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Complement-bound human antibodies to vaccinia virus B5 antigen protect mice from virus challenge

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Evaluation of: Benhnia MR, McCausland MM, Laudenslager J *et al.* Heavily isotype dependent protective activities of human antibodies against the vaccinia virus extracellular virion antigen B5. *J. Virol.* 83, 12355–12367 (2009).

The mechanism of protection afforded by vaccinia virus (VACV) – the smallpox vaccine – is a key issue for the development of modern vaccines and countermeasures. Antibodies to VACV antigens of the extracellular virion (EV) form play a central role in protection against poxvirus diseases in animal models, and contribute to the protection of immunized humans against poxviruses. B5, a viral EV protein, is conserved among different orthopoxviruses and antibodies to B5 that protect mice against VACV challenge. Antibodies to B5 are primarily responsible for neutralization of vaccinia EVs, yet the mechanism of EV neutralization by antibodies to B5 is not fully understood. The paper under evaluation demonstrates that most of the neutralization *in vitro* and protection *in vivo* in a mouse model, by monoclonal human anti-B5 IgGs, is heavily dependent on the ability of the IgGs to bind complement (C3 and C1q). Similarly, IgGs capable of complement binding control complement-dependent cytotoxicity of VACV-infected cells. Human polyclonal antibodies induced by the smallpox vaccine were similarly dependent on complement for EV neutralization and the complement-dependent destruction of infected cells. These findings not only contribute to a better understanding of the mechanism of protection by antibodies, but might also help in the development and evaluation of newly-developed therapeutic and prophylactic antibody-based products against virulent orthopoxviruses, and for the prevention or treatment of smallpox vaccine-related post-vaccinal adverse effects.

Keywords: B5 • complement • enveloped virus • neutralization • smallpox vaccine • vaccinia virus

The smallpox vaccine was the first, and so far the most successful, human vaccine, allowing for the worldwide eradication of smallpox disease in the late 1970s following an intensified worldwide campaign operated by the WHO [1]. Since the eradication of smallpox, vaccination efficacy and evaluation of new vaccines and other treatments relies on the use of the historical vaccine strains (vaccinia virus [VACV]) and on measures that were present before the eradication. These may include the appearance of 'clinical-take' – the typical papular–pustular lesions at the vaccine site, plaque reduction neutralization test [2] and vaccinia immune globulin (VIG) [3]. There are two major forms of infectious VACV: intracellular mature virions (MVs) and extracellular enveloped virions (EVs). The majority of the MV remains within the cell until lysis. Most of the EV remains attached to the outside of the plasma membrane and is responsible for direct cell-to-cell spread. However, some EVs (approximately 5% in most VACV strains in culture) are released from the infected cell and can cause the formation of comet-like satellite plaques in cultured cells [4]. In addition, EVs are important for virus dissemination *in vivo*, and thus contribute to virulence [5,6]. The outer membrane of the EV differs from that of the MV, harboring A33, B5, A56 and other unique viral proteins. EVs also bind cells differently than MVs, and do not undergo membrane fusion with the host cell surface [7]. Both the MV and EV viral forms are infectious; thus, neutralizing antibodies to both

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viral forms contribute to protection. Indeed, VIG, as well as the sera of immunized animals and humans, contains neutralizing antibodies to both MV- and EV-specific antigens [8–10].

B5 is one of five known EV-specific proteins and is highly conserved among different strains of VACV as well as in other orthopoxvirus species. B5 is a 42-kDa glycolysated type I membrane protein with a large ectodomain composed of four small domains that are similar to short consensus repeat domains of complement regulatory protein, although no complement regulatory activity has been demonstrated [11,12]. B5 is required for efficient envelopment of MV, as well as for actin tail formation, normal plaque size and virulence [13,14]. The B5 protein was identified as being the major target of VACV EV-neutralizing antibody [10,15,16], suggesting its importance for the protective immune response to VACV-based smallpox vaccines. Anti-B5 antibodies neutralize EV in plaque reduction tests, inhibit its spread from infected cells and inhibit 'comet formation' – the *in vitro* manifestation of cell-to-cell spread of EV [10,17]. The B5 protein is an important component of anti-EV responses and protective immunity [16,18,19]. Immunization of mice with subunit vaccines that include B5 elicited robust antibody responses and protected animals from severe disease symptoms following challenge with lethal doses of poxviruses [20–23]. An anti-B5 neutralizing monoclonal antibody alone may be sufficient to fully protect mice from lethal VACV challenge; chimpanzee/human monoclonal antibodies (mAbs) to vaccinia B5 protein neutralize VACV and variola virus (VARV) and protect mice against VACV. This monoclonal antibody inhibited the spread of VACV as well as VARV *in vitro* [18]. It is known that humans and animals immunized with the smallpox vaccine make antibodies against B5. Anti-B5 in VIG is responsible for most of the neutralizing activity against EV as measured by plaque reduction assay [24–28]. Thus, EVs, and more specifically B5, are important targets for antibodies, and antibodies to B5 have a major role in protection. It is, however, important to note that other viral antigens contribute to the protective immune response, and one of the most important EV antigens is A33. A33-based vaccines protect animal models against poxvirus challenge, and antibodies to A33 inhibit comets *in vitro* and protect against lethal challenge.

The paper under evaluation here elucidates the mechanisms of EV neutralization, focusing on human antibody responses to B5 [29]. The authors demonstrate that neutralization of VACV EVs by human antibodies to B5 is not simply dependent on antigen binding by the antibody, but is also heavily dependent on the ability of the antibody to bind complement. The ability to bind both B5 and complement positively correlates with *in vivo* protection.

Methods & results

EV neutralization in vitro *by human anti-B5 mAbs is complement dependent, & protection* in vivo *is affinity & isotype dependent*

In order to understand the mechanism of EV neutralization by B5 antibodies and to study the relationship between EV neutralization *in vitro* and protection *in vivo*, Benhnia *et al.* developed nine fully human mAbs against the B5 protein, using transchromosomic KM mice engineered to express human immunoglobulin. Nine human anti-B5 monoclonal antibodies were obtained from the KM mice, seven with high relative binding affinities and two with low binding affinities. The antibodies' isotypes were also determined. They demonstrated that only human IgG1 and IgG3 mAbs neutralize VACV EV, and the neutralization was complement dependent. Only human mAbs possessing complement-fixing Fc domains (IgG1 or IgG3) and having a high affinity to B5 had the ability to neutralize EV virions in the presence of complement. Those mAbs offered protection *in vivo.* Isotype switching of the same antibody to human IgG4 resulted in a loss of neutralization capacity *in vitro*, which was also manifested by much less efficient protection *in vivo*. Unlike human IgG1 and IgG3, which are potent activators of the classical complement pathway, IgG4 is incapable of binding complement. The ability to switch isotype while maintaining exact epitope specificity allowed for the clear demonstration of the complement dependency of the EV-neutralizing anti-B5 antibodies. Furthermore, C3 and C1q subunits of the complement are needed for EV neutralization but not C5, excluding virolysis as a mechanism of virus neutralization.

Although complement-mediated EV neutralization through the Fc domain of anti-B5 IgG seems to play a major role in EV neutralization and protection, modest neutralization is achieved regardless of complement. The authors suggest that *in vitro* complement-independent EV neutralization occurs by EV aggregation and cross-linking of EVs, while the suggested mechanism for the complement-independent modest *in vivo* protection with anti-B5 IgG is via antibody-dependent-cell-mediated cytotoxicity (ADCC). Neither the aggregation nor the ADCC mechanisms have been experimentally tested so far.

The authors suggest that the major mechanism of EV neutralization involves binding of C1q to the antibody, which increases the area occupied by the antibody on the EV surface, thus sterically hindering infection. A mechanism for EV infection points towards cell surface glycosaminoglycans (GAGs) that rupture the EV envelope, allowing for the interaction of the MV particle and the entry fusion complex with the cell membrane [30]. Whether C1q complexes interfere with EV–cell surface interaction or stabilize the EV membrane to prevent exposure of the MV is not currently understood.

Anti-B5 antibodies mediate complement-dependent destruction of VACV-infected cells

Enveloped virions are secreted from the plasma membrane, and B5 can be detected on the surface of VACV-infected cells and, as such, can mediate ADCC as a mechanism to clear intracellular pathogens [16]. The authors show that addition of human anti-B5 mAb (IgG1 or IgG3) with complement resulted in rapid and complete killing of VACV-infected cells. By contrast, an IgG4 isotype human anti-B5 that cannot mediate complement functions was unable to do so. Cell killing was only observed with the combination of complement and anti-B5 mAbs, was only active against virally infected cells, and was completely dependent on

the antibody isotype. When the mAb Fc domain was switched to IgG4, even though the antibody had identical specificity and affinity to B5, infected cells were not affected.

Complement & B5 antibodies confer protection against VACV challenge

Both MV and EV virions are infectious, and vaccinees exhibit immunity to antigens of both forms [31]. The double membrane of the EV form, and the fact that most of the virions in the inoculum used to infect animal models are MVs, poses unique challenges for protection, as virus neutralization requires antibodies for both MV and EV forms to control the infection [8]. In the case of anti-A33 (EV) and anti-L1 (MV), complement allows for EV destruction and exposure of the MV antigens to neutralizing antibodies (e.g., anti-L1) [32].

The ability of the anti-B5 antibodies, in combination with complement, to efficiently neutralize EVs challenged the authors to evaluate the protective efficacy of the human anti-B5 IgGs, to evaluate the therapeutic potential of these antibodies and to allow for correlation of *in vitro* results and *in vivo* protection. This would allow for the use of the EV neutralization test as an *in vitro* surrogate for *in vivo* protection studies. Indeed, IgG1- and IgG3-specified anti-B5 antibodies efficiently protected mice from intranasal VACV-Western Reserve challenge. Furthermore, depletion of complement in mice using complement-specific antibodies abrogated most of the protective efficacy provided by the murine anti-B5 mAb [16], suggesting that complement plays a protective role in EV neutralization by anti-B5 antibodies *in vivo*. In addition, the correlation between the *in vitro* and the *in vivo* results further substantiates the use of the EV neutralization as a surrogate for *in vivo* protection. The results, however, indicate that encoding a complement control protein (CCP) by VACV is unable to inhibit the antiviral activity of complement through anti-B5 antibodies. It is important to note, however, that the VARV CCP appears to be more potent and host adapted than the VACV ortholog [33], and that the presence of CCP in monkeypox virus correlates with strain virulence [34].

As the inoculating virus is mostly MV, and the antibodies are directed to an EV antigen, it was interesting to see that not only mortality, but also morbidity, were prevented using the anti-B5 IgGs (IgG1 or IgG3). The author's data suggest that not only EV neutralization, but also complement-dependent cell destruction, allows for such a protective response.

Expert commentary & five-year view

The amino acid sequence of B5 is conserved within the *Orthopoxvirus* family. Comparison between B5 and its VARV orthologue B6 shows divergence in 21 amino acids in the ectrodomain region [15]. These amino acid differences between B5 and B6 result in proteins with different antigenic properties, which was demonstrated using mAbs with varying target specificities. Finding antigenic differences between B5 and B6 would greatly impact both the ability to discriminate immunologically between VARV and VACV and the development of subunit vaccines. As subunit vaccines rely on a small number of proteins, antigenic differences might greatly affect vaccine efficacy, especially in the case of a smallpox vaccine that is intended to be used against several Orthopox species including VARV, monkeypox virus, cowpox virus and VACV. Among a panel of 26 mAbs that recognized B5 of VACV, ten failed to recognize B6, and of these, three have important anti-VACV biologic properties, including their ability to neutralize EV infectivity and block comet formation. Furthermore, one of these mAbs tested by passive immunization protected mice from a lethal VACV challenge [15]. These results highlight the importance of conservation of important and functional epitopes with therapeutic potential. Whether the human anti-B5R antibodies detect and protect against virulent orthopoxviruses such as VARV, monkeypox virus and cowpox virus is yet to be determined.

Out of several EV-specific viral proteins, B5 and A33 exhibit certain levels of protection and are included in experimental subunit vaccines and antibody preparations. Unlike B5, antibodies to A33 do not neutralize EVs, but efficiently inhibit comet formation. In addition, antibodies to A33 were previously shown to allow for complement-dependent lysis of the EV membrane. This resulted in exposure of the MV, allowing antibodies to L1 to neutralize infection. The antibodies to B5 with added complement seem to work differently, by EV neutralization rather then EV virolysis. Why A33 does not neutralize EV and B5 does is currently unclear.

Inhibition of comet formation by antibodies is considered an important *in vitro* correlate of protection, yet the phenomenon of comet formation is poorly understood and, as a consequence, the mechanism of inhibiting comet is also unclear. Antibodies to A33, as well as some antibodies to B5 that protect mice against VACV challenge, are capable of inhibiting comets *in vitro*. In the evaluated manuscript, using isotype-switched IgGs, the authors demonstrate that although both IgG1 and IgG3 can bind complement and neutralize EVs, only IgG1 can inhibit comet formation. As IgG4-based anti-B5, which does not bind complement, is unable to inhibit comets, the authors suggest that the comet reduction assay measures some complement fixation capacity, but also other IgG1-dependent functions. The mechanism underlying this IgG1 specificity is not yet known.

Lc16m8, a candidate replicating yet attenuated smallpox vaccine that was developed in Japan, carries a mutation resulting in the truncation of the B5 protein. This virus replicates in mammalian cells but spreads very inefficiently between cells. Vaccination with Lc16m8 resulted in a weaker antibody response against B5 compared with the antibody response following vaccination with the conventional Dryvax® (Wyeth Laboratories Inc., PA, USA) vaccine, while antibody response to other viral proteins, EV-neutralizing antibodies and protection of animal models were similar [35]. The results suggest that antibodies to the truncated region of B5 are not essential for virus neutralization and *in vivo* protection in the context of a whole-virus vaccine. It would be interesting to know whether antibodies against the truncated B5 can neutralize EVs and protect mice in a mechanism similar to that of the human anti-B5 presented here.

In summary, beside the potential use of the human anti-B5 IgGs as part of a therapeutic product, these antibodies allowed for elucidating the importance of isotype-dependent complement binding in anti-B5-mediated EV neutralization and target cell destruction. This was further correlated with *in vivo* protection, substantiating an important *in vitro* correlate for *in vivo* protection.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Key issues

- Isotype-dependent binding of complement to human antibodies to vaccinia virus B5 allows for efficient extracellular virus neutralization and the destruction of infected cells.
- *In vivo* protection efficacy of anti-B5 antibodies correlates with complement binding, suggesting an important role for complement in the immune response to the smallpox vaccine in humans.
- The correlation between the *in vitro* neutralization and *in vivo* protection makes it reasonable to suggest that anti-B5-dependent extracellular virion neutralization may be used in the future as an *in vitro* surrogate for *in vivo* protection studies.

References

1 Fenner FD, Henderson A, Arita I, Jezek Z, Ladnyi ID. *Smallpox and its Eradication.* World Health Organization, Geneva, Switzerland (1988).

2 Orr N, Forman M, Marcus H *et al.* Clinical and immune responses after revaccination and Israeli adults with Lister strain of vaccinia virus. *J. Infect. Dis.* 190, 1295–1302 (2004).

3 Hopkins RJ, Lane JM. Clinical efficacy of intramuscular vaccinia immune globulin: a literature review. *Clin. Infect. Dis.* 39, 819–826 (2004).

4 Blasco R, Moss B. Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. *J. Virol.* 66(7), 4170–4179 (1992).

5 Payne LG. Significance of extracellular enveloped virus in the *in vitro* and *in vivo* dissemination of vaccinia. *J. Gen. Virol.* 50, 89–100 (1980).

6 Smith GL, Vanderplasschen A, Law M. The formation and function of extracellular enveloped vaccinia virus. *J. Gen. Virol.* 83, 2915–2931 (2002).

7 Moss B. Poxvirus entry and membrane fusion. *Virology* 344 (1), 48–54 (2006).

8 Lustig S, Fogg C, Whitbeck JC, Eisenberg RJ, Cohen GH, Moss B. Combinations of polyclonal or monoclonal antibodies to proteins of the outer membranes of the two infectious forms of vaccinia virus protect mice against a lethal respiratory challenge. *J. Virol.* 79, 13454–13462 (2005).

9 Law M, Smith GL. Antibody neutralization of the extracellular enveloped form of vaccinia virus. *Virology* 280, 132–142 (2001).

10 Galmiche MC, Goenaga J, Wittek R, Rindisbacher L. Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. *Virology* 254, 71–80 (1999).

11 Engelstad MS, Howard T, Smith GL. A constitutively expressed vaccinia gene encodes a 42-kDa glycoprotein related to complement control factors that forms part of the extracellular virus envelope. *Virology* 188, 801–810 (1992).

12 Isaacs SN, Wolffe EJ, Payne LG, Moss B. Characterization of a vaccinia virus-encoded 42-kilodalton class I membrane glycoprotein component of the extracellular virus envelope. *J. Virol.* 66, 7217–7224 (1992).

13 Engelstad M, Smith GL. The vaccinia virus 42-kDa envelope protein is required for the envelopment and egress of extracellular virus and for virus virulence. *Virology* 194, 627–637 (1993).

14 Wolffe EJ, Isaacs SN, Moss B. Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. *J. Virol.* 67, 4732–4741 (1993).

15 Aldaz-Carroll L, Xiao Y, Whitbeck JC *et al.* Major neutralizing sites on vaccinia virus glycoprotein B5 are exposed differently on variola virus ortholog B6. *J. Virol.* 81, 8131–8139 (2007).

16 Benhnia MR, McCausland MM, Moyron J *et al.* Vaccinia virus extracellular enveloped virion neutralization *in vitro* and protection *in vivo* depend on complement. *J. Virol.* 83, 1201–1215 (2009).

17 Moss B, Eisenberg RJ, Cohen GH. Epitope-mapping studies define two major neutralization sites on the vaccinia virus extracellular enveloped virus glycoprotein B5R. *J. Virol.* 79, 6260–6271 (2005).

18 Chen Z, Earl P, Americo J *et al.* Chimpanzee/human mAbs to vaccinia virus B5 protein neutralize vaccinia and smallpox viruses and protect mice against vaccinia virus. *Proc. Natl Acad. Sci. USA* 103, 1882–1887 (2006).

19 Bell E, Shamim M, Whitbeck JC *et al.* Antibodies against the extracellular enveloped virus B5R protein are mainly responsible for the EEV neutralizing capacity of vaccinia immune globulin. *Virology* 325, 425–431 (2004).

20 Fang M, Cheng H, Dai ZP, Bu ZM, Sigal LJ. Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial protection from a lethal orthopoxvirus infection in a natural host. *Virology* 345(1), 231–243 (2006).

21 Hooper JW, Custer DM, Thompson E. Four-gene-combination DNA vaccine protects mice against a lethal vaccinia virus challenge and elicits appropriate antibody responses in nonhuman primates. *Virology* 306 (1), 181–195 (2003).

22 Heraud JM, Edghill-Smith Y, Ayala V *et al.* Subunit recombinant vaccine protects against monkeypox. *J. Immunol.* 177 (4), 2552–2564 (2006).

23 Fogg C, Lustig S, Whitbeck JC, Eisenberg RJ, Cohen GH, Moss B. Protective immunity to vaccinia virus induced by vaccination with multiple recombinant outer membrane proteins of intracellular and extracellular virions. *J. Virol.* 78(19), 10230–10237 (2004).

24 Davies DH, Liang X, Hernandez JE *et al.* Profiling the humoral immune response to infection by using proteome microarrays:

high-throughput vaccine and diagnostic antigen discovery. *Proc. Natl Acad. Sci. USA* 102, 547–552 (2005).

25 Davies DH, Molina DM, Wrammert J *et al.* Proteome-wide analysis of the serological response to vaccinia and smallpox. *Proteomics* 7, 1678–1686 (2007).

26 Edghill-Smith YH, Golding J, Manischewitz LR *et al.* Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. *Nat. Med.* 11, 740–747 (2005).

27 Lawrence SJ, Lottenbach KR, Newman FK *et al.* Antibody responses to vaccinia membrane proteins after smallpox vaccination. *J. Infect. Dis.* 196, 220–229 (2007).

28 Putz MM, Alberini I, Midgley CM *et al.* Prevalence of antibodies to vaccinia virus after smallpox vaccination in Italy. *J. Gen. Virol.* 86, 2955–2960 (2005).

29 Benhnia MR, McCausland MM, Laudenslager J *et al.* Heavily isotype dependent protective activities of human antibodies against the vaccinia virus extracellular virion antigen B5. *J. Virol.* 83, 12355–12367 (2009).

30 Carter GC, Law M, Hollinshead M, Smith GL. Entry of vaccinia virus intracellular mature virion and its interaction with glycosaminoglycans. *J. Gen. Virol.* 86, 1279–1290 (2005).

31 Moss B. Poxviridae: the viruses and their replication. In: *Fundamental Virology (5th Edition)*. Knipe DM, Howley P, Giriffin D, Lamb R (Eds). Lippincott Williams & Wilkins, Hagerstown, MD, USA, 2849–2883 (2006).

32 Lustig S, Fogg C, Whitbeck JC, Moss B. Synergistic neutralizing activities of antibodies to outer membrane proteins of the two infectious forms of vaccinia virus in the presence of complement. *Virology* 328, 30–35 (2004).

33 Rosengard AM, Liu Y, Nie Z, Jimenez R. Variola virus immune evasion design: expression of a highly efficient inhibitor of human complement. *Proc. Natl Acad. Sci. USA* 99(13), 8808–8013 (2002).

34 Jha P, Kotwal GJ. Vaccinia complement control protein: multi-functional protein and a potential wonder drug. *J. Biosci.* 28(3), 265–271 (2003).

35 Mesda CA, Mayer AE, Kumar A *et al.* Comparative evaluation of immune response and protection engendered by LC16m8 and Dryvax smallpox vaccines in mouse model. *Clin. Vaccine Immunol.* 16, 1261–1271 (2009).

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