Nocardia asteroides Recovery from a Dog with Steroid- and Antibiotic-Unresponsive Idiopathic Polyarthritis

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This report describes a fatal case of idiopathic polyarthritis in a dog that was partially responsive to vigorous immunosuppressive treatment. Synovial fluids were cultured for L-forms at the following stages of disease: (i) acute arthritic relapse, (ii) incomplete remission, and (iii) death. *Nocardia asteroides* UCD 1-581 was recovered from the L-form broth culture of the specimen taken during acute relapse, 5 weeks after inoculation, but not at any other stage of disease. Numerous conventional microbiological cultures were unproductive during all phases. Changes occurring in L-form plates included the formation of large irregular mineral deposits and many transferable bodies resembling pseudocolonies. Microscopic examination revealed the presence of many intracellular golden-brown granules and acid-fast bodies in macrophages of the lung and bronchial lymph node tissues. The granules are believed to be the variants embedded in calcium deposits similar to those which developed in the L-form cultures in vitro. Fluorescence of these acid-fast bodies with antibody specific for superoxide dismutase of *N. asteroides* GUH-2 and labeled anti-immunoglobulin G established their relationship to the isolate. The unrelenting course of disease and the persistence of *N. asteroides* as an L-form in this animal despite vigorous immunosuppression suggest that this organism plays a direct role in the etiology of this disease.

Idiopathic polyarthritis (IPA) is the most frequent immune mediated joint disease in the canine species (26, 27). It occurs in dogs between 2 and 8 years of age. In clinical presentation, it resembles the nonerosive arthritic components associated with systemic lupus erythematosus and chronic bacterial, e.g., actinomycete, infections in dogs (3, 26, 27). It differs from these diseases in that antinuclear antibodies and a primary infectious disease process are not demonstrable. In addition, the joint disease is the sole manifestation of illness. Treatment of IPA, as well as systemic lupus erythematosus, is directed toward control of the arthralgia with steroids and more potent drugs as necessary (3, 27).

The etiology of canine polyarthritis is unknown. Synovial fluids exhibit the characteristics of sepsis but are sterile when cultured for bacteria, mycoplasma, chlamydia, and viruses (27). The possible involvement of a bacterial L-form has not been fully explored. Methods for recovering the above agents would not necessarily be effective for isolation of an L-form (25).

In this paper, we document the isolation of *Nocardia asteroides* from a dog with IPA that

was refractory to steroids and required vigorous immunosuppressive therapy to provide a partial remission. The organism was isolated 5 weeks after inoculation of synovial fluid into a hyperosmolar broth medium enriched with fetal calf serum. The morphology of the transferable L-form variant colonies developing in all cultures and the efforts to achieve L-form reversion a second time are given in this paper. Intracellular acid-fast bodies were detected in macrophages of lung and bronchial lymph node tissues collected at death. Fluorescence of these bodies when stained with immunoglobulin G antibody specific for *N. asteroides* superoxide dismutase (SOD) and labeled anti-immunoglobulin G established a relationship of the isolate to the animal. The persistence of *N. asteroides* as an L-form in this animal despite vigorous immunosuppression suggests that *N. asteroides* has a direct role in the etiology of this disease. The characteristics of canine nocardiosis and of IPA are compatible with this view.

CASE REPORT

A 7-year-old male German shepherd-mix breed dog was referred to one of us (N.C.P.) on 30

September 1980 for treatment of shifting polyarthritis refractory to ampicillin and chloramphenicol. Periodically over the next 10 months, aspirates of the hock, stifle, or carpal joints or all three yielded thin fluids containing extracellular lipid and 30,000 to 80,000 leukocytes per mm³, most of which were polymorphonuclear neutrophils. Serum samples were excessively lipemic. Numerous cultures of blood, serum, and synovial fluids were negative for normal bacteria and mycoplasma during several relapses. Combinations of at least two and sometimes three drugs were required to produce and maintain incomplete remissions. Among the drugs used were prednisone, dexamethasone, cyclophosphamide, azathioprine, Leukeran, and a gold compound, aurothioglucose. Any alteration of the drug combination caused relapses that increased in severity as the disease progressed. L-form cultures of synovial fluid from the left hock were initiated during the first acute relapse, and *N. asteroides* was isolated 5 weeks after inoculation. The agent could not be isolated in its normal form during partial remission or at death. Trimethoprim-sulfamethoxazole was added to the ongoing drug regime for 8 weeks, resulting in the longest period of well-being. The dog died after 10 months of continuous immunosuppressive therapy. No masses, abscesses, or granulomatous tissues were evident in a thorough post-mortem examination. There was severe interstitial pneumonia with infiltration of alveoli by macrophages and neutrophils. The macrophages of the lung and bronchial lymph node tissues contained large numbers of golden-brown granules. Many phagocytic cells contained acid-fast bodies.

MATERIALS AND METHODS

L-form media. Various hyperosmolar broths and plate media were employed during the course of this work to encourage reversion of an L-form. Media I and II were similar to a medium described by Barile et al. (2). The following ingredients were dissolved in 150 ml of deionized distilled water: 7.4 g of brain heart infusion broth (Difco Laboratories, Detroit, Mich.) and an osmolar supplement, either 20 g of sucrose (type I) or 6 g of NaCl–10 g of sucrose (type II). The pH was adjusted to 6.9, and Difco Noble agar was added (0.02 g for broth; 2 g for plates). The basal mixtures were autoclaved, mixed briefly, and cooled to 56°C in a water bath. They were converted to the complete items by adding 40 ml of unheated, twice-filtered fetal calf serum (gift from J. R. Scientific Co., Woodland, Calif.) and 10 ml of yeast extract, pH 7.8 (freshly prepared from a 25% aqueous slurry of active dry yeast) (16).

Media III and IV were prepared in essentially the same manner as given above except for the use of 5.0 g of heart infusion broth (Difco) instead of brain heart infusion as the base. Medium III contained 10% sucrose, whereas medium IV contained 3% NaCl and 5% sucrose as osmotic stabilizers. Medium V was identi-

cal to medium IV except for the addition of 2.39 g of *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 0.176 g of putrescine to the base before autoclaving. Medium VI was similar to medium V with the additional supplements of 100 µg of valinomycin per ml and 0.01 M KCl. The pH of these media was 7.0 to 7.1. The assembly of the complete media, plating, and tubing was performed in a sterile room, using a hood. Plates were held upright overnight before placement in a refrigerator.

Control L-form broth and plates from each lot were incubated at 37°C for 8 weeks and were examined weekly with the test cultures for evidence of contamination and pseudocolony appearance.

Inoculation and incubation of cultures. The synovial fluid collected during the first acute relapse was cultured in medium I, whereas specimens collected during partial remission and at necropsy were cultured on all media. Samples were collected in syringes and delivered to the laboratory with the needles thrust into sterile rubber stoppers. They were processed in a laminar flow sterile hood within 30 min of collection. Droplets were placed on: Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) plates containing 5% sheep erythrocytes, Trypticase soy agar plates containing 5% laked sheep erythrocytes and 0.1 µg of menadione per ml, and two medium I plates, and one of all other types of L-form plates. Thioglycolate broth and L-form broths were inoculated with about 1 ml each of sample. The BA and L-form plates were incubated in two disposable zip-lock storage baggies (The Dow Chemical Co., Indianapolis, Ind.; 178 by 203 by 0.04 mm) along with two extra plates (any medium type) inoculated with *Bacillus cereus* to provide CO₂. The menadione-laked erythrocyte plate and one L-form plate were incubated in a GasPak anaerobic jar (BBL) with H₂ plus CO₂ generator envelopes and a freshly heat-rejuvenated palladium catalyst. The L-form broth cultures were incubated in a normal gaseous environment at 37°C. Plates were examined microscopically at 1, 8, 14, 21, and 28 days.

Identification of the isolate as *N. asteroides*. Gram and acid-fast staining reactions and differential biochemical tests were determined by accepted procedures (24). The cell wall chemotype was determined by the methods of Stanek and Roberts for analysis of whole-cell hydrolysates (31). Organisms employed as standards in the analysis included: *N. asteroides* 10905, *Actinomyces viscosus* ATCC 15987, and a xanthine-negative *Streptomyces* sp. that was encountered during the clinical survey for L-forms in animals (11; A. M. Buchanan, unpublished data).

Acridine orange fluorescence of L-form variant colonies. Impression smears of L-form variant colonies, grown for 4 weeks on a plate of L-form medium V, were fixed in absolute methanol for 3 min and dried. They were stained for 5 min with freshly prepared acridine orange in McIlvaine buffer, pH 3.8 (19), rinsed, and mounted in a drop of buffer. Fluorescence was observed with a Zeiss microscope equipped with a Ploem epi-illumination attachment and fluorescein isothiocyanate filters.

Immunofluorescent staining. The availability of the immune reagent against SOD of *N. asteroides* GUH-2 facilitated the identification of the intracellular acid-fast bodies in the tissues of the dog. The procedures for purification and characterization of SOD from *N.*

asteroides GUH-2, the production of antiserum in rabbits, the separation of the immunoglobulin G fraction, and the method of determining reactivity of this immune reagent against *Nocardia caviae* 112 and *Nocardia rubra* 721-A have been reported in detail previously by Beaman et al. (7). For the present study, the tests of specificity of anti-SOD were extended to include other strains of *N. asteroides*, *Nocardia brasiliensis*, and *Mycobacterium intracellulare*. Briefly, single-cell suspensions were prepared from log-phase cultures, grown in brain heart infusion or Middlebrook 7H9 broth containing 0.5% glycerol and 0.5% glucose, washed with phosphate-buffered saline (pH 7.2), and incubated with the purified unlabeled antibody (1:10 volume) at 37°C for 30 min. These cells were washed five times with the same buffer and treated with goat anti-rabbit immunoglobulin G (Miles Laboratories Inc., Elkhart, Ind.). Impression smears of lung and bronchial lymph node tissues and a control smear of normal dog lung tissues were fixed for 3 min with absolute methanol and dried. They were stained for the same interval as given above with rabbit anti-SOD, washed, and treated with fluorescein-labeled goat anti-rabbit immunoglobulin G reagent. Wet mounts were observed with a Zeiss research microscope equipped with Ploem epi-illumination and fluorescein isothiocyanate filter attachments.

Analysis of the serum for fatty acids. The fatty acids of the serum collected during incomplete remission were analyzed qualitatively for the presence of tuberculostearic acid by using a Hewlett-Packard 5880 gas chromatograph by methods described in detail previously (4).

RESULTS

Isolation of *N. asteroides* UCD 1-581 from the acute arthritic joint fluid. The left hock fluid was milky in appearance with 30,000 to 40,000 polymorphonuclear leukocytes per mm³ and contained occasional microscopic yellow-gold granules. No normal organisms were evident in droplets of the L-form culture that were examined by phase microscopy at 3, 7, and 10 days. The recovery of an actinomycete, strain UCD 1-581, was unexpected since previous efforts with L-form cultures of canine arthritic joint fluids were unrewarding (11). A hydrophobic layer floated on the surface of this culture 3 weeks after inoculation. Since it was not substantial, it was ignored. At 5 weeks, the amount and texture of this layer indicated the presence of an organism. A microcolony of the obligately aerobic isolate is shown in Fig. 1A. A Kinyoun stain revealed acid-fast branched filaments which tended to fragment. Aerial spores were produced, and the catalase reaction was positive. Chemical analysis revealed the presence of *meso*-diaminopimelic acid, arabinose, galactose, tuberculostearic acid, and nocardomycolic acids. Negative diagnostic reactions were obtained for decomposition of hypoxanthine, xanthine, starch, tyrosine, casein, and urea. The isolate produced acid from glucose, but not from rhamnose, maltose, mannitol, mannose, sucrose,

arabinose, galactose, or sorbitol. It was positive for esculin hydrolysis, citrate utilization, and growth on Sabouraud agar. These reactions are compatible for those reported for *N. asteroides* (20).

Morphological changes in other L-form cultures. Although revertants were not obtained from L-form cultures of serum collected during partial remission or of joint fluid or tissues collected at necropsy, the presence of a nonreverting L-form variant was suspected in all cases. Morphological changes occurred that were not seen with sham-inoculated plates. These developments included the formation of macroscopic granules and microcolonies of small spherical bodies in straight or curved lines oriented downward into the agar. These tended to become obscured by minerals, especially when incubated aerobically (Fig. 1B). To encourage reversion, the specimens were transferred to various media that differed in the type of infusion base, the serum or osmolar supplement, or the addition of 0.05 M HEPES-Cl buffer (pH 7.0)–0.01 M putrescine or all of the above. Three transfers were made at 3-week intervals. Although all of these techniques resulted in the transfer of the L-form variant colonies, no revertants appeared. Because these L-form variant colonies were not typical of L-forms, the following criteria are offered to prove their identity: (i) the vesicles of the colonies were gram negative; (ii) they were transferable, increasing in numbers with each transfer; (iii) they grew better on medium V plates containing HEPES and putrescine than on plates lacking these components; (iv) growth was inhibited on medium VI containing 100 µg of valinomycin per ml and 0.01 M KCl; and (v) they stained green for DNA and orange for RNA with acridine orange fluorescence microscopy (Fig. 1C).

Chemical analysis of the serum. To establish the presence of the L-form in the animal during partial remission, the serum lipid was analyzed chemically for the presence of tuberculostearic (10-methylstearic) acid, a lipid unique to the *Nocardia* spp. (or *Mycobacterium* spp.). Gas-liquid chromatographic analysis revealed a substantial amount of this bacterial lipid, indicating that the lipemia was not simply the normal expected response of this dog to steroid therapy.

Examination of necropsy tissues. All organs and tissues except for one synovial fluid specimen were contaminated with postmortem invaders so that most L-form cultures could not be interpreted. Intracellular acid-fast vesicles were detected in alveolar macrophages (Fig. 2A) and in phagocytic cells of the bronchial lymph node (not shown). They fluoresced intensely when stained by the indirect method, using immunoglobulin G antibody against SOD of *N. aster-*

FIG. 1. (A) Microcolony of *N. asteroides* UCD 1-581 grown on brain heart infusion plates for 48 h. (B) L-form variant colony consisting of a large central vesicle, obscured by minerals, with smaller vesicles equally spaced and oriented down into the agar. Other small vesicles are present in deeper focal planes (arrows). (C) Impression smear of L-form variant colony that was stained with acridine orange at pH 3.5. Green fluorescence was seen with the globular units at the periphery, whereas the interior and certain areas (white arrows) of the outside fluoresced orange. A filament that fluoresced orange is indicated by the black arrow.

oides GUH-2 (Fig. 2B). Virulent strains are known to secrete this enzyme (7). Therefore, the acid-fast vesicles are visible as C-shaped bodies

due to surface fluorescence (arrows), and there is considerable extracellular fluorescent material. No fluorescence was seen in stained smears

FIG. 2. (A) Intracellular acid-fast vesicles within macrophages of lung tissues. Note the regular spacing of these bodies. (B) Intracellular comma-shaped fluorescent structures and extracellular fluorescence are expected because the SOD antigen is located primarily on the surface and is secreted by some strains of *N. asteroides*.

of normal dog lung tissues. Anti-SOD of *N. asteroides* GUH-2 did not react with *N. asteroides* GUH-5, *N. caviae* 112, *N. brasiliensis*, or *M. intracellulare*.

DISCUSSION

N. asteroides has been characterized as a charlatan (12) because of the ability to masquerade obscure conditions. It is notorious for hydrophobicity and resistance to various antibiotics (12). It is frequently not detected by stains, when later cultures are positive, sometimes even after a delayed incubation (5, 12). L-forms have been proposed to account for these various properties (5, 12). Evidence for this conclusion includes repeated recoveries by Beaman of L-forms of *N. asteroides* from cerebrospinal fluid of a human patient who had developed pulmonary nocardiosis and a brain abscess while receiving steroids for polymyositis (5). L-forms of *N. asteroides* and *N. caviae* 112 evolve spontaneously in mice, persist, and cause some mycetomas containing L-forms as the sole agent present (5, 6). Finally, Beaman has fulfilled the postulates of Koch for pathogenicity of *N. caviae* 112 L-forms by transmitting infections to other mice (5).

SODs are cytoplasmic scavengers of O_2^- ; therefore, they serve a protective role in all aerobic organisms against this toxic anion (14). The enzyme from *N. asteroides* GUH-2 is unique among all known SODs because it has three metals bound to it instead of the usual two, as is the case in plants and animals, or one, as is the case in other bacteria (7, 14). Because it is a cytoplasmic enzyme, its presence in L-form variants is unrelated to the question of cell wall existence. Furthermore, the secretion of this enzyme by virulent strains of *N. asteroides* is considered a significant determinant in the pathogenesis of this organism (7). For all of these reasons, anti-SOD of *N. asteroides* GUH-2 was the ideal immune reagent for verifying the presence of noncultivable organisms in the tissues of this animal.

The transferable L-form variant colonies observed in the present study resembled the nonreverting L-form variant colonies described by others (6, 13). They were identical to those detected in earlier work from this laboratory (10, 11) in L-form cultures of synovial fluids from arthritic monkeys and dogs with steroid-responsive IPA and systemic lupus erythematosus. These colony-like bodies had to be ignored because of similarities with pseudocolonies of horse serum origin. Fetal calf serum was employed for the present study to avoid this control phenomenon. It is not unreasonable to assume that nocardia L-form variants, although incapable or reluctant to produce peptidoglycan, would

retain the ability to secrete protective lipids, giving them the appearance of pseudocolonies (16). The mineral deposits detected in alveolar macrophages, synovial fluids, and L-form cultures during incubation are believed to be Ca^{2+} deposits. Their formation, in vivo or in vitro, should be considered an indication of metabolic activity analogous to the uptake (or efflux) of this ion by mitochondria (18, 23).

Much has been said concerning the opportunistic nature of nocardiosis in humans; however, some reviews stress its occurrence in individuals with no preexisting disease (15, 34). The association of *N. asteroides* with neoplastic disease, as is seen in humans (34), is not substantiated in the canine cases of nocardiosis. With one possible exception (28), nocardiosis has not been seen concurrently with systemic lupus erythematosus in dogs. The frequency of nocardiosis is higher in animals under 1 year of age (8, 9, 15, 17, 22, 29, 31–33) than it is in adult dogs (21, 22). Prognosis is considered to be grave for systemic infections or cutaneous lesions (1, 30) even when treated. The grave nature of nocardiosis in young dogs as well as old dogs raises concern for the apparent resistance of animals older than 1 year. It is suggested that adult dogs are susceptible to infection with *Nocardia* spp. in an altered form that is maintained by the immune system.

In summary, the presence of an altered form of *N. asteroides* has been demonstrated in the lung tissues and synovial fluid of a dog with IPA. The necessity for innovative measures to achieve isolation, the persistence of the organism in an altered form in vivo despite vigorous immunosuppressive treatment, and the unrelenting and unresponsive course of the disease in this animal lead us to suggest that the organism played a direct role in this arthritic disease. The characteristics of nocardiosis and IPA are compatible with this hypothesis.

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