Ksiazek TG, Rollin PE, Williams AJ, Bressler DS, Martin ML, Swanepoel R, Burt FJ, Leman PA, Khan AS, Rowe AK, Mukunu R, Sanchez A, CJ P. 1999. Clinical virology of Ebola hemorrhagic fever (EHF): Virus, virus antigen, and IgG and IgM antibody findings among EHF patients in Kikwit, Democratic Republic of the Congo, 1995. J Infect Dis 179:S177–S187.

Martini GA, Knauff HG, Schmidt HA, Mayer G, Baltzer G. 1968. [On the hitherto unknown, in monkeys originating infectious disease: Marburg virus disease]. Dtsch Med Wochenschr 93:559–571.

Niikura M, Ikegami T, Saijo M, Kurane I, Miranda ME, Morikawa S. 2001. Detection of Ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. J Clin Microbiol 39:3267–3271.

Peters CJ, LeDuc JW. 1999. An introduction to Ebola: The virus and the disease. J Infect Dis 179:ix–xvi.

Saijo M, Niikura M, Morikawa S, Ksiazek TG, Meyer RF, Peters CJ, Kurane I. 2001. Enzyme-linked immunosorbent assays for detection of antibodies to Ebola and Marburg viruses using recombinant nucleoproteins. J Clin Microbiol 39:1–7.

Sanchez A, Ksiazek TG, Rollin PE, Miranda ME, Trappier SG, Khan AS, Peters CJ, Nichol ST. 1999. Detection and molecular characterization of Ebola viruses causing disease in human and nonhuman primates. J Infect Dis 179:S164–S169.

Smith DH, Johnson BK, Isaacson M, Swanapoel R, Johnson KM, Killey M, Bagshawe A, Siongok T, Keruga WK. 1982. Marburg-virus disease in Kenya. Lancet 1:816–820.

Clinical and Diagnostic Laboratory Immunology, Apr. 2005, p. 520–524
1071-412X/05/$08.00+0 doi:10.1128/CDLI.12.4.520–524.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.
Vol. 12, No. 4

Persisting Humoral Antiviral Immunity within the Japanese Population after the Discontinuation in 1976 of Routine Smallpox Vaccinations

Shuji Hatakeyama,[1*] Kyoji Moriya,[2] Masayuki Saijo,[3] Yuji Morisawa,[2] Ichiro Kurane,[3] Kazuhiko Koike,[1,2] Satoshi Kimura,[4] and Shigeru Morikawa[3]

Department of Infectious Diseases[1] and Department of Infection Control and Prevention,[2] Graduate School of Medicine, University of Tokyo, Bunkyo-ku, Special Pathogens Laboratory, Department of Virology I, National Institute of Infectious Diseases, Musashimurayama,[3] and AIDS Clinical Center, International Medical Center of Japan, Shinjyuku-ku,[4] Tokyo, Japan

Received 23 November 2004/Returned for modification 13 January 2005/Accepted 17 February 2005

Concerns have arisen recently about the possible use of smallpox for a bioterrorism attack. Routine smallpox vaccination was discontinued in Japan in 1976; however, it is uncertain exactly how long vaccination-induced immunity lasts. We sought to evaluate the seroprevalence and intensity of anti-smallpox immunity among representatives of the present Japanese population. The subjects included 876 individuals who were born between 1937 and 1982. Vaccinia virus-specific immunoglobulin G (IgG) levels were measured by enzyme-linked immunosorbent assay (ELISA), and 152 of 876 samples were also tested for the presence of neutralizing antibodies. Of the subjects who were born before 1962, between 1962 and 1968, and between 1969 and 1975, 98.6, 98.6, and 66.0%, respectively, still retained the vaccinia virus-specific IgG with ELISA values for optical density at 405 nm (OD_{405}) of ≥0.10. The corresponding figures for retained IgGs with OD_{405} values of ≥0.30 were 91.0, 90.3, and 58.2%, respectively. Neutralizing antibodies were also maintained. The sera with OD_{405} values of ≥0.30 showed 89% sensitivity and a 93% positive predictive value for detection of neutralizing antibodies (≥4). Thus, approximately 80% of persons born before 1969 and 50% of those born between 1969 and 1975 were also found to have maintained neutralizing antibodies against smallpox. A considerable proportion of the previous vaccinated individuals still retain significant levels of antiviral immunity. This long-lasting immunity may provide some protective benefits in the case of reemergence of smallpox, and the disease may not spread as widely and fatally as generally expected.

Smallpox was officially declared eradicated by the World Health Organization in 1980 after a worldwide mass vaccination campaign (22). Routine smallpox vaccination was discontinued in Japan in 1976, prior to the declaration. However, concerns have arisen recently about the possible use of variola virus, the causative agent of smallpox, as a bioweapon (14). A total of 37 million Japanese, accounting for approximately 30% of the total population, who were born after the discontinuation of the routine vaccination program are considered to be completely susceptible to smallpox (1), but the immune status of those who were vaccinated decades ago is uncertain. It had been believed that the full protective immunity conferred by smallpox vaccination lasts only 3 to 5 years and that even partial immunity fades substantially after 10 to 20 years (5, 14, 21). Recently, however, it has been suggested that the immunity may last much longer. Several epidemiological studies have shown that immunity to smallpox may still be present many years after the vaccination (8, 13, 15). The degree of residual protection in vaccinated cases was estimated (8) by analyzing data on the outbreak that occurred in Liverpool, in the United Kingdom, during 1902 to 1903 (13) and on smallpox epidemics that occurred following reintroduction to Europe between 1950 and 1971 (15). The authors concluded that protection against fatal smallpox disease was lost at the rate of 0.363% per year and, thus, that 77.6% of vaccinees were still protected even 70 years after vaccination (8). Furthermore, El-Ad et al. reported that the levels of virus-specific neutralizing antibody remain stable for at least 30 years after revaccination (9), and T-cell immunity in response to smallpox vaccination was also reported to remain constant for decades (7, 11).

It has recently been shown that B-cell and T-cell-deficient mice immunized with modified vaccinia virus Ankara, an attenuated vaccinia virus, are both protected against challenge with a pathogenic vaccinia virus, although depletion of a single component of the immune response can reduce the extent of protection (17, 24). In contrast, double-knockout mice deficient in major histocompatibility complex class I and II were not protected (24). These findings indicate that both humoral and cellular immunities make significant contributions to protection against smallpox. With respect to humoral immunities, neutralizing antibodies are believed to play a crucial role in the protection against smallpox (14, 16, 19). Several studies have shown that certain levels of neutralizing antibodies might be involved in preventing the disease or attenuating disease severity (4, 16, 19), although the actual neutralizing antibody titers considered sufficient to protect against smallpox remain to be determined. These data endorse the idea that adequate serum antibody levels might be one of the benchmarks of protective immunity.

* Corresponding author. Mailing address: Department of Infectious Diseases, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. Phone: 81 3 3815 5411 (ext. 33029 or 35335). Fax: 81 3 5800 8806. E-mail: shatake-tky@umin.ac.jp.

If people who were vaccinated some decades ago still maintain some immunity against smallpox, the morbidity, mortality, and transmission rates associated with the disease might be reduced significantly compared with present expectations (3, 18, 20). This might also affect future vaccination policy. Therefore, it is important to clarify whether individuals who were vaccinated decades ago maintain any immunity to smallpox, and if so, what fraction of the population possesses the immunity and how strong the immunity is. We have used enzyme-linked immunosorbent assays (ELISA) and neutralization assays to study the actual prevalence of virus-specific antibodies among representatives of the present Japanese population.

MATERIALS AND METHODS

Study population. We used stored anonymous serum samples that had been obtained from healthcare workers at the University of Tokyo Hospital in 2002 for serological screening of measles, rubella, mumps, and varicella-zoster virus. The sera were stored at 4°C until use. The present study was conducted with the approval of the Institutional Review Board of the University of Tokyo. It was impossible to identify the actual vaccination histories of each individual, because we used anonymous specimens. The ages of the 876 participants (257 males and 619 females) ranged from 20 to 65 years as of 2002 (mean ± standard deviation, 34.4 ± 10.3 years). Routine smallpox vaccination was discontinued in Japan in 1976, and the Immunization Law at that time recommended that individuals should receive three vaccinations against smallpox: the first vaccination was conducted in infancy, and subsequent vaccinations were given at the ages of 6 and 12 years. In this study, therefore, participants were divided into four birth cohorts according to the expected number of smallpox vaccinations they had received: those born after 1975 (younger than 26 years, as of 2002; the never-vaccinated group); those born between 1969 and 1975 (aged 27 to 33 years, as of 2002; the probable once-vaccinated group); those born between 1962 and 1968 (aged 34 to 40 years, as of 2002; the probable twice-vaccinated group); and those born before 1962 (older than 41 years, as of 2002; the probable thrice-vaccinated group). Before 1970, the most widely used strain of smallpox vaccine in Japan was the Ikeda strain, whereas the Lister strain was used during the 1970s.

Detection of vaccinia virus-specific IgG antibodies by ELISA. Levels of specific antibodies against the vaccinia virus, which was used for smallpox vaccines, were measured by ELISA. HeLa cells were infected with the vaccinia virus, Lister strain, at a multiplicity of infection of 1 and cultured for 48 h. They were then lysed in 1 ml of phosphate-buffered saline (PBS) containing 1% NP-40. The lysates were clarified by centrifugation at 10,000 ×g for 5 min, and the supernatant fraction was used as a positive vaccinia virus antigen. The mock-infected HeLa cells were also treated in the same way as those being used to prepare the vaccinia virus antigen to produce a negative-control antigen. Half of the wells of a flat-bottomed 96-well ELISA plate (Iwaki, Asahi Techno Glass, Chiba, Japan) were coated with the vaccinia virus-positive antigen, and the other half were coated with the negative-control antigen, followed by incubation at 4°C overnight. Both of the antigens were diluted 1:1,000 with PBS before coating, a dilution level that was determined by preliminary evaluations with box titration using the positive-control serum sample. After being washed three times with PBS containing 0.05% Tween 20 (T-PBS), the wells were blocked with 200 μl of PBS containing 5% skimmed milk and 0.05% Tween 20 (M-T-PBS) for 1 h and then washed three times with T-PBS. Samples of sera (100 μl per well), which were diluted 1:400 with M-T-PBS, were added, and the plates were incubated at 37°C for 1 h. The plates were washed three times with T-PBS and then incubated with 100 μl of M-T-PBS containing horseradish-peroxidase-conjugated goat anti-human IgG antibodies (ZYMED Laboratories, San Francisco, Calif.) (1:1,000 dilution) at 37°C for 1 h. After an additional washing step, 100 μl of the substrate reagent, ABTS [2,2'-azunobis (3-ethylbenzthiazoline sulfonic acid)] solution (Roche Diagnostics, Mannheim, Germany), was added to each well. The plates were incubated at room temperature for 30 min, and the optical density (OD) was measured at a wavelength of 405 nm (OD_{405}) with reference to that at 490 nm. The adjusted OD values (OD_{405}) were calculated by subtracting the OD values of the negative-control-antigen-coated wells from those of the corresponding wells. The negative- and positive-control sera were included for verification in each run.

Vaccinia virus neutralization assay. We conducted the neutralization assay on 152 serum samples, which were randomly selected from each of the birth cohorts (the pre-1962 cohort [n = 49], the 1962-to-1968 cohort [n = 22], the 1969-to-1975 cohort [n = 47], and the post-1975 cohort [n = 34]). Vaccinia virus, Lister strain, and the RK13 cell line grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), penicillin, and streptomycin were used. After heat inactivation at 56°C for 30 min, 100 μl of each of the sera serially diluted fourfold (beginning at 1:4) with DMEM containing 2% FCS and an equal volume of virus suspension containing 100 PFU per 100 μl were mixed and then incubated at 37°C for 2 h. RK13 cell monolayers in 24-well tissue culture plates were inoculated with 100 μl of the mixture in duplicate, and the cells were then incubated at 37°C for 1 h with frequent shaking. Thereafter, the inoculum was removed and the cells were washed once with PBS. They were then cultured in 1 ml of the overlay medium (DMEM containing 2% FCS, penicillin, streptomycin, and 0.5% methylcellulose) at 37°C in a humidified atmosphere of 5% CO_2 in air for 3 days. The cells were fixed with 10% formalin in PBS and stained with 0.1% crystal violet, and the number of plaques was counted. In each assay, the negative- and positive-control sera were included for verification. The neutralizing antibody titer (NT_{50}) was defined as a reciprocal of the highest dilution level of the serum demonstrating a >50% reduction in plaque count compared with the negative control results.

Statistical analysis. The sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated by standard methods (10). The differences in OD_{405} values and the proportion comparison between the birth cohorts were evaluated by an unpaired Student's t test and chi-square test, respectively, using Stat Flex version 5.0 software (Artech, Osaka, Japan). The level of statistical significance was set at $P < 0.05$. Receiver operating characteristics (ROC) and two-graph ROC (TG-ROC) curves were analyzed using the Stat Flex software. Vaccinia virus-specific NT_{50} values were analyzed by log transformation to linearize the relationship between variables. The relationship between antibody titers determined by neutralization assay and OD_{405} values determined by ELISA was evaluated by linear regression analysis.

RESULTS

Detection of vaccinia virus-specific antibodies by ELISA. We determined the cutoff values in the ELISA for the detection of specific antibodies against vaccinia virus by ROC and TG-ROC curves. The OD_{405} values of sera among subjects who were born in 1977 or after were treated as negative examples, because the routine smallpox vaccination program had already been discontinued. On the other hand, the serum samples of subjects who were born in or before 1968 were treated as positive examples with the vaccination history, because the vaccination program was being strictly enforced at that time, and so the majority of these subjects were considered to have been vaccinated. Logistic regression analysis ROC and TG-ROC curves under the aforementioned conditions are shown in Fig. 1. The cutoff value was set at 0.10, at which level the ELISA system exhibited optimal sensitivity (98.3%) and specificity (99.1%) for detecting the vaccinia virus-specific IgGs elicited by past smallpox vaccination. The area under the ROC curve was 0.988, which corresponds to "excellent probability" (12), allowing us to use this test to distinguish between vaccinated and unvaccinated individuals.

The OD_{405} values that were determined by subjecting serum samples (diluted 1:400) to ELISA are plotted for each birth cohort in Fig. 2A. Of the subjects born before 1962, those born between 1962 and 1968, and those born between 1969 and 1975, 98.6, 98.6, and 66.0%, respectively, retained vaccinia virus-specific IgGs (Table 1). There were significant differences (P < 0.0001) in the seropositivity rate between the 1969-to-1975 cohort and the older birth cohorts, but the difference between the 1962-to-1968 cohort and the pre-1962 cohort was not statistically significant (P = 0.984). The geometric mean for the OD_{405} values was 1.46 in the pre-1962 cohort, 1.36 in the 1962-to-1968 cohort, and 0.88 in the 1969-to-1975 cohort. Although there was no significant difference in these means

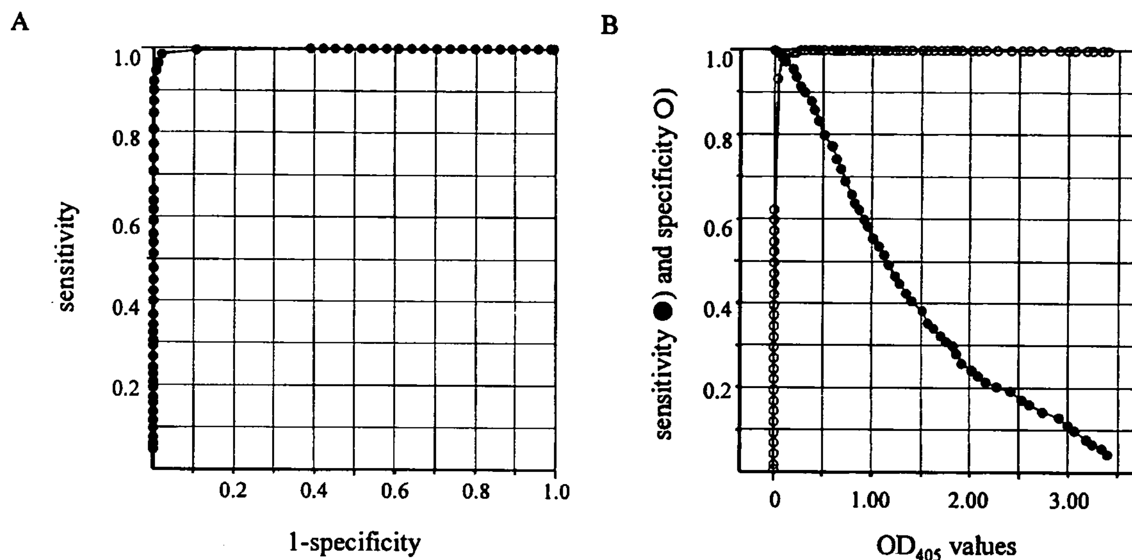

FIG. 1. Receiver operating characteristics (ROC) curve (A) and two-graph-ROC curve (B) for the detection of vaccinia virus-specific IgG. These graphs show the relationship between the sensitivity and specificity of the vaccinia virus-specific ELISA system for each cutoff OD_{405} value. The most optimal sensitivity (98.3%) and specificity (99.1%) are obtained when the cutoff OD_{405} value is set at 0.10. The area under the ROC curve is 0.988, which indicates the test has a good probability of distinguishing between vaccinated and unvaccinated individuals.

between the pre-1962 and 1962-to-1968 cohorts ($P = 0.371$), the mean OD_{405} value for the 1969-to-1975 cohort was significantly ($P < 0.0001$) lower than that for the pre-1969 cohorts (i.e., the pre-1962 and 1962-to-1968 cohorts). The year-on-year seropositivity rate for vaccinia virus-specific IgG among the 1969-to-1975 cohort was 90.0% for 1969 ($n = 29$), 93.3% for 1970 ($n = 30$), 76.2% for 1971 ($n = 42$), 88.6% for 1972 ($n = 44$), 79.3% for 1973 ($n = 58$), 45.8% for 1974 ($n = 48$), and 6.5% for 1975 ($n = 46$). The smallpox vaccination rate in Japan was reported to have already declined sharply in the final few years before the cessation of routine vaccination (information from the Ministry of Health, Labor and Welfare of Japan), which corresponds to the lower seropositivity rate in the 1974 and 1975 birth cohorts of this study. If we took into account the OD_{405} values of the seropositive subjects alone among the 1969-to-1975 cohort (i.e., excluding the seronegative subjects;

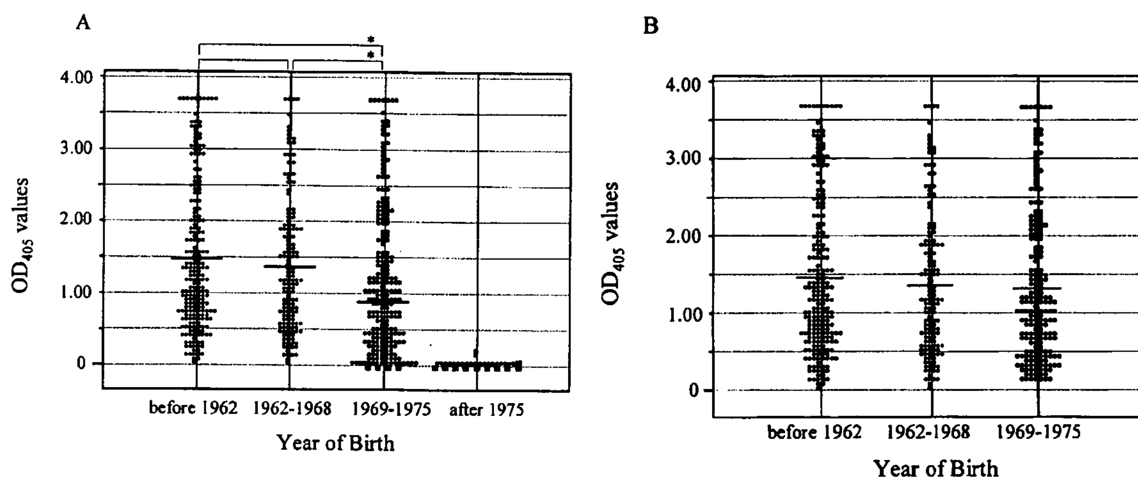

FIG. 2. Vaccinia virus-specific IgG determined by ELISA (the OD_{405} values). Panel A shows the ELISA OD_{405} values in each birth cohort. The bar indicates the geometric mean value, and the actual mean values are 1.46, 1.36, 0.88, and 0.02 for the pre-1962, 1962-to-1968, 1969-to-1975, and post-1975 birth cohorts, respectively. An asterisk indicates that the difference between the mean OD_{405} values is statistically significant. The pre-1962 and 1962-to-1968 cohorts exhibit significantly higher (unpaired Student's t test, $P < 0.0001$) mean OD_{405} values than the other two cohorts, but the difference between those of the pre-1962 and the 1962-to-1968 cohorts is not significant ($P = 0.371$). Each circle corresponds to one subject, and each square represents 20 subjects. Panel B shows the OD_{405} values in the pre-1962, 1962-to-1968, and 1969-to-1975 cohorts. Note, however, that only seropositive samples (OD_{405} values ≥ 0.10) are included for the 1969-to-1975 cohort. The mean values, between which there is no significant difference, are 1.46, 1.36, and 1.39, respectively. IgG, immunoglobulin G; ELISA, enzyme-linked immunosorbent assay; OD_{405}, adjusted optical density.

TABLE 1. Seroprevalence of vaccinia-virus-specific IgG, as determined by ELISA, in four different birth cohorts that cover the period from before 1962 to after the cessation of routine smallpox vaccination in Japan in 1976

Birth yr	No. of subjects	No. (%) of seropositive subjects	
		$OD_{405} \geq 0.10$	$OD_{405} \geq 0.30$
Before 1962	212	209 (98.6)	193 (91.0)
1962–1968	144	142 (98.6)	130 (90.3)
1969–1975	297	196 (66.0)	173 (58.2)
After 1975	223	2 (0.9)	0 (0)
Total	876	548	495

TABLE 2. Sensitivity, specificity, PPV, and NPV of ELISA with the designated reference value for neutralization assay for vaccinia virus within a subsample of 152 sera

ELISA OD_{405}	No. of NT_{50} results		Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
	≥4	<4				
≥0.10	96	17	97.0	67.9	85.0	92.3
<0.10	3	36				
≥0.30	88	7	88.9	86.8	92.6	80.7
<0.30	11	46				

OD_{405} values < 0.10), who were unlikely to have been vaccinated, the mean OD_{405} value was 1.32, which was not significantly different from those of the pre-1969 birth cohorts (Fig. 2B).

Relationship between the ELISA and the neutralization assay. Neutralizing antibodies were also maintained, and a significant linear correlation was observed between the neutralizing antibody titers (NT_{50}) and the OD_{405} values determined by the ELISA ($R^2 = 0.450$; $P < 0.0001$) (Fig. 3). The sensitivity, specificity, PPV, and NPV of the ELISA testing for the presence of neutralizing antibodies ($NT_{50} \geq 4$) were 88.9, 86.8, 92.6, and 80.7%, respectively, when the reference OD_{405} value in the ELISA was set at 0.30. The corresponding figures for a reference OD_{405} value of 0.10 were 97.0, 67.9, 85.0, and 92.3%, respectively (Table 2). Neutralizing antibodies were negative ($NT_{50} < 4$) in all of the 34 samples from the post-1975 birth cohort. On the basis of these analyses, the ELISA OD_{405} values of ≥0.10 provide good sensitivity and specificity (98.6 and 99.1%, respectively) for the prediction of smallpox vaccination history, and OD_{405} values of ≥0.30 provide a high PPV (92.6%) for detecting the retention of neutralizing antibodies. The OD_{405} values of ≥0.30 were demonstrated in 91.0% of subjects in the pre-1962 cohort, 90.3% of those in the 1962-to-1968 cohort, and 58.2% of those in the 1969-to-1975 cohort (Table 1). The OD_{405} values were below 0.30 in all samples from the post-1975 birth cohort. These results indicate that approximately 80% of subjects in the pre-1969 birth cohorts, and approximately 50% of those in the 1969-to-1975 cohort, also retained neutralizing antibodies against smallpox.

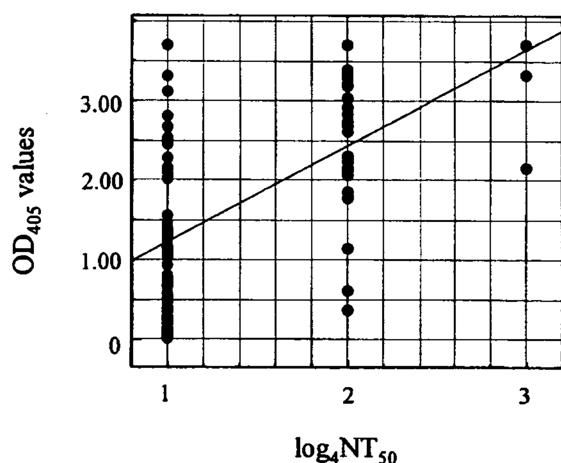

FIG. 3. Relationship between neutralizing antibody titers to vaccinia virus and ELISA OD_{405} values. The regression analysis yields a significant linear relationship ($R^2 = 0.450$; $P < 0.0001$).

DISCUSSION

Neutralizing antibodies have been thought to constitute an important correlate of protective immunity against smallpox (4, 14, 16, 19). To perform a test on a large number of samples, we developed a vaccinia virus-specific ELISA technique with high processing ability, because neutralizing assays are time and labor intensive and are not adequate for handling a large number of samples. Therefore, we evaluated the correlation between antibodies detected by the neutralization assay and those detected by ELISA, because the antibodies detected by ELISA do not always reflect the presence of neutralizing antibodies.

It has been shown that the majority of the Japanese population who were vaccinated against smallpox prior to the cessation of the routine vaccination program in 1976 still maintain certain levels of ELISA-detectable virus-specific antibodies. More than 98% of subjects in the pre-1969 birth cohorts and 66% of those in the 1969-to-1975 cohort still maintained detectable levels of IgG antibodies (OD_{405} values ≥ 0.10). One of the reasons why the subjects in the 1969-to-1975 cohort have a lower seropositivity rate than those in the older cohort is that the vaccination rate per se would have been lower, because they were given only one opportunity to receive a smallpox vaccination. In fact, the smallpox vaccination rate at each stage was reported to be around the 80% level, except in 1974 and 1975, when the rate declined markedly (information from the Ministry of Health, Labor, and Welfare of Japan). The mean OD_{405} value calculated only from the seropositive subjects among the 1969-to-1975 cohort was 1.32, and this value was not significantly different to that of the pre-1962 cohort or the 1962-to-1968 cohort. Although we do not have precise information on how many vaccinations each individual actually received, this result suggests that the additional vaccinations had little influence on the period or degree of IgG retention, as long as the first vaccination had "taken" successfully. Therefore, further investigations are required to determine whether or not multiple smallpox vaccinations are necessary for acquiring significant protection, although it is generally believed that additional vaccinations are likely to confer a stronger and

longer-lasting immune response (2, 9, 23). Hammarlund et al. reported that the mean antibody titer induced by double vaccinations was very slightly but significantly higher than that induced by a single vaccination but that additional (between 3 and 14) vaccinations did not result in any further increase in long-term antibody production (11).

In the present study, most of the individuals whose serum samples exhibited OD_{405} values of ≥0.30 also retained the neutralizing antibodies. Thus, it was demonstrated that a considerable proportion of the previously vaccinated individuals still retained neutralizing antibodies. Although lower ELISA OD_{405} values tend to associate with lower neutralizing antibody titers and higher OD_{405} values tend to associate with higher neutralizing antibody titers, the correlation between them was only moderately positive ($R^2 = 0.450$; $P < 0.0001$). It may be due to a number of epitopes on the viral proteins other than neutralizing epitopes on vaccinia virus.

This study has revealed that many individuals who were vaccinated 27 to 53 years ago retain a significant degree of antiviral humoral immunity. Although this remaining immunity may no longer provide full protection, it is highly likely to afford at least partial protection. Hammarlund et al. showed that virus-specific T-cell immunity could persist for a long time after smallpox vaccination, perhaps as long as 75 years, declining only slowly, with a half-life of 8 to 15 years (11). Furthermore, it was shown that virus-specific memory B-cells were maintained for more than 50 years after vaccination and correlated positively with circulating antibody levels (6). In addition, some epidemiological analyses have also indicated that the immunity achieved after smallpox vaccination may remain for several decades (1, 8, 13, 15). Taking these data together, it appears that the immunity conferred by smallpox vaccination persists for longer than had previously been expected.

In the present study, we found that more than 98% of the Japanese population in the pre-1969 birth cohorts and 66% of those in the 1969-to-1975 cohort still maintain the vaccinia virus-specific IgG, whereas approximately 80 and 50%, respectively, also retain detectable levels of neutralizing antibodies. These long-term persisting immunities may provide some protective benefits in the case of intentional smallpox reemergence. In addition, the present results may also contribute in making policy of vaccination priority, especially if vaccine supplies become limited in the event of a widespread outbreak.

ACKNOWLEDGMENTS

We thank M. Ogata, Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan, for her technical assistance.

This research was supported in part by a grant-in-aid from the Ministry of Health, Labor, and Welfare of Japan. There is no conflict of interest in connection with this paper.

REFERENCES

1. **Arita, I.** 2002. Duration of immunity after smallpox vaccination: a study on vaccination policy against smallpox bioterrorism in Japan. Jpn. J. Infect. Dis. **55:**112–116.
2. **Bartlett, J., L. Borio, L. Radonovich, J. S. Mair, T. O'Toole, M. Mair, N. Halsey, R. Grow, and T. V. Inglesby.** 2003. Smallpox vaccination in 2003: key information for clinicians. Clin. Infect. Dis. **36:**883–902.
3. **Bozzette, S. A., R. Boer, V. Bhatnagar, J. L. Brower, E. B. Keeler, S. C. Morton, and M. A. Stoto.** 2003. A model for a smallpox-vaccination policy. N. Engl. J. Med. **348:**416–425.
4. **Cherry, J. D., J. D. Connor, K. McIntosh, A. S. Benenson, D. W. Alling, U. T. Rolfe, J. E. Schanberger, and M. J. Mattheis.** 1977. Clinical and serologic study of four smallpox vaccines comparing variations of dose and route of administration. Standard percutaneous revaccination of children who receive primary subcutaneous vaccination. J. Infect. Dis. **135:**176–182.
5. **Cohen, J.** 2001. Bioterrorism. Smallpox vaccinations: how much protection remains? Science **294:**985.
6. **Crotty, S., P. Felgner, H. Davies, J. Glidewell, L. Villarreal, and R. Ahmed.** 2003. Cutting edge: long-term B cell memory in humans after smallpox vaccination. J. Immunol. **171:**4969–4973.
7. **Demkowicz, W. E., Jr., R. A. Littaua, J. Wang, and F. A. Ennis.** 1996. Human cytotoxic T-cell memory: long-lived responses to vaccinia virus. J. Virol. **70:**2627–2631.
8. **Eichner, M.** 2003. Analysis of historical data suggests long-lasting protective effects of smallpox vaccination. Am. J. Epidemiol. **158:**717–723.
9. **el-Ad, B., Y. Roth, A. Winder, Z. Tochner, T. Lublin-Tennenbaum, E. Katz, and T. Schwartz.** 1990. The persistence of neutralizing antibodies after revaccination against smallpox. J. Infect. Dis. **161:**446–448.
10. **Griner, P. F., R. J. Mayewski, A. I. Mushlin, and P. Greenland.** 1981. Selection and interpretation of diagnostic tests and procedures. Principles and applications. Ann. Intern. Med. **94:**557–592.
11. **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.
12. **Hanley, J. A., and B. J. McNeil.** 1982. The meaning and use of the area under a receiver operating characteristic (ROC) curve. Radiology **143:**29–36.
13. **Hanna, W., and D. Baxby.** 2002. Studies in smallpox and vaccination. 1913. Rev. Med. Virol. **12:**201–209.
14. **Henderson, D. A., T. V. Inglesby, J. G. Bartlett, M. S. Ascher, E. Eitzen, P. B. Jahrling, J. Hauer, M. Layton, J. McDade, M. T. Osterholm, T. O'Toole, G. Parker, T. Perl, P. K. Russell, and K. Tonat.** 1999. Smallpox as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. JAMA **281:**2127–2137.
15. **Mack, T. M.** 1972. Smallpox in Europe, 1950–1971. J. Infect. Dis. **125:**161–169.
16. **Mack, T. M., J. Noble, Jr., and D. B. Thomas.** 1972. A prospective study of serum antibody and protection against smallpox. Am. J. Trop. Med. Hyg. **21:**214–218.
17. **McCurdy, L. H., J. A. Rutigliano, T. R. Johnson, M. Chen, and B. S. Graham.** 2004. Modified vaccinia virus Ankara immunization protects against lethal challenge with recombinant vaccinia virus expressing murine interleukin-4. J. Virol. **78:**12471–12479.
18. **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.
19. **Sarkar, J. K., A. C. Mitra, and M. K. Mukherjee.** 1975. The minimum protective level of antibodies in smallpox. Bull. W. H. O. **52:**307–311.
20. **Smith, G. L., and G. McFadden.** 2002. Smallpox: anything to declare? Nat. Rev. Immunol. **2:**521–527.
21. **World Health Organization.** 1972. W.H.O. Expert Committee on Smallpox Eradication. Second report. WHO Tech. Rep. Ser. **493:**1–64.
22. **World Health Organization.** 1980. Declaration of global eradication of smallpox. Wkly. Epidemiol. Rec. **55:**145–152.
23. **World Health Organization.** 2002. Accession date, 20 November 2004. Smallpox vaccine. [Online.] http://www.who.int/vaccines/en/smallpox.shtml.
24. **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.

Journal of Medical Virology 77:83–88 (2005)

Antigen-Capture Enzyme-Linked Immunosorbent Assay for the Diagnosis of Crimean-Congo Hemorrhagic Fever Using a Novel Monoclonal Antibody

Masayuki Saijo,[1]* Qing Tang,[2] Bawudong Shimayi,[3] Lei Han,[2] Yuzhen Zhang,[4] Muer Asiguma,[4] Dong Tianshu,[4] Akihiko Maeda,[1] Ichiro Kurane,[1] and Shigeru Morikawa[1]

[1]*Special Pathogens Laboratory, Department of Virology 1, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo, Japan*
[2]*Second Division of Viral Hemorrhagic Fever, Institute of Infectious Disease Control and Prevention, Chinese Center for Disease Control and Prevention, P.O. Box 5, Beijing, P.R. China*
[3]*Bachu County Center for Disease Prevention and Control, Kashi District, the Xinjiang Autonomous Region, P.R. China*
[4]*Bachu People's Hospital, Bachu County, Kashi district, the Xinjiang Uygur Autonomous Region, P.R. China*

An antigen-capture enzyme-linked immunosorbent assay (ELISA) was developed for the diagnosis of Crimean-Congo hemorrhagic fever (CCHF) using a novel monoclonal antibody, 1B7, to the recombinant nucleoprotein (rNP) of CCHF virus (CCHFV) Chinese strain 8402. This ELISA detected at least 2 ng/100 µl of CCHFV rNP of 8402 and of the Nigeria strain Ibr 10200, and also detected authentic nucleoproteins (NP) of Chinese strains. Although the sensitivity of the ELISA was lower than that of nested reverse transcription polymerase chain reaction (RT-PCR), it was able to detect nucleoproteins in acute sera of CCHF patients. The presence of anti-CCHFV IgG decreased the sensitivity of the ELISA, possibly due to competition with 1B7, and this would tend to limit application of the ELISA. However, the method may be useful for the diagnosis of CCHF patients in the acute stage of illness, especially in laboratories not equipped with RT-PCR testing capabilities. ***J. Med. Virol. 77:83–88, 2005.***
© 2005 Wiley-Liss, Inc.

KEY WORDS: Crimean-Congo hemorrhagic fever; diagnosis; nucleoprotein; monoclonal antibody; ELISA

INTRODUCTION

Crimean-Congo hemorrhagic fever (CCHF) virus (CCHFV) is a member of the family Bunyaviridae, genus *Nairovirus* [Nichol, 2001]. Humans acquire infection primarily through tick (genus *Hyalomma*) bites or contact with fresh meat or blood from slaughtered viremic animals, including sheep, cattle, and goats [Nichol, 2001]. CCHFV infections have been reported in Africa, Eastern Europe, the Middle East, and Central and Southern Asia, although human cases are believed to be underreported because disease usually occurs in remote areas. Nosocomial outbreaks of CCHF have also been reported [Burney et al., 1980; Suleiman et al., 1980; Joubert et al., 1985; van de Wal et al., 1985; Fisher-Hoch et al., 1995; Papa et al., 2002, 2004].

Ribavirin has been shown to inhibit replication of CCHFV in vitro and in vivo [Watts et al., 1989; Tignor and Hanham, 1993]. Although controlled clinical trials have not been conducted, several groups have reported successful treatment of CCHF patients with ribavirin [van de Wal et al., 1985; Fisher-Hoch et al., 1995; Papa et al., 2002, 2004; Tang et al., 2003]. Therefore, rapid diagnosis of CCHF may result in early and effective treatment, as well as subsequent prevention of nosocomial infection.

A CCHFV recombinant nucleoprotein (rNP)-based enzyme-linked immunosorbent assay (ELISA) and immunofluorescent assay for detecting immunoglobulin (Ig) G antibodies to CCHFV were described previously [Saijo et al., 2002a,b]. Detection of CCHFV IgM antibodies is another reliable procedure for rapid diagnosis of CCHF [Tang et al., 2003; Saijo et al., 2005]. However, CCHFV-antigen detection is considered to be more

Grant sponsor: The Ministry of Health, Labor and Welfare of Japan.

*Correspondence to: Dr. Masayuki Saijo, Special Pathogens Laboratory, Department of Virology 1, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan. E-mail: msaijo@nih.go.jp

Accepted 4 May 2005
DOI 10.1002/jmv.20417
Published online in Wiley InterScience (www.interscience.wiley.com)

© 2005 WILEY-LISS, INC.

useful for rapid diagnosis of CCHF. In the past, CCHFV-antigen detection ELISA system was developed and used for diagnosis of CCHF [Logan et al., 1993; Khan et al., 1997]. Sheep antibody to CCHFV was used as a capture-antibody and mouse hyperimmune ascitic fluid against CCHFV was used as a detector-antibody in the system previously reported [Logan et al., 1993]. In the present study, a novel monoclonal antibody to the rNP of CCHFV Chinese strain 8402 was produced and characterized. An antigen-capture ELISA system using this monoclonal antibody was subsequently developed, and its use in the diagnosis of CCHF was compared with that of nested reverse transcription polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

Cell Culture

Hybridomas and their parental cell line, P3/Ag568, plus high five (Tn5) insect cells, were cultured as previously described [Niikura et al., 2001].

Recombinant Baculoviruses

Recombinant baculoviruses expressing 6X histidine-tagged rNP of CCHFV Chinese strain 8402 and Nigerian strain Ibr 10200 were used as described previously [Saijo et al., 2002a].

Authentic CCHFVs

Six CCHFV Chinese strains (66019, 7001, 7803, 78024, 88166, and 8402) were used. Viral antigens were prepared from newborn mouse brains inoculated with each of the virus strains. Antigens were inactivated by treatment with detergent, 1% Triton-X in phosphate buffered saline solution (PBS), and ultraviolet irradiation for more than 1 hr.

rNP of CCHFV

The full-length rNP of CCHFV, consisting of 482 amino acid residues, was expressed using a baculovirus system [Saijo et al., 2002a]. rNP was purified by utilizing the Ni^{2+}-resin purification system (Qiangen GmbH, Hilden, Germany). Protein concentrations of purified rNP were measured by the Bradford method using Protein Assay™ according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA).

Establishment of Monoclonal Antibodies

Monoclonal antibodies were generated as previously described except for the immunizing antigen [Niikura et al., 2001]; BALB/c mice were immunized with purified His-CCHFV rNP in the present study.

Polyclonal Antibodies

Polyclonal antibodies were induced in rabbits and in monkeys (*Macaca fascicularis*) by immunization with the purified rNP of CCHFV Chinese strain 8402 expressed in the baculovirus system [Saijo et al., 2002a]. Mouse hyperimmune ascites fluid (MHAF) against CCHFV (courtesy of T.G. Ksiazek), along with monoclonal antibodies to glutathione-*S*-transferase (GST) produced in our laboratory, were also used.

Antigen-Capture ELISA

Antigen-capture ELISA was performed as described previously [Niikura et al., 2001]. In this study, each of the purified monoclonal antibodies to CCHFV rNP were coated on 96-microwell immunoplates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ). For detection of antigen, each of the purified monoclonal antibodies was diluted in PBS solution and 100 µl adsorbed overnight at 4°C on to the immunoplates. Control wells were similarly adsorbed with PBS. The capture and control wells were washed three times with PBS supplemented with 0.05% tween-20 (PBS-T). Serum samples, positive antigen or negative antigens were diluted with 5% skimmed milk in PBS-T (M-PBS-T) and tested in twofold dilutions. The first of 6 wells coated with capture-antibody received 200 µl of samples diluted 1:4 or 1:8 in M-PBS-T and the other 5 wells 100 µl of M-PBS-T. Subsequently, 100 µl of material was diluted from the first well through the ensuing 5 wells. The same antigens were diluted similarly in 6 wells coated without capture-antibody in PBS. CCHFV rNP and negative control antigen were treated as above and used as positive and negative controls for each assay, respectively. Immunoplates were then incubated in a humidified condition for 1 hr at 37°C, washed three times with PBS-T, and rabbit anti-CCHFV rNP antibody was added in M-PBS-T at a dilution of 1:1,000 to all wells (100 µl/well). A further 1 hr incubation at 37°C was followed by three washes with PBS-T. One-hundred of goat anti-rabbit IgG antibody conjugated with horseradish peroxidase (Zymed) diluted 1:1,000 in M-PBS-T was added to each well, and incubated for 1 hr at 37°C. After three additional washes, 100 µl of [2,2′-azinobis(3-ethylbenzthiazolinesulfonic acid)] substrate (ABTS, Roche Diagnostics, Mannheim, Germany) was added to all wells, incubated for 30 min at room temperature, and the optical density (OD) read at 405 nm with reference read at 490 nm (OD_{405}) was obtained. The OD_{405} values of wells coated with the capture-monoclonal antibody were adjusted by subtracting the OD_{405} value for the corresponding well that had been coated without capture antibody. This adjusted OD_{405} was taken as a measure of the amount of antigen specifically bound.

Western Blotting

Monoclonal antibodies to rNP of CCHFV Chinese strain 8402 and polyclonal antibodies to CCHFV, were tested for reactivity to recombinant rNP fragments by Western blotting as previously described [Saijo et al., 2002a].

Clinical Specimens

Eleven serum samples were collected from nine suspected CCHF patients from the Xinjiang Uygur

Autonomous Region during an outbreak of the disease in 2001. Three samples were obtained from one patient on days 1, 5, and 9 of illness. The clinical course and virologic data of this patient were reported previously [Tang et al., 2003]. A single sample was collected from the eight remaining patients. Twenty-one serum samples were also collected from 12 suspected CCHF patients from the same region in 2002. Blood was collected twice (acute phase and 7 days later) in nine of the patients and once in the other three patients.

Informed Consent

All serum samples were collected from patients under informed consent. Consent from unconscious patients and children less than 20 years of age was obtained from family members.

Nested Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Nested RT-PCR was performed as previously described [Rodriguez et al., 1997; Burt et al., 1998; Tang et al., 2003].

Detection of CCHFV IgG and IgM Antibodies by rNP-Based ELISA

Antibodies to CCHFV were detected by CCHFV rNP-based IgG ELISA and IgM-capture ELISA as described previously [Saijo et al., 2002a, 2005; Tang et al., 2003].

RESULTS

Generation of Monoclonal Antibodies to CCHFV rNP

Ten hybridomas clones that secrete monoclonal antibodies to CCHFV rNP were generated. All antibodies had subclass G1 and kappa-type light chains. Of the 10 clones, eight reacted strongly with CCHFV rNP by antigen-capture ELISA, approximately 8 ng/ml of CCHFV rNP was detected using each of the eight monoclonal antibodies (Fig. 1A). Antigen-capture ELISA using each of these eight monoclonal antibodies resulted in similar reactivity in detecting CCHFV rNP (Fig. 1B). However, monoclonal antibodies of clones 1E10, 1F1, 2C4, 2E10, and 1B7 reacted more strongly to authentic viral NP of CCHFV strain 66019 than those of clones 1B8, 1E5, and 3B6 (Fig. 1C). Furthermore, antigen-capture ELISA using each of the five monoclonal antibodies (1E10, 1F1, 2C4, 1B7, 2E10) detected authentic viral NPs of all five CCHFV Chinese strains (7001, 7803, 78024, 88166, 8402) tested with similar reactivity patterns (data not shown).

Detection of CCHF Antigen in Human Serum Samples

Based on the above results, monoclonal antibody clone 1B7 was selected as a capture-antibody in the antigen-capture ELISA. Human serum samples collected from three CCHF cases in acute phase during the 2001 outbreak (01–2, 01–4, 01–8) showed positive reactions by nested RT-PCR (Fig. 2A). However, only sample 01–8 reacted positive by antigen-capture ELISA (Fig. 2B). Sample 01–8, collected on day 1 of illness, did not have either IgM or IgG antibodies. However, samples collected from the same patient on days 5 and 9 (01–8* and 01–8**, respectively) had both IgM and IgG antibodies [Tang et al., 2003]. Samples 01–2 and 01–4 had IgM antibodies but no IgG antibodies.

Among the samples collected in 2002, only samples 02–2 and 02–11 (acute serum from two CCHF cases) showed positive reactions by antigen-capture ELISA (Fig. 2E). CCHFV genome fragments were amplified in

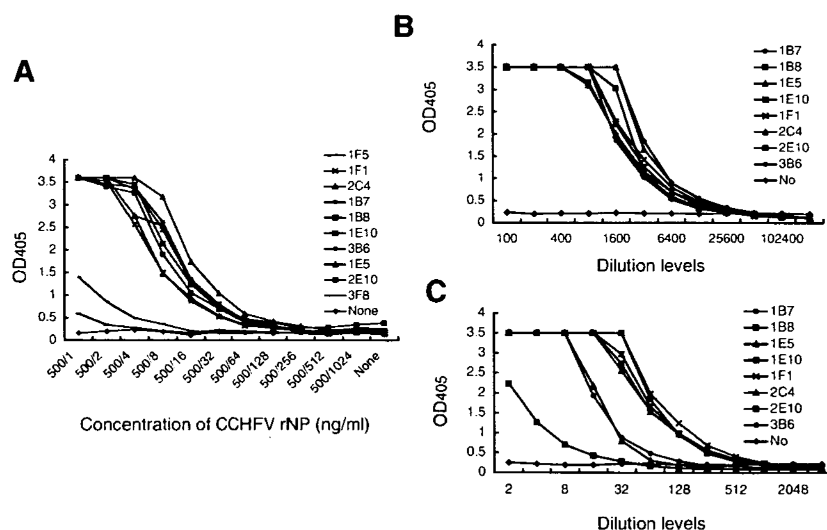

Fig. 1. Sensitivity of the antigen-capture ELISA using monoclonal antibodies in the detection of the CCHFV rNP (**A**, **B**) and the authentic nucleoprotein of CCHFV Chinese strain 66019 (**C**). Antigen-capture ELISA studies for B and C were conducted as part of the same experiment.

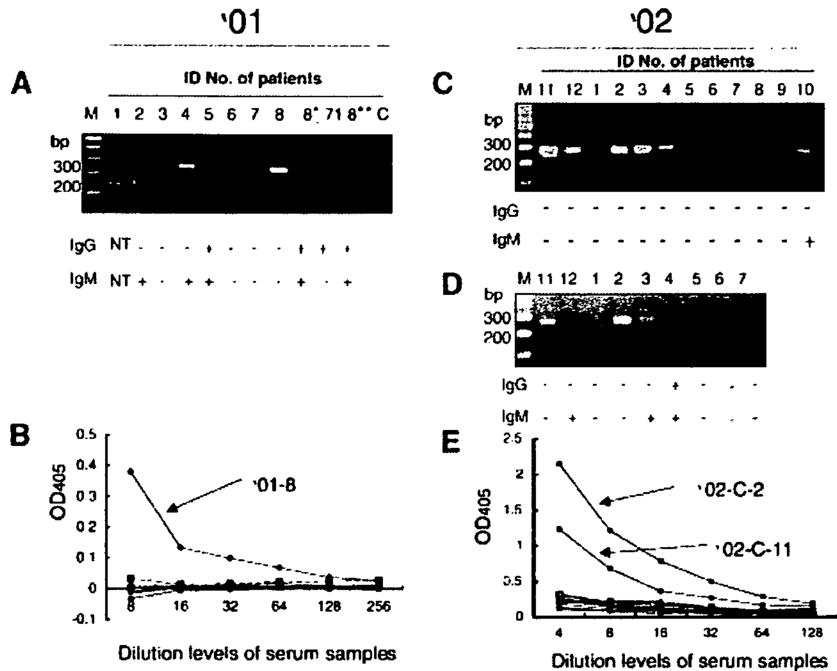

Fig. 2. Nested RT-PCR and IgG- and IgM-capture ELISA (**A**, **C**, **D**), and antigen-capture ELISA (**B**, **E**) results of serum samples collected from suspected patients with CCHF. Samples were collected from nine patients in 2001 ('01, **left panel**) and from 12 patients in 2002 ('02, **right panel**). Blood was drawn on days 1 ("8"), 5 ("8*"), and 9 ("8**") from patient No. 8. Samples in C were collected first and those in D were collected approximately 1 week after those in C. "NT" in A indicates "not tested." Samples collected in 2001 and 2002 were tested separately.

six of the 12 acute samples by nested RT-PCR (Fig. 2C). However, only 02–10 demonstrated an antibody response to CCHFV. Of the five samples (02–11, 02–12, 02–2, 02–3, 02–4) collected from patients with viremia as demonstrated by nested RT-PCR but without CCHFV antibody responses, only two samples exhibited positive reactions by antigen-capture ELISA (Fig. 2C).

Efficacy of the Antigen-Capture ELISA in Samples Containing Antibodies to CCHFV NP

The effect of the presence of anti-CCHFV antibodies on the sensitivity of the antigen-capture ELISA was investigated. CCHFV rNP diluted serially with TPBS-M solution was mixed with the monkey serum raised against CCHFV rNP (ID 3191 10Mar00) or with pre-immunized monkey serum, kept for 1 hr at room temperature, and tested for reactivity by antigen-capture ELISA. Monkey serum raised against CCHFV rNP possessed CCHFV rNP antibodies at a titer of approximately 1:6,400 (Fig. 3C). Reactivity of the ELISA decreased when antibodies to CCHFV rNP were present in the samples.

Reactivity of Monoclonal Antibody Clone 1B7 to rNP of CCHFV Strain Ibr10200

Reactivity of monoclonal antibody clone 1B7 to the rNP of CCHFV strain Ibr10200 and Chinese strain 8402 was compared by using both Western blotting and antigen-capture ELISA (Fig. 4). Both antigens reacted with monoclonal antibody 1B7 by Western blotting (Fig. 4A). Antigen-capture ELISA also detected both antigens with similar reactivity (Fig. 4B).

DISCUSSION

The antigen-capture ELISA using the monoclonal antibody 1B7 detected not only the authentic NP of CCHFV Chinese strains but also the CCHFV rNP of the Nigerian strain Ibr10200. These data suggest that other NPs of CCHFV strains besides those from Chinese strains may be detected by this ELISA.

Three of the 6 serum samples collected from patients, whose sera were RT-PCR positive and antibody-negative (01–8, 02–11, 02–12, 02–2, 02–3, and 02–4), showed positive reactions by the antigen-capture ELISA. Furthermore, 3 of 21 serum samples from suspected patients were positive by ELISA, although 9 were CCHFV genome positive by the nested RT-PCR. These data suggest that although ELISA may be useful for the diagnosis of CCHF, sensitivity is lower than nested RT-PCR.

The presence of antibodies to CCHFV NP in the samples decreased the reactivity of the antigen-capture ELISA (Fig. 3). None of the nested RT-PCR-positive and antibody-positive serum samples reacted positively by antigen-capture ELISA, suggesting that this method is useful for testing serum samples collected during the

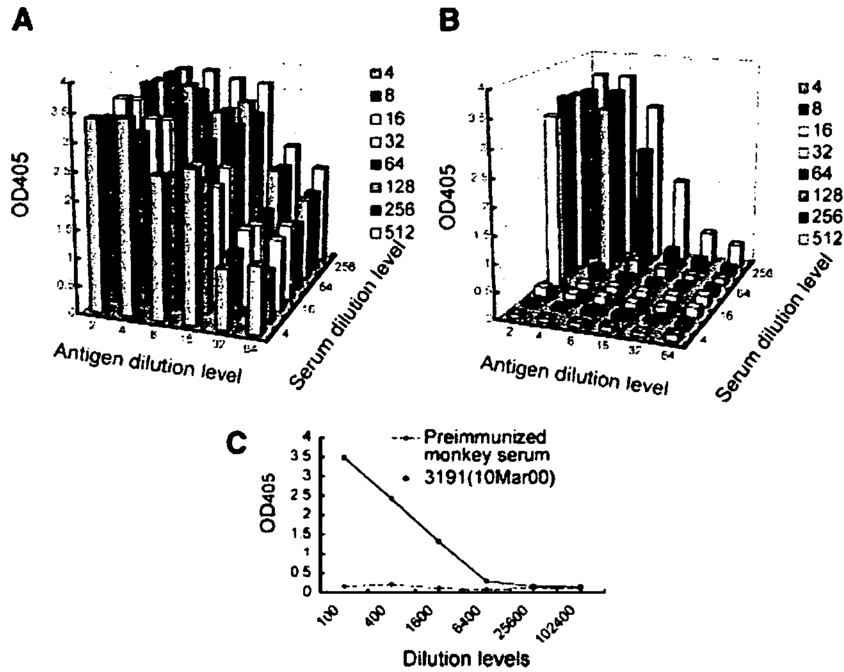

Fig. 3. Influence of CCHFV NP antibody presence toward detection of CCHFV NP by antigen-capture ELISA. CCHFV rNP mixed with monkey serum raised against CCHFV rNP were not as efficiently detected (**B**) as antigens mixed with pre-immunized monkey serum (**A**). OD_{405} values of CCHFV rNP antibody-positive and negative monkey sera are shown in figure **C**. The anti-CCHFV IgG antibody titer of sample 3191 (10Mar00) was 1:6,400, while the pre-immunized monkey serum contained no detectable antibodies (C).

acute phase of illness before antibody responses are detectable. The antigenic region is located within the CCHFV NP only in the middle section [Saijo et al., 2002a]. Monoclonal antibodies, including 1B7, recognized this region and its conformational epitopes (data not shown). It is possible that antibodies to the NP present in patients' sera inhibit the monoclonal antibodies from reacting with these epitopes. Furthermore,

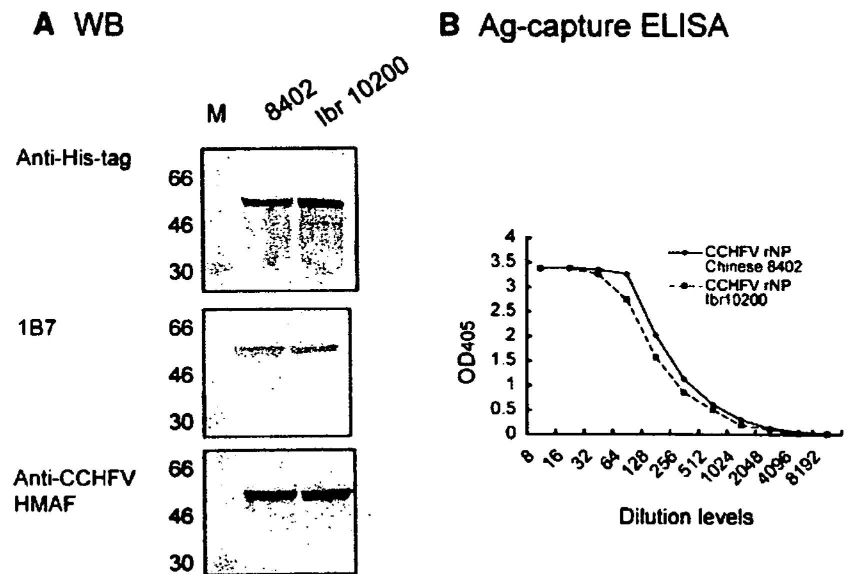

Fig. 4. Reactivity of monoclonal antibody clone 1B7 (**A, middle panel**), monoclonal antibody to His-tag (A, **upper panel**) and anti-CCHFV HMAF (A, **lower panel**) to CCHFV rNPs of CCHFV Chinese strain 8402 and CCHFV Nigerian strain Ibr10200 by Western blotting. Efficacy of the antigen-capture ELISA in detecting the CCHFV rNPs of CCHFV Chinese strain 8402 and CCHFV Nigerian strain Ibr10200 is shown in **B**. Protein concentrations of each sample were normalized in this experiment.

the amount of the CCHFV antigen decreases as time passes after illness onset. These findings may account for the low reactivity of the antigen-capture ELISA when testing antibody-positive serum samples.

The diagnostic sensitivity of the antigen-capture ELISA was verified in the present study using serum samples. However, mononuclear phagocytes are also known to be a primary target of CCHFV [Burt et al., 1997]. Therefore, it may be possible to increase the sensitivity of the ELISA by using whole blood samples instead of serum samples. Further studies are needed to address this issue.

In summary, CCHFV antigen-capture ELISA was developed using the novel monoclonal antibody clone 1B7. This ELISA was shown to be useful for the diagnosis of CCHF, especially when samples were collected during the acute phase of disease.

ACKNOWLEDGMENTS

The first and the second authors (Dr. M. Saijo, M.D., and Dr. Q. Tang, M.D.) contributed equally to this work. We thank all the staff at the Xinjiang Bachu People's Hospital, who contributed to the treatment of patients. We also thank Dr. Li Fan, Director of the AIDS Prevention and Control Center, Xinjiang Epidemic Prevention Station, Urumqi, the Xinjiang Uygur Autonomous Region, and his colleagues. We are grateful to Ms. M. Ogata, Special Pathogens Laboratory, Department of Virology 1, National Institute of Infectious Diseases, Tokyo, Japan, and Ms. X. Zhao and Ms. X. Tao, the Second Division of Viral Hemorrhagic Fever, Institute of Infectious Disease Control and Prevention, Chinese Center for Disease Control and Prevention, for their technical assistance with this project. Tests were performed in a high containment laboratory according to the regulations of the Institute of Infectious Disease Control and Prevention, Chinese Center for Disease Control and Prevention. We thank Dr. Pual Kitsutani, Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan, for helpful discussion. We thank Dr. T.G. Ksiazek, Chief of the Special Pathogens Branch, Division of Viral and Rickettsial Diseases, CDC, Atlanta, GA, for providing MHAF against CCHFV and the cDNA of the S-gene of CCHFV Nigerian strain Ibr 10200. This study was approved by both the Ethical Committee on Medical Research and Human Right and the Ethical Committee on Animal Experiments, National Institute of Infectious Diseases, Tokyo, Japan.

REFERENCES

Burney MI, Ghafoor A, Saleen M, Webb PA, Casals J. 1980. Nosocomial outbreak of viral hemorrhagic fever caused by Crimean Hemorrhagic fever-Congo virus in Pakistan, January 1976. Am J Trop Med Hyg 29:941–947.

Burt FJ, Swanepoel R, Shieh WJ, Smith JF, Leman PA, Greer PW, Coffield LM, Rollin PE, Ksiazek TG, Peters CJ, Zaki SR. 1997. Immunohistochemical and in situ localization of Crimean-Congo hemorrhagic fever (CCHF) virus in human tissues and implications for CCHF pathogenesis. Arch Pathol Lab Med 121:839–846.

Burt FJ, Leman PA, Smith JF, Swanepoel R. 1998. The use of a reverse transcription-polymerase chain reaction for the detection of viral nucleic acid in the diagnosis of Crimean-Congo haemorrhagic fever. J Virol Methods 70:129–137.

Fisher-Hoch SP, Khan JA, Rehman S, Mirza S, Khurshid M, McCormick JB. 1995. Crimean Congo-haemorrhagic fever treated with oral ribavirin. Lancet 346:472–475.

Joubert JR, King JB, Rossouw DJ, Cooper R. 1985. A nosocomial outbreak of Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part III. Clinical pathology and pathogenesis. S Afr Med J 68:722–728.

Khan AS, Maupin GO, Rollin PE, Noor AM, Shurie HH, Shalabi AG, Wasef S, Haddad YM, Sadek R, Ijaz K, Peters CJ, Ksiazek TG. 1997. An outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates, 1994–1995. Am J Trop Med Hyg 57:519–525.

Logan TM, Linthicum KJ, Moulton JR, Ksiazek TG. 1993. Antigen-capture enzyme-linked immunosorbent assay for detection and quantification of Crimean-Congo hemorrhagic fever virus in the tick, *Hyalomma truncatum*. J Virol Methods 42:33–44.

Nichol ST. 2001. Bunyaviruses. In: Knipe DM, Howley PM, editors. Fields virology. 4th edn. Philadelphia: Lippincott Williams & Wilkins. pp 1603–1633.

Niikura M, Ikegami T, Saijo M, Kurane I, Miranda ME, Morikawa S. 2001. Detection of Ebola viral antigen by enzyme-linked immunosorbent assay using a novel monoclonal antibody to nucleoprotein. J Clin Microbiol 39:3267–3271.

Papa A, Bino S, Llagami A, Brahimaj B, Papadimitriou E, Pavlidou V, Velo E, Cahani G, Hajdini M, Pilaca A, Harxhi A, Antoniadis A. 2002. Crimean-Congo hemorrhagic fever in Albania, 2001. Eur J Clin Microbiol Infect Dis 21:603–606.

Papa A, Christova I, Papadimitriou E, Antoniadis A. 2004. Crimean-Congo hemorrhagic fever in Bulgaria. Emerg Infect Dis 10:1465–1467.

Rodriguez LL, Maupin GO, Ksiazek TG, Rollin PE, Khan AS, Schwarz TF, Lofts RS, Smith JF, Noor AM, Peters CJ, Nichol ST. 1997. Molecular investigation of a multisource outbreak of Crimean-Congo hemorrhagic fever in the United Arab Emirates. Am J Trop Med Hyg 57:512–518.

Saijo M, Qing T, Niikura M, Maeda A, Ikegami T, Prehaud C, Kurane I, Morikawa S. 2002a. Recombinant nucleoprotein-based enzyme-linked immunosorbent assay for detection of immunoglobulin G antibodies to Crimean-Congo hemorrhagic fever virus. J Clin Microbiol 40:1587–1591.

Saijo M, Qing T, Niikura M, Maeda A, Ikegami T, Sakai K, Prehaud C, Kurane I, Morikawa S. 2002b. Immunofluorescence technique using HeLa cells expressing recombinant nucleoprotein for detection of immunoglobulin G antibodies to Crimean-Congo hemorrhagic fever virus. J Clin Microbiol 40:372–375.

Saijo M, Tang Q, Shimayi B, Han L, Zhang Y, Asiguma M, Tianshu D, Maeda A, Kurane I, Morikawa S. 2005. Recombinant nucleoprotein-based serological diagnosis of Crimean-Congo hemorrhagic fever virus infections. J Med Virol 75:295–299.

Suleiman MN, Muscat-Baron JM, Harries JR, Satti AG, Platt GS, Bowen ET, Simpson DI. 1980. Congo/Crimean haemorrhagic fever in Dubai. An outbreak at the Rashid Hospital. Lancet 2:939–941.

Tang Q, Saijo M, Zhang Y, Asiguma M, Tianshu D, Han L, Shimayi B, Maeda A, Kurane I, Morikawa S. 2003. A patient with Crimean-Congo hemorrhagic fever serologically diagnosed by recombinant nucleoprotein-based antibody detection systems. Clin Diagn Lab Immunol 10:489–491.

Tignor GH, Hanham CA. 1993. Ribavirin efficacy in an in vivo model of Crimean-Congo hemorrhagic fever virus (CCHF) infection. Antiviral Res 22:309–325.

van de Wal BW, Joubert JR, van Eeden PJ, King JB. 1985. A nosocomial outbreak of Crimean-Congo haemorrhagic fever at Tygerberg Hospital. Part IV. Preventive and prophylactic measures. S Afr Med J 68:729–732.

Watts DM, Ussery MA, Nash D, Peters CJ. 1989. Inhibition of Crimean-Congo hemorrhagic fever viral infectivity yields in vitro by ribavirin. Am J Trop Med Hyg 41:581–585.

JOURNAL OF VIROLOGY, Sept. 2005, p. 11873–11891 Vol. 79, No. 18
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.18.11873–11891.2005
Copyright © 2005, American Society for Microbiology. All Rights Reserved.

An Attenuated LC16m8 Smallpox Vaccine: Analysis of Full-Genome Sequence and Induction of Immune Protection§

Shigeru Morikawa,[1]† Tokuki Sakiyama,[2,3]† Hideki Hasegawa,[4]† Masayuki Saijo,[1] Akihiko Maeda,[1]‡ Ichiro Kurane,[1] Go Maeno,[3] Junko Kimura,[3] Chie Hirama,[3] Teruhiko Yoshida,[2,3] Yasuko Asahi-Ozaki,[4] Tetsutaro Sata,[4] Takeshi Kurata,[4] and Asato Kojima[4]*

Department of Virology I[1] and Department of Pathology,[4] National Institute of Infectious Diseases, and Genetics Division[2] and Center for Medical Genomics,[3] National Cancer Center Research Institute, Tokyo, Japan

Received 1 December 2004/Accepted 7 June 2005

The potential threat of smallpox bioterrorism has made urgent the development of lower-virulence vaccinia virus vaccines. An attenuated LC16m8 (m8) vaccine was developed in 1975 from the Lister strain used in the World Health Organization smallpox eradication program but was not used against endemic smallpox. Today, no vaccines can be tested with variola virus for efficacy in humans, and the mechanisms of immune protection against the major intracellular mature virion (IMV) and minor extracellular enveloped virion (EEV) populations of poxviruses are poorly understood. Here, we determined the full-genome sequences of the m8, parental LC16mO (mO), and grandparental Lister (LO) strains and analyzed their evolutionary relationships. Sequence data and PCR analysis indicated that m8 was a progeny of LO and that m8 preserved almost all of the open reading frames of vaccinia virus except for the disrupted EEV envelope gene B5R. In accordance with this genomic background, m8 induced 100% protection against a highly pathogenic vaccinia WR virus in mice by a single vaccination, despite the lack of anti-B5R and anti-EEV antibodies. The immunogenicity and priming efficacy with the m8 vaccine consisting mainly of IMV were as high as those with the intact-EEV parental mO and grandparental LO vaccines. Thus, mice vaccinated with 10^7 PFU of m8 produced low levels of anti-B5R antibodies after WR challenge, probably because of quick clearance of B5R-expressing WR EEV by strong immunity induced by the vaccination. These results suggest that priming with m8 IMV provides efficient protection despite undetectable levels of immunity against EEV.

Variola virus (VAR), a member of the orthopoxvirus (OPV) family, is the causative agent of smallpox and caused millions of deaths before its eradication. Today, smallpox is again becoming a potential threat to humans, with abuse of VAR as a bioterrorist weapon (10, 15, 20, 26, 30, 37, 40). The World Health Organization (WHO) program for smallpox eradication indicated that vaccinia virus (VV) vaccination is the most effective preventive measure against the disease. However, WHO recommended discontinuing the vaccination in 1980 (55) due to rare (around 20 cases/10^6 vaccinees) but severe complications, such as postvaccinial encephalitis, progressive vaccinia, and eczema vaccinatum with the primary vaccination (4, 17, 34, 57). Thus, after a lag time of more than 20 years, serious attempts have been urged to restart the development of lower-virulence vaccine strains (2, 3, 9, 43, 45, 50). A vaccinia ACAM1000 clone has recently been established using cell cultures from the Dryvax (NYBH strain) vaccine (50), but it may induce myocarditis (4, 11). Modified vaccinia virus Ankara (MVA) and NYVAC (modified Copenhagen strain) replication-incompetent viruses are certainly safer but may require high vaccine doses or boosting with replication-competent vaccines (2, 9).

One of the safest replication-competent vaccines, a vaccinia virus LC16m8 strain (m8), was developed and established in the early 1970s with cell culture systems (24, 25) through a temperature-sensitive and low-virulence LC16mO intermediate clone (mO) from the Lister (Elstree) original strain (LO) that was used worldwide in the WHO program. The m8 virus exhibited the lowest levels of neurovirulence and the mildest adverse events among several vaccine strains, such as NYBH, CV1, and EM63, in monkeys, rabbits, and cortisone-induced immunocompromised mice (24, 38, 39). Its antigenicity was as high as that of the LO vaccine, not only in animals, but also in approximately 50,000 Japanese children vaccinated from 1973 to 1974 (over 90,000 doses in 1974 and 1975) with no reports of severe complications (24, 57). Based on these studies, cell culture-derived m8 was licensed in 1975 in Japan as a second-generation smallpox vaccine, but it has never been confronted with VAR.

Recent progress in molecular genetics has demonstrated that m8 has a single-nucleotide deletion creating a termination codon at amino acid (aa) position 93 in the B5R envelope (*env*) gene (47). Several papers have indicated that the destruction of B5R contributes to attenuation of poxviruses (12, 36, 44, 46, 47, 54). In turn, the B5R Env protein was suggested to function as an antigen that induces neutralizing antibodies (NAbs) to the extracellular enveloped virion (EEV) form of poxviruses (12, 19, 44). EEVs are free virions released from infected cells and may cause long-range dissemination of infection, although

* Corresponding author. Mailing address: Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan. Phone: 81-3-5285-1189. Fax: 81-3-5285-1189. E-mail: akojima@nih.go.jp.

† S.M., T.S., and H.H. contributed equally to this work.

‡ Present address: Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan.

§ Supplemental material for this article may be found at http://jvi.asm.org.

they comprise less than 1% of the virus population, the majority being the intracellular mature virion (IMV) form (12, 41, 44). In addition, B5R is also a component of viral particles on the cell surface termed cell-associated enveloped virions, which are more abundant than EEV and are important for cell-to-cell spread (44). Consequently, the spread of these VVs seems to be prevented by anti-B5R NAbs.

However, little is as yet understood regarding the mechanisms of immune protection against EEVs, cell-associated enveloped virions, and IMVs of poxviruses. Thus, a concern has arisen that the B5R truncation and other possible mutations introduced into m8 during processes of attenuation of the LO vaccine reduce the generation of the enveloped virions and therefore might make the attenuated m8 vaccine less protective or nonprotective against VAR (5, 44, 45). No vaccines, however, can be tested for efficacy against VAR in humans. Alternatively, intranasal infection with a mouse-adapted and highly pathogenic vaccinia virus Western Reserve (WR) strain provides a mouse model well suited for evaluating protective efficacy (2, 32, 50, 51).

Here, we determined and compared the full-genome sequences of the licensed m8, parental mO, and grandparental LO strains to examine whether m8 has inherited the intact genome of LO or acquired other alterations in the EEV-related genes. We also examined antibody responses to B5R, EEV, and IMV in mice after a single vaccination with m8, mO, and LO and evaluated the protective efficacy against intranasal WR challenge in vaccinated mice. The results suggest that the genes, except for B5R, of m8 are similar to those of LO and that consequently, the immunogenicity and protective efficacy of m8 are similar to those of LO.

MATERIALS AND METHODS

Cells and viruses. RK13 cells were grown in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS). HeLa cells were cultured in Dulbecco's modified MEM containing 5% FBS. High five (Tn5) insect cells were cultured at 26°C in TC100 medium (JRH Bioscience, Inc.) supplemented with 10% FBS. LO, mO, m8, and WR strains of VV (kind gifts from S. Hashizume) were propagated and titrated on RK13 cell monolayers (58). The WR virus used was selected by sensitivity to 5-bromo-2-deoxyuridine before propagation. When a VV IHD-J strain was used as a high producer of EEV, the virus was freshly prepared, titrated, and inoculated into cells (41).

Purification of viral DNA. RK13 cells infected with m8, mO, or LO virus were harvested and disrupted by sonication in 10 mM Tris (pH 8.0)-1 mM EDTA buffer. Cell debris and nuclei were removed from cell lysates by low-speed centrifugation, and viruses were recovered by centrifugation at 15,000 × g for 40 min. Virions suspended in 0.1× Tris-EDTA were purified by centrifugation on 36% sucrose cushions and then on 20 to 40% linear sucrose density gradients, as described previously (29). After each centrifugation step, virion precipitates were resuspended by sonication to avoid virion aggregate formation. Genomic virus DNA was extracted from purified virions with sodium dodecyl sulfate-proteinase K and then with phenol-chloroform as described previously (42).

Sequence analysis of the complete viral DNA genomes. Purified viral DNA was fragmented with a HydroShear recirculating point-sink flow system (GeneMachines). DNA fragments of 1.5 to 2.5 kbp were recovered by 0.8% agarose gel electrophoresis, blunt ended, and cloned into pUC18. The inserts of the shotgun clones were amplified by PCR with primers (5′-CAGTCACGACGTTGTAAAACGAC-3′ and 5′-GTGTGGAATTGTGAGCGGATAAC-3′) and Ex Taq polymerase (TaKaRa Bio. Inc.). The amplified DNAs were sequenced with a BigDye Terminator v3.1 Cycle Sequencing Kit on PRISM 3700 automated DNA sequencers (Applied Biosystems). The net virus nucleotide sequences were collected with PHRED/PHRAP software and then assembled and edited with Sequencher 4.0 software (GeneCodes Corp.) (13, 14). Primer walking was done for filling gaps and for confirming the order and lengths of the preassembled contigs, as well as the approximately 6-kbp inverted terminal repeats (ITRs) of both genome ends. As the terminal hairpin loops were not sequenced, the leftmost nucleotide of the assembled sequences was arbitrarily designated base number 1. The final DNA sequences of m8, mO, and LO were represented at more than 9.2-, 7.8-, and 8.9-fold redundancy, respectively, at each base position. Open reading frames (ORFs) were identified using National Center for Biotechnology Information BLAST and compared to the GenBank files of the nonredundant protein sequence database, including OPVs and the vaccinia Copenhagen (CPN) strain (21). When there was a large gap between ORFs, mini-ORFs (more than 33 aa) were tentatively predicted for m8 and mO. Noncoding regions were examined for putative early, intermediate, and late promoters with MEME version 3.0 and MAST version 3.0.

PCR analysis. DNAs from LO and mO viruses were analyzed by PCR at six randomly selected loci of LO diversity, numbers L0202, L0403, L0638, L0640, L1000, and L1100, using combinations of the LO- or mO-specific forward primers and the common reverse primers. PCR mixtures were heat denatured at 95°C for 3 min and subjected to 30 cycles of 94°C for 20 s, 63°C for 40 s, and 72°C for 1 min. When the loci L0403 and L1000 were amplified, annealing was done at 61°C. The primers used were as follows: LO-0202 (5′-AGCTATTCTACCATAGCAAAT-3′), mO-0202 (5′-AGCTATTCTACCATAGCAGAA-3′), and R-0202 (5′-CTTGGTTGGTAGAAATGCGG-3′); LO-0403 (5′-TCTAGATAAAATCACTGACTTTC-3′), mO-0403 (5′-TCTAGATAAAATCACTGACTTTT-3′), and R-0403 (5′-AGGAATATGTATAAATGCGGG-3′); LO-0638 (5′-CATATTAGTAGTTCTGCGCAAT-3′), mO-0638 (5′-CATATTAGTAGTTCTGCGTAAG-3′), and R-0638 (5′-CATTATGGTGGCTAGTGATG-3′); LO-0640 (5′-CACCTCTACCGAATAGAGTA-3′), mO-0640 (5′-CACCTCTACCGAATAAAGTT-3′), and R-0630 (5′-GATCTAAATAGAATGCCGACC-3′); LO-1000 (5′-TTAATAGTTGATAGATACGCATTT-3′), mO-1000 (5′-AATAGTTGATAGATACGCGTTC-3′), and R-1000 (5′-CATTTATAACACTGTACTAAC-3′); and LO-1100 (5′-GAACTTCAGGCTGGTGAATC-3′), mO-1100 (5′-AGAACTTCAGGCTGGTAAATT-3′), and R-1100 (5′-CCATTAGTATCCATATACCATG-3′).

Comparison of EEV *env*-related genes. The B5R gene and other EEV *env*-related genes, A33R, A34R, A36R, A56R, and F13L, of a calf lymph Lister vaccine (ListerVAX), mO, and IHD-J were amplified by PCR, sequenced, and compared in amino acid alignment with the VV CPN (GenBank M35027), WR (GenBank AY243312), and MVA (GenBank, U94848) strains and also with other OPVs: VAR (strain Bangladesh-1975; GenBank L22579), monkeypox virus (MPV) (strain Zaire-96-I-16; GenBank AF380138), and cowpox virus (CPV) (strain GRI-90; GenBank X94355).

Preparation of B5R and vaccinia virus antigens. The ectodomain of B5R was amplified from ListerVAX DNA by PCR using primers B5R-Hisf-Bgl (5′-AGATCTACATGTACTGTACCCAC-3′) and B5R-ECTr-Bgl (5′-AGATCTATTCTAACGATTCTATTTCTTG-3′) and cloned into pGEM-Teasy (Promega). The B5R-ect insert was excised from the resultant pTe-Lis-B5R-ect and ligated into a pAcYM1 baculovirus transfer plasmid, pAcMel-His, modified with the melitin signal sequence and a six-His tag. A recombinant AcHis-Lister-B5R-ect baculovirus was constructed as described previously (33). Lysates of Tn5 insect cells were prepared with 1% NP-40 4 days after AcHis-Lister-B5R-ect infection. The lysates were clarified by centrifugation, and the recombinant B5R protein was purified by Ni column (Invitrogen) chromatography. For VV antigens, HeLa cells were infected with LO, harvested 4 days after infection, and lysed with 1% NP-40. The lysates were clarified by centrifugation.

Tests for immunogenicity and protective efficacy. All animal experiments were approved by the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases. Groups of 15 6-week-old female BALB/c mice were vaccinated with 10^5 or 10^7 PFU of m8, mO, or LO or with PBS. On day 21, five mice from each group were sacrificed to test for prechallenge antibody responses, and the other mice were challenged intranasally with 10^6 PFU of WR in 20 μl PBS (51). The mice were observed for clinical signs, examined for bodyweight, and sacrificed 14 days after WR challenge to test for postchallenge antibody responses. The immunogenicity of the recombinant B5R protein was confirmed by subcutaneous injection of BALB/c mice three times each with mixed-in aluminum adjuvant and with the B5R antigen adsorbed to Ni-agarose beads. The immunized mice were challenged with WR as described above 12 days after the last booster injection.

Anti-B5R and anti-vaccinia virus antibody ELISA. Enzyme-linked immunosorbent assay (ELISA) plates were coated with B5R or VV antigen and blocked with 5% skim milk. Dilutions of serum samples were reacted to the plates, and bound antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) (Zymed Laboratory), followed by a substrate (ABTS; Roche Diagnostics). The cutoff optical density at 405 nm

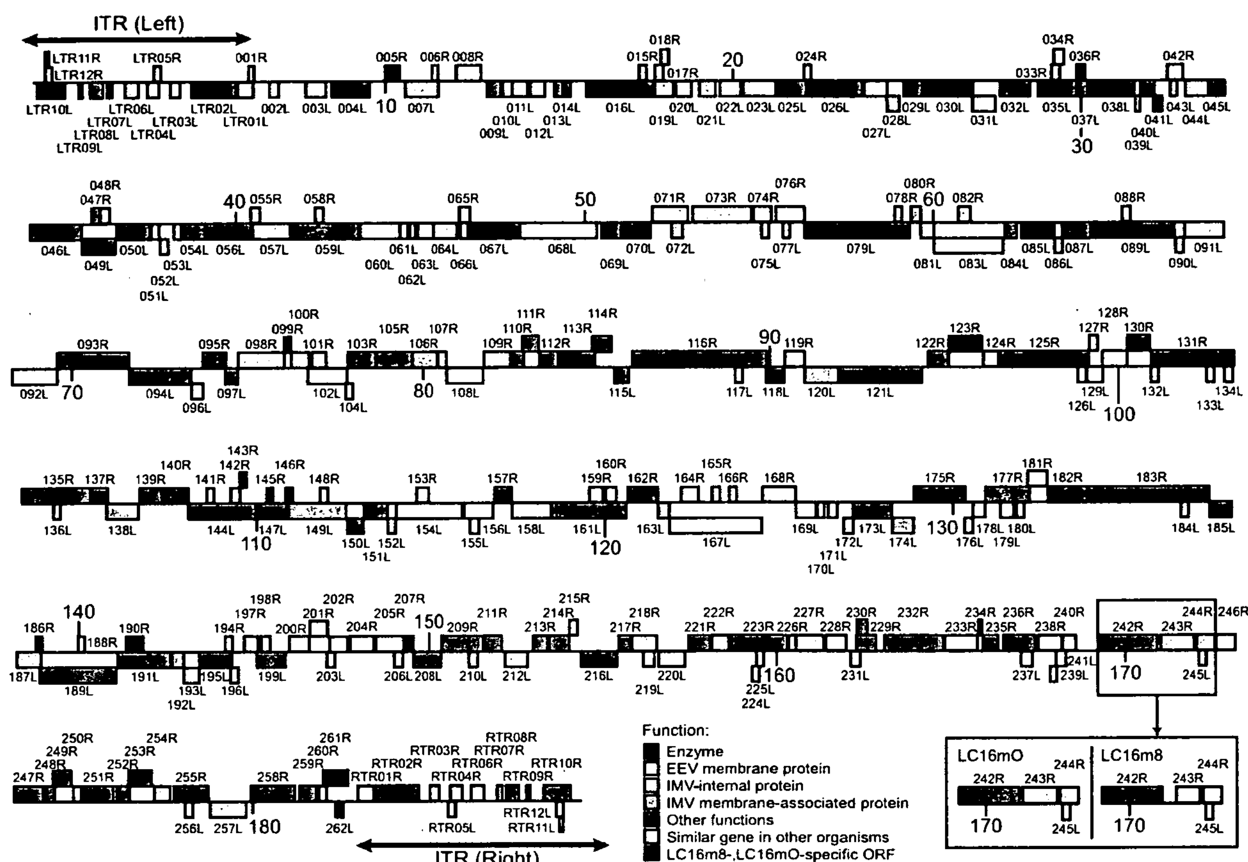

FIG. 1. ORF map of the LC16m8 and LC16mO strains. The ORFs transcribed rightward and leftward are presented above and below the horizontal centerlines, respectively. The major difference between the two strains is boxed. Putative functions of ORFs were evaluated or predicted by a BLAST search of the GenBank database and are expressed in different colors. The double-headed arrows indicate the regions of the ITRs of the left and right ends.

(OD_{405}) value of 0.2 was calculated from the average OD, plus three times the standard deviation, for five mock-immunized mouse sera.

Virus neutralization and comet inhibition assays. LO virus (100 PFU/100 μl determined on HeLa cells) was mixed with serially diluted mouse serum at 37°C for 1 h and then overnight at 4°C. HeLa cells in 24-well plates were infected with the serum-treated virus, cultured for 4 days, and stained with 0.1% crystal violet. The serum dilutions yielding a 50% plaque reduction were defined as IMV-neutralizing antibody titers. Comet-inhibiting activity in serum was examined as an indication of anti-EEV antibody responses (1). RK13 cells in 12-well plates were infected with IHD-J virus (100 PFU/well), incubated for 2 days in 2% FBS-Dulbecco's modified MEM containing mouse serum dilutions, and stained with crystal violet. The lengths of comets formed from primary plaques were measured under a microscope.

Histopathology and immunohistochemistry (IHC). The mouse nasal tissues were fixed in 10% buffered formalin and embedded in paraffin. Paraffin block sections were stained with hematoxylin and eosin (HE). VV antigens were immunohistochemically detected with a labeled-streptavidin-biotin complex staining system (DAKO). Rabbit polyclonal antibodies raised by LO infection were used as a primary antibody. A catalyzed signal amplification method (DAKO) was also used to detect VV antigens with enhanced sensitivity.

Nucleotide sequence accession numbers. The complete sequences of the vaccinia virus m8, mO, and LO strains have been deposited in GenBank under accession numbers AY678275, AY678277, and AY678276, respectively. The *env* gene sequences of IHD-J were deposited in DDBJ: A33R-A34R (accession no. AB191187), A36R (accession no. AB191188), A56R (accession no. AB191189), B5R (accession no. AB191190), and F13L (accession no. AB191191). As there were slight differences between the ListerVAX and compiled shotgun LO sequences, ListerVAX virus sequences were deposited in DDBJ as follows: B5R (accession no. AB191251), A56R (clone 1) (accession no. AB191252), and A56R (clone 3) (accession no. AB191253).

RESULTS

Complete genome sequences of m8, mO, and LO. Genomic DNA was prepared from purified m8, mO, and LO virions, shotgun sequenced, and confirmed by primer walking. As m8 and mO are clonal viruses, their genome sequences were easily assembled to 189,158 and 189,157 bp, respectively, and were analyzed with reference to the GenBank files, including the vaccinia virus CPN strain (21). Comparison of the m8 and mO genomes indicated that their gene structures and organizations were almost the same (Fig. 1 and Table 1). Notably, there were only six point mutations between m8 and mO (Fig. 2A). Three of them were in noncoding regions, probably in promoter regions. A single-amino-acid substitution was found in 4 ORFs out of 286 putative major, minor, and mini-ORFs: a T-to-G mutation caused the change from Ile to Leu in the LC16M098L (F12L for CPN) gene, and an A-to-T mutation caused the replacements of Thr with Ser in the LC16M105R (A ORF T for CPN) gene and Ser with Arg in the LC16M012L (A54L for CPN) gene. The most remarkable change was a deletion of G in the LC16M243R (B5R for CPN)

TABLE 1. ORF locations and features of the LC16m8 and LC16mO genomes

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	
LC16MLTR12R	300–503 (67)	–[c]		Hypothetical protein	Similar gene in other organisms	C ORF H	2e-36	CPN	C ORF H (2e-36)
LC16MLTR11R	307–420 (37)	–		Hypothetical protein	Similar gene in other organisms	C ORF G	4e-09	CPN	C ORF G (4e-09)
LC16MLTR10L	860–84 (258)	–		Major secreted protein	Other functions	VACWR001	e-113	WR	B29R (e-112)
LC16MLTR09L	1353–1249 (34)	–		Tumor necrosis factor receptor II fragment	Other functions	PredictadbyGaneMark	3e-17	CPN	PredictedbyGeneMark11 (3e-17)
LC16MLTR08L	1940–1572 (122)	–	L?	Tumor necrosis factor receptor II homologue	Other functions	VACWR004	4e-73	WR	C22L (3e-72)
LC16MLTR07L	2204–2058 (48)	–		K1R protein fragment	Other functions	VACWR005	4e-24	WR	PredictedbyGeneMark02 (5e-24)
LC16MLTR06L	2954–2568 (128)	–		Hypothetical protein	Similar gene in other organisms	VACWR007	4e-59	WR	C20L (1e-55)
LC16MLTR05R	3387–3599 (70)	–	L?	Hypothetical protein	Similar gene in other organisms	C ORF F	1e-29	CPN	C ORF F (1e-29)
LC16MLTR04L	3533–3204 (109)	–	L?,E	Hypothetical protein	Similar gene in other organisms	VACWR008	1e-62	WR	C19L (5e-57)
LC16MLTR03L	4141–3860 (93)	–		Hypothetical protein	Similar gene in other organisms	D4L	3e-41	Cowpox	PredictedbyGeneMark09 (3e-18)
LC16MLTR02L	5725–4475 (416)	–	L?	Host range protein	Other functions	C17L	0.0	CPN	C17L (0.0)
LC16M001R	6087–6242 (51)	–		Hypothetical protein	Similar gene in other organisms	TC18R	3e-65	Tian Tan	
LC16MLTR01L	6215–5772 (147)	–		Hypothetical protein	Similar gene in other organisms	C16L	4e-85	CPN	C16L (4e-85)
LC16M002L	6938–6669 (89)	–	L?	Hypothetical protein	Similar gene in other organisms	C15L	1e-35	CPN	C15L (1e-35)
LC16M003L	8281–7709 (190)	–		Hypothetical protein	Similar gene in other organisms	VACWR206	e-108	WR	C14L (3e-37)
LC16M004L	9505–8444 (353)	–	L?	Serine protease	Enzyme	C12L	0.0	CPN	C12L (0.0)
LC16M005R	9950–10372 (140)	–	L?	Growth factor	Other functions	MVA005R	3e-72	MVA	C11R (8e-69)
LC16M006R	11315–11512 (65)	–		Hypothetical protein	Similar gene in other organisms	C ORF E	e-14	CPN	C ORF E (e-14)
LC16M007L	11520–10525 (331)	–	L?	Hypothetical protein	Similar gene in other organisms	C10L	0.0	CPN	C10L (0.0)
LC16M008R	12034–12753 (239)	–	L?	Hypothetical protein	Similar gene in other organisms	C7R	e-105	Cowpox	
LC16M009L	13300–12826 (124)	–	L?	Interleukin 18 binding protein	Other functions	MVA008L	5e-64	MVA	
LC16M010L	13631–13359 (90)	–	E	Hypothetical protein	Similar gene in other organisms	ACAM3000_MVA_009	5e-50	ACAM3000	
LC16M011L	14072–13644 (142)	–	L?	Hypothetical protein	Similar gene in other organisms	ACAM3000_MVA_010	9e-80	ACAM3000	
LC16M012L	14574–14161 (137)	–	L?	Hypothetical protein	Similar gene in other organisms	VACWR015	5e-71	WR	
LC16M013L	15074–14841 (77)	–		Host range protein	Other functions	VACWR016	6e-41	WR	
LC16M014L	15311–15096 (11)	–	L?	Host range protein	Other functions	ACAM3000_MVA_013	9e-41	ACAM3000	
LC16M015R	17265–17477 (70)	–		Hypothetical protein	Similar gene in other organisms	C ORF D	8e-23	CPN	C ORF D (8e-23)
LC16M016L	17671–15767 (634)	–	L?,E	Host range protein	Other functions	C9L	0.0	CPN	C9L (0.0)
LC16M017R	17724–17972 (82)	–	L?	Hypothetical protein	Similar gene in other organisms	C ORF C	7e-33	CPN	C ORF C (7e-33)
LC16M018R	17697–18121 (74)	–		Hypothetical protein	Similar gene in other organisms	C ORF B	2e-37	CPN	C ORF B (2e-37)
LC16M019L	18247–17714 (177)	–	L?	Hypothetical protein	Similar gene in other organisms	VACWR020	e-102	WR	C8L (6e-99)
LC16M020L	18771–18319 (150)	–	L?	Hypothetical protein	Similar gene in other organisms	MVA018L	1e-88	MVA	C7L (2e-88)
LC16M021L	19455–19000 (151)	–	L?	Hypothetical protein	Similar gene in other organisms	MVA019L	6e-85	MVA	C6L (7e-85)
LC16M022L	20196–19582 (204)	–		Hypothetical protein	Similar gene in other organisms	C5L	e-120	CPN	C5L (e-120)
LC16M023L	21209–20259 (316)	–	L?,E	Hypothetical protein	Similar gene in other organisms	C4L	0.0	CPN	C4L (0.0)
LC16M024R	22010–22219 (69)	–	L?	Hypothetical protein	Similar gene in other organisms	C ORF A	2e-36	CPN	C ORF A (2e-36)
LC16M025L	22067–21276 (263)	–	L?	Complement regulatory protein	Other functions	C3L	e-159	CPN	C3L (e-159)
LC16M026L	23672–22134 (512)	–		Kelch-like protein	Other functions	C2L	0.0	CPN	C2L (0.0)
LC16M027L	24413–23739 (224)	–		Hypothetical protein	Similar gene in other organisms	C1L	e-120	CPN	C1L (e-120)

LC16M028L	24753–24400 (117)	-	L?	Hypothetical protein	Similar gene in other organisms	N1L	5e-66	CPN	N1L (5e-66)
LC16M029L	25416–24889 (175)	-		Putative alpha amanitin-sensitive protein	Other functions	N2L	e-100	CPN	N2L (e-100)
LC16M030L	26876–25458 (472)	-		Putative ankyrin isoform	Other functions	M1L	0.0	CPN	M1L (0.0)
LC16M031L	27516–26854 (220)	-	L?	Hypothetical protein	Similar gene in other organisms	M2L	e-132	CPN	M2L (e-132)
LC16M032L	28505–27651 (284)	-	E	Host range protein	Other functions	VACWR032	e-155	WR	K1L (e-153)
LC16M033R	29114–29359 (81)	-		Hypothetical protein	Similar gene in other organisms	K ORF A	4e-45	CPN	K ORF A (4e-45)
LC16M034R	29181–29483 (100)	-		Hypothetical protein	Similar gene in other organisms	K ORF B	1e-40	CPN	K ORF B (1e-40)
LC16M035L	29836–28727 (369)	-	L?,E	Serine protease inhibitor 3	Other functions	K2L	0.0	CPN	K2L (0.0)
LC16M036R	29843–30079 (78)	-	L?	Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M037L	30154–29888 (88)	-	L?,E	eIF-2 alpha protein	Other functions	MVA024L	2e-50	MVA	K3L (1e-49)
LC16M038L	31488–30214 (424)	-	L?,E	Phospholipase D-like protein	Enzyme	K4L	0.0	CPN	K4L (0.0)
LC16M039L	31649–31515 (44)	-		Hypothetical protein	Similar gene in other organisms	ACAM3000_MVA_026	9e-24	ACAM3000	
LC16M040L	32068–31664 (134)	-		Putative monoglyceride lipase	Enzyme	VACWR037	1e-72	WR	K5L (2e-60)
LC16M041L	32291–32037 (84)	-		Lysophospholipase-like protein	Enzyme	K6L	1e-45	CPN	K6L (1e-45)
LC16M042R	32430–32879 (149)	-	L?	Hypothetical protein	Similar gene in other organisms	K7R	2e-86	CPN	K7R (2e-86)
LC16M043L	32708–32514 (64)	-		Hypothetical protein	Similar gene in other organisms	K8	2e-21	WR	
LC16M044L	33624–32944 (226)	-		Hypothetical protein	Similar gene in other organisms	F1L	e-122	CPN	F1L (e-122)
LC16M045L	34079–33638 (147)	-	L?	dUTP pyrophosphatase	Enzyme	MVA030L	3e-76	MVA	F2L (4e-76)
LC16M046L	35545–34103 (480)	-	L	Kalch-like protein	Other functions	F3L	0.0	CPN	F3L (0.0)
LC16M047R	35827–36063 (78)	-		Ribonucleoside-diphosphate reductase	Enzyme	F ORF B	3e-40	CPN	F ORF B (3e-40)
LC16M048R	36075–36365 (96)	-		Hypothetical protein	Similar gene in other organisms	F ORF C	3e-55	CPN	F ORF C (3e-55)
LC16M049L	36515–35556 (318)	-	E	Ribonucleoside-diphosphate reductase	Enzyme	F4L	0.0	CPN	F4L (0.0)
LC16M050L	37512–36547 (321)	-	L?,E	Major membrane protein	Other functions	F5L	e-168	CPN	F5L (e-168)
LC16M051L	37766–37542 (74)	-	L?	Hypothetical protein	Similar gene in other organisms	MVA035L	5e-40	MVA	F6L (7e-40)
LC16M052L	38024–37782 (80)	-	E	Hypothetical protein	Similar gene in other organisms	MVA036L	3e-46	MVA	F7L (6e-43)
LC16M053L	38387–38190 (65)	-	L?	Hypothetical protein	Similar gene in other organisms	ACAM3000_MVA_037	9e-25	ACAM3000	F8L (3e-24)
LC16M054L	39085–38447 (212)	-	L	Putative membrane protein	Other functions	F9L	e-121	CPN	F9L (e-121)
LC16M055R	40370–40627 (85)	-	L?	Hypothetical protein	Similar gene in other organisms	F ORF D	1e-44	CPN	F ORF D (1e-44)
LC16M056L	40391–39072 (439)	-	L	Putative ser/thr protein kinase	Enzyme	F10L	0.0	CPN	F10L (0.0)
LC16M057L	41478–40414 (354)	-	L?,E	Hypothetical protein	Similar gene in other organisms	F11L	0.0	CPN	F11L (0.0)
LC16M058R	42203–42418 (71)	-	L?	Hypothetical protein	Similar gene in other organisms	F ORF E	2e-37	CPN	F ORF E (2e-37)
LC16M059L	43428–41521 (635)	-	L?	Putative EEV maturation protein	Other functions	F12L	0.0	CPN	F12L (0.0)
LC16M060L	44588–43470 (372)	-	L	Major envelope protein	EEV membrane protein	F13L	0.0	CPN	F13L (0.0)
LC16M061L	44827–44606 (73)	-	L?,E	Hypothetical protein	Similar gene in other organisms	MVA044L	3e-28	MVA	F14L (2e-27)
LC16M062L	45026–44877 (49)	-	L	Hypothetical protein	Similar gene in other organisms	PredictedbyGeneMark	7e-22	CPN	PredictedbyGeneMark04 (7e-22)

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] Name	BLASTP Score	Source	ORF corresponding to CPN
LC16M063L	45575–45099 (158)	–	L?,E	Hypothetical protein	Similar gene in other organisms	MVA045L	1e-78	MVA	F15L (6e-79)
LC16M064L	46277–45582 (231)	–	L?,E	Hypothetical protein	Similar gene in other organisms	MVA046L	e-122	MVA	F16L (e-121)
LC16M065R	46339–46644 (101)	–	L	Putative DNA-binding virion core protein	IMV internal protein	ACAM3000_MVA_047	8e-44	ACAM3000	F17R (2e-43)
LC16M066L	48586–46374 (70)	–		Hypothetical protein	Similar gene in other organisms	E ORF A	2e-27	CPN	E ORF A (2e-27)
LC16M067L	48080–46641 (479)	–	L?	Poly(A) polymerase large subunit	Enzyme	E1L	0.0	CPN	E1L (0.0)
LC16M068L	50290–48077 (737)	–		Hypothetical protein	Similar gene in other organisms	E2L	0.0	CPN	E2L (0.0)
LC16M069L	50989–50417 (190)	–		Double-stranded RNA-specific adenosine	Enzyme	MVA050L	2e-99	MVA	E3L (3e-99)
LC16M070L	51824–51045 (259)	–	L,E	DNA-directed RNA polymerese	Enzyme	E4L	e-139	CPN	E4L (e-139)
LC16M071R	51873–52898 (341)	–		Hypothetical protein	Similar gene in other organisms	E5R	0.0	CPN	E5R (0.0)
LC16M072L	52750–52430 (106)	–		Hypothetical protein	Similar gene in other organisms	E ORF B	4e-43	CPN	E ORF B (4e-43)
LC16M073R	53035–54738 (567)	–	L?	Hypothetical protein	Similar gene in other organisms	E6R	0.0	CPN	E6R (0.0)
LC16M074R	54805–55305 (166)	–	L	Hypothetical protein	Similar gene in other organisms	MVA054R	6e-89	MVA	E7R (7e-89)
LC16M075L	55236–55026 (70)	–		Hypothetical protein	Similar gene in other organisms	E ORF C	3e-38	CPN	E ORF C (3e-38)
LC16M076R	55430–56251 (273)	–	L?	Hypothetical protein	Similar gene in other organisms	MVA055R	e-161	MVA	E8R (e-160)
LC16M077L	55830–55630 (66)	–		Hypothetical protein	Similar gene in other organisms	E ORF D	5e-36	CPN	E ORF D (5e-36)
LC16M078R	58856–59053 (65)	–		Hypothetical protein	Similar gene in other organisms	E ORF E	2e-36	CPN	E ORF E (2e-36)
LC16M079L	59278–56258 (1006)	–	L,E	DNA-directed DNA polymerase	Enzyme	E9L	0.0	CPN	E9L (0.0)
LC16M080R	59310–59597 (95)	–	L	Putative redox protein	IMV membrane associated protein	MVA057R	2e-54	MVA	E10R (3e-53)
LC16M081L	59981–59592 (129)	–	L	Hypothetical protein	Similar gene in other organisms	MVA058L	3e-73	MVA	E11L (4e-73)
LC16M082R	60686–61033 (115)	–		Hypothetical protein	Similar gene in other organisms	E ORF F	3e-59	CPN	E ORF F (3e-59)
LC16M083L	61968–59968 (655)	–	E	Hypothetical protein	Similar gene in other organisms	O1L	0.0	CPN	O1L (0.0)
LC16M084L	62342–62016 (108)	–	L?	Glutaredoxin	Other functions	ACAM3000_MVA_061	8e-61	ACAM3000	O2L (1e-60)
LC16M085L	63426–62488 (312)	–	L,E	Putative DNA-binding virion care protein	Other functions	I1L	e-147	CPN	I1L (e-147)
LC16M086L	63654–63433 (73)	–	L	Hypothetical protein	Similar gene in other organisms	MVA063L	3e-28	MVA	I2L (4e-28)
LC16M087L	64464–63655 (269)	–	I	DNA binding phosphoprotein	Other functions	MVA064L	e-139	MVA	I3L (e-138)
LC16M088R	65372–65605 (77)	–		Hypothetical protein	Similar gene in other organisms	I ORF A	9e-34	CPN	I ORF A (9e-34)
LC15M089L	66862–64547 (771)	–	L?,E	Ribonucleoside-diphosphate reductase large subunit	Enzyme	I4L	0.0	CPN	I4L (0.0)
LC16M090L	67128–66889 (79)	–	L	Hypothetical protein	IMV membrane associated protein	I5L	3e-40	CPN	I5L (3e-40)
LC16M091L	68295–67147 (382)	–	L?	Hypothetical protein	Similar gene in other organisms	I6L	0.0	CPN	I6L (0.0)
LC16M092L	69559–68288 (423)	–	L	Hypothetical protein	IMV internal protein	I7L	0.0	CPN	I7L (0.0)
LC16M093R	69565–71595 (676)	–	I,L?	RNA helicase/NPH-I/NTPase II	Enzyme	I8R	0.0	CPN	I8R (0.0)
LC16M094L	73374–71599 (591)	–	L	Metalloprotease	Enzyme	G1L	0.0	CPN	G1L (0.0)
LC16M095R	73700–74362 (220)	–	L?	Putative transcriptional elongation factor	Other functions	G2R	e-127	CPN	G2R (e-127)
LC16M096L	73706–73371 (111)	–	L	Hypothetical protein	Similar gene in other organisms	G3L	2e-54	CPN	G3L (2e-54)
LC16M097L	74706–74332 (124)	–	L	Putative glutaredoxin	Other functions	MVA073L	3e-68	MVA	G4L (9e-69)
LC16M098R	74709–76013 (434)	–		Hypothetical protein	Similar gene in other organisms	G5R	0.0	CPN	G5R (0.0)

LC16M099R	76021–76212 (63)	–	L?,E	RNA polymerase	Enzyme	MVA075R	3e-26	MVA	Predicted by GeneMark05 (5e-26)
LC16M100R	76214–76711 (165)	–	I,L?	Hypothetical protein	Similar gene in other organisms	VACWR084	2e-96	WR	G6R (3e-95)
LC16M101R	76806–77204 (132)	–	L?,E	Hypothetical protein	Similar gene in other organisms	G ORF A	1e-60	CPN	G ORF A (1e-60)
LC16M102L	77791–76676 (371)	–	L	Putative virion core protein	IMV internal	G7L	0.0	CPN	G7L (0.0)
LC16M103R	77822–78604 (250)	–	I,L?	Late transcription factor	Other functions	G8R	e-151	CPN	G8R (e-151)
LC16M104L	77970–77752 (72)	–		Hypothetical protein	Similar gene in other organisms	G ORF B	3e-38	CPN	G ORF B (3e-38)
LC16M105R	78624–79646 (340)	–	L?	Myristytprotein	Other functions	G9R	0.0	CPN	G9R (0.0)
LC16M106R	79647–80399 (250)	–	L	Myristytated membrane protein	IMV membrane associated protein	L1R	e-142	CPN	L1R (e-142)
LC16M107R	80431–80688 (85)	–	E	Hypothetical protein	Similar gene in other organisms	MVA081R	2e-29	MVA	L2R (3e-29)
LC16M108L	81730–80678 (350)	–	L	Hypothetical protein	Similar gene in other organisms	L3L	0.0	CPN	L3L (0.0)
LC16M109R	81755–82510 (251)	–	L	Putative DNA-binding virion core protein	IMV internal protein	MVA083R	e-143	MVA	L4R (e-142)
LC16M110R	82520–82906 (128)	–	L	Putative membrane protein	Other functions	MVA084R	1e-60	MVA	L5R (2e-60)
LC16M111R	82863–83324 (153)	–	L	Dimeric Virion protein	Other functions	MVA085R	3e-82	MVA	J1R (9e-83)
LC16M112R	83340–83873 (177)	–	E	Thymidine kinase	Enzyme	J2R	2e-95	CPN	J2R (2e-95)
LC16M113R	83939–84940 (333)	–	L?,E	Poly(A) polymerase subunit	Enzyme	MVA087R	e-172	MVA	J3R (e-171)
LC16M114R	84855–85412 (185)	–	L?,E	DNA-directed RNA polymerase	Enzyme	J4R	e-104	CPN	J4R (e-104)
LC16M115L	85895–85494 (133)	–	L?	Membrane protein	Other functions	J5L	4e-69	CPN	J5L (4e-69)
LC16M116R	86002–89862 (1286)	–	L?,E	DNA-directed RNA polymerase subunit	Enzyme	J6R	0.0	CPN	J6R (0.0)
LC16M117L	89180–88965 (71)	–	L?	Hypothetical protein	Similar gene in other organisms	H ORF A	8e-36	CPN	H ORF A (8e-36)
LC16M118L	90374–89859 (171)	–	L	Tyrosine phosphatase	Enzyme	MVA091L	1e-91	MVA	H1L (6e-91)
LC16M119R	90388–90957 (189)	–		Hypothetical protein	Similar gene in other organisms	H2R	e-109	CPN	H2R (e-109)
LC16M120L	91934–90960 (324)	–	L	IMV membrane associated protein	IMV membrane associated protein	MVA093L	e-172	MVA	H3L (e-171)
LC16M121L	94322–91935 (795)	–	L	RNA polymerase-associated protein	Enzyme	H4L	0.0	CPN	H4L (0.0)
LC16M122R	94508–95119 (203)	–	L?	Late transcription factor	Other functions	MVA095R	1e-83	MVA	H5R (4e-83)
LC16M123R	95120–96064 (314)	–	L	DNA topoisomerase	Enzyme	H6R	0.0	CPN	H6R (0.0)
LC16M124R	96101–96541 (146)	–	L	Hypothetical protein	Similar gene in other organisms	MVA097R	6e-82	MVA	H7R (7e-82)
LC16M125R	96585–99119 (844)	–	L?,E	mRNA capping enzyme, large subunit	Enzyme	D1R	0.0	CPN	D1R (0.0)
LC16M126L	99049–98795 (84)	–		Hypothetical protein	Similar gene in other organisms	D ORF A	7e-43	CPN	D ORF A (7e-43)
LC16M127R	99133–99375 (80)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF B	1e-24	CPN	D ORF B (1e-24)
LC16M128R	99511–100224 (237)	–	L?	Structural protein	IMV Internal protein	VACWR108	e-141	WR	D3R (e-140)
LC16M129L	89518–99078 (146)	–	L?	Putative Virion protein	IMV internal protein	MVA099L	1e-81	MVA	D2L (2e-81)
LC16M130R	100224–100850 (218)	–	E	Uracil DNA glycosytase	Enzyme	MVA101R	e-124	MVA	D4R (e-123)
LC16M131R	100912–103269 (785)	–	L,E	Putative NTPase	Enzyme	D5R	0.0	CPN	D5R (0.0)
LC16M132L	101117–100908 (69)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF C	8e-26	CPN	D ORF C (8e-26)
LC16M133L	102713–102495 (72)	–		Hypothetical protein	Similar gene in other organisms	D ORF D	7e-38	CPN	D ORF D (7e-38)
LC16M134L	103247–103005 (80)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF E	3e-45	CPN	D ORF E (3e-45)
LC16M135R	103310–105223 (637)	–	L	Early transcription factor	Other functions	D6R	0.0	CPN	D6R (0.0)
LC16M136L	104388–104197 (63)	–		Hypothetical protein	Similar gene in other organisms	F-53	2e-21	WR	
LC16M137R	105250–105735 (161)	–	L	DNA-directed RNA polymerase subunit	Enzyme	MVA104R	2e-90	MVA	D7R (6e-91)
LC16M138L	106612–105698 (304)	–		Cell surface-binding protein	IMV membrane associated protein	VACWR113	e-161	WR	D8L (e-158)

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	
LC16M139R	106654–107295 (213)	–	E	MutT-like protein	Other functions	D9R	e-121	CPN	D9R (e-121)
LC16M140R	107292–108038 (248)	–	L	MutT-like protein	Other functions	VACWR115	e-144	WR	D10R (e-142)
LC16M141R	108556–108765 (69)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF F	4e-36	CPN	D ORF F (4e-36)
LC16M142R	109234–109506 (90)	–		Hypothetical protein	Similar gene in other organisms	D ORF G	8e-51	CPN	D ORF G (8e-51)
LC15M143R	109503–109688 (61)	–		Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M144L	109934–108039 (631)	–	L	Nucleoside triphosphate phosphohydrolase I, DNA helicase	Enzyme	D11L	0.0	CPN	D11L (0.0)
LC16M145R	110249–110437 (62)	–	L?	Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M146R	110794–111012 (72)	–	L?	Hypothetical protein	LC16m8, LC16mO specific gene				
LC16M147L	110832–109969 (287)	–	L,E	mRNA capping enzyme, small subunit	Enzyme	VACWR117	e-166	WR	D12L (e-165)
LC16M148R	111759–111993 (74)	–	L?	Hypothetical protein	Similar gene in other organisms	D ORF I	2e-43	CPN	D ORF I (2e-43)
LC16M149L	112518–110863 (551)	–	L?	Rifampicin resistance protein	IMV membrane associated protein	D13L	0.0	CPN	D13L (0.0)
LC16M150L	112994–112542 (150)	–	I,L	Late gene transactivator	Other functions	MVA111L	1e-84	MVA	A1L (5e-85)
LC16M151L	113689–113015 (224)	–	I,L?	Late gene transactivator	Other functions	A2L	e-131	CPN	A2L (e-131)
LC16M152L	113916–113586 (76)	–	L	Hypothetical protein	Similar gene in other organisms	MVA113L	6e-42	MVA	
LC16M153R	114510–114869 (119)	–		Hypothetical protein	Similar gene in other organisms	A ORF A	2e-69	CPN	A ORF A (2e-69)
LC16M154L	115865–113931 (644)	–	L?	Major care protein	IMV internal protein	A3L	0.0	CPN	A3L (0.0)
LC16M155L	116348–116088 (86)	–		Hypothetical protein	Similar gene in other organisms	A ORF B	e-24	CPN	A ORF B (e-24)
LC16M156L	116763–115918 (281)	–	L	Membrane associated core protein	IMV internal protein	A4L	e-116	CPN	A4L (e-116)
LC16M157R	116801–117295 (164)	–	L	DNA-directed RNA polymerase subunit	Enzyme	MVA116R	5e-72	MVA	A5R (6e-72)
LC16M158L	118410–117292 (372)	–	I,L?,E	Hypothetical protein	Similar gene in other organisms	A6L	0.0	CPN	A6L (0.0)
LC16M159R	119518–119904 (128)	–	L?	Hypothetical protein	Similar gene in other organisms	A ORF C	1e-68	CPN	A ORF C (1e-68)
LC16M160R	119986–120291 (101)	–	L?	Hypothetical protein	Similar gene in other organisms	A ORF D	3e-35	CPN	A ORF D (3e-35)
LC16M161L	120566–118434 (710)	–	L?	Early transcription factor	Other functions	A7L	0.0	CPN	A7L (0.0)
LC16M162R	120620–121486 (288)	–	E	Putative intermediate transcription factor	Other functions	MVA119R	e-165	MVA	A8R (e-164)
LC16M163L	121805–121479 (108)	–	L	Hypothetical protein	IMV membrane associated protein	VACWR128	6e-42	WR	A9L (3e-40)
LC16M164R	122149–122649 (166)	–		Hypothetical protein	Similar gene in other organisms	A ORF E	2e-82	CPN	A ORF E (2e-82)
LC16M165R	123031–123258 (75)	–		Hypothetical protein	Similar gene in other organisms	A ORF F	8e-39	CPN	A ORF F (8e-39)
LC16M166R	123525–123752 (75)	–		Hypothetical protein	Similar gene in other organisms	A ORF G	5e-43	CPN	A ORF G (5e-43)
LC16M167L	124481–121806 (891)	–	L	Major core protein	IMV internal protein	A10L	0.0	CPN	A10L (0.0)
LC16M168R	124496–125452 (318)	–	L	Hypothetical protein	Similar gene in other organisms	VACWR130	e-160	WR	A11R (e-159)
LC16M169L	126032–125454 (192)	–	L	Virion protein	IMV Internal protein	A12L	2e-79	CPN	A12L (2e-79)
LC16M170L	126268–126056 (70)	–	L	Putative IMV membrane protein	IMV membrane associated protein	A13L	2e-20	CPN	A13L (2e-20)
LC16M171L	126648–126376 (90)	–	L	Putative IMV membrane protein	IMV membrane associated protein	MVA125L	5e-44	MVA	A14L (6e-44)
LC16M172L	127100–126816 (94)	–	L,E	Hypothetical protein	Similar gene in other organisms	MVA126L	2e-52	MVA	A15L (3e-52)
LC16M173L	128217–127084 (377)	–	L?	Myristylprotein	Other functions	A16L	0.0	CPN	A16L (0.0)
LC16M174L	128831–128220 (203)	–	L	Putative phosphorylated IMV membrane protein	IMV membrane associated protein	A17L	6e-86	CPN	A17L (6e-86)