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Induction of Protective Immunity in Animals Vaccinated with Recombinant Vaccinia Viruses That Express PreM and E Glycoproteins of Japanese Encephalitis Virus

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A cDNA clone representing the genome of structural proteins of Japanese encephalitis virus (JEV) was inserted into the thymidine kinase gene of vaccinia virus strains LC16mO and WR under the control of a strong early-late promoter for the vaccinia virus 7.5-kilodalton polypeptide. Indirect immunofluorescence and fluorescence-activated flow cytometric analysis revealed that the recombinant vaccinia viruses expressed JEV E protein on the membrane surface, as well as in the cytoplasm, of recombinant-infected cells. In addition, the E protein expressed from the JEV recombinants reacted to nine different characteristic monoclonal antibodies, some of which have hemagglutination-inhibiting and JEV-neutralizing activities. Radioimmunoprecipitation analysis demonstrated that two major proteins expressed in recombinant-infected cells were processed and glycosylated as the authentic PreM and E glycoproteins of JEV. Inoculation of rabbits with the infectious recombinant vaccinia virus resulted in rapid production of antiserum specific for the PreM and E glycoproteins of JEV. This antiserum had both hemagglutination-inhibiting and virus-neutralizing activities against JEV. Furthermore, mice vaccinated with the recombinant also produced JEV-neutralizing antibodies and were resistant to challenge with JEV.

Japanese encephalitis (JE) is an acute viral encephalitis that is a serious public health problem for humans (19, 32). The etiologic agent, JE virus (JEV), is a flavivirus (27) composed of three structural proteins, capsid (C), membrane (M), and envelope (E) proteins (11, 30), and several nonstructural proteins. The C protein of about 14 kilodaltons (kDa) constitutes a nucleocapsid with the positive-strand RNA genome. Two envelope proteins are a nonglycosylated M protein of about 8 kDa and an E glycoprotein of about 53 kDa (30). A glycoprotein of about 19 kDa, a precursor to M (PreM), has also been found in JEV-infected chicken embryo cells (31). These structural proteins appear to derive from a polyprotein by its proteolytic processing (18, 35).

An effective inactivated JEV vaccine has been used for over 2 decades in Japan, Taiwan, and Korea, and JE has been considerably well controlled in these countries (24). In the endemic area, however, JE is still a serious problem for humans and cattle, and a safe, effective, low-cost vaccine is needed. The development of a new vaccine may be made possible by understanding of the mechanism of protection against the pathogenic agents. In flaviviruses, the E protein appears to play an important role in inducing protective immunity against flavivirus infection in animals as well as humans (28). Previous studies have suggested that the E protein of flaviviruses possesses at least three antigenic determinants; they are flavivirus cross-reactive, subgroup specific, and serotype specific (37, 38). Monoclonal antibodies to the JEV E glycoprotein have been characterized (7) and divided into nine groups (9, 10) based on hemagglutination inhibition (HI), neutralization, and reactivity to virions. These findings suggest that expression of the E protein of JEV in a biologically active form may be most conducive to the development of a new JE vaccine.

Nucleotide sequence analysis of the cDNA of JEV (18, 35, 36) has suggested that, as in other flaviviruses, the genome of JEV encodes one continuous open reading frame and that the immunologically important structural proteins are coded at 5′ termini of the genome. These findings enabled us to express the structural proteins of JEV by recombinant DNA techniques for new vaccine development. One expression vector, vaccinia virus, provides a eucaryotic vector system and opens up the possibility of using a recombinant as a novel live vaccine (6, 22, 25, 26, 33). Successful expression, immunization, and protection with infectious recombinant vaccinia viruses containing foreign genes have been reported (1, 15, 23, 40). We report here recombinant vaccinia viruses containing a cDNA that encodes the PreM and E glycoproteins of JEV. These recombinant vaccinia viruses efficiently expressed the PreM and E glycoproteins in a biologically active form in vitro and in vivo and induced both neutralizing antibodies and protection from JEV infection in vaccinated animals.

MATERIALS AND METHODS

Cells and viruses. RK13, Vero, and human 143TK$^-$ cells were grown in Eagle minimal essential medium supplemented with 5% fetal calf serum (FCS). Vaccinia virus strains WR and LC16mO (34) and their recombinants WRJ6 and mOJ6, respectively, were propagated with RK13 cells and purified from cytoplasmic extracts.

DNA manipulations. Plasmid DNA manipulations were performed essentially as summarized by Maniatis et al. (16), except that hybridization was carried out in a solution containing 6× SSC (1× SSC is 15 mM sodium citrate plus

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150 mM sodium chloride), 0.5% sodium dodecyl sulfate (SDS), and 2% nonfat dry milk. DNA sequencing analysis was done by the dideoxy-chain termination method described by Sanger et al. (29). Recombinant plasmids containing the cDNA of the Sagayama strain of JEV were constructed by insertion of cDNA into the *Pst*I site of pUC9 (K. Yasui et al., unpublished data). Two clones, pJE4118 and pJE2-20, were used to construct pBRJE203 containing about 2.9 kilobase pairs of cDNA that encodes from the middle of the C protein to the NS1 protein.

All restriction and modification enzymes and a random-primed labeling (5) kit were purchased from Boehringer Mannheim Biochemicals.

Antibodies. Rabbit antiserum and a panel of nine different kinds of monoclonal antibodies to the E protein were used to detect JEV-specific proteins throughout this work. Preparation and characterization of these monoclonal antibodies were described elsewhere (9, 10). Anti-JEV serum was prepared by repeated immunization of a rabbit with JEV purified from the brain homogenates of infected suckling mice by sequential centrifugation in discontinuous and linear density gradients.

Construction of recombinant vaccinia viruses. RK13 cells infected with wild-type vaccinia virus were transfected with calcium phosphate-precipitated plasmid DNA as already described (14). Thymidine kinase-negative (TK^-) viruses were isolated by plaque assay on $143TK^-$ cells in 5-bromo-2′-deoxyuridine at 25 μg/ml. TK^- viruses were amplified through RK13 cells and screened for recombinants by dot hybridization procedures (14). Recombinant vaccinia viruses of interest were subjected to plaque purification until more than 200 plaques on the infected RK13 cell monolayers were all shown to hybridize with a JEV-specific cDNA probe.

Labeling of proteins and immunoprecipitation. To prepare [^{35}S]methionine-labeled proteins, Vero cells infected with JEV or vaccinia viruses were incubated for 1 h in methionine-free minimal essential medium supplemented with 0.5% FCS and then labeled for 16 h with 100 μCi of [^{35}S] methionine per ml (1,150 Ci/mmol; Dupont, NEN Research Products) in methionine-free minimal essential medium supplemented with 2% FCS. The cells were washed three times with phosphate-buffered saline, harvested, and solubilized in 200 μl of lysing buffer (50 mM Tris hydrochloride [pH 7.5], 150 mM NaCl, 0.1% aprotinin [Sigma Chemical Co., St. Louis, Mo.], 0.5% Nonidet P-40). [^{14}C]glucosamine-labeled materials were prepared by a slight modification of the method described by Mackett et al. (14). Briefly, Vero cells infected with JEV or vaccinia viruses were incubated for 1 h in minimal essential medium supplemented with 2% FCS. The cells were washed with phosphate-buffered saline and incubated in glucose-deficient RPMI medium containing 0.1% fructose, 1.7% FCS, and 25 μCi of [^{14}C]glucosamine per ml (54 Ci/mmol; Dupont, NEN Research Products) with or without 1 μg of tunicamycin per ml. Labeled proteins of vaccinia virus- or JEV-infected cells were prepared 16 and 40 h later, respectively, in 40 μl of lysing buffer as described above.

Immunoprecipitation was performed as follows. A portion of radiolabeled material was incubated with 40 μl of nonimmune rabbit serum for 1 h on ice and adsorbed with 40 μl of protein A-Sepharose (Pharmacia) for 1 h at 4°C. The unbound materials were incubated with rabbit anti-JEV serum for 1 h on ice and then adsorbed with protein A-Sepharose for 1 h. The reaction mixture was centrifuged, and the pellet was washed, suspended in Laemmli electrophoresis loading buffer (12), and boiled for 5 min. The supernatants eluted from Sepharose were subjected to SDS-polyacrylamide gel electrophoresis, and the gels were dried and exposed to Hyperfilm, β-max, or ^{3}H (Amersham).

Detection of antigens. For detection of vaccinia virus- or JEV-specific antigens in vitro, subconfluent Vero cells grown on glass slides were infected with vaccinia viruses or JEV and cultured for 16 or 30 h, respectively, and acetone fixed. Viral antigens were detected by indirect immunofluorescence by using rabbit antiserum to vaccinia virus or JEV and a panel of nine different kinds of monoclonal antibodies to the E protein of JEV (7, 9).

Virus antigens on the cell surface were identified by fluorescence-activated flow cytometric analysis. Vero cell monolayers infected with vaccinia viruses or JEV were incubated for 14 or 24 h, respectively, washed three times with phosphate-buffered saline, and harvested. The cells were reacted with the appropriate antibodies diluted 1:100 for 45 min at 4°C. After being washed three times with phosphate-buffered saline containing 2% FCS, the cells were incubated with fluorescein-conjugated goat anti-rabbit immunoglobulin G (TAGO Inc., Burlingame, Calif.) or goat anti-mouse immunoglobulin G (TAGO) for 45 min at 4°C. The cells were washed and analyzed on an Epics PROFILE (Coulter Electronics, Inc., Hialeah, Fla.) flow cytometer.

To detect the JEV antigen expressed by JEV recombinant vaccinia virus in vivo, guinea pigs (Hartley strain; body weight, 250 g) were inoculated intradermally on the back with 3×10^7 PFU of either WR or WRJ6. After 3 days, a frozen section of the pock region was acetone fixed and incubated with anti-vaccinia virus- or anti-JEV hyperimmune serum (generously provided by K. Kitamura and A. Oya, respectively, National Institute of Health, Tokyo, Japan). Fluorescein-conjugated goat anti-rabbit immunoglobulin G (TAGO) was used to detect viral antigens.

Immunization and challenge. Female rabbits were vaccinated intradermally with 4×10^8 PFU of mOJ6 and bled at various times postvaccination. Antiserum specificity was examined by immunoprecipitation as described above by using antiserum taken from the rabbit at 8 days postinoculation with recombinant vaccinia virus. HI (20) and neutralization (8) tests against JEV were performed as previously described.

For the protection study, 4-week-old female C3H/He mice were used. Two groups of 20 mice were vaccinated subcutaneously with 3.5×10^7 PFU of either WR or WRJ6. After 2 weeks, half of the mice of the groups were bled for examination of JEV-neutralizing antibodies. The remaining mice were challenged intravenously with a lethal dose (3×10^2 PFU) of JEV and observed for 2 weeks.

RESULTS

Construction of recombinant plasmids and vaccinia viruses. Insertion plasmid pAK8 was constructed to facilitate expression of the JEV gene (Fig. 1A). Fifty-base synthetic nucleotides were ligated into the *Bam*HI site of pAK2, which contained the early-late promoter of the vaccinia virus 7.5-kDa polypeptide ($P_{7.5}$; 3, 13) in the *Eco*RI site of the *tk* region of the vaccinia virus genome. The synthetic DNA was designed to have the first ATG codon in its *Nco*I site downstream from the transcription start site (13) of $P_{7.5}$. It also has a cluster of termination codons of translation, thereby avoiding addition of any peptides behind the JEV polypeptide (Fig. 1B).

A 2.4-kilobase-pair fragment of JEV cDNA was prepared from pBRJE203 by *Hae*II digestion, DNA polymerase I

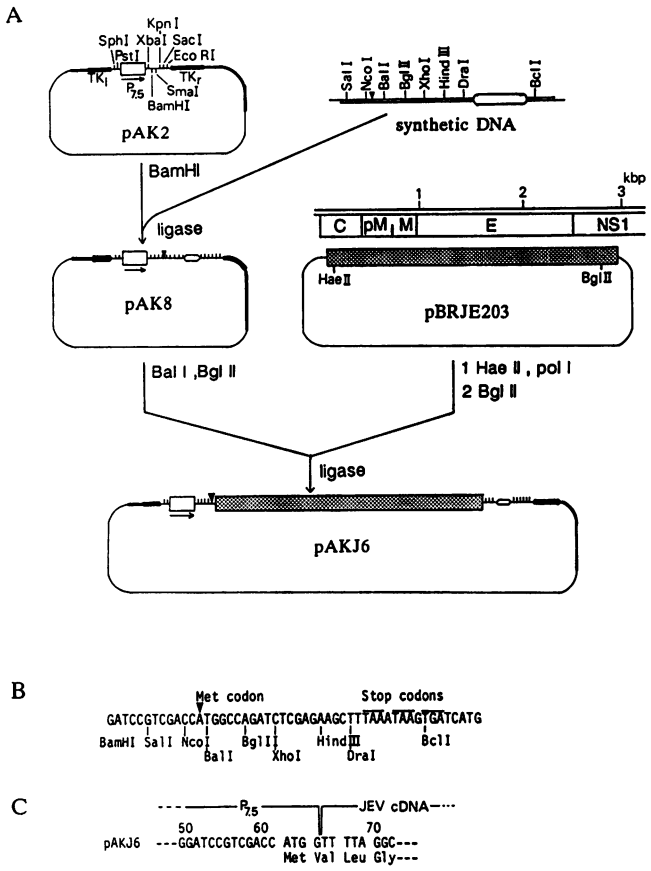

FIG. 1. (A) Construction of insertion plasmid pAKJ6. Plasmid vector pAK2 (A. Yasuda and A. Kojima, unpublished data), in which the 7.5-kDa vaccinia virus promoter ($P_{7.5}$) flanked by polylinker sequences had been inserted into the thymidine kinase (TK) gene (shown by the thick line and indicated by TK_l and TK_r) of vaccinia virus, was modified to pAK8 by insertion of a 50-base-pair synthetic polylinker sequence containing the translation initiation codon (solid triangle) and a cluster of translation termination codons (open oval). The cDNA of JEV in pBRJE203 (stippled box) and the corresponding partial genomic organization are shown. An about 2.4-kilobase-pair cDNA fragment of JEV was prepared by restriction with *Hae*II and treatment with DNA polymerase I, followed by *Bgl*II digestion, and inserted into pAK8 cleaved with *Bal*I and *Bgl*II to produce pAKJ6. Other symbols: thin line, pUC19 or pBR322 sequence; intermediate-thickness line, vaccinia virus DNA flanking the *tk* sequence; arrow, direction of transcription of $P_{7.5}$. The PreM protein is divided into pM and M. (B) Nucleotide sequence of a positive 50-base strand of synthetic oligonucleotides. Restriction sites, translation initiation codon ATG (▼), and translation stop codons are indicated. (C) Nucleotide and deduced amino acid sequences around the translation initiation codon identified by the dideoxy-chain termination method (29). The nucleotide number from the transcription site of $P_{7.5}$ (3) is also shown.

treatment, and subsequent *Bgl*II digestion. This sequence was inserted into the *Bal*I and *Bgl*II sites of pAK8 (Fig. 1A). Nucleotide sequence analysis revealed that the resulting plasmid, pAKJ6, had the ATG codon of the *Nco*I site in frame with the JEV cDNA sequence (Fig. 1C).

RK13 cells infected with vaccinia virus WR or LC16mO were transfected with pAKJ6. Recombinant vaccinia viruses WRJ6 and mOJ6 were isolated, plaque purified, and propagated. The genomic structures of WRJ6 and mOJ6 were examined by ethidium bromide staining analysis and blot analysis, and it was confirmed that the JEV cDNA was correctly integrated into the *tk* gene of the vaccinia virus genome and that no rearrangement or deletion had occurred during construction of the recombinant vaccinia viruses (data not shown).

Detection of JEV protein expressed by recombinants. Synthesis of JEV-specific protein by mOJ6 and WRJ6 was initially studied by indirect immunofluorescence. When acetone-fixed cells were treated with rabbit anti-JEV serum, the intensity of fluorescence of Vero cells infected with either WRJ6 or mOJ6 (Fig. 2B and C) was comparable to that of JEV-infected cells (Fig. 2A). Wild-type vaccinia virus-infected cells were not stained with this serum (Fig. 2D). Fluorescence-activated flow cytometric analysis was performed to examine the surface expression of JEV protein (Fig. 3). When JEV- and mOJ6-infected Vero cells were reacted with anti-JEV serum (Fig. 3B and C) or monoclonal antibody N.04 (Fig. 3E and F), a significant increase in relative fluorescence intensity was observed compared with cells infected with LC16mO and treated with these antibodies (Fig. 3A and D) or compared with mock-infected Vero cells (data not shown). These data showing that the JEV E protein was expressed on the surface of cells infected with the recombinant virus or JEV were essentially consistent with the results described by Matsuura et al. (17).

Nine different kinds of monoclonal antibodies to the JEV E protein were provided for further characterization of the products by the recombinant viruses (Table 1). Dilutions of monoclonal antibodies were determined to give fluorescence intensities similar to that of JEV-infected cells. They were then used at the indicated dilutions for comparison of fluorescence between JEV- and recombinant virus-infected cells. Table 1 summarizes the five independent results. Recombinant virus-infected cells reacted to four of nine monoclonal antibodies with intensities similar to that of JEV-infected cells. The other monoclonal antibodies, however, provided weaker fluorescence in recombinant virus-infected cells than in JEV-infected cells.

These results indicate that the JEV E protein was successfully expressed and transported to the recombinant vaccinia virus-infected cell surface, where antigenic determinants were confirmed as being recognized with monoclonal antibodies.

Characterization of the protein expressed by recombinants. The cDNA inserted into the vaccinia virus genome encodes an 86-kDa polyprotein. Immunoprecipitation analysis was used to determine which protein products were translated. The effects of tunicamycin and endoglycosidases on recombinant virus-derived proteins labeled with [^{35}S]methionine were investigated. In cells infected with mOJ6, two bands of 53 and 24 kDa which showed the same mobilities as authentic E and PreM proteins were immunoprecipitated (data not shown). Tunicamycin treatment and glycopeptidase F digestion reduced the mobilities of these proteins to about 51 and 21 kDa, respectively. To obtain direct evidence that the E protein expressed by the recombinant viruses was glycosylated, mOJ6-infected cells were labeled with [^{14}C]glucosamine with or without tunicamycin. Cell lysates were immunoprecipitated with rabbit anti-JEV serum and analyzed by SDS-polyacrylamide gel electrophoresis. Two bands, one at 53 kDa and the other at 24 kDa, corresponding to the E and PreM glycoproteins of JEV (Fig. 4, lane 5), respectively, were detected in recombinant virus-infected cell lysates (lane 3). The bands disappeared in tunicamycin (lanes 4 and 6), suggesting that the recombinant vaccinia viruses expressed not only the E protein but also the PreM protein in

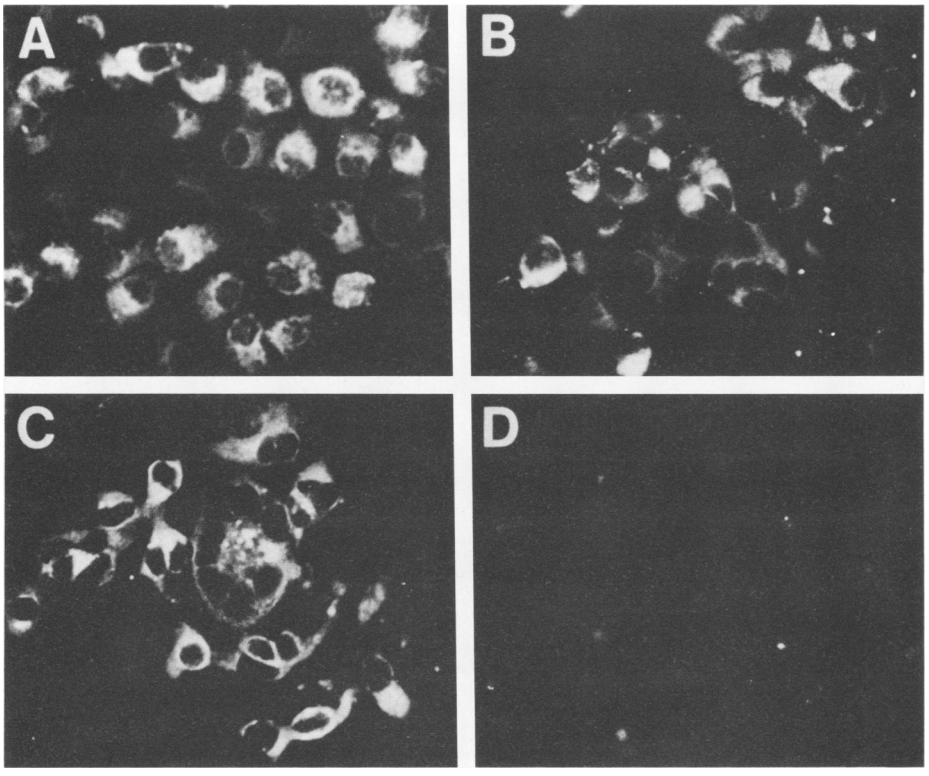

FIG. 2. Detection of JEV antigens in recombinant virus-infected cells. Vero cells were infected with JEV (A), WRJ6 (B), mOJ6 (C), and wild-type vaccinia virus (D). Antigens of acetone-fixed cells were detected by indirect immunofluorescence with anti-JEV serum.

the N-glycosylated form as JEV did. The amounts of PreM and E glycoproteins in recombinant virus-infected cells were less than those in JEV-infected cells (lanes 3 and 5).

Detection of JEV antigens in the dermis of guinea pigs inoculated with recombinant virus. Guinea pigs were inoculated intradermally on the back with WR or WRJ6 to determine whether the recombinant vaccinia virus also expressed JEV antigens in vivo. The viral antigens were detected by indirect immunofluorescence in frozen sections of skin taken 3 days after inoculation. When the section of the skin injected with WRJ6 was treated with anti-vaccinia virus serum, strong fluorescence was observed at the follicular epithelium (Fig. 5A). Expression and distribution of JEV antigens were similar to those of vaccinia virus antigens when a serial section of the skin was stained with anti-JEV serum (Fig. 5B). In contrast, in the skin of a guinea pig inoculated with WR, JEV antigens were not detected, while strong fluorescence of vaccinia virus antigens was observed (data not shown).

Induction of neutralizing and HI antibodies and protective immunity to JEV in vaccinated animals. Two rabbits were vaccinated intradermally with 4×10^8 PFU of mOJ6, and induction of antibodies to JEV and vaccinia virus was determined by immunoprecipitation, indirect immunofluorescence, HI, and neutralization assays. A rabbit produced antibodies capable of immunoprecipitation of PreM and E glycoproteins from JEV-infected cell lysates (Fig. 6). No detectable C or NS1 proteins were precipitated with this serum. High levels of HI and neutralization titers against JEV were induced in the serum of the rabbit (Table 2). HI and neutralizing antibody titers were highest on day 8 and then retained substantial levels for at least 3 months. Indirect immunofluorescence assays of serum samples revealed that

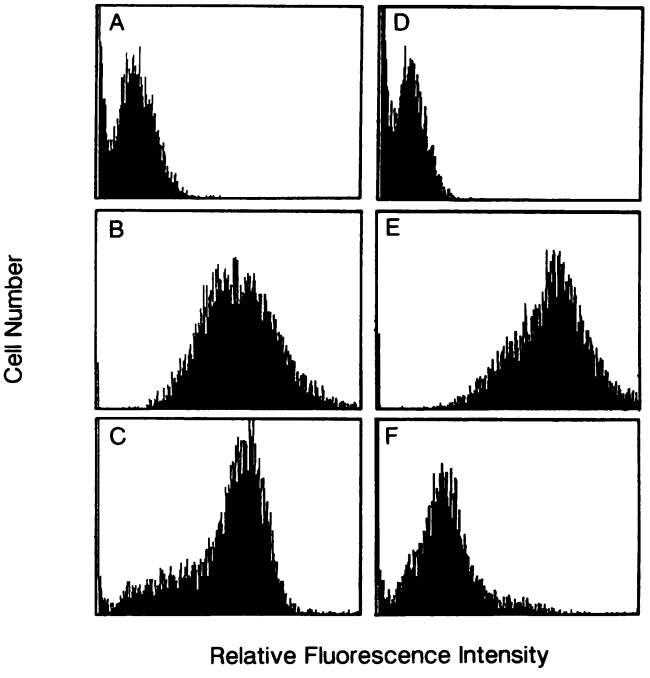

FIG. 3. Fluorescence-activated flow cytometric analysis of JEV antigens on Vero cells infected with LC16mO (A and D), JEV (B and E), or mOJ6 (C and F). Virus-infected cells were reacted with anti-JEV serum (A to C) or monoclonal antibody N.04 (D to F), incubated with a fluorescein-conjugated goat anti-rabbit or anti-mouse secondary antibody, and analyzed on an Epics PROFILE flow cytometer.

TABLE 1. Characterization of JEV proteins expressed by recombinant vaccinia virus with JEV-specific antibodies

MAb[a] (group) or antiserum	Characterization of MAb[b]			Relative immunofluorescent intensity of cells infected with[c]:		
	Cross-reactivity	Biological function	Dilution[d]	JEV	mOJ6	LC16mO
MAbs						
301 (1)	Flavivirus	HI	40	++	+	−
109 (2)	Subgroup	HI	80	++	+	−
112 (3)	JEV specific		80	++	+	−
503 (8)	JEV specific	N	80	++	+	−
N.04 (7)	Subgroup	HI, N	160	++	++	−
201 (6)	Subgroup		160	++	++	−
204 (5)	Subgroup		40	++	++	−
203 (4)	Subgroup		40	++	++	−
504 (9)	Flavivirus		80	++	+	−
Antisera						
Anti-JEV			150	+++	++	−
Anti-vaccinia virus			300	ND[e]	+++	+++

[a] MAb, Monoclonal antibody.
[b] The monoclonal antibodies used have already been described (7, 10). Specificity and biological activity against JEV (N, neutralization) are shown.
[c] +, Weak; ++, intermediate; +++, strong; −, no fluorescence.
[d] Reciprocal dilutions of monoclonal antibodies were determined to give similar intensities of fluorescence in JEV-infected cells.
[e] ND, Not determined.

TABLE 2. Antibody responses of a rabbit vaccinated with recombinant vaccinia virus mOJ6[a]

Time postvaccination	Antibody titer			
	Anti-vaccinia virus (IF)	Anti-JEV		
		IF	HI (log_2)	N (log_{10})
Preimmune	<40	<40	<1	<0.6
Day				
5	1,280	1,280	9	ND[b]
8	2,560	2,560	11	3.9
12	5,120	2,560	10	ND
15	5,120	2,560	10	ND
Week				
3	5,120	1,280	10	3.3
4	5,120	1,280	10	ND
6	2,560	640	10	ND
Month				
2	2,560	640	9	3.0
3	2,560	320	9	3.2
12	1,280	320	ND	ND
17	640	320	ND	ND

[a] A rabbit was vaccinated intradermally with 4×10^8 PFU of mOJ6 and bled at the times indicated. Reciprocal titers of antibodies in serum were determined by indirect immunofluorescence (IF), HI, or neutralization (N) assays. Neutralization titers against JEV were determined by 50% plaque reduction assays.
[b] ND, Not determined.

high titers of antibodies to JEV were maintained for more than 1 year in the rabbit (Table 2). A low but significant level of HI antibody was detected in the serum of another rabbit on day 8, although high levels of anti-vaccinia virus antibodies were detected and maintained in the serum of that rabbit (data not shown).

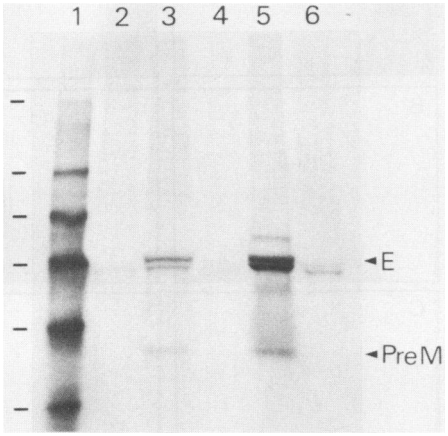

FIG. 4. Synthesis of JEV proteins by recombinant vaccinia viruses. Vero cell monolayers were infected with LC16mO (lane 2), mOJ6 (lanes 3 and 4), or JEV (lanes 5 and 6) and labeled with [^{14}C] glucosamine with (lanes 4 and 6) or without (lanes 2, 3, and 5) tunicamycin. Radiolabeled glycoproteins were immunoprecipitated with anti-JEV rabbit serum, and immune precipitates were resolved by SDS-polyacrylamide gel electrophoresis and autoradiographed. Lane 1 contained ^{14}C-labeled protein size markers. Positions of protein size markers are indicated on the left and (from the top) correspond to 200, 92.5, 69, 46, 30, and 14.3 kDa. Positions of JEV proteins are indicated at the right.

Inoculation of C3H/He mice with WRJ6 also resulted in rapid induction of neutralizing antibodies (Table 3). All mice vaccinated with WRJ6 were protected against challenge with JEV. In contrast, control mice and mice inoculated with wild-type vaccinia virus did not develope neutralizing antibodies, and almost all of them died of encephalitis during the 2 weeks after challenge with JEV.

DISCUSSION

The studies reported here demonstrated that the E protein of JEV could be expressed in biologically active form both in vitro and in vivo by recombinant vaccinia viruses.

WRJ6 and mOJ6 contain JEV cDNA that encodes a 790-amino-acid polyprotein (amino acids 65 to 854; 35). Two glycoproteins, E and PreM, were produced in recombinant virus-infected Vero cells. Investigators who studied the amino acid sequences of structural proteins C, M, and E of JEV (18) have suggested that the signal sequence motifs lie at the carboxyl-terminal ends of the sequences and therefore

TABLE 3. Protection of mice against challenge with JEV after vaccination with recombinant vaccinia virus WRJ6[a]

Vaccination	No. of mice that survived/no. tested	% Survival	Geometric mean neutralization antibody titer (log_{10})[b]
None	2/10	20	<0.6
WR	2/10	20	<0.6
WRJ6	11/11	100	2.2

[a] C3H/He mice 4 weeks old were vaccinated subcutaneously with 3.5×10^7 PFU of wild-type or recombinant vaccinia virus. At 2 weeks postvaccination, half of the mice in each group were challenged intravenously with 3×10^2 PFU of JEV per mouse and observed for 2 weeks.
[b] Neutralization antibody titers against JEV were determined by 50% plaque reduction.

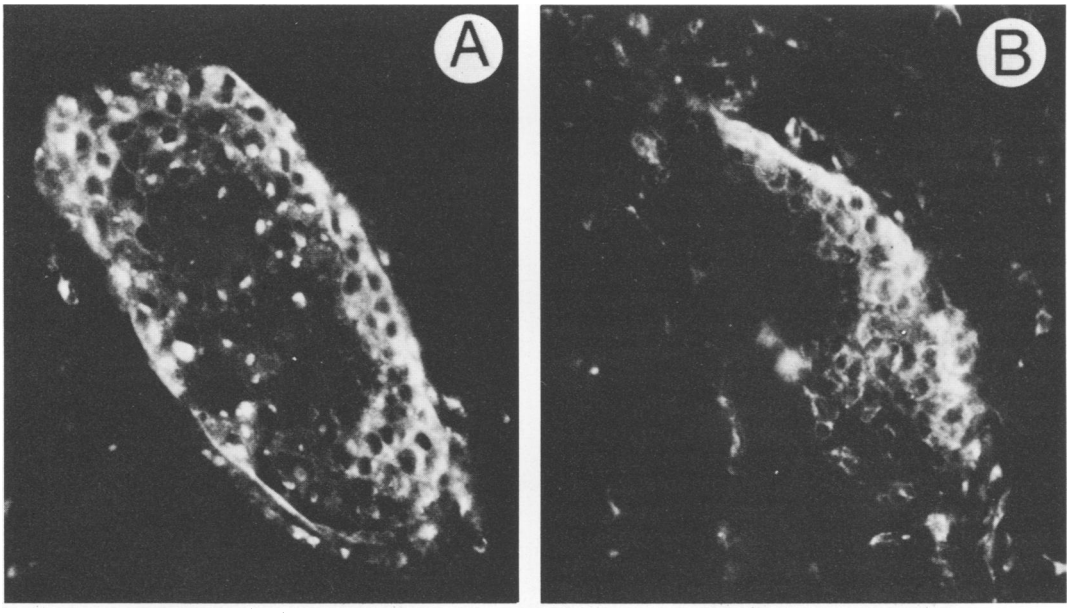

FIG. 5. Detection of vaccinia virus (A) and JEV (B) antigens in the pock region of a guinea pig inoculated with WRJ6. Viral antigens were detected by indirect immunofluorescence with anti-vaccinia virus (A) and anti-JEV (B) sera.

a cellular signalase might partly be responsible for the proteolytic cleavage of these structural proteins. The maturation of PreM and E proteins in cells infected with JEV recombinant vaccinia virus appears to occur by a similar processing mechanism. Similar expression and maturation of dengue virus proteins by recombinant vaccinia virus have been reported (2, 4, 41). We observed a band of about 8 kDa in recombinant virus-infected cells labeled with [^{35}S]methionine (data not shown). However, we could not verify that this molecule corresponds to M or to a fragment of C or NS1 protein, because the polypeptides encoded by the cDNA inserted into the vaccinia virus genome were similar in size.

E protein expressed by JEV recombinant vaccinia virus was shown to conform to biologically active epitopes of JEV by immunofluorescence analyses. The reasons for a slight difference in the reactivities of the epitopes to a panel of monoclonal antibodies in recombinant virus- and in JEV-infected cells are not clear. One possible explanation is that another condition, such as polymerization of the JEV proteins, may be needed to make all epitopes of the E protein like the authentic epitope.

A single inoculation with mOJ6 induced rapid production of HI antibody in two rabbits. Similar rapid induction of antibodies to hepatitis B virus surface antigen in rabbits inoculated with recombinant vaccinia viruses that express hepatitis B virus surface antigen has been demonstrated (39). Rabbit A then possessed high titers of both HI and neutralizing antibodies to JEV for at least 3 months. Unexpectedly, we could not detect HI antibody in rabbit B serum after 15 days postinoculation. The basis of the variable antibody responses of outbred rabbits is not well understood; we have also observed similar variable responses to hepatitis B virus surface antigen in rabbits inoculated with recombinant vaccinia viruses that express hepatitis B virus surface antigen (39; unpublished data). Inoculation of mice with WRJ6 induced high levels of JEV-neutralizing antibodies and protected mice from death due to encephalitis caused by JEV infection. Therefore, it is concluded that the E protein expressed from recombinant vaccinia virus conserves the

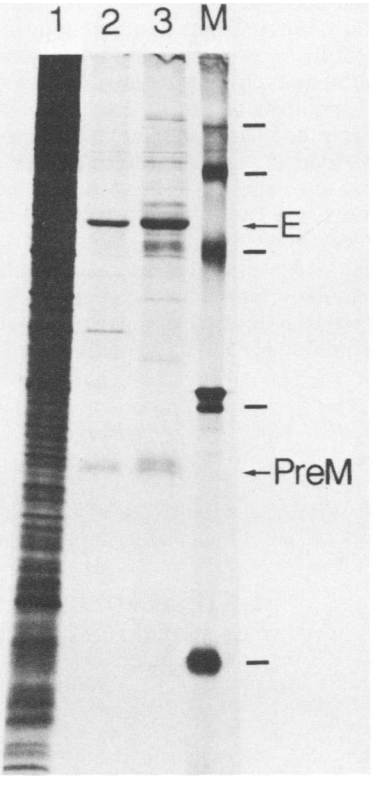

FIG. 6. Immune responses of a rabbit vaccinated with recombinant vaccinia virus. A rabbit inoculated with 4×10^8 PFU of mOJ6 was bled at 8 days postinoculation. Lysates of [^{35}S]methionine-labeled cells infected with LC16mO (lane 1) or JEV (lanes 2 and 3) were immunoprecipitated with anti-mOJ6 serum (lanes 1 and 2) or anti-JEV serum (lane 3). Immune precipitates were resolved by SDS–14% polyacrylamide gel electrophoresis and autoradiographed. Lane M contained ^{14}C-labeled protein size markers. The positions of the molecular size markers (from the top) of 92.5, 69, 46, 30, and 14.3 kDa are shown on the right. The positions of JEV proteins are also indicated at the right.

JEV-neutralizing epitopes in a highly immunogenic form and acts as a protective antigen. We observed tenfold higher neutralizing antibody titers in the sera of mice immunized with WRJ6 and boosted with inactivated JEV vaccine than in the sera of mice that received primary and secondary inoculations with inactivated JEV vaccine (Yasui et al., unpublished data), suggesting that JEV recombinant vaccinia viruses also have sufficient priming activities.

The biological activities of the JEV recombinants described here are quite different from those of dengue virus recombinant vaccinia virus (2, 4, 41) on two points. (i) Dengue virus proteins were not detected on the cell surface infected with dengue virus recombinant viruses. (ii) The most crucial difference between dengue virus and JEV recombinant vaccinia viruses is that dengue virus recombinants could not induce antibodies to E protein of dengue virus in vaccinated animals (2, 4, 41). Those researchers suggested that low-level expression of the E protein is responsible for the failure to induce antibodies to the E protein of dengue virus. However, they used the same $P_{7.5}$ promoter of vaccinia virus that we did for expression of the foreign genes. Our JEV recombinant could nevertheless express E protein on the cell surface and develop immune responses to the JEV E protein in vaccinated animals. Apparently, we could not fully neglect the possible difference in expression level between JEV and dengue virus E proteins by the corresponding recombinants. However, another possibility, that in terms of inherent properties, JEV E protein constitutes a stable conformation and therefore is more likely to construct neutralizing epitopes than is that of dengue virus, might be responsible for these discrepancies.

It should be noted that LC16mO, the parental strain of mOJ6, was isolated as a low-neurovirulence mutant from the Lister vaccinia virus strain and its recombinant retained low neurovirulence (21). The TK^- recombinant vaccinia virus mOJ6 produced milder erythema and pustules and had a shorter healing period than did its parental strain in inoculated animals (data not shown). In view of this result, together with those described above, it is suggested that mOJ6 will be potentially effective and safe as a live vaccine against JEV.

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