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Antibody-sensitive and antibody-resistant cell-to-cell spread by vaccinia virus: role of the A33R protein in antibody-resistant spread

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The roles of vaccinia virus (VV) intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV) and their associated proteins in virus spread were investigated. The plaques made by VV mutants lacking individual IEV- or EEV-specific proteins (vΔA33R, vΔA34R, vΔA36R, vΔA56R, vΔB5R, vΔF12L and vΔF13L) were compared in the presence of IMV- or EEV-neutralizing antibodies (Ab). Data presented show that for long-range spread, the comet-shaped plaques of VV were caused by the unidirectional spread of EEV probably by convection currents, and for cell-to-cell spread, VV uses a combination of Ab-resistant and Ab-sensitive pathways. Actin tails play a major role in the Ab-resistant pathway, but mutants such as vΔA34R and vΔA36R that do not make actin tails still spread from cell to cell in the presence of Ab. Most strikingly, the Ab-resistant pathway was abolished when the A33R gene was deleted. This effect was not due to alterations in the efficiency of neutralization of EEV made by this mutant, nor due to a deficiency in IMV wrapping to form IEV, which was indispensable for EEV formation by vΔA33R and vΔA34R. We suggest a role for A33R in promoting Ab-resistant cell-to-cell spread of virus. The roles of the different virus forms in the VV life-cycle are discussed.

Introduction

Vaccinia virus (VV) is the prototype orthopoxvirus and forms plaques on a wide range of cells in culture. The mechanisms of VV spread are complicated because of the presence of different forms of virions: intracellular mature virus (IMV), intracellular enveloped virus (IEV), cell-associated enveloped virus (CEV) and extracellular enveloped virus (EEV) (reviewed by Moss, 1996). IMV is the first type of virion formed and is present in the cytoplasm by 6 h post-infection (p.i.). A portion of IMV particles are wrapped by double membranes derived from the early endosome (Tooze *et al.*, 1993) or *trans*-Golgi network (Schmelz *et al.*, 1994) to form the IEV particles. IEV then migrate to the cell surface where the outermost membrane fuses with the plasma membrane to form CEV. As a result, CEV possesses one more membrane than IMV and one less than IEV. CEV particles induce the polymerization of actin tails from the cell surface (van Eijl *et al.*, 2000; Hollinshead *et al.*, 2001; Ward & Moss, 2001) that drive CEV into adjacent cells or into the extracellular environment as EEV. CEV is important for the cell-to-cell spread of virus (Blasco & Moss, 1991, 1992), whereas EEV is important for long-range spread of virus and is produced in varying amounts by different strains of virus (Payne, 1980). IMV, CEV and EEV are infectious but IEV with intact membranes has not been isolated.

VV forms clear round plaques in many different cell lines and some strains of VV form comet-shaped plaques under liquid overlay (Appleyard *et al.*, 1971; Payne, 1980), like herpes simplex virus (HSV) (Shinkai, 1975). This characteristic plaque phenotype is caused by the efficient long-range spread of virus, resulting in a series of secondary plaques (comet tails) distant from the primary infection site (comet heads). The comet-shaped plaque phenotype of VV can be blocked by antibodies (Ab) directed against EEV but not IMV (anti-comet assay) (Appleyard *et al.*, 1971; Appleyard & Andrews, 1974; Payne, 1980; Vanderplasschen *et al.*, 1997; Galmiche *et al.*, 1999). Anti-EEV, but not anti-IMV, Ab provides passive immunity against VV challenge (Madeley, 1968; Appleyard *et al.*,

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Table 1. Properties of VV WR mutants

Mutant	Plaque size	IMV	IEV	EEV	Actin tails	Virulence	References
vΔA33R	Tiny	2–3 × ↓	Incomplete wrapping	2–4 × ↑	No*	?	Roper *et al.* (1998)
vΔA34R	Tiny	Normal	Few	19–24 × ↑, specific infectivity 5 × ↓	No	Attenuated	Duncan & Smith (1992); McIntosh & Smith (1996); Wolffe *et al.* (1997); Sanderson *et al.* (1998a); Röttger *et al.* (1999)
vΔA56R	Normal	Normal	Normal	Normal	Normal	Virulent†	Flexner *et al.* (1987); Sanderson *et al.* (1998a)
vΔB5R	Small	Normal	Few	5 × ↓	Few	Attenuated	Engelstad & Smith (1993); Wolffe *et al.* (1993); Mathew *et al.* (1998); Sanderson *et al.* (1998a); Röttger *et al.* (1999)
vΔF13L	Tiny	Normal	Few	5 × ↓	Few	Attenuated	Blasco & Moss (1992); Sanderson *et al.* (1998a); Röttger *et al.* (1999)
vΔA36R	Small	Normal	Normal	3 × ↓	No	Attenuated	Parkinson & Smith (1994); Sanderson *et al.* (1998a); Wolffe *et al.* (1998); Röttger *et al.* (1999); van Eijl *et al.* (2000)
vΔF12L	Tiny	2 × ↓	Normal	7 × ↓	Few	Attenuated	Zhang *et al.* (2000); van Eijl *et al.* (2002)

*Short slender actin filaments were found instead of thick actin tails.
†By intranasal route (G. L. Smith, unpublished data).

1971; Boulter *et al.*, 1971; Turner & Squires, 1971; Appleyard & Andrews, 1974; Galmiche *et al.*, 1999).

IMV is assumed to be the virion responsible for the spread of VV between hosts because the EEV membrane is too fragile to survive the physical environment outside the host, and once broken, will release a fully infectious and relatively stable IMV particle (Ichihashi, 1996). IMV and EEV possess a different set of virus proteins on their surfaces (Payne, 1978, 1992) and use different cellular receptors (Vanderplasschen & Smith, 1997; Krijnse-Locker *et al.*, 2000) and pathways to enter cells (Payne & Norrby, 1978; Ichihashi & Oie, 1980; Vanderplasschen *et al.*, 1998a; Krijnse-Locker *et al.*, 2000).

CEV is physically indistinguishable from EEV and may be released from the cell surface by mild trypsin treatment (Blasco & Moss, 1992). The retention of CEV on the cell surface rather than its release as EEV contrasts with other viruses, e.g. measles virus, human immunodeficiency virus type 1 and influenza virus, where the cellular receptors are usually down-regulated or removed to facilitate virus release and prevent virus aggregation (Palese *et al.*, 1974; Firsching *et al.*, 1999; Piguet *et al.*, 1999). CEV mediates cell-to-cell spread, but the absolute level of CEV is not critical for plaque size because VV strains International Health Department (IHD)-J and Western Reserve (WR) form plaques of similar size despite WR retaining more CEV than IHD-J on the cell surface (Sanderson *et al.*, 1998a).

Several VV proteins are associated with only IEV (A36R and F12L) or with IEV/CEV/EEV (A33R, A34R, A56R, B5R and F13L). The study of virus mutants with these genes deleted or repressed, showed that these proteins are not needed for IMV production but are involved in the various stages of virus egress, such as wrapping of IMV, transport of IEV to the cell surface, actin tail formation, EEV release and plaque phenotype. The properties of these deletion mutants are summarized in Table 1. IEV proteins A36R (Röttger *et al.*, 1999; van Eijl *et al.*, 2000) and F12L (Zhang *et al.*, 2000; van Eijl *et al.*, 2002) are each non-glycosylated proteins that have the majority of their amino acids in the cytosol, whereas EEV proteins A33R (Roper *et al.*, 1996), A34R (Duncan & Smith, 1992), A56R (Shida & Dales, 1981; Shida, 1986) and B5R (Engelstad *et al.*, 1992; Isaacs *et al.*, 1992) are glycosylated integral membrane proteins with a type I (B5R and A56R) or type II (A33R and A34R) membrane topology. F13L is an acylated membrane-associated protein that is associated with the inner surface of the EEV outer envelope (Hiller & Weber, 1985; Hirt *et al.*, 1986).

Electron microscopy indicated that most EEV particles originate from IEV (Payne & Kristenson, 1979). Consistent with this proposal, levels of EEV were reduced when the formation of IEV was inhibited by drugs (Payne & Kristenson, 1979; Ulaeto *et al.*, 1995) or the deletion of gene B5R (Engelstad & Smith, 1993; Wolffe *et al.*, 1993) or F13L (Blasco & Moss, 1991). However, mutants lacking gene A33R or A34R produced higher levels of EEV despite the wrapping of IMV being less efficient (Duncan & Smith, 1992; Wolffe *et al.*, 1997) or incomplete (Roper *et al.*, 1998). This observation questions whether EEV made by these mutants is formed by an alternative pathway such as budding.

Here, we have investigated the roles of IMV, IEV, CEV and EEV in VV spread *in vitro*, by using a panel of virus mutants lacking individual IEV- or EEV-specific genes, and Abs that neutralize IMV or EEV. We demonstrate that comet-shaped plaques are probably made by convection currents, that VV

spreads from cell to cell by Ab-sensitive and Ab-resistant pathways, and that the A33R protein has a role in Ab-resistant spread.

Methods

■ **Cells and viruses.** RK_{13} and BS-C-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL) containing 5% heat-inactivated foetal bovine serum (HFBS). For infection of cells, virus was diluted in DMEM supplemented with 2% HFBS (DMEM/2%). VV strains IHD-J and WR (Alcamí & Smith, 1992), and deletion mutants vΔA33R (Roper *et al.*, 1998), vΔA34R (McIntosh & Smith, 1996), vΔA36R (Parkinson & Smith, 1994), vΔA56R (Sanderson *et al.*, 1998a), vΔB5R (Engelstad & Smith, 1993), vΔF12L (Zhang *et al.*, 2000) and vΔF13L (Blasco & Moss, 1992) originating from strain WR, were described previously. vRevA34R is the revertant virus of vΔA34R in which the A34R gene was inserted back into the virus genome (McIntosh & Smith, 1996). IMV was purified as described elsewhere (Law & Smith, 2001) and was used for all infections.

■ **Antibodies.** Mouse monoclonal Ab (mAb) 2D5 against the IMV L1R protein (Ichihashi & Oie, 1996), mAb AB1.1 against the IMV D8L protein (Parkinson & Smith, 1994), rabbit antiserum against the B5R protein (α-B5R) (Galmiche *et al.*, 1999) and VV-immune rabbit antiserum Rb-WR2 (Law & Smith, 2001) were described previously. Antisera were heat-inactivated at 56 °C for 30 min before use.

■ **Plaque assays.** (i) Liquid overlay for comet formation. IMV was diluted in DMEM/2% and adsorbed onto cells for 2 h at 37 °C. Unbound virus was washed away with PBS and the cells were overlaid with liquid medium (DMEM/2%) and stained 2 days later (unless specified otherwise) with 0·05% crystal violet in 15% ethanol. Antibodies and 10 μg/ml IMCBH (N_1-isonicotinoyl-N_2-3-methyl-4-chlorobenzoyl-hydrazine) (Payne & Kristenson, 1979) were included in the overlays where indicated. (ii) Semi-solid overlay. As for (i) except that virus was adsorbed for 1 h and cells were overlaid with DMEM/2% containing 1·5% carboxymethylcellulose (CMC).

■ **Titration of EEV.** The infectivity of EEV was quantified as described (Law & Smith, 2001). Briefly, fresh virus supernatants were collected at the indicated times, diluted and incubated with mAb 2D5 (diluted 1/2000) for 1 h at 37 °C to neutralize contaminating IMV. When specified, Rb-WR2 Ab was included to neutralize EEV. The virus was adsorbed onto cells for 1 h, washed, and overlaid with 1·5% CMC in DMEM/2%. After incubation, the plaques were stained as above.

■ **Microscopy.** Methods for indirect immunofluorescent staining have been described elsewhere recently (Law & Smith, 2001). VV-infected cells were detected using mAb AB1.1 (5 μg/ml) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (diluted 1/100, Jackson Laboratories). The methods for electron microscopy have been described elsewhere recently (Hollinshead *et al.*, 2001).

Results

VV long-range spread

The ability of VV to form a comet-shaped plaque under liquid overlay has been noted for years, but there is no explanation why the virus spreads unidirectionally to produce the comet tail. VV strains, such as IHD-J, that make high levels of EEV form comet-shaped plaques under liquid overlay (Fig. 1b), but round plaques under semi-solid overlay (Fig. 1c). In contrast, VV strains, such as WR, yielding less EEV form round, defined plaques under liquid overlay (Fig. 1a). The formation of comet-shaped plaques can be blocked by Ab raised against VV infection (Fig. 1d) or EEV proteins (Engelstad *et al.*, 1992; Galmiche *et al.*, 1999), resulting in a plaque phenotype similar to that produced under semi-solid overlay (Appleyard *et al.*, 1971; Payne, 1980). Immunofluorescent microscopy showed that comet-shaped plaques are formed by the distant spread of EEV (Fig. 1e) rather than by the migration of infected cells because infected cells moved $\leqslant$ 0·2 mm/24 h (Sanderson *et al.*, 1998b; Sanderson & Smith, 1999) far less than the 4·3 mm from the centre of the plaque to the end of the comet tail shown in Fig. 1(e). To examine why EEV spreads unidirectionally, the possible role of gravity was investigated. Cells infected with VV were incubated with the tissue culture plate tilted 10°. Surprisingly, instead of having comets pointing in different directions as when incubated flat (Fig. 1f), all the comet tails became parallel and moved uphill (Fig. 1g). This suggested that EEV was dispersed by an upward

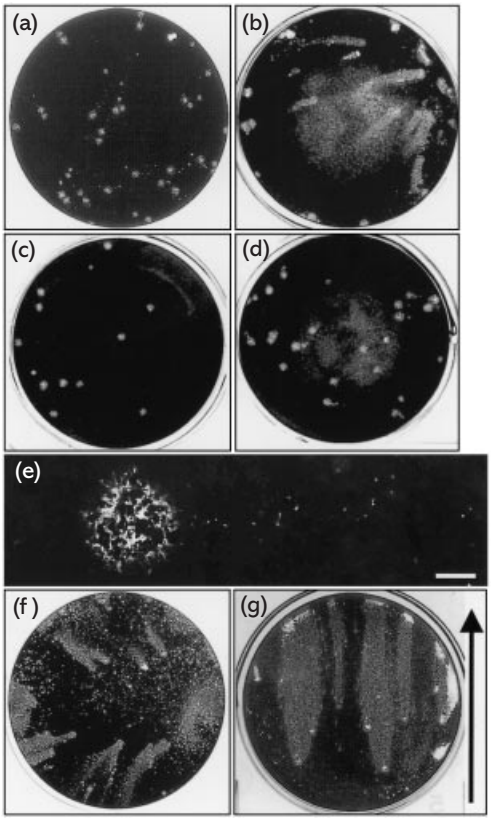

Fig. 1. VV plaque phenotypes. Plaques formed on RK_{13} cells infected with VV strains, WR (a) and IHD-J (b) under liquid overlay, IHD-J under CMC overlay (c) and liquid overlay with anti-VV Ab Rb-WR2 diluted at 1/100 (d). Plaques were stained 44 h p.i. (e) Indirect immunofluorescent staining of a VV IHD-J plaque on RK_{13} cells under liquid overlay 24 h p.i. Bar, 500 μm. (f) VV strain IHD-J plaques on RK_{13} cells under liquid overlay (44 h p.i.) with the plate incubated flat or (g) tilted 10°. The arrow points uphill, the direction of virus spread.

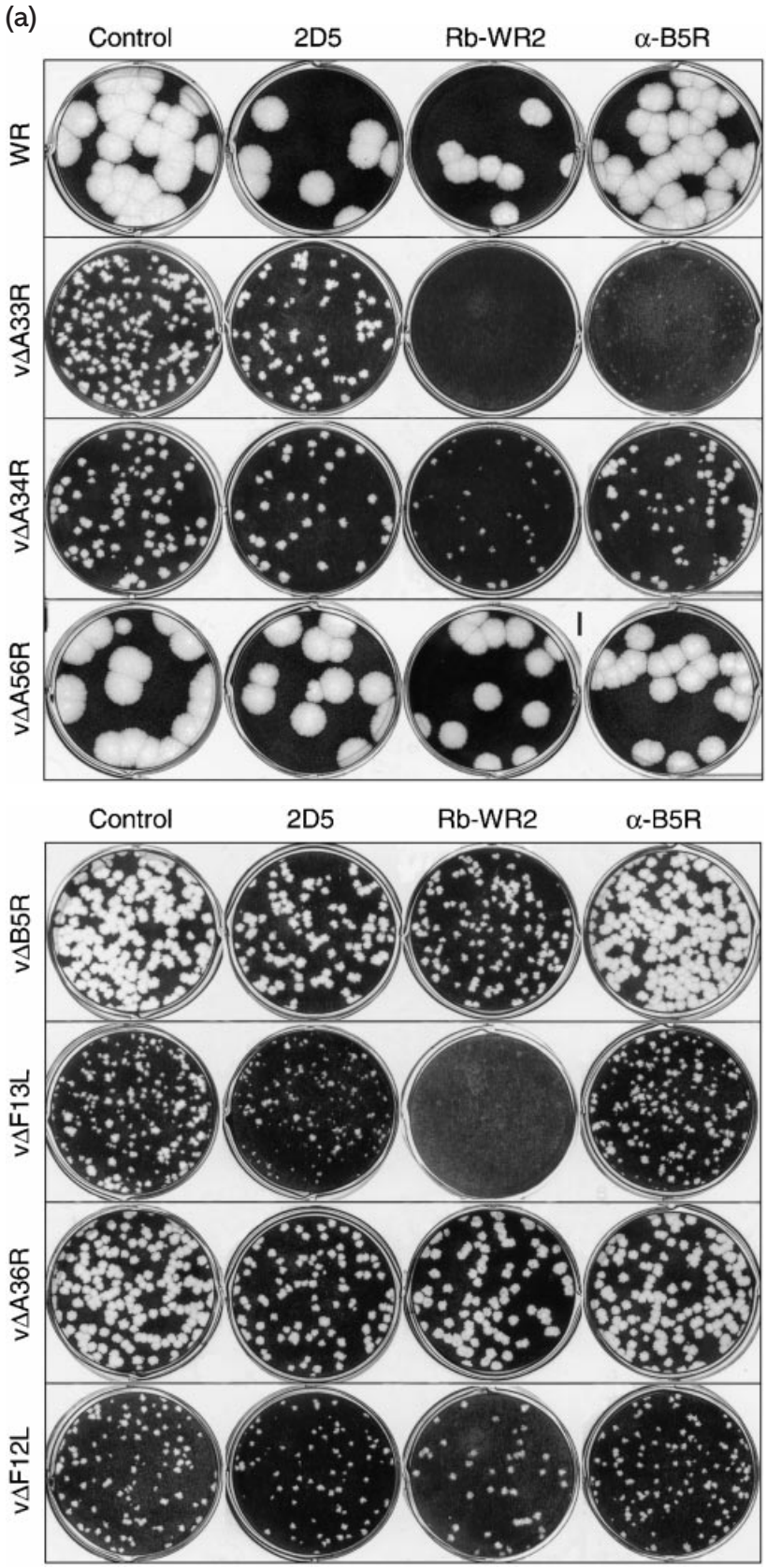

Fig. 2. For legend see facing page.

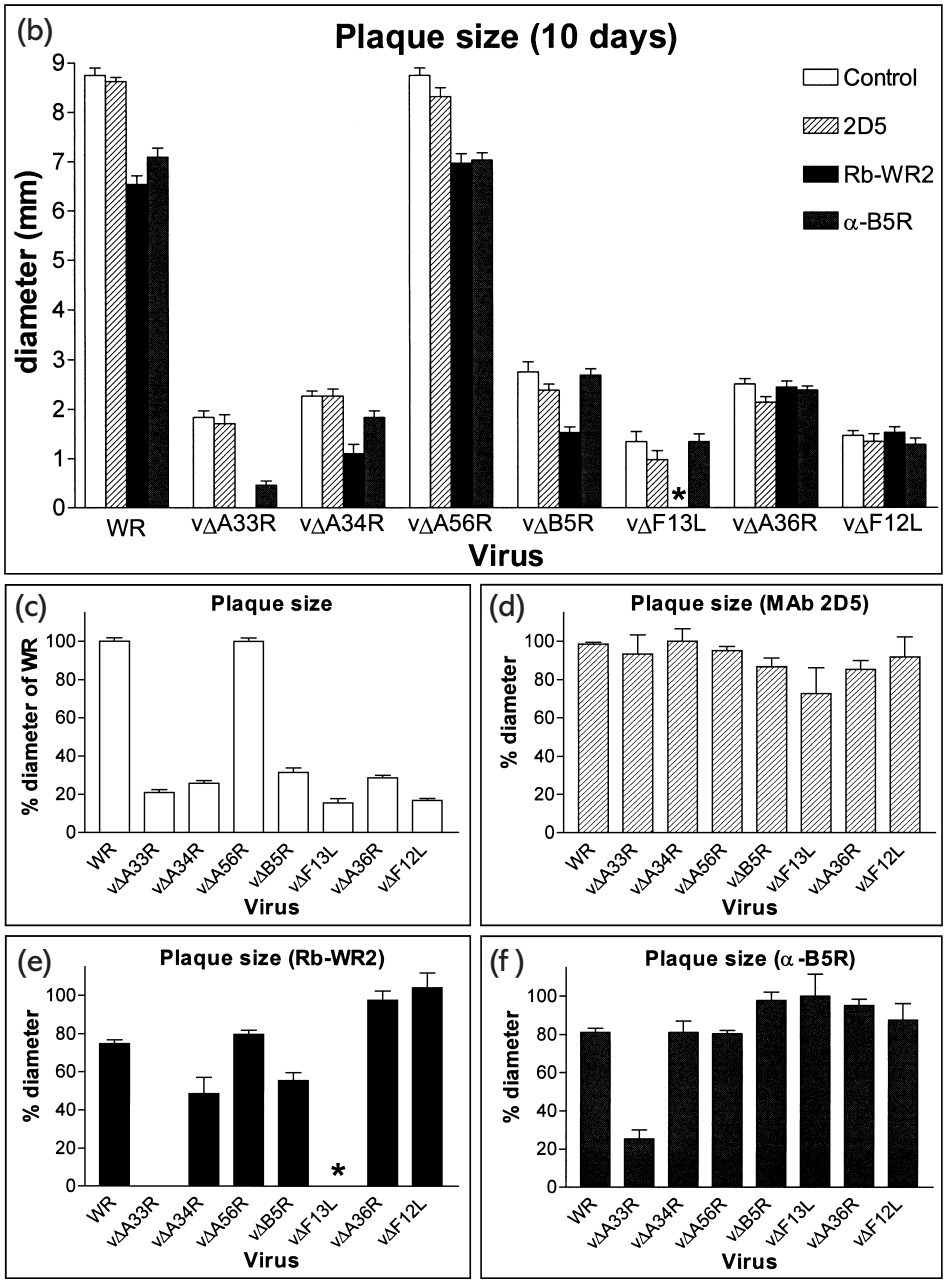

Fig. 2. (a) Cell-to-cell spread of VV in the presence of Ab. BS-C-1 cells were infected by WR or vΔA56R at 20 p.f.u. per dish and other mutants at 75 p.f.u. per well without Ab (control), or with mAb 2D5 diluted 1/500, Rb-WR2 or α-B5R antiserum diluted at 1/100. Cells were overlaid with CMC and stained with crystal violet at 10 days p.i. (b–f) Quantification of plaque sizes in the presence or absence of Ab. (b) The diameters of plaques made by the different viruses in (a) were measured. The data represent the mean ±SEM of the diameter of six plaques selected randomly. An asterisk indicates that vΔF13L plaques in the presence of Rb-WR2 were too faint to measure accurately. (c) The relative plaque size (diameter) of each mutant compared to WR is plotted. (d–f) The relative size of plaques formed by the mutants in the presence of mAb 2D5 (d), Rb-WR2 (e) or α-B5R (f), compared to control are shown.

convection current instead of gravity. Such currents might be generated by a temperature gradient resulting from evaporation or fluctuation of temperature inside the incubator.

Previously, anti-EEV Ab was reported to inhibit EEV release from cells (Ichihashi, 1996; Vanderplasschen *et al.*, 1997) and to neutralize EEV in solution (Boulter & Appleyard, 1973; Galmiche *et al.*, 1999; Law & Smith, 2001). To investigate if a single IEV or EEV protein was essential for this inhibition, we studied whether VV mutants lacking individual IEV- and EEV-specific genes would escape inhibition of virus

dissemination (formation of secondary plaques) in the presence of Rb-WR2, a rabbit Ab raised by repeated immunization with live VV WR (Law & Smith, 2001). This Ab reacted strongly with IMV proteins and also moderately with EEV proteins in immunoblot (see JGV Online for supplementary data, http://vir.sgmjournals.org). In assays using Rb-WR2 (diluted 1/100) and BS-C-1 cells, which gave clear plaques for all mutants, none of the mutants escaped inhibition of secondary plaque formation (data not shown). This is consistent with a recent study showing that EEV made by wild-type and each mutant virus were neutralized by Ab in solution (Law & Smith, 2001).

VV cell-to-cell spread

Ab Rb-WR2 not only inhibited the long-range spread of all the mutants but also caused significant reduction in the plaque size of some of the mutants. These observations suggest there are at least two mechanisms of virus cell-to-cell spread that are either sensitive or resistant to Ab. This was studied further by comparing the plaque size in the presence of Ab against different forms of VV and under a semi-solid overlay so as to measure virus cell-to-cell spread only. Fig. 2(a) shows the plaques formed by the mutants 10 days p.i. in the presence of mAb 2D5 (diluted 1/500), Rb-WR2 or α-B5R antisera (diluted 1/100), and their sizes are quantified in Fig. 2(b–f). MAb 2D5 is an IMV-neutralizing Ab (Ichihashi, 1996), Rb-WR2 inhibits both IMV and EEV (Law & Smith, 2001) whereas α-B5R neutralizes only EEV (Galmiche *et al.*, 1999; Law & Smith, 2001).

In the same conditions, plaques formed by all the mutants in the absence of Ab had at least a threefold reduced diameter compared with WR, except for those formed by vΔA56R that were equivalent (Fig. 2c). For the other mutants, the next largest were made by vΔA36R and vΔB5R, followed by vΔA34R and vΔA33R, and the smallest plaques were made by vΔF12L and vΔF13L.

Addition of mAb 2D5 had little effect on the plaque size of the mutants, although vΔF13L was reduced slightly more than the others (Fig. 2d). Possibly, IMV was released late during infection with vΔF13L and therefore its plaques were affected by mAb 2D5. These data indicate that IMV is not sufficient for VV cell-to-cell spread.

In contrast to mAb 2D5, the antiserum Rb-WR2 raised against a live infection had varying effects on the mutants (Fig. 2e). Most strikingly, plaques were not made by vΔA33R in the presence of Rb-WR2. Rb-WR2 also reduced the plaque size of WR, vΔA34R, vΔA56R and vΔB5R, but not vΔA36R or vΔF12L. Very faint plaques were seen with vΔF13L, but these were difficult to quantify. These results allow the viruses to be divided into three groups: complete blockage of virus spread (vΔA33R), partial blockage (WR, vΔA34R, vΔA56R, vΔB5R and vΔF13L) and no blockage (vΔA36R and vΔF12L). Since anti-IMV Ab did not affect cell-to-cell spread, the inhibition should be mediated by the anti-EEV Ab present in Rb-WR2.

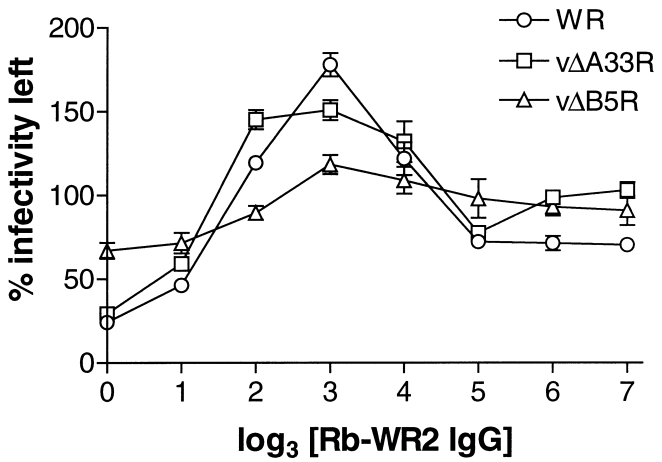

Fig. 3. Neutralization of EEV by Rb-WR2 Ab. EEV of the indicated viruses was grown in RK_{13} cells and incubated with Rb-WR2 IgG, which had been diluted serially in threefold steps, for 1 h at 37 °C in the presence of mAb 2D5 (diluted 1/2000). After washing, the cells were overlaid with 1% low-melting agarose in DMEM/2% and the plaques were counted at 40 h p.i. for WR, 4 days p.i. for vΔB5R and 5 days p.i. for vΔA33R. Data point '0' represents a concentration of 100 μg/ml of Rb-WR2 IgG. Two independent experiments gave similar results and each data point represents the mean ±SEM of duplicate measurements in one experiment.

The blockage of plaque formation by vΔA33R using Rb-WR2 is not due to an increase in Ab sensitivity of the mutant because EEV made by vΔA33R showed similar sensitivity to neutralization by antibody (Law & Smith, 2001) and further analyses using a range of dilutions of Ab confirmed that the sensitivity was the same as wild-type (Fig. 3). On the other hand, EEV of vΔB5R is less sensitive to Rb-WR2 because of the absence of major virus-neutralizing epitopes on the B5R protein (Law & Smith, 2001). Interestingly, antibody enhancement was observed in both vΔA33R and WR at similar Ab dilutions.

The effect of α-B5R also varied with different mutants (Fig. 3f). As expected, it had no effect on vΔB5R. Similar to Rb-WR2, it did not affect vΔA36R and vΔF12L, but inhibited WR, vΔA56R and vΔA34R slightly (~ 20%). Surprisingly, it did not affect vΔF13L at all, while tiny faint plaques were formed by vΔA33R.

Does abortive infection by vΔA33R occur in the presence of Rb-WR2?

The complete blockage of plaque formation by vΔA33R using Rb-WR2 Ab was unexpected. To investigate whether this blockage was a result of an Ab-sensitive spreading mechanism or a post-binding neutralization, we compared vΔA33R- and vΔA34R-infected cells by immunofluorescent microscopy (Fig. 4). Without Ab (Fig. 4a, d) or with mAb 2D5 (Fig. 4b, e), extensive cytopathic effect, such as cell rounding, cell flattening and projection formation (Sanderson *et al.*, 1998b), was found in cells infected by either mutant. At 72 h

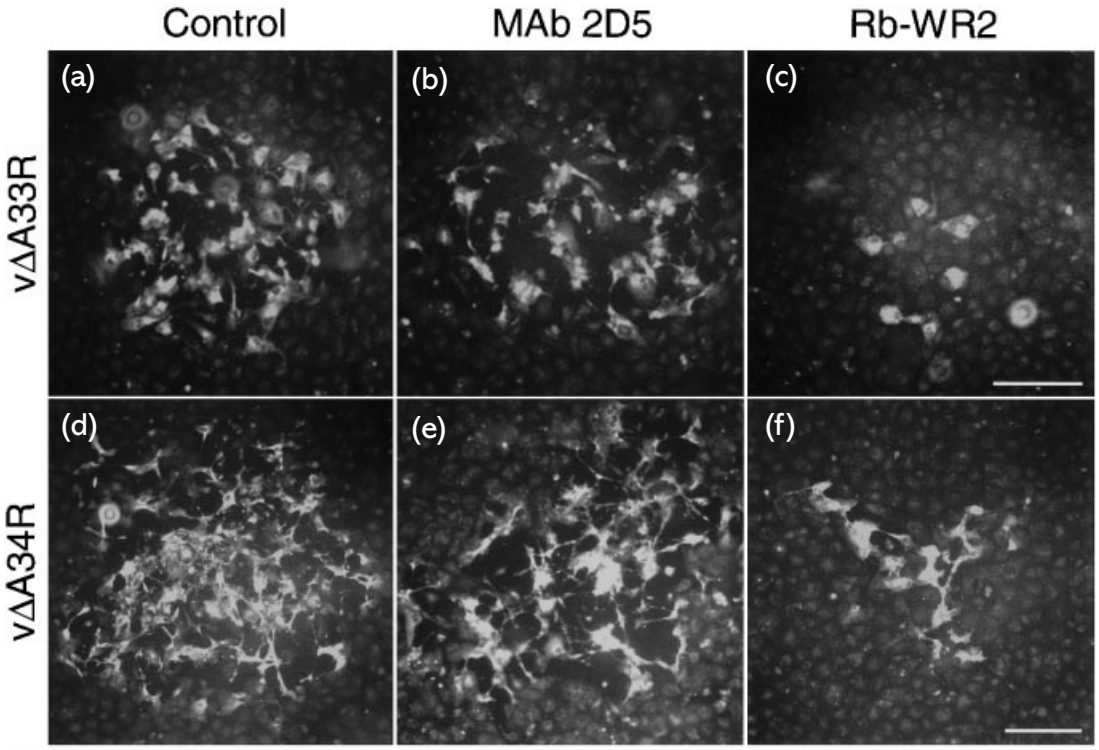

Fig. 4. Immunofluorescent microscopy of vΔA33R- and vΔA34R-infected cells. BS-C-1 cells growing on 22 mm glass coverslips were infected with vΔA33R or vΔA34R at 20 p.f.u. per coverslip for 2 h at 37 °C. The cells were covered with semi-solid overlay with or without Ab and were incubated for 72 h. Infected cells were detected using mAb AB1.1 followed by FITC-conjugated goat α-mouse IgG secondary Ab. (a, d) Control; (b, e) mAb 2D5; (c, f) Rb-WR2. Bar, 100 μm.

p.i., the infected foci were the same size with or without mAb 2D5 and were about 0·24 mm and 0·38 mm for vΔA33R and vΔA34R, respectively. In the presence of Rb-WR2, cells infected with vΔA34R developed similar cytopathic effect, although virus spread was restricted by the Ab (Fig. 4f). With vΔA33R in the presence of Rb-WR2 (Fig. 4c), very few infected cells were found in all the infected foci examined. In addition, the infected cells did not develop extensive cytopathic effect and were not clustered together. The individual infected cells within these atypical foci were unlikely to have derived from several separate infections because only a few plaque-forming units were used to inoculate the cell monolayer. Possibly, virus-induced cell movement might have caused the infected cells to disconnect from each other after the spread of the virus.

These results demonstrated that, in the presence of Rb-WR2, productive virus infection occurred in vΔA33R-infected cells but virus spread was greatly diminished.

Is IMV wrapping important for the Ab-resistant spreading mechanism?

vΔA33R and vΔA34R make higher levels of EEV (Table 1) despite the wrapping of IMV being either incomplete (Roper *et al.*, 1998) or inefficient (Duncan & Smith, 1992; Wolffe *et al.*, 1997). Therefore, we investigated whether an alternative mechanism for CEV and EEV formation, such as budding of IMV from the cell surface, occurred with vΔA33R and resulted in the increased susceptibility to Rb-WR2.

The wrapping of IMV to form IEV can be inhibited pharmacologically by IMCBH (Kato *et al.*, 1969; Payne & Kristenson, 1979; Hiller *et al.*, 1981), which targets the F13L protein (Hiller *et al.*, 1981; Schmutz *et al.*, 1991). Fig. 5(a) shows the plaques formed in the presence of IMCBH at 5 days p.i. As expected, IMCBH had no effect on vΔF13L due to the absence of the target protein, while WR and vΔA56R produced plaques similar in size to those of vΔF13L. All other mutants were inhibited severely and only tiny infected foci were seen.

The levels of EEV produced by these mutants in the presence of IMCBH were also studied (Fig. 5b). To measure EEV infectivity, fresh EEV was mixed with mAb 2D5 (diluted 1/2000) to neutralize contaminating IMV (Law & Smith, 2001). Without IMCBH (open bars), vΔA33R and vΔA34R produced 2·6 and 4·1 times more EEV than WR while vΔA36R, vΔB5R, vΔF12L and vΔF13L produced 2·5, 5·5, 2·9 and 5·3 times less EEV than WR, respectively, in broad agreement with published data (Table 1). The level of EEV made by vΔA56R is similar to WR (G. L. Smith, unpublished data). Here, we found that vΔA56R produced 1·8-fold less EEV than WR. In most reports, except vΔB5R and vΔF12L (Mathew *et al.*, 1998; Zhang *et al.*, 2000), EEV infectivity was measured as the total present in the supernatant. Here, only the infectivity of intact

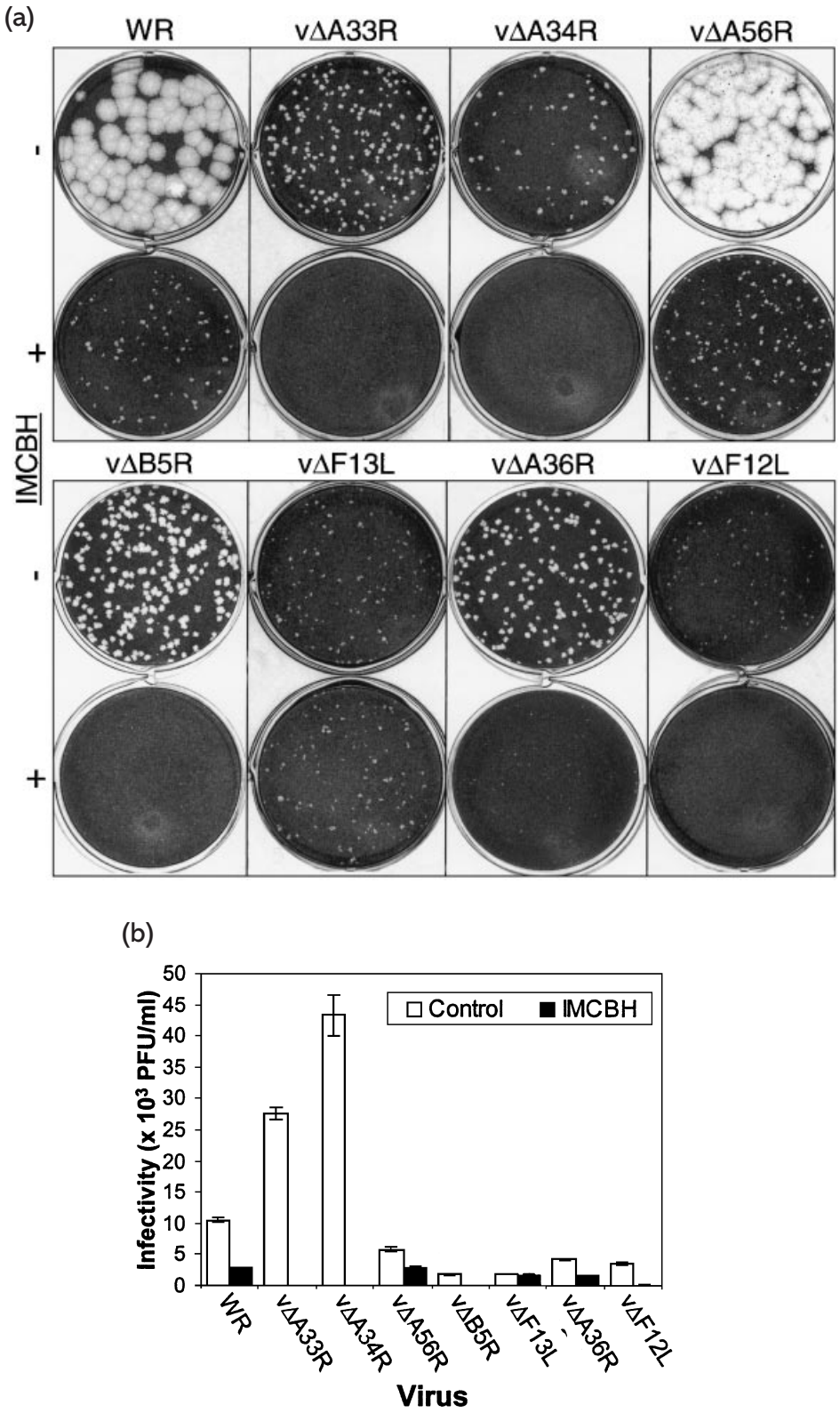

Fig. 5. (a) Cell-to-cell spread of VV mutants in the presence of IMCBH. BS-C-1 cells were infected by the indicated viruses at 75 p.f.u. per well for 2 h at 37 °C and the cells were covered with semi-solid overlay with (+) or without (–) IMCBH (10 μg/ml). The plaques were stained 5 days p.i. (b) EEV production in the presence of IMCBH. EEV of the indicated viruses was grown in RK_{13} cells with or without IMCBH (10 μg/ml) for 18 h and was titrated on BS-C-1 cells using mAb 2D5 (final dilution 1/2000). Two independent experiments gave very similar results. Each data point represents the mean ±SEM of triplicate measurements in one experiment.

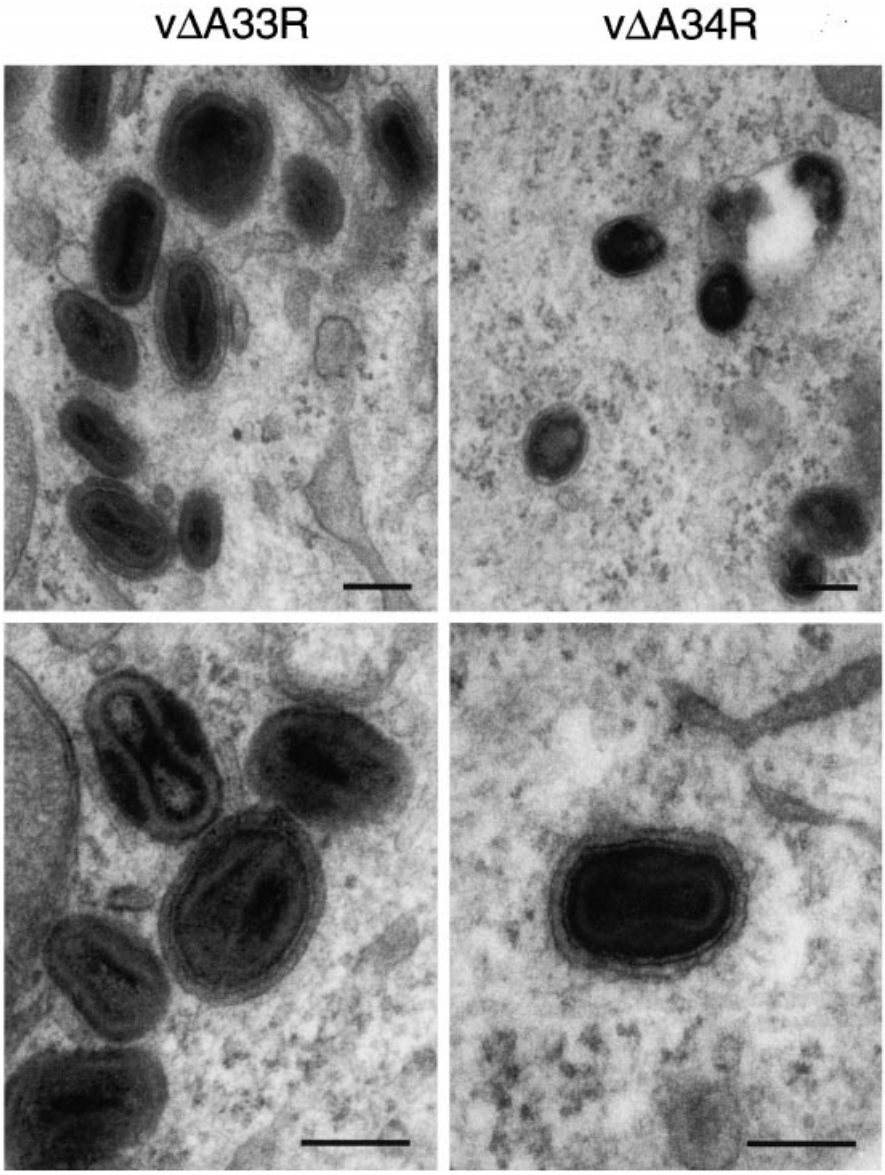

Fig. 6. Electron microscopy of vΔA33R- and vΔA34R-infected cells. RK_{13} cells were infected with WR, vΔA33R, vΔA34R or vRevA34R at 10 p.f.u. per cell. The cells were harvested at 6 h and were processed for thin section transmission electron microscopy. Wrapped particles of vΔA33R (left panels) and vΔA34R (right panels) are shown. Bars, 200 nm.

EEV was measured and this might account for the small variations observed.

In the presence of IMCBH (Fig. 5b, black bars), the levels of EEV made by vΔA33R and vΔA34R were reduced drastically, suggesting EEV were formed by the normal IMCBH-sensitive pathway. Similar results were noted for vΔB5R and vΔF12L. Some EEV were still made by WR, vΔA56R and vΔF13L, consistent with the observations on virus cell-to-cell spread above. Surprisingly, a significant level of EEV was made by vΔA36R in the presence of IMCBH, despite plaque formation being inhibited by this drug (Fig. 5a). These data may suggest that the inhibition of EEV production by IMCBH is also partially dependent on the presence of A36R in addition to F13L.

The formation of IEV by vΔA33R and vΔA34R was investigated further by electron microscopy. In RK_{13} cells infected with VV strain IHD-J most IMV wrapping occurred before 20 h p.i. (Payne & Kristenson, 1979). Since vΔA33R and vΔA34R make enhanced levels of EEV at 18–24 h p.i. (Table 1), the deletion of A33R or A34R might have accelerated the kinetics for IMV wrapping and EEV release. Therefore, RK_{13} cells infected with vΔA33R, vΔA34R, WR and vRevA34R (the revertant virus of vΔA34R) were analysed at 6 h and 8 h p.i. (Fig. 6) and intracellular virions (IMV and IEV) were quantified

Table 2. Intracellular virions (IMV and IEV) in VV-infected cells

	6 h p.i.				8 h p.i.			
	Particles counted (11 sections)	IMV	IEV	% IEV	Particles counted (10 sections)	IMV	IEV	% IEV
WR	202	77	125	61·9	312	150	162	51·9
vΔA33R	198	82	116	58·6	374	179	195	52·1
vΔA34R	170	128	42	24·7	191	146	45	23·6
vRevA34R	139	59	80	57·6	277	126	151	54·5

(Table 2). Fully wrapped IEV particles were found in cells infected with both mutants (Fig. 6) but, compared to the other viruses, lower levels of IEV were found in vΔA34R-infected cells at both 6 and 8 h p.i. This suggested that either IEV formation is less efficient or IEV dissemination is quicker. In all samples, we found no evidence that EEV were generated by budding and conclude that wrapping of IMV is necessary for the formation of CEV/EEV by vΔA33R and vΔA34R. Therefore, the sensitivity to Ab of vΔA33R cell-to-cell spread is unlikely to be caused by an alternative pathway of CEV/EEV formation.

Discussion

This paper describes an investigation of the mechanisms of cell-to-cell and long-range spread of VV in cell culture and the effects of antibody against IMV or EEV on these processes.

Long-range virus dissemination was analysed in cell culture by the formation of comet-shaped plaques and was shown to be mediated by EEV that is probably dispersed by convection currents to infect distant cells. By tilting the culture dish 10° all comets became parallel and went uphill. *In vivo*, EEV is released early in infection and is important for systemic spread (Payne, 1980).

The mechanism of cell-to-cell spread was investigated by a direct comparison of the plaques formed by all mutants lacking individual IEV or EEV proteins and the effects of anti-IMV and -EEV Ab. The plaque sizes of all mutants, except vΔA56R, was reduced at least threefold in diameter compared to WR. vΔA56R formed syncytia (Ichihashi & Dales, 1971; Sanderson *et al.*, 1998a) but had similar sensitivity to Ab compared with WR, indicating that syncytium formation did not contribute to virus spread. Syncytium formation by vΔA56R occurs late during infection and might occur between infected cells rather than between infected and uninfected cells.

After vΔA56R, the next largest plaque is made by vΔA36R followed by vΔB5R, vΔA34R, vΔA33R, vΔ12L and vΔF13L. All mutants make near normal levels of IMV (Table 1), demonstrating that IMV is insufficient for plaque formation. Plaque size is also not determined by whether or not IMV are wrapped to form IEV because mutants that can (vΔA36R) or cannot (vΔF13L) make IEV each form a small plaque. The levels of CEV and EEV also are not important for plaque size since viruses IHD-J and WR produce very different levels of CEV but make similar plaque sizes, and viruses with either enhanced (vΔA34R) or reduced (vΔB5R) EEV levels each make small plaques (Table 1). The major factor determining plaque size is the ability to make actin tails that mediate efficient cell-to-cell spread (Table 1).

The mechanism(s) of VV spread were dissected further using neutralizing Ab specific to IMV or EEV. A reduction in plaque size suggests that the virus spreads from cell to cell in a pathway that is exposed to Ab, whereas, if the plaque size is unchanged the virus spreads in a pathway that is protected from Ab. Plaques made by all viruses were largely unaffected by anti-IMV Ab (Fig. 2d). Therefore, cell-to-cell spread must involve the enveloped virions CEV/EEV, and consistent with this, anti-EEV Ab had varying effects on the different mutants (Fig. 2e, f). In the presence of Rb-WR2 or α-B5R Ab, WR and vΔA56R plaque sizes were inhibited to a similar extent (Fig. 2e), but the plaques were still bigger than those made by other mutants, even in the absence of the Ab (Fig. 2b). This reiterated the importance of actin tails for efficient cell-to-cell spread and showed that this mechanism of spread is resistant to Ab. Resistance to Ab was also observed in other mutants unable to make actin tails, e.g. vΔA36R and vΔF12L, and therefore an actin tail-independent and Ab-resistant pathway exists. Both A36R and F12L proteins are IEV-specific and not exposed to Ab, and so these results are unlikely to be due to the removal of neutralizing targets on CEV/EEV. Rb-WR2 reduced the size of plaques formed by vΔA34R and vΔB5R and only very faint plaques were formed by vΔF13L, implying that these viruses used both Ab-sensitive and Ab-resistant pathways for their spread. Most dramatic was the abrogation of vΔA33R plaque formation by Rb-WR2, showing this mutant spreads entirely via an Ab-sensitive pathway.

Anti-B5R Ab affected vΔA33R the most among all the mutants, showing B5R is a target in the Ab-sensitive pathway. However, other virus antigen(s) must be involved because α-B5R did not block vΔA33R completely, affected vΔA34R only

marginally and had no effect on vΔF13L (Fig. 2f). IMV wrapping by vΔF13L is inhibited severely and therefore this mutant makes very low levels of CEV and EEV (Table 1). vΔF13L was also slightly more sensitive to mAb 2D5 than other mutants (Fig. 2c), suggesting that IMV might contribute to some levels of virus spread in this mutant. This might account for the resistance to α-B5R. Cell-to-cell spread of vΔA34R was fairly resistant to α-B5R despite vΔA34R making 19- to 25-fold higher levels of EEV. This suggested A34R is required for the Ab inhibition via B5R. A34R may have a role in the conformation, recruitment or function of the B5R protein.

In summary, these data show that VV uses a combination of mechanisms to spread between cells. The mechanisms can be divided into (i) actin tail-dependent Ab-resistant pathway (WR and vΔA56R); (ii) actin tail-independent Ab-resistant pathway (vΔA36R and vΔF12L); and (iii) Ab-sensitive pathway (vΔA33R). Actin tails have a major role in the Ab-resistant pathway. This is probably because actin tails push the virions into the neighbouring cells directly without exposing the virions to Ab. WR and vΔA56R spread by a combination of these three pathways whereas vΔA34R, vΔB5R and vΔF13L use pathways (ii) and (iii).

The Ab-susceptibility of vΔA33R is analogous to HSV-1 gE and gI deletion mutants. HSV-1 entry kinetics and replication were not affected by deletion of either gene; however, cell-to-cell spread of these mutants was impaired (Dingwell *et al.*, 1994), and neutralizing Ab reduced the yields of the mutants but not wild-type virus. HSV gE and gI form a complex (Johnson *et al.*, 1988) that accumulates at sites of cell–cell contact, possibly by interacting with junctional components (Dingwell & Johnson, 1998), and which may mediate HSV transfer across the cell junctions. Similarly, varicellar-zoster virus gE expression in polarized epithelial cells altered the F-actin organization and accelerated the formation of tight junctions between cells (Mo *et al.*, 2000). The VV A33R protein expressed by Semliki forest virus or VV accumulated on microvillus-like cell surface projections (Lorenzo *et al.*, 2000). A33R might aid virus spread through cell junctions by interacting with junctional proteins in a similar fashion to HSV gE–gI. A33R might also interact with surface molecules of neighbouring cells to facilitate cell–cell contacts for virus spread. Deletion of A33R could disrupt these cell–cell interactions and permit vΔA33R to spread only in an Ab-sensitive pathway. Consistent with this proposal, the passive transfer of an anti-A33R mAb, and immunization with A33R DNA (Hooper *et al.*, 2000) or recombinant A33R protein (Galmiche *et al.*, 1999), protected mice from VV challenge. However, neither the anti-A33R mAb nor Ab raised against recombinant A33R proteins neutralized EEV *in vitro*.

The production of several infectious forms of VV is explained by these virions having different roles in the virus life-cycle. IMV is highly immunogenic (see JGV Online for supplementary data, http://vir.sgmjournals.org) (Law & Smith, 2001) and susceptible to neutralization by complement (Vanderplasschen *et al.*, 1998b), and therefore is poorly suited to virus spread within a host. It would be advantageous for the majority of VV infectivity (IMV) to be retained and protected within cells from Ab and complement. However, IMV particles are physically robust and are well suited for dissemination between hosts. To aid virus dissemination within a host, VV has exploited several features of the cell biology. It uses cellular membranes to wrap IMV particles and by the acquisition of host complement factors protects EEV particles from destruction by complement (Vanderplasschen *et al.*, 1998b), and uses virus-encoded proteins in the EEV outer envelope to bind to different receptors from IMV (Vanderplasschen & Smith, 1997) and to enhance the range of cell types that may be infected.

The role of IEV is less characterized and might appear unnecessary, for to make CEV or EEV an IMV might bud through the plasma membrane. Indeed, limited budding occurs late during infection in VV strain IHD-W (Tsutsui, 1983) and in fowlpox virus (Boulanger *et al.*, 2000). The apparent inefficiency of IMV wrapping but enhanced EEV production by mutants vΔA33R and vΔA34R (Duncan & Smith, 1992; McIntosh & Smith, 1996; Roper *et al.*, 1998) further questioned the significance of IEV in the VV life-cycle. However, by pharmacological and microscopic approaches, we demonstrated here that wrapping not only occurs with these mutants but is an essential step for both mutants (Figs 5 and 6). Two functions for IEV are proposed. First, asymmetric distribution of the A36R protein enables the unidirectional polymerization of actin after the outmost IEV membrane has fused with the plasma membrane (van Eijl *et al.*, 2000). This could not easily occur via budding. Second, both IMV and IEV utilize microtubules for intracellular movement (Sanderson *et al.*, 2000; Hollinshead *et al.*, 2001; Ward & Moss, 2001). The wrapping of IMV by additional membranes places different proteins on the IMV and IEV surface, and consequently these virions may have different interactions with microtubule components enabling movement towards (IMV) or away from (IEV) the site of wrapping.

Finally, we propose an explanation for the existence of CEV. CEV is structurally indistinguishable from EEV, but with most VV strains many enveloped virions are retained on the cell surface rather than being released. This appears curious since many viruses enhance virus release by down-regulating cell receptors. However, the efficient cell-to-cell dissemination of virus requires actin tail formation from the cell surface, and so enveloped virions need to be retained long enough at the surface to promote this activity. A34R and B5R are involved in the retention of CEV on the cell surface. The deletion of the entire B5R gene or fusion with the VV A56R extracellular domain reduced wrapping and EEV release (Engelstad & Smith, 1993; Wolffe *et al.*, 1993; Mathew *et al.*, 2001) while deletion of any of the short consensus repeat domains of B5R enabled wrapping and increased EEV release (Herrera *et al.*, 1998;

Mathew *et al.*, 1998). The deletion or mutation of A34R enhanced the release of EEV (Blasco *et al.*, 1993; McIntosh & Smith, 1996) and reduced wrapping compared to WR and vRevA34R (Table 2). B5R interacts with A34R (Röttger *et al.*, 1999) and, interestingly, α-B5R Ab did not inhibit the cell-to-cell spread of vΔA34R (Fig. 2f). A34R and B5R may work in concert to affect EEV release.

In summary, VV benefits from having four different virus forms, IMV, IEV, CEV and EEV, for efficient cell-to-cell spread (CEV and actin tails) and long-range spread (EEV) within the host, and reserving the majority of infectivity (IMV) for transmission between hosts.

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