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Virology 328 (2004) 30–35
VIROLOGY
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Rapid Communication

# Synergistic neutralizing activities of antibodies to outer membrane proteins of the two infectious forms of vaccinia virus in the presence of complement

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Received 25 June 2004; returned to author for revision 14 July 2004; accepted 26 July 2004
Available online 21 August 2004

**Abstract**

The two forms of infectious vaccinia virus particles, known as intracellular mature virions and extracellular enveloped virions, are liberated by cell lysis and exocytosis, respectively. The extracellular enveloped form, which is highly resistant to antibody neutralization, contains an outer membrane surrounding an intracellular mature form. We provide evidence that complement mediates antibody-dependent lysis of the outer membrane of extracellular virus, exposing the inner infectious virus to neutralization by a second antibody. These results can help explain the disparity between the in vitro neutralizing and in vivo protective effects of antibodies to extracellular envelope proteins as well as the enhanced protection afforded by specific combinations of antibodies.
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*Keywords:* Synergism; Neutralizing activities; Complement

## Introduction

Interest in smallpox vaccines has reemerged because of concern that variola virus may be released as a biological weapon (Henderson, 1999). Although the licensed vaccine comprised of live vaccinia virus provides excellent protection, its adverse side effects may prohibit general use without certain knowledge of an imminent smallpox outbreak (Fulginiti et al., 2003). To develop safer vaccines, it is important to understand the targets of orthopoxvirus immunity. Large numbers of infectious virus particles, called intracellular mature virions (IMV), are formed in factory regions within the cytoplasm. IMV can be released by cell lysis and their stability may enhance virus spread to other hosts. In addition, some IMV are double-wrapped by membranes derived from modified trans-Golgi or endosomal cisternae. These intracellular enveloped virions are transported to the periphery of the cell where they undergo exocytosis. The extracellular virions are classified into two types: (i) cell-associated enveloped virions (CEV), which remain adherent to the cell surface and induce the formation of actin-containing microvilli that facilitate direct cell-to-cell spread and (ii) extracellular enveloped virions (EEV), which detach from cells and mediate longer-range spread (Blasco and Moss, 1992; Smith et al., 2002). The ratio of CEV to EEV depends on the virus strain and host cell, but the former is usually more abundant.

The proteins of the outer membranes of IMV and CEV/EEV are distinct and thus present different targets to the immune system. In animal models, the poor protection of inactivated vaccines, comprised largely of IMV, has been attributed to the absence of antibodies to EEV (Boulter et al., 1971; Turner and Squires, 1971). Although neutralization of IMV by antibodies to IMV membrane proteins is

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0042-6822/$ - see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2004.07.024

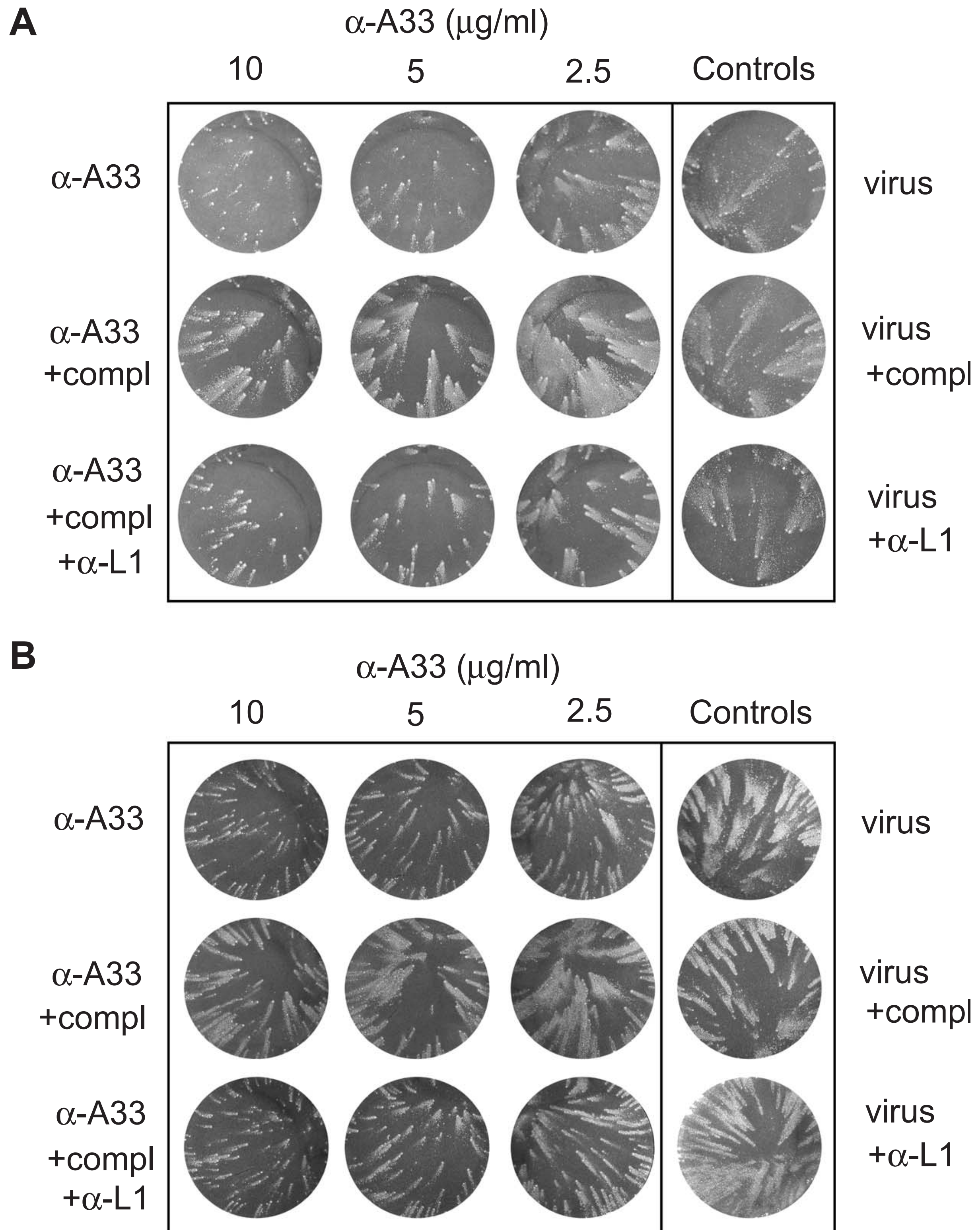


Fig. 1. Effects on satellite plaque formation of antibodies to IMV and EEV membrane proteins and complement. (A) Left side. BS-C-1 cells were infected with approximately 50 plaque-forming units of vaccinia virus strain IHD-J. After 2 h at 37 °C, the monolayer was washed and fresh medium containing heat-inactivated 2% fetal calf serum was added along with indicated concentrations of rabbit IgG to A33 or 10 μg/ml of rabbit IgG to L1 and guinea pig complement (diluted 1:40). After 48 h, the monolayers were stained with crystal violet. Right side. Controls without A33 antibody showing virus alone, virus plus guinea pig complement, and virus plus rabbit IgG to L1 as indicated on the right. (B) Left side. RK-13 cells were infected and incubated with rabbit IgG to A33 and L1 as in A, except that rabbit complement (diluted 1:40) was used. After 48 h, the monolayers were stained with crystal violet. Right side. Controls without antibody to A33 showing virus alone, virus plus rabbit complement, and virus plus rabbit IgG to L1 as indicated on the right. Abbreviations: α-A33, IgG to A33; α-L1, IgG to L1; compl, complement.

well documented, inhibition of EEV infectivity has been more difficult to demonstrate (Ichihashi, 1996; Law and Smith, 2001; Vanderplasschen et al., 1997). EEV also are more resistant than IMV to inactivation by complement (Vanderplasschen et al., 1998).

The present study was stimulated by two sets of observations. The first is that antibody to the A33 membrane protein component of extracellular virus does not neutralize EEV infectivity in vitro, but provides significant protection in vivo (Galmiche et al., 1999). The second is that the best protection is obtained when combinations of plasmids expressing IMV and EEV proteins or combinations of IMV and EEV proteins themselves are used for vaccination (Fogg et al., in press; Hooper et al., 2003). Here we provide evidence that complement plus A33 antibody lyses the EEV membrane, thereby exposing the IMV to a neutralizing antibody. This model can help explain the disparity between the in vitro neutralizing and in vivo protective effects of EEV antibodies and the enhanced in vivo effects of specific combinations of antibodies.

## Results and discussion

In confirmation of a report by Galmiche et al. (1999), we found that a polyclonal antibody prepared by immunizing rabbits with a recombinant A33 protein had little or no ability to neutralize EEV (shown later). Nevertheless, anti-A33 IgG reduced the size of satellite plaques made by the IHD-J strain of vaccinia virus, which have a comet shape on cell monolayers covered with a liquid medium (Fig. 1A). The latter may be explained by agglutination of progeny virions on the parental cell surface (Vanderplasschen et al., 1997). Because complement can enhance antibody-mediated neutralization of IMV in vitro (Isaacs et al., 1992; Takabayashi and McIntosh, 1973), we tested whether there would be a similar effect on neutralization of EEV. Addition of complement alone after virus infection had little or no effect on comet formation (Fig. 1A, controls). To our surprise, however, complement dramatically enhanced comet formation in the presence of all dilutions of A33 antibody compared to A33 antibody alone (Fig. 1A). The result was not specific for A33 antibody as a similar enhancement of comet formation resulted when antibody to B5, another EEV membrane protein, plus complement was used in a comet assay (not shown). Enhanced comet formation also occurred when A33 antibody and complement was added to the medium of cells infected with the WR strain of vaccinia virus, which normally makes very small comets (not shown). These effects of complement were eliminated by heat inactivation.

We considered that complement might have lysed the outer CEV or EEV membrane in the presence of antibody and released IMV, which formed satellite plaques. Because

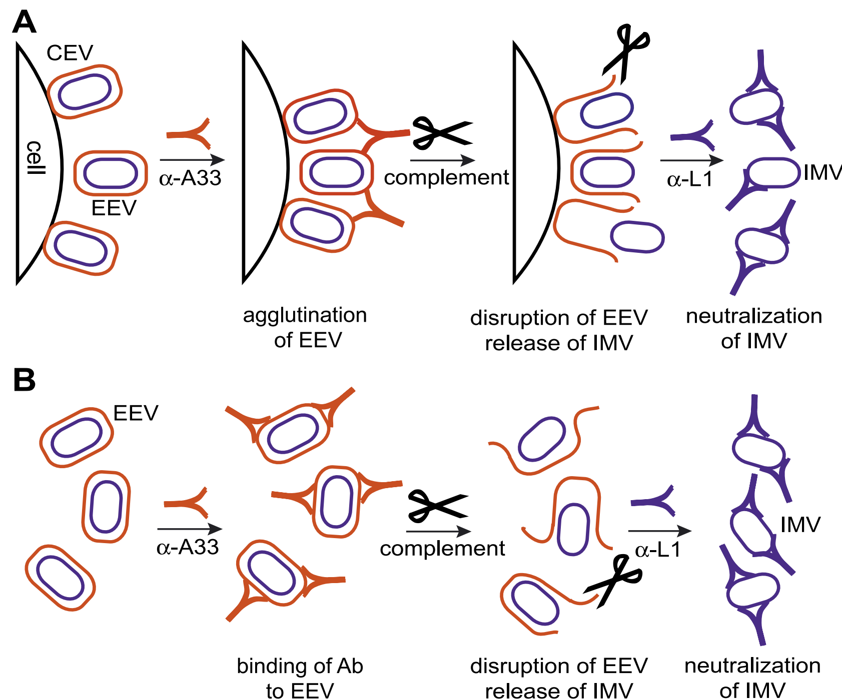


Fig. 2. Models depicting complement-mediated lysis of CEV or EEV membranes and neutralization of exposed IMV. (A) Inhibition of comet formation. The infected cell surface with progeny CEV and EEV is shown at the left. In the next panel, the virus particles are agglutinated by IgG to A33. Complement is activated in the presence of antibody and lyses the CEV/EEV outer membrane exposing IMV. In the final panel, the IMV are neutralized by IgG to L1. (B) Neutralization of EEV. EEV from the medium of infected cells are shown on the left. In the next panel, non-neutralizing IgG to A33 attaches to the surface of EEV. The outer membrane is lysed when complement is added, exposing the IMV to neutralizing polyclonal IgG to L1. Abbreviations: α-A33, IgG to A33; α-L1, IgG to L1; scissors, complement.

IMV can be neutralized by antibody to the L1 membrane protein (Wolffe et al., 1995), this hypothesis could be tested. Cells were infected as before, but this time we added anti-L1 IgG together with anti-A33 IgG and complement. Under these conditions, comets were sharply reduced in size (Fig. 1A). At the concentrations used, neither anti-L1 antibody by itself (Fig. 1A, Controls) or together with complement (not shown) had an inhibitory effect on comet formation. In the experiments shown in Fig. 1A, monkey kidney cells, rabbit anti-A33 antibody, and guinea pig complement were used. Because Vanderplasschen et al. (1998) reported that species-specific cellular complement inhibitory proteins are incorporated into the EEV membrane, the experiment was repeated with rabbit cells, rabbit antibody, and rabbit complement. Nevertheless, similar results were obtained (Fig. 1B).

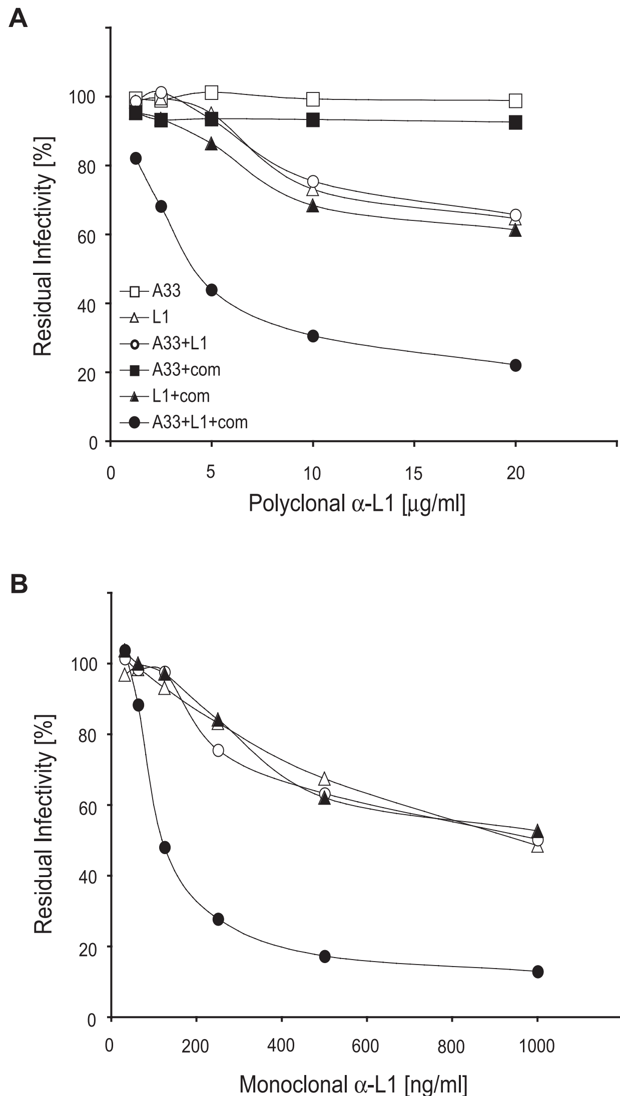


Fig. 3. Neutralization of EEV by antibodies to EEV and IMV membrane proteins in the presence of complement. (A) A fresh suspension of EEV was mixed with polyclonal antibody to A33 (A33), polyclonal antibody to L1 (L1), and guinea pig complement (com) as indicated by the symbols in the inset for 1 h at 37 °C. The virus titer was then determined by plaque assay on BS-C-1 cells to determine the residual infectivity. The concentration of polyclonal IgG to L1 (α-L1) is indicated. (B) The experiment was similar to that of panel A except that a mAb to L1 was used. The concentration of mAb to L1 (α-L1) is indicated. The reciprocal 50% IMV neutralization titers of the L1 polyclonal and monoclonal antibodies were 1.88 µg/ml and 113 ng/ml, respectively.

A model describing the above data is presented in Fig. 2A. In the first step, antibody to A33 binds to the outer membrane of EEV and CEV causing virus agglutination, which prevents comet formation. In the second step, complement is activated by antigen–antibody complexes on the surface of CEV or EEV resulting in lysis of the outer membrane and release of infectious IMV, which can form satellite plaques. Addition of anti-L1 antibody, however, neutralizes IMV infectivity and prevents satellite plaque formation. We also considered an alternate model, in which anti-A33 IgG and complement lysed the plasma membrane and released IMV from the cell. This was not supported by additional studies, as no evidence of cell lysis was found using an assay (Mosmann, 1983) based on the reduction of the tetrazolium salt 3,[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (data not shown).

Additional experiments were carried out to further test the hypothesis that the EEV membrane is lysed by complement in the presence of anti-A33 antibody, allowing anti-L1 antibody to neutralize IMV infectivity. Fresh medium of cells infected with the IHD-J strain of vaccinia virus was used as the source of EEV. The recommendation (Vanderplasschen et al., 1998) to avoid concentration and purification of EEV was followed because the outer membrane is fragile. Nevertheless, even such preparations are partially neutralized with high concentrations of IMV antibody, suggesting that the outer membranes of some EEV are not completely intact. Confirming the studies of Galmiche et al. (1999), the EEV were resistant to neutralization by anti-A33 IgG (Fig. 3A). Furthermore, no loss of infectivity occurred when complement was added with anti-A33 IgG (Fig. 3A). The combination of anti-A33 IgG and complement, however, allowed greatly enhanced virus neutralization by anti-L1 polyclonal (Fig. 3A) or monoclonal (Fig. 3B) IgG. In the absence of anti-A33 IgG, complement did not increase the sensitivity of the virus to anti-L1 polyclonal or monoclonal antibody (Figs. 3A, B). A model, consistent with the above data, is depicted in Fig. 2B. Here, we have eliminated any possible role of cell lysis so that our conclusion of EEV lysis by complement and anti-A33 antibody is unambiguous.

The encoding of a complement regulator by orthopoxviruses testifies to the importance of complement as a host defense mechanism (Isaacs et al., 1992; Kotwal et al., 1990). In addition, Vanderplasschen et al. (1998) reported the relative resistance of EEV to complement in the absence of specific antibodies, which was attributed to

the incorporation of cellular complement regulatory proteins in the outer viral membrane. Vanderplasschen et al. (1998) cited unpublished data that EEV infectivity was destroyed by complement in the presence of antivaccinia virus antibody. However, they did not indicate the target(s) of the antibody, which was presumably made against live vaccinia virus rather than individual proteins; neither did they comment on the role of complement or suggest synergism between EEV and IMV antibodies in the presence or absence of complement. Our finding, that complement is activated by antibodies to specific EEV membrane proteins and lyses the outer EEV membrane to expose infectious IMV, was not anticipated by previous studies. Moreover, as the EEV membrane no longer protects the IMV, the latter become sensitive to neutralization. The two-step mechanism proposed here can help explain the disparity between the poor in vitro neutralizing and good in vivo protective effects of antibodies to extracellular envelope proteins (Galmiche et al., 1999) as well as the enhanced protection afforded by immunization schemes that elicit antibodies to both EEV and IMV proteins (Fogg et al., in press; Hooper et al., 2000, 2003) or by passive administration of combinations of antibodies to IMV and EEV proteins (our unpublished data).

## Materials and methods

### *Antibodies and complement*

Rabbit polyclonal IgG to soluble recombinant A33 and L1 proteins, made in insect cells, will be described elsewhere. Mouse monoclonal IgG to L1 was made from a hybridoma generously given by Alan Schmaljohn and purified by protein A chromatography. Guinea pig and rabbit complement was purchased from Calbiochem.

### *Comet assays*

Vaccinia virus strain IHD-J or WR, diluted in Earle's modified Eagle medium with heat-inactivated 2% fetal bovine serum to give approximately 50 plaques, was incubated with monolayers of BS-C-1 or RK-13 cells in 12-well plates (COSTAR, Corning, Acton, MA). After 2 h at 37 °C, the inoculum was removed and the cells were washed. A liquid overlay, consisting of Earle's modified Eagle medium, supplemented with 2% heat-inactivated fetal bovine serum and one or more of the following components: rabbit polyclonal IgG to A33, rabbit polyclonal IgG to L1, and guinea pig or rabbit complement, was added. After 48 h, the cells were stained with crystal violet.

### *Preparation of EEV*

RK13 cells were infected with one to three plaque-forming units per cell of vaccinia virus strain IHD-J and the medium containing EEV was harvested after 24 h. The EEV were used immediately or stored at 4 °C for a maximum of 24 h.

## Acknowledgments

We thank Norman Cooper for cells and virus, Gary Cohen and Roselyn Eisenberg for rabbit IgG to A33 and L1, and Alan Schmaljohn for hybridoma cells. The work was supported by intramural NIAID funding and by NIH grants R21-AI053404 and U54-AI57168.

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