| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| LC16M175R | 128846–130327 (493) | 128845–130326 | L? | DNA helicase | Enzyme | A18R | 0.0 | CPN | A18R (0.0) |
| LC16M176L | 130541–130308 (77) | 130540–130307 | L | Hypothetical protein | Similar gene in other organisms | MVA130L | 3e-42 | MVA | A19L (4e-42) |
| LC16M177R | 130894–132174 (426) | 130893–132173 | E | Putative DNA polymerase processivity factor | Other functions | A20R | 0.0 | CPN | A20R (0.0) |
| LC16M178L | 130895–130542 (117) | 130894–130541 | L? | Hypothetical protein | Similar gene in other organisms | MVA131L | 6e-57 | MVA | A21L (7e-57) |
| LC16M179L | 131714–131328 (128) | 131713–131327 | L? | Hypothetical protein | Similar gene in other organisms | A ORF H | 6e-52 | CPN | A ORF H (6e-52) |
| LC16M180L | 132017–131796 (73) | 132016–131795 | | Hypothetical protein | Similar gene in other organisms | A ORF I | 2e-39 | CPN | A ORF I (2e-39) |
| LC16M181R | 132104–132667 (187) | 132103–132668 | L?,E | Hypothetical protein | Similar gene in other organisms | VACWR142 | e-100 | WR | A22R (1e-99) |
| LC16M182R | 132687–133835 (382) | 132686–133834 | L? | Putative intermediate transcription factor | Other functions | A23R | 0.0 | CPN | A23R (0.0) |
| LC16M183R | 133832–137326 (1164) | 133831–137325 | L? | DNA-directed RNA polymerase subunit | Enzyme | A24R | 0.0 | CPN | A24R (0.0) |
| LC16M184L | 136716–138495 (73) | 136715–136494 | | Hypothetical protein | Similar gene in other organisms | A ORF J | 2e-28 | CPN | A ORF J (2e-28) |
| LC16M185L | 137963–137331 (210) | 137962–137330 | E | DNA-directed RNA polymerase subunit | Enzyme | A26L | 1e-64 | Cowpox | A26L (4e-45) |
| LC16M186R | 138773–138958 (61) | 138772–138957 | | Hypothetical protein | LC16m8, LC16mO specific gene | | | | |
| LC16M187L | 138918–138235 (227) | 138517–138234 | E | Hypothetical protein | Similar gene in other organisms | VACWR147 | e-128 | WR | |
| LC16M188R | 139964–140146 (60) | 139963–140145 | | Hypothetical protein | Similar gene in other organisms | TA30R | 3e-18 | Tian Tan | |
| LC16M189L | 141055–138878 (725) | 141054–138877 | L | A-type inclusion protein | Other functions | VACWR148 | 0.0 | WR | |
| LC16M190R | 141327–141827 (166) | 141326–141826 | | Hypothetical protein | LC16m8, LC16mO specific gene | | | | |
| LC16M191L | 142607–141099 (502) | 142606–141098 | L | Structural protein | Other functions | VACWR149 | 0.0 | WR | A26L (e-115) |
| LC16M192L | 142989–142657 (110) | 142988–142656 | L | Cell fusion protein | IMV membrane associated protein | MVA138L | 2e-52 | MVA | A27L (5e-52) |
| LC16M193L | 143430–142990 (146) | 143429–142989 | L | Hypothetical protein | Similar gene in other organisms | VACWR151 | 2e-84 | WR | A28L (7e-84) |
| LC16M194R | 144164–144376 (70) | 144163–144375 | L? | Hypothetical protein | Similar gene in other organisms | A ORF K | 1e-38 | CPN | A ORF K (1e-38) |
| LC16M195L | 144348–143431 (305) | 144347–143430 | L? | DNA-directed RNA polymerase subunit | Enzyme | A29L | e-178 | CPN | A29L (e-178) |
| LC16M196L | 144544–144311 (77) | 144543–144310 | L | Hypothetical protein | Similar gene in other organisms | A30L | 2e-28 | CPN | A30L (2e-28) |
| LC16M197R | 144704–145087 (127) | 144703–145086 | L? | Hypothetical protein | Similar gene in other organisms | MVA142R | 1e-61 | MVA | A31R (2e-61) |
| LC16M198R | 145175–145441 (88) | 145174–145440 | L? | Hypothetical protein | Similar gene in other organisms | A ORF L | 1e-46 | CPN | A ORF L (1e-46) |
| LC16M199L | 145866–145054 (270) | 145865–145053 | L?,E | ATP/GTP-binding protein | Other functions | A32L | e-151 | CPN | A32L (e-151) |
| LC16M200R | 145984–146541 (185) | 145983–146540 | L? | EEV glycoprotein | EEV membrane protein | A33R | 5e-96 | CPN | A33R (5e-96) |
| LC16M201R | 146565–147071 (158) | 146564–147070 | L,E | EEV glycoprotein | EEV membrane protein | VACWR157 | 2e-85 | WR | A34R (8e-85) |
| LC16M202R | 147115–147645 (176) | 147114–147644 | E | Hypothetical protein | Similar gene in other organisms | MVA146R | 1e-93 | MVA | A35R (2e-93) |
| LC16M203L | 147275–147045 (76) | 147274–147044 | L? | Hypothetical protein | Similar gene in other organisms | A ORF M | 7e-40 | CPN | A ORF M (7e-40) |
| LC16M204R | 147712–148377 (221) | 147711–148376 | L?,E | EEV membrane protein | EEV membrane protein | A36R | e-106 | CPN | A38R (e-106) |
| LC16M205R | 148441–149232 (263) | 148440–149231 | L? | Hypothetical protein | Similar gene in other organisms | VACWR150 | e-143 | WR | A37R (e-141) |
| LC16M206L | 149213–148962 (83) | 149212–148961 | | Hypothetical protein | Similar gene in other organisms | A ORF O | 1e-41 | CPN | A ORF O (1e-41) |
| LC16M207R | 149322–149510 (62) | 149321–149509 | L?,E | Hypothetical protein | LC16m8, LC16mO specific gene | | | | |
| LC16M208L | 150340–149507 (277) | 150339–149506 | L? | CD47 antigen/integrin-associated protein | Other functions | A38L | e-149 | CPN | A38L (e-149) |
| LC16M209R | 150357–151568 (403) | 150356–151567 | L? | Semaphorin | Other functions | A39R | 0.0 | CPN | A39R (0.0) |
| LC16M210L | 151402–151133 (89) | 151401–151132 | | Hypothetical protein | Similar gene in other organisms | A ORF P | 3e-51 | CPN | A ORF P (3e-51) |

*Continued on following page*

TABLE 1—*Continued*

| ORF | Position in LC16m8 (aa length) | Position in LC16m0 | Promoter type[a] | Putative function | Category | Best-matching ORF[b] | | | ORF corresponding to CPN |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Name | BLASTP Score | Source | |
| LC16M211R | 151594–152073 (159) | 151593–152072 | L?,E | Natural killer cell receptor homologue | Other functions | VACWR165 | 4e-86 | WR | A40R (5e-70) |
| LC16M212L | 152830–152171 (219) | 152829–152170 | L? | Hypothetical protein | Similar gene in other organisms | MVA153L | e-131 | MVA | A41L (e-129) |
| LC16M213R | 152994–153395 (133) | 152993–153394 | L? | Profilin-like protein | Other functions | A42R | 1e-75 | CPN | A42R (1e-75) |
| LC16M214R | 153433–154017 (194) | 153432–154016 | L,E | Membrane glycoprotein | Other functions | A43R | e-112 | CPN | A43R (e-112) |
| LC16M215R | 154025–154261 (78) | 154024–154260 | E | Hypothetical protein | Similar gene in other organisms | MVA156R | 6e-23 | MVA | PredictedbyGeneMark06 (1e-15) |
| LC16M216L | 155397–154357 (346) | 155396–154356 | L? | Hydroxysteroid dehydrogenase | Enzyme | A44L | 0.0 | CPN | A44L (0.0) |
| LC16M217R | 155444–155821 (125) | 155443–155820 | L? | Superoxide dismutase (Cu-Zn)-related protein | Enzyme | VACWR171 | 1e-70 | WR | A45R (5e-69) |
| LC16M218R | 155811–156533 (240) | 155810–156532 | L?,E | Hypothetical protein | Similar gene in other organisms | MVA159R | e-127 | MVA | A46R (e-105) |
| LC16M219L | 155454–156137 (105) | 156453–156136 | | Hypothetical protein | Similar gene in other organisms | A ORF Q | 6e-39 | CPN | A ORF Q (6e-39) |
| LC16M220L | 157339–156581 (252) | 157337–156579 | L? | Hypothetical protein | Similar gene in other organisms | VACWR173 | e-129 | WR | A47L (e-125) |
| LC16M221R | 157439–158053 (204) | 157437–158051 | L? | Thymidylate kinase | Enzyme | A48R | e-115 | CPN | A48R (e-115) |
| LC16M222R | 158101–158589 (162) | 158099–158587 | L,E | Hypothetical protein | Similar gene in other organisms | A49R | 2e-90 | CPN | A49R (2e-90) |
| LC16M223R | 158622–160280 (552) | 158620–160278 | L | ATP-dependent DNA ligase | Enzyme | A50R | 0.0 | CPN | A50R (0.0) |
| LC16M224L | 159491–159291 (66) | 159489–159289 | | Hypothetical protein | Similar gene in other organisms | A ORF R | 7e-38 | CPN | A ORF R (7e-38) |
| LC16M225L | 159610–159407 (67) | 159608–159405 | | Hypothetical protein | Similar gene in other organisms | A ORF S | 3e-36 | CPN | A ORF S (3e-36) |
| LC16M226R | 160333–160554 (73) | 160331–160552 | L? | Hypothetical protein | Similar gene in other organisms | A51R | 3e-40 | CPN | A51R (3e-40) |
| LC16M227R | 160533–161333 (266) | 160531–161331 | | Hypothetical protein | Similar gene in other organisms | A51R | e-150 | CPN | A51R (e-150) |
| LC16M228R | 161403–161975 (190) | 161401–161973 | | Hypothetical protein | Similar gene in other organisms | VACWR178 | 4e-92 | WR | A52R (3e-91) |
| LC16M229R | 162275–162835 (186) | 162273–162833 | | Tumor necrosis factor receptor | Other functions | A53R | 1e-50 | VV | A53R (1e-50) |
| LC16M230R | 162291–162587 (98) | 162289–162585 | | Tumor necrosis factor receptor | Other functions | A ORF T | 5e-40 | CPN | A ORF T (5e-40) |
| LC16M231L | 162383–162111 (90) | 162381–162109 | | Hypothetical protein | Similar gene in other organisms | A54L | 8e-49 | CPN | A54L (8e-49) |
| LC16M232R | 163083–164777 (584) | 163081–164775 | L?,E | Kelch-like protein | Other functions | A55R | 0.0 | CPN | A55R (0.0) |
| LC16M233R | 164827–165759 (310) | 164825–165757 | L? | Hemagglutinin | EEV membrane protein | A56R | e-142 | CPN | A56R (e-142) |
| LC16M234R | 165777–165890 (37) | 165775–165888 | L | Guanylate kinase fragment | Other functions | PredictedbyGeneMark | 2e-18 | CPN | PredictedbyGeneMark07 (2e-18) |
| LC16M235R | 165904–166359 (151) | 165902–166357 | | Guanylate kinase | Enzyme | A57R | 1e-82 | CPN | A57R (1e-82) |
| LC16M236R | 166510–167412 (300) | 166508–167410 | L?,E | Putative ser/thr protein kinase | Enzyme | MVA167R | e-178 | MVA | B1R (e-177) |
| LC16M237L | 167333–167010 (107) | 167331–167008 | | Hypothetical protein | Similar gene in other organisms | B ORF A | 2e-60 | CPN | B ORF A (2e-60) |
| LC16M238R | 167502–168161 (219) | 167500–168159 | L? | Hypothetical protein | Similar gene in other organisms | B2R | e-130 | CPN | B2R (e-130) |
| LC16M239L | 168029–167829 (66) | 168027–167827 | | Hypothetical protein | Similar gene in other organisms | B ORF B | 1e-35 | CPN | B ORF B (1e-35) |
| LC16M240R | 168197–168571 (124) | 168195–168569 | | Hypothetical protein | Similar gene in other organisms | B3R | 2e-62 | CPN | B3R (2e-62) |
| LC16M241L | 168292–168005 (95) | 168290–168003 | | Hypothetical protein | Similar gene in other organisms | B ORF C | 1e-52 | CPN | B ORF C (1e-52) |
| LC16M242R | 169227–170903 (558) | 169225–170901 | L?,E | Ankyrin repeat protein | Other functions | B4R | 0.0 | CPN | B4R (0.0) |
| LC16M243R | | 171004–171957[d] | L? | Plaque-size/Host range protein precursor | EEV membrane protein | MVA173R | 0.0 | MVA | B5R (e-179) |
| | 171293–171958 (221)[d] | | | Plaque-size/Host range protein precursor | EEV membrane protein | MVA173R | e-123 | MVA | B5R (e-122) |
| LC16M244R | 172040–172561 (173) | 172039–172560 | I,L?,E | Hypothetical protein | Similar gene in other organisms | MVA174R | 2e-99 | MVA | B5R (3e-99) |
| LC16M245L | 172317–172102 (71) | 172316–172101 | E | Hypothetical protein | Similar gene in other organisms | B ORF D | 4e-37 | CPN | B ORF D (4e-37) |
| LC16M246R | 172599–173147 (182) | 172598–173146 | L | Hypothetical protein | Similar gene in other organisms | B7R | e-107 | CPN | B7R (e-107) |
| LC16M247R | 173202–174020 (272) | 173201–174019 | L? | Interferon-gamma receptor | Other functions | VACWR190 | e-163 | WR | B8R (e-161) |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| LC16M248R | 174107–174340 (77) | 174106–174339 | L? | Putative ER-localized apoptosis regulator | Other functions | VACWR191 | 1e-42 | WR | B9R (3e-42) |
| LC16M249R | 174303–174803 (166) | 174302–174802 | | Kelch-like protein | Other functions | B10R | 5e-82 | CPN | B10R (5e-82) |
| LC16M250R | 174875–175093 (72) | 174874–175092 | L? | Hypothetical protein | Similar gene in other organisms | VACWR193 | 5e-25 | WR | B11R (3e-23) |
| LC16M251R | 175160–176011 (283) | 175159–176010 | | Protein kinase | Enzyme | B12R | e-160 | CPN | B12R (e-160) |
| LC16M252R | 176116–176466 (116) | 176115–176465 | | Serine protease inhibitor | Other functions | ACAM3000_MVA_161 | 2e-63 | ACAM3000 | B13R (1e-61) |
| LC16M253R | 175441–177109 (222) | 176440–177108 | | Serine protease inhibitor | Other functions | B14R | e-127 | CPN | B14R (e-127) |
| LC16M254R | 177186–177635 (149) | 177185–177634 | | Hypothetical protein | Similar gene in other organisms | B15R | 4e-89 | CPN | B15R (4e-89) |
| LC16M255R | 177748–178728 (326) | 177747–178727 | L? | Interleukin-1 binding protein precursor | Other functions | VACWR197 | 0.0 | WR | B16R (e-166) |
| LC16M256L | 178289–178062 (75) | 178288–178061 | | Hypothetical protein | Similar gene in other organisms | B ORF F | 4e-29 | CPN | B ORF F (4e-29) |
| LC16M257L | 179796–178774 (340) | 179795–178773 | L? | Hypothetical protein | Similar gene in other organisms | B17L | 0.0 | CPN | B17L (0.0) |
| LC16M258R | 179936–181177 (413) | 179935–181176 | | Ankyrin-like protein | Other functions | B18R | 0.0 | CPN | B18R (0.0) |
| LC16M259R | 181307–181810 (187) | 181306–181809 | L? | CrmE protein | Other functions | crmE | 2e-74 | USSR strain | |
| LC16M260R | 181859–182080 (73) | 181858–182079 | L? | Hypothetical protein | Similar gene in other organisms | CMP6L | 1e-80 | Camalpox | |
| LC16M261R | 181978–182691 (237) | 181977–182690 | L? | Hypothetical protein | LC16m8, LC16mO specific gene | | | | |
| LC16M262L | 182555–182328 (75) | 182554–182327 | | Hypothetical protein | LC16m8, LC16mO specific gene | | | | |
| LC16MRTR01R | 182972–183415 (147) | 182971–183414 | | Hypothetical protein | Similar gene in other organisms | B22R | 4e-85 | CPN | B22R (4e-85) |
| LC16MRTR02R | 183462–184712 (418) | 183461–184711 | L? | Host range protein | Other functions | B23R | 0.0 | CPN | B23R (0.0) |
| LC16MRTR03R | 185046–185327 (93) | 185045–185326 | | Hypothetical protein | Similar gene in other organisms | D4L | 3e-41 | Cowpox | PredictedbyGeneMark09 (3e-18) |
| LC16MRTR04R | 185654–185983 (109) | 185653–185982 | L?,E | Hypothetical protein | Similar gene in other organisms | VACWR211 | 1e-62 | WR | B25R (5e-57) |
| LC16MRTR05L | 185800–185588 (70) | 185799–185587 | L? | Hypothetical protein | Similar gene in other organisms | B ORF G | 1e-29 | CPN | B ORF G (1e-29) |
| LC16MRTR06R | 186233–185619 (128) | 186232–186618 | | Hypothetical protein | Similar gene in other organisms | VACWR212 | 4e-59 | WR | B26R (1e-55) |
| LC16MRTR07R | 186983–187129 (48) | 186982–187128 | | K1R protein fragment | Other functions | VACWR214 | 4e-24 | WR | PredictedbyGeneMark02 (5e-24) |
| LC16MRTR08R | 187247–187615 (122) | 187246–187614 | L? | Tumor necrosis factor receptor II homologue | Other functions | VACWR215 | 4e-73 | WR | B26R (3e-72) |
| LC16MRTR09R | 167834–187938 (34) | 187833–187937 | | Tumor necrosis factor receptor II fragment | Other functions | PredictedbyGeneMark | 3e-17 | CPN | PredictedbyGeneMark11 (3e-17) |
| LC15MRTR10R | 188327–189103 (258) | 188326–189102 | | Major secreted protein | Other functions | VACWR218 | e-113 | WR | B29R (e-112) |
| LC16MRTR11L | 188880–188767 (37) | 188879–188766 | | Hypothetical protein | Similar gene in other organisms | B ORF H | e-10 | CPN | B ORF H (e-10) |
| LC16MRTR12L | 188887–188684 (67) | 188886–188683 | | Hypothetical protein | Similar gene in other organisms | B ORF I | 2e-36 | CPN | B ORF I (2e-36) |

[a] Regulatory sequences upstream of the ORFs were classified into early (E), intermediate (I), late (L) and putative late (L?) promoters.
[b] Best-matching ORF from BLASTP analysis of nonredundant protein database.
[c] Broken lines indicate that LC16mO ORFs were in the same positions and had the same amino acid lengths as those of LC16m8.
[d] LC16M243R ORF was full-size (317 aa) in LC16mO but was truncated (221 aa) in LC16m8.

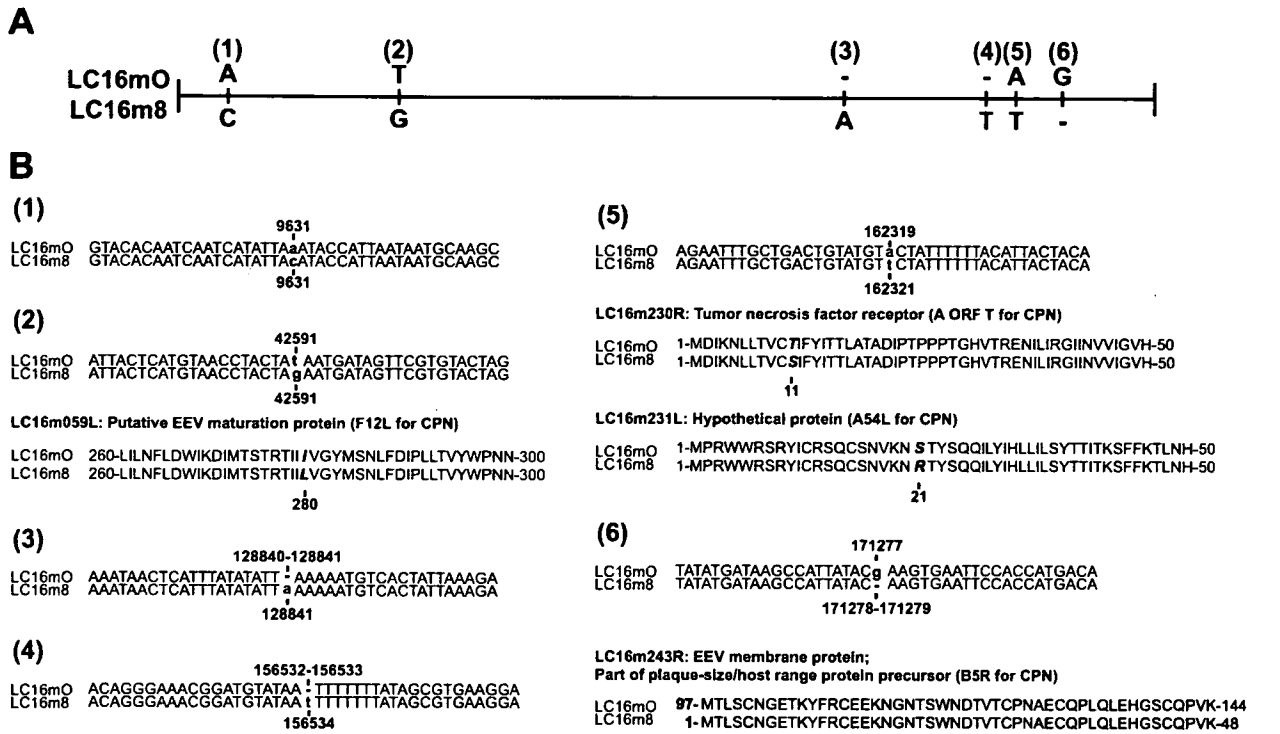


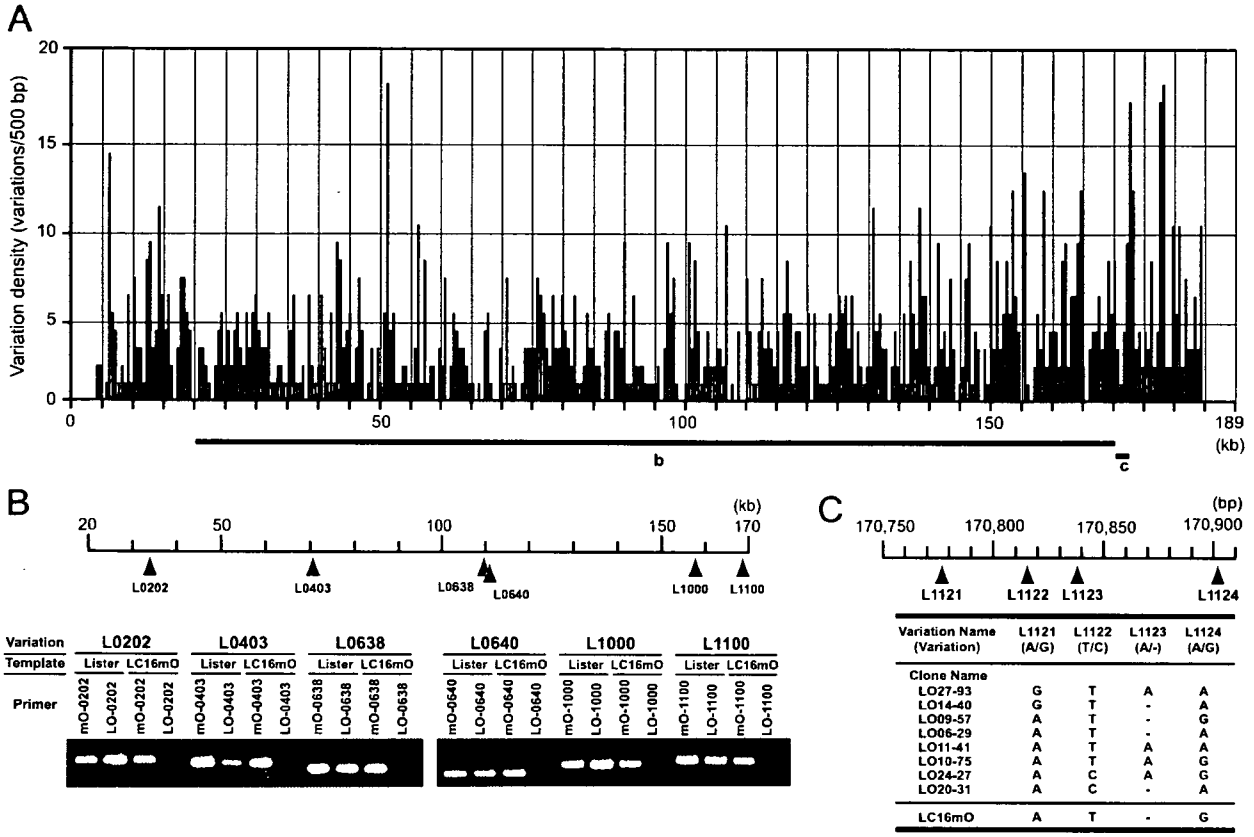

FIG. 2. Differences in nucleotide sequences between the LC16m8 and LC16mO strains. (A) The locations (1 to 6) of nucleotide point mutations in the genomes are shown schematically. (B) The nucleotide changes are shown in boldface lowercase letters. The resultant amino acid changes in ORFs are indicated by shaded boldface italics in loci (2, 5, and 6). Putative gene functions and the ORFs corresponding to the CPN strain are also shown.

gene, which generated a termination codon and truncated the B5R Env protein of m8 EEV at amino acid position 93 (Fig. 2B), as described previously (47).

Almost all of the m8 ORFs best matched those of OPV, mainly the vaccinia virus CPN strain. Therefore, m8 and CPN were strikingly similar in their genomic organizations and ORF orientations (Fig. 1 and Table 1) (21). The m8 virus retained 192 out of 198 major CPN ORFs (60 out of 65 minor CPN ORFs), including other EEV *env*-related genes, A33R, A34R, A36R, A56R, F12L, and F13L. Only a few differences were observed. CPN C21L/B27R and C19L/B24R were absent in the ITR regions of m8, although they appear to be nonessential and presumably do not represent functional genes (21). The m8 genome lacked nonessential ORFs C13L, B19R, and B20R of unknown function in the regions neighboring the ITR termini and A25L in the central coding region, which encodes a short fragment (65 aa) (21) homologous to an A-type inclusion protein of CPV (1,284 aa) (18). ORF LC16M191L (502 aa), however, corresponded to CPN A26L, also encoding a truncated homologue (322 aa) of the CPV inclusion protein (18, 21).

As LO had no history of virus cloning, nucleotide polymorphisms were observed at 1,264 sites in the genome putatively assembled by 4,913 sequencing reactions. This diversity was mapped from L0001 to L1264 along the whole genome (Fig. 3A; see Table S1 in the supplemental material). Sequences of the only marginal region spanning the diversity numbers from L1121 to L1124 (150 bp) revealed at least eight genotypes in LO, whereas mO possessed the "AT-G" genotype, which was the same as the LO09-57 clone in the region (Fig. 3C). Furthermore, PCR analysis of other randomly selected loci demonstrated that mO-specific primers amplified template LO DNA, but not vice versa (Fig. 3B). These results indicate that LO consists of a huge divergent virus population but likely contains the ancestors of mO. Because of the diversity of LO, however, it was impossible to exactly assign its consensus full-genome sequence and all ORFs. Thus, the LO shotgun sequences with major hits were tentatively assembled, compiled as an artificial genome sequence, and deposited in GenBank.

**Analysis of the EEV *env*-related genes.** The evolutionary relationships of the EEV *env*-related genes in Lister-related viruses were further analyzed by sequencing of PCR amplicons from ListerVAX, another batch of mO and m8, and WR and IHD-J, which were stored in our laboratory. Because the mO and m8 sequences were identical except for B5R, the resultant amino acid alignments of A33R, A34R, A36R, A56R, F13L, and B5R of ListerVAX and mO were presented with reference to those of CPN and compared to other VV strains and OPVs deposited in GenBank (Fig. 4). ListerVAX had the same amino acid alignment in A33R as wild-type (wt) VV CPN or WR. On the other hand, mO A33R had two amino acid substitutions: Asn at amino acid position 165 ($Asn^{165}$) was unique to mO, but $Thr^{141}$ was found in mO and MVA, and also in VAR, MPV, and CPV of OPV (Fig. 4A). A34R was rather conserved in OPV, and no substitution was observed between ListerVAX and mO. Interestingly, however, $Lys^{165}$ seems to be specific to VV ($Arg^{165}$ for VAR, MPV, and CPV), and aa 110




| Variation Name (Variation) | L1121 (A/G) | L1122 (T/C) | L1123 (A/-) | L1124 (A/G) |
|---|---|---|---|---|
| Clone Name | | | | |
| LO27-93 | G | T | A | A |
| LO14-40 | G | T | - | A |
| LO09-57 | A | T | - | G |
| LO06-29 | A | T | - | A |
| LO11-41 | A | T | A | A |
| LO10-75 | A | T | A | G |
| LO24-27 | A | C | A | G |
| LO20-31 | A | C | - | A |
| LC16mO | A | T | - | G |

FIG. 3. Polymorphism of the Lister strain genome. (A) Nucleotide sequence variations are presented in each 500-bp length along the central coding region of the Lister genome. (B) Six divergent loci, L0202, L0403, L0638, L0640, L1000, and L1100, were randomly selected. LO and mO genomic DNAs were amplified at the selected sites by PCR with the forward primers specific for LO or mO and the common reverse primers. (C) The marginal (150-bp) region spanning diversity numbers L1121 to -1124 of LO virus DNA were cloned, sequenced, and classified into eight genotypes. The genotype of LC16mO is also shown.

(Asn or Asp) may classify OPV into two groups (Fig. 4B). Similarly, A36R was almost conserved in VV strains but divergent in other OPVs. ListerVAX, mO, WR, and IHD-J strains of VV, however, had a common $Glu^{146}$-to-$Lys^{146}$ substitution from CPN. An additional $Met^{104}$-to-$Ile^{104}$ change occurred in mO, although this was also the case in VAR (Fig. 4C).

As for A56R, ListerVAX was a mixture of wt-like VV (clone 3) and an mO-type mutant (clone 1) that possessed a 5-aa deletion from $Ala^{245}$ to $Asp^{249}$ and a conversion of $Tyr^{302}$ to $Cys^{302}$, which may be an ancestor clone of mO. Another difference between ListerVAX and mO was aa 19, which was Phe and Ser in ListerVAX and mO, respectively (Fig. 4D). $Lys^{291}$ in F13L was unique to the Lister family viruses, whereas it was $Arg^{291}$ in other VVs and OPVs, supporting the Lister lineage of mO. F13L $Pro^{6}$ and $Ser^{6}$ of ListerVAX and mO, respectively, seem to be within the divergence of OPV, because there was $Pro^{6}$ in MVA and IHD-J and $Ser^{6}$ in CPN, WR, VAR, and MPV (Fig. 4F). B5R is located close to the right-terminal end, and therefore, it was most divergent among the EEV *env* genes. ListerVAX differed from the compiled shotgun LO sequence in 3 nucleotides. However, the differences resulted in one amino acid substitution, from $Ile^{82}$ to $Val^{82}$, which also occurred in other OPVs. There were four amino acid changes in B5R between ListerVAX ($Ile^{82}$, $Asn^{87}$, $Ile^{153}$, and $Val^{233}$) and mO ($Val^{82}$, $Asp^{87}$, $Met^{153}$, and $Ile^{233}$) (Fig. 4E).

Altogether, these results confirm the notion that mO, and consequently m8, are the progeny of LO and not so divergent from LO, wt VV, or OPV, except for B5R.

**Antibody responses by vaccination.** The truncated m8 and intact LO B5R proteins were compared for antigenic activity in initial experiments. BALB/c mice were subcutaneously immunized six times with the recombinant B5R proteins adsorbed to aluminum adjuvant or Ni-agarose beads. The mice were challenged by intranasal infection with $10^{6}$ PFU of mouse-pathogenic WR virus 20 weeks after the first immunization and 12 days after the last booster injection. The LO B5R protein partially protected mice from death, with a survival rate of 78% after the appearance of severe clinical symptoms, such as ruffled fur, hunched posture, and weight loss, peaking at around 7 to 9 days after challenge. However, mice receiving the truncated m8 protein similarly developed symptoms, lost bodyweight, and died (100%) within 9 days (data not shown). These results confirm the immunogenicity of the intact B5R protein and also suggest a lack of antigenic activity of the truncated B5R protein.

Thus, B5R-defective m8 was compared with B5R-intact mO

**A: A33R**

| aa position | 20 | 34 | 59 | 73-5 | 81 | 95 | 97 | 112 | 117-8 | 120 | 127-8 | 141 | 149 | 164-5 | 171 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CPN | G | L | L | AAV | S | D | K | L | QL | S | TA | I | E | SD | V |
| Lister | * | * | * | *** | * | * | * | * | ** | * | ** | * | * | ** | * |
| LC16mO | * | * | * | *** | * | * | * | * | ** | * | ** | T | * | *N | * |
| WR | * | * | * | *** | * | * | * | * | ** | * | ** | * | * | ** | * |
| IHD-J | * | * | * | *** | * | * | * | * | ** | * | ** | * | * | ** | I |
| MVA | R | * | * | *** | * | * | * | * | ** | * | ** | T | K | ** | * |
| Variola | * | I | * | -*T | L | K | Q | F | ** | * | AT | T | * | T* | * |
| Monkeypox | * | * | Q | S** | * | * | * | * | KS | E | A* | T | * | ** | * |
| Cowpox | * | * | * | *** | * | * | * | F | ** | * | ** | T | * | ** | * |

**B: A34R**

| aa position | 11-3 | 19 | 24 | 39 | 84 | 110 | 138 | 151 | 165 |
|---|---|---|---|---|---|---|---|---|---|
| CPN | MFK | A | I | K | R | N | A | K | K |
| Lister | R** | * | * | * | * | * | * | * | * |
| LC16mO | R** | * | * | * | * | * | * | * | * |
| WR | R** | * | * | * | * | D | * | * | * |
| IHD-J | R** | * | * | * | * | * | * | E | * |
| MVA | R** | V | * | * | * | D | * | * | * |
| Variola | R** | * | * | * | * | * | * | * | R |
| Monkeypox | R*R | * | L | R | G | D | S | * | R |
| Cowpox | R*R | * | * | * | * | D | S | * | R |

**C: A36R**

| aa position | 2 | 35 | 49 | 60 | 75 | 87 | 104 | 109 | 120 | 126 | 129 | 138 - 47 | 155-7 | 180 | 188 | 205-6 | 207 - 21 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CPN | M | D | S | S | N | D | M | E | L | R | O | VINETETVEV | NPN | T | E | EO | DHEHDDIESSVVSLV |
| Lister | * | * | * | * | * | * | * | * | * | * | * | ********K* | *** | * | * | ** | *************** |
| LC16mO | * | * | * | * | * | * | I | * | * | * | * | ********K* | *** | * | * | ** | *************** |
| WR | * | * | * | * | * | * | * | * | * | * | * | ********K* | *** | * | * | ** | *************** |
| IHD-J | * | * | * | * | * | * | * | * | * | * | * | ********K* | *** | * | * | ** | *************** |
| MVA | * | * | * | * | * | * | * | * | * | * | * | *---------- | *** | * | * | ** | *********----** |
| Variola | I | N | * | N | * | * | I | * | R | C | * | -******I** | **S | * | * | *- | ---********** |
| Monkeypox | LYIEQSE* | * | P | * | Y | N | * | K | * | * | L | ***D*****I | I*S | A | D | -- | ---------- |
| Cowpox | | * | * | * | * | * | * | * | * | * | * | ********** | **S | * | * | **E | Q********** |

**D: A56R**

| aa position | 2-5 | 16 - 22 | 32 | 35 | 39-41 | 96 | 102 | 110 | 124 | 144-7 | 150-2 | 155-6 | 158 | 160 | 164 | 172 | 178 | 180 | 183 | 190-1 | 196-200 | 206-9 | 213-6 | 234 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CPN | TRLP | ATPFPOT | L | N | TND | R | V | P | T | THS | SSE | DY | D | S | S | E | V | D | T | DS | SATSG | ETPE | DKEE | G |
| Lister (cl-1) | **** | ******* | * | * | *** | * | * | * | * | *** | *** | ** | * | * | * | * | * | * | * | ** | **S** | **** | **** | * |
| (cl-3) | **** | ******* | * | * | *** | * | * | * | * | *** | *** | ** | * | * | * | * | * | * | * | ** | **S** | **** | **** | * |
| LC16mO | **** | ***S*** | * | * | *** | * | * | * | * | *** | *** | ** | * | * | * | * | * | * | * | ** | **S** | **** | **** | * |
| WR | **** | ******* | * | * | *** | * | * | T | * | *** | **K | ** | * | * | * | * | * | * | * | ** | **S** | **** | ***- | * |
| IHD-J | **** | ******* | * | * | *** | * | * | T | * | *** | *** | ED | * | F | * | * | * | * | * | ** | **S** | **** | **** | * |
| MVA | **** | ******* | * | * | *** | * | * | * | * | *** | *** | ** | * | * | * | * | * | * | * | ** | **S** | **** | **** | * |
| Variola | ***S | S**Y**IOI | * | S | I** | K | * | T | * | S** | *** | ** | N | F | L | G | * | N | * | *I | *TS** | K*SG | N**- | E |
| Monkeypox | *O** | V**S*** | I | S | **Y | G | I | T | I | *-- | I** | ED | * | * | * | * | * | * | * | ** | NAS** | **** | **** | * |
| Cowpox | A*** | S**S*** | * | * | *T* | * | * | T | * | *** | *** | ** | * | * | * | * | E | * | - | EN | *T**R | **** | **** | * |

| aa position | 242 - 55 | 258-9 | 263 - 7 | 270-3 | 277-80 | 284-5 | 302 | 305-6 |
|---|---|---|---|---|---|---|---|---|
| CPN | TDDADLYDTYNDND | PS | GSSTT | SNYK | FVEI | TA | C | RS |
| | ***------****** | *P | *G*** | **** | **** | ** | * | ** |
| | ************** | *P | *G*** | **** | **** | ** | Y | ** |
| | ***------****** | *P | *G*** | **** | **** | ** | * | ** |
| | ************** | *P | *G*** | **** | **** | ** | Y | ** |
| | ************** | *P | *G*** | **** | **** | ** | Y | ** |
| | ************** | ** | *G*** | **** | **** | ** | Y | ** |
| | AN**---HND*EPS | SP | KNI*K | GK*S | Y*KV | A* | * | ** |
| | ************** | *P | *G*** | **** | **** | *T | * | HP |
| | ************** | *P | *G*** | **** | **** | ** | * | ** |

**F: F13L**

| aa position | 5-7 | 72 | 98 | 126 | 173 | 181-3 | 228 | 250 | 291 |
|---|---|---|---|---|---|---|---|---|---|
| CPN | ASV | A | C | D | A | CSA | R | N | R |
| Lister | *P* | * | * | * | * | *** | K | * | K |
| LC16mO | *** | * | * | * | * | *** | K | * | K |
| WR | *** | * | * | * | * | *** | K | * | * |
| IHD-J | *P* | * | * | * | * | *** | K | * | * |
| MVA | *P* | * | * | N | * | *** | K | * | * |
| Variola | T*A | * | * | * | V | Y*S | * | * | * |
| Monkeypox | V** | V | S | * | * | *** | K | S | * |
| Cowpox | *Q* | * | * | * | * | *** | K | * | * |

**E: B5R**

| aa position | 40-1 | 50 | 53 | 55 | 82 | 87 | 95-7 | 100-3 | 132 | 136 | 145 | 152-3 | 166 | 170 | 188 | 216 | 233 - 40 | 243 - 8 | 260 | 283 | 296 | 304 | 317 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| CPN | NN | Q | H | S | I | N | STM | SCNG | P | E | E | YM | A | S | I | I | VLPICVRT | EFDPVD | L | V | V | D | P |
| Lister | DK | * | * | L | * | * | *** | **** | * | * | * | *I | * | * | M | T | ***T***S | K****** | * | * | * | * | * |
| LC16mO | DK | * | * | L | V | D | *** | **** | * | * | * | ** | * | * | M | T | I**T***S | K****** | * | * | * | * | * |
| WR | DK | * | * | * | * | * | *** | **** | * | * | * | ** | * | * | M | T | ******** | ****** | * | * | * | * | * |
| IHD-J | DK | * | * | L | V | D | *** | **** | * | * | * | ** | * | * | * | * | I**T***S | K****** | * | * | * | * | * |
| MVA | ** | * | * | * | * | * | *** | **** | * | * | * | *I | * | * | * | * | I**T***S | K****** | * | * | * | * | * |
| Variola | DK | S | Y | L | V | * | AII | I*KD | S | D | G | HI | * | T | * | * | *****I*S | *****E | * | * | * | N | L |
| Monkeypox | DK | S | * | L | V | D | *** | **** | * | * | * | ** | V | * | * | T | I**T***S | ****** | * | M | I | * | * |
| Cowpox | DK | * | * | L | V | D | *** | **** | * | * | * | ** | * | * | * | T | I**T***S | ***L** | I | * | I | * | * |

FIG. 4. Comparison of amino acid alignments of the EEV Env-related proteins in six vaccinia virus strains and other OPVs. The numbers at the top of each panel indicate the amino acid positions of the EEV proteins of vaccinia virus CPN strain. The asterisks and dashes show conserved and deleted amino acids, respectively, with reference to CPN. The vaccinia viruses compared are CPN, Lister (calf lymph Lister vaccine), LC16mO, WR, IHD-J, and MVA strains. Variola, monkeypox, and cowpox viruses shown for reference are Bangladesh-1975, Zaire-96-I-16, and GRI-90 strains, respectively.

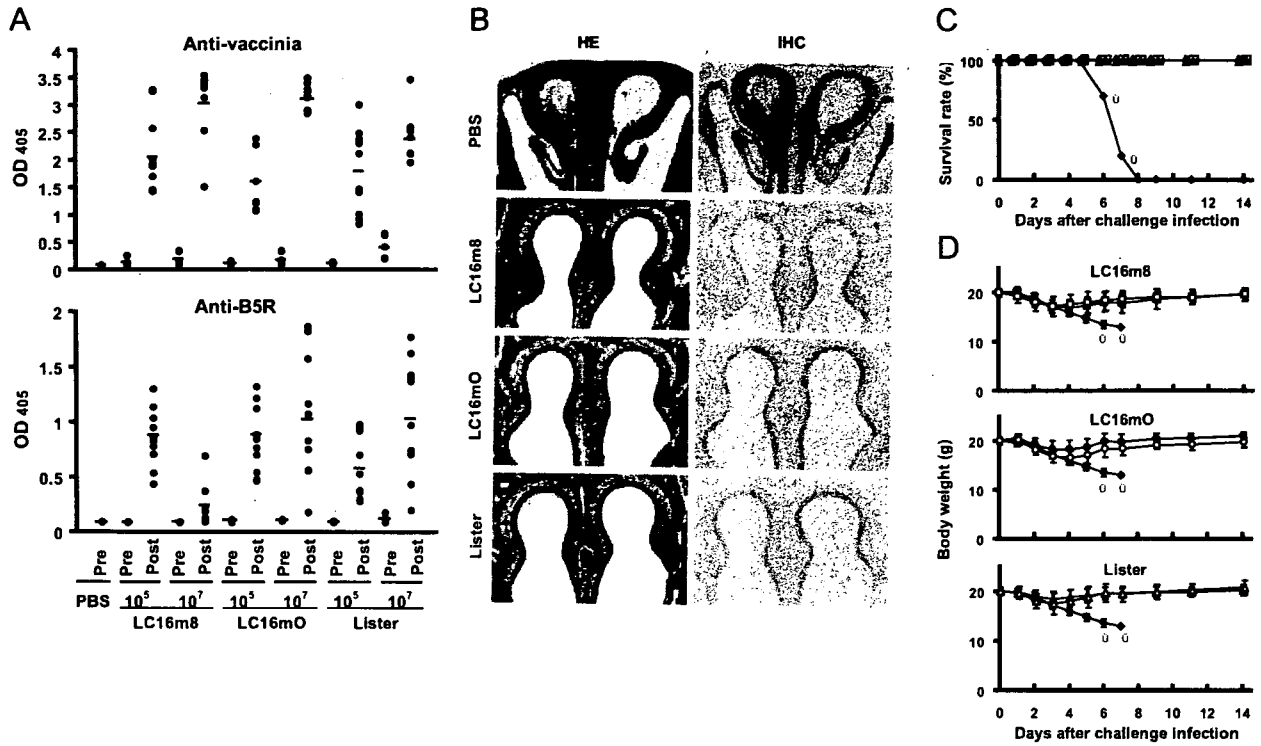



FIG. 5. Protection against lethal WR challenge by vaccination with LC16m8. Groups of 6-week-old BALB/c mice were subcutaneously vaccinated and intranasally challenged as for Table 2. (A) Levels of antibodies in pre- and postchallenge sera of individual mice. Sera were examined by ELISA for vaccinia virus- and B5R-specific antibodies, and the results are shown with $OD_{405}$ values at 1:400 and 1:100 dilutions, respectively. The horizontal bars indicate the averages. (B) Histopathology by HE staining and IHC by peroxidase staining of the nasal tissue collected from nonimmunized and vaccinated mice 9 and 14 days after challenge infection, respectively. (C) Survival and (D) bodyweights of mice after WR challenge. The mice had been vaccinated with $10^5$ (open symbols) or $10^7$ (solid symbols) PFU of LC16m8 (□ and ■), LC16mO (○ and ●), or Lister (△ and ▲) strain or PBS (◆). To avoid confusion, the average bodyweight ± standard deviation is shown in separate panels in comparison with the PBS group. The crosses indicate the deaths of mice.

and LO for the ability to prime or induce anti-B5R and anti-EEV antibody responses before and after pathogenic-WR infection. BALB/c mice were vaccinated subcutaneously with a low ($10^5$ PFU) or high ($10^7$ PFU) dose of the vaccine strains. On day 21 after vaccination, one-third of the mice were bled to determine prechallenge antibody levels, and the other mice were challenged intranasally with $10^6$ PFU of WR. Sera were collected 14 days later to test for postchallenge antibodies. Representative ELISA antibody levels in individual mice are shown in Fig. 5A, and the results of antibody responses examined are summarized in Table 2. ELISA antibody levels at prechallenge were low against VV antigens and undetectable against the B5R protein in all vaccinated mice. The titers and seroprevalences, if any were present, tended to be higher in $10^7$

TABLE 2. Antibody responses in vaccinated mice at pre- and postchallenge infection[a]

| Vaccination (day 0) | | Prechallenge (day 21) | | | | Postchallenge (day 35) | | | |
|---|---|---|---|---|---|---|---|---|---|
| Strain | Dose (PFU) | IgG ELISA (positive/total) | | NAb | Comet inhibition | IgG ELISA (positive/total) | | NAb | Comet inhibition |
| | | Anti-vaccinia virus[b] | Anti-B5R[b] | | | Anti-vaccinia virus[c] | Anti-B5R[b] | | |
| PBS | | 0.10 (0/5) | 0.08 (0/5) | <4[d] | <10[d] | ND[e] | ND | ND | ND |
| Lister | $10^5$ | 0.20 (3/5) | 0.09 (0/5) | <4 | <10 | 1.78 (10/10) | 0.56 (10/10) | 4 | <10 |
| | $10^7$ | 1.00 (5/5) | 0.11 (0/5) | 16 | <10 | 2.42 (10/10) | 1.06 (10/10) | 64 | <10 |
| LC16mO | $10^5$ | 0.19 (2/5) | 0.09 (0/5) | <4 | <10 | 1.60 (10/10) | 0.83 (10/10) | 16 | <10 |
| | $10^7$ | 0.52 (4/5) | 0.10 (0/5) | 4 | <10 | 3.18 (10/10) | 1.03 (9/10) | 64 | <10 |
| LC16m8 | $10^5$ | 0.39 (2/5) | 0.08 (0/5) | <4 | <10 | 2.08 (10/10) | 0.85 (10/10) | 64 | <10 |
| | $10^7$ | 0.53 (4/5) | 0.08 (0/5) | <4 | <10 | 3.14 (10/10) | 0.21 (3/10) | 64 | <10 |

[a] Mice vaccinated with a single dose were challenged intranasally with $10^6$ PFU of WR strain on day 21 and sacrificed on day 35.
[b] Averages of $OD_{405}$ values at a 1:100 dilution.
[c] Averages of $OD_{405}$ values at a 1:400 dilution.
[d] The highest serum dilutions yielding a 50% plaque reduction or inhibitory comet formation.
[e] ND, not determined.

PFU vaccination groups than in those vaccinated with $10^5$ PFU. Comet inhibition activity in sera, which is an indicator of anti-EEV antibodies, was negative in each of the vaccinated groups. NAb titers to VV, that is, IMV, were also low or undetectable; titers as low as 1:4 and 1:16 were detected only in groups of mice immunized with $10^7$ PFU of mO and LO, respectively (Table 2).

Upon lethal challenge with virulent WR, however, high levels of anti-vaccinia virus ELISA antibodies were induced in all groups of mice vaccinated with m8, mO, and LO. Substantial levels of anti-B5R antibodies were also detected in all groups, except for that receiving $10^7$ PFU of m8, where only 3 out of 10 mice developed anti-B5R antibodies (Fig. 5A and Table 2). Therefore, mice immunized with $10^7$ PFU of m8 produced significantly ($P < 0.0008$) lower levels of anti-B5R antibodies after WR infection than did those immunized with $10^5$ PFU of m8, $10^7$ PFU of mO, or $10^7$ PFU of LO (Fig. 5A), when compared by an unpaired Student's $t$ test. The lethal challenge with WR did not elicit comet inhibition activity against EEV in vaccinated mice but induced and/or augmented NAb titers to IMV ranging from 1:4 to 1:64 (Table 2). Levels of antibodies after WR challenge were higher in mice immunized with $10^7$ PFU than in those immunized with $10^5$ PFU, indicating that mice were effectively primed with a higher dose of vaccine and boosted by WR infection. The exception was anti-B5R antibody titers in groups receiving B5R-defective m8 (Table 2 and Fig. 5A), probably because B5R-expressing EEV of WR was more quickly cleared before eliciting anti-B5R antibodies by stronger immunity induced with $10^7$ PFU of m8 than with $10^5$ PFU of m8.

**Pathological findings.** The immunogenicities of the m8, mO, and LO vaccines were evaluated by histopathological and immunohistochemical analyses of the nasal tissue of mice, the primary infection site for pathogenic WR. The specimens from mice mock vaccinated with PBS demonstrated massive destruction and necrosis of the mucosal epithelium of the nasal cavity. The severe necrosis of olfactory epithelial cells was widespread in the nasal-cavity tissue (Fig. 5B, HE). VV antigens were distributed widely and intensively, colocalizing at the damaged areas of the epithelium (Fig. 5B, IHC). In contrast to nonimmune mice, severe epithelial destruction was rarely observed in the nasal cavities of mice vaccinated with a lower dose ($10^5$ PFU) of m8, mO, or LO. Their nasal specimens showed intact tissue morphology without evidence of recovery from tissue necrosis. In addition, no VV antigens were detected in nasal mucosal epithelial cells when examined by enhanced immunohistochmical staining (Fig. 5B, IHC). Similarly, no pathological changes were detectable after intranasal WR challenge in mice vaccinated with a higher dose ($10^7$ PFU) of m8, mO, or LO (data not shown).

**Protection by m8, mO, and LO vaccines.** The immunological and histopathological studies described above suggest that m8 is as effective as mO and LO against pathogenic-OPV infection. Therefore, the protective efficacies of the m8, mO, and LO vaccine strains were further estimated in additional WR challenge experiments. Groups of 10 BALB/c mice vaccinated as for immunogenicity studies were examined for survival rate (Fig. 5C) and bodyweight loss (Fig. 5D) after intranasal inoculation with $10^6$ PFU of WR. As this WR dose represented 10 $LD_{50}$ for 6-week-old BALB/c mice (data not shown), the nonimmunized mice receiving PBS developed clinical symptoms, lost bodyweight, and died within 9 days after WR challenge. In contrast, none of the mice in the m8, mO, or LO vaccination group died (Fig. 5C). Vaccinated mice developed only a transient and slight loss of bodyweight, peaking at 3 or 4 days after challenge, but looked healthy without ruffled fur, inactivity, or respiratory distress and promptly gained weight thereafter (Fig. 5D). Notably, there were no significant differences in bodyweight between the low-dose ($10^5$ PFU) and high-dose ($10^7$ PFU) vaccination groups nor among the m8, mO, and LO vaccination groups (Fig. 5D).

## DISCUSSION

In this study, we suggest that an attenuated vaccinia virus m8 strain that was licensed in 1975 in Japan as the second-generation smallpox vaccine is as efficacious as the first-generation LO vaccine that was used worldwide in the WHO smallpox eradication program.

The m8 vaccine was not used in a large population in areas of endemicity because smallpox was almost eradicated when it was developed. Today, no vaccines under development or in human trials can be tested for protective efficacy against smallpox by infection of humans with the causative virus, VAR. However, a pathogenic vaccinia virus WR strain provides an alternative small-animal model suited for evaluating protective immunization (2, 32, 50, 51). VV has rather low infectivity for mice, but WR is an exception, because it is adapted to mice by repeated passages in the mouse brain (27). Intranasal inoculation with as little as $10^5$ PFU of WR elicited severe illness and 50% death in BALB/c mice, although they were less susceptible to VV infection than C57BL/6 and C3H/He mice (unpublished data). Thus, BALB/c mice vaccinated with the LO and LO-derived vaccine strains failed to develop definite erythema or pustules at the inoculated skin sites, which is classified as a "take" that is indicative of viral replication and therefore successful immunization in other vaccinia virus-sensitive hosts, such as humans, cows, and rabbits. Anti-B5R, -EEV, or -IMV antibodies were certainly undetectable or at low levels in vaccinated BALB/c mice. Nevertheless, the m8, mO and LO vaccines all protected mice comparably and completely against challenge with $10^6$ PFU of WR. Notably, a single subcutaneous vaccination with m8 primed mice to render them as protective as vaccination with mO and LO, even at a low dose ($10^5$ PFU). Furthermore, with an increased WR challenge dose ($10^7$ PFU), 100% of mice vaccinated percutaneously with m8 ($10^5$ PFU) survived, while they lost significant weight temporarily and comparably to those vaccinated with the LO or NYBH strains (unpublished data) that had been used in humans.

OPVs are known to be highly cross-reactive among themselves in immune protection. Indeed, the m8 vaccine protected monkeys against MPV challenge (unpublished data), as recently described for the MVA vaccine (9). On the basis of these historical and experiential facts, CPV is thought to have been used in 1798 as the first human vaccine against VAR, and VV became the smallpox vaccine in the modern era. Similarly, OPVs are genetically highly conserved. Complete OPV genome sequences from VV, VAR, CPV, MPV, ectromelia virus, and camelpox virus have recently been investigated for phylo-

genetic analyses, with results indicating that CPV (strain GRI) is closely related to VV and that the genetic distances from VAR were lowest for camelpox virus (<0.0155), next lowest for VV (<0.0259), high for MPV (<0.0307), and highest for ectromelia virus (<0.0354) (22). These analyses may lead to the prediction that complete genome sequence data from VVs or OPVs will provide insight into the efficacy of smallpox vaccine strains.

Therefore, we determined the complete genome sequences of the licensed m8, parental mO, and grandparental LO strains. Our data may be interpreted to mean that the LO-related vaccines have similar abilities that would induce immune protection, supporting the above-mentioned prediction. Only four missense mutations occurred among the >280 deduced ORFs of m8 during evolution from the parental mO strain. The major change was a truncating mutation of the B5R gene. It is therefore noted that B5R was the only destroyed gene in m8 compared to mO. Furthermore, m8 and mO possessed almost all ORFs corresponding to the vaccinia virus CPN strain (21). As the grandparental LO strain has never been plaque cloned, its genome sequence exhibited huge polymorphisms, which were previously suggested by analyses of restriction enzyme fragments and pock or plaque size (46, 52, 53). However, our PCR sequencing of the EEV *env*-related genes indicated that they were all preserved in mO, and in LO as well, and that m8 was probably derived from a low-virulence clone of divergent LO. This genomic background of m8 suggests that it functions like LO as a smallpox vaccine, except for B5R.

B5R is the only NAb-inducing antigen of EEV so far identified (19). EEVs are extracellular free virions released from infected cells and seem to be prevented by NAbs (12, 19, 44). Destruction of B5R reduced the formation of EEV 5- to 10-fold (36, 44, 54), although they comprise less than 1% of the total virus population (41). In light of these findings, a concern has arisen that the m8 vaccine seems to contain reduced amounts of EEV that lacks the B5R antigen and might not be protective against long-range spread of VAR EEV (5, 44, 45). Our study of multiple immunizations with recombinant B5R proteins adsorbed to adjuvant showed that antigenic activity was absent in the truncated B5R protein of m8 but present in the intact protein of LO. In addition, infection or vaccination with live VV induced very few anti-EEV NAbs, and repeated inoculations were required to induce moderate NAb levels (19, 44), probably because of the small EEV population. Alternatively, low levels of the antibodies may be due to the low sensitivity of conventional assay systems. Wyatt et al. recently reported that NAbs can be produced after a single percutaneous vaccination (56). They recently developed and used a highly sensitive system, a semiautomated flow cytometric assay with recombinant VV expressing enhanced green fluorescent protein (8).

It was therefore important to examine the levels of protection against virulent WR infection in m8-vaccinated mice, irrespective of the absence of EEV B5R-specific antibody responses. Our results confirmed that a single vaccination with m8, mO, and LO failed to induce detectable levels of anti-EEV and anti-B5R antibodies. Nevertheless, mice immunized with these vaccines were 100% protected against pathogenic WR challenge as early as 3 weeks after vaccination. Moreover, m8 with the whole B5R gene deleted protected mice from lethal WR challenge (32). These findings suggest that many viral antigens other than B5R are also involved in protective immunity to EEV. In this regard, antibodies to the A33R Env antigen did not neutralize EEV but provided mice with 100% protection (19). Anti-A33R might disrupt fragile EEV Env and convert to IMV, which is easily neutralized by anti-vaccinia antibodies (19, 28). Alternatively, A33R-specific cellular immunity may be crucial for protective immunity.

We have only limited knowledge about the protective immune mechanisms against smallpox. Experience with worldwide vaccination, however, has suggested that the protective mechanisms involve innate immunity, including interferons, natural killer cells, and complements, and also acquired immunity, including specific antibody- and T-cell-mediated immune responses (12). Indeed, recent papers have revealed the involvement of gamma interferon-expressing CD8 and CD4 T cells, vaccinia-specific cytotoxic T cells, and T-helper type 1 memory in humans (6, 7, 31, 48) and mice (16, 35, 49). Several studies conducted out of urgency in the last few years using smallpox vaccine candidates came to similar conclusions with regard to the contribution of overall immunity to smallpox protection (2, 9, 50, 56). Moreover, priming effects in vaccinated persons were recently shown to be long-lived or long-lasting, for as long as 75 years after vaccination (23). These historical and most recent studies imply that vaccine priming for immunological memory is important so that effecter components, such as NAbs, $CD4^+$ or $CD8^+$ T cells, and various cytokines can promptly be induced or boosted to protective levels by VAR infection, regardless of whether they are above measurable levels before infection. In support of this hypothesis, we found that mice that received a single dose of LO-related vaccines could not fully develop antibody responses as early as 3 weeks after vaccination but could produce enhanced levels of antibodies and complete immune protection after pathogenic-virus infection.

The need to produce safer and more effective vaccines may increase in the future. Here, we determined the nucleotide sequences of the whole genomes from the m8, intermediate mO, and original LO vaccine strains. The accumulating information on complete genome sequences of attenuated or pathogenic VVs and other OPVs will provide a basis for producing new genetically engineered vaccines. The double-stranded DNA genomes of OPVs are known to be highly stable. However, a single nucleotide insertion just upstream of the m8 B5R mutation site has recently been reported to restore the ORF to the parental mO phenotype after repeated (10 or more) virus passages. Although the repaired viruses were a marginal population, attenuation that is achieved by a deletion of the whole B5R gene prevented the reversion of m8- to mO-type viruses (32), which have, however, much lower virulence than LO and NYBH (24, 25, 39). In turn, the genetic manipulation of m8 to replace genes related to protective immunity, but not to pathogenicity, with the counterpart genes of VAR may make m8 more efficacious. It will be necessary to study in detail the correlation between individual gene functions and antigenicity of the gene products for inducing protective immunity in the future.

## ACKNOWLEDGMENTS

We thank S. Hashizume for smallpox vaccine strains of vaccinia virus, LC16m8, LC16mO, and Lister Original (Elstree); Y. Sato for technical assistance; and N. Fujita, A. Kikuchi, M. Kudo, Y. Kuroda, S. Mimaki, M. Ohsawa, N. Okada, R. Sasaki, and S. Shinohara for assistance in sequencing and data processing.

This work was supported in part by grants from the Ministry of Health, Labor, and Welfare.

## REFERENCES

1. **Appleyard, G., A. J. Hapel, and E. A. Boulter.** 1971. An antigenic difference between intracellular and extracellular rabbitpox virus. J. Gen. Virol. **13**:9–17.
2. **Belyakov, I. M., P. Earl, A. Dzutsev, V. A. Kuznetsov, M. Lemon, L. S. Wyatt, J. T. Snyder, J. D. Ahlers, G. Franchini, B. Moss, and J. A. Berzofsky.** 2003. Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. Proc. Natl. Acad. Sci. USA **100**:9458–9463.
3. **Birmingham, K., and G. Kenyon.** 2001. Smallpox vaccine development quickened. Nat. Med. **7**:1167.
4. **Chen, R. T., and J. M. Lane.** 2003. Myocarditis: the unexpected return of smallpox vaccine adverse events. Lancet **362**:1345–1346.
5. **Cohen, J.** 2002. Looking for vaccines that pack a wallop without the side effects. Science **298**:2314.
6. **Combadiere, B., A. Boissonnas, G. Carcelain, E. Lefranc, A. Samri, F. Bricaire, P. Debre, and B. Autran.** 2004. Distinct time effects of vaccination on long-term proliferative and IFN-producing T cell memory to smallpox in humans. J. Exp. Med. **199**:1585–1593.
7. **Drexler, I., C. Staib, W. Kastenmüller, S. Stevanovi, B. Schmidt, F. A. Lemonnier, H.-G. Rammensee, D. H. Busch, H. Bernhard, V. Erfle, and G. Sutter.** 2003. Identification of vaccinia virus epitope-specific HLA-A*0201-restricted T cells and comparative analysis of smallpox vaccines. Proc. Natl. Acad. Sci. USA **100**:217–222.
8. **Earl, P. L., J. L. Americo, and B. Moss.** 2003. Development and use of a vaccinia virus neutralization assay based on flow cytometric detection of green fluorescent protein. J. Virol. **77**:10684–10688.
9. **Earl, P. L., J. L. Americo, L. S. Wyatt, L. A. Eller, J. C. Whitbeck, G. H. Cohen, R. J. Eisenberg, C. J. Hartmann, D. L. Jackson, D. A. Kulesh, M. J. Martinez, D. M. Miller, E. M. Mucker, J. D. Shamblin, S. H. Zwiers, J. W. Huggins, P. B. Jahrling, and B. Moss.** 2004. Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. Nature **428**:182–185.
10. **Enserink, M.** 2002. How devastating would a smallpox attack really be? Science **296**:1592–1595.
11. **Enserink, M.** 2004. Smallpox vaccines: looking beyond the next generation. Science **304**:809.
12. **Esposito, J. J., and F. Fenner.** 2001. Poxviruses, p. 2885–2921. *In* D. M. Knipe and P. M. Howley (ed.), Fields virology, 4th ed., vol. 2. Lippincott Williams & Wilkins, Philadelphia, Pa.
13. **Ewing, B., L. Hillier, M. C. Wendl, and P. Green.** 1998. Base-calling of automated sequencer traces using phred. I. Accuracy assessment. Genome Res. **8**:175–185.
14. **Ewing, B., and P. Green.** 1998. Base-calling of automated sequencer traces using phred. II. Error probabilities. Genome Res. **8**:186–194.
15. **Ferguson, N. M., M. J. Keeling, W. J. Edmunds, R. Gani, B. T. Grenfell, R. M. Anderson, and S. Leach.** 2003. Planning for smallpox outbreaks. Nature **425**:681–685.
16. **Fogg, C., S. Lustig, J. C. Whitbeck, R. J. Eisenberg, G. H. Cohen, and B. Moss.** 2004. Protective immunity to vaccinia virus induced by vaccination with multiple recombinant outer membrane proteins of intracellular and extracellular virions. J. Virol. **78**:10230–10237.
17. **Fulginiti, V. A., A. Papier, J. M. Lane, J. M. Neff, and D. A. Henderson.** 2003. Smallpox vaccination: a review, part II. Adverse events. Clin. Infect. Dis. **37**:251–271.
18. **Funahashi, S., T. Sato, and H. Shida.** 1988. Cloning and characterization of the gene encoding the major protein of the A-type inclusion body of cowpox virus. J. Gen. Virol. **69**:35–47.
19. **Galmiche, M. C., J. Goenaga, R. Wittek, and L. Rindisbacher.** 1999. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. Virology **254**:71–80.
20. **Gani, R., and S. Leach.** 2001. Transmission potential of smallpox in contemporary populations. Nature **414**:748–751.
21. **Goebel, S. J., G. P. Johnson, M. E. Perkus, S. W. Davis, J. P. Winslow, and E. Paoletti.** 1990. The complete DNA sequence of vaccinia virus. Virology **179**:247–266.
22. **Gubser, C., S. Hue, P. Kellam, and G. L. Smith.** 2004. Poxvirus genomes: a phylogenetic analysis. J. Gen. Virol. **85**:105–117.
23. **Hammarlund, E., M. W. Lewis, S. G. Hansen, L. I. Strelow, J. A. Nelson, G. J. Sexton, J. M. Hanifin, and M. K. Slifka.** 2003. Duration of antiviral immunity after smallpox vaccination. Nat. Med. **9**:1131–1137.
24. **Hashizume, S., H. Yoshizawa, M. Morita, and K. Suzuki.** 1985. Properties of attenuated mutant of vaccinia virus, LC16m8, derived from Lister strain, p. 421–428. *In* G. V. Quinnan (ed.), Vaccinia viruses as vectors for vaccine antigens. Elsevier Science Publishing Co., Amsterdam, The Netherlands.
25. **Hashizume, S., M. Morita, H. Yoshizawa, S. Suzuki, M. Arita, T. Komatsu, H. Amano, and I. Tagaya.** 1973. Internal symposium on smallpox vaccines, Bilthoven. Symp. Ser. Immunobiol. Stand. **19**:325–331.
26. **Henderson, D. A.** 1999. The looming threat of bioterrorism. Science **283**:1279–1282.
27. **Henderson, D. A., and B. Moss.** 1999. Smallpox and vaccinia, p. 74–97. *In* S. A. Plotkin and W. A. Orenstein (ed.), Vaccines. Saunders, Philadelphia, Pa.
28. **Ichihashi, Y.** 1996. Extracellular enveloped vaccinia virus escapes neutralization. Virology **217**:478–485.
29. **Joklik, W. K.** 1962. The purification of four strains of poxvirus. Virology **18**:9–18.
30. **Kaplan, E. H., D. L. Craft, and L. M. Wein.** 2002. Emergency response to a smallpox attack: the case for mass vaccination. Proc. Natl. Acad. Sci. USA **99**:10935–10940.
31. **Kennedy, J. S., S. E. Frey, L. Yan, A. L. Rothman, J. Cruz, F. K. Newman, L. Orphin, R. B. Belshe, and F. A. Ennis.** 2004. Induction of human T cell mediated immune responses after primary and secondary smallpox vaccination. J. Infect. Dis. **190**:1286–1294.
32. **Kidokoro, M., M. Tashiro, and H. Shida.** 2005. Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8. Proc. Natl. Acad. Sci. USA **102**:4152–4157.
33. **Kitts, P. A., M. D. Ayres, and R. D. Possee.** 1990. Linearization of baculovirus DNA enhances the recovery of recombinant virus expression vectors. Nucleic Acids Res. **18**:5667–5672.
34. **Lane, J. M., F. L. Ruben, J. M. Neff, and J. D. Millar.** 1969. Complications of smallpox vaccination, 1968. National surveillance in the United States. N. Engl. J. Med. **281**:1201–1208.
35. **Legrand, F. A., P. H. Verardi, K. S. Chan, Y. Peng, L. A. Jones, and T. D. Yilma.** 2005. Vaccinia viruses with a serpin gene deletion and expressing IFN-γ induce potent immune responses without detectable replication in vivo. Proc. Natl. Acad. Sci. USA **102**:2940–2945.
36. **Martinez-Pomares, L., R. J. Stern, and R. W. Moyer.** 1993. The ps/hr gene (B5R open reading frame homolog) of rabbitpox virus controls pock color, is a component of extracellular enveloped virus, and is secreted into the medium. J. Virol. **67**:5450–5462.
37. **Meltzer, M. I., I. Damon, J. W. LeDuc, and J. D. Millar.** 2001. Modeling potential responses to smallpox as a bioterrorist weapon. Emerg. Infect. Dis. **7**:959–969.
38. **Morita, M., K. Suzuki, A. Yasuda, A. Kojima, M. Sugimoto, K. Watanabe, H. Kobayashi, K. Kajima, and S. Hashizume.** 1987. Recombinant vaccinia virus LC16m0 or LC16m8 that expresses hepatitis B surface antigen while preserving the attenuation of the parental virus strain. Vaccine **5**:65–70.
39. **Morita, M., Y. Aoyama, M. Arita, H. Amano, H. Yoshizawa, S. Hashizume, T. Komatsu, and I. Tagaya.** 1977. Comparative studies of several vaccinia virus strains by intrathalamic inoculation into cynomolgus monkeys. Arch. Virol. **53**:197–208.
40. **O'Toole, T., M. Mair, and T. V. Inglesby.** 2002. Shining light on "Dark Winter." Clin. Infect. Dis. **34**:972–983.
41. **Payne, L. G.** 1980. Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. J. Gen. Virol. **50**:89–100.
42. **Rice, C. M., C. A. Franke, J. H. Strauss, and D. E. Hruby.** 1985. Expression of Sindbis virus structural proteins via recombinant vaccinia virus: synthesis, processing, and incorporation into mature Sindbis virions. J. Virol. **56**:227–239.
43. **Rosenthal, S. R., M. Merchlinsky, C. Kleppinger, and K. L. Goldenthal.** 2001. Developing new smallpox vaccines. Emerg. Infect. Dis. **7**:920–926.
44. **Smith, G. L., A. Vanderplasschen, and M. Law.** 2002. The formation and function of extracellular enveloped vaccinia virus. J. Gen. Virol. **83**:2915–2931.
45. **Smith, G. L., and G. McFadden.** 2002. Smallpox: anything to declare? Nat. Rev. Immunol. **2**:521–527.
46. **Takahashi-Nishimaki, F., K. Suzuki, M. Morita, T. Maruyama, K. Miki, S. Hashizume, and M. Sugimoto.** 1987. Genetic analysis of vaccinia virus Lister strain and its attenuated mutant LC16m8: production of intermediate variants by homologous recombination. J. Gen. Virol. **68**:2705–2710.
47. **Takahashi-Nishimaki, F., S. Funahashi, K. Miki, S. Hashizume, and M. Sugimoto.** 1991. Regulation of plaque size and host range by a vaccinia virus gene related to complement system proteins. Virology **181**:158–164.
48. **Terajima, M., J. Cruz, G. Raines, E. D. Kilpatrick, J. S. Kennedy, A. L. Rothman, and F. A. Ennis.** 2003. Quantitation of CD8+ T cell responses to newly identified HLA-A*0201-restricted T cell epitopes conserved among vaccinia and variola (smallpox) viruses. J. Exp. Med. **197**:927–932.
49. **Tscharke, D. C., G. Karupiah, J. Zhou, T. Palmore, K. R. Irvine, S. M. M. Haeryfar, S. Williams, J. Sidney, A. Sette, J. R. Bennink, and J. W. Yewdell.** 2005. Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines. J. Exp. Med. **201**:95–104.
50. **Weltzin, R., J. Liu, K. V. Pugachev, G. A. Myers, B. Coughlin, P. S. Blum, R.**

**Nichols, C. Johnson, J. Cruz, J. S. Kennedy, F. A. Ennis, and T. P. Monath.** 2003. Clonal vaccinia virus grown in cell culture as a new smallpox vaccine. Nat. Med. **9**:1125–1130.

51. **Williamson, J. D., R. W. Reith, L. J. Jeffrey, J. R. Arrand, and M. Mackett.** 1990. Biological characterization of recombinant vaccinia viruses in mice infected by the respiratory route. J. Gen. Virol. **71**:2761–2767.
52. **Wittek, R., A. Menna, D. Schümperli, S. Stoffel, H. K. Müller, and R. Wyler.** 1977. *Hin*dIII and *Sst*I restriction sites mapped on rabbit poxvirus and vaccinia virus DNA. J. Virol. **23**:669–678.
53. **Wittek, R., H. K. Müller, A. Menna, and R. Wyler.** 1978. Length heterogeneity in the DNA of vaccinia virus is eliminated on cloning the virus. FEBS Lett. **90**:41–46.
54. **Wolffe, E. J., S. N. Isaacs, and B. Moss.** 1993. Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. J. Virol. **67**:4732–4741.
55. **World Health Organization.** 1980. The global eradication of smallpox. Final report of the Global Commission for the Certification of Smallpox Eradication. World Health Organization, Geneva, Switzerland.
56. **Wyatt, L. S., P. L. Earl, L. A. Eller, and B. Moss.** 2004. Highly attenuated smallpox vaccine protects mice with and without immune deficiencies against pathogenic vaccinia virus challenge. Proc. Natl. Acad. Sci. USA **101**:4590–4595.
57. **Yamaguchi, M., M. Kimura, and M. Hirayama.** 1975. Report of the National Smallpox Vaccination Research Committee: study of side effects, complications and their treatments. Clin. Virol. **3**:269–278. (In Japanese.)
58. **Yasuda, A., J. Kimura-Kuroda, M. Ogimoto, M. Miyamoto, T. Sata, C. Takamura, T. Kurata, A. Kojima, and K. Yasui.** 1990. Induction of protective immunity in animals vaccinated with recombinant vaccinia viruses that express PreM and E glycoprotein of Japanese encephalitis virus. J. Virol. **64**:2788–2795.

JOURNAL OF VIROLOGY, June 2006, p. 5179–5188 Vol. 80, No. 11
0022-538X/06/$08.00+0 doi:10.1128/JVI.02642-05


# LC16m8, a Highly Attenuated Vaccinia Virus Vaccine Lacking Expression of the Membrane Protein B5R, Protects Monkeys from Monkeypox

Masayuki Saijo,[1]* Yasushi Ami,[2] Yuriko Suzaki,[2] Noriyo Nagata,[3] Naoko Iwata,[3] Hideki Hasegawa,[3] Momoko Ogata,[1] Shuetsu Fukushi,[1] Tetsuya Mizutani,[1] Tetsutaro Sata,[3] Takeshi Kurata,[3] Ichiro Kurane,[1] and Shigeru Morikawa[1]

*Special Pathogens Laboratory, Department of Virology 1,[1] Laboratory of Animal Experimentation,[2] and Laboratory of Infectious Disease Pathology, Department of Pathology,[3] National Institute of Infectious Diseases, Tokyo, Japan*

Received 18 December 2005/Accepted 6 March 2006

**The potential threat of smallpox as a bioweapon has led to the production and stockpiling of smallpox vaccine in some countries. Human monkeypox, a rare but important viral zoonosis endemic to central and western Africa, has recently emerged in the United States. Thus, even though smallpox has been eradicated, a vaccinia virus vaccine that can induce protective immunity against smallpox and monkeypox is still invaluable. The ability of the highly attenuated vaccinia virus vaccine strain LC16m8, with a mutation in the important immunogenic membrane protein B5R, to induce protective immunity against monkeypox in nonhuman primates was evaluated in comparison with the parental Lister strain. Monkeys were immunized with LC16m8 or Lister and then infected intranasally or subcutaneously with monkeypox virus strain Liberia or Zr-599, respectively. Immunized monkeys showed no symptoms of monkeypox in the intranasal-inoculation model, while nonimmunized controls showed typical symptoms. In the subcutaneous-inoculation model, monkeys immunized with LC16m8 showed no symptoms of monkeypox except for a mild ulcer at the site of monkeypox virus inoculation, and those immunized with Lister showed no symptoms of monkeypox, while nonimmunized controls showed lethal and typical symptoms. These results indicate that LC16m8 prevents lethal monkeypox in monkeys, and they suggest that LC16m8 may induce protective immunity against smallpox.**

Three decades have passed since the global eradication of smallpox (variola). This eradication was made possible by the development of effective vaccinia virus vaccines (VVs), such as strains Lister and Dryvax. Unfortunately, we now face the potential threat of bioterrorism with variola virus, the causative agent of variola. This threat has led to the production and stockpiling of vaccinia virus-based vaccines in several countries. Human monkeypox (MPX), infection of humans with monkeypox virus (MPXV), is endemic to central and western Africa (18), and the first human MPX outbreaks outside Africa were reported in the United States in 2004 (6, 9, 30). Most human MPX patients in this outbreak acquired the virus from prairie dogs (*Cynomys* spp.) that became ill after contact with various exotic rodents shipped from Ghana, Africa (30). Therefore, VVs are still of great importance, although variola has already been eradicated.

LC16m8, a highly attenuated VV strain, was developed in the early 1970s by multiple passages in cell culture through a temperature-sensitive and low-virulence strain, LC16mO, from the original Lister strain (Elstree) (11, 36). LC16m8 forms smaller plaques than Lister in the chicken chorioallantoic membrane. LC16m8 is temperature sensitive, as demonstrated by the fact that LC16m8 does not grow well in primary rabbit kidney (PRK) cells cultured at 41°C, while Lister grows efficiently (36). The fact that LC16m8 grows efficiently in PRK cells but not in African green monkey kidney (Vero) cells, while the parental strain Lister grows well in both cell lines, suggests that LC16m8 has a narrow host cell range, growing in a cell-selective manner (36).

We recently determined the complete genome sequences of LC16m8, the parental LC16mO strain, and the original Lister strain (GenBank accession no. AY678275, AY678277, and AY678276, respectively) (24). It was revealed that there was a single nucleotide deletion of guanosine (G) at the 274th position from the initiation codon in the membrane protein gene *B5R* (GenBank accession no. M55434 and AY678275) that generated a premature termination codon and truncated the B5R membrane protein of LC16m8 extracellular enveloped virions (EEV) at amino acid position 93. LC16m8 may possess nearly all the open reading frames corresponding to the VV strains Copenhagen and Lister except for the membrane protein B5R. Because Lister had no history of virus cloning, nucleotide polymorphisms were observed at more than 1,000 sites in the whole genome, indicating that it is difficult to make a simple comparison between the nucleotide sequences of LC16m8 and Lister. However, alignments of the EEV-related membrane proteins in LC16m8 and Lister indicated that there were only 1, 1, 1, and 2 amino acid differences in the EEV-related membrane proteins A36R, F13L, A56R, and A33R, respectively, and that the EEV-related membrane protein A34R of LC16m8 was identical to that of Lister. Although the genetic background responsible for the temperature sensitivity has not been elucidated, it has been confirmed that mutation in the membrane protein gene *B5R* is responsible for small-plaque formation and cell-selective growth of this strain (35).

* Corresponding author. Mailing address: Department of Virology 1, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. Phone: 81-42-561-0771, ext. 320. Fax: 81-42-561-2039. E-mail: msaijo@nih.go.jp.

LC16m8 has very low neurovirulence in animal models (11). More than 100,000 people were vaccinated with LC16m8 in Japan, but no LC16m8-associated adverse events such as serious complications and/or death were reported. The currently available VVs, such as strains Lister and Dryvax, are known to be efficacious. However, severe adverse events, such as encephalitis, encephalopathy, progressive and generalized vaccinia, ocular vaccinia virus infections, and cardiac dysfunction, have been reported for recipients and are of great concern (1, 2, 4, 10, 16, 22, 23, 25, 31, 33, 38). These observations suggest that LC16m8 is safer than the currently used VVs derived from bovine skin (20, 21). Thus, LC16m8 is considered a potentially useful replacement for the currently available VVs.

Unfortunately, the protective efficacy of LC16m8 against variola has not been evaluated, because variola had already been eradicated at the time of its development. LC16m8 lacks expression of the full-length membrane protein B5R, one of the most immunogenic proteins, because of a frameshift mutation in the membrane protein gene *B5R* (19, 35). It is expected that LC16m8 does not pass through the EEV stage in the viral life cycle, because the membrane protein B5R is essential in packaging the intracellular mature virion with the *trans*-Golgi membrane or endosomal cisternae to form intracellular enveloped virions (13, 32, 34) and this protein is also involved in the release of cell-associated enveloped virions from the cell surface to form EEV in cooperation with proteins A36R and A33R (17, 29). Furthermore, the membrane protein B5R induces protective neutralizing antibodies to EEV in vaccinia (8, 14, 15, 34). Recently, it was reported that LC16m8 induced protective immunity to vaccinia virus challenge in mice and rabbits (19, 24). However, the protective efficacy of LC16m8 against variola and human MPX has not been confirmed in humans.

If LC16m8 is as efficacious as Lister and Dryvax, it will be of great benefit to humans, because it is expected to induce much less severe VV-associated adverse events. A nonhuman-primate model for MPXV infections is expected to mimic natural variola virus infection in humans. In the present study, the protective efficacy of LC16m8 against MPX was evaluated in comparison with that of Lister VV in cynomolgus monkeys (*Macaca fascicularis*). The present study was performed to examine the protective efficacy of LC16m8 against variola in humans.

## MATERIALS AND METHODS

**Virus, vaccinia virus vaccines, and cells.** MPXV strains Liberia and Zr-599, used in challenge experiments, and MPXV Congo-8, used in the neutralizing antibody assay, had been kept in the National Institute of Infectious Diseases. Strain Liberia was originally isolated from a patient with MPX in Liberia, and strain Zr-599 was from a patient with MPX in the Democratic Republic of Congo (formerly Zaire), suggesting that the former originated from West Africa and the latter from the Congo Basin. It is suggested that MPXV originating from West Africa is less virulent than MPXV originating from the Congo Basin (3). The virus was confirmed to be MPXV by determining the specific nucleotide sequence of the ATI gene of MPXV (28). The infectious dose of the virus was determined by plaque assays on Vero cells, which were purchased from the American Type Cell Collection (Manassas, VA). Vero cells were grown in Eagle's minimum essential medium supplemented with penicillin G and streptomycin and with 5% fetal bovine serum (MEM-5FBS). LC16m8 vaccine was produced by the Chiba Serum Research Institute, Chiba, Japan, and Lister vaccine was produced by the Kitasato Research Institute, Kanagawa, Japan. The titers of the two vaccines were higher than $1 \times 10^8$ PFU/ml.

**Nonhuman primates and vaccination.** One male and 14 female cynomolgus monkeys (*Macaca fascicularis*), aged 3 to 4 years and weighing 2.180 to 3,100 g, were used in the experiments (Table 1). These monkeys were born and raised in the Tsukuba Primate Center for Medical Research, National Institute of Infectious Diseases, Tsukuba, Japan. They were assigned to six groups as shown in Table 1: group IN-Naïve, consisting of naïve monkeys challenged intranasally with MPXV (monkeys 4595 and 4596), group IN-Lister, consisting of monkeys immunized with Lister and challenged intranasally with MPXV (monkeys 4597, 4598, and 4599), group IN-LC16m8 (monkeys 4600, 4601, and 4602), group SC-Naïve, consisting of naïve monkeys challenged subcutaneously with MPXV (monkeys 4651 and 4653), group SC-Lister (monkeys 4575 and 4576), and group SC-LC16m8 (monkeys 4577, 4525, and 4526). Monkeys were immunized with each of the vaccines by the multiple-puncture method with standard bifurcated needles in the same way as immunization is performed for humans. Briefly, a bifurcated needle holding a drop of vaccine was pressed more than 15 times into the skin at the vaccination site.

**Assays of IgG antibody.** Levels of vaccinia virus-specific antibody were measured by an enzyme-linked immunosorbent assay (ELISA) using the entire vaccinia virus proteins as antigens, as reported previously, except for the secondary antibody conjugated with horseradish peroxidase (24). The secondary antibody was a goat anti-human immunoglobulin G (IgG) antibody conjugated with horseradish peroxidase purchased from Zymed Laboratories (South San Francisco, CA).

**Neutralizing antibody assay.** Levels of neutralizing antibody to MPXV Congo-8 in the plasma samples were measured as reported previously with some modifications (12). Briefly, about 30 PFU of MPXV strain Congo-8 in 100 μl of MEM-2FBS was mixed with 100 μl of serially diluted heat-inactivated plasma samples and incubated at 4°C overnight. The mixtures were inoculated into Vero cell monolayers seeded in a 24-well culture plate and were incubated for 2 h for adsorption. The inocula were then removed, and the cells were cultured with MEM-2FBS supplemented with 0.5% methylcellulose. After a 3-day incubation, plaque numbers were counted. The neutralizing antibody titer was defined as the reciprocal of the dilution level at which the plaque number decreased to less than 50% of that in the control.

**Cytokine assays.** The concentrations of tumor necrosis factor alpha (TNF-α), gamma interferon (IFN-γ), interleukin-2 (IL-2), IL-4, IL-6, and IL-10 in sera were determined using rhesus monkey TNF-α, monkey IFN-γ, rhesus monkey IL-2, monkey IL-4, human IL-6, and rhesus monkey IL-10 (BioSource International Inc., Camarillo, CA), respectively, according to the manufacturer's instructions.

**Virus isolation from PBMCs or buffy coat fraction.** Virus was isolated using Vero cells from peripheral blood mononuclear cells (PBMCs) and buffy coat fractions obtained from monkeys inoculated intranasally and subcutaneously, respectively. PBMCs were isolated from peripheral blood by the Ficoll centrifugation method. Aliquots of $10^6$ PBMCs were cocultivated with Vero cells in MEM-2FBS for 2 weeks, when PBMCs were used. The whole buffy coat fraction collected by centrifugation of 4 ml of peripheral blood was washed twice with a phosphate-buffered saline solution and then cocultivated with Vero cells as described above. When a cytopathic effect was observed in cell culture, the cytopathic effect agent was confirmed to be MPXV by an indirect immunofluorescence assay with an anti-vaccinia virus antibody prepared in our laboratory and by amplification of the ATI gene and sequencing of the amplicon (28). Furthermore, the plaque number was also counted.

**Determination of MPXV loads in total peripheral blood by quantitative PCR.** DNAs were isolated from total peripheral blood using a Viral Nucleic Acid purification kit (Roche Diagnostics, Mannheim, Germany) according to the supplier's instructions. The primers and probes were designed based on the specific ATI gene on the MPXV genome. The sequences of primers and probes were as follows: forward primer, 5′-GAGATTAGCAGACTCCAA-3′; fluorescein probe, 5′-GCAGTCGTTCAACTGTATTTCAAGATCTGAGAT-3′-fluorescein; LCRed640 probe, 5′-LCRed640-CTAGATTGTAATCTCTGTAGCATTTCCACGGC-3′-phosphorylation; reverse primers, 5′-TCTCTTTTCCATATCAGC-3′ for amplification of the MPXV Liberia genome and 5′-GATTCAATTTCCAGTTTGTAC-3′ for amplification of the MPXV Zr-599 genome. The internal controls for determination of viral genome copy numbers of MPXV Liberia and MPXV Zr-599 were pGEM-T Easy vectors (Promega Cooperation, Madison, WI) carrying the ATI gene of MPXV strain Liberia or Zr-599, respectively, and were included in each quantitative real-time PCR (qPCR) assay. The reverse primer sequences were designed according the nucleotide sequences of the ATI genes of MPXV Liberia and Zr-599. Amplification conditions were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 57°C for 10 s, and 72°C for 6 s, and a melting reaction.

TABLE 1. Characteristics, MPX-associated symptoms, and viremia in mock-immunized monkeys and those immunized with Lister or LC16m8

| Group | ID[a] | Vaccination | Sex[b] (wt [g]) | MPXV challenge strain (route,[c] dose [PFU]) | Virus isolation[d] (no. of plaques at the indicated day of collection) | MPX-associated symptoms | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Symptom(s) at the site of MPXV inoculation | No. of papulovesicular lesions | Outcome |
| Expt 1 | | | | | | | | |
| IN-Naïve | 4595 | Mock | F (2,500) | Liberia (IN, $10^6$) | Positive (20 at day 4, 10 at day 6, 10 at day 8) | Rhinorrhea | 10 | Survival |
| | 4596 | | F (2,320) | | Positive (10 at day 6, 10 at day 13) | Rhinorrhea | 16 | Survival |
| IN-Lister | 4597 | Lister | F (2,360) | | Negative | None | 0 | Survival |
| | 4598 | | F (2,580) | | Negative | None | 0 | Survival |
| | 4599 | | F (2,700) | | Negative | None | 0 | Survival |
| IN-LC16m8 | 4600 | LC16m8 | F (2,650) | | Negative | None | 0 | Survival |
| | 4601 | | F (2,800) | | Negative | None | 0 | Survival |
| | 4602 | | F (2,700) | | Negative | None | 0 | Survival |
| Expt 2 | | | | | | | | |
| SC-Naïve | 4651 | Mock | F (2,560) | Zr-599 (SC, $10^6$) | Positive (4 at day 3, 5 at day 7, 33 at day 9, 30 at day 14) | Erythema, papulovesicles, ulcer | 390 | Death |
| | 4653 | | M (3,100) | | Positive (176 at day 3, 18 at day 7, 3 at day 9, 23 at day 11, 9 at day 14, 3 at day 18) | Erythema, papulovesicles, ulcer | 1,150 | Death |
| SC-Lister | 4575 | Lister | F (2,980) | | Negative | None | 0 | Survival |
| | 4576 | | F (3,100) | | Negative | None | 0 | Survival |
| SC-LC16m8 | 4577 | LC16m8 | F (2,640) | | Positive (1 at day 3) | Erythema | 0 | Survival |
| | 4525 | | F (2,180) | | Positive (7 at day 3, 1 at day 6) | Erythema, papulovesicles, ulcer | 0 | Survival |
| | 4526 | | F (2,730) | | Negative | Erythema, papulovesicles, ulcer | 0 | Survival |

[a] ID, monkey identification number.
[b] F, female; M, male.
[c] IN, intranasal inoculation; SC, subcutaneous inoculation.
[d] Positive or negative, MPXV was or was not isolated, respectively, during the observation period from challenge to sacrifice. In experiment 1, the intranasal inoculation model, virus isolation was attempted from aliquots of $10^6$ purified PBMCs collected from monkeys on days 0, 2, 4, 6, 8, 10, 13, 16, and 20 after virus challenge. In experiment 2, the subcutaneous inoculation model, virus isolation was attempted from the buffy coat fractions collected from 4-ml aliquots of total peripheral blood collected on days 0, 3, 7, 9, 11, 14, and 18.

**Challenge with MPXV.** All the challenge experiments with MPXV were conducted in a highly contained laboratory at the National Institute of Infectious Diseases, Tokyo, Japan. The monkeys that were mock immunized or immunized with vaccines (Lister and LC16m8) were anesthetized and either inoculated intranasally with 0.5 ml of a virus solution containing $1 \times 10^6$ PFU of MPXV strain Liberia by using an atomizer (Keytron Co., Tokyo, Japan) to atomize the virus solution or inoculated subcutaneously with 0.5 ml of a virus solution containing $1 \times 10^6$ PFU of MPXV strain Zr-599. Blood samples were collected every week after immunization up to the time of challenge. After the challenge, blood was drawn every 2 to 4 days. Clinical manifestations, such as volume of food and water consumed, the appearance of feces, etc., were observed every day. When monkeys were anesthetized for drawing of blood, the skin surface was observed carefully, and body temperature and weight were measured.

**Schedule for immunization and challenge.** In the present study, day zero was defined as the day on which monkeys were challenged with MPXV. All of the monkeys were challenged with MPXV at 5 weeks after immunization. Monkeys were challenged with MPXV (strain Liberia or Zr-599) on day zero and were observed for about 3 weeks.

**Histopathological examination.** After sacrifice under deep anesthesia using ketaral, skin, lymph nodes, brain, lungs, heart, liver, spleen, pancreas, kidneys, bladder, gastrointestinal organs, and genitourinary tract structures were excised, fixed in 10% formalin in phosphate-buffered saline, and embedded in paraffin. Macroscopic and histological examinations were performed on the excised tissues and organs. Paraffin sections, 4 μm thick, were stained with hematoxylin and eosin (H&E) and with Luxol-Fast Blue for the brain. Immunohistochemistry (IHC) for the MPXV antigens was performed using paraffin sections according to the method described previously (26, 27). For detection of MPXV antigens, a rabbit anti-vaccinia virus serum was used.

## RESULTS

**Skin lesions after immunization with LC16m8 or Lister.** Immunization with LC16m8 or Lister induced "vaccine take" (pustules, scabs, and scarring) as shown in Fig. 1. The lesions reached a maximum size at about 2 weeks after immunization. On day 13 postimmunization, the area was $27 \pm 11$ mm$^2$ with LC16m8, significantly smaller than the area of lesions induced by Lister ($115 \pm 65$ mm$^2$) (Fig. 1B). The lesions induced by Lister were more exudative and granulomatous than those induced by LC16m8. Satellite lesions appeared with Lister but not with LC16m8. Pigmentation of the scars was apparent with Lister, but not with LC16m8, on day 28.

**Protection of monkeys from intranasal MPXV challenge by immunization with LC16m8.** The challenge experiment included six groups: (i) the IN-Naïve group, comprising two monkeys vaccinated with mock vaccine (negative control) and challenged intranasally with MPXV strain Liberia; (ii) the IN-

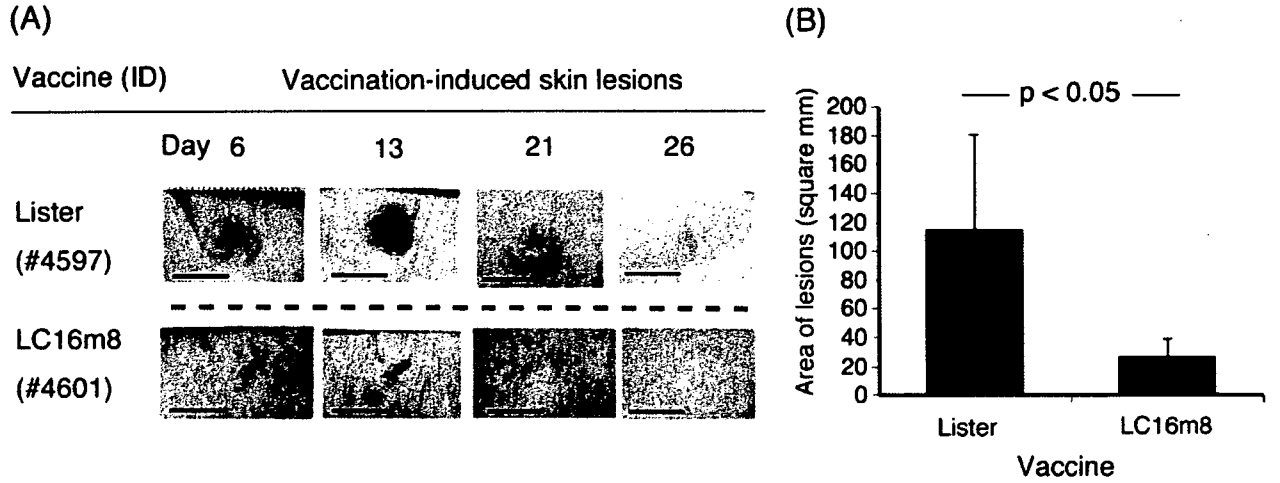



FIG. 1. Local cutaneous lesions at the site (upper left arm) of vaccination with Lister or LC16m8. (A) Typical vaccine-induced local lesions on the designated days postimmunization. Bars, 10 mm. (B) Sizes (areas) of the vaccine-induced lesions with Lister ($n$ = 5) or LC16m8 ($n$ = 6), measured on day 13 and shown as averages and standard deviations.

Lister group, comprising three monkeys vaccinated with Lister and challenged intranasally with MPXV strain Liberia; (iii) the IN-LC16m8 group, comprising three monkeys vaccinated with LC16m8 and challenged intranasally with MPXV strain Liberia; (iv) the SC-Naïve group, comprising two monkeys vaccinated with mock vaccine (negative control) and challenged subcutaneously with MPXV strain Zr-599; (v) the SC-Lister group, comprising two monkeys vaccinated with Lister and challenged subcutaneously with MPXV strain Zr-599; and (vi) the SC-LC16m8 group, comprising three monkeys immunized with LC16m8 and challenged subcutaneously with MPXV Zr-599.

The identification numbers of the monkeys and their sex, weight, and vaccination histories are shown along with summarized results in Table 1. The monkeys were challenged intranasally with MPXV 5 weeks after mock immunization or immunization with VVs (Table 1, experiment 1). Body weight decreased sharply after challenge, by approximately 10%, in group IN-Naïve but not in group IN-Lister or IN-LC16m8 (Fig. 2). In the IN-Naïve group, symptoms including loss of appetite, rhinorrhea and conjunctival discharge, diarrhea, skin rash (papulovesicular and ulcerative lesions, as shown in Fig. 3A), irritability, and decreased activity appeared around day 10 after challenge and continued for approximately 5 days. All the MPX-associated symptoms disappeared by day 20 after challenge except for the skin lesions. All animals in the IN-Lister and IN-LC16m8 groups survived and showed no symptoms associated with MPXV infection.

Histopathological examinations of the nasal cavity were carried out at the inoculation site to compare the efficacies of the vaccines in conferring protection against MPXV challenge. In IN-Naïve monkeys, the structures of the mucous membranes were damaged due to necrosis, inflammatory cell accumulation was seen, and MPXV antigens were detected in the lesions. In contrast, the nasal structures of the mucous membranes were maintained, and no MPXV antigens were detected, in the IN-Lister and IN-LC16m8 groups. Inflammation in the mucous membranes was detected in the IN-LC16m8 group but not in the IN-Lister group (Fig. 4).

**Protection of monkeys against lethal subcutaneous MPXV challenge by immunization with LC16m8.** The efficacy of LC16m8 in the lethal MPXV infection model was then evaluated (Table 1, experiment 2). Subcutaneous infection with MPXV Zr-599 was fatal to nonimmunized monkeys in the SC-Naïve group (Table 1). Body weight was decreased by approximately 15% after challenge in the SC-Naïve group. However, the monkeys in the SC-Lister and SC-LC16m8 groups maintained their body weight (Fig. 2). Papulovesicular skin lesions appeared on day 7 after challenge in the SC-Naïve group; monkeys 4651 and 4653 showed 390 and 1,150 lesions, respectively. The symptoms of both of the monkeys in the SC-Naïve group were so severe that they were euthanized for ethical reasons. On the other hand, the monkeys in the SC-LC16m8 group did not develop any MPX-associated symptoms, except for local cutaneous lesions at the site of MPXV inoculation (Fig. 4). The lesions consisted of erythema, vesicles, and ulceration and were much milder than those for the SC-Naïve group. The SC-Lister group showed no MPX-associated symptoms, not even cutaneous lesions at the site of MPXV inoculation (Fig. 4).

**Histopathological examination.** All the monkeys were sacrificed for virological and histopathological examination 3 weeks after MPXV challenge. After intranasal inoculation, nodular and granulomatous lesions were detected in the lungs in the IN-Naïve group, while no lesions were detected in the lungs of any of the monkeys in the IN-Lister and IN-LC16m8 groups (Fig. 3A). IHC examination with an anti-vaccinia virus antibody revealed the presence of MPXV in the nodular lesions (Fig. 3A). Similar nodular lesions caused by MPXV were also detected in the pancreas of monkey 4595 in the IN-Naïve group (Fig. 3A). Macroscopic and histological examination revealed that the thymus, tonsil, and lymph node structures were affected by MPXV in the IN-Naïve monkeys. In contrast, no MPX-associated lesions were detected in any of the monkeys in the IN-Lister and IN-LC16m8 groups by histopathological examination.

After subcutaneous inoculation with MPXV, MPX-associ-

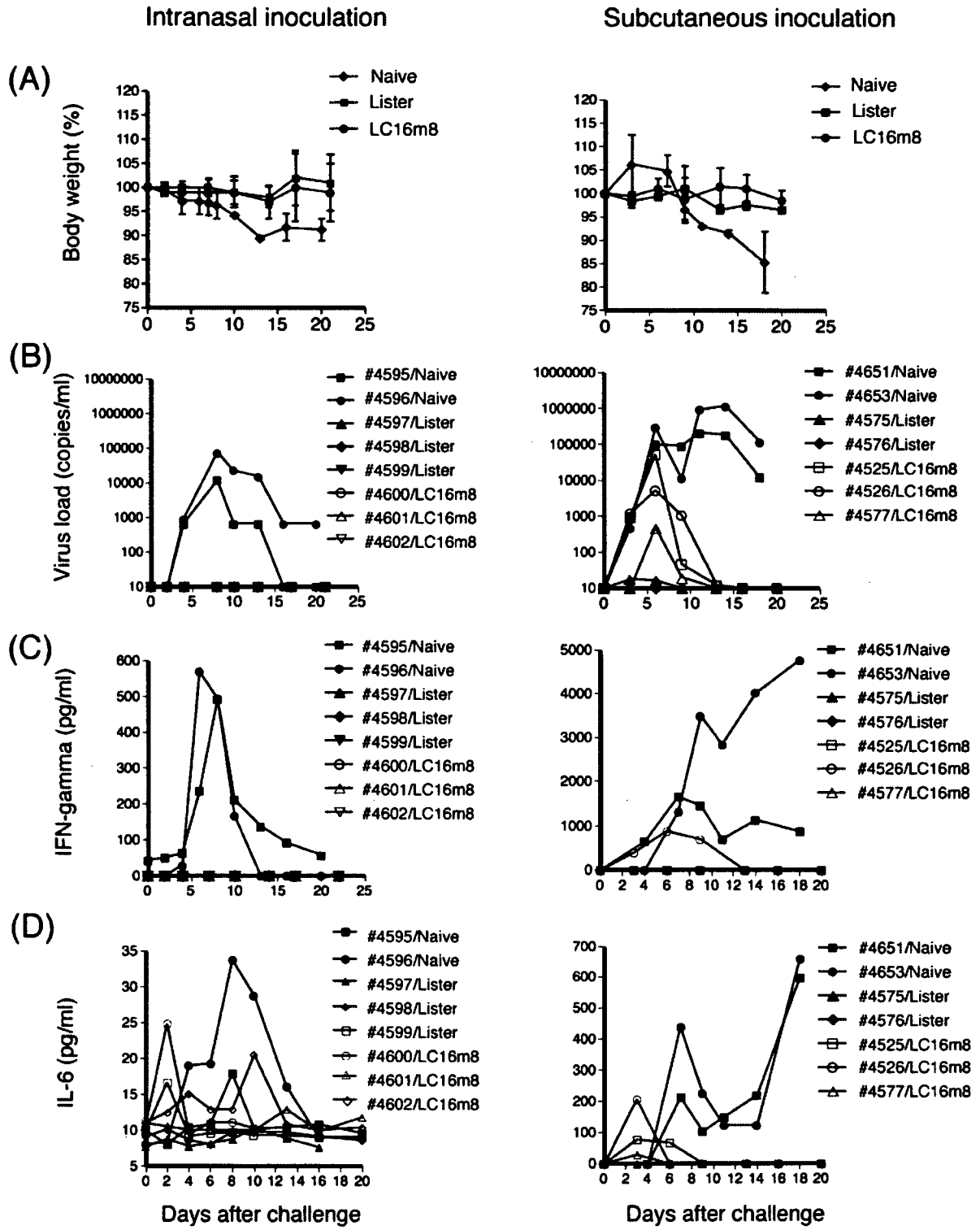



FIG. 2. Changes in body weight, MPXV loads in total peripheral blood, and cytokine responses. (A) Body weight expressed as a percentage of that measured at the time of MPXV challenge. (B) Viral loads in total peripheral blood as measured by qPCR. (C) IFN-γ response. (D) IL-6 response. Left and right panels show these indicators for monkeys challenged intranasally with MPXV strain Liberia and subcutaneously with MPXV Zr-599, respectively.

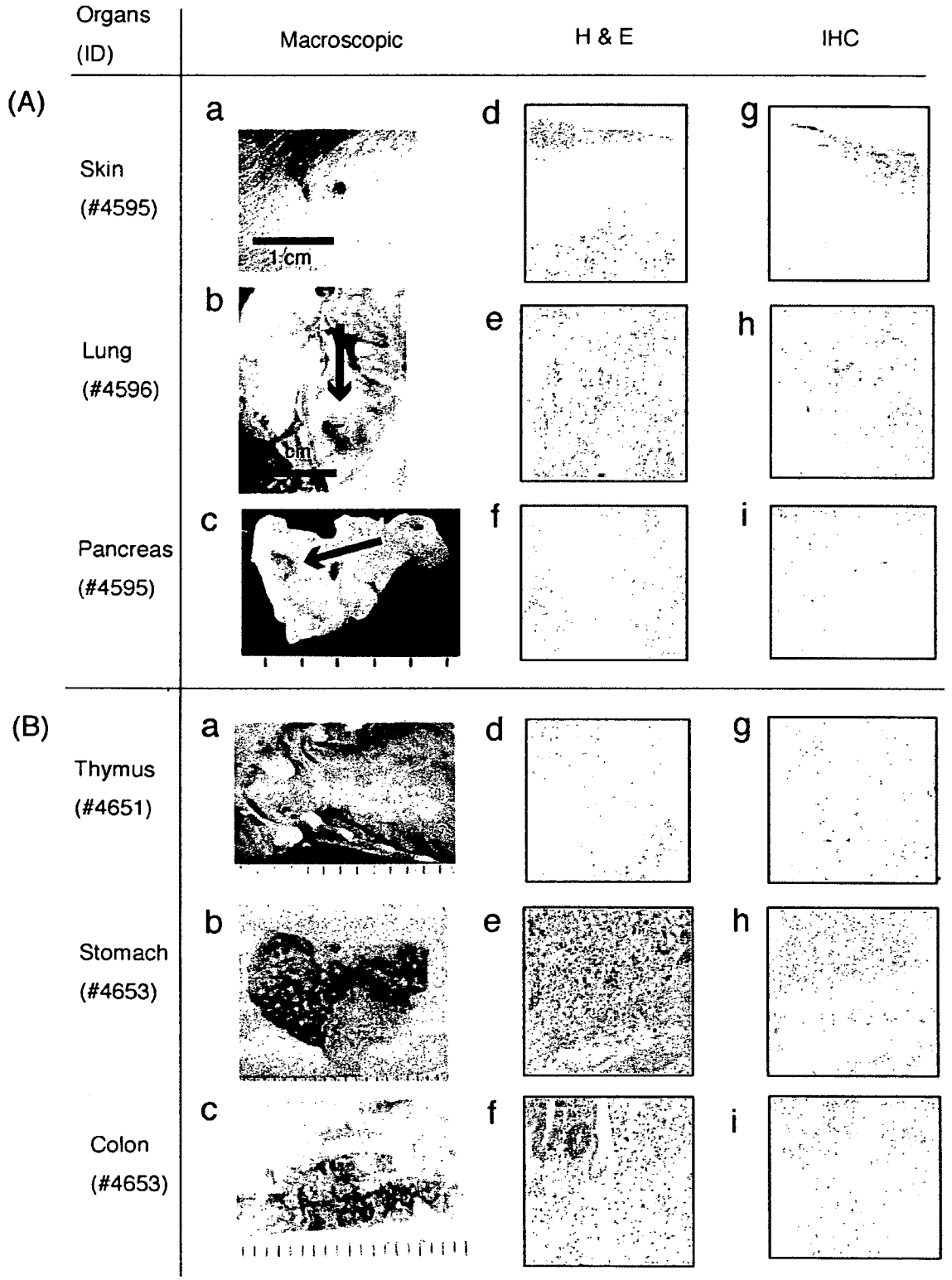



FIG. 3. Macroscopic and histological lesions observed in naïve monkeys infected with MPXV. (A) Macroscopic and histological lesions in the skin, lungs, and pancreas in the IN-Naïve group. Papulovesicular lesions were observed in the skin (a), and nodular and granulomatous lesions were present in the lungs (b) and pancreas (c). (d) The edges of the cutaneous lesions were characterized by epithelial cell swelling, epidermal hyperplasia, hyperkeratosis, necrosis, and infiltration of inflammatory cells. (e) Nodular and granulomatous lesions in the lungs were characterized by destruction of alveolar structures, necrosis, edema, proliferating fibroblasts, and infiltration of inflammatory cells. (f) Nodular and granulomatous lesions in the pancreas were characterized by extensive necrosis with infiltration of inflammatory cells and proliferating fibroblasts. (g to

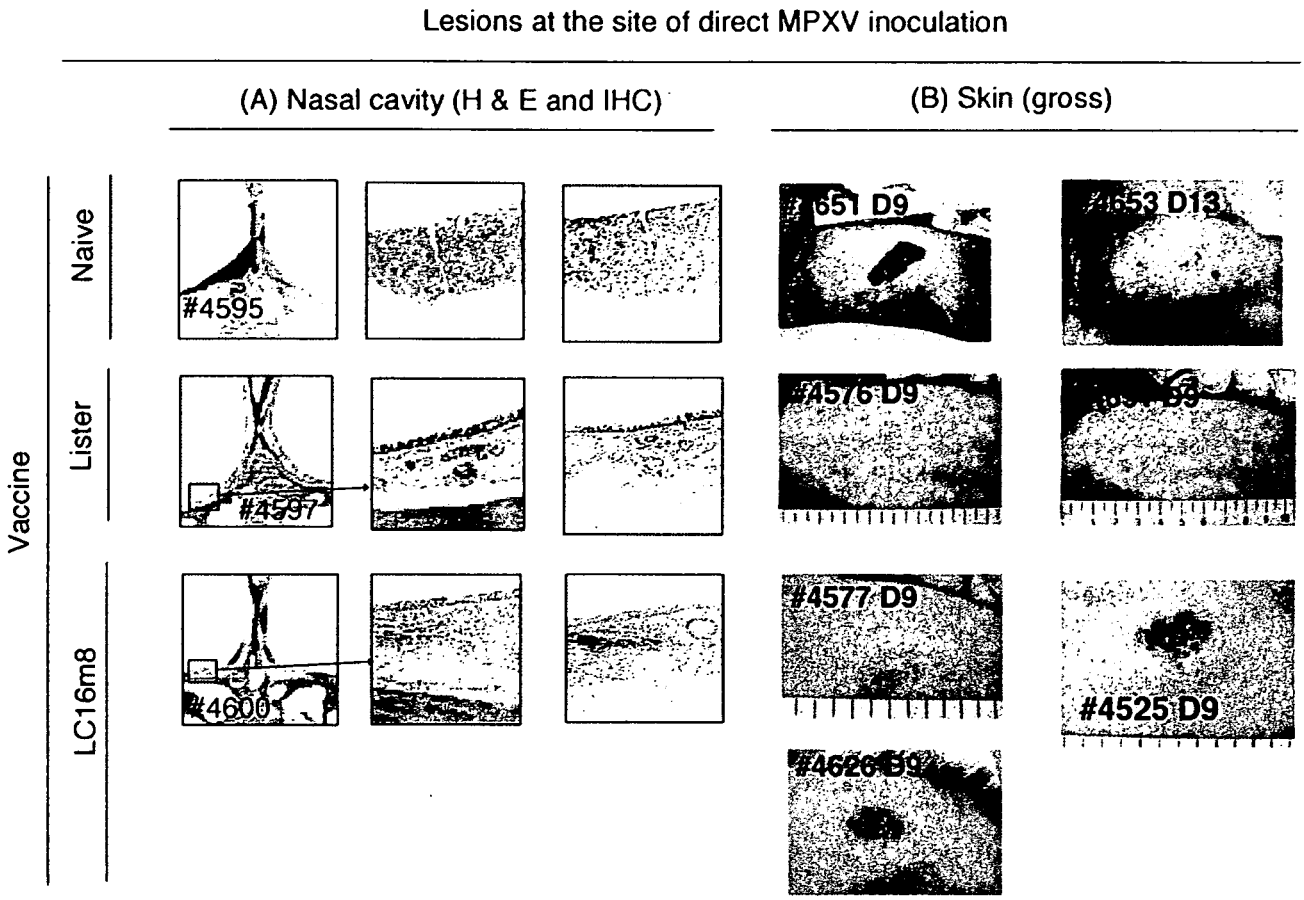



FIG. 4. Histology of the nasal cavity caused by intranasal challenge with MPXV strain Liberia (A) and macroscopic lesions at the site of subcutaneous inoculation with MPXV strain Zr-599 (B). The identification numbers of monkeys are given. Analyses by H&E staining (A, low and high magnifications) revealed that the lesions of naïve monkey 4595 were characterized by destruction of mucous membrane structures, disappearance of mucosal epithelial cells resulting in ulcer formation, necrosis, and hyperplasia. MPXV antigens were present in the lesions. In contrast, the mucous membranes of the nasal cavity into which MPXV was inoculated in Lister-immunized monkey 4597 were normal, and MPXV antigens were not detected. Although the mucous membranes of the LC16m8-immunized monkey 4600 showed infiltration of inflammatory cells, the structure was maintained without necrosis. Furthermore, MPXV antigens were not detected. (B) Erythematous, vesicular, and ulcerative lesions appeared in the SC-Naïve group. The maximum diameter of the lesions exceeded 10 cm on day 14 postchallenge. In the SC-LC16m8 group, similar but milder lesions were observed, while no obvious lesions were detected at the site of inoculation in the SC-Lister group.

ated lesions were detected in the lymphoid systems (lymph nodes, thymus, and tonsils), respiratory tract structures (lung and trachea), digestive organs (stomach, small intestine, colon, rectum, and liver), urogenital tract (bladder, uterus, and ovary), or skin in the SC-Naïve group (Fig. 3B and 4B). On the other hand, no MPX-associated lesions were detected in any internal organs of any of the monkeys in the SC-Lister and SC-LC16m8 groups, except for skin lesions at the site of MPXV inoculation in the SC-LC16m8 group (Fig. 4B).

**Laboratory findings and cytokine responses.** C-reactive protein (CRP) levels were measured as an indicator of inflammation. CRP levels were significantly increased in groups IN-Naïve and SC-Naïve but not in any of the animals immunized with either vaccine (data not shown). Furthermore, lymphocytopenia and thrombocytopenia were also detected in mock-immunized naïve monkeys but were not apparent in any of the animals immunized with either vaccine (data not shown). The levels of IFN-γ, TNF-α, IL-2, IL-4, IL-6, and IL-10 in plasma

i) In these lesions, MPXV antigens were demonstrated by IHC analyses, indicating that they were caused by MPXV infection. (B) Macroscopic and histological lesions in the thymus, stomach, and colon in the SC-Naïve group. (a to c) Multiple nodular lesions were present in the thymus, and papilliform and granular lesions with hemorrhaging were seen in the lumens of the stomach and colon. (d) The lesions in the thymus were characterized by granulomatous inflammation and proliferation of fibrous tissue consisting of fibroblastic cells, histiocytes, and microvascular structures. (e) The histology in the stomach consisted of necrotic changes with inflammatory cells including neutrophils. (f) The submucosal area of the colon consisted of fibroblastic tissues with granulomatous inflammation characterized by infiltration of inflammatory cells. The mucosal membranes showed ulceration. (g to i) In these lesions, MPXV antigens were demonstrated by IHC analyses, indicating that they were caused by MPXV infection.

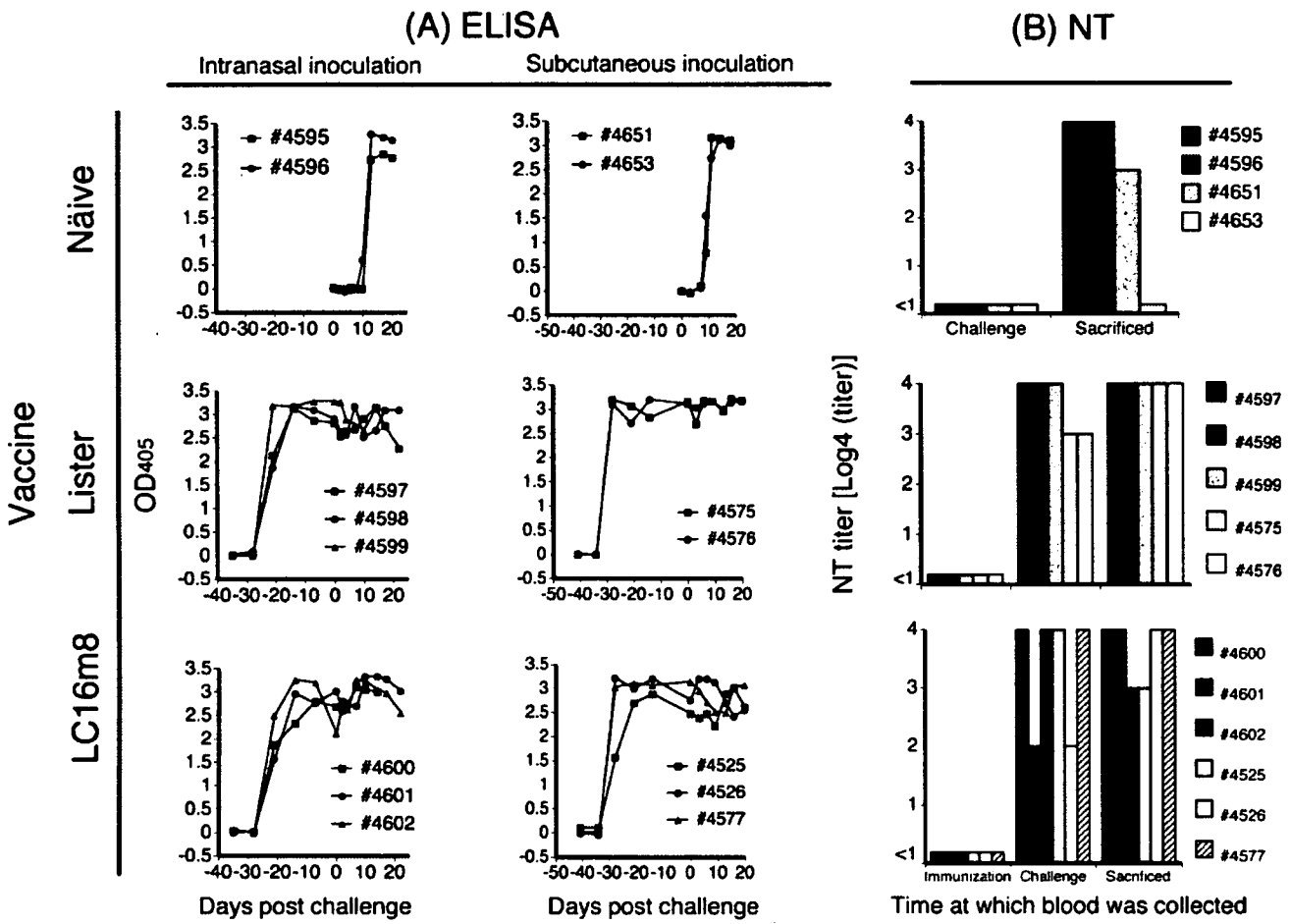



FIG. 5. Vaccinia virus-specific IgG responses determined by IgG ELISA (A) and a neutralizing assay (B) for MPXV. The optical densities at 405 nm ($OD_{405}$) at serum sample dilutions of 1:100 are shown. (A) Development of specific IgG antibody responses to vaccinia virus antigens in plasma samples collected on different days after vaccination and/or challenge, as measured by IgG ELISA. The day of MPXV challenge was taken as day zero. (B) MPXV-specific neutralizing antibody titers (NT) in plasma samples were determined by plaque reduction assay on different days after vaccination and/or challenge. The neutralizing antibody titers were determined for plasma collected from mice in the SC-Naïve group at the times of MPXV challenge and sacrifice and for plasma collected from mice in the SC-Lister and SC-LC16m8 groups at the times of immunization with Lister or LC16m8, challenge with MPXV, and sacrifice. The day of MPXV challenge was defined as day zero.

were examined. IFN-γ and IL-6 levels increased after MPXV challenge in groups IN-Naïve and SC-Naïve (Fig. 2). The levels of IFN-γ and IL-6 were higher in group SC-Naïve than in group IN-Naïve. The monkeys immunized with Lister or LC16m8 showed very low level or no detectable cytokine responses (Fig. 2).

**Viremia determined by virus isolation and qPCR.** Virus isolation results are summarized in Table 1. MPXV was isolated from purified PBMCs from group IN-Naïve between days 4 and 13 after challenge but not from PBMCs collected from any of the monkeys in groups IN-Lister and IN-LC16m8. MPXV was also isolated from the buffy coat fractions obtained from 4 ml of peripheral blood collected between days 3 and 14 from monkey 4651 and between days 3 and 18 from monkey 4653 in the SC-Naïve group. MPXV was isolated from the buffy coat fractions of two of the three SC-LC16m8 monkeys, but the plaque number was small and the isolation period was short. MPXV was not isolated from any of the monkeys in the SC-Lister group.

The levels of viremia were assessed by qPCR with LightCycler using in-house primer sets and fluorescent probes. In the intranasal-inoculation model, viremia was demonstrated for group IN-Naïve but not for group IN-Lister or IN-LC16m8 (Fig. 2). In the subcutaneous-inoculation model, viremia was demonstrated for all monkeys in groups SC-Naïve and SC-LC16m8 and for one of the two monkeys in group SC-Lister. The levels and durations of viremia were highest and longest in group SC-Naïve, followed by group SC-LC16m8. These results were consistent with those of virus isolation experiments (Table 1 and Fig. 2).

**IgG and neutralizing antibody responses.** VV antigen-specific IgG became detectable by IgG-ELISA in monkeys immunized with Lister or LC16m8 within 2 weeks postimmunization (Fig. 5). The time courses and levels of IgG response deter-

mined by ELISA were similar for monkeys immunized with LC16m8 and those immunized with Lister (Fig. 5). IgG reactive to VV antigens became detectable by IgG-ELISA in the IN-Naïve and SC-Naïve groups within 2 weeks after MPXV challenge. The levels of neutralizing antibody to MPXV were tested before and after challenge with MPXV. At the time of challenge with MPXV, neutralizing antibody was detected in the monkeys immunized with LC16m8 or Lister. The titers were not increased after the challenge. Neutralizing antibody was demonstrated in both of the animals in the IN-Naïve group and in one of the two animals in the SC-Naïve group.

## DISCUSSION

The protective efficacy of LC16m8 was evaluated in a mild-MPX nonhuman-primate model, in which monkeys were intranasally inoculated with MPXV strain Liberia, and in a lethal-MPX nonhuman-primate model, in which monkeys were subcutaneously inoculated with MPXV strain Zr-599. Monkeys subcutaneously inoculated with MPXV strain Liberia developed relatively milder symptoms of MPX than those subcutaneously inoculated with MPXV strain Zr-599. Monkeys intranasally inoculated with MPXV strain Liberia also developed relatively milder symptoms than those intranasally inoculated with MPXV strain Zr-599. These data suggest that MPXV strain Liberia, one of the West African strains, is less virulent than MPXV Zr-599, one of the Congo Basin strains (unpublished data).

A single vaccination with LC16m8 protected monkeys from MPX, as did a single vaccination with Lister. The results of the present study indicate that LC16m8 confers sufficient protection against MPX in monkeys, even in the lethal-MPX nonhuman-primate model. LC16m8 completely protected nonhuman primates from MPX in the intranasal-inoculation model. The protective efficacy of LC16m8 was confirmed not only with regard to clinical symptoms but also by virological assays, such as determination of the lymphocyte and thrombocyte counts, CRP level, and interleukin levels, viremia level determination by qPCR, virus isolation, and histopathological examinations. Differences between LC16m8 and Lister were observed only with respect to the viremia level and the cutaneous lesions at the site of virus inoculation after subcutaneous MPXV inoculation.

LC16m8 was reported to cause few adverse events when tested in a preliminary trial in which about 30,000 children were immunized in Japan in the 1970s (11). No serious complications were reported. Fever was observed in fewer cases than for Lister- or CV1-78-immunized individuals (11). The results of the present study strongly suggest that LC16m8 is as efficacious as Lister in protecting humans from smallpox or MPX and support the suggestion that LC16m8 may be useful as a replacement for currently available VVs, such as Lister and Dryvax. Especially, the risk of VV-related casualties must be minimized and avoided if VVs are used today, when there are no variola outbreaks. LC16m8 can also be used for the treatment of people in regions in which human MPX is endemic.

It has been reported that some of the characteristics of LC16m8, such as small-pock formation in chicken chorioallantoic membrane and a narrow host range for replication, are due to a mutation in the membrane protein B5R (35). A single-nucleotide deletion in the *B5R* gene results in a deficiency in expression of full-length and intact B5R membrane protein (24, 35). There was a single-base deletion of G at the 274th position from the initiation codon, resulting in expression of a truncated B5R membrane protein. Although some poxvirus researchers have shown interest in the protective efficacy of LC16m8 (5), LC16m8 was shown to induce protective immunity to MPX in nonhuman primates in the present study. It was reported that the membrane protein B5R is not essential for protection against vaccinia virus infection in mice (19). Lister showed a stronger preventive effect against the local reactions at the site of MPXV inoculation in monkeys and resulted in lower levels of MPXV viremia than LC16m8. These observations suggest a role for the membrane protein B5R in induction of immunity to MPXV in nonhuman primates; however, the presence of the membrane protein B5R is not essential. Although the results of the present study cannot exclude the importance of the membrane protein B5R, LC16m8 induces sufficient protective immunity to MPX and probably induces protective immunity against variola.

Humans are usually infected with MPXV through the skin surface by bites from infected animals (9), while infection with variola occurs through the respiratory tract (7). In the present study, the monkeys were infected with MPXV by either the intranasal or the subcutaneous route in order to design an appropriate nonhuman-primate model not only for MPX but also for variola. The symptoms associated with MPX in naïve monkeys challenged intranasally with MPXV were somewhat milder than those reported in previous studies (15, 37, 39). This may have been due to the differences in MPXV strains, infection routes, or virus doses used for challenge. Subcutaneous infection of naïve monkeys with MPXV was fatal, but a single vaccination with LC16m8 prevented fatal infection. It must be emphasized that LC16m8 sufficiently protects monkeys even from lethal MPX.

In summary, a single vaccination with LC16m8 induced protective immunity against MPX, as did immunization with Lister, in nonhuman primates. These results strongly suggest that LC16m8 is also effective in the induction of high levels of protective immunity against variola.

### ACKNOWLEDGMENTS

All animal procedures were approved by the Committees on Biosafety and Animal Handling and Ethical Regulations of the National Institute of Infectious Diseases, Japan. We thank A. Harashima, Department of Pathology, National Institute of Infectious Diseases, for technical assistance.

This study was supported financially by a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan.

### REFERENCES

1. **Cassimatis, D. C., J. E. Atwood, R. M. Engler, P. E. Linz, J. D. Grabenstein, and M. N. Vernalis.** 2004. Smallpox vaccination and myopericarditis: a clinical review. J. Am. Coll. Cardiol. **43**:1503–1510.
2. **CDC.** 2004. Update: adverse events following civilian smallpox vaccination—United States, 2003. Morb. Mortal. Wkly. Rep. **53**:106–107.
3. **Chen, N., G. Li, M. K. Liszewski, J. P. Atkinson, P. B. Jahrling, Z. Feng, J. Schriewer, C. Buck, C. Wang, E. J. Lefkowitz, J. J. Esposito, T. Harms, I. K. Damon, R. L. Roper, C. Upton, and R. M. Buller.** 2005. Virulence differences between monkeypox virus isolates from West Africa and the Congo basin. Virology **340**:46–63.
4. **Chen, R. T., and J. M. Lane.** 2003. Myocarditis: the unexpected return of smallpox vaccine adverse events. Lancet **362**:1345–1346.