

# Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence

(complement binding protein/virus neutralization/attenuation/immunity)

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**ABSTRACT** The role of a viral gene product in evasion of the host immune response was investigated. The antibody-dependent complement-enhanced neutralization of vaccinia virus infectivity was prevented by the culture medium from vaccinia virus-infected cells. The vaccinia virus complement-control protein (VCP) was identified as the secreted product of vaccinia virus gene C21L and has homology to a group of eukaryotic genes encoding regulators of complement activation. Thus, the culture medium from cells infected with a C21L deletion mutant was VCP deficient and had little or no effect on antibody-dependent complement-enhanced neutralization. In addition, the anticomplement effect was associated with the C21L-encoded protein partially purified from the medium of cells infected with wild-type virus. Antibody-dependent, complement-enhanced neutralization of vaccinia virus occurred with a complement source that was deficient in the classical pathway complement component C4 and required the alternative pathway complement factor B. Furthermore, the presence of VCP abrogated the complement-enhanced neutralization in C4-deficient serum. Together with previous hemolysis data, the present result suggests that VCP can inhibit both the classical and alternative pathways of complement activation. Skin lesions caused by the C21L deletion mutant were smaller than those caused by wild-type virus, demonstrating an important role for VCP in virulence. The C21L deletion mutant also was attenuated in C4-deficient guinea pigs, consistent with *in vitro* studies. Vaccinia virus appears to have acquired the ability to regulate the complement cascade for the purpose of evading the host immune response.

Higher eukaryotic organisms have developed an array of specific and nonspecific mechanisms to combat virus infections. Some viruses, however, have acquired genes encoding proteins that can modulate host responses. For example, down regulation of class I major histocompatibility antigens by an adenovirus protein may diminish cell-mediated immune responses (1), whereas membrane-associated glycoproteins of herpes simplex virus bind the Fc ends of immunoglobulins and may deflect humoral immune responses (2). The importance of the complement system in host defense against viruses is emphasized by the discovery that members of at least two virus families, herpesviruses (3, 4) and poxviruses (5, 6), encode proteins that interact with complement components.

The complement system has historically been divided into classical and alternative pathways that lead to the formation of a common membrane attack complex (7, 8). Both pathways may be activated by the binding of complement components to immune complexes that form on viruses or virus-infected cells, although the alternative pathway can

bypass this step (9). *In vitro* studies suggest that virus particles coated with complement components may not attach to or enter cells efficiently or may be blocked at a subsequent stage of uncoating (10, 11). Also, formation of the membrane attack complex can result in lysis of virions or infected cells. *In vivo*, additional mechanisms including the binding of complement-coated virus to the CR1 complement receptors of phagocytes and other cells and the release of anaphylactic and chemotactic complement peptides could also contribute to the immune response. Nevertheless, beneficial antiviral effects of complement have been more difficult to document *in vivo* than *in vitro*. Several studies have shown that mice depleted of complement may have prolonged viremia and high virus titers in affected organs (12). Genetic deficiencies in complement components, however, are not generally associated with a greatly increased susceptibility to virus infections (13).

The difficulty in demonstrating antiviral effects of complement *in vivo*, particularly by genetic methods, may be due at least in part to the host's multiplicity of specific and nonspecific immune defenses. Thus, the ability to clear viruses in the absence of complement does not mean that complement ordinarily plays no role in host defense but rather that there are efficient alternative mechanisms. Viruses, however, may have fewer options than their more complex hosts. Not only are viruses simpler than eukaryotic organisms but they must shield themselves against all of the weapons brought to bear against them. Therefore, if a virus has evolved a specific gene to counter a host defense, the loss of that gene might result in an obvious decrease in virulence.

Vaccinia virus, a member of the poxvirus family of cytoplasmic DNA viruses (14), appears particularly suitable to test the above hypothesis. The C21L gene of vaccinia virus [the 21st open reading frame starting from the left end of the *Hind*III C fragment of the genome (15)] is predicted to encode a protein of 28.6 kDa with homology to a group of complement control proteins (5). The family of eukaryotic proteins that regulate complement activation (16) includes two receptors (CR1 and CR2) and four inhibitors (factor H, C4b binding protein, membrane cofactor protein, and decay-accelerating factor). These proteins are composed largely of tandem arrays of a short consensus repeat of  $\approx 60$  amino acids and can bind to complement components C3 and/or C4 (17). The vaccinia virus complement-control protein (VCP) contains four short consensus repeats that are most similar in sequence (38% identity) to the first four repeats of the C4b binding protein (5), a regulator of the classical complement pathway (18). VCP is secreted from infected cells after cleavage of a signal peptide and can inhibit the classical

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Abbreviations: VCP, vaccinia virus complement-control protein; pfu, plaque-forming units.

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pathway of complement activation as measured by a hemolysis assay (6). VCP is not required for virus replication since mutants with the C21L gene partially or completely deleted propagate like wild-type virus in tissue culture (6). In this report, we present evidence that antibody-dependent complement-enhanced neutralization of vaccinia virus can be prevented by concentrations of VCP present in the culture medium of infected cells. Furthermore, skin lesions produced by a mutant virus unable to synthesize VCP are smaller than those produced by wild-type virus, suggesting that the viral protein plays a significant role in suppressing the host immune response.

## MATERIALS AND METHODS

**Viruses.** Vaccinia virus strain WR (American Type Culture Collection VR1354) was used for all neutralization studies. Mutant vaccinia virus vSIGK3 was derived from the WR strain by replacing a 70-base-pair segment of the C21L gene with the selectable marker gene *gpt* (6).

**Source of VCP.** Confluent monolayers of RK-13 cells (American Type Culture Collection CCL37) in flasks with a 150-cm<sup>2</sup> surface area were washed with serum-free Opti-MEM (GIBCO) and then were infected with five plaque-forming units (pfu) per cell of vaccinia virus WR or vSIGK3 or were mock infected. The infected cells were maintained in 20 ml of serum-free Opti-MEM for 24 h at 37°C in a 5% CO<sub>2</sub>/95% air incubator. The medium was then collected from each flask and cell debris and extracellular virus were pelleted by ultracentrifugation at 13,500 rpm for 80 min in a Beckman SW27 rotor. The clarified medium was stored on ice or frozen at -20°C. VCP was purified as described (6).

**Antibody to Vaccinia Virus.** A rabbit (New Zealand White) was inoculated intradermally at multiple sites with both wild-type and vSIGK3 mutant vaccinia virus. After 6 weeks, the rabbit was given an intradermal inoculation of 10<sup>8</sup> pfu of wild-type virus at two sites. Two weeks later, the rabbit was bled by cardiac puncture. The immune serum was heated at 56°C for 30 min to inactivate complement and was stored in aliquots at -70°C for use as antibody in the neutralization assays. An immunoglobulin G fraction was prepared with the AvidChrom antibody kit (BioProbe International, Richmond, CA) following the manufacturer's instructions and was used where indicated.

**Complement.** If received frozen, sera containing complement were thawed once and then stored as single-use aliquots at -70°C. Lyophilized sera were reconstituted in the recommended volume of cold deionized water and then aliquoted and frozen at -70°C. Rabbit complement (fresh frozen; GIBCO), guinea pig complement (lyophilized; Sigma), guinea pig C4-deficient serum (fresh frozen; Sigma), and human complement (lyophilized; Sigma) were used where indicated. Human serum depleted of factor B by immunoadsorption was purchased frozen from Sigma. Purified human complement component factor B was from Calbiochem.

**Standard Neutralization Assay.** Vaccinia virus was diluted to a concentration of 2-4 × 10<sup>5</sup> pfu/ml in fresh Opti-MEM and sonicated. Volumes of 0.1 ml of the virus suspension were pipetted into microcentrifuge tubes, to which 1 μl of heat-inactivated serum containing polyclonal antibody to vaccinia virus (final dilution, 1:100) and/or 5 or 10 μl of the complement source (final dilution, 1:10 or 1:20) were added. After incubation at 37°C for 2 h, aliquots were removed from each tube and serial dilutions in 1 ml of cold serum-free MEM (Quality Biologicals, Gaithersburg, MD) were made in duplicate. Several dilutions of the virus suspensions were inoculated on to BSC-1 cell monolayers, which were then incubated at 37°C in a 5% CO<sub>2</sub>/95% air incubator. After 2 h, the inoculum was removed and 3 ml of fresh MEM containing 2.5% heat-inactivated fetal bovine serum was added to each

well. The plates were incubated for 36 h and stained with 0.2 ml of a 0.015% crystal violet solution. Plaques were counted at two dilutions under magnification (×6.5).

**Determination of Virus Pathogenicity.** Dilutions (10-fold) of sucrose gradient-purified wild-type and vSIGK3 virus were made in sterile phosphate-buffered saline (Quality Biologicals) and 0.1-ml aliquots containing 10<sup>3</sup>-10<sup>6</sup> pfu were injected intradermally down either side of the spine of the freshly shaved backs of New Zealand White rabbits. The diameters of the skin lesions were measured with calipers. A similar procedure was used to inoculate 2/NCR (National Cancer Institute, Frederick, MD) and C4-deficient guinea pigs (small animal section, National Institutes of Health) except that separate animals were used for wild-type and mutant viruses.

## RESULTS

**VCP Prevents Antibody-Dependent Complement-Mediated Enhancement of Neutralization.** Enhanced neutralization of virus infectivity in the presence of antibody is considered to be an important antiviral function of complement. At a dilution of 1:100, rabbit polyclonal antibody to vaccinia virus reduced the infectious titer by ≈1 log<sub>10</sub> over a 30-min period, after which no further drop occurred (Fig. 1). Rabbit complement enhanced the effect of antibody by ≈100-fold during a 180-min incubation but had only a small effect by itself (Fig. 1). As expected, enhanced neutralization did not occur when the complement source was heat inactivated or when the incubation was performed in the presence of 10 mM EDTA (data not shown).

VCP is secreted into the medium of cells infected with vaccinia virus strain WR (5). Addition of such medium prevented the complement-enhanced neutralization of vaccinia virus, whereas enhanced neutralization occurred in medium lacking VCP obtained from uninfected cells or from cells infected with the vaccinia virus deletion mutant vSIGK3 (Fig. 2). These data implied that the product of the C21L gene interfered with complement-enhanced neutralization of vaccinia virus and that sufficient quantities of VCP were present in unconcentrated culture medium. A significant effect was obtained even when the culture medium was diluted 1:10 or when partially purified VCP (6) was used (data not shown).

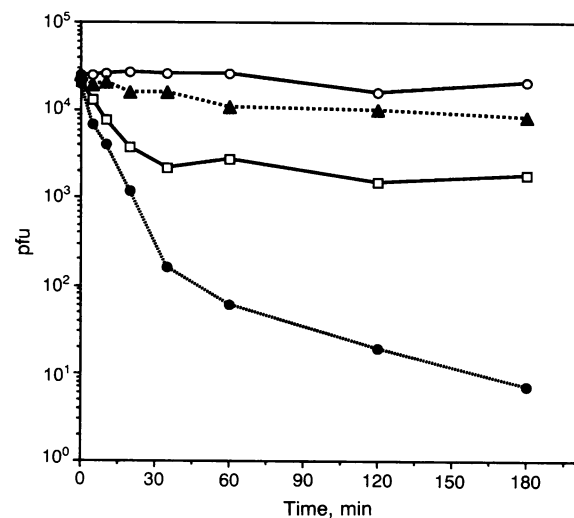


FIG. 1. Time course of antibody and complement-mediated neutralization of vaccinia virus. Vaccinia virus at 10<sup>5</sup> pfu/ml was incubated at 37°C without any additives (○), with rabbit complement source at a final dilution of 1:10 (△), with antiserum at a final dilution of 1:100 (□), or with both antiserum and complement (●). Aliquots were removed at various times and the infectious virus was titered on BSC-1 monolayers.

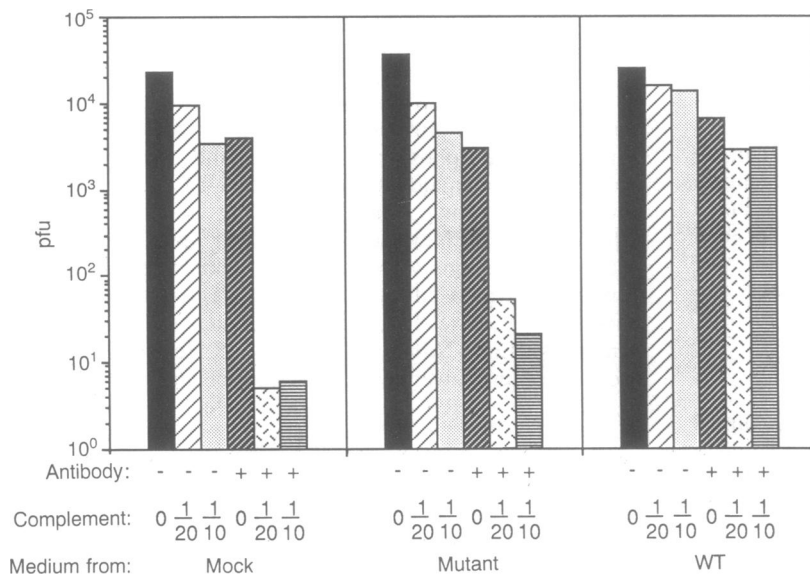


FIG. 2. Medium from cells infected with wild-type virus abrogates complement-enhanced virus neutralization. Vaccinia virions (10<sup>4</sup> pfu) were incubated in 100- $\mu$ l aliquots of medium from uninfected cells (Mock) or in medium from cells infected with VCP<sup>-</sup> virus (Mutant) or VCP<sup>+</sup> virus (WT) at 37°C for 2 h with the additives indicated below the bar graphs. The antiserum was at a final concentration of 1:1000. Rabbit complement source was either absent (0) or at the final dilution indicated.

**Effect of Deletion of the VCP Gene on Virus Pathogenicity in Rabbits.** Previous studies (ref. 6; S.N.I., unpublished data) showed that deletion of the VCP gene had no effect on the growth of virus in tissue culture, consistent with a speculative role for this protein in defense against the immune system rather than for replication *per se*. Intradermal inoculation of vaccinia virus results in characteristic skin lesions, which persist over a 3-week period. We sought to determine whether expression of VCP was important for the development of these lesions. Accordingly, 1:10 dilutions of wild-type and mutant viruses were injected intradermally into the shaved backs of three rabbits. By using the same rabbits simultaneously for both wild-type and mutant viruses, we avoided differences due to animal variation. The sizes of the lesions were measured throughout the course of infection and the results obtained with 10<sup>6</sup> pfu of virus are presented in Fig. 3. For the first few days, the lesions formed by wild-type and mutant viruses were similar, suggesting little or no difference in the innate abilities of these viruses to replicate in rabbit skin. From day 5 on, however, lesions of the mutant virus were smaller in size when compared to those produced by the parental virus. Similar results were also obtained with the other dilutions of virus (data not shown). Analysis of sera from two of the infected rabbits indicated that antibody capable of mediating complement-enhanced neutralization *in vitro* was detectable at day 6 and increased significantly thereafter (data not shown). These results suggested that VCP plays a significant role in evading the host immune response.

**VCP Inhibits Antibody-Dependent, Complement-Enhanced Neutralization by a C4-Independent Pathway of Complement Activation.** Based on previous studies (19), we anticipated that the complement-enhanced neutralization of vaccinia virus was mediated by the classical pathway of complement activation. To test this, we used serum from normal and C4-deficient guinea pigs as complement sources. Unexpectedly, both sources of complement enhanced virus neutralization in an antibody-dependent fashion (Fig. 4). Moreover, this enhancement was prevented by medium from wild-type but not from uninfected or mutant virus-infected cells (Fig. 4). These results, which were obtained with commercial C4-deficient guinea pig serum, were replicated by using well characterized serum from a colony of guinea pigs with a total genetic deficiency of C4 (20) maintained at the National Institutes of Health (a gift of M. M. Frank). To ensure that the 1:100 dilution of heat-inactivated polyclonal antiserum was not providing a minute amount of C4, the immunoglob-

ulin G fraction was purified and used as the antibody source with similar results (data not shown).

To assess the role of the alternative pathway in complement-enhanced neutralization of vaccinia virus, we used standard or factor B-depleted human serum as the complement source. As shown in Fig. 5, the undepleted complement source had some neutralization activity alone but activity was

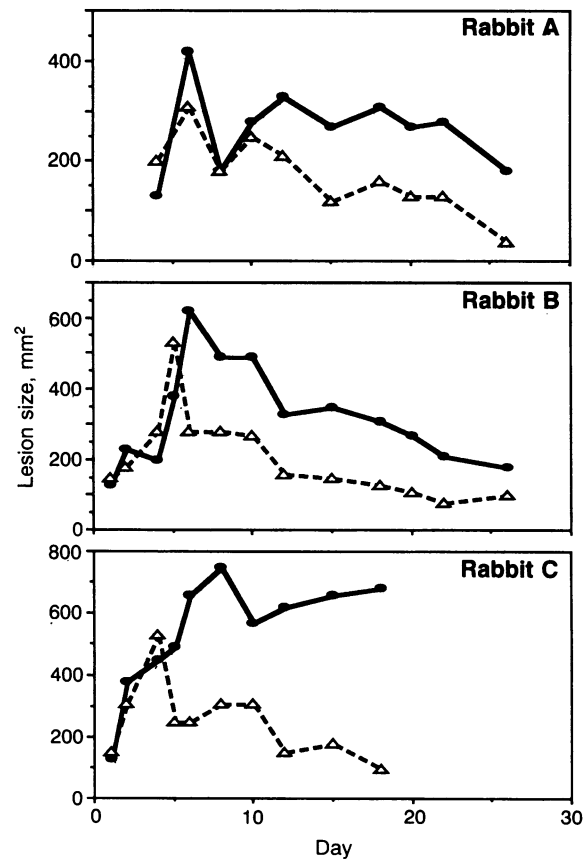


FIG. 3. Skin lesion size after intradermal injection of VCP<sup>+</sup> and VCP<sup>-</sup> viruses. Ten-fold dilutions from 10<sup>6</sup> to 10<sup>1</sup> pfu of VCP<sup>+</sup> and VCP<sup>-</sup> viruses were injected intradermally down either side of the dorsal spine. The size of the lesion formed by 10<sup>6</sup> pfu of VCP<sup>+</sup> virus (●) and VCP<sup>-</sup> virus (Δ) was recorded by day for each rabbit. The areas of the lesions were calculated from the measured diameters. Rabbit C was sacrificed on day 19 because of poor health.

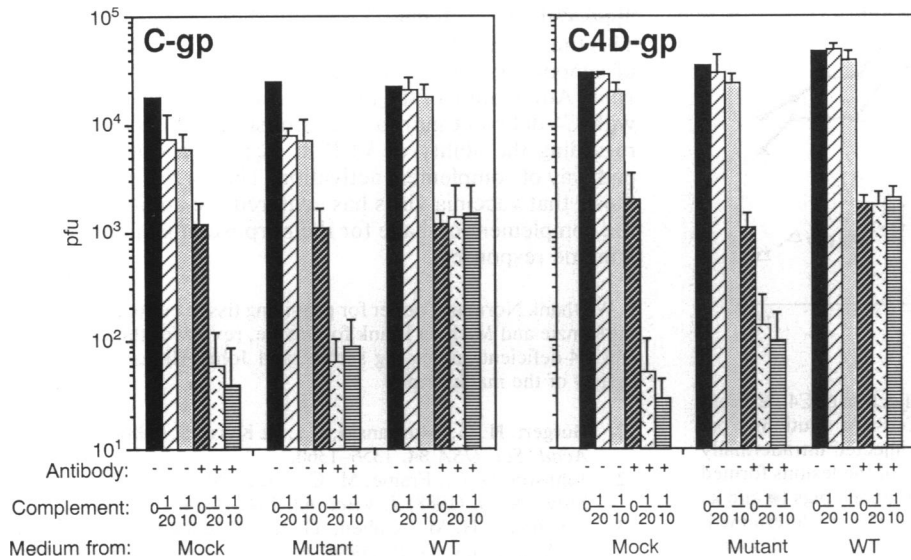


FIG. 4. VCP inhibited enhanced neutralization mediated by normal and C4-deficient guinea pig complement. Vaccinia virions were incubated in medium from Mock, Mutant, or WT-infected cells as described in Fig. 2. Antiserum was at a final dilution of 1:100. Normal guinea pig (C-gp) or C4-deficient guinea pig (C4D-gp) complement sources were either absent (0) or at the final dilution indicated below the graph. The data for C-gp and C4D-gp represent the mean ( $\pm$ SD) of six and five experiments, respectively. In each individual experiment, the data were standardized to the titers of the virus without antibody or complement.

greatly enhanced in the presence of antibody. The antibody-dependent activity was largely abrogated by medium from wild-type vaccinia virus-infected cells when a 1:20 dilution of complement was used and was partially abrogated when the 1:10 dilution of complement was used. As expected, medium from uninfected cells or from cells infected with the mutant virus had little effect. The factor B-depleted human serum, however, had little or no ability to neutralize vaccinia virus itself or to enhance neutralization in the presence of antibody (Fig. 5). The complement-enhancing activity of the factor B-depleted human serum was restored by addition of physiological amounts of factor B (Fig. 5), indicating a role for alternative pathway components in this system.

**Effect of VCP on Virus Pathogenicity in C4-Deficient Guinea Pigs.** The availability of C4-deficient guinea pigs (20) allowed us to investigate the role of VCP in animals with a genetic lesion resulting in the absence of the classical complement pathway. Ten-fold dilutions of wild-type or mutant (vSIGK3) virus were injected intradermally and the progression of skin lesion sizes was measured. As observed with rabbits, the lesions formed by mutant virus on the backs of the C4-deficient guinea pigs were smaller than those formed by the parental virus (Fig. 6). Similar results were obtained when normal 2/NCR strain guinea pigs were studied under the same conditions (data not shown).

**DISCUSSION**

The purpose of the present study was to evaluate the possibility that VCP has a role in regulating the host immune response to vaccinia virus. The data reported indicate that VCP can prevent antibody-dependent enhanced neutralization of vaccinia virus mediated by complement derived from rabbits, guinea pigs, or humans. Unexpectedly, enhanced neutralization of vaccinia virus did not require the C4 component of the classical pathway of complement activation. Thus, antibody-dependent, complement-enhanced neutralization was achieved with C4-deficient guinea pig serum, which has no classical pathway complement activity (20), but was not achieved with factor B-depleted human serum, which is defective in the alternative pathway of complement activation. In the latter case, neutralization-enhancing ability was restored by addition of purified factor B. This indicates that components of the alternative pathway of complement activation play an important role in our assay system. Further studies are needed to resolve differences between our results and those of Leddy *et al.* (19), who reported that the classical pathway of complement activation makes a major contribution to enhanced neutralization of vaccinia virus. The poxviruses, of which vaccinia is the prototype, are arguably the most complex of all animal viruses, with 100 or more polypeptides in the infectious particle (21). It is possible that our

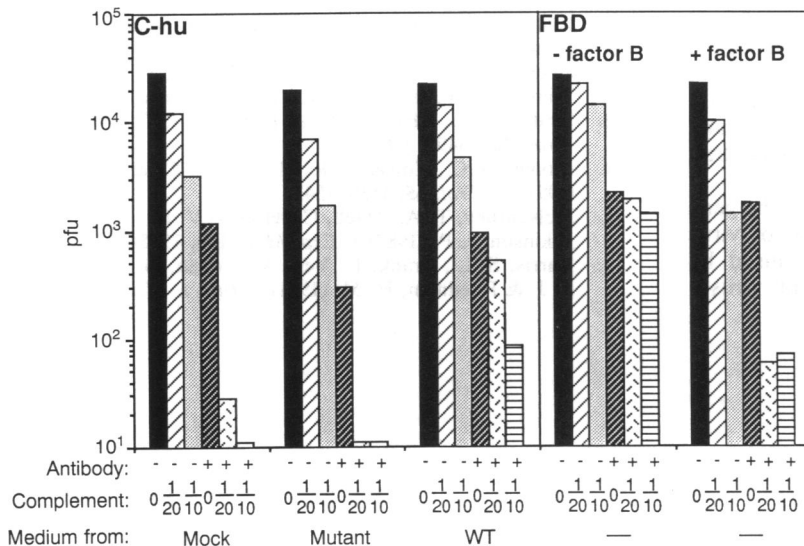


FIG. 5. Components of the alternative pathway of complement activation were required for neutralization of vaccinia virus. (Left) The extent of neutralization of vaccinia virions, incubated in the presence of the indicated amount of human serum as the complement source (C-hu) and antiserum (1:100) in medium from Mock, Mutant, or WT-infected cells, was determined as described in Fig. 2. (Right) Factor B-depleted human serum (FBD) was used as the complement source with or without addition of 4  $\mu$ g of purified active factor B. This amount of factor B was calculated to be twice the amount expected in the 1:10 dilution of normal human serum. A similar effect was achieved with 2  $\mu$ g of added factor B (data not shown). Data for - and + factor B are the means of five and two experiments, respectively.

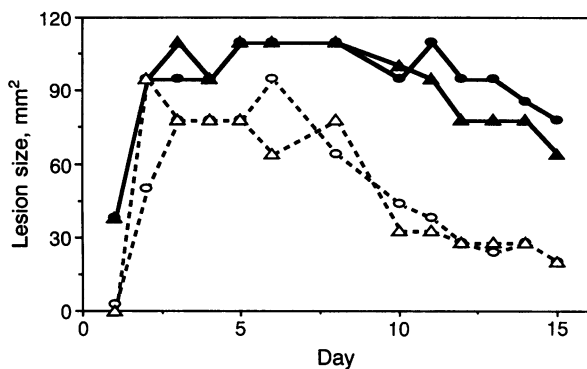


FIG. 6. Skin lesion size after intradermal injection of C4-deficient guinea pigs with VCP<sup>+</sup> and VCP<sup>-</sup> viruses. Ten-fold dilutions from 10<sup>6</sup> to 10<sup>1</sup> pfu of VCP<sup>+</sup> or VCP<sup>-</sup> virus were injected intradermally down either side of the dorsal spine. The sizes of the lesions formed by 10<sup>6</sup> pfu of VCP<sup>+</sup> virus on two C4-deficient guinea pigs (● and ▲) and 10<sup>6</sup> pfu of VCP<sup>-</sup> virus on two others (○ and △) were determined. The areas of the lesions were calculated from the measured diameters.

antibody, which was obtained from rabbits infected with live vaccinia virus, and that of Leddy *et al.* (19), which was from rabbits immunized with virus that had been emulsified with Freund's adjuvant, targeted different viral proteins, leading to different findings regarding the mechanisms of neutralization.

Hemolysis assays had provided evidence that VCP can block the classical pathway of complement activation (6). The present study demonstrated that VCP can modulate complement activation in C4-deficient serum, indicating that VCP can interact with complement components other than C4. The recent findings that VCP binds to both C4b and C3b components of the complement cascade and accelerates the decay of classical and alternative pathway convertases provide a physical basis for the dual action (R. McKenzie and M. M. Frank, personal communication). Binding to C4b and C3b is not unprecedented since several eukaryotic members of a complement regulatory family also display this property. The ability to regulate both pathways of complement activation would be an obvious advantage to vaccinia virus. Vaccinia virus may even encode additional complement regulatory proteins. Structural similarities between the predicted products of several other vaccinia virus genes and complement components have been noted (22–24), but functional studies have not yet been reported. Based on sequence, one of the latter proteins was predicted to be an integral membrane protein. The presence of a complement regulatory protein in the membrane of vaccinia virus or virus-infected cells might provide a second line of defense and influence the relative contributions of classical and alternative pathways of complement activation. Herpes simplex virus glycoprotein C is a component of viral and infected cell membranes and prevents complement-mediated cell lysis and virus neutralization (25, 26).

Most significant was our finding that deletion of the gene encoding VCP results in measurable attenuation of virus pathogenicity in rabbits. Initially, the lesions caused by intradermal inoculation of wild-type and mutant viruses

appeared similar, but after day 5 the latter diminished in size. This timing correlated with the detection of antibody capable of supporting complement-enhanced virus neutralization *in vitro*. Attenuation of the mutant virus was also demonstrated with C4-deficient guinea pigs, consistent with *in vitro* data regarding the ability of VCP to regulate a C4-independent pathway of complement activation. These results support the thesis that vaccinia virus has acquired the ability to regulate the complement cascade for the purpose of evading the host immune response.

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