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Posterior Paresis and Osteolysis in Guinea Pigs (*Cavia porcellus*) Secondary to Freund's Adjuvant Immunization

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Bilateral hindlimb paresis occurred in 3 guinea pigs after immunization with an adjuvant–antigen mixture containing complete Freund's adjuvant. Doses were injected into unanaesthetized animals, divided among 3 or 4 sites, and given slightly off midline in the subcutaneous tissues of the back. Neurologic examination of affected animals revealed intact flexor and panniculus responses and limited voluntary movement of the hindlimbs. Histopathologic interpretation of 2 affected animals showed fibrogranulomatous material effacing the skeletal muscle and vertebral bone, with marked bone lysis and infiltration into the marrow space and spinal canal. In addition, multiple granulomas in the pulmonary parenchyma were noted. A postmortem radiograph of the excised thoracolumbar spine of 1 animal revealed a soft tissue swelling and 'moth-eaten' and geographic osteolysis of 2 spinous processes. Hindlimb paresis and osteolysis likely resulted from accidental injection of the adjuvant–antigen mixture into the epaxial musculature and subsequent extension of injection site granulomas into the spinal canal.

Abbreviations: CFA, complete Freund's adjuvant; IFA, incomplete Freund's adjuvant

For more than 70 y, adjuvants have been used to augment the immune response of animals to antigen inoculation. Of all adjuvants, complete Freund's adjuvant (CFA) is the most widely used and effective for experimental antibody production.[15] CFA is a water-in-oil emulsion containing heat-killed and dried mycobacterial cells. The mineral oil used in CFA establishes a depot that extends the period of antigenic stimulation[11] and provides a vehicle for antigen transport through the lymphatic system.[15] The inclusion of killed mycobacteria in CFA recruits an aggregation of macrophages to the injection site, which culminates in a delayed-type hypersensitivity reaction.[6] The resulting inflammatory reaction increases cellular interactions with antigen and enhances antibody production. Incomplete Freund's adjuvant (IFA) is often used subsequent to CFA and has a similar mechanism of action but lacks killed mycobacterial cells. Despite their popularity, use of CFA and IFA is fraught with problems. Complications include injection site granulomas; subpleural, hepatic, and renal granulomas; necrotizing dermatitis;[2,14] and spinal cord compression from extension of injection site granulomas.[7] In our present report, we describe clinical signs and lesions associated with the use of Freund's adjuvant in 3 guinea pigs.

Case History

A 10-wk-old, 440-g, nulliparous Hartley guinea pig was presented to a facility veterinarian for non-weight-bearing on both rear limbs of 1 day's duration. On physical examination, moderate to severe urine and fecal staining extending from the perineum to the abdomen were noted. Neurologic examination revealed bilateral hindlimb paresis with intact flexor and panniculus responses; limited voluntary movement of the hindlimbs was noted. The guinea pig was able to move about the primary enclosure and used the forelimbs to obtain food and water. The affected animal was part of a group of 15 nulliparous guinea pigs simultaneously received from the same commercial, US vendor (Charles River Laboratories International, Wilmington, MA). Animals were pair-housed in suspended, polycarbonate cages (Allentown Caging Equipment, Allentown, NJ) and provided a commercially milled guinea pig chow (Harlan, Indianapolis, IN) and water ad libitum. Physical examination of the affected animal's cagemate was unremarkable. All guinea pigs were received as specific pathogen-free from the vendor, but no additional serologic monitoring was performed in the facility. Animals were used pursuant to a single University of Tennessee Health Science Center Animal Care and Use Committee-approved protocol to elucidate immune injury mechanisms involved in tubulin-induced autoimmune hearing loss. Animals were housed and cared for in compliance with Animal Welfare Act regulations and the *Guide for the Care and Use of Laboratory Animals*[10] in a program accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International.

To establish a guinea pig model for beta-tubulin hearing loss, animals were immunized with varying doses of tubulin mixed with CFA for primary immunization and mixed with IFA for booster immunization. CFA was prepared in the laboratory of the principle investigator and was made by grinding 2 mg of H37Ra *Mycoplasma tuberculosis* in 1 ml IFA and adding an additional 99 ml IFA. IFA was composed of 85 parts paraffin oil and 15 parts mannide monooleate. The final concentration of mycobacteria in the prepared emulsion was 0.02 mg/ml.

Fourteen days prior to the onset of paresis in the affected animal, all colony animals had received injections of 200 µg

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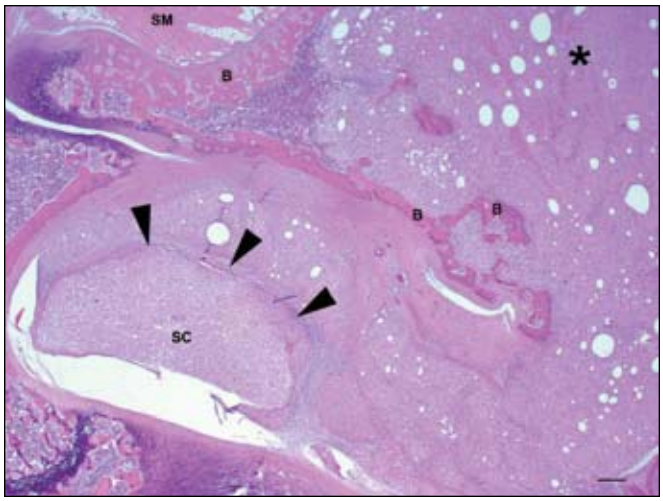

Figure 1. Fibrogranulomatous material effaces the skeletal muscle (*) and bone (B) with significant bone lysis (arrows) and infiltration into the marrow space and the spinal canal. SC, spinal cord; SM, normal skeletal muscle. Hematoxylin and eosin stain; magnification, ×20; bar, 400 µm.

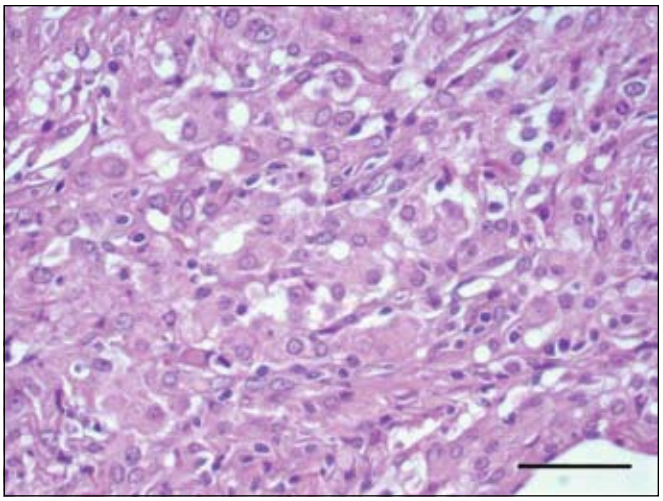

Figure 2. The inflammatory infiltrate is composed of macrophages and fibroblasts, with few lymphocytes and neutrophils. Hematoxylin and eosin stain; magnification, ×400; bar, 50 µm.

tubulin and 0.4 ml CFA, yielding a total injection volume of 0.4 ml. The injections were administered to manually restrained animals, divided among 3 to 4 sites (0.1 to 0.13 ml per site), and given slightly off midline in the subcutaneous tissues of the back. The day prior to presentation of the affected animal, each animal received a booster injection with 2 µg tubulin and 0.4 ml IFA; again, the total volume injected was 0.4 ml. Booster injections were performed as described for initial injections. No precautions were taken to avoid previous injection sites. The affected guinea pig was euthanized via CO_2 overdose 3 d after the onset of paresis. Prior to euthanasia, blood was collected for a health assessment serologic profile. Whole-body radiographs were taken after euthanasia. No significant serologic titers were detected, and whole-body radiographs were unremarkable.

Approximately 2 mo prior to presentation of the affected animal, another guinea pig with a similar history of CFA and tubulin immunization was presented with bilateral paresis and subsequently was euthanized via CO_2 overdose. Both animals were necropsied. In both animals, there was a firm, white mass approximately 3.81 cm in length that was adhered to the skeletal muscle and extended over cervical and thoracic segments of the vertebral column; no other gross lesions were identified.

Heart, lungs, liver, spleen, kidney, small and large intestines, and vertebral column with attached epaxial musculature from both animals were examined microscopically. In sections of the vertebral column and associated skeletal muscle, massive proliferation of fibroblasts, macrophages, lymphocytes, plasma cells, and neutrophils created an expansile and infiltrative mass that invaded and effaced the vertebral bone. Bone lysis was extensive and accompanied by infiltration and replacement of the bone marrow by fibrogranulomatous inflammation. Within the spinal canal, the fibrogranulomatous tissue expanded and compressed the spinal cord; no infiltration of nervous tissue was observed (Figures 1 and 2).

In addition to the local reaction, multiple granulomas were identified in the pulmonary interstitium. The pulmonary granulomas each were composed of a core of macrophages admixed with few neutrophils, often bordered by cuffs of lymphocytes and plasma cells. Fibroplasia was not a prominent feature (Figure 3). Histologic lesions were not identified in any other organ system examined.

Nine days after the presentation of the animal 1st discussed, a 3rd guinea pig with similar signs was presented. Neurologic examination of the 3rd animal also revealed bilateral hindlimb paresis with intact flexor and panniculus responses. The 3rd guinea pig was euthanized via CO_2 overdose and necropsied. Gross lesions were as described for the 2 previous animals. Histopathologic examination was not performed. Postmortem lateral radiographs (Figure 4) of the excised thoracolumbar spine were made using a cabinet x-ray unit (Faxitron MX20DC2, Faxitron X-Ray, Wheeling, IL). A soft tissue swelling was seen dorsal to the spine, centered on the T11 vertebral body and extending from the level of T8 through T13. There was also moth-eaten osteolysis of the spinous process of T10 as well as geographic osteolysis of the T9 spinous process. In addition, the caudal margin of the T7 spinous process was irregularly marginated, although this change was thought to represent chronic remodeling of doubtful clinical significance.

Discussion

Of the 15 animals injected with the tubulin–CFA mixture, 3 developed bilateral hindlimb paresis. Bilateral hindlimb paralysis and paresis induced by CFA in guinea pigs have previously been reported. Freund and colleagues demonstrated that a single subcutaneous injection of rabbit or guinea pig brain combined with killed acid-fast bacilli incorporated in a water-in-oil emulsion produced paralysis and central nervous system lesions in guinea pigs. Histologic examination of the affected animals revealed histocytic infiltration of the meninges, brain, and spinal cord. Freund concluded that the presence of acid-fast bacilli in the sensitizing injection was essential for production of clinical signs and lesions, because paralysis did not occur in animals given brain suspended in salt solution or in a water-in-oil emulsion without mycobacteria.[4]

More recently (1993), Kleinman and colleagues reported posterior paresis in 5 guinea pigs injected subcutaneously with antigen and 0.5 ml CFA. Histopathologic evaluation of the animals showed 0.5- to 1.0-cm granulomas consisting primarily of lymphocytes, fibroblasts, and histiocytes in the epaxial muscle mass, with inflammatory infiltrate into the spinal canal and displacement of the spinal cord.[7] Adjuvant-associated pulmonary granulomas after subcutaneous injection of CFA have also been reported to occur in the guinea pig.[12] Our report appears to be the 1st to describe osteolysis after subcutaneous injection of CFA in the guinea pig. Osteolysis secondary to presumed

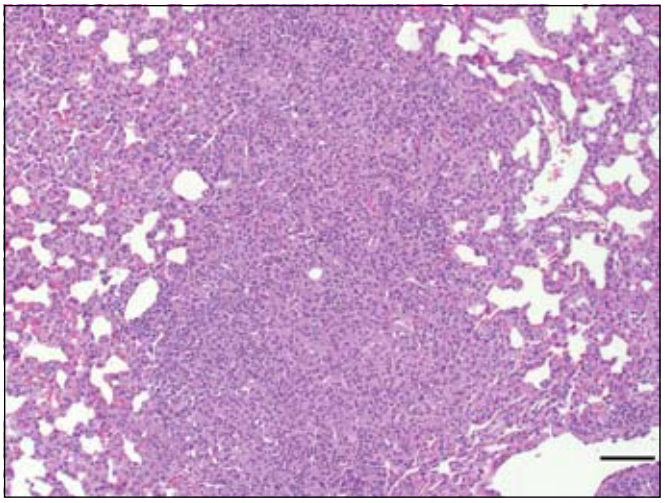

Figure 3. Multiple granulomas and fibrogranulomas centered on vessels were present throughout the pulmonary parenchyma. In the lung, granulomas were composed of macrophages cuffed by lymphocytes with or without fibroblast infiltration. Hematoxylin and eosin stain; magnification, ×100; bar, 100 µm.

mycobacterial infection in horses has been reported.[13]

In the animals described in the present report, hindlimb paresis and osteolysis likely resulted from accidental injection of the CFA–antigen mixture into the epaxial musculature and subsequent extension of injection-site granulomas into the spinal canal. The pathology associated with using CFA and IFA results from localization of the nonbiodegradable oily fraction in the tissue and the inflammatory response invoked by the mycobacteria in CFA.[2] Steiner and colleagues suggest 2 mechanisms for extension of injection-site granulomas—lymphohematogenous dissemination and local hypersensitivity reactions.[14] Most of the undesirable effects associated with the use of CFA can be eliminated by careful selection of injection sites and adjuvant amount.[1] The Canadian Council on Animal Care notes that the concentration of mycobacteria in CFA should be <0.5 mg/ml, to minimize associated inflammatory reactions.[3] The concentration of mycobacteria received by guinea pigs described in the present report were well within published recommendations.

The Institute for Laboratory Animal Research's *Institutional Policies and Guidelines on Adjuvants and Antibody Production*[5] recommends that dorsal cervical and scapular areas, tails of rodents, and sites prone to self-mutilation be avoided when possible. The Institute for Laboratory Animal Research and Canadian Council on Animal Care both also suggest that hair should be clipped from injection sites and that sites should be aseptically prepared to reduce potential infections and to facilitate visualization of lesions if they develop.[3,5] Hair clipping also would delineate previous injection sites to avoid repeat injection, which may have occurred in our guinea pigs.

In guinea pigs, a ≤20-gauge needle should be used for injection of antigen.[9] Subcutaneous injection volumes in rodents should not exceed 0.1 to 0.2 ml per site.[5,8,9] Furthermore, Kleinman and colleagues suggest using anesthesia to prevent sudden movement during injection and selection of injection sites on the sides of the thorax to prevent accidental injection into the epaxial muscles.[7] In addition, Broderson notes that injection into muscles in the lumbar area is not suitable in small animals because of the proximity of spinal nerves and insufficient muscle mass.[2] Use of anesthesia may not be necessary if the handler is comfortable with and experienced in proper guinea pig handling and restraint. However, selection of injection sites on the sides of the thorax likely would have prevented the adverse events we observed. In addition, animals should be monitored daily[3,9] and examined for side effects at least 3 times weekly after adjuvant immunization.[8] Standard operating procedures with detailed criteria for euthanasia of animals that present with unrelievable distress should be developed and used during post immunization monitoring.[3] Finally, when depot-forming adjuvants such as CFA are used, the preferred interval between priming and booster immunization is ≥4 wk.[9]

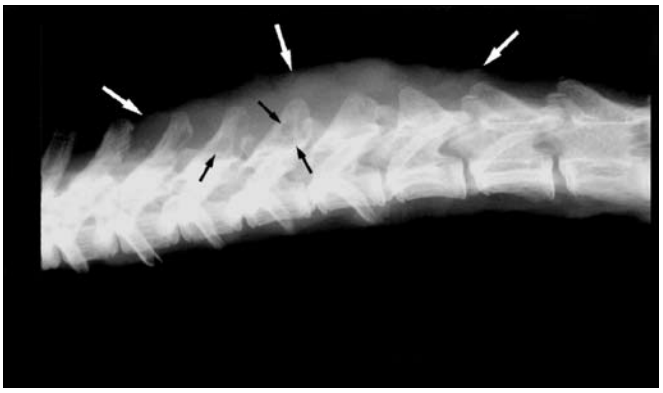

Figure 4. Lateral radiograph of the spine from T6 through L1. A soft tissue swelling is seen dorsal to the spine, extending from T8 to T13 (white arrows). Lucencies in the spinous processes of T9 and T10 (black arrows) represent osteolysis.

Production of polyclonal and monoclonal antibodies likely will continue to necessitate the use of adjuvants, and CFA remains the 'gold standard' for adjuvants.[15]

The decision as to whether an adjuvant is required should be judiciously considered and justified.[3] Possible adverse clinical events associated with adjuvants such as CFA should be discussed during experimental planning and the institutional animal care and use committee's review process and monitored during the experiment. Further, animal care and use committees are charged with oversight of institutional training programs. Sufficient training and competence of animal users and animal care staff are of paramount importance in minimizing pain and distress associated with adjuvant use.[3]

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