Comparative Medicine
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Vol 53, No 1
February 2003
Pages 85-88

Administration of Vaccinia Virus to Mice May Cause Contact or Bedding Sentinel Mice to Test Positive for Orthopoxvirus Antibodies: Case Report and Follow-up Investigation

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Routine testing of bedding sentinels from a barrier room revealed one mouse seropositive to ectromelia virus (EV). Results of hemagglutination-inhibition testing and western blot analysis were confirmatory for orthopoxvirus antibodies. Additional seropositive animals were not identified. Interviews indicated that replication-competent vaccinia virus (VV), Western Reserve strain (VV-WR), recently had been given to mice. Although VV-WR was not expected to spread by contact or via fomites, the case evidence suggested transmission of vaccinia via soiled bedding. In a follow-up experiment, 15 index mice were inoculated with 10^7 plaque-forming units of VV by either subcutaneous or intrarectal instillation. A dedicated contact sentinel and a bedding sentinel were provided for each index mouse. All 15 index mice were positive for antibodies when tested 22 days after inoculation. One mouse, inoculated by the subcutaneous route, appeared ill and developed lesions on the proximal portion of the tail. The contact sentinel mouse housed with this index mouse was the only sentinel to seroconvert. We conclude that VV-WR can spread to contact sentinels and potentially to bedding sentinels. The ability of other VV strains to be transmitted horizontally and the susceptibility of different mouse strains to infection merit further investigation. The use of VV in animal facilities must be managed carefully since the available serologic tests do not distinguish between VV and EV, an exotic agent of major concern to laboratory animal facilities.

Vaccinia virus (VV) and ectromelia virus (EV) are members of the *Orthopoxvirus* genus of the poxvirus family (Poxviridae). Vaccinia virus is commonly used in animal models for gene therapy because of its historical safety when used in humans, and because the large genome and viral structure allow ready insertion of exogenous DNA (1). Ectromelia virus is a natural pathogen of mice that spreads readily between mice and can cause a range of clinical signs of disease and death in many strains of experimental mice (2). Although EV is not endemic in mice in the United States, quality assurance programs for mice commonly include testing for EV antibody because EV has been accidentally introduced into animal facilities several times over the past several decades by the use of mouse serum in tissue culture preparation (3) and the introduction of mice from other institutions (4). Detection of EV-seropositive sentinel mice in an animal facility is cause for institution of maximal quarantine measures or destruction of valuable or irreplaceable mice. We describe detection of an EV-seropositive sentinel mouse apparently due to indirect exposure to VV via soiled bedding and provide preliminary experimental evidence that VV may spread to sentinel mice. We also raise the issue that increased use of VV in biomedical research will require improved serodiagnostic testing to discriminate between VV and EV exposures.

Received: 1/11/02. Revision requested: 2/26/02, 8/05/02. Accepted: 10/21/02.
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Case Report

The quality-assurance program for rodent barrier facilities at the Albert Einstein College of Medicine includes quarterly serologic screening of sentinel mice from each animal room. Mice belonging to multiple investigators are housed in each room in low-profile static isolator cages, bedded with corncob bedding, provided irradiated commercial rodent chow (Pico Mouse 5058, PMI Nutrition, International, Brentwood, Mo.) and autoclaved water in water bottles. Cage bedding is changed weekly, and cages are opened only in Biosafety Level 2 (BSL-2) cabinets. Swiss Webster mice purchased from a commercial colony (Taconic, Germantown, N.Y.) are used as bedding sentinels. Sentinel mice are exposed and tested under protocols approved by the institutional animal care and use committee (IACUC). Typically, sentinels are housed in pairs, with one cage of sentinels provided per rack in each animal room. Each cage of sentinels is exposed weekly for three months to pooled soiled bedding from an alternating one-sixth of the cages housed on a 112-cage rack. Thus, within any quarterly period, all cages are sampled and used to expose sentinel mice. Sentinels are tested for fur mites and pinworms. They also are tested by use of enzyme-linked immunosorbent assay (ELISA) and immunofluorescent assay (IFA) for antibody against a panel of 19 murine infective agents, including Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, Theiler's murine encephalomyelitis virus, reovirus 3, *Mycoplasma pulmonis*, lymphocytic choriomeningitis virus, ectromelia virus, K virus, polyoma virus, mouse adenovirus, epidemic diarrhea virus of infant mice, murine cytomegalovirus, hantavirus, *Encephalitozoon cuniculi*,

murine T-cell lymphoma virus, cilia-associated respiratory bacillus, and murine parvovirus antigens NS1 and VP2.

In one multi-investigator barrier room, containing approximately 520 cages of transgenic and knockout mice, serologic testing by a commercial laboratory (Charles River Laboratories, Wilmington, Mass.) of seven bedding sentinels from four cages detected a single mouse positive for antibody against EV by use of the ELISA and IFA. The serum from this mouse was retested by use of the IFA at an independent laboratory (Section of Comparative Medicine, Yale University School of Medicine, New Haven, Conn.), and results were positive. The cage mate of this sentinel had been humanely killed several weeks earlier, but its serum was not tested.

At the time the initial mouse tested positive for EV, the room had been under quarantine and treatment for three months for fur mite (*Myobia* sp.) infestation. However, additional quarantine procedures were initiated and included restricted, supervised room access, confinement of all live animals to the room, and decontamination of all materials leaving the room by use of 10% sodium hypochlorite solution.

Each of 10 research groups housing mice in the room was interviewed to identify possible sources of EV exposure, such as murine cell lines or other biological products. Two investigators reported that replication-competent, attenuated VV (derived from the Western Reserve strain; VV-WR) had been administered to mice by intravenous or intra-tumor injection, or by intrarectal instillation on several occasions over the preceding six months. The VV had been genetically modified to carry carcinoembryonic antigen (CEA), a well-characterized oncofetal glycoprotein antigen common to human gastrointestinal tumors and was intended to stimulate tumor rejection as a candidate cancer vaccine (5, 6). The VV-WR strain, included as a control, was not expected to spread by mouse-to-mouse contact or by bedding fomites. Discussions with investigators failed to reveal any other probable source of poxvirus exposure, such as use of untested cell lines or of mouse serum.

The original positive serum was additionally tested by use of hemagglutination-inhibition (HAI) and western blot (WB) analyses, and positive results were confirmed. Both diagnostic laboratories, however, reported that serologic testing for EV, including ELISA, IFA, HAI, and WB tests, used VV, rather than EV as the substrate antigen due to the extensive antigenic similarity of VV with EV and safety of VV. Thus, serologic testing did not distinguish between EV and VV seropositivity (7, 8). Tissues were not available from the seropositive animal that could be used for molecular diagnostic or virus isolation testing. Polymerase chain reaction (PCR) testing for EV and VV was performed at a third laboratory (Research Animal Diagnostic and Investigative Laboratory, College of Veterinary Medicine, University of Missouri, Columbia, Mo.) on serum from the original test-positive sentinel and three human tumor cell lines being used by investigators in the VV study. The stock of VV, which had been administered to mice in the room, was included as a positive control. All PCR test results for EV were negative, and only the VV stock was PCR positive for VV.

To determine the prevalence of EV-seropositive mice in the room, one mouse from each of 504 cages containing immunocompetent mice was tested for orthopoxvirus antibodies. In addition, sentinels were placed in 11 cages of immune-deficient mice for one week and were tested for seroconversion 3 weeks later. Results of all 515 serology tests were negative for antibodies to orthopoxvirus. Bedding sentinels in all other rooms in the facility also were tested and were negative for orthopoxvirus antibodies and have remained seronegative in the intervening 24 months. We concluded that the initial finding of an EV-positive bedding sentinel was most likely a result of fomite transmission of VV via pooled bedding. We also hypothesized that excoriations and ulcerations due to concurrent fur mite infestation or other causes could have enhanced transmission of VV to the bedding sentinels.

Experimental Study

A follow-up study was conducted to determine whether fomite transmission of VV occurs from experimentally inoculated mice. It was designed to determine, specifically, whether VV could be transmitted to contact or bedding sentinels after subcutaneous injection or intrarectal instillation of index mice, two routes used in the IACUC-approved vaccination research protocol. Scarification of the skin also was used to test the effect of prior integumentary damage on transmissibility from index mice.

Materials and Methods

Animals and husbandry. Mice were purchased and used under a protocol approved by the IACUC and the institutional biosafety committee. Forty-five, six-week-old female Swiss Webster mice were purchased from a commercial colony (Taconic, Germantown, N.Y.) and were housed individually in an isolation cubicle during a two-week acclimatization period, as described previously. All animal manipulations were performed by one of the authors (DJG), and daily animal observations were made by animal caretakers and DJG. Gloves were sprayed with 10% bleach between cage handling.

Virus. Virus was propagated and titrated in BSC-1 cells (5). Frozen aliquots were thawed and diluted in Dulbecco's modified Eagle medium (Gibco, Grand Island, N.Y.) supplemented with 2.5% fetal bovine serum (Gibco) on the day of inoculation.

Experimental inoculations and sentinel exposures. Three groups of five index mice were each inoculated with 10^7 plaque-forming units (PFU) of the VV-WR in a volume of 100 µl, the same dose used in the aforementioned research vaccination protocols. Animals of two groups (A and B) were inoculated subcutaneously in the scruff of the neck. Animals of another group (C) were given VV intrarectally by placing the tip of a p-100 pipetman 0.5 cm into the rectum. This route was used by vaccine investigators in an attempt to promote mucosal immunity to the vaccine, since prevention of colorectal cancer was one aim of their study. Mice were anesthetized with isoflurane prior to intrarectal instillation.

Inoculated mice were ear-punched in the right ear, contact sentinels were ear-punched in the left ear, and bedding sentinels were not ear-punched. An additional 15 mice served as contact sentinels, and 15 mice served as bedding sentinels. Each index mouse was added to the cage of a contact sentinel 24 h after exposure to VV, and each soiled cage bedding from an index mouse was transferred to a dedicated bedding sentinel mouse at the same time. Eight and 15 days after inoculation of index mice, they and their contact sentinels were placed in clean cages and bedding sentinels were placed individually in the soiled cages. Additionally, contact and bedding sentinels for group B had induced breaks in the skin. They were induced dur-

ing isoflurane anesthesia by puncture of the proximal two centimeters of tail skin, using a 21-gauge needle. Fifty small punctures per mouse were induced at two time points to mimic fight wounds or the excoriations that might occur in mice with acariasis. Punctures were placed on the ventral surface of the tail because it would be in contact with soiled bedding. The wounds completely healed within five days.

Necropsy, serologic testing, and histologic examination. On day 22 after inoculation, the index mice were humanely killed with CO_2, exsanguinated, and necropsied. Serum samples were obtained and submitted for serologic testing for antibody against a comprehensive panel of 18 murine viral and mycoplasmal agents (nine sera) or for EV antibody only (36 sera). All animals were examined for gross lesions, and abnormal tissues were fixed in formalin, embedded, sectioned at five-micron thickness, and stained with hematoxylin and eosin. Sections were observed by DJG and a veterinary pathologist by use of light microscopy.

On days 29 and 35 after inoculation of index mice, contact sentinels and bedding sentinels, respectively, were humanely killed, necropsied, and tested for exposure to virus as described previously. These time points allowed each sentinel an additional week following the end of the exposure period for seroconversion to occur.

Results

Clinical observations. A small amount of VV-containing media leaked from the rectum of one mouse shortly after installation. On day 5, two index mice given VV by subcutaneous injection were hunched and scruffy. By day 8, one of these mice appeared normal, but one remained hunched, with eyes partially closed. The proximal two centimeters of the mouse's tail was covered by small scabs (Fig. 1), which coalesced by day 10. However, this mouse improved clinically thereafter. All other index and sentinel mice remained normal.

Pathologic changes. The affected index mouse had healing scabs on the tail base, and the proximal two centimeters of the tail was narrower in diameter than the adjacent more distal area. Microscopically, the tail skin had focal and diffuse infiltration of mononuclear cells in the dermis, with hyperplasia and hyperkeratosis of the epidermis. Inclusion bodies were not seen. Immunohistochemical analysis (9) failed to detect viral VV antigen in skin sections (10). Internal organs of all index mice as well as those of contact and bedding sentinels were normal.

Serologic test results. Sera from three index mice, three contact sentinels, and three bedding sentinels tested negative for antibody against a panel of 18 viral and mycoplasmal agents other than poxviruses. Anti-poxvirus antibody was detected in all 15 index mice by use of the ELISA and IFA. Of 15 contact sentinels, only the contact sentinel for the mouse that had developed tail skin lesions, tested positive for orthopoxvirus antibodies. All 15 bedding sentinels were seronegative for poxviruses.

Discussion

Results of the clinical case and follow-up experiment, indicated that VV-WR can cause clinical signs of disease and be transmitted by cage contact or contaminated bedding. Although the bedding sentinels in the follow-up experiment did not seroconvert, this was the only mechanism by which the original sentinel mouse could have been exposed. The limited scope of the follow-up experiment did not allow us to compare the frequency of transmission by contact with that of bedding. Sentinel mice with induced breaks in tail skin did not develop VV antibodies, so it remains unclear as to whether skin wounds facilitate exposure to virus. Similarly, contact or bedding sentinels for mice given VV intrarectally did not become seropositive, even though such mice were more likely to release virus into the bedding immediately after inoculation. In contrast, the contact sentinel for the index mouse that developed tail skin scabs became seropositive. This finding suggests that the virus was transmitted from pox lesions, despite the fact that histologic and immunostaining evidence did not prove that the skin lesions were caused by VV. This result may have been favored by the fact that skin lesions were examined at a late, healing stage, after viral replication and shedding had ceased (11). Future studies should include larger numbers of mice per group and should include biopsy of suspect lesions when they are first observed.

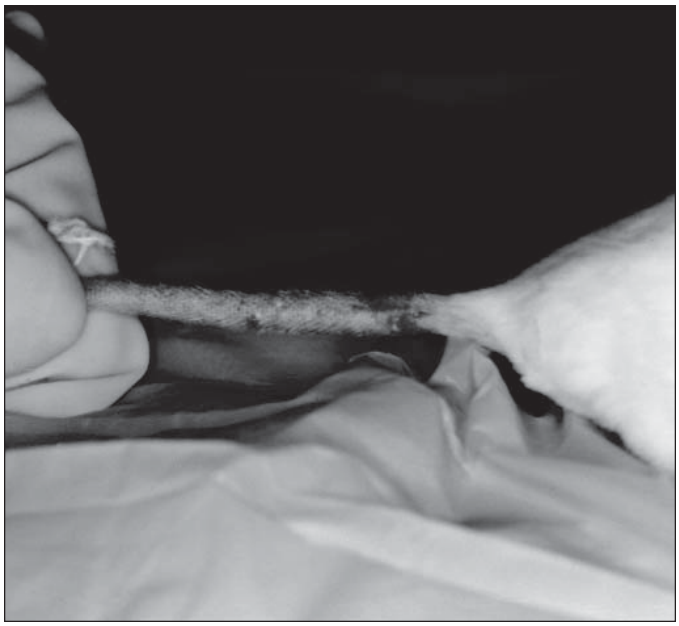

Figure 1. Mouse A5, eight days after inoculation subcutaneously in the scruff of the neck with 10^7 plaque-forming units of the Western Reserve strain of vaccinia virus. Notice small scabs encircling the proximal two centimeters of the tail and cyanotic color of this portion of the tail. When tested 20 days later, the cage contact sentinel of this mouse was seropositive for antibody against orthopoxviruses.

Transmission and severity of disease in mice infected with EV varies widely among mice with different genetic backgrounds (9, 12). Cages that previously housed mice infected with EV were inconsistently infective to naive mice (13). Transmission of EV was high in cages where infected mice died and were cannibalized but was low to moderate in cages where there was no cannibalism (13). Published information on the transmission of VV from mouse to mouse is limited (14, 15). One report (14) indicated transmission of VV from mice immunized by intradermal scarification with the IHD-E strain. Transmissibility varied depending on the strains inoculated and contact mice. Respiratory transmission was postulated because contacts did not develop skin lesions. However, naïve mice housed in open cages within the same room did not develop infection (14). In a second report (15), the NYCBH strain of VV was used and nine genetically

engineered strains with variable virulence were inoculated into BALB/c mice by nasal instillation, intraperitoneal inoculation, subcutaneous inoculation, and scarification of the denuded skin on the back. Results indicated that route of inoculation altered the likelihood of systemic dissemination. Pox skin lesions that developed following skin scarification transmitted infection by contact to naive cagemates (15). That study did not examine transmission of VV by bedding. Our study suggests that VV transmission can occur following subcutaneous inoculation and by bedding. However, prior studies of EV imply that virus shedding and transmission of VV, will vary with strain of the infected and/or exposed mice as well as route of inoculation.

The species of origin of VV is not known, and the virus is remarkable for its wide host range, including pathogenicity for rabbits (12, 16). Because of the long history of use in the laboratory and as a smallpox vaccine for humans, there are a large number of VV strains (16). These strains are known to differ in virulence when evaluated by intracranial inoculation into infant mice (16).

The likelihood that other VV strains will cause illness or be transmitted is unknown. Tests of other VV strains and mice of other genetic backgrounds would be useful to better judge the frequency of transmission of VV. Since 1991, VV has been suggested as a vector to carry genes for tumors (5, 17, 18) and infective agents (19-22). Thus, use of VV in experimental mice has increased rapidly. Caution should be exercised when VV is used as a vector to avoid spurious transmission and seroconversions that raise false concerns about EV infection. It is unfortunate that serologic tests currently available do not distinguish between VV and EV. Because VV use is increasingly prevalent, serodiagnostic tests that readily distinguish between exposure to VV and EV are essential.

Acknowledgments

We thank Robert G. Russell, Albert Einstein College of Medicine, for interpreting histologic sections and to Elizabeth Johnson, Section of Comparative Medicine, Yale University School of Medicine for performing immunohistochemical staining and interpretation of sections.

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