 41). Antibodies against Lf may also impair polymorphonuclear leukocytes (11), and it has been reported that neutrophil functions can be affected during autoimmune events of leprosy (33, 52, 74).

By immunogold ultracytochemistry, we found that the *M. tuberculosis* and *M. leprae* receptors for the anti-Lf IgG are present in all cellular compartments of the pathogenic mycobacteria. The cell wall location of these receptors makes them accessible to immune cells. This may be of particular importance for the immune cells that have surface receptors for Lf, as it is the case of B and T lymphocytes (14, 39) and macrophages (8). We found some anti-Lf marking around the *M. leprae* bacilli in human lepromas. Since the 65-kDa protein is a heat shock element, this extrabacillar labeling may be due to an increase in the secretion of the mycobacterial protein by the bacilli because of adverse environmental conditions (53, 54, 63).

A significant number of the *M. leprae* bacilli found in human lepromas were in a state of advanced degradation, a state in which they are commonly found in leprosy lesions (30, 57, 59). Those altered mycobacteria frequently consisted of cell wall remnants. This is because the cell wall of *M. leprae*, like those of other mycobacterial species (56), is not easily digested by the lysosomal armory of phagocytes. Consequently, "ghosts" of mycobacteria remain in lesions long after the death of the microbes. In this study, these mycobacterial remnants were heavily labeled by the anti-Lf antibodies, indicating that the bacterial debris kept its antigenicity for the anti-Lf probe well after the death of *M. leprae*. It is therefore possible that the persistence of mycobacterial remnants in patients leads to potentially harmful consequences resulting from antigenic cross-reactivity even after all of the mycobacteria are dead.

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FIG. 7 AND 8. Cell-wall debris of *M. leprae* in the skin tissue of a patient with leprosy under multidrug therapy. The mycobacterial remnants are labeled by anti-Lf antibodies in Fig. 7 and by antibodies against the recombinant 65-kDa mycobacterial protein in Fig. 8. The labeling is visualized by the immunogold method (black dots). The *M. leprae* cell wall debris is heavily labeled by both types of antibodies. Notice that the gold marking follows comparable patterns in the two samples, although they are labeled with distinct antibodies; this result is compatible and in agreement with the occurrence of epitope sharing between the anti-Lf and the anti-65-kDa protein antibodies. Magnification: Fig. 7, $\times 55,000$; Fig. 8, $\times 76,000$. Bars, 0.3 μm .

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