Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8

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A highly attenuated LC16m8 (m8) smallpox vaccine has been licensed in Japan because of its extremely low neurovirulence profile, which is comparable to that of replication incompetent strains of vaccinia virus. From 1973 to 1975, m8 was administrated to >100,000 infants where it induced levels of immunity similar to that of the originating Lister strain, without any serious side effects. Recently, we observed that m8 reverts spontaneously to large plaque forming clones that possess virulence equivalent to that of LC16mO, a parental virus strain of m8. Here, we report that the *B5R* gene is responsible for the reversion, and that we could construct a more genetically stable virus by deleting *B5R* from m8. The protective immunogenicity of the vaccine candidate proved to be equivalent to that of the U.S.-licensed product Dryvax, and much superior to modified vaccinia Ankara in a mouse model. Furthermore, the vaccine strain never elicited any symptoms in severe combined immunodeficiency disease mice, even at a dose 1,000-fold greater than that used in the immune protection experiments, which is in contrast to the lethal pathogenicity induced by Dryvax inoculation of severe combined immunodeficiency disease mice. Our results suggest that this vaccine strain is a good candidate as a suitable smallpox vaccine and a vector virus, and that B5R is not essential for protective immunity against smallpox.

B5R gene | reversion | Lister strain | extracellular enveloped virion

Although smallpox was eradicated >20 years ago (1), the necessity of a smallpox vaccine has been reawakened by concerns of bioterrorism using the smallpox virus (2) and outbreaks of monkeypox (3). However, the current vaccine in the United States, Dryvax, occasionally elicits serious adverse effects, including postvaccinal encephalitis (4). Accordingly, a safer smallpox vaccine is much needed.

In Japan, a highly attenuated form of vaccine referred to as LC16m8 (m8) was administrated to >100,000 infants without any serious adverse events and proved to be as immunogenic as the Lister (LO) strain (5, 6), a once widely used vaccine. m8 was indirectly isolated from LO through intermediate strains, such as LC16mO (mO) and LC16. m8, a variant that forms small-sized pocks, is a direct descendant of mO, which itself is a clone that forms medium-sized pocks, isolated from the LC16 strain (5). LC16 was selected from LO based on its temperature sensitivity (5, 7, 8). In rabbit and monkey models, the neurovirulence of m8 was markedly reduced in comparison with other vaccine strains (5, 7–9), including LO and Dryvax (10, 11), and comparable to the replication-defective mutant DIs (Dairen I-derived small-sized pock variant) (12). Moreover, m8 exhibited a markedly diminished dermal reaction in both rabbits and humans and a lower fever ratio compared with mO in clinical trials (5, 6). Therefore, m8 was finally adopted as a vaccine strain instead of mO (6).

Takahashi-Nishimaki *et al.* (13) first identified the vaccinia virus (VV) gene *B5R* as responsible for large plaque formation and proliferating ability in Vero cells. m8 has lost the *B5R* function as the result of a frameshift mutation brought about by a single base deletion in the ORF. *B5R* encodes a 42-kDa glycoprotein that is involved in packaging the intracellular matured virion with trans-Golgi membrane or endosomal cisternae to form an intracellular enveloped virion (IEV) (14–16). IEV is transported along microtubules to the cell periphery (17, 18) where it adheres to the cell surface as a cell-associated enveloped virion (CEV). B5R, in cooperation with the A36R and A33R proteins, also participates in the Src kinase-dependent process of forming of actin-containing microvilli and releasing CEV from the cell surface to form an extracellular enveloped virion (EEV) (19, 20). Despite its relative paucity of whole progeny virions, EEV plays an important role in dissemination within the host (21). Because anti-B5R antibodies can neutralize EEV, expression of *B5R* has been proposed as an effective smallpox vaccine (14, 22–25). In contrast, the results of the field trial in Japan showed that neutralizing (NT) antibody titers induced by m8 were similar to a conventional LO vaccine (5, 6).

Recently, we found that m8 reverted spontaneously to large plaque-forming clones (LPCs).§ The content of LPCs seemed to increase rapidly in proportion to passage number of the virus. Because LPCs emerged from plaque-purified m8, their generation appears to be an intrinsic property of m8. We were concerned that LPC contamination might ruin the safety of the m8 vaccine. Therefore, to improve the m8 strain, we tested whether *B5R* was the gene responsible for the reversion, because this gene has been correlated with large plaque formation. We then constructed genetically more stable virus by deleting *B5R*. Moreover, by using this virus, we were able to evaluate the contribution of *B5R* to protective immunity against smallpox.

Methods

Virus Preparations. m8 was obtained from Chiba Serum Institute (Chiba, Japan). m8rc (plaque-purified m8 to minimize contamination by revertants) and the revertant viruses (LPCs) were isolated from the m8 stock by three serial plaque purifications in RK13 cells. The modified VV Ankara (MVA) (26, 27) and Western Reserve (WR) viruses were obtained from S. Morikawa (National Institute of Infectious Diseases, Tokyo). MVA was propagated and titrated in chicken embryo fibroblasts. Other viruses were propagated and titrated in RK13 cells, and purified by sedimentation through a 36% sucrose cushion. A vial of Dryvax vaccine, obtained from I. K. Damon and J. Becher (Centers for Disease Control and Prevention, Atlanta), was

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Abbreviations: m8, LC16m8; LO, Lister; mO; LC16mO; VV, vaccinia virus; EEV, extracellular enveloped virion; NT, neutralizing; LPC, large plaque-forming clone; MVA, modified VV Ankara; WR, Western Reserve; PRK, primary rabbit kidney; SCID, severe combined immunodeficiency disease; ErD_{50}, 50% erythema dose; RED_{50}, 50% rash expression dose; pfu, plaque-forming units.

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dissolved in the enclosed solvent, aliquoted, and stored at −80°C. Construction of m8B5R, which harbors the intact *B5R* gene, m8Δ, which lacks the entire *B5R* gene, and m8dTM, which expresses only the ectodomain of the B5R protein, and characterization of their properties, including the structures of *B5R* in the viruses used, are described in more detail (*Supporting Text*, Table 1, and Fig. 5, which are published as supporting information on the PNAS web site).

Western Blotting. We performed immunoblotting by using an antiserum from rabbits that were immunized with baculovirus-expressed recombinant B5R protein. The anti-B5R sera were used at a dilution of 1:200, and detected with a horseradish peroxidase-labeled secondary antibody and an ECL Plus kit (Amersham Pharmacia Biosciences, Piscataway, NJ).

Evaluation of Genetic Stabilities of VVs. We passaged the VVs in primary rabbit kidney (PRK) cells that are used for vaccine production 7 times at 30°C or 34°C, then in Vero cells 2 times at 34°C to amplify LPCs, or 10 times in PRK at 30°C or 34°C. We estimated the fraction of LPCs as the ratio of plaque counts on Vero cells to those on RK13 cells.

Animals. Severe combined immunodeficiency disease (SCID) mice (female, 6 weeks old) and BALB/c mice (female, 6 weeks old) were purchased from Charles River Japan (Kanagawa, Japan). Female Japan white rabbits (16 weeks old) were obtained from Kitayama Labes (Nagano, Japan). All animal experiments were approved by the National Institute of Infectious Diseases Animal Experiment Committee and were performed in accordance with guidelines for animal experiments performed at the National Institute of Infectious Diseases.

Skin Reaction Test in Rabbits. We conducted a skin reaction test as described (10). Briefly, after inoculating tenfold serial dilutions of VVs intradermally on rabbit backs, the diameters of erythema were measured daily for 1 week. Two animals were used for each viral strain, and each rabbit received two injections of the serial dilution series of a virus. Erythemas >10 mm in diameter was scored as positive. The time at which erythemas reached their peak was determined for each animal, and the 50% erythema dose (ErD_{50}) was calculated by the Beherns and Karber method (28).

SCID Mice Infection Test. To establish an index for pathogenicity of VV against SCID mice, we defined a 50% rash expression dose (RED_{50}), which indicates the virus dose needed to induce a rash in 50% of the animals. After inoculating 10-fold serial dilutions

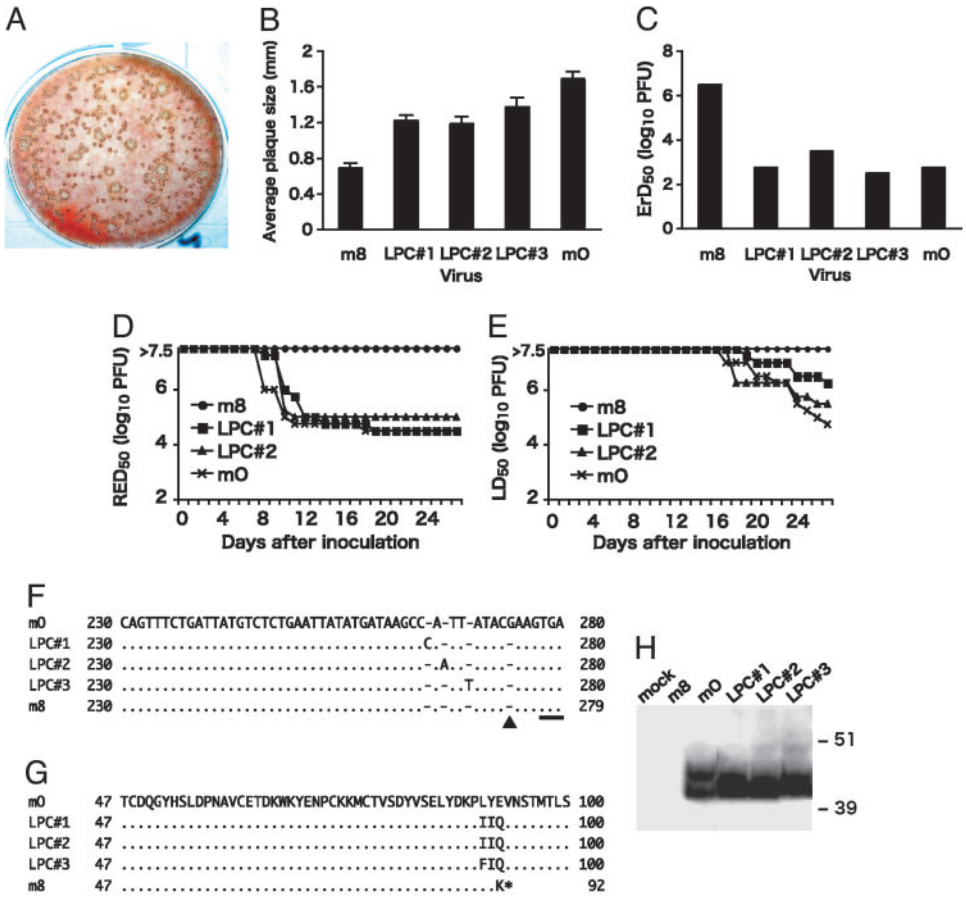

Fig. 1. Biological properties of LPC viruses. (*A*) The plaque configurations of LPCs contaminating an m8 virus stock. LPC viruses make considerably larger plaques than m8. (*B*) The mean plaque sizes of m8, mO, and plaque-purified LPCs. LPCs were isolated from an m8 stock solution. The data are presented as mean ± SD ($P < 0.05$). (*C*) The dermal reaction scores (ErD_{50}) of the LPCs intradermally inoculated in rabbits. (*D* and *E*) Pathogenicity of LPCs against SCID mice. The graphs show temporal changes of RED_{50} (*D*) and LD_{50} (*E*) for a 4-week period after inoculation. The m8 strain was asymptomatic even at the highest viral doses in this experiment (10^7 pfu). If all mice are killed by inoculation of 10^8 pfu of m8, its LD_{50} is $10^{7.5}$ pfu. Therefore, pathogenicity of asymptomatic group ought to be $>10^{7.5}$ pfu. (*F* and *G*) Alignment of the *B5R* nucleotide sequences (*F*) and amino acid sequences (*G*) of mO, m8, and three LPC viruses. Numbers at both ends of the alignments indicate residue numbers. Dots, hyphens, and black triangles in the alignments show identical sequences, gaps, and the single-nucleotide deletion of m8, respectively. The bar and asterisk in the alignments indicate the termination codon. (*H*) Western blots of B5R in VV-infected RK13 cell-lysates. Duplex bands of B5R may be the result of differential glycosylation. Molecular weight markers are shown in kDa.

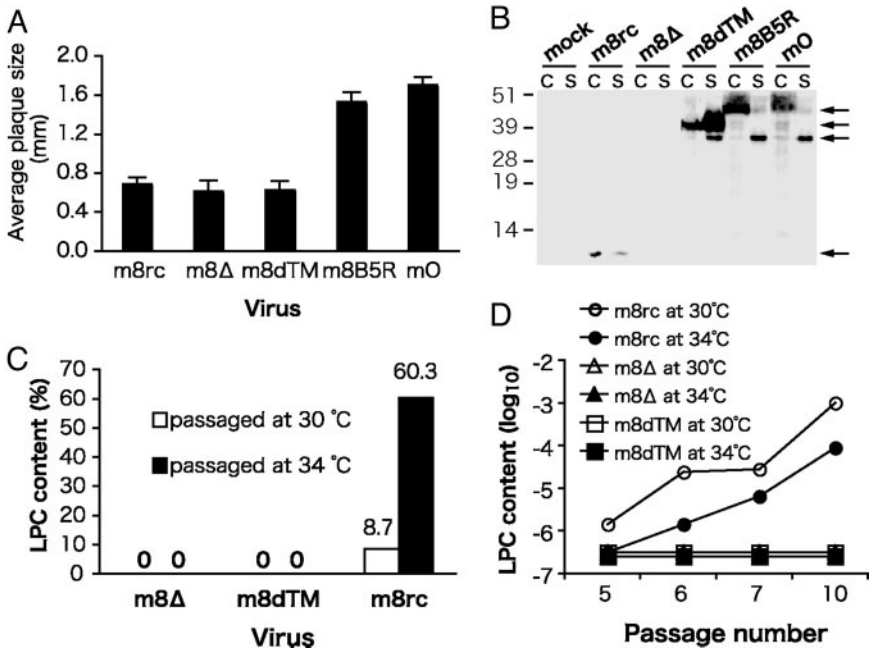

Fig. 2. Characterization of *B5R*-defective viruses. (*A*) The average plaque sizes of m8rc-, m8Δ-, m8dTM-, and *B5R*-positive viruses (m8B5R and mO). The data are presented as mean ± SD. (*B*) Western blots of VV-infected RK13 cell lysates (lane C) and supernatants (lane S). m8rc expresses a short peptide (10 kDa) in the cell and supernatant lanes. The soluble ectodomain of B5R (38 kDa) is expressed from m8dTM. The smaller molecule (35 kDa) in the supernatant lanes of m8dTM, m8B5R, and mO is proteolytically cleaved B5R by cellular proteases. (*C* and *D*) Evaluation of genetic stability by serial passages in PRK and Vero cells under different temperatures (at 30°C or 34°C). The revertant contents of viruses that were passaged seven times in PRK cells and two times in Vero cells are shown in *C*, and those contents that were passaged in PRK cells are shown in *D*.

[10^3 to 10^7 plaque-forming units (pfu)] of VVs i.p. into a series of SCID mice, we calculated the viral doses required for inducing rash (RED_{50}) or killing (LD_{50}) in 50% of the animals by the Reed–Muench method, and followed both values for 4 weeks and 8 weeks.

BALB/c Protection Study. BALB/c mice (eight animals per group) were injected intramuscularly with a single dose of 10^4 to 10^6 pfu of VVs, bled at the tail artery 3 weeks later, and then challenged intranasally with 10^6 pfu of the WR strain 4 weeks after vaccination. Individual body weight was measured daily for 3 weeks, and animals with a weight loss of >30% were killed.

Neutralization Assays. Serial 4-fold dilutions (from 2^{-1} to 2^{-7}) of heat-inactivated mouse serum were mixed with solution containing ≈200 pfu of the WR strain, incubated for 16 h at 37°C, and inoculated on RK13 cells cultured in 48-well plates. Antibody titers were defined as the reciprocal of serum dilution that reduces viral plaques by 50%. All assays were performed in triplicate. The antibody titers of sera from a mock-immunized group were <2 in our assay system.

Statistical Methods. We used Microsoft EXCEL and ORIGIN (OriginLab, Northampton, MA) for statistical analysis. The differences in the mean plaque sizes and in body weight changes measured 5 days after viral challenge in the mouse model were determined by Student's *t* test, with $P < 0.05$ as the criterion for statistical significance. The results are summarized in Table 2, which is published as supporting information on the PNAS web site.

Results

We isolated three LPC clones from an m8 stock and compared several biomarkers with m8 and mO (Fig. 1). All of the clones exhibited phenotypical characteristics similar to mO, such as plaque size (Fig. 1*B*), dermal reactions in rabbits (ErD_{50}) (Fig. 1*C*), and pathogenicity to SCID mice (Fig. 1 *D* and *E*). Specifically, i.p. injection of 10^7 pfu of m8 elicited no overt symptoms over a 4-week period, whereas mO and two LPC clones induced a severe rash and then killed mice, even when administered at a dose (10^5 pfu) 100-fold lower than that of m8 (Fig. 1 *D* and *E*). The accelerated viral replication of LPCs in Vero cells (data not shown) also supported the similarity of the mO and LPC clones. Because the growth ability of mO has been linked to the *B5R* gene product, we hypothesized that the *B5R* gene might be involved in the reversion. Sequencing the LPC genomes revealed that the *B5R* ORF was restored in all of the LPCs, by a one-base insertion at sites just upstream of the deletion site in the m8 *B5R* (Fig. 1 *F* and *G*). Western blotting confirmed the expression of B5R proteins from these LPCs (Fig. 1*H*).

To prevent the reversion of the m8 *B5R* gene, we constructed *B5R*-knockout viruses (see *Supporting Text*). First, we constructed a $B5R^+$ virus (named m8B5R) from m8 by introducing the complete *B5R* cloned from mO (*Supporting Text*, Fig. 5, and Table 1). We then deleted the entire *B5R* sequence from m8B5R to construct m8Δ (*Supporting Text*, Fig. 5, and Table 1). The resultant knockout virus formed plaques as small as the m8rc plaques that were then plaque-purified from m8 stock to minimize LPC contamination (Fig. 2*A*), and did not express the B5R protein in infected RK13 cells, whereas m8B5R and mO did (Fig. 2*B*).

One method by which to augment the immunogenicity of VV without increasing its pathogenicity may be the construction of VV that overexpresses a B5R derivative, which is fully immunogenic but loses its original function in the formation of EEV. The ectodomain of B5R has been reported to possess all epitopes necessary for induction of NT antibody production (22, 29), whereas B5R must be anchored in the membrane for EEV formation (30). We constructed a VV named m8dTM (*Supporting Text*, Fig. 5, and Table 1) that expresses only the ectodomain

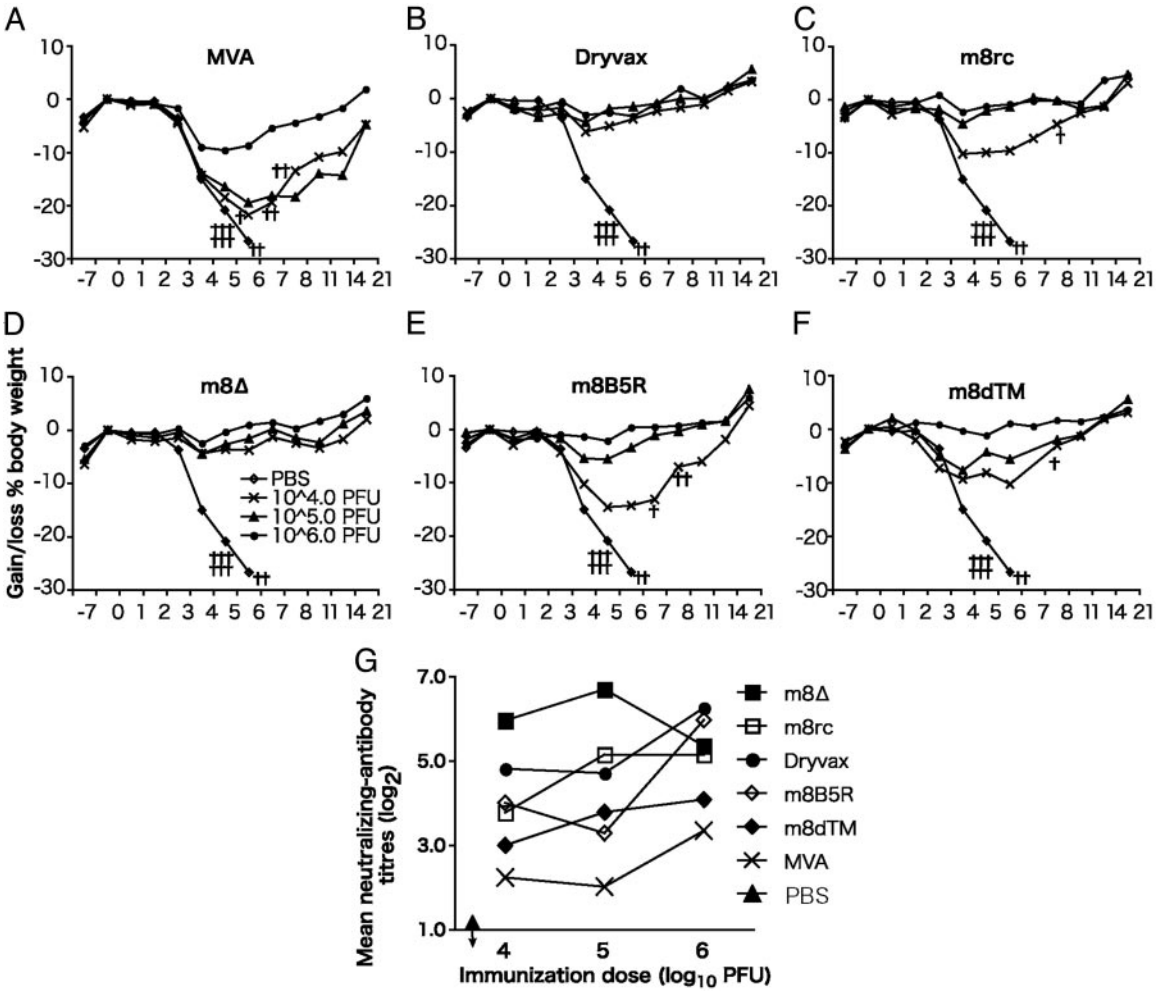

Fig. 3. Protective immunogenicity of vaccine candidate virus in mice. (*A–F*) Average body weight of mice immunized with $10^{4.0}$ to $10^{6.0}$ pfu of VVs intramuscularly and challenged intranasally with the WR strain. Cross marks indicate the mice that died or were killed because of a 30% weight loss. (*G*) Average NT antibody titers in mouse sera collected 3 weeks after immunization with VVs. The titers in sera from a Sham-immunized group were below the limit of detection.

of the B5R protein by replacing the whole *B5R* region of m8B5R with the *B5R* ectodomain sequence placed downstream of the strong promoter PSFJ1–10 (31). m8dTM also formed as small plaques as m8Δ, suggesting that this truncated B5R was not functional for EEV formation. As expected, m8dTM expresses a large quantity of a 38-kDa truncated protein in the culture medium of infected cells (Fig. 2*B*).

To evaluate the genetic stability of the viruses and m8rc, we serially passaged the viruses in PRK cells and Vero cells. Under all conditions tested, including that for vaccine production (passage in PRK cells at 30°C), detectable levels (i.e., levels of $>10^{-6}$) of LPCs failed to emerge from either m8Δ or m8dTM, which is in contrast to the LPC generation from m8rc (Fig. 2 *C* and *D*). Each of the three viruses propagated at similar levels in the cultured cells. It should be noted that once LPCs appeared in the cultures, the fraction of LPCs derived from m8rc rapidly increased with the number of passages (Fig. 2*D*), suggesting it is of vital importance to prevent the emergence of LPCs for optimum quality control of the vaccine.

The protective immunogenicities of smallpox vaccine candidates were compared with other vaccine strains by using a mouse model challenged with a highly pathogenic VV, the WR strain (32) (Fig. 3 and Table 2). All mice immunized with doses of m8Δ or Dryvax survived, whereas all Sham-immunized mice, and 5/8, 3/8, 1/8, and 1/8 mice immunized with 10^4 pfu of MVA, m8B5R, m8rc, or m8dTM, respectively, died or were killed because of a 30% weight loss (Fig. 3 *A–F*). At the lower doses, the mice immunized with m8Δ or Dryvax did not exhibit any significant differences in weight in a challenge after 5 days (*t* test, $P < 0.05$, Table 2). Moreover, the m8Δ-immunized group lost less weight than the Dryvax-immunized group at the highest dose (Table 2). In contrast, the groups immunized with 10^4 pfu of m8rc, m8B5R, and all mice immunized with MVA, experienced a significant weight loss in comparison to m8Δ ($P < 0.05$, Table 2). The m8dTM-immunized group also showed significant weight loss by days 4 and 6 ($P = 0.012$ and 0.038, respectively, data not shown). Measurement of the NT antibody titers elicited in the mice at 3 weeks after immunization (Fig. 3*G*) showed that m8Δ induced the highest titers among the viral strains at lower doses than the other immunizations. The next group, including Dryvax, m8rc, m8B5R, and m8dTM, induced NT antibodies with an efficiency intermediate between m8Δ and MVA. MVA was the least immunogenic virus: 10^6 pfu of MVA was required to induce significant NT antibodies.

The pathogenicity of the *B5R*-defective viruses was examined by ErD_{50} in rabbits (Fig. 4*A*) and by RED_{50} and LD_{50} in SCID mice (Fig. 4 *B* and *C*). m8Δ and m8dTM exhibited an ErD_{50} in rabbits similar to that of m8rc, whereas m8B5R induced the most

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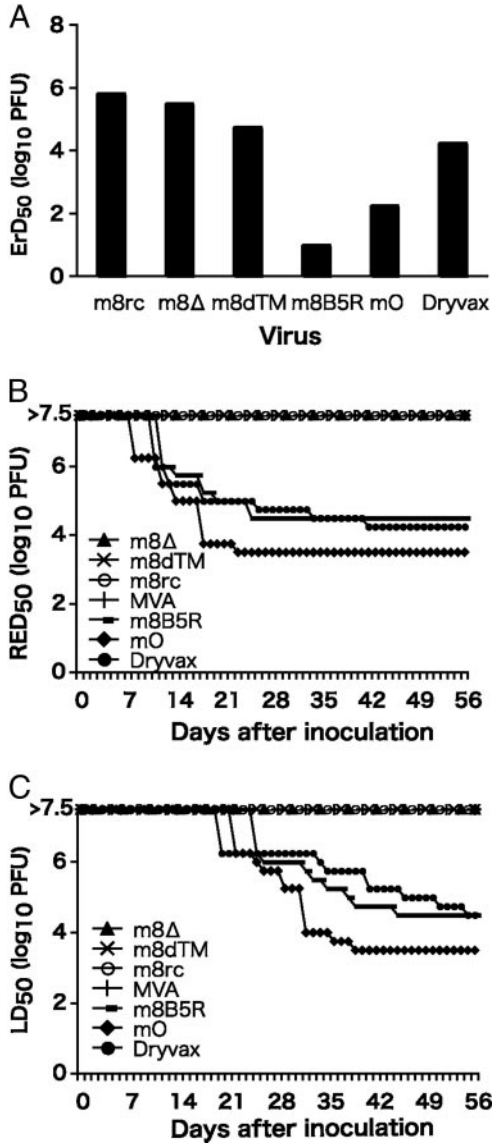

Fig. 4. Pathogenicity of vaccine candidate virus in animals. (*A*) The dermal reaction scores (ErD_{50}) of the *B5R*-defective viruses in rabbits. (*B* and *C*) Virulence of the *B5R*-defective viruses in SCID mice. RED_{50} and LD_{50} are shown in *B* and *C*, respectively.

severe dermal reaction among the strains examined (Fig. 4*A*). The pathogenicity of m8Δ and m8dTM to SCID mice was particularly weak, as demonstrated by the fact that 10^7 pfu of m8Δ or m8dTM did not elicit any symptoms in SCID mice over a period of 56 days (Fig. 4 *B* and *C*). It should be noted that this dose is 1,000-fold higher than that conferring protective immunity (see Fig. 3*D*). MVA and m8rc were also safe in SCID mice, whereas mO, m8B5R, and Dryvax exhibited lethal pathogenicities at lower doses ($10^{3.5}$, $10^{4.5}$, and $10^{4.5}$ pfu, respectively) (Fig. 4 *B* and *C*).

Discussion

One of our goals is to develop a safe and effective smallpox vaccine and vector virus. The m8 strain could be used as a prototype because it has been proven to induce an effective immune response without serious complications in humans (5, 6). It is important to note that the neurovirulence of these strains was separable from their dermal replicability, as suggested by previous experiments with LO-derived strains expressing the envelope protein of human T cell Leukemia virus type 1 (10).

A major safety drawback of m8 is its spontaneous reversion to the mO-like viruses. We identified the *B5R* gene as being responsible for the reversion, and constructed the *B5R*-defective viruses, m8Δ and m8dTM. These viruses are genetically stable and evidently retain the properties of the highly attenuated m8. Moreover, m8Δ shows a level of immunogenicity similar to that of Dryvax.

A previous study (33) reported that the m8-derived recombinant virus, which expresses the hepatitis B surface antigen, maintained its plaque size during 10 passages in the cell culture. The discrepancy between these data and our results in this study may be due to differences in the cell types used to measure plaque sizes. In the earlier study, plaque assays were performed on PRK cell monolayers on which m8 and mO form plaques that are indistinguishable in size, in contrast to the RK13 cell line used in our study. This may be one reason why the reversion of m8 has been previously undocumented.

Another purpose of this study was to evaluate the importance of *B5R* in generating an immune response that confers protection against smallpox infection. B5R protein and a DNA vaccine expressing *B5R* have been reported to induce production of NT antibodies and achieve partial protection against the virus (22–25). Therefore, we assessed the ability of *B5R*, which is expressed during viral replication in mice, to induce protective immunity. The *B5R*-defective virus (m8Δ) was able to elicit NT antibodies, leading to protection comparable to that of the wild-type *B5R*-harboring vaccine, Dryvax. Moreover, the protective efficacies of m8dTM and m8B5R were, unexpectedly, never superior to the *B5R*-defective virus (Fig. 3 and Table 2), and m8B5R was statistically inferior to m8Δ. Western blotting confirmed that B5R proteins were expressed by m8dTM, m8B5R, and Dryvax, and that these proteins were immunogenic and could induce anti-B5R antibody production in mice (data not shown). The subtle difference in protection between m8rc and the *B5R*-knockout virus may be due to the 10-kDa truncated B5R protein synthesized by m8rc (Fig. 2*B*). These data indicate that B5R does not play a major role in inducing protective immunity in response to live vaccinia inoculation in mice. The clinical trial data on m8 in Japan (5, 6) also support our conclusion.

However, the NT antibody titers induced in mice were correlated with body weight changes to some extent, but not completely. Quantitation of antibody titers by ELISAs against the outer membrane proteins of intracellular matured virion (34, 35), which includes L1R, a major target of NT antibodies, showed a similar tendency with NT antibody titers (data not shown). Moreover, the levels of A33R EEV-specific antibodies in mice, which had been suggested to be important for protective immunity (22, 23), did not correlate with the protection level (data not shown). These results may suggest that there may be a contribution of cell-mediated immunities to the protection (36, 37).

Recently, several groups have reevaluated the available vaccinia strains, including the replication-defective MVA, in a search for safer smallpox vaccines (37–40). Although 10^9 pfu of MVA was shown to be safe in monkeys (41), a large quantity of virus, 10^8 pfu, an amount that is 1,000-fold more than a conventional vaccination dosage, was necessary to induce protective immunity (40). Because m8Δ can replicate in the host, it can induce protective immunity comparable to that of the Dryvax strain at a 100-fold lower dose of the virus, making it clearly more effective than MVA. Moreover, m8Δ was not pathogenic in SCID mice at a dose 1,000-fold greater than the lethal dose of Dryvax, a dose that was also 1,000-fold greater than the dose required for its effective protective immunity. m8Δ replicated at the injection site in rabbit skin and caused temporary viremia in SCID mice (data not shown). The preliminary experiments suggested that the viral loads of VV correlate with their pathogenicity to SCID mouse. The virus seems to be eliminated rapidly thereafter and seldom replicates in the CNS (5);

therefore, the magnitude and the region of replication should be restricted, which may explain its safety and efficacy. Therefore, m8Δ should be eminently suitable as a safe and effective vaccine virus and viral vector.

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