A Single Vaccination of Nonhuman Primates with Highly Attenuated Smallpox Vaccine, LC16m8, Provides Long-term Protection against Monkeypox

INTRODUCTION

Smallpox is caused by the variola virus (genus: Orthopoxvirus and family: Poxviridae). The global vaccination program against smallpox has now ceased because of its eradication (1); therefore, new generations lack protective immunity against smallpox. The monkeypox virus (MPXV; Orthopoxvirus), which also belongs to the genus Orthopoxvirus, of the family Poxviridae, causes a similar disease to smallpox, not only in nonhuman primates (NHPs) but also in humans (2-4). Recently, the incidence of human monkeypox (MPX), as suggested by the immediate viremia reduction and the response of the IgG antibody. The results demonstrated that the vaccination of monkeys with a single dose of LC16m8 provided durable protection against MPXV for longer than one year after immunization. The results suggest that the vaccination of humans with LC16m8 could induce long-term protection against MPXV infection.

METHODS

Virus, smallpox vaccine, and cells: MPXV strain Zr-599, a highly virulent strain, was used in the challenge experiments (12, 17). The infectious doses of MPXV were determined via plaque assays, using Vero cells obtained from American Type Cultures

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Collection (ATCC) Cefaclor (CCL). Vero cells were grown in Eagle’s minimum essential medium (MEM), supplemented with 5% fetal bovine serum, penicillin G, and streptomycin (MEM-5FBS). The smallpox vaccines, LC16m8 and Lister, were used in this study. The naïve group monkeys were challenged with MPXV without vaccination. The LC16m8-6M (3 monkeys) comprised those infected with MPXV 6 months after immunization with LC16m8. The LC16m8-12M (2 monkeys) comprised those infected with MPXV 12 months after immunization with LC16m8. Lister-6M (2 monkeys) comprised those infected with MPXV 6 months after immunization with Lister, and the Lister-12M (2 monkeys) comprised those infected with MPXV 12 months after immunization with Lister. The naïve group comprised 4 monkeys without vaccination that were challenged subcutaneously with 10^6 PFU of MPXV Zr-599 (Table 1). Notably, only one (#4626) of 4 naïve group monkeys was inoculated with MPXV in parallel with the LC16m8-6M-/Lister-6M-group monkeys and the LC16m8-12M-/Lister-12M-group monkeys in the present study. Another monkey (#4683), which was used in a separate experiment in which LC16m8-efficacy was evaluated, was included in this study (Table 1.5). The other 2 naïve group monkeys, #4651 and #4653, were the monkeys described in a previous study (12), and were inoculated with 10^6 PFU of MPXV Zr-599 in the same manner as in the present study. The 3 naïve group monkeys (#4683, #4651 and #4653) were included in this study for ethical reasons to reduce the number of monkeys used. It should be noted that the virological analyses of these monkeys were performed simultaneously using the stored samples and those collected from the monkeys that were infected with MPXV in the present study. The viral dose used for inoculation was the dose predicted by back-titration confirmation in each case. The data obtained from the naïve group monkeys were also used in the previous study to compare the virulence and pathophysiology of the Congo Basin strain (Zr-599) and West African strain (Liberia strain) (17).

Long-Term Efficacy of LC16m8 against Monkeypox

<table>
<thead>
<tr>
<th>Group</th>
<th>ID[1]</th>
<th>Sex/Body weight (g)</th>
<th>Vaccination</th>
<th>Month before infection</th>
<th>Virus isolation[2]</th>
<th>No. of plaques on the indicated day of collection[3]</th>
<th>Symptom(s)</th>
<th>No. of papulovesicular lesions</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Positive</td>
<td>3 + on day 3, 3 + on day 7, 2 + on day 10</td>
<td>Oral cavity, papulovesicles, decreased activity, loss of appetite</td>
<td>95</td>
<td>Survived</td>
</tr>
<tr>
<td></td>
<td>#4626</td>
<td>M/4,080</td>
<td>Mock</td>
<td>0</td>
<td>Positive</td>
<td>2 + on day 4, 3 + on day 6, 3 + on day 10, 3 + on day 13</td>
<td>Diarrhea, papulovesicles, decreased activity, loss of appetite</td>
<td>388</td>
<td>Sacrificed</td>
</tr>
<tr>
<td></td>
<td>#4683</td>
<td>M/3,460</td>
<td></td>
<td></td>
<td>Positive</td>
<td>1 + on day 3, 1 + on day 7, 3 + on day 9, 3 + on day 14</td>
<td>Diarrhea, rhinorrhea, depressed, ulcer, loss of appetite</td>
<td>390</td>
<td>Sacrificed</td>
</tr>
<tr>
<td></td>
<td>#4651</td>
<td>F/2,660</td>
<td></td>
<td></td>
<td>Positive</td>
<td>3 + on day 3, 2 + on day 7, 1 + on day 9, 3 + on day 11, 2 + on day 14, 1 + on day 18</td>
<td>Diarrhea, severely decreased activity, papulovesicles, ulcer, loss of appetite</td>
<td>1,150</td>
<td>Sacrificed</td>
</tr>
<tr>
<td>LC16 m8-6M</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>0</td>
<td>Survived</td>
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<tr>
<td></td>
<td>#4636</td>
<td>M/3,980</td>
<td>LC16m8</td>
<td>6mo</td>
<td>Positive</td>
<td>2 + on day 7</td>
<td>None</td>
<td>0</td>
<td>Survived</td>
</tr>
<tr>
<td></td>
<td>#4637</td>
<td>M/4,260</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>#4638</td>
<td>M/4,460</td>
<td></td>
<td></td>
<td>Positive</td>
<td>1 + on day 3</td>
<td>None</td>
<td>0</td>
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</tr>
<tr>
<td>Lister-6M</td>
<td></td>
<td></td>
<td></td>
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<td>Survived</td>
</tr>
<tr>
<td></td>
<td>#4642</td>
<td>M/3,440</td>
<td>Lister</td>
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<td></td>
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<td></td>
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</tr>
<tr>
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<tr>
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<td>12mo</td>
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<td>#4632</td>
<td>M/2,560</td>
<td></td>
<td></td>
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<tr>
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<td>#4633</td>
<td>M/4,633</td>
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<tr>
<td>Lister-12M</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td>None</td>
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<td>Survived</td>
</tr>
<tr>
<td></td>
<td>#4634</td>
<td>M/4,300</td>
<td>Lister</td>
<td></td>
<td>Negative</td>
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<td>None</td>
<td>0</td>
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</tr>
<tr>
<td></td>
<td>#4635</td>
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<td></td>
<td></td>
<td>Negative</td>
<td></td>
<td>None</td>
<td>0</td>
<td>Survived</td>
</tr>
</tbody>
</table>

[2] “Positive” and “Negative” indicate that MPXV isolation was positive or negative from the buffy coat fractions, respectively, during the observation period from challenge until sacrifice. Virus isolation was attempted using the buffy coat fractions obtained from 4-mL aliquots of total peripheral blood collected from monkeys every 3 or 4 days during the observation period after viral challenge.
[3] No. of plaques on the indicated day of collection, 1 + indicates 1–5 plaques, 2 + indicates 6–20 plaques, and 3 + indicates >21 plaques observed by virus isolation.
[4] Monkey, #4626, was used in this study as a control subject.
[5] Monkey, #4683, which was used in the other study, in which efficacy of LC16m8 was evaluated (data not shown). This monkey was included in this study.
[6] Monkeys, #4651 and #4653, were used in a previous study, which evaluated the efficacy of LC16m8 (12).
Vaccination with smallpox vaccines and challenge with MPXV: The monkeys were vaccinated and challenged (12), and were observed until the humane endpoints after a maximum of 3 weeks. Body temperature and weight were measured after the challenge. Peripheral blood was drawn, and a throat swab was collected every 3–4 days, as described previously (12). Clinical manifestations, including skin lesions, were also monitored daily.

Assays of IgG antibody against orthopoxvirus: The antibody levels against orthopoxivirus were measured using enzyme-linked immunosorbent assay (ELISA), using the entire vaccinia virus proteins as antigens (12).

Virus isolation from peripheral blood mononuclear cells: Virus isolation was performed (12). The peripheral blood samples were collected from the monkeys in each group between Days 0 and 21, and were tested for the virus isolation and for the determination of the viremia level.

Determination of MPXV loads in total peripheral blood by quantitative polymerase chain reaction (PCR): The viremia level was determined using the Light Cycler PCR-based quantitative real-time PCR (qPCR) method (12,18).

Scoring system for determining the severity of MPX: To compare the level of MPX severity, we used the scoring system developed in a previous study (17).

Histopathological examination: After sacrificing the monkeys by placing them under deep anesthesia using ketalar, the skin and several organs were excised, fixed in 10% formalin in PBS, and embedded in paraffin. Macroscopic and histological examinations were performed using the excised tissues and organs. Paraffin sections that measured 4 µm in thickness were stained with hematoxylin and eosin, and with Luxol-Fast Blue for the brain tissues. Immunohistochemistry was performed using the paraffin sections, in order to detect MPXV antigens, according to a previously described method (12).

Laboratory data and cytokine responses: Total peripheral blood cell counts and serum biochemical tests, including tests for aspartate transaminase, alanine transaminase, lactate dehydrogenase, and C-reactive protein (CRP), were performed (17). The concentrations of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), and IL-6 in the serum samples were determined using a Human Cytokine 24-plex Antibody Bead Kit (BioSource Invitrogen, Camarillo, CA), according to the manufacturer’s instructions. Samples were analyzed using a Luminex 100™ (Luminex Corporation, Austin, US) system.

Statistical analysis: The Student’s t-test was performed to compare the maximum viremia level between the LC16m8-6M and Lister-6M group monkeys, and that between LC16m8-6M and LC16m8-12M group monkeys. The statistical analyses were performed as 2-tailed tests using JMP 11 (SAS institute, Cary, NC). The results were considered significant when the p value (p) was less than 0.05.

RESULTS

Protection of monkeys from MPX by immunization with LC16m8 or Lister: The naïve group monkeys developed severe symptoms (Table 1, Fig. 1), where their body weight decreased by approximately 10% at 7 days post-challenge (Fig. 1A, left panel). In contrast, the monkeys in the vaccinated groups (LC16m8-6M, Lister-6M, LC16m8-12M, and Lister-12M) maintained their body weights during the observation period (Figs. 1B and 1C, left panel). There was no significant difference in the changes in body weight between the LC16m8-vaccinated and the Lister-vaccinated monkeys.

Papulovesicular skin lesions appeared from day 7 post-challenge in the naïve group (Table 1 and Fig. 2). In contrast, neither the LC16m8-vaccinated nor the Lister-vaccinated monkeys developed any MPX-associated skin lesions (Table 1), except for ulcerative lesions that appeared locally at the site of the MPXV inoculation in all the LC16m8-6M and LC16m8-12M monkeys. The Lister-6M and Lister-12M group monkeys did not exhibit any MPX-associated symptoms, even at the site of MPXV inoculation (Fig. 2).

Viremia determined by virus isolation: The virus isolation results obtained using peripheral blood samples are summarized in Table 1. MPXV was isolated from the buffy coat fractions of the naïve group monkeys between Days 3 and 14. The plaque number was so high that the number could not be counted, particularly during the acute phase of infection in the naïve monkeys (Table 1). In contrast, MPXV isolation was positive in a limited number of monkeys in the groups vaccinated with LC16m8 or Lister. The buffy coat fractions of 2 (#4636 and 4638) of the 3 LC16m8-6M monkeys were MPXV-isolation positive during Days 3 to 7. The buffy coat fractions collected from monkeys in the LC16m8-12M, Lister-6M, and Lister-12M groups were negative for virus isolation (Table 1).

Viremia determined by qPCR: In the naïve group monkeys, viremia was more than 10⁵ copies/mL at either Days 3 or 4. The level peaked at Day 10, when the concentration was more than 10⁶ copies/mL (Fig. 1A, right panel). All the LC16m8-6M group monkeys exhibited viremia, with a peak level of approximately 10⁶ copies/mL, and a shorter viremia period than in the naïve monkey group (Fig. 1, right panel). The Lister-6M group monkeys also exhibited viremia (maximum level approximately 10⁶ copies/mL) during Days 3 to 7 (Fig. 1B, right panel). The viremia level was higher in the LC16m8-6M group monkeys than in the Lister-6M group monkeys. Furthermore, the viremia-positive period was significantly longer in the LC16m8-6M group monkeys than in the Lister-6M group monkeys (Fig. 1B, right panel). All the LC16m8-12M group monkeys exhibited viremia, where the peak level was approximately 10⁶ copies/mL in 3 monkeys from the LC16m8-12M group and in one monkey from the Lister-12M group, on Day 3 (Fig. 1C, right panel). There was no significant difference in the maximum viremia levels between the LC16m8-6M group and the LC16m8-12M group monkeys (p = 0.42).

Pathological examination: All the monkeys were sacrificed to perform virological and pathological examinations at the end of the observation period. The results of the pathological examinations in the naïve monkey group have been reported in a previous study (12,17). MPX-associated lesions were detected in most of the organs, including skin, such as in the lymphoid...
Long-Term Efficacy of LC16m8 against Monkeypox

The optical density (OD) value measured at 405 nm (OD\textsubscript{405}) represented the level of IgG antibody to vaccinia virus antigen in the ELISA. The OD values for the Lister-6M and Lister-12M groups remained at the same level throughout the observation period, whereas those of the LC16m8-6M and LC16m8-12M group monkeys decreased in most of the monkeys (Fig. 3B).

The kinetics of the antibody responses in the LC16m8-vaccinated monkeys differed from those in the Lister-vaccinated monkeys, where the antibody titer exhibited a substantial increase from the baseline level, systems (lymph nodes, thymus, and tonsils), respiratory tract structures (lung and trachea), digestive organs (stomach, small intestine, colon, rectum, and liver), and urogenital tract (bladder, uterus, and ovary) of the naïve monkey group. In contrast, no MPX-associated lesions were detected in any of the organs of the monkeys vaccinated with Lister or LC16m8, except for skin lesions at the site of the MPXV challenge in the LC16m8-6M and LC16m8-12M groups (Fig. 2).

IgG antibody response to vaccinia virus after vaccination and MPXV challenge: The pre-challenge sera of the naïve group monkeys were negative for IgG to vaccinia virus antigens (Fig. 3A). The optical density (OD) value measured at 405 nm (OD\textsubscript{405}) represented the level of IgG antibody to vaccinia virus antigen in the ELISA. The OD values for the Lister-6M and Lister-12M groups remained at the same level throughout the observation period, whereas those of the LC16m8-6M and LC16m8-12M group monkeys decreased in most of the monkeys (Fig. 3B).

The kinetics of the antibody responses in the LC16m8-vaccinated monkeys differed from those in the Lister-vaccinated monkeys, where the antibody titer exhibited a substantial increase from the baseline level,
reaching the maximum level approximately 28 days post-immunization in all monkeys, before subsequently decreasing slowly (Figs. 3B and 3C). The OD values for the antibody titer in the LC16m8-6M group were significantly lower than those for the Lister-6M group at 185 days (approximately 6 months) after vaccination. The OD values reached their maximum 4 weeks after vaccination, before decreasing gradually until the
Long-Term Efficacy of LC16m8 against Monkeypox

MPXV challenge, only in the LC16m8-vaccinated monkeys (both the LC16m8-6M and LC16m8-12M groups).

After infection, the OD values measured by IgG-specific ELISA increased in the plasma of the naïve monkeys, and they reached their maximum level at approximately 10 days after MPXV challenge (Fig. 3A). In contrast, the antibody responses were induced immediately in both the LC16m8- and Lister-vaccinated groups within 7 days of the challenge (Figs. 3B and 3C; right panel).

**Laboratory findings and cytokine responses:**
The CRP levels increased in the naïve group but not in the animals immunized with either of the vaccines (Table 2). The Luminex assay showed that there was no increase in IFN-γ in the plasma of monkeys from both the naïve (#4626) and vaccinated groups (data not shown). However, the levels of inflammatory cytokines, such as IL-6 and TNF-α, increased in the naïve monkey group but not in the vaccination groups (Fig. 4), except for a positive TNF-α response in one (#4637) of the 3 LC16m8-6M monkeys (Fig. 4C).

**Severity of MPX-associated symptoms in LC16m8-6M, LC16m8-12M, and naïve group monkeys:** The clinical severity of the symptoms in the LC16m8-6M and LC16m8-12M monkeys were compared with those in the naïve monkey group using a scoring system (17). The mean ± standard deviation (SD) of the scores for the

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Fig. 3. (Color online) Vaccinia virus-specific IgG responses determined by IgG-ELISA. The optical densities measured at 405 nm (OD405) at serum sample dilutions of 1:100 are shown for naïve-group monkeys after MPXV challenge (A, right), LC16m8-6M and Lister-6M group monkeys (B, left panel: after vaccination until MPXV challenge; right panel: after MPXV challenge), and LC16m8-12M and Lister-12M group monkeys (C, left panel: after vaccination until MPXV challenge; right panel: after MPXV challenge). The day of MPXV challenge was taken as day 0. The graph for the naïve-group monkeys (A) is exactly the same as that presented in a previous study (17).
LC16m8-6M and LC16m8-12M groups were 4.3 ± 0.5 and 2.2 ± 1.0, respectively, which were both much lower than that for the naïve monkeys, at 30.0 ± 10.6 (Table 2). Ulcerative lesions appeared at the site of MPXV inoculation in the LC16m8-6M and LC16m8-12M monkeys, whereas no lesions appeared in the Lister-6M and Lister-12M groups. More severe lesions appeared in all monkeys in the naïve group (Fig. 2).

**DISCUSSION**

In this study, for the first time, we confirmed that a single vaccination of NHPs with LC16m8 induced long-lasting protective immunity against MPXV infection, where the protective efficacy persisted for at least 12 months. The protective efficacy exhibited 6 and 12 months after LC16m8 vaccination was similar to the shorter period (12). These results suggest that a single vaccination with LC16m8 provides long-term immunity against MPX, and that that a single vaccination of humans with LC16m8 might also confer protection from MPXV infections or smallpox for a relatively longer period. However, more studies are needed to elucidate the duration of conferring protection by a single vaccination with LC16m8 against these specific virus infections in humans.

We also demonstrated that Lister conferred greater protective efficacy against MPX in NHPs than LC16m8 (12). The viremia levels determined by qPCR were consistent with those detected in virus isolation experiments using naïve, LC16m8-vaccinated, and Lister-vaccinated groups (Table 1, Fig. 1), according to the difference in the severity of skin lesions at the site of MPXV inoculation and the maximum viremia levels between the LC16m8 group and the Lister group monkeys (Figs. 1 and 2).

To evaluate the efficacy of LC16m8 in terms of the immune response, we measured the levels of circulating IgG against vaccinia virus antigens by ELISA. The data obtained in previous studies were included to analyze the IgG values for naïve monkeys. The kinetics of the IgG response in naïve monkeys differed substantially between individuals (12, present study). The plasma specimen from monkey #4626 (the naïve group monkey), which survived, exhibited a strong antibody response, where the maximum was reached within 7 days post-challenge, according to the IgG-specific ELISA, whereas the IgG response reached the

![Fig. 4.](image) Changes in the serum IL-6 levels in each group of monkeys inoculated with MPXV 6 months after vaccination, i.e., LC16m8-6M and Lister-6M groups (A), and those 12 months after vaccination, i.e., LC16m8-12M and Lister-12M groups (B). Changes in TNF-α serum levels in LC16m8-6M and Lister-6M groups (C) and those at 12 months after vaccination, i.e., LC16m8-12M and Lister-12M groups (D). The response in the naïve-group monkey (#4626) is included as the control.
maximum level at approximately 10 days post-infection in the other 3 monkeys (Fig. 3A). It was evident that the clinical symptoms and viremia level observed in monkey #4626 were milder than those observed in the previous monkeys (Table 1, Fig. 1). Thus, it is possible that the rapid IgG response in this monkey (#4626) protected it from death due to MPX, although the underlying mechanism of the response in this monkey was unclear.

The body weight of the monkeys used in the present study varied and was not standardized among the groups, because of the difficulty in selection of monkeys for the study. However, all the naïve group monkeys showed MPX-related symptoms, but not all the monkeys immunized with LC16m8 or Lister, suggesting the difference in body weight among the groups did not influence the conclusions.

The kinetic OD values used to represent the antibody titer were monitored for 12 months in the LC16m8 and Lister group monkeys. The OD values reached their maximum level after one month before decreasing gradually in the LC16m8 group monkeys (Fig. 3). However, the protective efficacy remained at the same level as that challenged with MPXV at 4–5 weeks post-vaccination, thereby suggesting that cellular immunity as well as humoral immunity plays a role in protecting NHPs from lethal MPX.

The mode of protection by smallpox vaccination in humans has not been fully defined. Edghill-Smith et al. reported that vaccinia-specific B-cell responses were essential for protection of macaques from MPX and that antibody-mediated depletion of B cells, but not CD4+ or CD8+ T cells, abrogated vaccine-induced protection from a lethal intravenous challenge with MPXV. Furthermore, they reported that passive transfer of human vaccinia-neutralizing antibodies protected nonimmunized macaques from severe disease (19). On the other hand, Chaudhri et al. recently reported that CD4 T-cell help was necessary for neutralizing antibody production and virus control during a secondary ectromelia virus infection, which is a disease infecting mice, and is similar to smallpox in humans, suggesting that cellular immunity plays a prominent role in the protection of humans from orthopoxvirus infections including smallpox and human MPX (20). We demonstrated that the protective efficacy of LC16m8 vaccination 12 months post-vaccination was at a similar level to that one month post-vaccination in NHPs, but that the antibody level decreased at 6 months and 12 months post-vaccination. These results appear to be consistent with the previous reports (19,20).

Human MPX is endemic to central and eastern Africa. Furthermore, there is a possibility that smallpox will re-emerge in the future. Although smallpox has been eradicated, the smallpox vaccine is still important and necessary. LC16m8 might be a suitable choice for protection of people in the human MPX-endemic regions from human MPX and for stockpiling in preparation for a possible outbreak of smallpox in the future.

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Conflict of interest None to declare.

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