Safety of Attenuated Smallpox Vaccine LC16m8 in Immunodeficient Mice

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Freeze-dried live attenuated smallpox vaccine LC16m8 prepared in cell culture has been the sole smallpox vaccine licensed in Japan since 1975 and was recently recommended as a WHO stockpile vaccine. We evaluated the safety of recently remanufactured lots of LC16m8 using a series of immunodeficient mouse models. These models included suckling mice, severe combined immunodeficiency disease (SCID) mice, and wild-type mice treated with cyclosporine. LC16m8 showed extremely low virulence in each of the three mouse models compared with that of its parental strains, Lister and LC16mO. These results provide further evidence that LC16m8 is one of the safest replication-competent smallpox vaccines in the world and may be considered for use in immunodeficient patients.

Smallpox was eradicated using vaccinia virus vaccines through the smallpox eradication program led by the World Health Organization (WHO); the WHO certified the eradication of smallpox in 1980 (1, 2). Vaccinia virus strains used in this program were mostly derived from the Lister and New York City Board of Health (NYCBH) strains. Data gathered in 1968 before eradication showed that the rate of serious adverse events, such as post-vaccinal encephalitis, was about 20 per 1 million primary subjects (3). Therefore, the WHO recommended that vaccinations be stopped after eradication.

The risk of bioterrorism using smallpox or other lethal agents is a major concern that has increased since the 11 September 2001 incident in New York, New York, USA. Among the pathogens that might be used in bioterrorism, variola virus is one of the most feared. Therefore, a safer smallpox vaccine is an important goal.

Serious attempts have been made to develop lower-virulent replication-competent vaccines, such as ACAM2000, derived from NYCBH Dryvax (Wyeth Pharmaceuticals) (4), and modified vaccinia Ankara (MVA) (5, 6). A nonreplicating vaccine has also been developed from the NYCBH strain. However, ACAM2000 was reported to induce myopericarditis (7, 8) and probably has virulence similar to that of other NYCBH vaccines. The replication-incompetent vaccines may have relatively poor immunogenicity (5, 6), and replication-competent MVA requires repeated vaccination for optimal immunity and maintains high levels of serum antibody for a relatively short period.

In the late 1970s, Hashizume and coworkers developed one of the safest replication-competent vaccines, LC16m8, from the original Lister strain (9, 10). This vaccine is a freeze-dried vaccine prepared in cell culture and is the sole smallpox vaccine licensed in Japan. In the original study of Hashizume et al., LC16m8 was selected as a temperature-sensitive small plaque- and small pock-forming clone (9, 10). A rabbit skin proliferation study and a neurovirulence study in which LC16m8 and Lister viruses were inoculated into the thalamus of cynomolgus monkeys showed very low pathogenicity of LC16m8 compared with that of Lister. A clinical evaluation of 90,000 infants immunized during the initial development of LC16m8 from 1973 to early 1976 showed no encephalitis or other serious adverse events after vaccination. No major differences exist in the immunogenicity of LC16m8 compared with that of conventional first-generation smallpox vaccines (3).

The LC16m8 vaccine has now been manufactured and maintained as a stockpile in Japan. A 2013 WHO Strategic Advisory Group of Experts (SAGE) meeting on immunization recommended both licensed ACAM2000 (second-generation vaccine) and LC16m8 (third-generation vaccine) as the preferred WHO stockpile vaccines (11). Based on this WHO recommendation, use of LC16m8 as a WHO stockpile vaccine for many subjects, including immunodeficient subjects in the future, is possible.

A wide epidemic of monkeypox was reported in areas of western and central Africa where a high prevalence of HIV infection exists (12). In this case, whether LC16m8 is applicable to the population, including immunodeficient subjects such as those infected with HIV, has become an important question to be answered. We report here the safety of this vaccine, focusing on experiments done with three immunodeficient mouse models inoculated with newly manufactured LC16m8 to address this question.

MATERIALS AND METHODS

Vaccine and virus strains. We used Kaketsuken-manufactured vaccine (LC16-Kaketsuken, LC16m8) prepared in a culture of rabbit kidney cells and freeze-dried. We obtained Lister (Elstree) and LC16mO, the parental strains of LC16m8, from the Chiba Serum Institute (Chiba, Japan) and propagated them in VeroE6 (Vero C1008) cells (ATCC CRL-156) and RK-13 cells, respectively. All three of these vaccine strains were titrated in VeroE6 cells and were used to vaccinate control groups of mice in the experiments.

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TABLE 1 Mouse groups used for CsA experiments

| Group | Vaccine               | CsA dose (mg/kg/day) |
|-------|-----------------------|----------------------|
| 1     | Nothing               | 0                    |
| 2     | Vaccine diluent       | 25                   |
| 3     | Vaccine diluent       | 50                   |
| 4     | LC16m8                | 0                    |
| 5     | LC16m8                | 25                   |
| 6     | LC16m8                | 50                   |
| 7     | Lister                | 0                    |
| 8     | Lister                | 25                   |
| 9     | Lister                | 50                   |

* CsA, cyclosporine.

Animals. We purchased a total of 120 ICR suckling mice (3 to 5 days old) from Japan SLC (Shizuoka, Japan). A total of 40 severe combined immunodeficiency disease (SCID) mice (CD-1/Scid, 6-week-old females) and a total of 54 wild-type C57BL/6 mice (7-week-old females) were obtained from Charles River Japan (Kanagawa, Japan). All mouse studies were approved by the Kaketsuken Institutional Animal Care and Use Committee.

Immunosuppressive agent. We obtained cyclosporine (CsA) from Wako Pure Chemical Industries (Osaka, Japan). We prepared dosing solutions daily by diluting the CsA solution with a water solution containing 5% ethanol and 1% Tween 80 for the final injection.

Neurovirulence test with suckling mice. Ten suckling mice in each of three groups were inoculated intracerebrally with $10^{1.3}$ to $10^{3.3}$ PFU of either LC16m8, LC16mO, or Lister. These mice were observed every day for 21 days after inoculation. We calculated the survival ratio and mean survival time (days) by observing the mice for 21 days after inoculation. Statistical analyses were done using Fisher's exact method and the log rank method. The neurovirulence test was done according to the WHO recommendations (13).

LD$_{50}$ test with suckling mice. Groups of 10 suckling mice were each inoculated intracerebrally with graded doses of $10^{1.3}$ to $10^{3.3}$ PFU of LC16m8 and from $10^{1.3}$ to $10^{3.3}$ PFU of LC16mO and of Lister. These mice were observed every day for 21 days after inoculation. The 50% lethal dose (LD$_{50}$) was calculated by the probit method in mice that died up to 21 days after inoculation.

Test with SCID mice. Ten mice in each of the three SCID mouse groups were inoculated intraperitoneally with $10^{3.3}$ PFU of either LC16m8, LC16mO, or Lister. Ten additional mice were inoculated intraperitoneally with a saline as a control. These mice were observed for 120 days after inoculation.

Test with CsA-treated mice. Mice were divided into a total of 9 groups with 6 mice each as shown in Table 1. Mice were injected subcutaneously every day for 15 days (from day 1 before inoculation to day 14 after inoculation) with $25$ or $50$ mg/kg of body weight/day CsA or a water solution containing 5% ethanol and 1% Tween 80 as a control. The mice in groups 1, 2, and 3 did not receive vaccine inoculation throughout the experimental period, and the changes in immune cell numbers after treatment with CsA were examined on day 14 after CsA injection. The mice in groups 4, 5, 6, 7, 8, and 9 were inoculated intraperitoneally with $10^{5.0}$ PFU of Lister or LC16m8 (total of six groups). The clinical symptoms of the mice in all 9 groups were recorded every day during the 14-day experimental period. Table 2 shows the scoring system used in this study, which was designed by us based on the scoring system used for general acute toxicity tests in wild-type mice but with minor modifications.

The change in immunocyte numbers for the three control groups with no CsA treatment or CsA treatment, but not receiving any vaccine, on day 14 after inoculation was evaluated by staining splenocytes with (i) anti-CD4$^+$ T cell antibodies conjugated with phycoerythrin (PE) (anti-CD4-PE), (ii) anti-CD3 antibodies conjugated with fluorescein isothiocyanate (FITC) (anti-CD3-FITC), (iii) anti-CD8$^+$ T cell antibodies conjugated with PE (anti-CD8-PE) together with anti-CD3-FITC antibodies, and (iv) anti-B cell antibodies [anti-CD45R(B220)-PE]. Only spleen was studied because CsA is reported to affect the spleen and lymph nodes in a similar way (14). These four antibodies were purchased from Becton Dickinson, NJ. Stained splenocytes were analyzed by using a fluorescence-activated cell sorter (FACScan, Becton, Dickinson).

RESULTS AND DISCUSSION

We prepared new stockpile LC16m8 lots and used them in a series of safety tests that were essentially the same as the tests done by Hashizume and coworkers (9, 10) with rabbits and monkeys. Our results were also essentially the same as the results of Hashizume et al. (data not shown).

Survival study in suckling mice. After we inoculated suckling mice intracerebrally with $10^{3.3}$ PFU of LC16m8, LC16mO, or Lister, the survival ratios during the 21 days of observation were $7:10$ (70%) for LC16m8, $1:10$ (10%) for LC16mO, and $1:10$ (10%) for Lister (Fig. 1). The difference in the survival ratios between LC16m8 and either of the two unattenuated strains was significant ($P = 0.02$, Fisher’s exact test). The mean survival times were 17.1 days for LC16m8 versus only 6.1 days for LC16mO and 6.3 days for Lister ($P = 0.001$, log rank test). The LD$_{50}$ calculated at 21 days after inoculation were $10^{3.3}$ PFU for LC16m8 versus $10^{1.6}$ PFU for LC16mO and $10^{0.4}$ PFU for Lister. Thus, the LD$_{50}$ for LC16m8 was $>1,000$-fold higher than that for the unattenuated strains (Table 3).

Survival and clinical symptoms of SCID mice. The three vaccine strains were inoculated intraperitoneally into SCID mice. Figure 2A shows the survival curve. The survival ratios were $0:10$ (0%) for Lister and for LC16mO and 10:10 (100%) for LC16m8. The increase in mean survival times between LC16m8 ($>120$ days) and Lister (30.8 days) was statistically significant ($P < 0.001$, log rank test); the difference between LC16m8 ($>120$ days) and LC16mO (24.5 days) was also statistically significant ($P < 0.001$, log rank test).

Figure 2B shows the group’s average percentage of weight gain or loss patterns from each animal’s weight on the inoculation day. Lister- and LC16mO-inoculated mice showed a marked decrease in body weight from day 12 after inoculation. All mice were euthanized by CO$_2$ gas when the weight loss reached $\geq$25%. No significant weight loss was observed in LC16m8-inoculated mice nor in placebo-inoculated mice.

Figure 2C shows the clinical symptom data for these mice. Lister- and LC16mO-inoculated mice began to show clinical symptoms around day 10 after inoculation followed by an abrupt increase in severity, reaching maximal levels between 20 and 30 days after inoculation, whereas the LC16m8-inoculated mice showed no recognizable clinical symptoms.

TABLE 2 Criteria for scoring clinical observations of mice

| Score | Clinical symptom criteria                                      |
|-------|----------------------------------------------------------------|
| 0     | No clinical symptoms                                          |
| 1     | Rough coat, rash, nervous behavior                             |
| 2     | Spiky hair, pock formation (no suppuration)                    |
| 3     | Pock formation (suppuration), loss of hair, hunched over, inactive, slow moving |
| 4     | Paralysis, moribund, abnormal behavior (stumbling, etc.)       |
| 5     | Dead                                                           |

* The evaluation of clinical symptoms was expressed as a score for each mouse. Each group’s total clinical score for every observation day was expressed as the sum of each mouse score.
Clinical symptoms of CsA-treated mice. Lister and LC16m8 were inoculated intraperitoneally into mice in which the immune system was suppressed by CsA. For the mice not receiving any vaccine, the effects of CsA on the immunocyte numbers were evaluated: the numbers of CD4$^+$ T cells and CD8$^+$ T cells decreased by about 20% compared with those in the non-CsA-treated mice, but little change was observed in the numbers of B cells (Fig. 3A). No mice died during the observation period. Lister-inoculated mice treated with CsA developed severe vaccinia-related symptoms, including pock formation, rash, or both. LC16m8-inoculated mice in either CsA-treated or nontreated groups developed no clinical symptoms (Fig. 3B).

These studies indicate that the LC16m8 virus has highly attenuated properties in immunodeficient mice. This attenuation is mainly caused by a mutation in the $B5R$ gene coding for an extracellular envelope protein necessary for rapid propagation of the virus (15–17). Although LC16m8 lacks the normal $B5R$ gene product (B5 protein), it has a strong protective ability against infection by virulent viruses in various immunodeficient mouse models (18–20). In this regard, we previously studied (18) the effect of Dryvax or LC16m8 by administration by skin scarification to macaques depleted systemically of T or B cells. B cell depletion did not affect the size of the skin lesions induced by either vaccine. However, while depletion of both CD4$^+$ and CD8$^+$ T cells had no adverse effects on LC16m8-vaccinated animals, it caused progressive vaccinia in macaques immunized with Dryvax. As both Dryvax and LC16m8 vaccines protect healthy macaques from a lethal monkeypox intravenous challenge, our data identified LC16m8 as a safer and effective alternative to ACAM2000 and Dryvax vaccines for immunocompromised individuals.

We have found no report on pathogenesis directly comparing LC16m8 and MVA. However, a smallpox vaccine strain (m8Δ) constructed by modifying LC16m8 showed lower pathogenicity similar to that of MVA in SCID mice (19). This modification only augmented the genetic stability by lowering the risk of the occurrence of a $B5R$ gene revertant without affecting its pathogenicity, suggesting that LC16m8 and MVA have comparable pathogenicity. As noted above, MVA has poor immunogenicity (20).

Based on the above-mentioned facts, we consider LC16m8 to be one of the best candidate vaccines usable in the case of attack by bioterrorists, and the SAGE recommended both ACAM2000 (extended-spectrum vaccine) and LC16m8 (broad-spectrum vaccine) as preferred WHO stockpile vaccines (11).

This study on the safety of LC16m8 by use of suckling mice and immunocompromised mice not only confirms the safety of the LC16m8 vaccine but also supports the idea that LC16m8 could be usable for immunodeficient subjects, including those infected with HIV, which is important in relation to the recent epidemics of monkeypox in western and central Africa.

Infection with monkeypox has increased greatly in areas of
FIG 2 Effects of vaccination with Lister, LC16mO, or LC16m8 vaccine on SCID mice. Each vaccine was inoculated intraperitoneally (see details in the text). The numbers in parentheses show the numbers of animals in each experimental group. (A) Survival curve; (B) changes in body weight shown as percentages; (C) clinical score.
western and central Africa where a high prevalence of HIV infection exists (12). LC16m8 protects monkeys from monkeypox (21), and because monkeypox also infects humans and causes smallpox-like disease, vaccination of subjects with LC16m8 is warranted in this region. Our studies have shown that LC16m8 vaccine may be safely used in immunodeficient subjects and that LC16m8 can also serve as a safe vector for a recombinant virus expressing foreign antigens (22).

In conclusion, we have shown that LC16m8 vaccine, recommended by the WHO SAGE as a preferred stockpile vaccine, can be safely used in suckling mice and immunodeficient mice. Our results also open the way for its use to protect against monkeypox infection in areas, such as western and central Africa, where there are many immunodeficient subjects.

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