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Vaccinia virus strain LC16m8 defective in the *B5R* gene keeps strong protection comparable to its parental strain Lister in immunodeficient mice

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ABSTRACT

Background: Attenuated vaccinia virus strain, LC16m8, defective in the *B5R* envelope protein gene, is used as a stockpile smallpox vaccine strain in Japan against bioterrorism: the defect in the *B5R* gene mainly contributes to its highly attenuated properties.
Methods: The protective activity of LC16m8 vaccine against challenge with a lethal dose of vaccinia Western Reserve strain was assessed in wild-type and immunodeficient mice lacking CD4, MHC class I, MHC class II or MHC class I and II antigens.
Results: The immunization with LC16m8 induced strong protective activity comparable to that of its parent strain, Lister (Elstree) strain, in wild-type mice from 2 days to 1 year after vaccination, as well as in immunodeficient mice at 2 or 3 weeks after vaccination. These results implicated that the defect in the *B5R* gene hardly affected the potential activity of LC16m8 to induce innate, cell-mediated and humoral immunity, and that LC16m8 could be effective in immunodeficient patients.
Conclusion: LC16m8 with truncated B5 protein has an activity to induce immunity, such as innate immunity and subsequent cell-mediated and humoral immunity almost completely comparable to the activity of its parental strain Lister.
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1. Introduction

The success of smallpox eradication by the World Health Organization (WHO) program in the 1970s is a landmark in the history of biomedical science. Since the terrorist attack on the World Trade Center in New York in September 2001, however, concern about bioterrorism has increased. Among many pathogens that could be used potentially in bioterrorism, variola virus is one of the most recognized and the most feared. Under such circumstances, serious attempts have been made to restart the development of vaccine strains, including a vaccinia ACAM2000 clone established from Dryvax derived from the New York City Board of Health (NYCBH) strain [1,2], modified vaccinia Ankara (MVA) and replication-incompetent viruses derived from the NYCBH strain [3,4]. However, replication-competent vaccine strains derived from the NYCBH strain may have a side effect of inducing myopericarditis [1,2], and replication-incompetent viruses may have the potential problem of relatively poor immunogenicity [3,4].

In the early 1970s, Hashizume et al. [5,6], developed one of the safest replication-competent vaccines, LC16m8 strain, from the Lister (Elstree) strain that was used worldwide in the WHO smallpox eradication program. Freeze-dried live attenuated smallpox vaccine of LC16m8 prepared in cell culture has been the sole smallpox vaccine licensed in Japan since 1975. LC16m8 was selected as a temperature- sensitive small-plaque- and small-pock-forming clone [6]. 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 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 when compared with conventional first generation smallpox vaccines such as its parental vaccine

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strain Lister. The LC16m8 vaccine is now produced by the Chemo-Sero-Therapeutic Research Institute (Kaketsuken), Japan, and is stockpiled as a smallpox vaccine against bioterrorism in Japan. According to the November 2013 conclusions and recommendations of the Meeting of the Strategic Advisory Group of Experts on immunization [7], both licensed ACAM2000 (2nd generation vaccine) and LC16m8 (3rd generation vaccine) are preferred for the WHO stockpile.

A molecular biological study showed that one gene of envelope proteins is defective, contributing to smaller plaque size and host range and was accordingly called as *ps/hr*) gene [8]: the *ps/hr* gene is a homologue of *B5R* gene of Copenhagen (CPN) strain, and now this gene is usually called as *B5R* gene. The *B5R* gene product, B5 protein, is a 42-KDa envelope glycoprotein composed of 317 amino acids. Vaccinia virus produces two typical types of virion from each infected cell called intracellular mature virus and extracellular enveloped virus (EEV) [9]. B5 protein is necessary for the formation of EEV, and therefore LC16m8 does not form EEV resulting in small-plaque size and less spreading ability, which is considered to mainly attribute to its attenuated properties. The analysis of the full-genome sequence of LC16m8 and its parental strain Lister showed that LC16m8 preserved almost of all the open reading frames of vaccinia virus except for the disrupted EEV protein B5 [10].

A truncated B5 protein (approximately 8 kDa) was expressed abundantly in LC16m8-infected cells, and both murine immune sera and human vaccinia virus immunoglobulin recognized the truncated recombinant B5 protein in antigen-specific enzyme-linked immunosorbent assays [11].

Of major concern, however, is whether LC16m8 vaccine has enough immunogenicity to confer strong protection, because LC16m8 lacks normal type of the *B5R* gene product, B5 protein, which is a target epitope of neutralizing antibodies contributing to protection in mice [12]. Furthermore, LC16m8 fails to induce enough anti-B5 antibody responses in the human [13]. Although previous studies showed that in normal animals and humans LC16m8 keeps strong immunological and protective activity comparable to that of its parent strain Lister [6,10,14], whether it keeps such activity also in immunodeficient animals remains to be clarified. This point is important in relation to the problem of whether LC16m8 is usable in areas of Africa where many immunodeficient patients, such as human immunodeficiency virus-infected patients, live.

To address this problem, this study investigated if LC16m8 with truncated B5 protein has immunological and protective activity comparable to that of its parental strain Lister by using wild-type and immunodeficient mice lacking CD4, MHC class I, MHC class II antigen or MHC class I and II antigens. Immunological and protective activity was assessed in intranasally challenged mice with a lethal dose of a highly pathogenic vaccinia virus (VACV) strain, Western Reserve (WR) strain.

2. Material and methods

2.1. Vaccine and virus strains

Freeze-dried smallpox vaccine (LC16-KAKETSUKEN; LC16m8) manufactured under good manufacturing practice (GMP)-compliant condition by Kaketsuken in cell culture using primary rabbit kidney cells was used as the source of LC16m8 VACV strain. The vaccine lot used in this study is one (Lot V06) of the lots of smallpox vaccine stockpiled now in Japan against bioterrorism using smallpox. In the current manufacturing process, the occurrence of *B5R* revertant virus which was reported by Kidokoro et al. [15] was well controlled and the content rate in the Lot V06 is under 1%. The Lister (Elstree) VACV strain, which was used for the WHO smallpox eradication program, and the parental strain of the LC16m8 strain were transferred to Kaketsuken from the Chiba Serum Institute (Chiba) when this institute closed. UV-inactivated Lister virus was prepared by placing 1×10^7 PFU/mL of Lister virus under UV lumps for approximately 18 h and the inactivation was confirmed by standard plaque assay. The WR VACV strain (ATCC VR-1354) used for the challenge was purchased from the American Type Culture Collection (ATCC).

2.2. Mice

Wild-type BALB/c mice (female, 4 or 8-week old) were purchased from Charles River Laboratories Japan (Kanagawa). The following immunodeficient mice (female, 4- to 7-week old) were purchased from the Taconic Farms (New York): Cd4 (001055-MF) CD4-deficient, B2 m (B2MN12-MF) MHC class I-deficient, Abb (ABBN12-MF) MHC class II-deficient, and Abb/β_2 m (004080-MMF) MHC class I and II-double deficient mice.

2.3. Vaccination and WR challenge

Groups of wild-type or immunodeficient mice received a single vaccination at 2.5×10^5 PFU/mouse of LC16m8 or Lister virus by a 15 times-puncture by using standard bifurcated needles at the base of the tail or a single intraperitoneal vaccination with UV-inactivated Lister virus (the virus titer before inactivation was 1×10^7 PFU/mouse). See Table 1 and Table 2 for the number of mice in each experimental group. After vaccination, the mice were challenged intranasally with a dose of 10^5 PFU/mouse or 10^6 PFU/mouse WR viruses, either of which was amply lethal, at appropriate time point after vaccination in order to achieve a highly-lethal condition in the non-immunized animals per each model. The mice were observed and weighed each day for 14 days after the challenge. Mice that had showed severe weight loss and survived to the end of this study, that is, on day 14 after the challenge were euthanized by heart exsanguination under anesthesia. Statistical analysis on mean survival time was done using the log rank method. All mice studies were approved by the Kaketsuken Institutional Animal Care and Use Committee.

2.4. Plaque reduction neutralizing (PRN) assay

The level of neutralizing antibody against vaccinia WR virus in the serum samples was assessed by PRN assay as follows. WR-virus infected Vero E6 cells (ATCC CRL-1586, purchased from ATCC) received three times repeated freeze-thaw and the resultant crude virus fluid was used as a source of WR virus. This virus fluid may contain mainly intracellular mature viruses, with some other virus forms such as intracellular enveloped viruses, cell-associated enveloped viruses and extracellular enveloped viruses [9]. WR virus at 240 PFU in 0.12 mL of Eagle's minimal essential medium (EMEM) (Nissui, Tokyo) containing 1% fetal bovine serum (FBS) (Life Technologies, Maryland) was mixed with 0.12 mL of serially diluted heat-inactivated serum samples collected from test animals and was incubated at 37 °C for 15–18 h. The mixtures were inoculated in VeroE6 cell (ATCC CRL-1586, purchased from ATCC) monolayers in 12-well culture plates and were incubated in EMEM containing 3% FBS and 0.8% agarose ME (Iwai Chemicals Company, Tokyo) after 2 h of adsorption. After 4 days of incubation, the number of observed plaques was counted. The neutralizing antibody titer was calculated based on the plaque number by using the Probit analysis method with Minitab software (Kozo Keikaku Engineering Inc., Tokyo) and was defined as the reciprocal of the dilution level

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