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Extracellular Enveloped Vaccinia Virus Escapes Neutralization

YASUO ICHIHASHI

Department of Virology, Faculty of Medicine, Niigata University, Asahimachi, Niigata 951, Japan

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The vaccinia virus forms two morphologically distinct infectious virus particles: intracellular mature virus (IMV) and extracellular enveloped virus (EEV). The envelope of EEV is a Golgi-derived membrane (wrapping membrane). A mutant (vRB10) lacks the ability to form the EEV. In medium containing a neutralizing antibody (2D5mAb), the vRB10 mutant was diluted out from infected cells, whereas the IHD-J strain of vaccinia virus replicated well. The result indicated that the 2D5mAb specifically neutralized the IMV. The 2D5-resistant EEV appeared at 6–7 hr postinfection, and over 65% of infectious virus in the culture fluid was EEV at 48 hr after infection. The EEV was resistant not only to the 2D5mAb but also against several neutralizing antibodies, including polyclonal antivaccinia serum reactive with proteins of the wrapping membrane. Freeze–thawing and other procedures that may damage the wrapping membrane converted the EEV to a form susceptible to the antibodies. Since specific infectivity was not affected by the damage or by exposure to antibody against the wrapping membrane proteins, the wrapping membrane did not directly participate in penetration. The infection process of vaccinia virus was analyzed by comparison of responses to acid treatment between normal IMV and trypsin-treated IMV. Proteolytically activated IMV infected rapidly responding to acid. The protected form virus, which was noninfectious under usual conditions, was proteolytically activated on cell membrane then responded to the acid. Proteolysis activated the virus, and an acidic condition accelerated fusion between the activated IMV and plasma membrane. The virus in the EEV wrapping membrane was the activated form that has the capacity to fuse with the cell membrane. However, the infection of intact EEV was more sensitive against lysosomotropic agents (NH_4Cl, neutral red) than that of the trypsin-activated IMV. Resistance to the 2D5mAb, sensitivity to lysosomotropic agents, and acceleration of infection by acid suggested that the intact EEV penetrated by virus–endosome membrane fusion. The combined effect of the presence of wrapping membrane and the process of internalization via an endocytic mechanism rendered EEV resistant to neutralizing antibodies.

INTRODUCTION

Morphological studies of vaccinia virus biogenesis have revealed two forms of infectious virus: intracellular mature virus (IMV) and extracellular enveloped virus (EEV). Spherical immature virus, which assembles in the cytoplasmic viral foci, transforms to brick-shaped IMV, and then a fraction of the IMV is disseminated by an intracellular transportation process. Golgi-derived membrane cisternae associate with the IMV, and the cisternae fuse each other to form two layers of membrane wrapping around the virus. The wrapped virus migrates to the cell boundary where the outer-layer membrane fuses with the plasma membrane, releasing EEV. Thus EEV contains an additional cellular membrane (wrapping membrane) derived from the Golgi (Dales, 1963; Ichihashi *et al.,* 1971; Morgan, 1976; Schmelz *et al.,* 1994). Vaccinia-specific proteins are located in the wrapping membrane (Payne, 1978; Hiller *et al.,* 1981), and a vaccinia gene (F13L) encodes a major 37-kDa membrane protein (Hirt *et al.,* 1986). A mutant (vRB10) lacking this gene demonstrates that the 37-kDa protein is nonessential for replication of the virus but is required for the membrane envelopment (Blasco and Moss, 1991). Dissemination of EEV results in cell-to-cell spreading and the long-distance distribution of the virus (Blasco and Moss, 1992). These results indicate that the EEV is transported across the plasma membrane by the enveloping system.

Both EEV and IMV are infectious, but the EEV has higher relative infectivity than IMV (Boulter and Appleyard, 1973; Payne and Norrby, 1978). On the other hand, analysis of the IMV's infectivity by proteolysis and a protease inhibitor (PMSF) indicates that the purified IMV sample is composed of viral populations having different infectivity (Ichihashi and Oie, 1980). The protected form does not infect cells in the presence of the PMSF, whereas the activated form can do so. The protected form can be converted to the activated form after *in vitro* exposure to trypsin or to isolated plasma membrane (Ichihashi and Oie, 1982a,b), and the infectiousness of the virus increases two- to fivefold. The trypsinized IMV infects cells more quickly than the normal IMV, and it penetrates cells by means of virus–plasma membrane fusion (Ichihashi *et al.,* 1994). As infection with vaccinia virus establishes by core and lateral bodies entering the cytoplasm (Dales, 1963), the penetration mechanism of EEV should differ from that of the activated IMV. The EEV must uncoat its wrapping membrane in addition to the surface membrane of virus. Roles of the wrapping mem-

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brane for the high infectivity and for the entry of EEV are unclear.

We produced a monoclonal antibody (2D5mAb) that inhibits the penetration step of infection (Ichihashi *et al.,* 1994). Despite having high neutralizing activity against IMV (50% plaque reduction titer of 10^6), the mAb demonstrated incomplete neutralization against the virus disseminated into culture fluid. The resistant virus was EEV. Specific neutralization of IMV by 2D5mAb provides a means of distinguishing EEV. In this study we analyzed the infective process of EEV and the functions of the EEV wrapping membrane.

TABLE 1

mAb	Reactive viral protein in immunoblots	Neutralization titer, 50% plaque reduction: IMV	Neutralization titer, 50% plaque reduction: Trypsin-treated IMV	Adsorption inhibition %: IMV
3A5	VP17-25K	7.5×10^4	5.5×10^4	12
1C1	VP13.8K	8.5×10^2	$<10^1$	16
11A2	VP23-29K	4.0×10^5	8.7×10^5	2
2D5	VP23-29K	6.2×10^6	5.0×10^7	−6
Anti-vac	Polyclonal	2.1×10^3	1.0×10^4	28

MATERIALS AND METHODS

Cells and viruses

The IHD-J strain vaccinia virus was inoculated into Vero cells with a multiplicity of infection (m.o.i.) of 0.5 PFU/cell. Infection with a higher m.o.i. resulted in an earlier CPE and a lower rate of EEV. The cells were incubated at 4° for 45 min, washed with Dulbecco modified Eagle's minimum essential medium (DMEM), and incubated in DMEM containing 10% fetal calf serum at 37° for 48 hr. The culture medium was collected and centrifuged at 1000 *g* for 15 min. The supernatant was used as the EEV sample. For purification of EEV, the supernatant was centrifuged at 8000 *g* for 60 min and the pellet was applied to CsCl equilibrium centrifugation. IMV was purified from cell-associated virus as described (Joklik, 1962; Ichihashi *et al.,* 1994). The purified IMV and EEV sedimented as single peaks with a buoyant density of 1.278 and 1.230 g/ml, respectively. The vRB10 mutant was a gift from Dr. B. Moss (Blasco and Moss, 1991).

Titration of virus

The virus titer was assayed by plaque formation in Vero cell monolayer cultures using 12-well plate. Since low pH accelerated the penetration of the virus, the assay was performed at pH 7.4. The virus was diluted and the cells were washed with phosphate-buffered saline (PBS).

Virus samples were assayed under four different conditions to distinguish the viral fractions differing in their infectivity. The virus sample to be assayed was divided to two portions, and one was treated with TPCK-treated trypsin (Sigma, 20 μg/ml) at 37° for 10 min and the other was mock treated with PBS. The samples were assayed in the presence or absence of a protease inhibitor (PMSF, phenylmethylsulfonyl fluoride, Sigma, 2 m*M*) as described (Ichihashi and Oie, 1980). Titers usually line up in order of [titer of trypsin-treated sample assayed in absence of PMSF (I)] $\geqq$ [titer of trypsin-treated sample assayed in the presence of PMSF (II)] $\gg$ [titer of normal virus assayed in the absence of PMSF (III)] $>$ [titer of normal virus assayed in the presence of PMSF (IV)]. A fraction of the virus determined by IV was termed the activated form virus. A fraction of the virus determined by (I − III) was termed the protected form virus. A fraction of the virus determined by (III − IV) represents the protected form virus that infects after proteolytic activation on the plasma membrane (Ichihashi and Oie, 1980, 1982a,b).

Antibodies

BALB/c strain mice were immunized with the IHD-J strain of vaccinia virus collected from the culture medium of infected Vero cells. The immunization was repeated four times with 2-week intervals. About 20% of inoculated mice died at Day 4. Sera of the surviving mice were prepared 4 weeks after the last shot and pooled as polyclonal antivaccinia mouse serum. The neutralization antibody titer was assayed by plaque reduction as described (Ichihashi *et al.,* 1994). The 50% plaque reduction titer of the polyclonal antivaccinia serum was 2.1×10^3 against purified IMV. The serum was reactive with proteins of the EEV wrapping membrane prepared by the method of Payne (1978) and showed an immunoblotting profile similar to that presented in the paper. The monoclonal antibodies (3A5, 11A2, 1C1, and 2D5) were prepared as described (Ichihashi *et al.,* 1994). The target molecules, neutralization titers, and adsorption inhibitory activity of the mAbs are presented in Table 1. The 2D5 and 11A2 mAbs reacted with different epitopes of VP23-29K.

The viral sensitivity against a fixed concentration of the mAbs was assayed. As mouse normal serum and ascites fluid showed nonspecific interference against vaccinia virus infection at a dose higher than 10%, antibodies and normal mouse serum were used as 1% solution. The viruses to be compared were serially diluted, and equal volumes of 1% antibody solution were added to each of the diluted virus samples. The mixture was inoculated onto Vero cell monolayers in 12-well plates (50 μl/well). The virus was adsorbed to Vero cell culture at 4° for 45 min, washed with cold PBS, and overlaid with DMEM containing 1% agar and 2% fetal calf serum (1 ml/well). After a 2-day incubation, agar medium supplemented with neutral red was added (1 ml/well). The per-

TABLE 2

Neutralization of IHD-J Strain Vaccinia Virus

IHD-J virus	Titer (PFU/50 μl)	Effect of neutralizing Abs: Escaped virus % 2D5	11A2	3A5	Anti-vac
Culture fluid of infected cells	7.5×10^3	37.3	36.0	39.8	30.3
Cell-associated virus	1.4×10^6	1.2	15.7	14.1	8.8
Purified EEV	8.3×10^7	0.01	24.6	27.4	14.5
Purified IMV	9.5×10^7	0.01	9.4	7.1	5.3

Note. Vero cells were infected with the IHD-J strain of vaccinia virus. Culture fluid and cells were harvested 48 hr after infection. EEV and IMV were purified by CsCl equilibrium centrifugation, where the densities were 1.230 and 1.278, respectively. The virus samples were serially diluted with PBS. Antibodies were 1/100 diluted with PBS. Equal volumes of virus and antibody were mixed and inoculated onto Vero cell cultures for plaque assay. The virus that escaped neutralization is presented as a ratio (%) to the control virus titer. Each value represents the mean of four wells.

centage of virus that escaped neutralization [(titer of the sample with antibody/titer of the corresponding control sample) $\times$ 100] was calculated as an index expressing the sensitivity of the virus to the tested antibody.

Adsorption inhibitory activity of the antibodies was assayed using [^{35}S]methionine-labeled purified IMV. The virus was adjusted to 10^5 dpm/100 μl and mixed with equal volume of 1/100 diluted antibody or normal mouse serum and incubated at 37° for 60 min. The mixture (100 μl/well) was added to Vero cell culture at 4°. After a 60-min incubation at 4° with gentle shaking, the culture was washed with cold PBS three times. The washout solutions were pooled and centrifuged at 1000 *g* for 15 min. The [^{35}S] recovered in the supernatant of the washout solution was assayed by gamma well scintillation counter. The difference between the inoculum dpm and that of the recovered solution represents adsorbed virus. Percentage reduction against the normal mouse serum was calculated as the adsorption inhibitory activity of the antibody. The 2D5mAb did not show the adsorption inhibitory activity. The amount of virus remaining in the culture medium was 6% lower than that of normal mouse serum control, probably by increased adsorption to the Fc receptor.

RESULTS

2D5mAb does not neutralize intact EEV

Vero cells were infected with the IHD-J strain of vaccinia virus with a low multiplicity of infection (10 PFU/well, about m.o.i. 10^{-5}) and incubated in the presence of 1% 2D5mAb. Although the neutralization activity of the 2D5mAb was high, the virus replicated well and spread to surrounding cells. This was not due to mutation of the virus or to exhaustion of the 2D5mAb, because purified progeny IMV was sensitive to the 2D5mAb and the culture fluid maintained high neutralization activity against purified IMV. In contrast, the vRB10 mutant virus, with which Vero cells were infected with an m.o.i. of 0.1, diluted out during three subculture passages of the infected cells in the 1% 2D5mAb. Since the mutant virus produced similar amount of IMV as the parental IHD-J strain virus but lacked the ability to produce EEV, this result indicated that the 2D5mAb did not neutralize EEV.

To confirm the above findings, purified EEV and IMV were compared using several neutralizing antibodies (Table 2). The virus in the original culture fluid was more resistant against all antibodies tested than purified IMV. The 11A2mAb, 3A5mAb, and polyclonal anti-IHD-J serum neutralized the purified IMV more effectively than purified EEV. But, the ratio of the resistant virus was higher in the original culture fluid than in the purified EEV, and the 2D5mAb neutralized the purified EEV almost as well as it did IMV. As the purified EEV was prepared from the culture fluid, the resistant virus seemed to be converted to a more sensitive form during the purification process.

The culture medium and cell-associated virus samples were fresh and processed as described in Table 3. Sixty-five percent of the virus in the original culture fluid was

TABLE 3

Differences in Sensitivity against 2D5mAb

IHD-J grown in Vero cells 48 hr postinfection	Titer (PFU/50 μl)	Titer after exposure to 2D5	Escaped virus %
Virus in culture medium	2.6×10^4	1.7×10^4	65
Ultrasonic	2.8×10^4	9.2×10^3	33
Hypotonic	2.7×10^4	7.2×10^3	27
Trypsin	3.1×10^4	3.8×10^3	12.3
Freeze–thaw $\times$1	2.8×10^4	6.2×10^2	2.2
Trichloro-trifluoroethane	2.4×10^4	2.0×10^2	0.8
pH 5	2.7×10^4	1.5×10^4	55.5
Cell-associated virus	3.5×10^6	3.4×10^4	0.9
Hypotonic	5.0×10^6	2.5×10^4	0.5
Trichloro-trifluoroethane	4.4×10^6	3.9×10^3	0.1
pH 5	3.3×10^6	7.8×10^3	0.2

Note. Fresh culture medium and cells were collected 48 hr postinfection. The cells suspended in PBS were sonicated for 1 min and centrifuged at 1000 *g* for 10 min. The supernatant was used as cell-associated virus. Hypotonic: virus samples were 1/10 diluted with distilled water, stored for 20 min, and then serially diluted with PBS. Ultrasonic: 5-min treatment in a 200W Kubota sonicator. Trypsinization: culture fluid was 1/10 diluted in TPCK-treated trypsin solution (20 μg/ml, PBS) and incubated at 37° for 10 min. Trichloro-trifluoroethane: virus solution was mixed with 3 volumes of 1,1,2-trichloro-1,2,2-trifluoroethane and shaken gently for 10 min. After low-speed centrifugation, the virus fraction was collected. pH 5: virus solution was adjusted to pH 5 with 0.1 *N* HCl and readjusted to pH 7.4 with 0.1 *N* NaOH after 5 min.

TABLE 4

Treatment of EEV and IMV with Antibodies

	Percentage of virus that infected after exposure to a mixture of antibodies					
	Normal serum	2D5	11A2	3A5	1C1	Anti-vac serum
(A) Normal serum	100					
2D5	64.7	64.1				
11A2	61.8	61.2	78.2			
3A5	69.4	68.2	69.4	67.6		
1C1	77.6	68.2	61.3	68.2	78.8	
Anti-vac serum	82.0	67.1	72.9	88.2	78.6	84.7
(B) Normal serum	100					
2D5	0.02	0.01				
11A2	5.0	0.03	4.2			
3A5	6.3	0.02	ND	5.6		
Anti-vac serum	1.0	0.39	1.1	4.2	ND	0.7

Note. (A) Culture fluid of Vero cells infected with the IHD-J strain of vaccinia virus was collected at 48 hr postinfection. Antibodies and normal BALB/C mouse serum were diluted with PBS (1/100). An equal volume of two antisera was mixed. The culture fluid was mixed with the antisera for 60 min and then titrated in Vero cell cultures. The control titer of the culture fluid was 8.5×10^3 PFU/50 μl. (B) Purified IMV was mixed with antibodies for 60 min and then titrated in Vero cell cultures. The control titer of the purified IMV was 1.9×10^7 PFU/50 μl. The titer of the sample exposed to antibody is expressed as a ratio (%) to the control. Each value represents the mean of four assays.

resistant to the 2D5mAb. The virus in the freeze–thawed culture fluid maintained a titer similar to that of the original culture fluid, but the ratio of the 2D5-resistant virus dropped from 65 to 2.2%. The virus collected from the freeze–thawed culture fluid banded in CsCl equilibrium centrifugation with a peak at the EEV position ($p = 1.242$ g/ml). Apparently the EEV wrapping membrane was associated with the virions even after the change to the 2D5-sensitive form. Shaking the virus with trichloro-trifluoroethane most effectively converted the EEV to the susceptible form. When stored at 4°, the virus in the culture fluid maintained the titer, but lost resistance to the 2D5mAb. The ratio of the 2D5-resistant EEV declined to 10% after 3 days of storage and to 2% after 4 weeks. These results confirmed that the 2D5mAb did not neutralize fresh intact EEV and that the EEV lost the resistance against 2D5mAb as a result of damage to the wrapping membrane.

The mechanism to yield "residual virus fraction" that escapes neutralization by antibody has been explained by low neutralization activity of the antibody, a few virus particles that escaped antibody binding, or by the notion that neutralization occurs after binding of antibodies to two or more epitopes of the virus. These explanations do not fit our results. We assayed the neutralization activity of various antibodies against EEV and IMV (Table 4). All the tested antibodies neutralized IMV with various levels of efficiency, and a mixture of two antibodies produced a neutralization value intermediate between those of the paired antibodies. In contrast, over 60% of virus in the culture fluid was resistant to all the antibodies. Mixing two mAbs did not elevate the neutralization rate, even when the mAbs were mixed with the polyclonal antivaccinia serum containing antibody against wrapping membrane proteins. This indicated that the EEV was resistant to all the tested antibodies and that the antibody against EEV wrapping membrane had no neutralization activity. The adsorption inhibitory activity of antivaccinia polyclonal antibody did not effectively increase neutralization rate. The neutralized virus in the culture fluid seemed to be IMV and EEV with a damaged wrapping membrane. A fresh intact wrapping membrane protected the virus against neutralizing antibody.

Time course of EEV formation

Vero cell cultures were infected with the IHD-J strain of vaccinia virus, and then the culture fluid and cells were collected at various times after infection. The cells were briefly sonicated to liberate the associated virus. The samples were centrifuged at 1000 *g* for 15 min, and virus in the supernatant was titrated before and after exposure to 2D5mAb (Fig. 1). The 2D5-reactive epitope of the virus was masked by the wrapping membrane. Therefore, 2D5mAb will eliminate all the IMV and leave the infectious intact EEV. The 2D5-resistant EEV appeared at first as cell-associated virus 6 hr after infection. The amount increased and reached a plateau at 18–20 hr. The percentage of the cell-associated EEV against the total cell-associated virus was less than 3% throughout the infection process. The EEV in the culture fluid was first detected 7 hr after infection. The ratio of EEV in the culture fluid reached 67% at 48 hr after infection. The time lag between the growth of cell-associated virus and that of cell-associated EEV was about 6 hr, which corresponded to the period required for wrapping with Golgi membrane

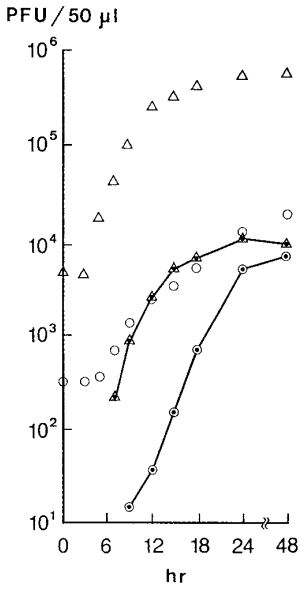

FIG. 1. Growth of vaccinia virus in Vero cell culture. Vero cells were inoculated with the IHD-J strain of vaccinia virus at an m.o.i. of 3. After a 60-min adsorption at 4°, the cultures were washed with cold PBS and incubated in DMEM containing 10% fetal calf serum. Culture fluid and cells were collected at the indicated times and stocked at 4° until assay. The cells were sonicated for 1 min to liberate associated virus. The culture fluid and cell homogenates were centrifuged at 1000 *g* for 10 min. The supernatants were serially diluted and mixed with an equal volume of 2D5mAb (1/100 diluted) or with normal mouse serum (1/100 diluted) and then titrated in Vero cell monolayer cultures. Every point represents the mean of four wells. (○) Virus in the culture medium. (⊙) Virus in the culture medium incubated with 2D5mAb. (△) Virus in the cell homogenate. (▲) Virus in cell homogenate incubated with 2D5mAb.

cisternae and for transportation of the EEV across the plasma membrane. The time lag between the growth of cell-associated EEV and that of culture fluid EEV was about 5 hr, which corresponded to the period required for transition of the cell-associated EEV to the culture medium.

The virus within the wrapping membrane is activated form

The culture fluid of cells infected with the IHD-J strain virus (48 hr postinfection) was centrifuged (1000 *g*, 15 min). The supernatant was freeze–thawed to disrupt the wrapping membrane of EEV and divided into two portions. One was digested with TPCK-treated trypsin (20 μg/ml) at 37° for 10 min, and the other portion was incubated under the same conditions, but with DMEM. The viral titer in the culture fluid doubled and that of purified IMV increased about threefold after the trypsinization (Table 5). The increase indicated that the samples contained the protected form virus. Titer of the IMV was reduced by PMSF to 25% that of the control. About 75% of the infectious IMV was the protected form virus that infects after activation on cell surface. The PMSF did not affect the virus titer of the culture fluid even when wrapping membrane of EEV was disrupted by freeze–thawing. This indicated that the virus in the EEV wrapping membrane had been activated. Differing from the IMV sample, the culture fluid contained only a trace amount of the virus that infected after activation on cell surface. Since the majority of IMV was protected form, the wrapping membrane activated it. This explained the origin of naturally activated virus and the high infectiousness of EEV.

Penetration mechanism of EEV

When IMV is trypsinized, it becomes activated and rapidly infects Vero cells by means of virus–cell membrane fusion (Ichihashi *et al.,* 1994). EEV was found to accommodate the activated form virus inside; however, the penetration mechanism of intact EEV should be different from that of the activated IMV. Infection of vaccinia virus establishes by entry of core and lateral bodies into the cytoplasm. If virus–plasma membrane fusion was the exclusive pathway of penetration, the EEV would have to remove its wrapping membrane prior to the penetration. This ecdysis may result in the neutralization of the virus when there is 2D5mAb. The EEV is resistant to the 2D5mAb throughout the penetration process, therefore, the most probable infection route of EEV is entry through the endosome membrane.

All known viruses that infect with a pH-dependent mode infect through the endosome (Marsh and Helenius, 1989); therefore, the acid-induced virus–cell membrane fusion has been considered to suggest endosomal infection. The fusion of vaccinia-infected cells is inducible by treatment of the cells with pH 5.0 solution (Gong *et al.,* 1990), and the 2D5mAb inhibits the cell fusion (Ichihashi

TABLE 5

Effect of PMSF on Infection of EEV

IHD-J strain vaccinia virus	Time of the virus × 10^{-3} PFU/50 μl			
	Control		Trypsin-treated	
	−PMSF	+PMSF	−PMSF	+PMSF
Culture fluid	5.7	5.5	13.0	12.1
Culture fluid freeze–thaw	5.7	5.1	10.3	11.1
IMV	118	31	285	259

Note. Culture fluid was collected 48 hr after infection and divided into two portions. One was incubated with trypsin (TPCK-treated trypsin, 20 μg/ml) at 37° for 10 min, and the other portion was incubated with PBS under the same conditions. Vero cells were infected with the viruses in the presence or absence of a serine protease inhibitor (PMSF, 3 m*M*) as described (Ichihashi and Oie, 1980). Values represent the mean of four wells. Statistical tests showed a significant increase of virus titer caused by trypsin ($P < 0.01$) and a significant decrease of the IMV titer assayed in the presence of PMSF ($P < 0.01$).

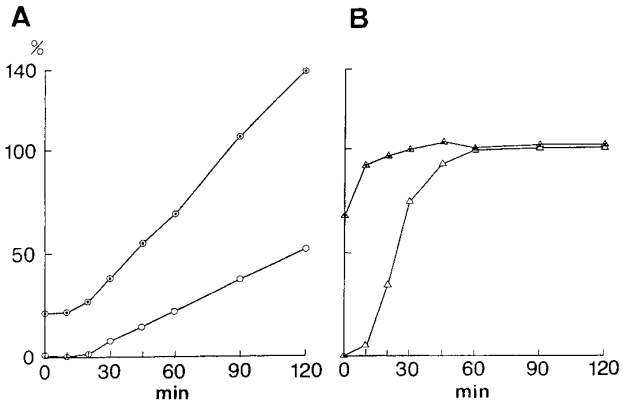

FIG. 2. Acid-induced acceleration of infection. The first group of Vero cell monolayer cultures in 24-well plates was infected with about 200 PFU/50 μl of the IHD-J strain of vaccinia virus or trypsinized virus. After adsorption at 4° for 60 min, the cells were washed with cold DMEM to remove unabsorbed virus. At time 0, medium (37°) was added to all cultures (0.5 ml/well). Diluted 2D5mAb was added at the indicated times (1/100 dilution, 50 μl/well). Normal mouse serum was added to control cultures. All cultures were washed with cold PBS 120 min after infection and overlaid with agar medium. The second group of cultures was treated with an acidic solution (RPMI 1640, pH 5, 10 m*M* HEPES, 10 m*M* MES, 37°, 0.5 ml/well) at the indicated time. After 3 min, the acidic RPMI was replaced with DMEM containing 1% 2D5mAb and 3% fetal calf serum. All cultures were washed with cold DMEM at 120 min and overlaid with agar medium. Plaques were counted after 3 days. The mean number of plaques formed in cultures is indicated in the ordinate as a ratio (%) of the mean number of plaques formed in the control cultures assayed without 2D5mAb treatment. The titer of normal IMV was 1.8×10^6 PFU/50 μl, and that of the trypsinzed IMV was 7.4×10^6 PFU/50 μl. (A) ○, purified IMV; ⊙, purified IMV treated with RPMI, pH 5.0. (B) △, trypsinized IMV; ◬, trypsinized IMV treated with RPMI, pH 5.0. Every point represents the mean of four wells.

et al., 1994). Since the 2D5mAb reacts specifically with virion, the cell fusion is suggested to be triggered by virus–cell membrane fusion. We analyzed the direct effect of acid on IMV infection.

Infection kinetics of normal IMV and trypsinized IMV were assayed by addition of 2D5mAb at various times after inoculation. In the parallel assays, the virus-infected Vero cell cultures were treated with RPMI (pH 5) for 3 min at 37° at various times after infection and then incubated in medium containing 1% 2D5mAb and 3% fetal calf serum. Control cultures were mock-treated with RPMI (pH 7.4). The cultures were overlaid with agar medium 120 min after infection, and plaques developed 3 days later. Percentages of the plaque numbers against control value were plotted (Fig. 2). Plaque formation signified that the seed virus of the plaque completed the 2D5-sensitive step of penetration by the time the 2D5mAb was added. The control titer of IMV, which was assayed without 2D5mAb treatment, increased about threefold by trypsinization. Fifty percent of the trypsin-activated IMV infected within about 30 min, whereas normal IMV required 2 hr. The results confirmed the findings that the trypsinization of IMV increased infectivity of the virus and that the penetration of the trypsinized virus occurred at neutral pH (Ichihashi *et al.,* 1994).

The brief exposure to acid accelerated the penetration process. Twenty-three percent of the adsorbed normal IMV responded to the acidic conditions at time 0 (Fig. 2A). The amount of responder virus increased during incubation, and the titer exceeded the control value. In contrast, 65% of the trypsinized IMV responded to the acidic conditions at time 0 (Fig. 2B). After an additional 10-min incubation at 37°, all the adsorbed trypsinized IMV completed the 2D5-sensitive step of penetration in response to the acid. Differing from normal IMV, the penetration of trypsinized IMV was only accelerated, since the titer obtained upon the acid exposure did not exceed that of control cultures.

The acid treatment accelerated the infection process, but it did not explain the increase of the titer over control value that was observed in infection of normal IMV. When the experiment presented in Fig. 2A was performed in the presence of PMSF (2.5 m*M*), the acid treatment did not increase efficiency of infection or accelerate the infection process (Fig. 3). Since the PMSF prevents proteolytic activation of the virus on the cell surface, only the virus that has been already activated develops plaque in the presence of PMSF. The suppression of activation on the cell surface by PMSF resulted in the suppression of acid-induced acceleration of infection. It indicated that the activated virus responded to the acid treatment. This agreed with the finding that a large portion of the trypsinized IMV responded to the acid at time 0. In the normal IMV, the virus responding to acid at time 0 seemed to be naturally activated virus. The increase over control titer was, therefore, not a direct effect of the acid treatment but a consequence of the proteolytic activation of IMV which proceeded continuously on the cell surface. The activation on the cell surface was not sufficient to induce virus–cell fusion at neutral pH by which the trypsin-activated virus infects.

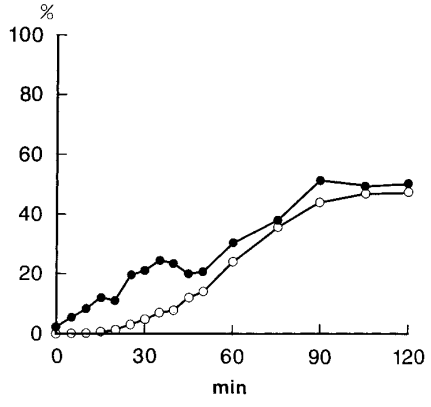

FIG. 3. Inhibition of the acid-induced acceleration. Vero cell cultures in 24-well plates were infected with IHD-J strain virus. At time 0, medium containing 2.5 m*M* PMSF was added to all cultures (0.5 ml/well). The other experimental procedures were the same as that of Fig. 2A. (○) Purified IMV. (●) Purified IMV treated with RPMI, pH 5.0.

The finding that the virus penetration occurred immediately in response to the exposure to acid supported the view that the activated IMV fuses with the cell surface membrane. The acid-induced process was temperature-dependent and sensitive to 2D5mAb because there was only a slight acceleration when the RPMI (pH 5) was cold (4°) and the acceleration was blocked when 2D5mAb was added to the RPMI (pH 5) solution.

It was concluded that the acid accelerated membrane fusion between the activated virus and the cell surface membrane. The acid-induced acceleration of infection indicated that the vaccinia virus had the pH-dependent step in its infection process and that the virus has the capacity to infect through the endosome membrane.

Effect of ammonium chloride and neutral red

A weak base such as ammonium chloride reduces the endosome-dependent infection by elevating the endosomal pH (Maxfield, 1982; Marsh and Helenius, 1989). The effect of the NH_4Cl was compared between trypsin-activated IMV and EEV. Vero cell cultures were infected with the viruses and then overlaid with agar medium just after adsorption at 4°. When NH_4Cl was added to the overlay agar medium (final concentration, 15 m*M*), the number of proteolytically activated IMV plaques was reduced from 3.1×10^3 to 2.5×10^2 PFU/50 μl, and that of EEV reduced from 1.9×10^3 to 11 PFU/50 μl (mean of six wells). The EEV was about 10 times more sensitive to NH_4Cl than trypsinized IMV. The plaques decreased to about $\frac{1}{5}$ of the normal size. A lower dose (10 m*M*) did not reduce the number of plaques but they were small. A higher concentration of NH_4Cl (20 m*M*) completely inhibited plaque formation and the phagocytosis of neutral red.

Endocytosis was apparently impaired by the ammonium chloride, but it might possibly be an effect of toxicity to other organelles. The inhibition rate drifted in repeated assays. As an alternative, Vero cells were incubated in medium containing neutral red (final, 1/10,000) for 60 min. Endosomes of the cells became saturated with it. The Vero cell cultures were infected with trypsin-activated IMV or intact EEV for plaque assays. The EEV was prepared by treating culture fluid of infected cells with 2D5mAb. Titer of the EEV decreased from 2.1×10^3 to 1 PFU/50 μl (mean of six wells). The titer of the activated IMV was not affected even though the plaque size was smaller than that of the control culture.

The differences in reactions to the 2D5mAb, to acid, and to the suppression of endocytosis imply that the trypsin-activated IMV penetrated the cell surface membrane, whereas EEV penetrated the endosome membrane, both by virus–cell membrane fusion.

DISCUSSION

The vaccinia virus matures in the cytoplasm as protected form IMV. It is wrapped by the membrane of Golgi cisternae and disseminated as EEV. This process requires about 6 hr, and the EEV stays on the cell surface for an additional 5 hr and then liberates to culture fluid. Electron micrographs of infected cells show large amounts of cell-associated EEV at this stage of infection, and some EEV lose the wrapping membrane in the culture medium (Ichihashi *et al.,* 1971). About 65% of the infectious virus in the culture medium remains as intact EEV at 48 hr after infection. During an encounter with an adequate host cell, the EEV is endocytosed and the wrapping membrane may be disrupted in the endosome. Thereafter the virus fuses with the endosome membrane assisted by the low-pH conditions of the endosome. The wrapping membrane is multifunctional. It transports the virus, protects the virus against antibodies, activates the virus for penetration, and probably opsonizes the EEV.

The IMV seems to remain in protected form in the cytoplasm. It will prevent fusion of the virus with the cellular membrane from inside. Trypsinization of the virus increases the titer of the IMV two- to threefold, indicating that 50–70% of the IMV is noninfectious to Vero cells under normal culture conditions. An infectivity index (physical particle number/PFU) of the normal IMV is about 40 at best, and the ratio of the trypsinized IMV is about 10. There is a large fraction of noninfectious virus even after the trypsin-induced activation. When sufficiently activated, the virus infects by direct virus–cell fusion (Chang and Metz, 1976). It may facilitate rapid spread of infection, but the activated IMV is vulnerable to antibodies and serum inhibitors. Under the circumstance where such inhibitory factors are abundant, cell-to-cell transmission and long-distance dissemination of the virus are achieved by the EEV.

The vaccinia virus has a stable DNA genome and consequently, stable immunogenicity. Almost all monoclonal antibodies to the structural proteins are cross-reactive among orthopoxviruses (Ichihashi and Oie, 1988). The stable immunogenicity represents the identity of the virus, but the immune state for stable epitopes will easily prevail on a susceptible host population. RNA viruses mutate frequently to escape neutralization by antibody. In contrast, vaccinia virus compensates for the disadvantage by acquiring a mechanism to escape neutralization. The wrapping membrane of EEV and infection through the endosome effectively block the function of neutralizing antibodies. This finding explains the complications of vaccinia infection in Bruton-type immunodeficient individuals who produce antibody but lack cytotxic T cell activity. Both humoral antibody that neutralizes IMV and cytotoxic T cells that recognize vaccinia-specific cell membrane proteins are required for recovery from infection.

For the virus–cell membrane fusion to occur, the vaccinia virus must be in the activated form, and there is a difference between requirements for the virus–plasma membrane and the virus–endosome membrane fu-

sions. The trypsin-activated virus could infect by direct virus–plasma membrane fusion under neutral pH, whereas the virus activated on the cell surface required acidic conditions. Since cell surface hemagglutinin (HA) blocks cell fusion at the late stage of infection (Oie *et al.,* 1990; Seki *et al.,* 1990), HA has a capacity to block fusion protein under neutral pH. In the trypsin-treated IMV, the epitope reactive to the 2D5mAb is in a more exposed condition probably by removal of virus-associated HA (Ichihashi *et al.,* 1994). The incomplete activation of IMV on cell surface may due to insufficient removal of the HA on the virus. The acidic condition seems to inactivate the fusion inhibitor on the virus. Serpin-like protease inhibitors also control cell fusion (Law and Smith, 1992; Turner and Moyer, 1992; Zhou *et al.,* 1992), probably by limiting the proteolytic activation of the IMV and/or degradation of HA.

Cells infected with the vRB10 mutant do not fuse in response to the anti-HA B2D10 mAb or at low pH (Blasco and Moss, 1991). If EEV as well as IMV can fuse with cells at neutral pH (Doms *et al.,* 1990) and if IMV-induced cell-to-cell fusion from without occurs at low pH (Gong *et al.,* 1990), then the nonresponsiveness of the cells infected with the vRB10 mutant cannot be explained, because the mutant produces a similar amount of IMV as the parental IHD-J strain virus. This difficulty arises from the notion that the EEV is stable and that the infecting efficiency of IMV is homogeneous. This study showed that the EEV described in the literature may not be intact, but has a disrupted wrapping membrane which will behave as activated virus. Thus the nonresponsiveness in cells infected with the vRB10 mutant is attributable to a lack of sufficient numbers of viruses on the cell surface. The wrapping transportation of the virus provides abundant numbers of the activated virus on the surface of surrounding cells. Presence of the activated virus induces cell fusion in response to the anti-HA B2D10 mAb or low pH. Therefore, there is no difference in the requirement for virus–cell and cell–cell fusion. The common basic mechanism is activated form virus–cell membrane fusion, and the process remains to be elucidated.

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