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Experimental Production of Actinomycetoma in BALB/c Mice

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Chronic actinomycetoma associated with grain production was induced in BALB/c mice by subcutaneous inoculation of live *Nocardia brasiliensis* in Freund incomplete adjuvant into the hind footpads. Similar inoculation of *N. asteroides* and *N. caviae* resulted in local tumor formation which healed spontaneously after 5 months, the disease disseminating into the peritoneum, where masses or organisms could be detected. Grains were recovered from superficial skin lesions of *N. caviae*, but not from the *N. asteroides*-infected mice. Mycetoma lesions, appearing as early as 1 month after inoculation of 1.2×10^7 colony-forming units of *N. brasiliensis* per ml or as late as 3 months with inoculation of 1.0×10^5 colony-forming units per ml, became persistent and were readily detectable even 6 months after inoculation. No spontaneous healing occurred, and grains were recovered at different stages of the disease. Saline suspensions of *N. brasiliensis* also produced typical mycetoma lesions, although the incubation period was ca. 6 months. Adjuvant addition appeared to accelerate the onset of the disease. Experimental production of actinomycetoma in laboratory animals allows the study of many unanswered aspects of the disease and also provides a suitable model for therapeutic trials in the search for new and more effective chemotherapeutic agents.

Classical mycetoma is a chronic, progressive tumor of the subcutaneous tissues characterized by the production of grains and ultimate bone destruction (8). This disease, prevalent in some tropical and subtropical areas of the world, can be caused by several fungi from various genera and species, as well as by aerobic *Actinomadura* and *Nocardia* (9).

Mycetoma arises from soil contamination of skin wounds, usually on the feet and hands of laborers. Its development is slow and insidious, being characterized by localized subcutaneous abscesses that spread by direct extension after breaking through the skin surface to form chronic, draining, ulcerated, crusted lesions (6). The disease is extremely disfiguring and only amenable to chemotherapy when *Nocardia* and *Actinomadura* are the causative agents and it is diagnosed early. Otherwise, surgical excision of the infected areas is the only other treatment available and is often followed by a high rate of recurrence (7).

Although mycetoma has been recognized as a disease since 1861 (2), its study has been greatly hindered by the lack of a suitable animal model. Experimental production of mycetoma in laboratory animals would offer: (i) the possibility of analyzing those aspects of the disease not yet understood (e.g., route of infection, incubation period, immune status of the host, etc.), and (ii) a suitable model for therapeutic trials in the search for new and more effective chemotherapeutic agents.

Several investigators (1, 4, 10-13) have inoculated different causative organisms into various laboratory animals and in most instances have failed to obtain the chronic mycetoma lesions seen in humans. In 1967, González-Ochoa and Kumico-Hojyo were able to produce, after a single inoculation of *N. brasiliensis* into the footpad of albino mice, typical progressive mycetoma with sinuses, grain production, and no tendency to spontaneous cure (5). Their significant findings, however, have not been reproduced. Therefore, we decided to reexamine González-Ochoa's model and attempt to produce actinomycetoma in laboratory mice.

MATERIALS AND METHODS

Bacterial strains. *N. brasiliensis* 27-78 and 267-78 were isolated from human cases of mycetoma and were given to us by Pedro Lavalle; *N. caviae* 4420 was obtained from González-Ochoa's collection. *N. asteroides* N-58 was isolated from a case of pulmonary nocardiosis and was provided by the Center for Disease Control (A332).

Experimental animals. Six-week-old BALB/c mice were purchased from the Institute for Cancer Research (Philadelphia, Pa.). They were divided into five groups of either five or four animals, kept at room temperature, and fed Purina Chow and given water ad libitum.

Culture and collection of organisms. *N. brasi-*

liensis, N. caviae, and *N. asteroides* were grown in Sabouraud broth until the stationary phase. Growth was aseptically collected by filtration through 0.22-μm membrane filters (Millipore Corp.) and washed twice with sterile saline. Cell clumps were disrupted in a tissue grinder, and the homogeneous suspensions obtained were used to prepare serial dilutions. These were plated on Sabouraud plates, and the concentrations of the original cell suspensions were determined as the number of colony-forming units per milliliter (Table 1).

Animal inoculation and follow-up. Two groups of BALB/c mice were inoculated with the two strains of *N. brasiliensis*; the other three groups received *N. asteroides, N. caviae,* and saline, respectively. Inocula were emulsions of equal volumes of these organisms in Freund incomplete adjuvant. A 0.1-ml volume was used to inoculate each of the two hind footpads of the animals. The footpads were checked each week for signs and symptoms of the disease, and the clinical observations were recorded.

Four months after inoculation, the localized open lesions present in the infected animals were skin scraped and tested for the presence of grains. This was done by pressing clean glass slides on the exposed infected areas and by adding potassium hydroxide to the recovered material. The preparations were covered with cover slips, cleared by heat, and observed under the microscope.

RESULTS

Table 1 shows the results obtained for the different groups of animals at 1, 3, and 6 months after inoculation. As can be seen, the two strains of *N. brasiliensis* used resulted in typical mycetoma lesions with grains (Fig. 1 and 2). The inoculum of *N. asteroides*, 10 times lower than the dose of *N. brasiliensis* 267 used, also produced an infection with early necrosis of the toes and feet (Fig. 3), but no grains could be recovered from the infected sites. The animals that received *N. caviae* exhibited a clinical pattern similar to that seen in the animals infected with *N. brasiliensis*; however, there was a more apparent early inflammatory response associated to severe necrosis.

It is interesting to note that the dose of *N. brasiliensis* 27 used did not produce any detectable infection initially (Fig. 4). However, 2 months after inoculation, inflammation became apparent, and by 3 months, draining, ulcerated lesions could be detected in the infected animals (Fig. 1).

Some animals were sacrificed during the course of the experiment, and in all cases the infection tended to metastasize, invading the peritoneal cavity. Histopathological studies of the material obtained from skin lesions revealed a dense granulomatous infiltration. In the case of *N. brasiliensis* and *N. caviae* infections, the center of this granulomatous formation was occupied by the actinomycete granules (Fig. 5).

Five months after inoculation, those animals infected with *N. asteroides* and *N. caviae* showed involution of the mycetoma lesions at the local site of inoculation. The disease had disseminated to the stump and peritoneal cavity, where masses of the organisms could be detected by palpation. The animals inoculated with *N. brasiliensis*, especially those receiving the low dose of the organism, exhibited a different pattern for the evolution of the disease. After 5 months, the lesions were largely confined to the feet and the inflammatory response began to

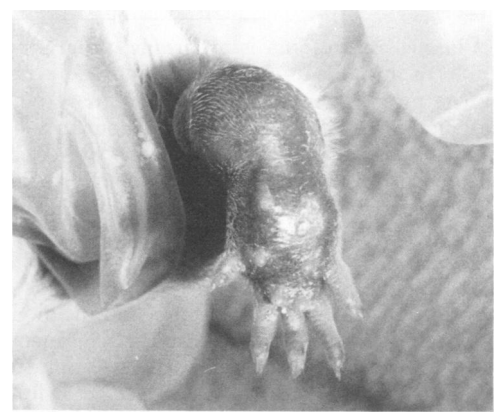

FIG. 1. *N. brasiliensis 27-infected mouse 3 months after footpad inoculation with 0.1 ml of 1.0 × 10⁵ colony-forming units per ml in Freund incomplete adjuvant.*

TABLE 1. *Presence of mycetoma lesions and grains in the N. asteroides-, N. brasiliensis- and N. caviae-infected mice*

Group	Inocula	Dose[a] (CFU/ml)	Mycetoma lesions after			Presence of grains in skin lesions
			1 mo	3 mo	6 mo	
I	*N. brasiliensis* 27	1.0×10^5	−	+	+	+
II	*N. brasiliensis* 267	1.2×10^7	+	+	+	+
III	*N. caviae* 4420	2.4×10^7	+	+	+	+
IV	*N. asteroides* N-58	1.9×10^6	+	+	±	−
V	Saline (control)		−	−	−	−

[a] A 0.1-ml amount was used to inoculate the hind footpads of each mouse. CFU, Colony-forming units.

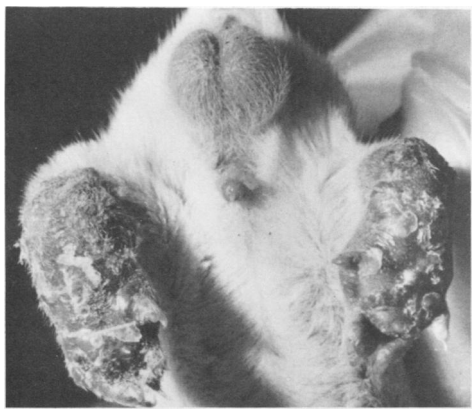

FIG. 2. *N. brasiliensis 267-infected mouse showing typical mycetoma lesions 1 month after footpad inoculation with 0.1 ml of 1.2 × 10^7 colony-forming units per ml in Freund incomplete adjuvant.*

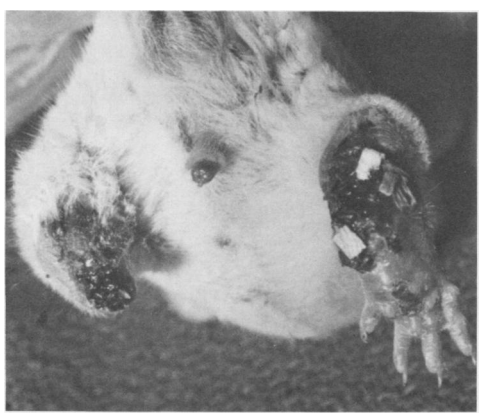

FIG. 3. *N. asteroides-infected mouse 1 month after footpad inoculation. Note early necrosis of toes and foot.*

reach its climax (Fig. 6).

All the *Nocardia*-infected animals exhibited an abnormal growth pattern as indicated by their reduced size when compared to the controls. Also, most started to lose hair a few months after infection; this was probably due to a hypersensitivity reaction to some of the components of the microorganisms.

Two animals inoculated with saline suspensions of *N. brasiliensis* and *N. caviae* were also followed for signs and symptoms of the disease. Approximately 6 months after inoculation they began to exhibit a local inflammatory response that was followed by the appearance of mycetoma lesions. It appears that mycetoma induction does not require the presence of adjuvant; however, its addition accelerates the onset of the disease.

DISCUSSION

The results presented in this study are in good agreement with the animal model described by González-Ochoa and Kumico-Hojyo in 1967. The only difference between their procedure and ours is in the nature and concentration of the initial inoculum, since they used milligram amounts of both in vitro cultured organisms and bacteria isolated from patients with mycetoma. It is also worth noting that we used a different strain of mice. However, interestingly enough, the disease pattern for those BALB/c mice inoculated with *N. brasiliensis* correlates well with that described by González-Ochoa for the albino mice.

The fact that the dose of *N. brasiliensis* 27 used took a longer time to produce detectable lesions in the infected animals seems to correlate with what actually happens in humans. It is possible that still lower doses might produce mycetoma, as has been suggested by González-Ochoa (5).

Our finding that inocula consisting of only live *N. brasiliensis* organisms in saline are still capable of causing the disease indicates that adjuvant addition is not required for the production of the infection. However, its addition accelerates the onset of the clinical symptoms with the same final outcome—chronic mycetoma lesions with no tendency to spontaneous cure.

Histopathological findings for both *N. caviae*- and *N. brasiliensis*-infected mice resemble those observed in human cases of mycetoma. We did not detect any major bone damage in them, but it seems likely to expect it much later in the course of the disease (3).

The fact that the *N. asteroides*-infected mice

FIG. 4. *N. brasiliensis 27-infected mouse 1 month after footpad inoculation. No inflammatory or necrotic response was detected at this stage of the infection.*

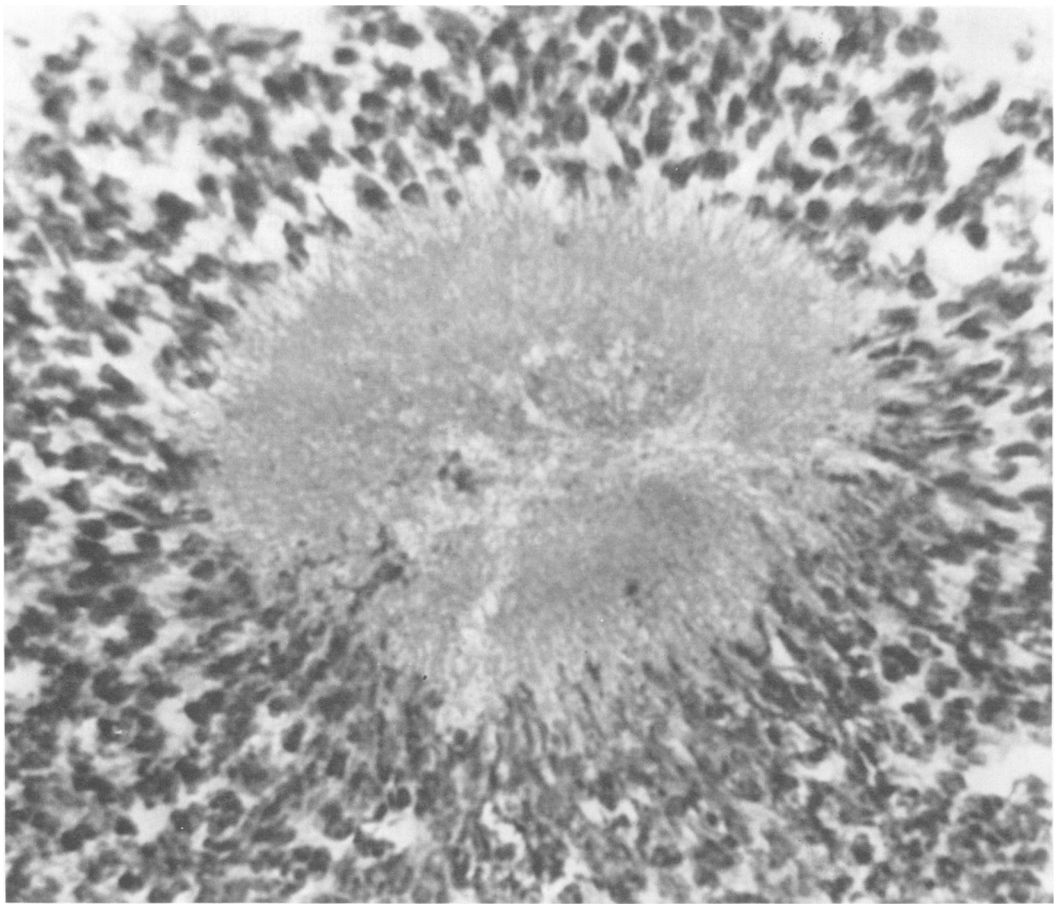

FIG. 5. *Tissue section from N. brasiliensis 27-infected mouse showing a typical grain surrounded by a wide zone of polymorphonuclear leukocytes (stained with hematoxylin and eosin, ×400).*

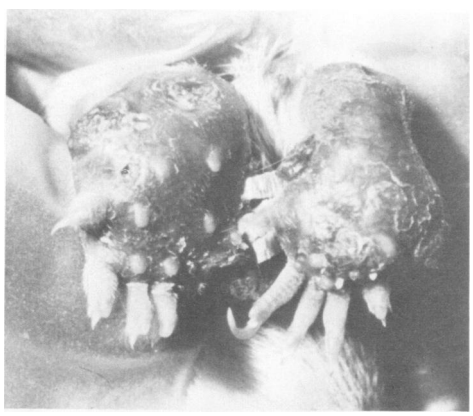

FIG. 6. *N. brasiliensis 27-infected mouse six months after footpad inoculation with 1.0 × 10^5 colony-forming units per ml in incomplete Freund adjuvant.*

showed local healing reactions 5 months after inoculation might be due to the dose of organisms used, since *N. asteroides* is considered to be one of several actinomycetoma causative agents (8).

Our knowledge of the mycetoma disease process is rather incomplete. An animal model, such as the one described here, may represent the beginning for a better understanding of this disease process and, most importantly, might provide a suitable model for therapeutic trials in the search for new and more effective chemotherapeutic agents.

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