

## A chemoattractant cytokine associated with granulomas in tuberculosis and silicosis

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**ABSTRACT** Chronic inflammation and granuloma formation are associated with mononuclear cell infiltrates and are characteristic pathologic responses in tuberculosis. To identify host cell genes involved in tuberculous pathology, we screened macrophage cDNA libraries for genes induced by mycobacterial infection. One gene isolated in this screen, osteopontin (also known as early T lymphocyte activation protein 1 or Eta-1), was of particular interest because it is a cytokine and macrophage chemoattractant. Further study revealed that *Mycobacterium tuberculosis* infection of primary human alveolar macrophages causes a substantial increase in osteopontin gene expression. Osteopontin protein was identified by immunohistochemistry in macrophages, lymphocytes, and the extracellular matrix of pathologic tissue sections of patients with tuberculosis. Increased osteopontin expression also was found to be associated with silicosis, another granulomatous disease. The association of osteopontin with granulomatous pathology, together with the known properties of the protein, suggest that osteopontin may participate in granuloma formation. The strategy of identifying host genes whose expression is altered by infection thus can provide valuable clues to disease mechanisms and will be increasingly valuable as additional human genome sequences become available.

Mycobacterial infections are among the most numerous in the world, with *Mycobacterium tuberculosis* believed to have infected one-third of the world's population (1). Aggravating the worldwide pandemic have been the emergence of resistant organisms and the concurrent HIV epidemic. These events have renewed interest in understanding the fundamental biology of the interactions between pathogenic mycobacteria and their host.

Exposure to *M. tuberculosis* can lead to a pulmonary infection characterized by macrophage recruitment to the site of infection, followed in many cases by granuloma formation (2–4). *M. tuberculosis* is a facultative intracellular pathogen whose cellular habitat is the macrophage (5–9). Thus, the *M. tuberculosis*–macrophage relationship is of considerable interest. Previous investigations have revealed that interleukin 1, tumor necrosis factor- $\alpha$ , interleukin 6, transforming growth factor- $\beta$ , and interleukin 8 production by macrophages is increased by exposure to mycobacteria (10–14). Negative effects of mycobacteria on macrophage antigen presentation (15) and responsiveness to cytokines (16, 17) also have been documented. However, little is known about the molecular mechanisms involved in granuloma formation.

To gain insight into the host response to tuberculosis, we used differential screening of cDNA libraries to compare mRNAs of infected and uninfected macrophages. We reasoned that the identification of macrophage genes whose

expression is altered after phagocytosis of *M. tuberculosis* might provide clues to pathogenesis. Using a model system that used a macrophage cell line (18, 19), we surveyed the population of macrophage mRNAs for those altered by mycobacterial infection. Genes identified with this strategy then were studied in human cells exposed to *M. tuberculosis* and in tissues from patients with tuberculosis. One gene identified repeatedly in this screen was particularly interesting because its protein product, osteopontin, is a cytokine and macrophage chemoattractant (20, 21). Osteopontin gene expression was induced by mycobacterial infection of human macrophages, and osteopontin protein was found in human tissue specimens from patients with clinical tuberculosis.

### METHODS

**Bacteria.** *Mycobacterium bovis* bacillus Calmette–Guérin (American Type Culture Collection no. 35734) was grown from a frozen stock for 3 days (to OD<sub>600</sub>  $\approx$  1.2) in Middlebrook 7H9 broth with 0.5% glycerol, 0.05% Tween 80, and ADC enrichment (Difco). BCG diluted 1:10 in RPMI medium 1640 with 1% fetal calf serum was added to J774 cells (bacteria:macrophage ratio approximately 10:1). The streptomycin-dependent strain of *Escherichia coli*, sd-4 (American Type Culture Collection no. 11143), was cultured in Luria–Bertani medium with streptomycin at 624  $\mu$ g/ml. Frozen stocks in 20% glycerol were made from stationary-phase cultures after 2 days growth in Luria–Bertani medium containing 25  $\mu$ g/ml streptomycin. On the day of infection these bacteria were thawed, were resuspended in 7H9 medium, and were diluted as for BCG (bacteria:macrophage approximately 1:1). These two ratios were required to achieve comparable percentages of infected macrophages; limited growth of the *E. coli* in the absence of streptomycin necessitated a lower ratio. Latex beads (0.8  $\mu$ m, carboxylate-modified, Sigma no. L-1398) were treated with 70% ethanol before use.

**Macrophage Culture and Infection.** J774 cells (American Type Culture Collection no. TIB 67) were grown without antibiotics in RPMI medium 1640 with 10% low-endotoxin fetal calf serum (GIBCO/BRL) until infection. These cells were infected by incubation with either bacteria or beads for 4 hr, were washed three times with Hanks' balanced salt solution, and were cultured for an additional 20 hr before RNA harvest. During and after infection, cells were maintained in 1% low-endotoxin fetal calf serum as previously described (18). Alveolar macrophages were obtained from consenting human donors. Bronchoalveolar lavage cells were harvested by bronchoscopy and plated at a density of 500,000 cells/ml. Alveolar macrophages were purified by adherence to plastic after growing 4 days in RPMI medium 1640 with 10% fetal calf serum. Cells were infected as described above except that cultures of *M. tuberculosis* H37Rv and BCG were

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Abbreviations: BCG, bacillus Calmette–Guérin; MIP-1 $\alpha$ , macrophage inflammatory protein 1- $\alpha$ .

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passed through a 25-gauge needle before addition to macrophages.

**Library Construction and Screening.** RNA was isolated using the guanidinium isothiocyanate/CsCl method (22). Five micrograms of poly(A)<sup>+</sup> RNA isolated by oligo(dT) chromatography was used to construct a cDNA library from BCG-infected J774. The Superscript cDNA kit (GIBCO/BRL) was used to generate first-strand cDNA using oligo(dT). Second-strand cDNA was synthesized using a modification of the protocol of Gubler and Hoffman (23). The resulting cDNA was ligated into  $\lambda$ gt10 using *EcoRI*–*NotI* linkers. Recombinant phage were selected and amplified using standard techniques. The cDNA library prepared from BCG-infected J774 cells contained  $1.1 \times 10^6$  independent recombinants with an average insert size of greater than 1 kb. Of six random phage isolates, all contained inserts.

A nonradioactive detection method, the Genius System (Boehringer Mannheim) was used for library screening. Preparation of digoxigenin-labeled cDNA probes from poly(A)<sup>+</sup> RNA, hybridization, and detection with Lumi-Phos 530 or NBT/X-phosphate were used as per the manufacturer's protocol. Plaques were screened by incubating duplicate filters with digoxigenin-labeled cDNA from *E. coli* or BCG-infected macrophages.

**RNA Analysis.** Ten micrograms of total cellular RNA was run per lane of a 1% glyoxal/dimethyl sulfoxide agarose gel and transferred to nylon membranes (Amersham). Hybridization, washing, and probe stripping were as directed by the membrane manufacturer. Probe fragments isolated from agarose gels by using DEAE membrane (Schleicher & Schuell) or by using silica adherence (GeneClean II, SUN Bioscience) were labeled by random priming (PrimeIt II, Stratagene). Relative signals were quantitated with a phosphorimager (Fuji) and were normalized to the actin signals.

**Quantitative PCR Analysis.** One microgram of total RNA was reverse-transcribed using Superscript reagents (GIBCO/BRL). The PCR used 1/10th of the reverse-transcribed cDNA in a 100- $\mu$ l volume, and included 10  $\mu$ l of  $10\times$  *Taq* buffer, 4  $\mu$ l of 5 mM dNTPs, 50 pmol of each primer, 1  $\mu$ l of competitive template, and 1  $\mu$ l (5 units) of *Taq* DNA polymerase (Perkin-Elmer). The reaction was incubated for 35 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 1 min. The competitive template was generated by insertion of a 333-bp DNA fragment from an *NdeI* digest of the yeast *RPB1* gene to the unique *NdeI* site of the human osteopontin gene. The primers used were 5'-CACCTGTGCCATACCAGTTAAACAG-3' and 5'-CATGGCTGTGAAATTCATGGCTGTG-3'. These primers allowed amplification of an 830-bp cDNA fragment and a 1,163-bp fragment from the competitive template. No product was seen when these primers were used to amplify this fragment from 1  $\mu$ g of purified genomic DNA. Commercially available actin primers were used (Stratagene).

**Immunohistochemical Staining.** Tissue sections were obtained from the Gaensler Lung Archive at the Boston University Pulmonary Center and the Boston Veterans Affairs Hospital. These specimens are paraffin-embedded tissue processed using standard clinical techniques. Immunohistochemical staining was done on a Ventanna ES automated stainer (Ventanna, Tucson, AZ). Sections 5  $\mu$ m thick were baked for 1 hr at 60°C onto positively charged slides and then deparaffinized with xylene and hydrated in graded alcohol washes to water. Slides were then processed with Ventanna's protease I reagent for 4 min and incubated with primary antibodies for 32 min at 42°C. The manufacturer's protocol was used for the diaminobenzidine kit (osteopontin) and the fast red kit (CD68). Images were photographed with Kodak Ektachrome or Ektar film and scanned into Adobe Photoshop to create composite figures.

mAbs MPIIB10<sub>1</sub>, specific for rat and human osteopontin, and QH1, specific for quail vascular endothelial cells, were obtained from the Developmental Studies Hybridoma Bank

(Iowa City, Iowa) and used at 1:200 dilution of hybridoma supernatant (400 and 500 ng/ml, respectively). mAb KP-1 (24) recognizing human CD68 was obtained from Dako.

## RESULTS

**Identification of Genes Associated with Infection of Macrophages.** The effect of bacterial infection on macrophage gene expression was investigated by differential screening of cDNA libraries. The murine macrophage cell line J774 was used in these initial experiments because previous work has established its usefulness for studying interactions between mycobacteria and macrophages (18, 19). Differential screening of libraries has the advantage of detecting smaller changes in relative mRNA levels than subtractive hybridization.

The screening strategy is summarized in Fig. 1. The J774 cells were exposed to latex beads, or *E. coli*, or *M. bovis* BCG, and recombinant cDNA libraries and probes were prepared from the macrophage poly(A)<sup>+</sup> mRNA. The phage library representing mRNAs of macrophages infected with BCG was plated with host bacteria, and duplicate filters were placed on the plaques. These filters then were hybridized with cDNA probes prepared from J774 cells infected with either *E. coli* or BCG, and plaques with different signal strengths were isolated. This screening strategy was designed to identify genes whose expression differed when macrophages were exposed to BCG versus *E. coli*. The library constructed with mRNA from BCG-infected J774 cells was screened to maximize the chance of identifying genes whose expression was specifically increased by BCG.

Approximately 730,000 recombinant phage were screened by probing duplicate filters with labeled cDNA prepared from J774 cells that had phagocytized BCG or *E. coli*. Three clones were isolated that produced stronger signals with cDNA from macrophages infected with *E. coli*. Sequencing of their insert DNAs revealed that one cDNA clone encoded macrophage inflammatory protein 1- $\alpha$  (MIP-1 $\alpha$ ) (25) and two encoded ferritin (26). Ten clones were isolated that produced stronger signals with cDNA from macrophages infected with BCG. All of these encoded osteopontin (27).

**Differential Expression of MIP1 $\alpha$ , Ferritin, and Osteopontin mRNAs.** To confirm that the three genes represented by the cDNA clones identified by the screening process were indeed differentially expressed, radiolabeled insert DNAs from the clones were used to probe macrophage mRNA immobilized on nylon filters (Fig. 2A). The mRNAs of all three genes accumulated to higher levels when macrophages phagocytized the two bacterial species versus the latex beads.

Osteopontin mRNA accumulated to similar levels in macrophages exposed to latex beads or *E. coli*, but accumulated to

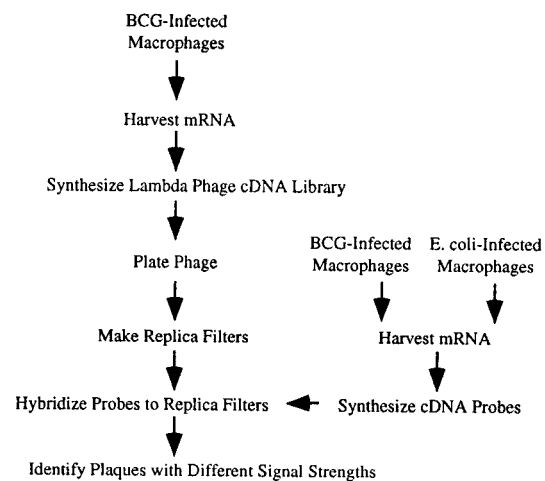


FIG. 1. Screening strategy using lambda phage cDNA library analysis.

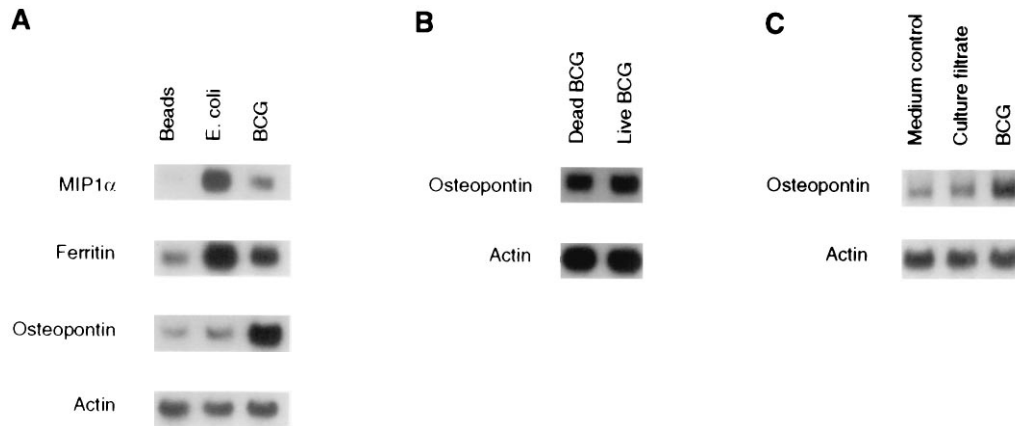


FIG. 2. Analysis of gene expression by Northern hybridization of total cellular RNA prepared from J774 cells stimulated with various agents. (A) Confirmation of differential expression of genes identified by phage library screening. J774 macrophages were stimulated with latex beads, *E. coli*, or BCG and RNA was probed with labeled MIP-1 $\alpha$ , ferritin, osteopontin, and actin cDNAs. (B) Osteopontin mRNA levels are equal in J774 macrophages that have phagocytized dead or live BCG. Heat-killed BCG were prepared by heating a 1-ml culture of BCG in Middlebrook 7H9 broth for 15 min at 65°C. This treatment reduced bacterial viability more than 10,000-fold. (C) Osteopontin mRNA is induced in J774 macrophages by the BCG bacillus but not Middlebrook 7H9 medium or a BCG culture filtrate prepared with a 0.2- $\mu$ m filter.

at least 5-fold higher levels after infection of macrophages with BCG (Fig. 2A). There were similar increases in osteopontin mRNA levels in macrophages that had phagocytized live and heat-killed BCG (Fig. 2B). As shown in Fig. 2C, neither 7H9 medium nor supernatant from a BCG culture was capable of eliciting the increase in osteopontin expression caused by BCG organisms. Thus, osteopontin mRNA levels increase substantially in murine macrophages that phagocytize BCG.

**Osteopontin Expression in Human Lung Macrophages.** We investigated whether increased osteopontin gene expression occurs in primary human cells in response to phagocytosis of virulent *M. tuberculosis*. Human alveolar macrophages were exposed to latex beads, BCG, and *M. tuberculosis* strain H37Rv. To determine precisely the number of osteopontin mRNA molecules present per cell before and after infection, a reverse transcriptase-PCR assay was used with varying levels of competing template. The results demonstrate that the levels of osteopontin mRNA were higher in alveolar macrophages infected with BCG (Fig. 3, lane B) and virulent *M. tuberculosis* (Fig. 3, lane Rv) than in macrophages that had phagocytized latex beads (Fig. 3, lane L). The human alveolar macrophages that had phagocytized latex beads contained approximately 1 molecule of osteopontin mRNA per cell. In contrast, the alveolar macrophages that had

phagocytized BCG and *M. tuberculosis* had about 10 molecules of osteopontin mRNA per cell. This magnitude of increase in osteopontin mRNA after exposure to mycobacteria was similar to that observed with the murine macrophage model.

**Osteopontin in Human Granulomatous Diseases.** The increase in osteopontin mRNA observed in infected alveolar macrophages predicts that osteopontin protein levels should increase in human tissues infected by *M. tuberculosis*. We therefore investigated whether the presence of osteopontin protein in patient tissues correlated with the histopathology of tuberculosis. Tissue sections from normal and diseased lungs were stained with a mAb specific for osteopontin, MPIIB10<sub>1</sub>, with a mAb specific for the macrophage antigen CD68 (24), or with an isotype control, QH 1 (28). The MPIIB10<sub>1</sub> mAb has been used extensively to identify osteopontin by immunohistochemistry (29, 30) and immunoprecipitation (31, 32).

Osteopontin was readily identified throughout specimens of tuberculous lung. The architecture of a tuberculosis lesion with necrosis (N) and with a lymphoid aggregate (L) above alveolar air spaces was evident in sections treated with the isotype control antibody and the hematoxylin counterstain (Fig. 4A). No staining (brown color) of this specimen was observed with the isotype control (Fig. 4A). However, when an adjacent serial section was probed with the MPIIB10<sub>1</sub> antibody, a strong osteopontin signal was detected in the inflammatory border surrounding the necrosis, and immediately adjacent to and within the areas of caseating necrosis (Fig. 4B). At higher power, macrophages were identified in a serial section with anti-CD68 antibody and the fast red reagent (Fig. 4C). These cells also expressed osteopontin (Fig. 4D). Lymphocytes identified at low power and at high power in Fig. 4A and C (L) demonstrated intense osteopontin signal singly and in aggregates (Fig. 4B and D). Giant cells expressed CD68 (Fig. 4E) but did not universally stain for osteopontin; in general, epithelioid giant cells expressed osteopontin (Fig. 4F). Bronchiolar epithelium (Fig. 4G) and small blood vessels (Fig. 4H and below columnar epithelium in Fig. 4G) stained with the MPIIB10<sub>1</sub> antibody though they have not been known to express osteopontin in normal adult tissue (33–36). In addition, osteopontin appeared in layers near caseation (Fig. 4B) and was intimately associated with fibroblast-like cells in a number of views (Fig. 4F, G, H). In summary, osteopontin was identified in a variety of cell types within the tuberculosis specimens.

Specimens from normal lung, an acute inflammatory condition (bacterial bronchopneumonia), and two chronic inflammatory conditions (silicosis and granulation tissue of a pilonidal cyst) were studied to assess the variety of situations in

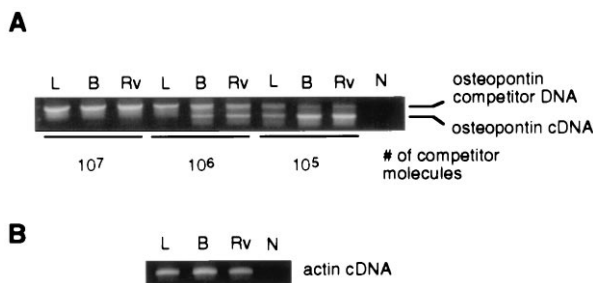


FIG. 3. Quantitative analysis of osteopontin mRNA expression by human alveolar macrophages using competitive reverse transcriptase-PCR. (A) Competitive PCR using primers specific for osteopontin cDNA. Osteopontin competitor cDNA had binding sites for the PCR primers but contained an inserted DNA sequence to allow for the generation of a larger-sized PCR product. L, cDNA from human alveolar macrophages that phagocytized latex beads; B, cDNA from human alveolar macrophages that phagocytized BCG; Rv, cDNA from human alveolar macrophages that phagocytized *M. tuberculosis* H37Rv; N, no cDNA added to the PCR. cDNA from approximately 100,000 cells was used in each reaction. (B) PCR using primers specific for control actin cDNA.

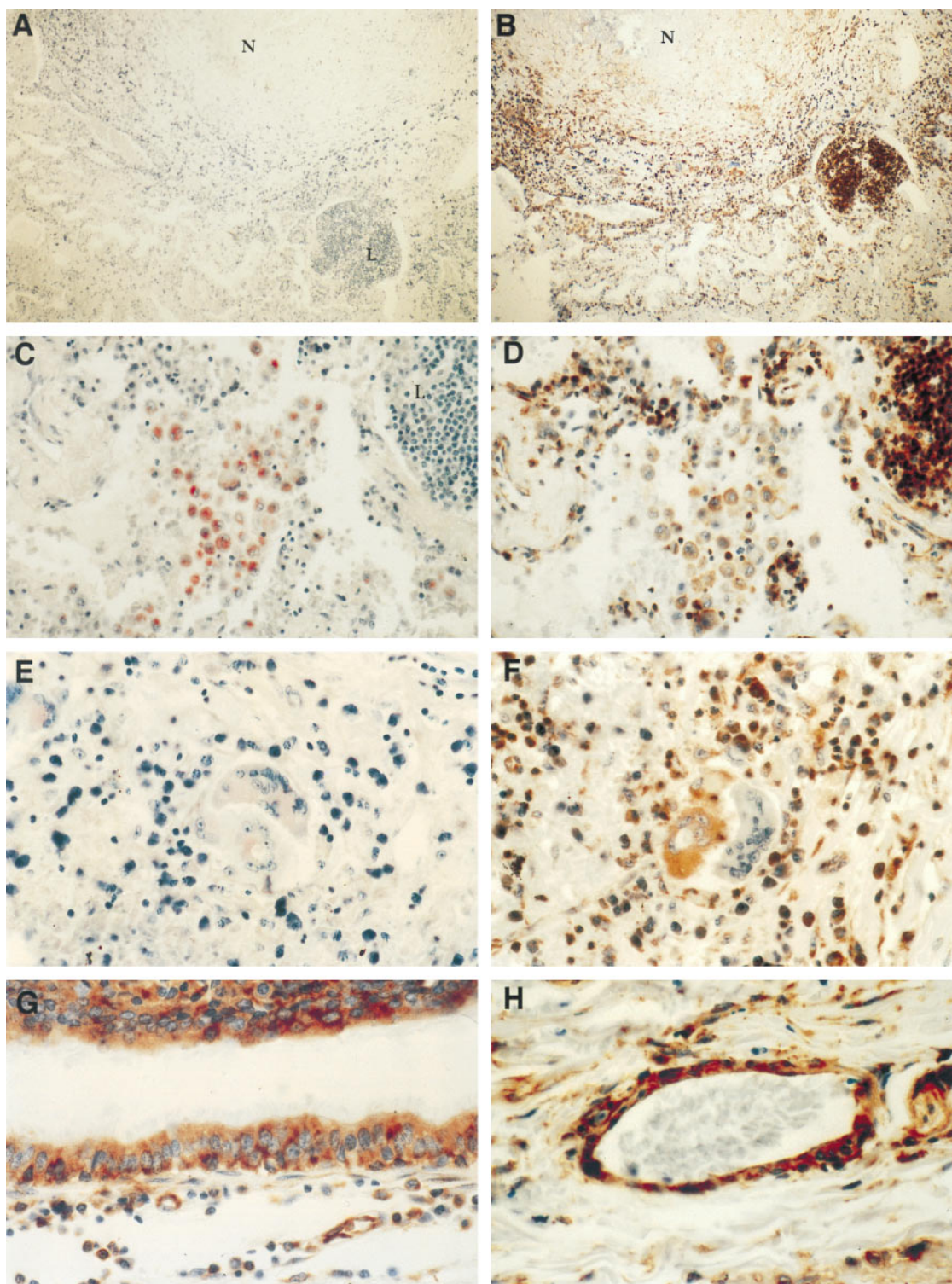


FIG. 4. Immunohistochemical stain of human tuberculosis specimen. (A) Tuberculosis granuloma stained with isotype control antibody showing hematoxylin counterstaining of nuclei, ×90. Area of caseating necrosis (N), lymphoid aggregate (L). (B) Serial section of granuloma in A, stained for osteopontin with mAb MPIIB10<sub>1</sub>, ×90. (C) Serial section of same specimen stained with anti-CD68 first-step antibody demonstrating macrophage aggregate, ×360. (D) Higher power view of B showing same macrophage aggregate staining for osteopontin, ×360. (E) Giant cells in specimen stained for CD68, ×900. (F) Higher power view of B showing osteopontin staining of epithelioid, but not Langhans, giant cell, ×900. (G) Small airway respiratory epithelium stained for osteopontin; same section though different field of B, ×900. (H) Small vessel staining with MPIIB10<sub>1</sub>, ×900. Similar results were obtained with sections from multiple specimens and patients.

which osteopontin could be detected. Normal lung did not show significant staining for osteopontin either in alveolar networks (Fig. 5A) or in airways (not shown). The bronchopneumonia specimen had scattered cells of macrophage and lymphocyte morphology that produced some signal with

MPIIB10<sub>1</sub> (Fig. 5B). Lymphoid aggregations associated with the pneumonia were also positive for osteopontin (not shown). The two chronic inflammatory conditions showed dichotomous results for the presence of osteopontin. Silicosis is a noninfectious granulomatous disease (37). The silicotic nodule

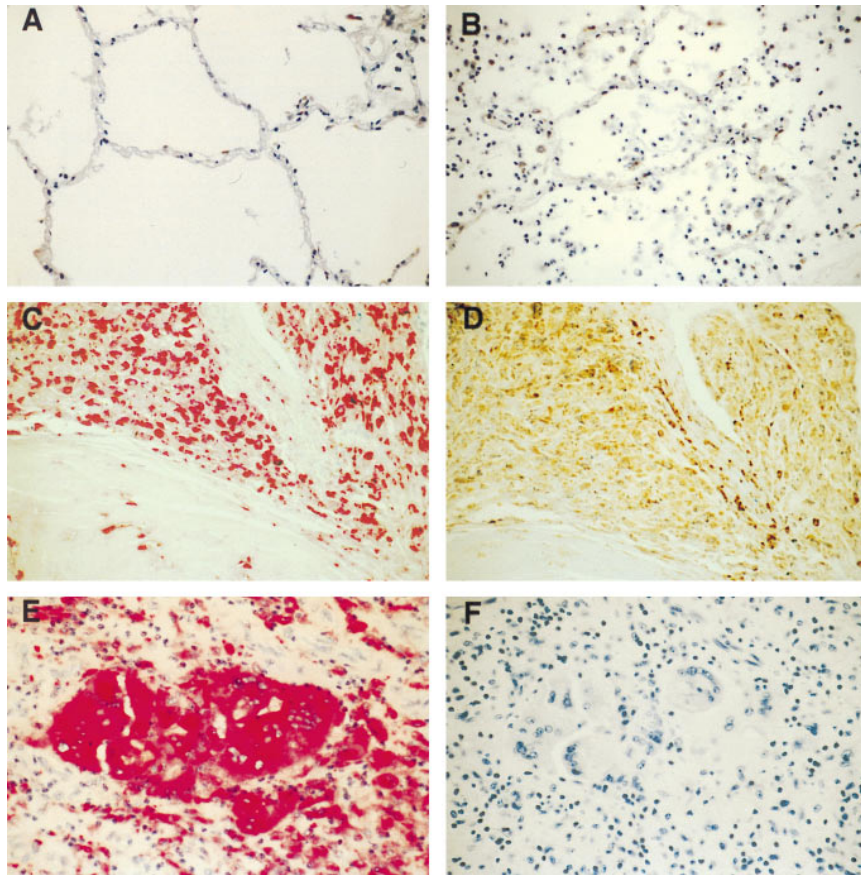


FIG. 5. Immunohistochemical stain for osteopontin in other clinical states. (A) Normal lung alveoli stained for osteopontin with mAb MPIIB101. (B) Bacterial bronchopneumonia stained with MPIIB101. (C) Lung section from silicosis patient stained for CD68. Intense red color corresponds to fast red color substrate. (D) Silicosis specimen stained with MPIIB101. (E) Granulation tissue stained for CD68. (F) Granulation tissue from pilonidal cyst stained for osteopontin. (All images  $\times 260$ .)

with its characteristic fibrosis showed an abundance of macrophages that stained with CD68 (Fig. 5C). These cells, and presumably some of the extracellular matrix, showed heavy signal for osteopontin on immunohistochemical staining (Fig. 5D). Granulation tissue is another pathologic process characterized by mononuclear cell infiltration but is distinct from granulomatous inflammation (37). While the granulation tissue of the pilonidal cyst showed a large aggregation of giant cells and numerous macrophages based on CD68 staining (Fig. 5E), there was no osteopontin detected (Fig. 5F). Thus, only tuberculosis and silicosis, the two granulomatous conditions, expressed high levels of osteopontin in the tissue pathology.

### DISCUSSION

The experimental approach used here, employing differential screening of cDNA libraries from infected cell lines, should continue to be useful in future studies of host-pathogen interactions. A model system using a macrophage cell line was essential to permit infections under controlled conditions and to provide the numbers of cells necessary to generate cDNA libraries and probes. This model system, employing the macrophage cell line used by Rastogi and colleagues (18), had excellent predictive value. Our results agree with previous reports linking MIP-1 $\alpha$  and ferritin expression with exposure to lipopolysaccharide and BCG (38–40). The J774 cell line also has been used in a PCR-based strategy to determine gene expression changes after exposure to *Listeria monocytogenes* (41). In contrast to previous work, we have extended the observations made with the *in vitro* model to confirm the presence of elevated levels of osteopontin and MIP-1 $\alpha$  (data not shown) in human tissue pathology.

Using this approach we have identified a novel association between osteopontin and two granulomatous diseases, tuberculosis and silicosis. Infection of primary human alveolar macrophages with *M. tuberculosis* causes a substantial increase in osteopontin gene expression. Elevated levels of osteopontin protein are found in macrophages and lymphocytes in pathologic tissue sections from patients with tuberculosis. The association of osteopontin with tuberculosis and granulomatous disease, together with previous evidence that osteopontin is a cytokine and macrophage chemoattractant, implicate osteopontin in granuloma formation.

Though osteopontin gained its name from its presence in bone (42, 43), it is expressed in a variety of cell types (27, 34, 44), including T cells (refs. 45, 46; reviewed in ref. 21), and is likely to be important in host responses to infection. This secreted glycosylated phosphoprotein has been shown to promote macrophage migration and adhesion (47, 48). The discovery of a receptor-ligand interaction between CD44 and osteopontin has led to the proposal that this interaction may mediate migration of activated lymphocytes and monocytes out of the bloodstream into sites of inflammation (49). Osteopontin may enhance intercellular interactions and facilitate binding to the extracellular matrix via CD44 and integrins (21). This interaction could anchor CD44<sup>+</sup> cells because osteopontin can be covalently linked to fibronectin by transglutaminase (50).

The accumulation of osteopontin in tissues infected with *M. tuberculosis*, and osteopontin's role in macrophage migration and adhesion, suggests a model for granuloma formation. In this model, phagocytosis of *M. tuberculosis* by alveolar macrophages leads to induction of osteopontin gene expression and protein secretion. The secreted osteopontin stimulates accumulation of

monocytes and macrophages, perhaps by promoting chemotaxis, adhesion, and anchoring of these cells, to initiate granuloma formation. This model is attractive because it can account for the genesis and maintenance of the unusual tissue pathology associated with granulomas. However, granuloma formation appears to require more than the simple expression of osteopontin. Low levels of osteopontin were observed in a subset of cells of bacterial bronchopneumonia tissue sections (Fig. 4B) and in certain cell types in normal specimens (34) in the absence of granuloma formation. The duration and level of expression of osteopontin, the specific cells producing the protein, and other factors in the tissue environment, all may determine whether granuloma formation is the histologic outcome of an inflammatory process.

Future studies with differential screening strategies should reveal additional associations between specific gene products and infectious processes. The identification of genes that are differentially expressed in infected host cells might use differential cDNA screening, differential display (41, 51), or high-density oligonucleotide arrays (52). As human genome sequences become more available, these approaches will be even more valuable in identifying important elements of host defenses.

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