Japanese encephalitis virus (JEV) is a mosquito-borne flavivirus that is pathogenic for humans. In Southeast Asia, epidemics of JEV have been serious hazards to public health. Formalin-inactivated or attenuated live-virus vaccines for JEV are in use, but they are accompanied by high cost and the possibility of allergic effects. Flaviviruses cross-react in hemagglutination inhibition (HI) tests and weakly in neutralization (NT) tests. Antibody-dependent enhancement of viral infectivity induced by cross-reactive antibodies may be responsible for hemorrhagic fever and shock in severe dengue (DEN) disease. There is no direct evidence that severe dengue disease is caused by antibody-dependent enhancement, but DEN-infected monkeys that are pretreated with anti-DEN serum show increased viremia. Epidemics of JEV and DEN viruses overlap in Southeast Asia. Using monoclonal antibodies (MAbs) against the envelope (E) protein of JEV, we reported that cross-reactive MAbs, including HI and DEN, are directed against the E protein, which is one of three structural proteins; the other two are capsid (C) and membrane (M) proteins. Previously, we characterized MAbs directed against the E protein of JEV in vitro and in vivo, and found that JEV-specific MAb 503 (group 8) had the highest level of NT and the most protective activity against JEV infection. The critical NT site recognized by MAb 503, which is the most important for protective immunity, is distinct from the HI and virus receptor binding sites. Despite the importance of NT antibody, the NT mechanism of MAB 503 is not clearly understood.

In the present study, we analyzed the effects of MAB 503 on the sequential steps of viral infection, which include viral adsorption, internalization, and membrane fusion steps. A number of studies provide evidence that flaviviruses enter cells by endocytosis involving pH-induced fusion with endosomal membranes, which is a common mode of entry for enveloped viruses. On the basis of the results presented here, the NT mechanism of JEV by MAB 503 is discussed.

MATERIALS AND METHODS

Cells and viruses. Vero cells and the JEV strain Bejing-I were used in this study. Culture medium was Eagle’s minimum essential medium (MEM) containing 5% fetal bovine serum (FBS). Propagation and titration of JEV were done with Vero cells as previously described. Purified radiolabeled JEV was prepared as described previously. Briefly, Vero cells were infected with JEV at a multiplicity of infection (MOI) of one plaque-forming units (PFU)/cell. Twenty-four hours after infection, the medium was replaced with 15 ml of methionine and cysteine–free medium containing 740 kBq/ml of 35S-methionine and 35S-cysteine (New England Nuclear Research Products, Du Pont, Wilmington, DE). Radiolabeled JEV was purified from the infected culture fluid 40-hr postinfection. For labeling with 3H-uridine, Vero cells were infected with JEV at an MOI of 10 PFU/cell. After 18 hr, the medium was replaced with labeling medium containing 925 kBq/ml of 3H-uridine (American Radiolabeled Chemicals, Inc., St. Louis, MO), 1 μg/ml of actinomycin D, and 0.2 M thymidine. The viruses were purified from the culture medium 48-hr postinfection.

Monoclonal and polyclonal anti-JEV antibodies. The MAbs against the E protein of JEV used in this study have been described. They were purified from ascitic fluid using protein A-Sepharose 4B. Purification was confirmed by a single band in sodium dodecyl sulfate–polyacrylamide gel electrophoresis without β-mercaptoethanol treatment, and protein concentration was determined by the bicinchoninic acid method (Pierce, Rockford, IL). The IgG fraction of polyclonal anti-JEV serum was purified from BALB/c mice immunized with three intraperitoneal injections of formalin-inactivated vaccine as described previously. Purified normal mouse IgG (NMG; Cappel Products, Aurora, OH) was used as a control.

Neutralization test assays before or after adsorption to Vero cells. The NT assay before adsorption of JEV was performed as previously described. Briefly, the infectivities of mixtures of JEV and a serial dilution of each MAb were assayed on confluent Vero cell monolayers. The JEV-infected cell monolayers were cultured under overlay medium consisting of Eagle’s MEM supplemented with 5% FBS and 1.25% methylcellulose at 37°C in a CO2 incubator. After three days, the cells were stained overnight with 0.2% neutral red in MEM and the plaques were counted. The NT titer was expressed as the maximum dilution of MAb that resulted in 90% plaque reduction of the virus inoculum.
The cell-associated JEV or virus-antibody complexes, purified with an ImmunoPure Fab Preparation Kit (Pierce) scintillation spectrometer. The Fab fragments of MAbs were dioactivity of each sample was quantitated using a liquid sulfate (SDS) in phosphate-buffered saline (PBS). The ra-
times with cold MEM and lysed with 2% sodium dodecyl
bath. Precooled monolayers of Vero cells (1.2
3
PFU/cell) for 1 hr at 4
were adsorbed with MAB-JEV mixtures at an MOI of 20 PFU/cell at 4°C for 1 hr. The cells were washed with cooled MEM, and incubated in 350 µl of medium containing each MAb for 1 hr at 4°C. The MAbs were used at a concentration of 2 ng/1 PFU, which is more than is required for JEV neu-
ralization. After the cells were washed with cold MEM, 3,000–4000 cpm of 35S-JEV was bound/3 × 10^5 cells before warming. The number of cpm bound to untreated cells (no antibody) was used to determine 100% binding. The washed cells were added with prewarmed MEM, and incubated at 37°C for 0–90 min. At certain intervals, the medium (A) was removed to measure the radioactivity. The cells were then washed with cold PBS and treated with 0.125% trypsin
at 37°C for 15 min. After removal of the trypsin solution by cen trifugation, the cell pellet was lysed with 2% SDS in PBS. The radioactivity of the lysed cell pellet (B) was measured to determine cell-associated JEV as a percent of the initial binding count (100 × B/total binding count). Percent release of 35S-JEV was calculated from the radioactivity in the medium (100 × A/total binding count).

**TABLE 1** Neutralization (NT)* of Japanese encephalitis virus (JEV) by anti–envelope (E) monoclonal antibodies (MAbs) before or after adsorption of virus to Vero cells

<table>
<thead>
<tr>
<th>Groups, MAbs, and antibodies</th>
<th>IgG subclass</th>
<th>NT titer (log_{10}) before adsorption</th>
<th>NT titer (log_{10}) after adsorption</th>
</tr>
</thead>
<tbody>
<tr>
<td>MABs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.301</td>
<td>2a</td>
<td>1.1</td>
<td>&lt;1.1</td>
</tr>
<tr>
<td>2.109</td>
<td>2a</td>
<td>&lt;1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>3.112</td>
<td>2a</td>
<td>&lt;1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>8.503</td>
<td>2a</td>
<td>3.2</td>
<td>3.2</td>
</tr>
<tr>
<td>7.9.04</td>
<td>2b</td>
<td>1.8</td>
<td>1.8</td>
</tr>
<tr>
<td>6.201</td>
<td>2a</td>
<td>&lt;1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>5.204</td>
<td>2a</td>
<td>&lt;1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>4.302</td>
<td>2b</td>
<td>&lt;1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>9.504</td>
<td>2a</td>
<td>&lt;1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Polyclonal anti-JEV IgG</td>
<td>2.5</td>
<td>1.8</td>
<td></td>
</tr>
</tbody>
</table>

* Purified MAb IgG and polyclonal IgG antibodies were adjusted to 100 µg/ml. 90% reduction NT titer was examined by serial dilution of purified antibodies using 100 plaque-forming units of JEV.

\[ \sqrt{\text{binding}} = \text{binding assay}, 5 \text{ the epitopes of reciprocal pairs of MAb groups 2 and 3 and MAb groups 6 and 8 may be adjacent to or overlap each other. The site of MAb group 7 seems to be related to those of MAB groups 2, 3, 6, and 8. On the basis of these results, the order of the nine MAB groups was arranged.} \]

**Inhibition of JEV-induced cell fusion by MAbs.** To determine the effects of MAbs on JEV-induced cell fusion, a fusion from within (FFWI) assay was performed by a modiﬁed method of Randolph and Stoller. Vero cells (2 × 10^4 cells/eight-well chamber slide; Nunc, Roskilde, Denmark) were infected with JEV at an MOI of 10 PFU/cell and cultured for two days at 37°C. Then the cells were washed, placed in 25 mM 2-(morpholino) ethanesulfonic acid (MES, pH 5.5) in MEM without NaHCO₃ for 30 min at 37°C, and replaced in growth medium. After incubation for 2 hr at 40°C, cell-cell fusion was observed. The number of nuclei and the number of cells in five microscopic visual ﬁelds (magniﬁcation 100×) were counted using a phase-contrast microscope, and the percentage of fused cells was calculated.

**Binding of JEV to cells in the presence of MAbs.** The effect of MAbs on JEV-binding to cells before adsorption was examined. 35S-JEV (approximately 1 × 10^4 counts per minute [cpm]/2.4 × 10^5 PFU) was preincubated with 250 µg of each MAb for 1 hr at 37°C and then chilled in an ice bath. Precocooned monolayers of Vero cells (1.2 × 10^5 cells) were adsorbed with MAB-JEV mixtures at an MOI of 20 PFU/cell at 4°C. The cells were then washed three times with cold MEM and lysed with 2% sodium dodecyl sulfate (SDS) in phosphate-buffered saline (PBS). The radioactivity of each sample was quantitated using a liquid scintillation spectrometer. The Fab fragments of MAbs were prepared with an ImmunoPure Fab Preparation Kit (Pierce) as described previously. 6

**Assay for cell-associated 35S-labeled JEV.** To investigate the cell-associated JEV or virus-antibody complexes, puriﬁed 35S-JEV (approximately 2.5 × 10^4 cpm 6 × 10^5 PFU) was prebound to monolayers of Vero cells (3 × 10^4 cells on 3.5-cm diameter plastic dish; Nunc) at an MOI of 20 PFU/ cell at 4°C for 1 hr. The cells were washed with cooled MEM, and incubated in 350 µl of medium containing each MAb for 1 hr at 4°C. The MAbs were used at a concentration of 2 ng/1 PFU, which is more than is required for JEV neutralization. After the cells were washed with cold MEM, 3,000–4000 cpm of 35S-JEV was bound/3 × 10^5 cells before warming. The number of cpm bound to untreated cells (no antibody) was used to determine 100% binding. The washed cells were added with prewarmed MEM, and incubated at 37°C for 0–90 min. At certain intervals, the medium (A) was removed to measure the radioactivity. The cells were then washed with cold PBS and treated with 0.125% trypsin
at 37°C for 15 min. After removal of the trypsin solution by centrifugation, the cell pellet was lysed with 2% SDS in PBS. The radioactivity of the lysed cell pellet (B) was measured to determine cell-associated JEV as a percent of the initial binding count (100 × B/total binding count). Percent release of 35S-JEV was calculated from the radioactivity in the medium (100 × A/total binding count).

**Sensitivity of 3H-uridine-labeled JEV to RNase.** The RNase sensitivity of 3H-uridine-labeled JEV was examined to determine uncoating of JEV as previously described. 13 The 3H-JEV (approximately 1 × 10^6 cpm 6 × 10^5 PFU) was bound to monolayers of Vero cells (3 × 10^4 cells on 3.5-cm diameter plastic dish; Nunc) at an MOI of 20 PFU/cell at 4°C for 1 hr. After the cells were washed with cold MEM, they were incubated with MAbs (2 ng/1 PFU) in virus diluent at 4°C for 1 hr. After washing with cold MEM, 700–1,000 cpm of 3H-JEV was bound/3 × 10^5 cells before warming. The cpm bound to untreated cells served as 100% binding. Prewarmed medium was then added and the cells were incubated at 37°C. At various time intervals, the culture me-
Table 2
Fusion inhibition by anti-envelope monoclonal antibodies (MAbs)*

<table>
<thead>
<tr>
<th>Groups and MAbs</th>
<th>Fusion</th>
</tr>
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<tbody>
<tr>
<td>1.301</td>
<td>+</td>
</tr>
<tr>
<td>2.109</td>
<td>+</td>
</tr>
<tr>
<td>3.112</td>
<td>++</td>
</tr>
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<td>8.503</td>
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<td>7.N.04</td>
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<tr>
<td>6.201</td>
<td>+++</td>
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<tr>
<td>5.204</td>
<td>+++</td>
</tr>
<tr>
<td>4.302</td>
<td>+++</td>
</tr>
<tr>
<td>9.504</td>
<td>+++</td>
</tr>
<tr>
<td>NMG</td>
<td>+</td>
</tr>
</tbody>
</table>

* In the absence of antibodies, more than 25% of Vero cells were fused. Fifty micrograms of MAb was used in this assay. Fusion activity was scored as follows: - = no fusion; + = <5% of cells fused; ++ = 5–25%; +++ = 25–50%; ++++ = 50–75%; NMG = normal mouse IgG.

dium (A) was removed from the cells and used to measure released radioactivity. The cells were then washed once with cold PBS, scraped from the plate, precipitated by centrifugation, and incubated in 0.2 ml of low salt buffer (10 mM Tris-HCl, pH 8.0, 0.01 M NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 0.2 mM benzamide) at 4°C for 15 min. The cells were mechanically lysed by passing 30 times through a 26-gauge needle, resulting in more than 90% lysis as judged by microscopic examination. Seventy microliters of RNase A (10 mg/ml) was added to each sample and incubated at 37°C for 30 min. The samples were then chilled on ice, precipitated with 10% trichloroacetic acid (TCA) at 4°C for 30 min, and centrifuged for 15 min. Radioactivity of the supernatant (B) was counted to determine RNase-sensitive viral RNA as a percent of the total binding count (100 × B/total binding count). Radioactivity in the culture medium was also counted to determine the release of 3H-JEV (100 × A/total binding count).

Form of viral RNA released from the cells. To determine the form of viral RNA released from the cells into the

Figure 3. A, effects of monoclonal antibodies (MAbs) 503 and 204 on 35S-Japanese encephalitis virus (JEV) binding to Vero cells following trypsin treatment. In the presence of MAb 503, a significant level of radioactivity was trypsin resistant before shifting the temperature to 37°C as well as for 2 hr following the temperature shift. B, release of 35S-JEV from the cells at 37°C. The release in the presence of MAb 503 (O) or 204 (Δ) was almost same as that with normal mouse IgG (■). Each value is the mean from triplicate wells. Error bars indicate the standard deviation of values obtained from two independent experiments. CPM = counts per minute.
FIGURE 4. A, level of RNase-sensitive $^3$H-Japanese encephalitis virus (JEV) RNA associated with Vero cells following temperature shift to 37°C. Monoclonal antibody (MAb) 503 decreased the amount of RNase-sensitive $^3$H-JEV RNA associated with the cells. B, release of $^3$H-JEV from Vero cells at 37°C. MAb 503 (O) increased the release of $^3$H-JEV from the cells during 30–90 min of incubation at 37°C. The release in the presence of MAb 204 (A) was the same as that with normal mouse IgG (■). Each value is the mean from triplicate wells. Error bars indicate the standard deviation of values obtained from two independent experiments. CPM = counts per minute.

medium, Vero cells preadsorbed with JEV (MOI = 20 PFU) were treated with MAbs at 4°C for 1 hr. The cells were then washed and incubated at 37°C for 60 or 120 min. At various time intervals, the culture medium was collected and divided into two portions. The first portion (A) was used to measure radioactivity in the medium. The second portion was centrifuged at 100,000 $\times$ g for 1 hr. Radioactivity of the resultant pellet (B) was calculated as a percent of the total count of the released $^3$H-JEV (100 × B/A). To examine the infectivity of the JEV released into the culture medium, plaque assays were performed.

Observations with confocal laser microscopy (CLM). Vero cells ($5 \times 10^4$ cells) were plated on a glass coverslip that was coated with poly-L-lysine (0.1 mg/ml) using silicon wells (Flexiperm® 70 mm$^2$/well; Heraeus Instruments, Osterode am Harz, Germany). After one day, JEV was adsorbed to the Vero cells at an MOI of 1,800 at 4°C for 1 hr. After the cells were washed with cold MEM, MAbs (503 and 204) and NMG at a concentration of 1 mg/well were added to the cells, which were then kept at 4°C for 1 hr. It was confirmed by plaque assay that JEV was neutralized at this concentration of MAb 503.

To detect virus adsorbed to the cell surface, the cells were washed with cold MEM, incubated in fresh MEM at 37°C for 0–30 min, and then washed with cold MEM containing 0.05% sodium azide. The cells were stained by treatment with rabbit anti-JEV, followed by Texas red–labeled anti-rabbit IgG at 4°C in the presence of sodium azide. The stained cells were observed in the presence of sodium azide by CLM (Molecular Dynamics, Sunnyvale, CA) and fluorescence microscopy (Axioplan; Zeiss, Oberkochen, Germany).

An endocytosis assay was performed by incubating virus-adsorbed cells at 37°C for 30 min in the presence of lucifer yellow (1 mg/ml; Molecular Probes, Eugene, OR). If JEV particles are internalized by endocytosis, lucifer yellow in the culture medium is taken into the cells. The cells were washed with cold MEM containing 0.05% sodium azide to prevent further endocytosis and block further steps in the cells. The stained cells were observed in the presence of sodium azide by CLM.

Electron microscopic (EM) study. Vero cells ($5 \times 10^4$ cells) were plated in plastic dish compartments with Flexiperm® (Heraeus). The JEV was adsorbed to Vero cells at an MOI of 7,200 for 1 hr at 4°C. Cells were then rinsed with cold MEM and incubated with 1 mg/well of NMG or MAbs 503 and 204 for 1 hr at 4°C. After the cells were washed with cold MEM, prewarmed growth medium was added, and

FIGURE 5. Form of $^3$H-Japanese encephalitis virus RNA released from Vero cells after the temperature shift to 37°C. The total count of the released $^3$H radioactivity was calculated as 100%. Cross-hatched and white columns indicate the proportion of total radioactive label pelleted following centrifugation of medium from monoclonal antibody 503– and normal mouse IgG–treated cells, respectively. Error bars indicate the standard deviation of values obtained from two independent experiments. CPM = counts per minute.
the cells were incubated for 5 and 30 min at 37°C. The EM studies were done as previously described. Briefly, the cells were fixed in a mixture of 2% (w/v) paraformaldehyde and 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 2–6 hr at 4°C, rinsed, treated with OsO₄, block-stained, dehydrated, and embedded by standard procedures. Sections (60 nm) were cut, stained with lead citrate and uranyl acetate, and subsequently examined with a Hitachi (Hitachi, Japan) 7000 electron microscope at 80 kv. Neutralization of most JEV (> 90%) was confirmed by plaque assay at this concentration of MAb 503.

**Statistical analysis.** In all experiments using radiolabeled JEV, each value is the mean of 2–3 samples and was confirmed in at least three independent experiments. The data were screened for statistical significance by the Student’s unpaired, two-tailed t-test, and P values < 0.05 were considered to be significant.

**RESULTS**

**Neutralization test activity of MAbs.** The NT activities of nine MAbs against the E protein of JEV were examined on Vero cell monolayers by either the standard plaque assay method or treatment of the cells with MAb after virus-adsorption. The NT activity was obtained with two MAbs (N.04 and 503) at dilutions of log10 1.8 and log10 3.2 that
corresponded to 1.6 µg and 64 ng of purified MAb per 100 PFU of JEV, respectively (Table 1). The NT activities of these two MAbs showed no difference between preadsorption and postadsorption treatments, while the NT activity of polyclonal anti-JEV antibody before adsorption was somewhat higher than that after adsorption. Monoclonal antibody 301 showed a slight NT activity only in the preadsorption assay.

**Effect of MAb 503 on JEV binding to Vero cells.** To determine the effect of MAb 503 on virus binding to Vero cells, 35S-JEV was incubated with MAbs for 1 hr before adsorption. The binding assay was performed at 4°C to prevent virus-internalization. All nine MAbs failed to inhibit JEV-binding to the cells (data are shown only for MAbs 503, 301, and 204 in Figure 1). Only the Fab fragment of MAb 301 inhibited about 60% of the JEV-binding to cells. Similar results were obtained with different concentrations of MAbs (500 and 50 µg). These results showed that neutralization of JEV by MAb 503 was not due to inhibition of JEV binding to the cells, but to an effect on a subsequent step of JEV infection. To avoid an effect of MAb on the initial binding of JEV to cells, the cells in subsequent experiments were pre-bound with JEV prior to MAb treatment.

**Inhibition of JEV-induced cell fusion by MAbs.** The JEV-induced cell fusion at low pH provides a model system for assaying viral membrane fusion. Cell fusion assays using Vero cells was performed with nine MAbs. Photographs of cell fusion in the presence of MAb 503 or NMG are shown in Figure 2. As shown in Table 2, MAb 503 and the three HI MAbs (301, 109, and N.04) were able to inhibit cell-cell fusion. Monoclonal antibody 503 showed the highest inhibition. Using C6/36 mosquito (Aedes albopictus) cells, FFWI and fusion from without (FFWO) assays were examined with these MAbs and similar results were obtained. Monoclonal antibody 503 has inhibited FFWI and FFWO in C6/36 cells, but no biological activity has been demonstrated for MAb 204.

**Trypsin sensitivity of MAb-JEV on the cell surface.** To determine the effect of MAB 503 on the next step of virus adsorption, JEV-bound Vero cells were treated with trypsin at time intervals following the temperature shift to 37°C. Figure 3A shows that about 90% of 35S-labeled JEV bound
to the cells at 0 min was removed from the cell surface with 0.125% trypsin in the presence of NMG or MAb 204. At 30–60 min after the temperature shift, trypsin-resistant radioactivity increased to 15–30% for these two MAbs. At 120 min at 37°C, 35–39% resistance was observed, suggesting that 35S-methionine labeled-JEV internalized within the cells. In the presence of MAb 503, a significant level of radioactivity (39%) was trypsin resistant even at 4°C, and a 30–39% level of trypsin resistance occurred for 120 min following the temperature shift to 37°C. A shift of temperature to 37°C causes internalization of adsorbed virus within cells and results in trypsin resistance of radioactively labeled virus. Internalization of JEV occurs via the endocytosis process and requires a temperature shift. Thus, internalization of virus should not occur at 4°C. Temperature shift for trypsin digestion at 37°C might cause some internalization of JEV, but the trypsin-resistant radioactivity in the presence of MAb 503 at 4°C was significantly higher than the control (NMG).

Figure 3B shows the release of 35S-labeled JEV virion or viral proteins from the cells into the culture medium after the temperature shift to 37°C. In the case of MAB 204 or NMG, the release of radioactivity increased 30–90 min after the temperature shift. The release of label associated with MAB 503 treatment 30–120 min after the temperature shift was slightly higher than occurred with MAB 204 or NMG. Statistical analysis was examined using the Student’s unpaired, two-tailed t-test. A statistically significant difference was not observed between MAB 503 and NMG or MAB 204 (P = 0.07 and P = 0.1, respectively) even at 120 min.

Sensitivity of 3H-uridine-labeled JEV to RNase. To identify the effects of the MAbs on subsequent infection steps, RNase sensitivity of 3H-uridine-labeled JEV was examined. After internalization and uncoating, 3H-labeled JEV RNA in the cells should be RNase sensitive. In the presence of NMG or MAb 204 (Figure 4A), 31–33% of the labeled intracellular JEV RNA was RNase sensitive after 5 min incubation at 37°C, indicating uncoating of viral RNA. In the culture medium of these cell samples, 20–30% of the radioactivity was released from the cells by 90 min following the temperature shift (Figure 4B). In the presence of MAB 503, 27% of the radioactivity of intracellular JEV-RNA was RNase sensitive at 5 min, decreasing to 17% by 90 min (Figure 4B), while the released radioactivity from the cells was significantly higher than that of NMG or MAB 204 (Figure 4B). The RNA of 3H-JEV treated with MAB 503, and then with RNase, was resistant to degradation by the RNase.

Form of JEV-RNA released from the cells. To examine the form of JEV-RNA released from the cells, culture medium containing the released 3H radioactivity of JEV-RNA was centrifuged or precipitated with TCA. The JEV virions (specific gravity = 1.2 g/ml) should be precipitated by centrifugation at 100,000 × g. When labeled JEV RNA is uncoated from the viral particle, it results in radioactivity that is not precipitated by centrifugation. As shown in Figure 5, only about 13–15% of the labeled JEV-RNA in the control (NMG) was pelleted by centrifugation at 100,000 × g. Treatment with MAB 503 resulted in the pelleting of 45–62% of the total labeled JEV RNA in the medium by centrifugation at 100,000 × g (Figure 5), as well as a significant increase in the total radioactive label in the medium (Figure 4B) relative to NMG. Results with MAb 204 were nearly equal to that of NMG. The total count of the released 3H-radioactivity in the presence of MAB 503 was 2–3-fold higher than that of NMG at 60–120 min, as partly shown in Figure 4B. The total radioactivity of initial binding of JEV to the cells in the presence of MAB 503 was almost same as with NMG (Figure 1). These results suggested that MAB 503 might cause the release of most of the JEV-RNA from the cells in the form of viral particles or associated with viral membrane.

Although 3H-radioactivity of the culture medium containing released JEV was lower, treatment with NMG or MAb 204 resulted in the release of infectious JEV (1.5 × 101 or 1.3 × 102 PFU/ml, respectively) from the cells 1 hr after the temperature shift to 37°C. But when treated with MAB 503, infectious JEV (<5 PFU/ml) was not detected in the culture medium, which contained higher 3H-radioactivity.

Observations of JEV on the cell surface and endocytosis by CLM. To determine whether or not JEV internalized into the cells in the presence of MAB 503, JEV particles on the cell surface were observed by CLM. As shown in Figure 6a, c, and e, JEV binding to the cell surface in the presence of MAB 503 was similar to the binding in the presence of NMG and MAB 204 at 0 min. After 30 min at 37°C in the presence of MAB 503, a large proportion of the JEV remained on the cell surface as large aggregates (Figure 6f). In the presence of NMG or MAB 204, significant amounts of JEV particles disappeared from the cell surface (Figure 6b and d). In the presence of MAB 204, the remaining viral particles made aggregates preferably as compared with the control. After 60–120 min of incubation at 37°C, the number of MAB 503-treated JEV particles on the cells surface decreased and the cell surface staining was similar to that of NMG or MAB 204 treatment.

As shown in Figure 7, numerous endocytotic vesicles containing lucifer yellow were observed in JEV-adsorbed cells in the control (NMG) or MAB 204 treatment (Figure 7b and d), whereas fewer vesicles were observed in both uninfected cells and MAB 503-treated, JEV-adsorbed cells (Figure 7a and c respectively). This indicates that MAB 503 inhibited endocytosis induced by JEV in Vero cells (Figure 7c).

Electron microscopic observations. The fate of JEV was observed by EM. In the control (NMG) or MAB 204-treated, JEV-adsorbed cells, JEV particles were observed on the cell surface and entered the cells via an endocytosis pathway (results are shown for NMG in Figure 8a–d). Virus internalization was not observed in the cells treated with MAB 503, and aggregates of numerous JEV particles remained on the cell surface (Figure 8e–g). Treatment with MAB 503 showed the location of the viral particles to be rather distant from the cell membrane as compared with the control (NMG), as shown in Figure 8. Further incubation at 37°C did not result in internalization of JEV particles treated with MAB 503.

DISCUSSION

From the results of NT assays of JEV before or after adsorption to Vero cells, NT activities of polyclonal anti-JEV antibody and MAb 301 indicated that inhibition of virus binding to the cells is one mechanism of neutralization (Table 1), but other mechanisms of neutralization might be im-
FIGURE 8. Electron microscopic observations of Japanese encephalitis virus (JEV) adsorption and internalization into Vero cells. In the control (normal mouse IgG), adsorption of JEV particles (a) at 5 min and viral internalization into the cells (b–d) at 30 min after the temperature shift to 37°C was observed. Treatment with monoclonal antibody 503 inhibited internalization of JEV, and viral particles remained on the cell surface (e–g) even after the temperature shift for 30 min. Magnifications: a and b, ×160,000; c, ×122,000; d, ×121,000; e, ×170,000; f, ×105,000; g, ×80,000.
neutralization. As previously reported,\textsuperscript{11} we confirmed that the NT activity of MAb 503 had no effect on the binding of JEV to the cells (Figure 1). Because the NT activity of MAb 503 is greater than that of the other MAbs and polyclonal antibody, the epitope recognized by MAb 503 should be important for neutralization.

Table 2 showed that the inhibitory effect of MAb 503 on JEV-induced cell fusion was greater than the other MAbs. The HI MAbs 301, 109, and N.04 also inhibited JEV-induced cell fusion. As shown in Figure 1, the binding of JEV to Vero cells is inhibited by the Fab fragment of MAB 301, but not by its whole IgG, which suggests that the E protein epitope recognized by MAB 301 interacts with a cellular receptor site for JEV. The binding of JEV to the cell surface via this epitope might result in membrane fusion. Because the HI MAbs 301 and 109 show little or no NT activity, JEV may enter the cells via other receptor sites. Antibody virus binding of several flaviviruses has been proposed to enter the cell by the Fc receptor.\textsuperscript{16, 17} The epitope recognized by MAb 503 might be a fusion site or close to such a site.

Treatment with MAb 503 induced trypsin-resistant binding of \textsuperscript{35}S-labeled JEV to cells even before the temperature shift to 37°C (Figure 3A). Using \textsuperscript{3}H-uridine-labeled JEV, significant uptake of RNase-sensitive JEV-RNA by the cells was observed in the treatment with MAB 503, but the level of this uptake was lower than that of NMG or MAB 204. Monoclonal antibody 503 significantly increased the release of \textsuperscript{3}H-JEV RNA from the cells into the culture fluid. These observations suggested two possibilities: 1) MAB 503-JEV complexes were internalized within the cells but later released from the cells without undergoing a normal uncoating process, or 2) MAB 503-JEV complexes were not internalized in the cells, but were released from the cell surface. To investigate these possibilities, JEV particles were observed by CLM and EM. Observations by CLM and EM (Figures 6–8) indicated that MAB 503-JEV complexes were not internalized within the cells and remained on the cell surface. The JEV treated with NMG or MAB 204-JEV complexes were internalized into the cells via an apparent endocytosis pathway. Anti-clathrin (Bio Makor, Rehovot, Israel) staining showed that MAB 503 did not affect clathrin expression in Vero cells. When treated with MAB 503, supernatant containing 3H-JEV RNA from the cells was precipitated by centrifugation (Figure 5), which indicated that its specific gravity was the same as intact JEV particles. This suggested that JEV-RNA might be released from the cell surface in the form of viral particle or associated with viral membrane.

The effect of MAB 503 on the E protein might involve a conformational change of the E glycoprotein including a fusion site. The first uptake of RNase sensitive JEV-RNA treated with MAB 503 might be caused by some conformational change of E protein, but not by an uncoating process. In flaviviruses, several reports have indicated that acid treatment induces irreversible conformational changes of E protein, which involve both a trypsin-resistant form and an oligomeric rearrangement.\textsuperscript{18–20} These observations suggest the possibility that the E protein changes induced by MAB 503 are similar to those induced by acid treatment. Further experiments are needed to better define this phenomenon.

In this study, we have shown that the highly NT and protective MAB 503 does not prevent the early step of JEV binding to cells but rather inhibits later steps of JEV infection. Monoclonal antibody 503 appears to affect internalization and fusion steps between JEV and the cellular membrane, which leads to the uncoating of viral RNA. These observations may provide important information for elucidation of JEV neutralization and development of improved JEV vaccines.

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REFERENCES

14. Swanson J, Burke E, Silverstein C, 1987. Tubular lysosomes...