Postexposure Immunization with Modified Vaccinia Virus Ankara or Conventional Lister Vaccine Provides Solid Protection in a Murine Model of Human Smallpox

Nir Paran,[1,a] Yasemin Suezer,[2,a] Shlomo Lustig,[1,a] Tomer Israely,[1] Astrid Schwantes,[2] Sharon Melamed,[1] Lior Katz,[1] Thomas Preuß,[2] Kay-Martin Hanschmann,[2] Ulrich Kalinke,[2] Noam Erez,[1] Reuven Levin,[1] Baruch Velan,[1] Johannes Löwer,[2] Avigdor Shafferman,[1] and Gerd Sutter[2]

[1]Israel Institute for Biological Research, Ness-Ziona, Israel; [2]Paul-Ehrlich-Institut, Langen, Germany

Background. Decades after the cessation of smallpox vaccination, the potential of the deliberate release of pathogenic orthopoxviruses has forced a reconsideration of using these extremely efficient human vaccines. Scenarios of sudden biothreats have prompted demand for rapidly protective vaccination. However, the feasibility of short-term vaccination (i.e., vaccination shortly before exposure) with vaccinia virus (VACV) is uncertain.

Methods. We tested the rapid protective capacity of vaccines based on VACV strain Lister (VACV-Lister) and on modified VACV Ankara (MVA) in different mouse models, comparing lethal infections with VACV strain Western Reserve (VACV-WR) or ectromelia virus (ECTV).

Results. In contrast to VACV-WR challenge, we found extended incubation periods after ECTV challenge, allowing successful therapeutic immunization with VACV-Lister and MVA when applied 2–3 days after exposure. Rapid protection from respiratory tract ECTV infection was significantly affected by vaccine dose and was associated with occurrence of poxvirus-specific antibodies. Vaccinations in type I interferon receptor–deficient mice were protective, whereas recombination activating gene 1–deficient mice lacking mature T and B cells failed to mount immunity after short-term vaccination, confirming an essential role of adaptive immune responses.

Conclusions. ECTV infection in mice models the course of human smallpox. Our data provide evidence to substantiate historical data on the usefulness of postexposure vaccination with conventional VACV and the new candidate MVA to protect against fatal orthopoxvirus infections.

Several strains of vaccinia virus (VACV) have been successfully used for immunization against human smallpox, and global eradication of the natural causative agent, variola virus (VARV), was achieved in 1980 [1]. Recently, a reevaluation of VACV vaccines became a priority because of the increasing threat of the intentional use of orthopoxviruses as agents of bioterrorism [2].

Conventional smallpox vaccines based on VACV strain Lister (VACV-Lister) were the most frequently used vaccines in the World Health Organization smallpox eradication campaign. Local application of these vaccines (by skin scarification) was highly efficacious in providing solid protective immunity against smallpox [1]. Yet severe adverse effects associated with these vaccines in certain populations have spurred an interest in obtaining safer vaccines, for example, ones based on the highly attenuated modified VACV Ankara (MVA) and on the Lister-derived VACV LC16m8 [3]. MVA is a safety-tested and replication-deficient VACV that was derived from conventional VACV-Ankara and that is undergoing clinical testing as a replacement smallpox vaccine and as a candidate vector for vaccine development against various infections and cancer diseases [4–9].

The conventional smallpox vaccines are known to have several adverse effects, but the effectiveness of safer next-generation vaccines has never been tested in actual smallpox outbreaks. This dilemma is potentiated by the uncertainty regarding the time, place, and dimension of a bioterrorism event. It makes prophylactic vaccination of large populations against smallpox less feasible and underscores the need for alternative measures. One such approach relies on short-term (i.e., vaccination shortly before exposure) or postexposure vaccination.

The noted long incubation time of smallpox disease after infection suggests that vaccination immediately after exposure could be effective, yet data supporting this hypothesis are quite limited. Before smallpox eradication, several anecdotal studies of people who were vaccinated with VACV apparently after exposure to VARV were conducted [10]. These data suggest that vaccination with VACV can be effective, at least in part, up to 4 days after exposure to VARV. Because of the absence of an accepted animal model for VARV infection and the eradication of the human disease, there is no supporting experimental data that would enable the determination of the window of protection and the efficiency of newer vaccines compared with the conventional ones. However, respiratory tract infection of mice with the neurovirulent VACV strain Western Reserve (VACV-WR) is a well-established model for characterizing the protective capacity of orthopoxvirus-specific immunization [11–13]. Short-term but not postexposure protection against a lethal challenge with VACV-WR has been achieved using MVA vaccine [14], and recent data have suggested that therapeutic MVA vaccination against ECTV, the causative agent of mousepox, might be effective [15].

Here, we further investigate the possibility of short-term and postexposure protection in mice infected with VACV-WR and ECTV. We demonstrate different disease profiles in each model and suggest that ECTV infection more closely resembles human smallpox. Using this model, we show that, at equal doses, both MVA and the conventional vaccine VACV-Lister protect mice even when administered 3 days after infection and that protection requires the rapid induction of adaptive immunity.

METHODS

Cells and viruses. Vero (ATCC CCL-81), BS-C-1 (ATCC CCL-26), HeLa (ATCC CCL-2), BHK-21 (ATCC CCL-10), and CEF cells were used and were maintained by routine methods, as described elsewhere [16]. Ectromelia virus (ECTV) strain Moscow (ATCC VR-1374), VACV-WR (ATCC VR-119), VACV-Lister (Elstree; provided by the Israeli and German Ministries of Health), and MVA clonal isolate F6 [4, 9, 17–19] at the 584th CEF passage were used and were titrated in plaque-forming units, as described elsewhere [16, 20]. Virus inactivation was performed by UV irradiation (2.5 mL of virus suspension exposed with horizontal agitation for 15 min). Complete inactiva-

tion was verified by immunostaining and plaque assay, as described elsewhere [21].

Measurement of VACV-specific antibodies. Serum anti-VACV IgG and IgM titers were measured by ELISA, as described elsewhere [22].

Immunization experiments in mice. Female BALB/c and C57BL/6 mice (6–10 weeks old) were purchased from Charles River Laboratories. C57BL/6J-Rag1[tm1Mom] mice (recombination activating gene 1 deficient [RAG-1$^{-/-}$]) [23] and B6.129S7-*Ifnar1tm1Agt* mice (type I interferon receptor deficient [IFNAR$^{-/-}$]) [24] were bred under specific pathogen–free conditions at the central animal facility of the Paul-Ehrlich-Institut. For experimental work, mice were housed in an ISOcage unit (Techniplast) and were handled in compliance with the regulations for animal experimentation of the Paul-Ehrlich-Institut and the Israel Institute for Biological Research.

Intradermal (ID) vaccination was done by tail scarification [21]. A droplet of 10 μL of virus suspension (containing 1×10^6 pfu) was deposited on the mouse skin at the tail base. The skin was then scratched with the tip of a 26-gauge needle (Braun) through the droplet to allow virus uptake. For intramuscular (IM) vaccination, 50 μL of virus suspension containing 1×10^6 or 1×10^8 pfu were injected into the right hind leg. Intranasal (IN) immunization was performed as described elsewhere [12, 14]. Serum samples were obtained and treated as described elsewhere [21].

Challenge experiments. Respiratory tracts were infected by IN instillation of 20 μL of virus suspension [14, 21]. For challenge, VACV-WR was used at $1\times$, $3\times$, $5\times$, and $10\times$ LD$_{50}$ (where $1\times$ LD$_{50}$ corresponds to $\sim 5 \times 10^4$ pfu in BALB/c and C57BL/6 mice). ECTV was applied at $3\times$, $5\times$ or $10\times$ LD$_{50}$ (where $1\times$ LD$_{50}$ corresponds to ~ 1 pfu in BALB/c mice and ~ 80 pfu in C57BL/6 mice). After challenge, signs of illness, weight loss, and death were monitored daily for 14–22 days.

Determination of viral load in mouse organs. Organs were homogenized in PBS at 10% tissue weight to buffer volume in an IKA-Werke tissue homogenizer. The suspensions were sonicated ($3\times$, 30 s, 3000 J) and centrifuged (250 g, 5 min, 4°C), and virus in supernatants was titrated on BS-C-1 cells.

Analysis of viral load in blood. Viral DNA was isolated from blood samples by means of the QIAamp DNA-Mini Kit (Qiagen). ECTV DNA was amplified by means of the Artus Orthopox LC PCR Kit (Qiagen), in accordance with the manufacturer's recommendations, on a LightCycler 1.5 instrument (Roche). Data were analyzed with LightCycler SW3.5 software.

Statistical analysis. Statistical comparison of different vaccination groups was performed by means of the area under the weight curve, adjusted for individual weight differences at baseline (day of infection). Experiments with different mouse types and vaccination strategies were analyzed by means of a 2-factorial analysis of variance model. For multiple compari-
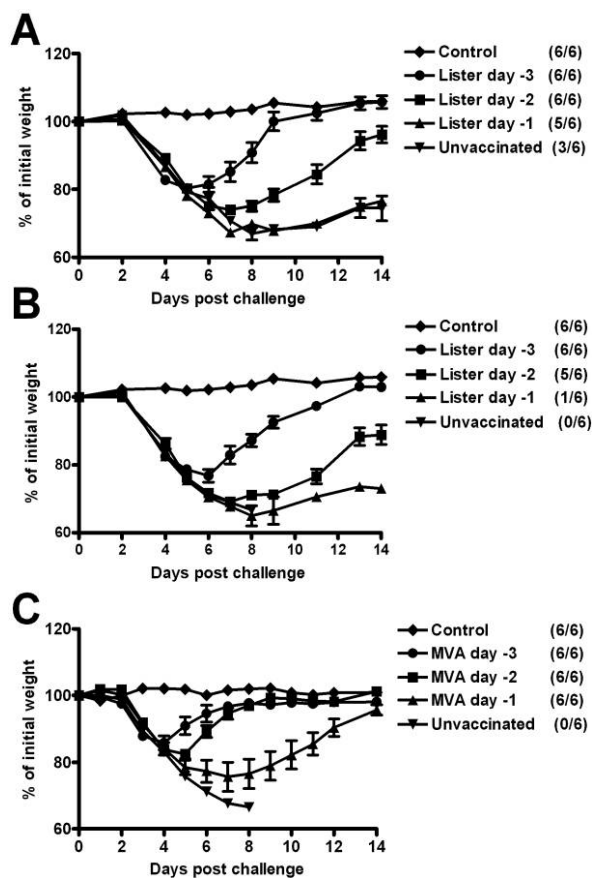
Figure 1. Protective capacity of short-term vaccination with vaccinia virus (VACV) strain Lister (VACV-Lister) and modified VACV Ankara (MVA) against VACV strain Western Reserve (VACV-WR) challenge. In panel A, mice were vaccinated by tail scarification with 1×10^6 pfu of VACV-Lister 3, 2, or 1 day before challenge with VACV-WR at $1 \times LD_{50}$. In panel B, the experiment was performed as described for panel A except that VACV-WR was used at $3 \times LD_{50}$ for the challenge. In panel C, mice were vaccinated by intramuscular injection of 1×10^8 pfu of MVA 3, 2, or 1 day before challenge with VACV-WR at $3 \times LD_{50}$. In all experiments, weight loss was monitored daily, using unchallenged (control) and unvaccinated mice as controls. The nos. of surviving mice per the total no. of mice in each group are indicated in brackets. Error bars indicate SEs.

sons, *P* values were adjusted by means of the Bonferroni method. Survival was evaluated by means of Kaplan-Meier curves and the log-rank test. Analyses were performed with SigmaPlot software (version 8; Systat) and SAS/STAT software (version 9.2; SAS System for Windows).

RESULTS

Short-term vaccination against VACV-WR challenge. In light of previous studies, we set out to more closely examine the efficacy of short-term vaccination of BALB/c mice with VACV-Lister or MVA against IN VACV-WR challenge with varying doses of $1 \times$ and $3 \times LD_{50}$ (figure 1). VACV-Lister alleviated morbidity (lower weight loss and shorter morbidity periods)

and conferred full protection (100% survival) against $1 \times LD_{50}$ IN challenge when administered 2 days before challenge. Vaccination 1 day before challenge was still protective (83% survival), yet morbidity was similar to that in unvaccinated mice (figure 1A). Vaccination with VACV-Lister was not effective when done on the day of challenge. In contrast, MVA vaccination on the day of challenge conferred full protection (data not shown). When mice were challenged with $3 \times LD_{50}$, vaccination with VACV-Lister 2 days before challenge effectively protected from death (83% survival); however, severe morbidity was alleviated only when vaccination was applied 3 days before challenge ($P < .002$) (figure 1B). In comparison, MVA vaccination protected more efficiently against the $3 \times LD_{50}$ challenge (figure 1C), with 100% survival and with significant reduction of morbidity in all groups ($P < .001$). Mice vaccinated 3 or 2 days before challenge exhibited only mild to moderate symptoms of illness (with loss of 12%–18% of their initial body weight), whereas mice vaccinated 1 day before challenge had more severe disease and >20% weight loss. Altogether, these results in the VACV-WR respiratory tract infection model clearly demonstrate the possibility of providing protection via short-term vaccination with MVA and VACV-Lister.

Disease progression in VACV-WR– and ECTV-infected mice. To determine the protective capacity of VACV vaccination against ECTV challenge, we wished to test the 2 prototype inbred mouse models BALB/c and C57BL/6. IN ECTV infection of BALB/c mice with $10 \times LD_{50}$ resulted in a lethal disease with a mean time to death (MTTD) of 10 days, much longer than that observed for VACV-WR infection (MTTD, 6 days) ($P < .002$) (figure 2A). Furthermore, the disease induced by ECTV infection had a 7-day lag period, whereas the illness caused by VACV-WR infection began 2–3 days after infection (figure 2B). The accumulation of virus in target organs correlated well with the rate of disease progression. Infection was characterized by early extensive proliferation in the lungs, which was then followed by virus accumulation in the spleen and liver, reaching peak levels by day 8 (figure 2C). It is interesting to note that rates of virus accumulation in the spleen and liver after IN infection were similar to those after footpad inoculation (data not shown and [25]). In accordance with its known neurovirulence, we readily found infectious VACV-WR in brain tissues, but we failed to detect ECTV in the brains of infected mice (figure 2C).

ECTV-infected C57BL/6 mice revealed very similar mortality and morbidity profiles (figure 2D and 2E), confirming the substantial difference with VACV-WR infection ($P < .001$ for the comparison of MTTD). Analysis of viral loads in C57BL/6 mice demonstrated high copy numbers of ECTV genomes in blood samples from days 5–7 after infection, whereas no viral DNA was detected in blood samples from VACV-WR–infected mice, suggesting that VACV-WR infection failed to generate genuine viremia (data not shown).
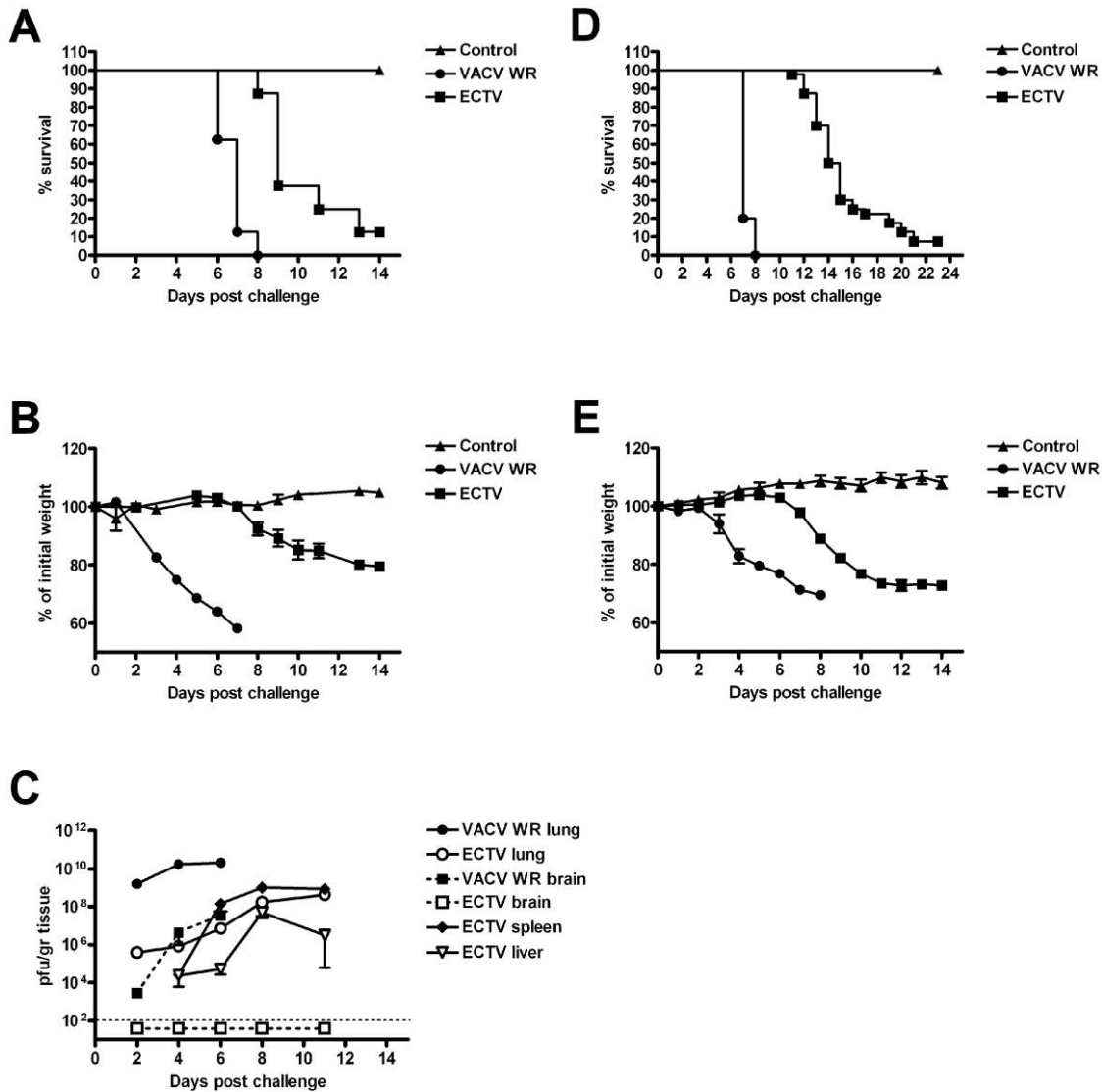
Figure 2. Disease progression in mice infected with vaccinia virus (VACV) strain Western Reserve (VACV-WR) or ectromelia virus (ECTV). Shown are survival of *(A)* and morbidity in *(B)* BALB/c mice after intranasal (IN) infection with VACV-WR and ECTV at $10\times$ LD$_{50}$, survival of *(D)* and morbidity in *(E)* C57BL/6 mice IN infected with VACV-WR at $5\times$ LD$_{50}$ or ECTV at $3\times$ LD$_{50}$, and viral loads *(C)* in tissues of BALB/c mice IN infected with VACV-WR or ECTV at $10\times$ LD$_{50}$. Control mice were left uninfected. The dotted line indicates the detection limit, and error bars indicate SEs.

These apparent differences between the ECTV and VACV-WR infection models in BALB/c and C57BL/6 mice recommend ECTV infection as a suitable mouse model of human smallpox.

Short-term and postexposure vaccination against ECTV infection. BALB/c and C57BL/6 mice were inoculated with VACV-Lister or MVA vaccine by different routes (ID, IM, or IN) at various time points before or after lethal respiratory track challenge with ECTV at $3\times$ LD$_{50}$. In contrast to the results obtained with VACV-WR, vaccination of BALB/c mice with VACV-Lister fully protected when given on day -2 or -1 (before challenge) and even when given on the day of challenge, regardless of the vaccination route (table 1). Moreover, using the ECTV model, we could demonstrate effective postexposure protection with VACV-Lister. Survival was ~80% when vaccination was done 1 day after challenge

(ID and IM) but was reduced to 16%–50% 2 days after exposure, depending on the route of administration (table 2). In contrast, MVA vaccination 2 days after exposure conferred full protection from death, and 60% survival was found for vaccination 3 days after challenge (table 1). Furthermore, IM vaccination with MVA prevented weight loss even when given 1–2 days after infection ($P < .002$) (figure 3*B*–3*D*), whereas VACV-Lister could prevent it only via short-term vaccination ($P < .002$) (figure 3*A*). Also, IN immunizations appeared to be highly efficient and particularly attractive for MVA vaccination. In the ECTV-infected C57BL/6 mouse model, we could demonstrate full protective capacity of MVA given IN on days -2 to $+1$ with a significant reduction in morbidity in all vaccinated mice ($P < .001$ in figure 3*E*–3*G* and $P < .02$ in figure 3*H*), and substantial protec-

Table 1. Protective capacity of short-term and postexposure vaccination against ectromelia virus (ECTV) challenge.

| Day of vaccination | BALB/c mice | | | C57BL/6 mice,[a] MVA at 10^8 pfu, IN |
| | VACV-Lister at 10^6 pfu | | MVA at 10^8 pfu, IM | |
	ID	IM		
−2	100 (6/6)	100 (5/5)	100 (5/5)	100 (6/6)
−1	100 (6/6)	100 (5/5)	100 (5/5)	100 (6/6)
0	100 (6/6)	100 (5/5)	100 (5/5)	100 (6/6)
+1	83 (5/6)	80 (4/5)	100 (5/5)	100 (6/6)
+2	16 (1/6)	40 (2/5)	100 (5/5)	50 (3/6)
+3	ND	20 (1/5)	60 (3/5)	ND
+4	ND	20 (1/5)	20 (1/5)	ND

NOTE. Data are percentage (proportion) of survivors. Mice were challenged with ECTV ($3\times$ LD$_{50}$) by intranasal (IN) instillation. Unvaccinated mice did not survive the challenge (0/6). MVA, modified vaccinia virus Ankara; ND, not determined; VACV-Lister, vaccinia virus strain Lister.

[a] Additional data on C57BL/6 mice after vaccination with VACV-Lister: after intradermal (ID) vaccination on day −2, 84% (5/6) of the mice survived the challenge; after intramuscular (IM) vaccination on day −1, 100% (6/6) of the mice survived the challenge.

tion was still found 2 days after challenge (50% survival) (table 1).

Contribution of antigenic mass to vaccine potency. To evaluate the possible contribution of vaccine dose, we infected mice with ECTV at $5\times$ LD$_{50}$ and, after exposure, vaccinated them IM with equal doses (1×10^6 or 1×10^8 pfu) of either MVA or VACV-Lister (table 2). Given that VACV-Lister can replicate efficiently in mammalian cells while MVA cannot, we also included a control group that was vaccinated with UV-inactivated VACV-Lister at a dose equivalent to 1×10^8 pfu. Inoculation of either vaccine on day 0 and +1 provided full protection against death independent of dose. Vaccination at later days (+2 and +3) clearly revealed that increasing the dose of VACV-Lister and MVA from 1×10^6 to 1×10^8 pfu improved the survival rates. Interestingly, vaccination with inactivated VACV-Lister could protect at levels similar to those obtained with live vaccine of the same dose (1×10^8 pfu)

($P < .002$) (table 2 and figure 4A–4D). Two days after exposure, inoculation of 1×10^6 pfu of VACV-Lister produced 40% survival, with 80% of the mice exhibiting weight loss and other signs of illness (figure 4 and data not shown). Although morbidity was not significantly improved ($P = .86$), MVA immunization with 1×10^6 pfu achieved 80% survival ($P < .002$) (figure 4). None of the mice that were vaccinated with 1×10^8 pfu of MVA or with the inactivated VACV-Lister experienced morbidity or died ($P < .002$) (figure 4D). The MVA vaccine lost its apparent advantage when immunizations were done 3 days after challenge (table 2).

In general, the protective capacities of the MVA and VACV-Lister vaccines became markedly more similar when equal doses of the vaccines were used. Both MVA and VACV-Lister can effectively provide postexposure protection against a lethal host-specific orthopoxvirus infection, and in this scenario the initial antigenic mass introduced by vaccination obviously has a major influence on vaccine potency.

Requirement of adaptive immunity for protective immunization. To elucidate to some extent the possible contribution of innate or adaptive responses to protective immunization, we vaccinated IFNAR$^{-/-}$ mice with 1×10^8 pfu of MVA (day −2, IN) and challenged them with ECTV at $3\times$ LD$_{50}$ (figure 5A). The IFNAR$^{-/-}$ mice were effectively protected ($P < .001$), but, unlike in C57BL/6 mice (figure 5C), we observed slight symptoms of disease, suggesting an important but not essential role for type I IFN in MVA-induced immunity. However, in the same experimental setting, no protection whatsoever was observed in RAG-1$^{-/-}$ mice, which lack mature B and T cells, the key effectors of adaptive immune responses (figure 5B). Thus, unlike type I IFN–dependent innate responses, adaptive immunity is indispensable to achieving rapid protection against ECTV.

Protection against secondary poxvirus infection by adaptive immunity is mainly antibody mediated [26–28]. Thus, we followed the kinetics of antibody responses (IgM and IgG development) in BALB/c mice after vaccination with VACV-Lister or MVA by IM application, the route showing the highest protec-

Table 2. Protective capacity of postexposure vaccination against ectromelia virus (ECTV) challenge ($5\times$ LD$_{50}$).

| Day of vaccination | VACV-Lister | | | MVA | |
	1×10^6 pfu	1×10^8 pfu	1×10^8 pfu, inactivated	1×10^6	1×10^8
0	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)
+1	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)	100 (5/5)
+2	40 (2/5)	80 (4/5)	100 (5/5)	80 (4/5)	100 (5/5)
+3	40 (2/5)	80 (4/5)	80 (4/5)	60 (3/5)	80 (4/5)

NOTE. Data are percentage (proportion) of survivors. Both modified vaccinia virus Ankara (MVA) and vaccinia virus strain Lister (VACV-Lister) vaccines were administered intramuscularly. Unvaccinated mice did not survive the challenge (0/6).
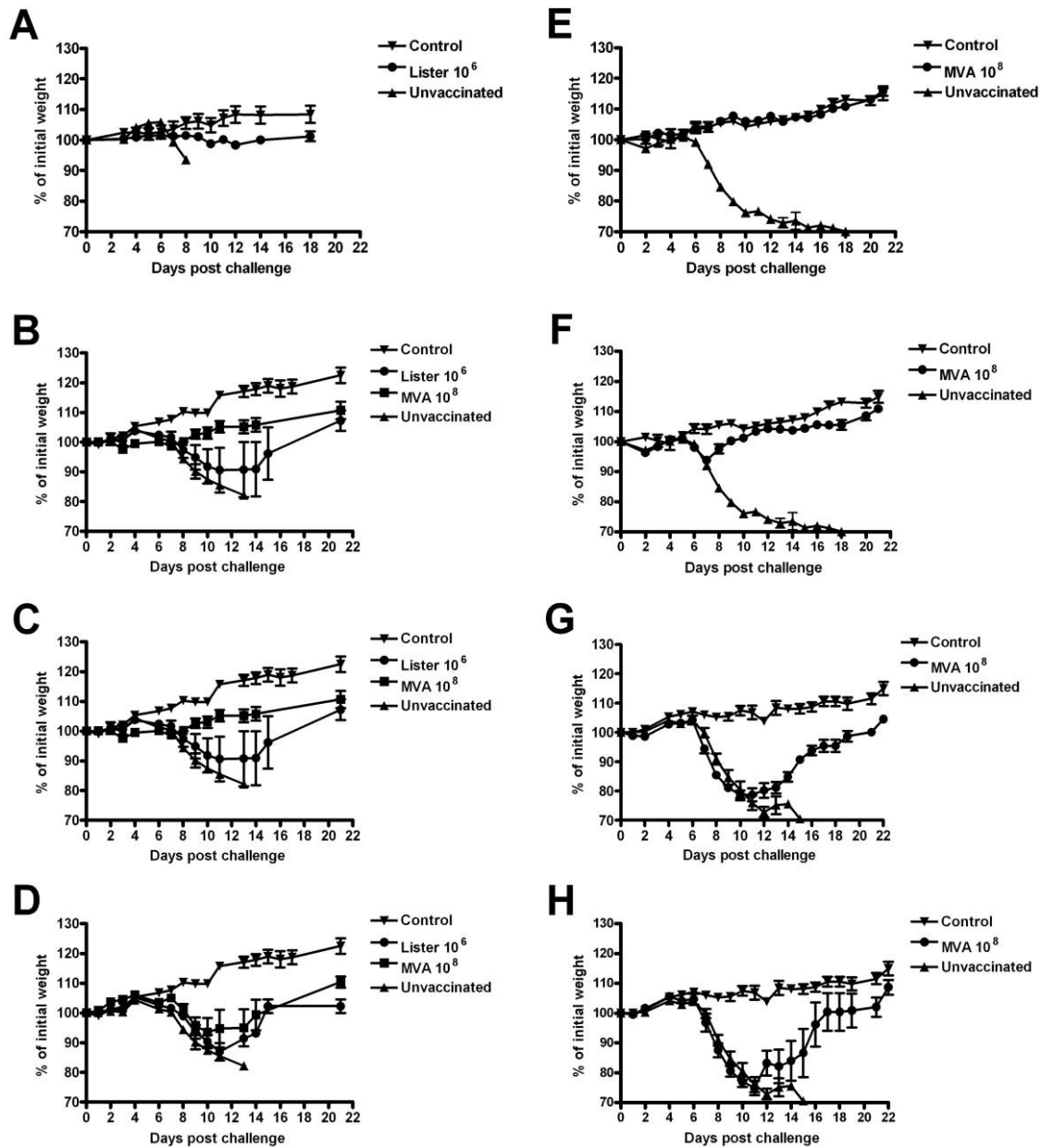
Figure 3. Protective capacity of postexposure vaccination against ectromelia virus (ECTV) infection. Shown are morbidity profiles for ECTV-infected ($3\times$ LD_{50}) BALB/c *(A–D)* or C57BL/6 *(E–H)* mice vaccinated on day -1 (*A* and *E*), day 0 (*B* and *F*), day $+1$ (*C* and *G*), or day $+2$ (*D* and *H*) relative to infection with vaccinia virus (VACV) strain Lister (1×10^6 pfu intradermally *[A–D]*) or modified VACV Ankara (MVA) (1×10^8 pfu intramuscularly *[A–D]* or intranasally *[E–H]*). Unchallenged (control) and unvaccinated mice served as controls. Error bars indicate SEs.

tive efficacy in our experiments (figure 5*D* and 5*E*). Vaccination with 1×10^6 pfu of either VACV-Lister or MVA resulted in rather modest responses, but 1×10^8 pfu of either vaccine induced increased amounts of IgM antibodies, which peaked at similar levels on day 9 (figure 5*D*). Nevertheless, MVA appeared to have some advantage over VACV-Lister in inducing a more rapid response. Vaccine dose also clearly influenced the kinetics of IgG induction (figure 5*E*). Elevating the immunization dose to 1×10^8 pfu markedly increased the IgG titers, more prominently for MVA than for VACV-Lister, which also seemed to

induce high-level IgG at a quicker rate. Interestingly, vaccination with UV-inactivated VACV-Lister also induced IgM and IgG responses, resembling the response induced by an equivalent dose of live vaccine (data not shown).

To estimate the antibody levels required for protection, we passively transferred to mice varying doses (3×10^3–3×10^4 ELISA geometric mean titer [GMT]) of rabbit anti-vaccinia hyperimmune serum and challenged them 1 day later with ECTV at $3\times$ LD_{50}. Full protection was achieved by antibody titers of at least 1×10^4 ELISA GMT, suggesting that effective passive im-
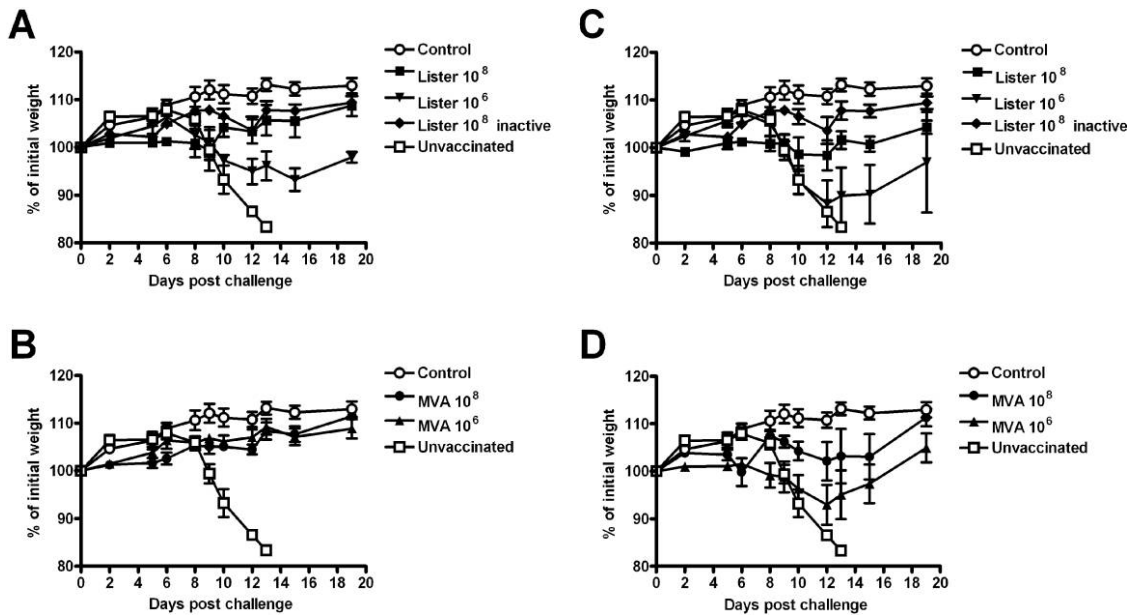
Figure 4. Role played by dose in postexposure protection mediated by vaccinia virus (VACV) strain Lister (VACV-Lister) and modified VACV Ankara (MVA). BALB/c mice were infected by intranasal instillation of ectromelia virus (ECTV; $5\times$ LD_{50}) and, after exposure, were vaccinated intramuscularly with either VACV-Lister (*A* and *C*) or MVA (*B* and *D*). Vaccinations were given on day +1 (*A* and *B*) or day +2 (*C* and *D*) after infection. Vaccines were used at dose equivalents of 1×10^6 or 1×10^8 pfu. Morbidity was monitored daily. Unchallenged (control) and unvaccinated mice served as controls. Error bars indicate SEs.

munization requires titers of antibodies higher than those induced by protective vaccination (data not shown).

DISCUSSION

A collection of historical anecdotal studies indicated that vaccination of previously unvaccinated individuals up to 4 days after exposure to VARV reduced, at least to some degree, the rate of mortality and the severity of smallpox [1, 10]. The purpose of the present study was to generate further experimental evidence for this observation. Because smallpox has been eradicated, generation of new data must rely on animal models. The most prevalent experimental model for orthopoxvirus infection is based on VACV-WR, which causes rapid encephalitic disease on IN delivery of rather high infectious doses [29–31] and thereby appears to differ from human smallpox, a host-specific systemic infectious disease produced by very low infectious doses of VARV [1].

Indeed, our previous work [14] and data from the present study (figure 1) show that vaccination with MVA or VACV-Lister can confer protection when administered before challenge or on the day of challenge but is not effective as a postchallenge treatment, which may result from inherent drawbacks of the VACV-WR model in mice. Alternatively, ECTV infection of mice is considered an excellent surrogate for a small-animal model of smallpox, because mouse-restricted ECTV is infectious at very low doses and causes a fatal systemic disease [32].

As shown here (figure 2), IN infection with ECTV in BALB/c and C57BL/6 mice is characterized by long incubation periods,

viremic phases, delayed death, and, in comparison with VACV-WR, longer times required to reach maximal viral loads in the lungs. In contrast, VACV-WR infections seem to lack extensive viremia but produce substantial viral loads in the brain. Interestingly, in both respiratory tract infection models the onset of morbidity appeared to correlate with virus accumulation in the lungs. However, after ECTV infection we found similar virus loads in the spleen and liver, other relevant target organs in the pathogenesis of mousepox. Thus, it remains unclear whether ECTV lung infection results in a somewhat different disease pattern from that described for the classic footpad infection model. Comparable data on viral loads during human smallpox are not available, yet the VARV entry route via the respiratory tract, the requirement of a low infectious dose, the rather long disease-free period during smallpox, and the lack of encephalitis support the relevance of the ECTV model in research for smallpox therapies [32–35].

Importantly, by means of this model we could clearly demonstrate the efficacy of the VACV-Lister and MVA vaccines in postexposure applications (tables 1 and 2 and figures 3 and 4). Vaccine dose had a major influence on the success and timing of protective vaccination. The observed effect of dose brings forward the role played by vaccine virus replication in protection. In contrast to VACV-Lister, MVA is unable to productively replicate in vivo, and vaccination with MVA apparently results in a lower and transient concentration of antigen at the site of inoculation [18, 36]. When comparing the protection conferred by
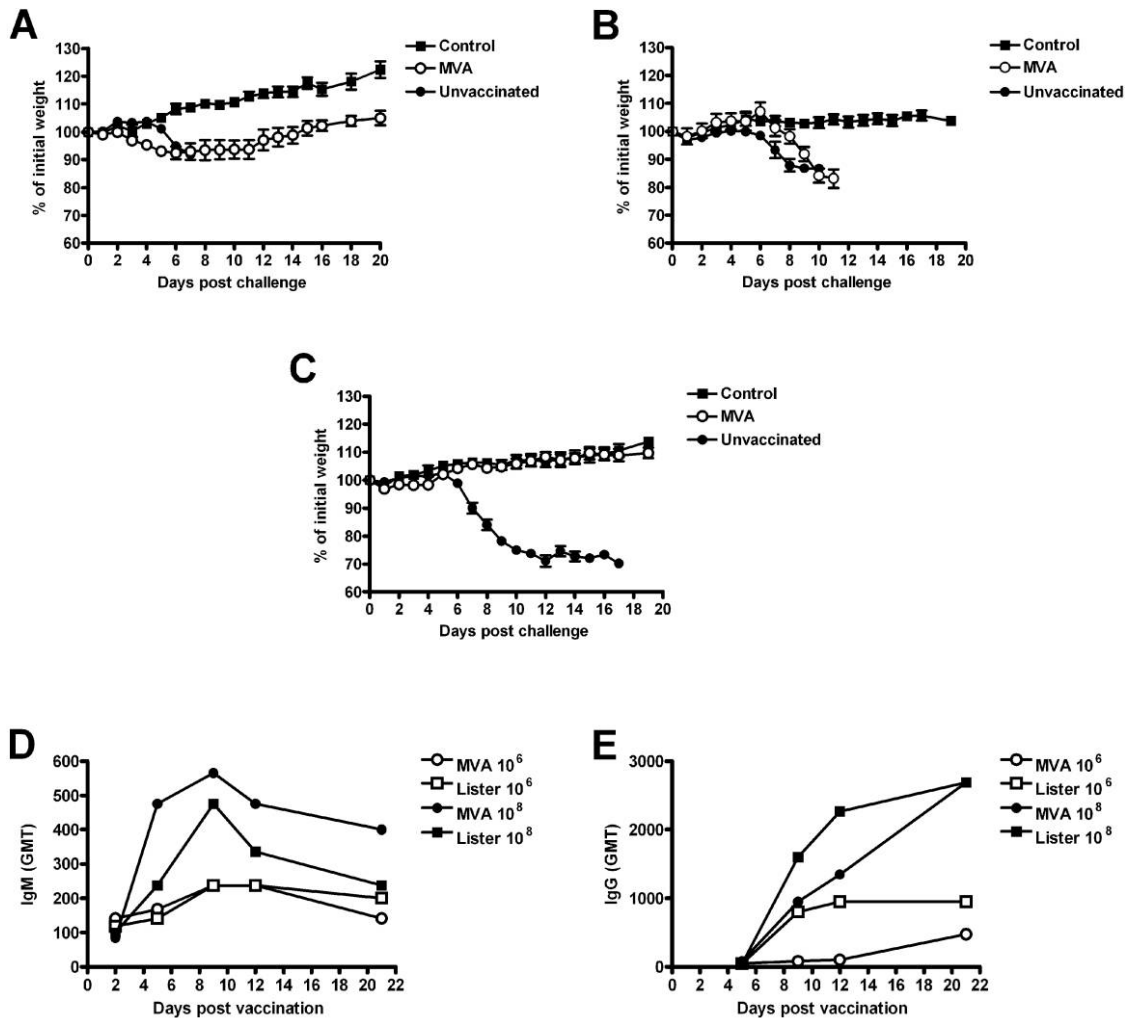
Figure 5. Requirement of the induction of adaptive immunity for protection against ectromelia virus (ECTV). *A–C,* Vaccination of mice lacking activities of type I interferon (IFN) or mature B and T cells. IFNAR$^{-/-}$ *(A),* RAG-1$^{-/-}$ *(B),* or C57BL/6 control *(C)* mice were immunized intranasally (IN) with 1×10^8 pfu of modified vaccinia virus (VACV) Ankara (MVA) 2 days before IN infection with ECTV at $3\times$ LD$_{50}$. Unchallenged (control) and unvaccinated mice served as controls. Error bars indicate SEs. *D* and *E,* Development of antibody responses after vaccination. Shown are anti-VACV IgM *(D)* and IgG *(E)* levels in serum of mice vaccinated intramuscularly with MVA or VACV-Lister at doses of 1×10^6 or 1×10^8 pfu. Data are geometric mean titers (GMT) of antibody ($n = 6$).

inactivated VACV-Lister with that conferred by live VACV-Lister or MVA, we found similar efficacies as long as equally high antigen doses (equivalent to 1×10^8 pfu) were administered (table 2). This observation is intriguing in view of the accepted belief that the efficacy of a VACV vaccine largely depends on its ability to stimulate antibody responses against both infectious forms of virions, intracellular mature virus and extracellular enveloped virus (EEV) [37–39]. However, because the EEV outer envelope is highly fragile, EEV antigen is easily lost during vaccine production. The EEV antigen content in our inactivated VACV-Lister vaccine preparation is unclear, and we cannot exclude the possibility that EEV-specific responses contributed to the protection mediated by our inactivated vaccine. Alternatively, one could argue that the high antigen dose may play a central role in the onset of rapid postexposure immunity and

may override the need for EEV-specific antibodies. Thus, postexposure vaccination could have antigen requirements different from those for immunizations with VACV vaccines tested in conventional models that challenge mice weeks or months after vaccination [40, 41].

That RAG-1$^{-/-}$ mice were not protected against ECTV infection (figure 5*B*) indicates that a functional B and/or T cell response is essential to rapidly mount solid immunity. To overcome ECTV infection, the critical roles of both antibody and cell-mediated responses have been well established [26, 27, 42–44]. The result of a comparison between active and passive immunization in our model of postexposure protection against ECTV was in compliance with this assumption. We observed that the net antibody levels required for passive protection are higher than the antibody titers attained by active immunization.

This suggests that other immune effectors (e.g., CD8 and CD4 T cells) are likely also involved in the protection conferred by postexposure vaccination. In addition to allowing time for adaptive immunity to develop, initial innate immune responses with anti-ECTV functions (e.g., involving natural killer cells, Th1 cytokines, and IFNs) are likely implicated [45–48]. Although recent work has corroborated the capacity of MVA to efficiently induce type I IFN [49], the efficacy of MVA vaccination in both IFNAR$^{-/-}$ (figure 5A) and Toll-like receptor 9 (TLR9)$^{-/-}$ mice [15] implies that type I IFN– and TLR9-dependent innate immunity is not essential in the protection conferred by short-term and postexposure vaccination. After VACV-WR infection, MVA-vaccinated RAG-1$^{-/-}$ mice developed disease significantly later than did unvaccinated mice, which might result from innate immunity induced by the vaccine [14]. Surprisingly, this short-lasting protective effect was not evident in ECTV-infected RAG-1$^{-/-}$ mice, suggesting that ECTV can efficiently counteract the mouse innate immune response, a notion that is also supported by the identification of ECTV type I IFN– binding protein as an essential virulence factor [48] and the inhibition of TLR9 signaling by ECTV [15].

Taken together, our observations broadly highlight the importance of appropriate animal models for vaccine evaluation. Although caution is needed in extrapolating from mice to humans, our data strongly support the potential of VACV for postexposure immunization in the event of emerging VARV or other orthopoxvirus infections. This is of practical relevance because swift vaccinations would be a major challenge if an outbreak were to occur. Postexposure vaccination, in combination with other treatment modalities, should provide more flexibility in being prepared for an epidemic in previously unvaccinated populations.

Acknowledgments

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