Postexposure Prevention of Progressive Vaccinia in SCID Mice Treated with Vaccinia Immune Globulin

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A recently reported case of progressive vaccinia (PV) in an immunocompromised patient has refocused attention on this condition. Uniformly fatal prior to the licensure of vaccinia immune globulin (VIG) in 1978, PV was still fatal in about half of VIG-treated patients overall, with a greater mortality rate in infants and children. Additional therapies would be needed in the setting of a smallpox bioterror event, since mass vaccination following any variola virus release would inevitably result in exposure of immunocompromised people through vaccination or contact with vaccinees. Well-characterized animal models of disease can support the licensure of new products when human studies are not ethical or feasible, as in the case of PV. We chose vaccinia virus-scarified SCID mice to model PV. As in immunocompromised humans, vaccinia virus-scarified SCID animals develop enlarging primary lesions with minimal or no inflammation, eventual distal virus spread, and lethal outcomes if left untreated. Postexposure treatment with VIG slowed disease progression, caused local lesion regression, and resulted in the healthy survival of most of the mice for more than 120 days. Combination treatment with VIG and topical cidofovir also resulted in long-term disease-free survival of most of the animals, even when initiated 7 days postinfection. These results support the possibility that combination treatments may be effective in humans and support using this SCID model of PV to test new antibody therapies and combination therapies and to provide further insights into the pathogenesis and treatment of PV.

The licensed smallpox vaccine is administered to people at potential risk of exposure in the event of a bioterrorism attack with variola virus. These include medical personnel, as well as designated civilian first responders (39). Life-threatening but rare complications of smallpox vaccine include encephalitis, eczema vaccinatum, and progressive vaccinia (PV). For routine nonemergency vaccination, screening for predisposing conditions minimizes but cannot completely prevent the exposure of all at-risk individuals (7, 32, 38). PV is a severe adverse event with a poor prognosis even following treatment and has been reported in patients with HIV, cancer (with and without chemotherapy), and primary cellular and humoral immunodeficiencies and in patients with connective tissue disorders receiving steroid treatment (2, 5, 13). Historically, mortality was highest in infants and young children with primary combined cellular and humoral immunodeficiencies. Adults with secondary immunodeficiency had a greater survival rate, which was presumed to be due to residual or returning immune function during the course of disease (5). Vaccinia immune globulin (VIG) has been the only approved therapy for PV since 1955, and while positive outcomes were attributed to its use, no placebo-controlled studies have been performed. Since human clinical trials to determine the efficacy of anti-vaccinia virus treatments are not ethical or feasible, the mechanisms by which VIG works in immunocompromised patients and methods to improve VIG efficacy have been insufficiently explored.

The recent occurrence of PV in a young member of the U.S. military has renewed concern about the condition (7). The patient was treated over a period of 2 months with a variety of therapies, including multiple doses of VIG, oral and topical ST-246, CMX001, and topical imiquimod, with eventual lesion resolution and clearance of infection. In such situations, little insight can be achieved concerning the effects of individual therapies or combinations of therapies; moreover, patients may be exposed to unnecessary drug toxicities. Studies with animal models should provide more straightforward evidence to inform treatment options, as well as facilitate the identification of new treatments for PV. Specifically, we sought to further study and test a relevant, accessible model of PV that could help to optimize existing VIG treatments and provide a platform for testing new therapeutics and combination therapies.

Animal models of PV were described by the early 1960s and have included mice with primary immune defects, such as SCID and nude mice, as well as immunocompetent animals given immunosuppressants such as corticosteroids or cyclophosphamide (6, 23–26, 31, 34, 37, 40). In two recent studies, postexposure VIG treatment delayed but did not prevent death in SCID mice receiving intravenous Dryvax or intranasal Western Reserve virus strains (23, 34). However, continuous

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combination treatment with VIG and a nucleoside derivative after intradermal vaccinia virus inoculation in SCID mice prevented lethal disease but did not eliminate infection and was only effective while treatment was ongoing (19).

We selected SCID mice as a worst-case model, representative of human PV patients with combined cellular and humoral immunodeficiencies (34). Various experimental models of poxvirus infection have utilized neurovirulent vaccinia virus or ectromelia (mousepox) virus strains, which produce lethal infections in immunocompetent or immunocompromised animals (22, 41). Previous studies of vaccinia virus infection in nude, SCID, or cyclophosphamide-treated mice have demonstrated slowly progressive infection, with some similarities between human PV and murine models (19, 25, 37). SCID mice were selected as a model for combined T and B cell immunodeficiencies, to provide a stringent challenge for therapy, and to replicate combined deficiencies seen in historical PV cases. The lack of anti-human antibody responses and the prolonged half-life of human antibodies in SCID mice also replicates the human situation when VIG preparations are used and eliminates the possibility of confounding anti-human antibody responses (42). The VIG doses administered in our studies were up to 342,000 U/kg, which is 57 times higher than the initial recommended dose of VIG for PV treatment (6,000 U/kg) but comparable in scale to the amount used in the recent PV case (186,000 U/kg) and likely to compare to many historical cases, where large volumes of VIG were administered (7, 32).

We utilized the New York City Board of Health vaccinia virus strain (Dryvax) that has caused PV in humans, and mice were scarificed with the same bifurcated needles used to administer smallpox vaccine to people. As in humans with PV, scarificed SCID mice developed a subacute, slowly progressive infection characterized by expanding primary lesions, distal satellite lesions, anorexia, weight loss, and inevitable death if left untreated. As also described in humans, the cutaneous lesions contained relatively few inflammatory cells even late in the course of disease (5, 13, 21). Postinfection treatment with licensed VIG modified the disease course and resulted in the healthy survival of most mice long after cessation of treatment, suggesting that antibody alone can prevent PV in the setting of intradermal infection and that combination treatment may entirely resolve a more established infection. To our knowledge, this study expands the characterization of a murine PV model in a strain that faithfully replicates the combined T and B cell immunodeficiencies seen in the most severe cases of PV in the 1950s and 1960s. Characterization of this model provides a platform for better understanding of treatment dosing and timing and suggests testable hypotheses for determining correlates of disease progression and amelioration. The unexpected finding of the long-term healthy survival of mice treated only with VIG provides a means of elucidating how antibodies may function to inhibit indolent viral disease in the absence of cellular immunity.

### MATERIALS AND METHODS

#### Mice.
SCID/NCr mice were purchased from the NCI Frederick Animal Production Program and were 6 to 12 weeks old unless otherwise noted. Mice were housed and cared for according to guidelines of the Animal Research Advisory Committee of NIH. Protocols for experiments were approved by the Center for Biologics Evaluation and Research Intramural Animal Care and Use Committee.

**Vaccinia virus strain and preparation.** A HeLa cell passage of the smallpox vaccine virus (Dryvax; derived from the New York City Board of Health strain and produced as a vaccine by Wyeth) was obtained from the laboratory of Michael Merchlinsky via Hana Golding (CBER, FDA). Vero E6 cells (ATCC, Manassas, VA) were infected at a low multiplicity of infection (0.2) for 1 h at 37°C and then incubated at 37°C and monitored for a cytopathic effect daily. On day 3 postinfection, cells and cell culture supernatant were moved to centrifuge tubes and spun for 15 min at 2,000 x g at 4°C in an Eppendorf 5404R centrifuge.
equipped with a swinging bucket rotor. The resulting pellets were processed with a chilled Dounce homogenizer, resuspended in cell culture medium, and clarified by centrifugation at 750 × g for 10 min at 4°C. The supernatant was disrupted using a cup horn sonicator for 4 cycles each composed of 15 s at 100% power, 50% duty cycle, followed by 15 s on ice. Aliquots were removed to check sterility and viral titer, and the remainder was aliquoted and frozen at −70°C.

VIG. VIG intravenous (human) is a sterile, purified solution containing antibodies to vaccinia virus provided and manufactured by Cangene Corporation. This preparation is made from human plasma obtained from donors who have been immunized with smallpox vaccine. It is supplied in vials containing 40 to 70 mg/ml total protein and >50,000 U/vial vaccinia virus antibody as determined by a plaque neutralization assay. The same lot of VIG was used for all experiments, and it contained 53 mg/ml total protein and 9,145 U/ml vaccinia virus neutralizing activity.

Vaccination, VIG, and cidofovir treatment. Aliquots of vaccinia virus were removed from −70°C storage, thawed on ice, and sonicated for 3 cycles, each consisting of 15 s at 90% power at a 50% duty cycle and 15 s on ice. Virus was diluted to the required concentration in cDMEM (Dulbecco’s minimal essential medium containing 10% fetal bovine serum, 4.5 mg/ml D-glucose, 110 μg/ml sodium pyruvate, 100 μM nonessential amino acids, 100 U/ml penicillin-streptomycin, and 0.25 μg/ml amphotericin) and kept on ice prior to scarification. To perform scarification, mice were anesthetized with ketamine-xylazine and the fur was removed from the lower back area using clippers. Ten microliters of the virus inoculum (i.e., 10⁶ PFU/ml for a 10⁶ PFU challenge, 10⁵ PFU/ml for a 10⁵ PFU challenge, and 10⁴ PFU/ml for a 10⁴ PFU challenge) was pipetted onto the skin covering the cartilaginous area proximal to the base of the tail. A bifurcated vaccination needle (Precision Medical Products, Denver, PA) was used to deliver 20 punctures through the inoculum solution. Mice were monitored for clinical

FIG. 3. Primary lesion histopathology in mice scarified with 10⁴ PFU of vaccinia virus. Early lesion samples are from days 15 to 20 postchallenge, and late infection samples are from days 27 to 37 postchallenge. The stains used are H+E (a, b, c), Wright’s Giemsa stain to visualize hematopoietic cells and specifically identify the mast cell population (d, e, f), Masson’s trichrome to highlight connective tissue (blue) (g, h, i), and rabbit anti-vaccinia virus antibody to visualize cells expressing vaccinia virus antigens (j, k, l). Results are representative of two or three mice for each time point. Scale bars, 100 μm.
Results are representative of two or three mice. Scale bars, 100 μm.

Lesion area analysis was performed using digital photographs and a calibrated reference.

**Vaccinia virus plaque assay for tissues.** Individual organs collected from euthanized mice were weighed and homogenized in cDMEM. The homogenized samples were sonicated for 3 cycles, each consisting of 15 s at 80% power at a 50% duty cycle and 15 s on ice, and were then serially diluted with cDMEM and plated onto confluent VeroE6 cells. After a 1-h adsorption at 37°C, an overlay consisting of 1.5% methylcellulose in cMEM was added. Plates were incubated for an additional 3 days or until plaques were observed and were then stained with crystal violet in ethanol-formalin, and plaques were counted.

**Histology and immunohistochemistry.** Mouse tissues were fixed in 10% neutral buffered formalin and then embedded in paraffin blocks for processing. Five-micrometer sections on positively charged glass slides were deparaffinized using consecutive xylene washes and dehydrated using an ethanol gradient and then rehydrated prior to staining with hematoxylin and eosin (H&E). For immunohistochemistry, heat-induced epitope retrieval was performed with citrate buffer. Tissues were incubated with dilutions of rabbit antiserum detecting vaccinia virus antigens (BEI Resources, Manassas, VA), anti-human IgG (Jackson Immunoresearch, West Grove, PA), or an irrelevant control (rabbit anti-hepatitis B core antigen; Zymed, San Francisco, CA) in albumin blocking buffer. Bound antibodies were detected with appropriate species-specific peroxidase-labeled secondary antibody (Jackson Immunoresearch) followed by diaminobenzidine peroxidase substrate (Sigma, St. Louis, MO).

**Statistical analysis.** Statistical analysis was performed using Prism 5 (GraphPad Software).

**RESULTS**

**Clinical and histopathological progression of intradermally acquired vaccinia virus infection in SCID mice.** SCID mice scarified with 10⁶ PFU at the base of the tail developed an expanding ulcerating primary lesion that failed to resolve (Fig. 1). After infection, the primary lesion enlarged continuously over the course of the next 30 to 40 days, and mice eventually developed distal satellite lesions, lethargy, anorexia, weight loss, and increasingly severe morbidity and died (Fig. 2). By day 15, primary lesions of infected mice showed epidermal keratinocyte proliferation at the edge of areas of skin necrosis. This epidermal proliferation continued to increase through the disease course (Fig. 3a to c); vaccinia virus antigens colocalized with proliferating keratinocytes and sebaceous gland cells (Fig. 3j to l). Vaccinia virus-infected SCID mouse skin maintained epidermal and dermal structural integrity, and minimal inflammatory cell infiltration was observed. Tissue mast cells appeared to be increased at later time points postinfection, compared with those in control samples (Fig. 3d to f). Ongoing infection was also associated with accumulation of collagen in the dermis, suggestive of fibroblast activity (Fig. 3g to i). Neutrophilic inflammation was apparent only at late time points postinfection, at the base of ulcerated skin lesions (Fig. 4). In mice challenged with 10⁴ PFU, plaque-forming virus was detected only in the ovaries and skin and rarely in the lungs (Fig. 5). In experiments using a larger challenge dose (10⁶ PFU), virus was sporadically detected at low levels in other tissues, including the heart, liver, spleen, kidneys, brain, and gastrointestinal tract (data not shown).

**Effects of treatment with VIG.** To further examine the SCID model and assess its relevance to human disease, mice were treated postexposure with VIG. Preliminary experiments were conducted using dose and schedule variations to determine a feasible regimen that resulted in increased survival of treated mice. Unless otherwise noted, mice received 10 mg of VIG i.p. on days 2, 5, 10, and 15 postscarification with virus doses...
ranging from $10^4$ to $10^6$ PFU (Fig. 6). Mice were monitored for lesions and death until the termination of experiments. Survival of VIG-treated mice but not control mice increased as the virus challenge dose was reduced. Most VIG-treated mice that received a $10^4$ or $10^5$ PFU viral challenge remained healthy for more than 120 days. Interestingly, while all of the mice developed early vaccinial lesions at the primary immunization site, these regressed in a proportion of the VIG-treated mice but never in the control mice (Fig. 7 and 8). Lesion regression was observed between day 4 and day 15 after vaccinia virus infection and correlated with long-term survival. This finding was dose dependent with respect to viral challenge (Table 1). Detection of human antibody in VIG-treated mice in the skin confirmed effective transudation of human antibody into the epidermis and colocalization with epidermal cells and sebaceous glands (Fig. 9).

**Clearance of vaccinia virus in mice treated with cidofovir and VIG.** Once infection is established, vaccinia virus has been difficult to eradicate in immunocompromised mice receiving VIG monotherapy (34), as well as in human patients receiving extensive monitoring and aggressive treatment (7). To test whether combined drug and antibody treatment might prove effective after established infection, mice received a combination of cidofovir and VIG at 7 days postinfection, a time when scarification lesions are well established. Treatment was stopped at 20 days postinfection, and mice were monitored for 16 additional weeks. All of the treatments increased the time to death, compared to that of untreated mice (Fig. 10). However, only the combination of VIG and topical cidofovir resulted in long-term survival (50%).

**DISCUSSION**

It is striking that VIG treatment can prevent the development of PV in mice that are completely lacking in adaptive immunity, if given early enough in the course of disease. We observed dose-dependent frequencies of lesion regression and long-term survival when VIG treatment was started at 2 days postinfection, and a proportion of the mice survived disease free for at least 120 days postinfection without receiving VIG beyond day 15. Results of combination treatment with VIG and topical cidofovir provide the first demonstration that an established vaccinia virus infection is cleared in SCID mice after the cessation of treatment by any regimen. Cellular immunity has been deemed necessary for effective vaccinia virus clearance since the 1960s, when it was recognized that PV patients had cellular, as well as humoral, immunodeficiencies. Supporting the contention that antibodies are not essential for controlling primary vaccinia virus infections are observations that the same patients with agammaglobulinemia have been vaccinated and have not developed PV, that mice can clear poxvirus infections in the absence of B cells, and that mice and humans with defective cellular immunity seem unable to resolve vaccinia virus infection (3, 5, 18, 25). In immunocompetent mice, both antibodies and T cells are involved in the resolution of primary vaccinia virus infections, although in NYVBOH strain-scarified mice, no single immune cell type among CD4⁺, CD8⁺, B, and NK cells is essential (3). However, an absolute role for cellular immunity is controversial since B cells, but not CD4⁺ or CD8⁺ T cells, are required for vaccine-induced protection from monkeypox virus in a nonhuman primate model. Moreover, passive transfer of anti-vaccinia virus antibodies protects primates from severe monkeypox disease (11). This divergence of opinion may be partially explained by the diversity of experimental models used to explore the relative importance of adaptive immune responses. Model systems, as well as human observations, vary with respect to the type of underlying immunodeficiency. In animal studies, the vaccinia virus strain, dose, and route of challenge also differ. Additionally, a strong innate immune response may...
be beneficial in the clearance of ongoing disease (a treatment scenario) while an adaptive immune response may be sufficient for prevention of disease. Our observations suggest that aggressive antibody treatment, even in the absence of adaptive cellular immune responses, can treat a developing indolent vaccinia virus infection. Since the use of VIG can be considered late prophylaxis or early treatment in our model, the high levels of antibodies being administered may delay or limit infection to the extent that innate effectors are sufficient for clearance of virus.

The precise mechanism of action of VIG is not known; however, the correlation between primary lesion regression and survival and the benefits of topical over parenteral cidofovir support Neyts' contention that the skin is an important platform for the eventual spread of vaccinia virus to organs (25, 30, 37). The vaccinia virus dose-response relationship to survival in mice treated with the same dose of VIG suggests that early antibody treatment limits virus spread from the skin and also that early postexposure prophylaxis in humans, as soon as immunocompromised status is recognized and when the viral burden is relatively light, may be prudent. It is possible that antibody Fc region functions such as complement binding or accessory cell activation (macrophages, NK, or mast cells) help to resolve local or systemic vaccinia virus infection in SCID mice. Complement-dependent efficacy of an anti-B5 monoclonal antibody has recently been demonstrated (4), and NK cells can be important for limiting vaccinia virus infection (14, 20). Our ongoing work is focused on Fc region functions of VIG to identify effector mechanisms. If Fc-mediated effects are important, VIG product potency enhancement strategies could be envisioned and potency could be further characterized and assured for new antibody preparations.

In SCID mice inoculated with the NYCBOH strain of vaccinia virus, the expanding primary lesion, development of distal lesions, and slow pace of infection mimic clinical descriptions of PV in humans (5, 13, 15). Similar to reported human lesions, SCID mouse lesions contained few inflammatory cells other than neutrophils in underlying ulcerated skin in later infection. Local satellite lesions were not observed in SCID mice; these are variably observed in PV patients. It is difficult to know whether the time course of disease is different in SCID mice and human patients, in part because the more well-documented human cases had interventional treatments and medical support for recent cases was intensive. Unfortunately, further comparisons of the human and murine versions of the disease cannot be made because specimens from historical PV cases are unavailable for study using modern techniques. Important information that would further support the relevance of the SCID model to human PV includes the timing of the distribution of vaccinia virus infection beyond the skin and understanding of the fundamental mechanism(s) of death. In the SCID mouse, dissemination appears to be related to the inoculum dose. A small challenge dose (10^4 PFU) results in a very limited dissemination of virus, while a larger challenge dose (10^6 PFU) results in a more widespread, albeit sporadic, distribution of virus, with titers in the affected organs apparently much lower than those described for other mouse orthopox disease models (12, 28, 36). In humans, very little

### TABLE 1. Primary lesion resolution in and survival of mice that received VIG

| Vaccinia virus dose (PFU) | No. of VIG-treated mice | No. (%) with primary lesion healing | No. (%) of survivors at 120 days |
|--------------------------|------------------------|-----------------------------------|--------------------------------|
| 10^6                     | 66                     | 36 (55)                           | 0                              |
| 10^5                     | 14                     | 10 (71)                           | 6 (43)                         |
| 10^4                     | 18                     | 15 (83)                           | 13 (72)                        |

* Mice received VIG (10 mg/mouse on days 2, 5, 10, and 15 after vaccinia virus infection) and were challenged with 10^6, 10^5, or 10^4 PFU of vaccinia virus. Using a two-tailed chi-square test, the primary lesion healing endpoint was significantly different between the 10^6 and 10^5 PFU challenge groups (P = 0.0266) but not between the 10^6 and 10^4 PFU (P = 0.2458) or the 10^5 and 10^4 PFU (P = 0.4190) groups. Likewise, survival differed significantly (P < 0.001) between the 10^6 PFU challenge group and either the 10^5 PFU or the 10^4 PFU challenge group but not between the 10^5 and 10^4 PFU groups (P = 0.0934).
The increase in mast cells observed at late time points in this model, similar to findings in vaccinia virus-scarified guinea pigs (10), is of uncertain significance and possibly specific to the rodent models selected. There are no data regarding mast cell recruitment to PV lesions in humans and limited information on how mast cells may impact the course of viral infections generally. Recent studies show that mast cells can speed the development of adaptive immunity (9, 35), which could enhance antipathogen responses in immunocompetent animals. Moreover, mast cells bear FcR (8, 27) and can be activated and/or degranulated in the presence of antibody, raising the possibility of synergy with VIG. Additional work is required to explore these possibilities.

In comparing our studies to prior studies of VIG in immunocompromised mice, we note a number of differences that might explain the unexpected observation of long-term survival of VIG-treated SCID mice. Variations include different routes of administration which are shown to impact vaccinia virus pathogenesis (23, 34), different strains of vaccinia virus (19, 23, 40), different VIG preparations whose potencies relative to that of the VIG we used or relative to each other are not known (19, 23, 34), and different models of immunocompromised status. One previous study showed that combining VIG treatment with a nucleoside derivative resulted in prolonged survival of scarified SCID mice while treatment was continued, although vaccinia virus persisted at the inoculation site and the mice were not followed long term off therapy (19). Prior work demonstrated that cidofovir alone and VIG alone can both delay death, which provided a rationale for attempting combination treatments (19, 23, 25, 34, 37).

Given the limited knowledge of human PV pathogenesis, use of the SCID mouse in conjunction with licensed smallpox vaccine given intradermally seems to be a relevant and informative model for studying PV antibody therapy, with or without additional drugs. This study further confirms the in vivo effects of VIG, provides new information about the efficacy potential of combination treatments in severely immunocompromised patients, and supports the SCID mouse model as one that should be useful in understanding how VIG suppresses vaccinia virus in the immunocompromised host.

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REFERENCES

1. Ahmad, N., P. Cheng, and H. Mukhtar. 2000. Cell cycle dysregulation by green tea polyphenol epigallocatechin-3-gallate. Biochem. Biophys. Res. Commun. 275:328–334.
2. Barbero, G. J., A. Gray, T. F. Scott, and C. H. Kempe. 1955. Vaccinia gangrenosa treated with hyperimmune vaccinal gamma globulin. Pediatrics 16:609–618.
3. Belyakov, I. M., et al. 2003. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. Proc. Natl. Acad. Sci. U. S. A. 100:9458–9463.
4. Benhnia, M. R., et al. 2009. Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. J. Virol. 83:1201–1215.
