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Inhibition of the Complement Cascade by the Major Secretory Protein of Vaccinia Virus

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The complement system contributes to host defenses against invasion by infectious agents. A 35-kilodalton protein, encoded by vaccinia virus and secreted from infected cells, has sequence similarities to members of a gene family that includes complement control proteins. Biochemical and genetic studies showed that the viral protein binds to derivatives of the fourth component of complement and inhibits the classical complement cascade, suggesting that it serves as a defense molecule to help the virus evade the consequences of complement activation.

THE COMPLEMENT SYSTEM IS COMposed of more than 20 plasma proteins that participate in host defenses against infectious agents. The proposed antiviral mechanisms of complement components include virus neutralization and opsonization, lysis of virus-infected cells, and amplification of inflammatory and specific immune responses (*1*). Some viruses may have evolved defenses against the complement system. The envelope glycoprotein gC of herpes simplex viruses types 1 and 2 acts as a receptor for fragment C3b of the third component of complement and thereby modulates the alternative complement pathway in vitro (*2*). Epstein-Barr virus, another herpesvirus, also may regulate activation and processing of the third component of complement (*3*). The 35-kD major secretory protein of vaccinia virus, a poxvirus, contains conserved elements of the 60 amino acid repeating unit of structurally related eukaryotic proteins with the greatest similarity to a human protein (C4bp) that binds the C4b fragment of the fourth component of complement and inhibits the classical pathway of complement activation (*4*).

The classical pathway for activation of the complement system starts with the binding of the C1 complex via C1q to the Fc region of immunoglobulin (Ig). Activation of bound C1 leads to sequential cleavages of C4 and C2, forming C4b2a, the classical pathway C3 convertase. Cleavage of C3 by the bound convertase activates the terminal lytic mechanism that is common to both the

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classical and alternative pathways. We adapted the sheep red blood cell (RBC) hemolysis assay (*5*) to determine whether the vaccinia virus 35-kD major secretory protein can regulate the complement cascade. In this assay, IgM-sensitized sheep RBCs interact with and activate components of the classical complement pathway in fresh serum leading to hemolysis. Hemolysis was inhibited by medium from RK_{13} cells infected with wild-type (strain WR) virus, but not with an attenuated mutant (designated 6/2) that lacks a cluster of more than a dozen genes, including the one that encodes the 35-kD protein (*6*). To determine the role of the 35-kD protein, we constructed recombinant vaccinia virus, vSIGK1 (Fig. 1A), with the entire gene for the 35-kD protein and some flanking DNA replaced by the selectable marker gene, xanthine-guanine phosphoribosyltransferase (*gpt*) regulated by a vaccinia promoter (*7*). A second recombinant, vSIGK3 (Fig. 1A), had a 70-bp segment within the 35-kD protein gene replaced by the *gpt* cassette (*7*). Analysis of the viral genomes confirmed the deletions and the presence of the selectable marker in the desired locations. A labeled 35-kD protein was not secreted from [^{35}S]methionine-labeled RK_{13} cells infected with either vSIGK1 or vSIGK3, but was secreted from the wild-type virus–infected cells (Fig. 1B). Also, whereas cells infected with mutant 6/2 secreted neither the 35-kD protein nor a 12-kD secretory protein encoded by another vaccinia virus gene (*8*), cells infected with vSIGK1 and vSIGK3 still secreted the smaller protein. Immunoblotting with an antibody to the COOH-terminal peptide (amino acids 237 to 248), confirmed the presence of the 35-kD protein in the medium of cells infected with the wild-type virus, but not in medium of 6/2, vSIGK1, or vSIGK3 (Fig. 1C).

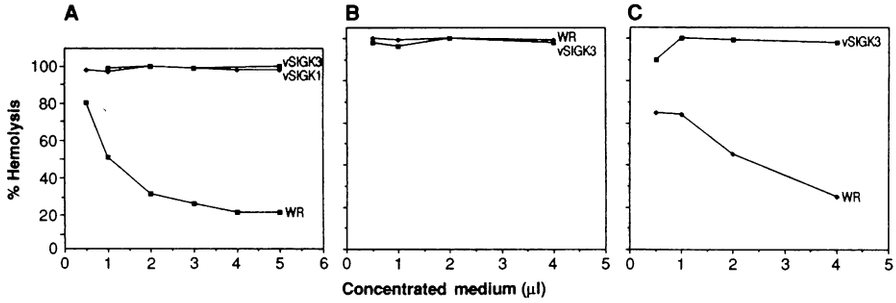

Fig. 2. Inhibition of complement-mediated hemolysis of sheep RBC. (**A**) Sensitized sheep RBCs were incubated with the highest dilution of human serum that gave 100% hemolysis and concentrated (×50) medium from cells infected with wild-type vaccinia virus (WR) or with mutants vSIGK1 or vSIGK3. The 100% hemolysis value was obtained by addition of water. (**B**) Unsensitized rabbit RBCs were incubated with C4-deficient guinea pig serum that had been preadsorbed with protein A Sepharose, at the highest dilution that resulted in 100% hemolysis and with medium from cells infected with wild-type vaccinia virus (WR) or vSIGK3. Similar results were obtained with serum dilutions that resulted in a lower percent hemolysis. (**C**) Sensitized sheep RBCs with bound C4 were incubated with C4-deficient guinea pig serum and medium from cells infected with wild-type vaccinia virus (WR) or vSIGK3. In each panel, experiments were carried out in triplicate; the individual results differed by less than 5%, and mean values were plotted.

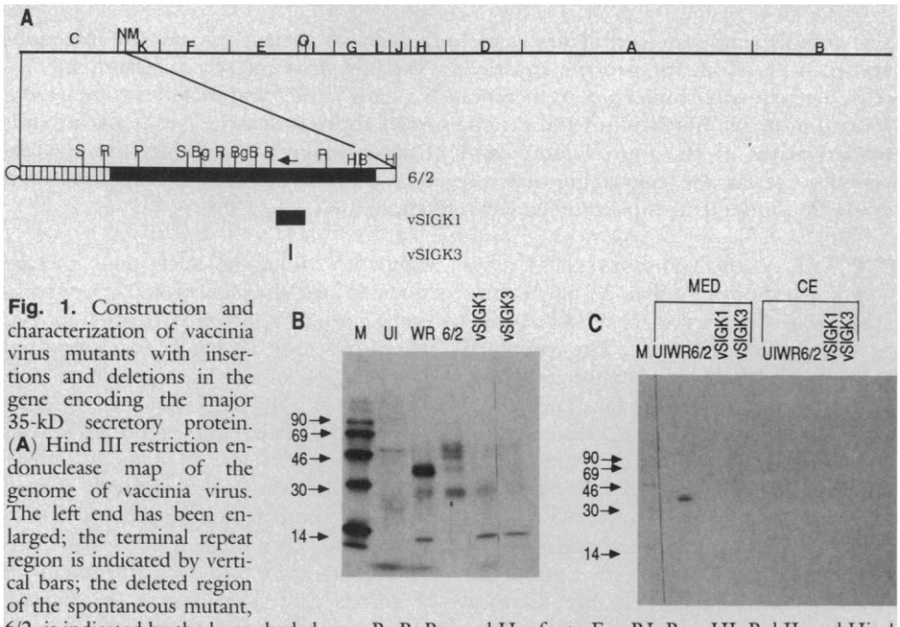

Fig. 1. Construction and characterization of vaccinia virus mutants with insertions and deletions in the gene encoding the major 35-kD secretory protein. (**A**) Hind III restriction endonuclease map of the genome of vaccinia virus. The left end has been enlarged; the terminal repeat region is indicated by vertical bars; the deleted region of the spontaneous mutant, 6/2, is indicated by the large shaded area; R, B, Bg, and H refer to Eco RI, Bam HI, Bgl II, and Hind III sites, respectively. The arrow indicates the direction and position of the open reading frame corresponding to the 35-kD protein. The filled horizontal bars indicate the sizes and locations of the deletions in the recombinant viruses vSIGK1 and vSIGK3. (**B**) An autoradiograph after SDS–polyacrylamide gel electrophoresis (SDS-PAGE) showing [^{35}S]methionine-labeled proteins secreted by uninfected RK_{13} cells (UI) or cells infected with 50 plaque-forming units per cell of wild-type vaccinia virus (WR), 6/2, vSIGK1, and vSIGK3. Metabolic labeling was in minimal RPMI medium from 1 to 15 hours after infection. M, ^{14}C-labeled relative molecular size marker proteins in kilodaltons. (**C**) Autoradiograph of a nitrocellulose membrane on to which unlabeled proteins secreted into the medium (MED) or remaining associated with cells (CE) were transferred after electrophoresis. The membranes were incubated with an antibody raised in rabbits to peptide Gly-Tyr-Lys-Leu-Ser-Gly-Ser-Ser-Ser-Ser-Thr-Cys, derived from the COOH-terminus of the 35-kD protein, and ^{125}I-labeled protein A.

Human complement–mediated lysis of IgM-sensitized RBCs was progressively inhibited with increased amounts of medium from cells infected with vaccinia virus WR, but not recombinant viruses vSIGK1 or vSIGK3 (Fig. 2A). Virtually identical results were obtained when guinea pig serum was used as the source of complement. Thus, the 35-kD protein is necessary for vaccinia-mediated inhibition of the classical complement cascade.

We also tested whether the medium from vaccinia virus–infected cells could inhibit the alternative pathway of complement activation, which does not require C1, C2, or C4 components of the classical complement pathway to make a C3 convertase. With unsensitized rabbit RBCs and C4-deficient guinea pig serum depleted of IgG, a test system in which lysis is mediated by the alternative pathway, hemolysis was not significantly inhibited by medium from cells infected with vaccinia virus WR or vSIGK3 (Fig. 2B). Thus, neither the 35-kD protein nor any other protein secreted from vaccinia virus–infected cells inhibited the alternative complement pathway under conditions in which the classical one was affected.

In the above experiments, hemolysis via the classical complement pathway might be inhibited by preventing formation or accelerating decay of the classical C3 convertase or by blocking attachment of C4b to RBCs. To evaluate the latter possibility, we first verified that hemolysis occurred after mixing sensitized sheep RBCs with human C4b already bound to their surfaces (*9*) and C4-deficient guinea pig serum, which provides

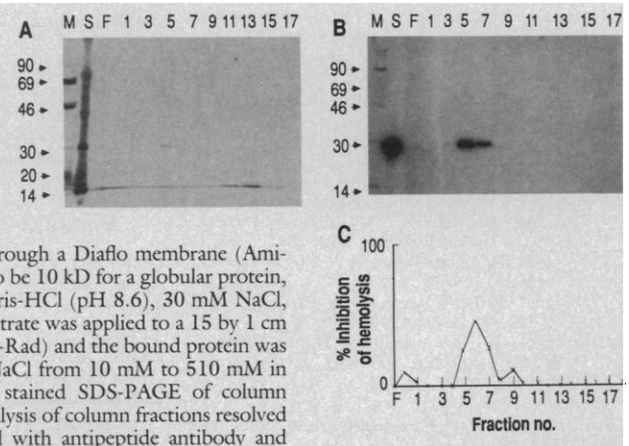

Fig. 3. Copurification of 35-kD secreted protein and complement inhibitory activity. Serum-free medium (OPTI-MEM I, Gibco), from 15 150-cm^2 flasks of RK_{13} cells that had been infected with 50 plaque-forming units per cell of vaccinia virus WR, was concentrated 2000-fold by ultrafiltration through a Diaflo membrane (Amicon) with a cut-off estimated to be 10 kD for a globular protein, and dialyzed against 10 mM tris-HCl (pH 8.6), 30 mM NaCl, and 5 mM EDTA. The concentrate was applied to a 15 by 1 cm column of DEAE-BioGel (Bio-Rad) and the bound protein was eluted with 30-mM steps of NaCl from 10 mM to 510 mM in the above buffer. (**A**) Silver stained SDS-PAGE of column fractions. (**B**) Immunoblot analysis of column fractions resolved by SDS-PAGE and incubated with antipeptide antibody and ^{125}I-labeled protein A. (**C**) Hemolysis assay as in the legend to Fig. 2A. M, marker proteins in kilodaltons; S, starting material; F, flow through; and 1 to 17, alternate column fractions.

the other complement components. Hemolysis was inhibited by prior addition of medium from cells infected with vaccinia virus WR, but not by medium from cells infected with vSIGK3 (Fig. 2C). Thus, the virus-induced inhibitor specifically prevents the formation or stability of the classical C3 convertase. Preliminary experiments demonstrated that the inhibitor accelerated the decay of the classical convertase formed by human C4b and C2a (*10*) with kinetics similar to those of human C4bp (*11*).

The failure of media from cells infected with mutant viruses vSIGK1 and vSIGK3 to inhibit complement-mediated hemolysis demonstrated that a functional gene encoding the 35-kD protein was required for this activity. These results do not prove, however, that the 35-kD protein is directly involved in inhibition of the complement cascade or that it is sufficient to mediate this effect without other viral proteins. Therefore, concentrated serum-free medium from vaccinia virus–infected RK_{13} cells was chromatographed on a DEAE-Biogel column (Fig. 3). The majority of material with complement-mediated hemolysis-inhibitory activity eluted at 180 mM NaCl (Fig. 3C), which correlated precisely with the 35-kD protein, as detected by silver staining (Fig. 3A) and immunoblotting with antibody to peptide serum (Fig. 3B). In addition, no other proteins coeluted with the complement inhibitory activity when [^{35}S]methionine-labeled proteins secreted from virus-infected cells were chromatographed. The complement inhibitory activity also coeluted with the 35-kD immunoreactive protein in a Sephadex G-100 gel filtration step that was introduced after DEAE-Biogel chromatography. These results are consistent with a direct effect of the viral protein on the classical complement cascade.

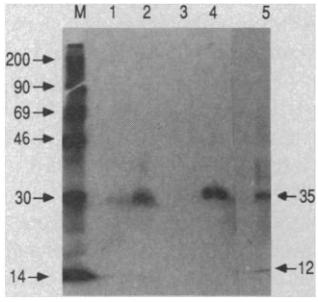

Fig. 4. Binding of the 35-kD protein to RBCs with attached C4b. The [^{35}S]methionine-labeled 35-kD protein was purified from the medium of vaccinia virus–infected RK_{13} cells as described in Fig. 3. Pooled fractions from the DEAE-BioGel column were then applied to a Sephadex G-100 column and the fractions were assayed as in the legend to Fig. 3. Equal samples of purified 35-kD protein were then incubated with 10^8 sheep RBCs with (lanes 2 and 4) or without (lanes 1 and 3) bound C4b in 0.2 ml of dextrose veronal buffer for 16 hours at 4°C. The RBCs were washed five times, suspended in distilled water, and analyzed by SDS-PAGE (10 to 20%) and fluorography. M, marker proteins in kilodaltons; lanes 1 and 3, proteins associated with RBCs without attached C4b; lanes 2 and 4, proteins associated with RBCs with attached C4b; and lane 5, total ^{35}S-labeled proteins from the medium of infected cells. Twice the number of RBCs was applied to lanes 3 and 4 compared to lanes 1 and 2.

Additional assays were used to investigate the interaction of the 35-kD protein with complement components. Direct binding of the purified [^{35}S]methionine-labeled viral protein to cells with C4b attached to their surfaces, but not to cells without C4b, was demonstrated (Fig. 4). The ability of sheep RBCs with bound C4b to adhere to human erythrocytes, which have the surface C3b/C4b receptor (CR1), is the basis of a rosette assay (*9*). Medium from cells infected with vaccinia virus WR or purified 35-kD protein blocked rosetting, but medium from cells infected with the vaccinia virus mutant 6/2 or Sephadex fractions without the 35-kD protein did not. Thus, both assays provided evidence for the interaction of the 35-kD protein with C4b.

The activity of the 35-kD major secretory protein of vaccinia virus is consistent with its structural resemblance (38% amino acid identity) to the first half of C4bp (*11*), a classical pathway complement control protein. C4bp's from mouse, guinea pig, and human plasma have subunits of about 70 kD and exist as multimeric molecules of about 550 kD (*12*). These proteins bind C4b and cause accelerated decay of the C3 convertase by dislocating C2a and also serve as cofactors for cleavage of C4 by serum factor I (*13*). The vaccinia protein also has homology with other regulators of complement activation, including the precursor of human membrane cofactor protein (*14*) and decay-accelerating factor (*15*), for which the amino acid identities are 35% and 31%, respectively.

Some vaccinia virus genes are not required for replication in tissue culture cells and may function to enhance infection or evade immune responses in the host animal (*16*). Thus, the predicted biological role of the 35-kD secretory protein is to diminish the antiviral effects of the host complement system. Consistent with this possibility, skin lesions in rabbits produced by 35-kD protein mutants were smaller and healed more rapidly than those caused by wild-type virus, indicating attenuation of viral pathogenicity (*17*). The significance of the 35-kD protein in virus-host interactions is supported by the finding of similar secretory proteins in the media of cells infected with two other members of the orthopoxvirus genus, cowpox virus and ectromelia (*18*). Ectromelia virus, which causes a fatal disease in mice, should be particularly suitable for further studies of the role of the 35-kD protein as a virus-encoded defense molecule.

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tized rabbit RBCs were prepared as described by T. A. E. Platt-Mills and K. Ishizaka [*J. Immunol.* **113**, 348 (1974)].
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Expression of Interleukin-10 Activity by Epstein-Barr Virus Protein BCRF1

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Cytokine synthesis inhibitory factor (CSIF; interleukin-10), a product of mouse T_H2 T cell clones that inhibits synthesis of cytokines by mouse T_H1 T cell clones, exhibits extensive sequence similarity to an uncharacterized open reading frame in the Epstein-Barr virus BCRF1. Recombinant BCRF1 protein mimics the activity of interleukin-10, suggesting that BCRF1 may have a role in the interaction of the virus with the host's immune system.

INTERLEUKIN 10 (IL-10) IS A CYTOKINE produced by one class of mouse helper T cell clone (TH2) that inhibits synthesis of cytokines [notably interferon-γ (IFN-γ)] by activated TH1 clones (*1*). Because TH1 cells preferentially mediate delayed type hypersensitivity (DTH) and macrophage activation (*2*), whereas TH2 cells provide superior help for B cell (antibody) responses (*3*), IL-10 may represent a mechanism whereby TH2 cells can inhibit the effector functions of TH1 cells. This possibility could help explain why DTH responses and antibody responses are often mutually exclusive (*4*).

Complementary DNA clones that encode mouse IL-10 (mIL-10) (*5*) reveal that the mature, secreted IL-10 polypeptide has approximately 70% amino acid identity to an uncharacterized open reading frame in the Epstein-Barr virus (EBV) BCRF1 (*6*). We therefore cloned and expressed the *BCRF1* gene, and demonstrated that the expressed BCRF1 protein, like IL-10, inhibits IFN-γ synthesis by activated lymphoid cells.

As a source of BCRF1 DNA, we used either whole EBV genomic DNA prepared from infectious virus isolated from the marmoset cell line B95-8 (*7*), or plasmid subclones of the EBV Bam HI C fragment (*6*). The predicted protein-coding region of the *BCRF1* gene (*5, 6*) was amplified by polymerase chain reaction (PCR) with oligonucleotide primers that also contained Eco RI sites for subsequent cloning into a modified form of the pcDSRα296 expression vector (*8*). The BCRF1 insert in the expression plasmid used in these experiments was derived from the Bam HI C fragment subclones, but EBV genomic DNA isolated from infectious virus also gave a PCR-amplified fragment of the expected size. The complete DNA sequence of the resulting BCRF1 insert was determined and was identical to the published sequence (*6*). COS-7 cells transiently transfected with this plasmid were cultured in the presence of [^{35}S]methionine, with or without tunicamycin B_2 (TcB_2) (*5*), and supernatants were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1). The ^{35}S-labeled supernatant from BCRF1-expressing cells contained an ~17 kD polypeptide not present in supernatants from mock-transfected cells. The BCRF1 polypeptide is approximately the same size as the unglycosylated form of mIL-10. The mobility of BCRF1 in SDS-PAGE was not altered when TcB_2 was included in the culture, suggesting that, unlike mIL-10 (*5*), BCRF1 contains little or no N-linked oligosaccharide. Because BCRF1 lacks the N-linked glycosylation site at Asn^{11} of mIL-10 (*5*),

Table 1. BCRF1 inhibits IFN-γ synthesis by antibody to CD3 (anti-CD3)–stimulated PBMC. Occasionally enhancement by IFN-γ synthesis by COS-7 (mock) supernatant was observed (experiments 2 and 3). This result was not uniformly obtained among various donors (experiment 1).

Stimulation	IFN-γ (ng/ml)		
	Experiment 1	Experiment 2	Experiment 3
None	<0.30	<0.30	<0.30
Anti-CD3	18.65	3.95	7.87
Anti-CD3 + BCRF1	<0.30	1.04	<0.30
Anti-CD3 + mock	19.29	9.00	14.67

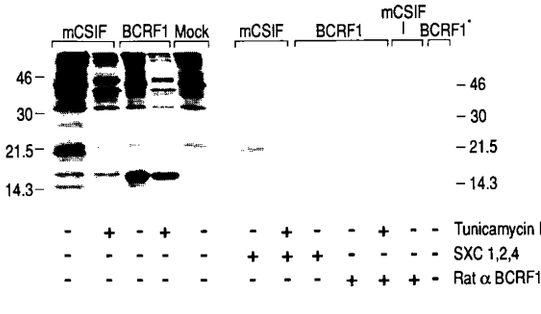

Fig. 1. Expression of the BCRF1 gene (*22*). Lanes show either total ^{35}S-labeled COS-7 supernatants (mCSIF, BCRF1, mock) or immunoabsorbed mCSIF or BCRF1 as indicated. *, Immunoabsorption was carried out with preimmune rat serum (*23*). SXC 1,2,4 are monoclonal antibodies to rat mIL-10 (*9*).

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