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Viral Inhibition of Inflammation: Cowpox Virus Encodes an Inhibitor of the Interleukin-1β Converting Enzyme

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Summary

Cowpox virus effectively inhibits inflammatory responses against viral infection in the chick embryo. This study demonstrates that one of the viral genes necessary for this inhibition, the *crmA* gene (a cytokine response modifier gene), encodes a serpin that is a specific inhibitor of the interleukin-1β converting enzyme. This serpin can prevent the proteolytic activation of interleukin-1β, thereby suppressing an interleukin-1β response to infection. However, the modification of this single cytokine response is not sufficient to inhibit inflammatory responses. This suggests that cowpox virus encodes several cytokine response modifiers that act together to inhibit the release of proinflammatory cytokines in response to infection. These viral countermeasures to host defenses against infection may contribute significantly to the pathology associated with poxvirus infections.

Introduction

Inflammatory processes constitute one of the primary lines of defense against virus infection. These processes are rapidly induced to limit the spread of the virus during the first hours and days after the infection, when the host may not yet be capable of mounting effective cellular and humoral immune responses against the invading virus. This suggests that the ability to inhibit host inflammatory processes would be advantageous to the virus. However, such inhibition might be difficult to achieve, because of the multiplicity of mediators effecting these processes. The specific inhibition of each mediator would necessitate an array of inhibitors, requiring a genetic coding capacity that few viruses possess.

The poxviruses are among the more complex viruses that do possess the capacity to encode an array of inhibitors of host defenses. The poxviruses, whose properties have recently been reviewed by Moss (1990), are each capable of encoding about 200 proteins. Many of these proteins are not essential for virus replication in tissue culture, but their conservation suggests that they are advantageous for virus replication in vivo. Potential functions for these proteins include countermeasures to restrictions on viral replication in vivo, including most inflammatory and immune defenses against viral infection, which would be inoperative in vitro. Successful inhibition of host defenses might be expected to increase the pathology associated with virus infection. In this context, it is noteworthy that some of the poxviruses, the smallpox viruses, are among the most virulent of all human viruses. In the first half of this century, smallpox viruses infected millions of people each year; virus infection rapidly produced a severe disease with a case-fatality rate of up to 30% (reviewed by Fenner et al., 1989). The mechanisms by which smallpox viruses induced these effects are poorly understood, but the extreme pathogenicity of these viruses suggests that the host defenses against these viruses were often fatally ineffective.

One of the first of the poxvirus genes shown to affect host responses to infection was the cowpox virus gene that exerts a profound effect upon the type of lesion produced in the chorioallantoic membrane of the chick embryo (Pickup et al., 1986). This gene, which was informally designated the 38K gene (Palumbo et al., 1989), encodes a 38 kd protein whose amino acid sequence is similar to those of members of the serpin superfamily (Pickup et al., 1986). This superfamily includes several protein inhibitors of serine proteinases, such as antithrombin III and α_1–proteinase inhibitor (Carrell et al., 1987). Initially, this similarity, and the correlation between the expression of this gene and the production of hemorrhage in the red lesions (pocks) induced by the virus (Figure 1), suggested that the viral protein might inhibit serine proteinases involved in the blood coagulation pathway. However, several lines of evidence indicated that this was unlikely. First, the predicted reactive site of this putative inhibitor is not similar to that of any known serine proteinase inhibitor. Second, the protein appears to be localized in the cytoplasm of the cell. Third, this protein is an early gene product; maximum synthesis of this protein occurs during the first 5 hr of the viral replication cycle (Pickup et al., 1986). And fourth, subsequent studies indicated that the hemorrhage, evident 3 days after infection, was not a primary effect of the expression of this gene, but a secondary effect stemming from the lack of inflammatory responses to cowpox viruses expressing this gene (Palumbo et al., 1989; Chua et al., 1990). Histological examination of sections through lesions induced by wild-type cowpox virus in the chorioallantoic membranes of chick embryos demonstrated a marked lack of inflammatory cells. In contrast, analysis of the white pocks induced by a cowpox virus that did not synthesize the 38 kd serpin showed an influx of large numbers of inflammatory cells (mainly heterophils and macrophages) and a proliferation of cells in the mesodermal and endodermal layers. This induction of host inflammatory responses by the variant cowpox virus resulted in a reduction in the number of virus-infected cells, less damage to the tissue, and lower yields of virus in comparison with similar infection with the wild-type virus (Palumbo et al., 1989).

The magnitude of the effect of this one viral protein on

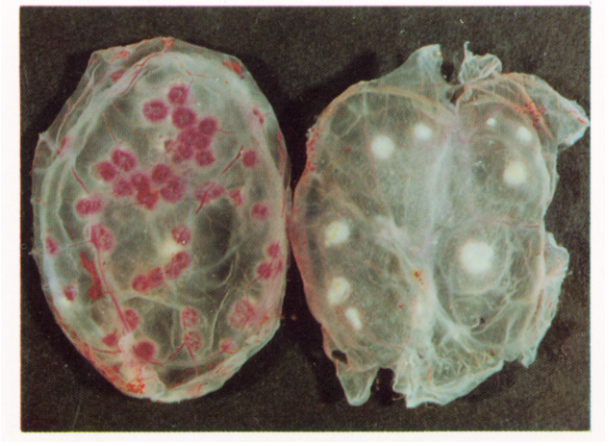

Figure 1. Pocks Produced on the Chorioallantoic Membranes of 14-Day-Old Chick Embryos

Chorioallantoic membranes were infected either with wild-type cowpox virus (producing red pocks) or with cowpox virus BR.D1 (producing white pocks). Cowpox virus BR.D1 is similar to the wild-type virus, except that the gene encoding the 38 kd serpin has been inactivated (Palumbo et al., 1989).

host inflammatory processes suggested that its target might be one of the major cytokine responses to infection. Among the many cytokines involved in inflammatory processes, interleukin-1α and interleukin-1β are mediators of cardinal importance. The multiple effects of these interleukins have recently been reviewed (Dinarello, 1988, 1991). Interleukin-1β, the predominant secreted interleukin-1, is produced by cells of many different types. It may be synthesized in response to virus infection (Flamand et al., 1991; Wano et al., 1987; Ensoli et al., 1989). And it can mediate several processes that contribute to the host defenses against virus infection.

The expression of the interleukin-1β gene is governed transcriptionally, translationally, and posttranslationally (reviewed by Dinarello, 1991). A variety of stimuli, including virus infection, can induce the transcription of the interleukin-1β gene. The interleukin-1β mRNAs may be translated immediately, or they may remain untranslated until certain stimuli release a translational repression (Schindler et al., 1990). Translation generates an inactive 31 kd precursor, pro-interleukin-1β, which is subsequently proteolytically processed to the active 17.5 kd form (Mosley et al., 1987; Black et al., 1988; Hazuda et al., 1989). The specific proteinase responsible for the processing, the interleukin-1β converting enzyme, has been identified and purified from human cells (Black et al., 1989; Kostura et al., 1989). And the recent characterization of a cDNA encoding this proteinase has confirmed that it is a novel proteinase (Cerretti et al., 1992). In vitro, this enzyme generates a 28 kd form of interleukin-1β as well as the 17.5 kd mature form (Black et al., 1989; A. D. Howard et al., 1991), but the physiological significance of the 28 kd form is unknown. In a cell, the accumulation of either untranslated interleukin-1β mRNA or unprocessed pro-interleukin-1β (Hazuda et al., 1988) may facilitate the rapid generation of active interleukin-1β in response to appropriate stimuli. This pathway of synthesis suggests that viral inhibition of host cell transcription and host mRNA translation might not be sufficient to inhibit an interleukin-1β response. To achieve effective inhibition the virus also may have to prevent the activation of the pro-interleukin-1β, or inhibit the activity of mature interleukin-1β, or do both.

These considerations led us to investigate whether the cowpox virus 38K gene, now formally designated *crmA* (cytokine response modifier), is one of several viral genes encoding modifiers of cytokine responses to infection. In this study we demonstrate that the *crmA* gene can modify the interleukin-1β response to infection by encoding a specific inhibitor of the interleukin-1β converting enzyme.

Results

Cytoplasmic Extracts from Cells Infected with Cowpox Virus Inhibit Processing of Pro-Interleukin-1β

Extracts of soluble proteins from the cytoplasm of virus-infected cells were examined for their ability to inhibit the processing of human pro-interleukin-1β by human interleukin-1β converting enzyme. As shown in Figure 2, although extracts from uninfected cells did not affect processing, extracts from cells infected with the wild-type cowpox virus completely inhibited processing even after 32-fold dilution. In contrast, extracts from cells infected with a recombinant cowpox virus, BR.D1 (Palumbo et al., 1989), which is similar to wild-type virus except that it contains an inactivated *crmA* gene, failed to inhibit processing. This demonstrated that significant amounts of a factor that inhibits the proteolytic processing of pro-interleukin-1β were present in the cytoplasm of cowpox virus–infected cells within 4 hr of infection, consistent with the predicted physiological role of this viral gene product. It also demonstrated that the inhibition of proteolytic processing of pro-interleukin-1β was dependent upon the expression of the *crmA* gene.

Purification of the Protein Encoded by the *crmA* Gene

To determine whether the *crmA* gene product inhibits the interleukin-1β converting enzyme directly, the 38 kd protein was purified. The *crmA* gene is an early gene whose product is not abundant in the infected cell. Therefore, to obtain greater yields of functional protein, a vaccinia virus vector directing high levels of expression of this gene was employed. To facilitate the purification of the protein, the method of Smith and Johnson (1988) was adopted, whereby the coding region of the *crmA* gene was fused in-frame via a peptide linker segment to the C-terminal portion of the Schistosoma japonicum gene encoding glutathione S-transferase (GST). This fusion gene was then expressed by means of the binary vaccinia virus expression system of Elroy Stein et al. (1989), which employs the phage T7 RNA polymerase to gain efficient transcription of the inserted gene, and the leader sequence of the RNA of encephalomyocarditis virus (EMCV) to gain cap-independent translation of the T7 RNA polymerase-generated transcripts. Yields of up to 0.5 mg of fusion

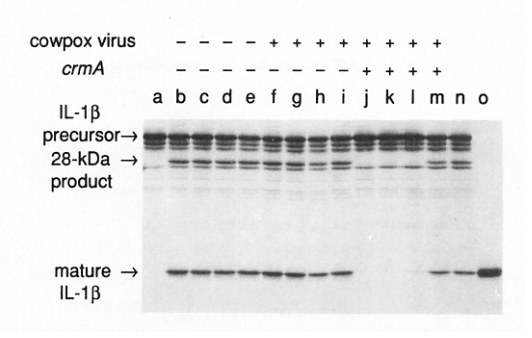

Figure 2. The Inhibition of Human Interleukin-1β Converting Enzyme by a Factor Present in Cells Infected with Cowpox Virus Is Dependent upon the Expression of the *crmA* Gene

Ten microliters of 10 mM Tris–HCl (pH 8.0) buffer, or 10 μl of desalted cell extract, diluted with this buffer as described below, were mixed with 10 μl of human interleukin-1β converting enzyme, and the mixtures were incubated for 25 min at 37°C. Then 50 ng of human pro-interleukin-1β was added, and the incubation was continued for 90 min. Processing of the pro-interleukin-1β was then analyzed by Western blot as described in Experimental Procedures, using a monoclonal antibody specific for the C-terminal region of interleukin-1β. Lane a: pro-interleukin-1β alone. Lanes b–e: interleukin-1β converting enzyme preincubated with extracts, diluted 1:8, 1:16, 1:32, and 1:64, from uninfected cells. Lanes f–i: interleukin-1β converting enzyme preincubated with extracts, diluted 1:8, 1:16, 1:32, and 1:64, from cells infected with cowpox virus BR.D1, which contains an insertionally inactivated *crmA* gene. Lanes j–m: interleukin-1β converting enzyme preincubated with extracts, diluted 1:8, 1:16, 1:32, and 1:64, from cells infected with wild-type cowpox virus. Lane n: interleukin-1β converting enzyme preincubated with sample buffer. Lane o: mature interleukin-1β.

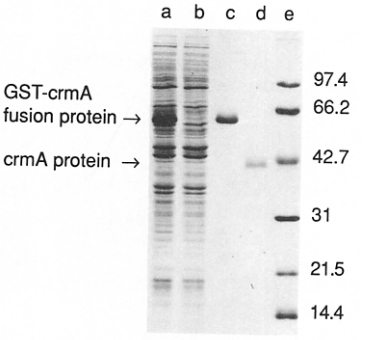

Figure 3. Purification of the CrmA Protein

Cells were infected with the vaccinia virus vector expressing a fusion gene encoding the C-terminal region of GST connected by a thrombin/trypsin cleavage-site linker to the N-terminus of the 38 kd protein. Proteins (Coomassie blue–stained) were resolved by electrophoresis through a 12% polyacrylamide gel. Lane a: total soluble proteins in the extract of the cytoplasm of virus-infected cells. Lane b: proteins in this extract that failed to bind to the glutathione–Sepharose. Lane c: fusion protein eluted from the glutathione–Sepharose. Lane d: the CrmA protein after trypsin cleavage from the fusion protein GST moiety, and the removal of GST proteins and trypsin by chromatography over DEAE–Sephacel and glutathione–Sepharose. Lane e: size standards in kd.

protein per 10^6 cells were obtained with this expression system (Figure 3, lane a). The fusion protein, which was soluble, was efficiently recovered from extracts of the cytoplasm by affinity chromatography over glutathione–Sepharose (Figure 3, lanes b and c).

The region at the peptide linker in the fusion protein comprises the amino acids Lys-Ser-Asp-Leu-Val-Pro-Arg-Gly-Ser-Ile-Met, where the methionine residue corresponds to the initiation codon of the *crmA* gene. Although this segment contains a thrombin cleavage site, thrombin failed to cleave the fusion protein, even at high ratios of thrombin to fusion protein, presumably because it was unable to gain access to this site. However, trypsin did cleave the fusion protein within the linker sequence, without cleavage of the 38 kd moiety. Therefore, trypsin was used to remove the 38 kd moiety from the fusion protein. After trypsin digestion, the 38 kd cleavage product was separated from the trypsin, GST, and residual fusion protein by chromatography over DEAE–Sephacel and glutathione–Sepharose (Figure 3, lane d). This method produced up to 0.15 mg of CrmA protein from 10^8 cells. Sequence analysis of the purified CrmA protein showed that its N-terminal sequence corresponded to that predicted for the authentic protein, together with the addition of 9 aa derived from the trypsin-cleaved peptide linker segment (Ser-Asp-Leu-Val-Pro-Arg-Gly-Ser-Ile). This short extension is not expected to have any effect upon the activities of the CrmA protein; the N-terminal regions of serpins, which are not conserved, do not play a direct role in the inhibition of proteinases (Carrell et al., 1987).

A sample of the purified CrmA protein was quantified by amino acid compositional analysis. The calculated composition agreed with that predicted from the nucleotide sequence with deviations of only 1%–10% per residue, confirming the purity and identity of the protein. The sample of purified material contained 91 μg/ml of protein, in good agreement with results of quantification by the dye-binding assay used routinely to quantify the CrmA protein (110 μg/ml of protein).

The CrmA Protein Is a Specific Inhibitor of the IL-1β Converting Enzyme

Incubation of the purified CrmA protein with the interleukin-1β converting enzyme, before the addition of pro-interleukin-1β, inhibited the processing of the precursor (Figure 4). These results showed that under the conditions of this assay, 4 ng of the purified CrmA protein inhibited the processing capacity of approximately 0.5 ng of the interleukin-1β converting enzyme by 50%; and between 7.5 and 15 ng of the 38 kd protein completely inhibited this amount of enzyme. The purified CrmA protein was also capable of inhibiting the ability of the interleukin-1β converting enzyme to cleave a synthetic peptide representing the cleavage site, Ala-112 to Ser-121, in pro-interleukin-1β (Figure 5). This suggests that the viral protein inhibits processing of the precursor by interacting with the proteinase rather than by interacting with the precursor.

To examine the specificity of the viral proteinase inhibitor, the purified CrmA protein was tested for its ability to inhibit the activity of representative members of several major families of serine proteinases: trypsin, chymotrypsin, cathepsin G, pig and human elastases, thrombin, plasmin, human tissue plasminogen activator (t-PA), and human urinary plasminogen activator (u-PA). It was also

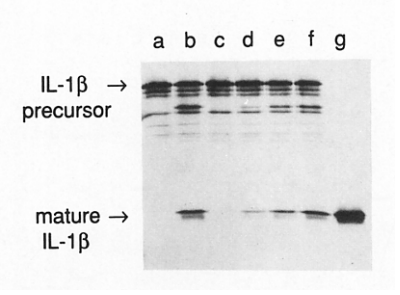

Figure 4. The Purified CrmA Protein Directly Inhibits the Human Interleukin-1β Converting Enzyme

Ten microliters of 10 mM Tris–HCl (pH 8.0) buffer, or 10 μl of a solution of purified CrmA protein were mixed with 10 μl (about 0.5 ng) of human interleukin-1β converting enzyme and incubated for 25 min at 37°C. Then 50 ng of human pro-interleukin-1β was added, and the incubation was continued for 90 min. Processing of the pro-interleukin-1β was then analyzed by Western blot as described in Experimental Procedures. Lane a: pro-interleukin-1β alone. Lane b: pro-interleukin-1β incubated with interleukin-1β converting enzyme that had been preincubated with buffer. Lanes c–f: pro-interleukin-1β incubated with interleukin-1β converting enzyme that had been preincubated with 15, 7.5, 4, or 2 ng of purified CrmA protein. Lane g: mature interleukin-1β.

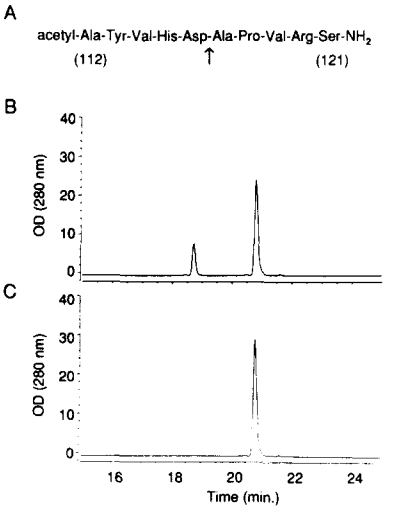

Figure 5. The CrmA Protein Inhibits Interleukin-1β Converting Enzyme from Cleaving a Synthetic Peptide Substrate

A synthetic peptide (A) representing Ala-112 to Ser-121 of pro-interleukin-1β was incubated with the purified human interleukin-1β converting enzyme for 4.5 hr in the absence (B) or presence (C) of purified CrmA protein. The samples were then analyzed by high performance liquid chromatography as described (Sleath et al., 1990). The cleavage site in the peptide substrate corresponding to the cleavage site in pro-interleukin-1β is indicated by the arrow. The two peaks in (B) correspond to the cleavage fragment Ala-112 to Asp-116 and the intact peptide. The single peak in (C) corresponds to the intact peptide.

tested against papain, a cysteine proteinase. The *crmA* gene product failed to cause detectable inhibition of any of these proteinases, even when the ratio of 38 kd protein to proteinase was 100:1 (w/w). Although it is difficult to rule out the possibility that a given protein has some inhibitory effect upon a particular proteinase, it is possible to determine the limits of any inhibition (Salvesen and Nagase, 1989). In these assays, all reaction mixtures contained greater than 4×10^{-7} M CrmA protein. Therefore, assuming that 0%–10% inhibition of activity was undetectable, the maximum limit for the K_a was 10^6 M^{-1}, a value indicating that the CrmA protein is not a physiologically significant inhibitor of either cysteine proteinases of papain-like specificity or serine proteinases of trypsin-, chymotrypsin- or elastase-like specificities.

Discussion

The *crmA* gene encodes a novel, specific inhibitor of the human interleukin-1β converting enzyme. When the synthesis of interleukin-1β is induced in the cell as a result of the virus infection, the action of this viral protein could reduce the secretion of active interleukin-1β, thereby modifying the ability of the cell to generate an interleukin-1β response to infection.

The observed effects of the expression of the *crmA* gene on the type of lesions produced by cowpox virus on the chorioallantoic membrane of the chick embryo are all consistent with the predicted modification of interleukin-1β-mediated autocrine and paracrine responses to infection. These effects include: reduced emigration of inflammatory cells into the lesion, decreased local proliferation of cells, an increase in the number of cells supporting viral replication, increased damage to the tissue, and a decrease in the production of chemotactic factors (Palumbo et al., 1989; Chua et al., 1990; Fredrickson et al., 1992). Each of these effects will assist the virus in establishing a productive infection before the induction of target-specific immune defenses.

The mechanism by which the CrmA protein inhibits the interleukin-1β converting enzyme appears to be novel. This virus protein strongly resembles proteins that are grouped in the serpin superfamily, most members of which are known to be inhibitors of serine proteinases (Carrell et al., 1987). However, preliminary characterizations of the interleukin-1β converting enzyme suggest that this is not a serine proteinase but a cysteine proteinase, albeit one whose properties differ from those of the cysteine proteinases similar to papain (Black et al., 1989). The inhibition of a cysteine proteinase by a protein of the serpin family has not been described previously. Therefore, the nature of the interaction between these two proteins is not yet understood. The interleukin-1β converting enzyme is a highly specific endopeptidase (Sleath et al., 1990; A. D. Howard et al., 1991), as might be expected of a proteinase that controls the activation of such a potent cytokine. The *crmA* gene product appears to be highly specific also; its only known target is the interleukin-1β converting enzyme.

The failure of the CrmA protein to inhibit representative members of the major families of serine proteinases is consistent with the dissimilarity between the predicted reactive site of this protein and those of known serpin inhibitors. In general, residues to the N-terminal side of the P_1 residue in the reactive site align well between serpins, whereas several insertions are required to enable alignment of the conserved regions in the C-terminal side (Huber and Carrell, 1989). The P_1 residues of most charac-

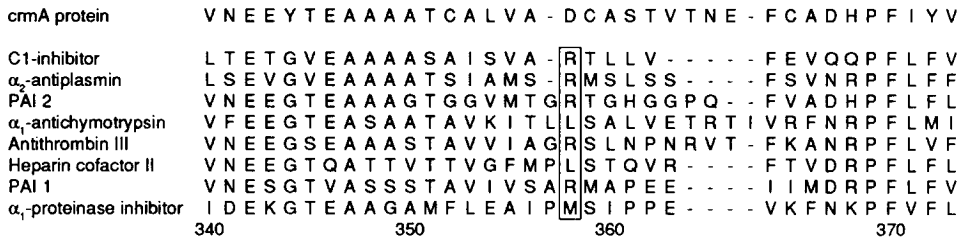

Figure 6. The Predicted Reactive Site of the CrmA Protein Is an Aspartate or a Cysteine Residue

Residues 340–373 of the human α_1–proteinase inhibitor, which contain the reactive site loop, are aligned with the corresponding regions of the CrmA protein and a number of human serpins whose reactive sites have been determined. The P_1 residues, which impart the primary specificity of each of these inhibitors, are boxed. Data are from Salvesen et al. (1985), Shieh and Travis (1987), Kiso et al. (1988), Morii and Travis (1983), Jornvall et al. (1979), Griffith et al. (1985), Andreasen et al. (1986), and Johnson and Travis (1978).

terized serine proteinase inhibitors are identical to the P_1 residues of the corresponding substrates of the proteinase. As Figure 6 shows, depending on whether the CrmA protein sequence is aligned with the sequences of serpins of the α_1–proteinase inhibitor type or C1 inhibitor type, its P_1 residue would be an aspartate or cysteine. Significantly, the P_1 residue of pro-interleukin-1β cleaved by the interleukin-1β converting enzyme is aspartate (Black et al., 1989; Kostura et al., 1989). Moreover, analysis of the substrate specificity of the interleukin-1β converting enzyme indicates that the proteinase requires the presence of aspartate in the P_1 position (Sleath et al., 1990; A. D. Howard et al., 1991). Therefore, the amino acid sequence of the predicted reactive site of the CrmA protein is consistent with its apparent ability to act as a pseudosubstrate of the interleukin-1β converting enzyme.

The importance of the interleukin-1β converting enzyme suggests that the *crmA* gene encodes the major viral modifier of the interleukin-1β response to virus infection. Genetic evidence is consistent with this interpretation; analyses of the genomes of ten different cowpox virus variants that are defective in their ability to inhibit inflammatory responses showed that each variant lacked an intact copy of the *crmA* gene (Pickup et al., 1986). Nonetheless, additional viral proteins may contribute to the modification of the interleukin-1β-mediated responses. For example, vaccinia virus (WR strain) was recently shown to contain an open reading frame with the potential to encode a protein that resembles the ligand-binding domain of the type II interleukin-1 receptor (McMahan et al., 1991; Smith and Chan, 1991). Such a viral protein may be capable of acting as an antagonist of interleukin-1. Cowpox virus appears to have a gene encoding an equivalent protein (J. E. Sims, personal communication); this gene is only 0.7 kb downstream of the *crmA* gene. Physical maps of the genomes of several spontaneously generated, whitepock variants of cowpox virus show that each virus has lost this gene in addition to the inactivation of the *crmA* gene (Pickup et al., 1984, 1986). However, the loss of this gene by the whitepock variants is probably a function of the position of the gene relative to the *crmA* gene. The deletions generating the genomes of the whitepock variants all appear to be deletions extending from one end of the viral DNA through the *crmA* gene. Therefore, selection for the loss of *crmA* would also select for the loss of genes between *crmA* and the end of the viral DNA, irrespective of their function. Furthermore, the insertion of a fragment of DNA containing the putative interleukin-1 receptor gene into the genome of a cowpox virus that lacked the *crmA* gene failed to generate a virus capable of inhibiting inflammatory processes (Pickup et al., 1986). Conceivably, one function of viral interleukin-1-binding proteins might be to augment the inhibition effected by the *crmA* gene either by binding to any interleukin-1β generated before the viral inhibition of the interleukin-1β converting enzyme, or by binding to any active interleukin-1β that might be generated adventitiously by nonspecific cleavage of the accumulated precursor form of interleukin-1β. In vitro activation of interleukin-1β by proteinases other than the interleukin-1β converting enzyme has been reported previously (Black et al., 1988; Hazuda et al., 1989).

Viral inhibition of the interleukin-1β response alone is unlikely to be sufficient to inhibit inflammatory processes effectively, because virus infection almost certainly will induce multiple mediators of inflammatory processes. This suggests that cowpox virus must encode modifiers of other responses to infection. One observation supporting this suggestion is that the vaccinia virus (WR strain), which is closely related to cowpox virus, does not inhibit inflammatory processes to the same degree as cowpox virus (Fredrickson et al., 1992). It produces white pocks on the chorioallantoic membranes of chick embryos, even though it has a gene (open reading frame B13R) that is almost identical to the cowpox virus *crmA* gene (Kotwal and Moss, 1989; Smith et al., 1989). Moreover, although the insertion of the intact cowpox virus *crmA* gene into the genome of the W2 whitepock variant of cowpox virus generated viruses producing red pocks in which inflammatory responses were inhibited (Pickup et al., 1986), similar insertion of this *crmA* gene construct into the genome of vaccinia virus generated viruses producing white pocks similar in appearance to those produced by the wild-type vaccinia virus (data not shown). This suggests that the viral inhibitor of the interleukin-1β converting enzyme is only one component of a viral system capable of inhibiting the generation or activities of multiple mediators of inflammatory responses.

Further evidence is now accumulating in support of this

model. For example, cowpox virus might be expected to modify responses mediated by tumor necrosis factor (TNF), whose proinflammatory effects are similar to those induced by IL-1 (reviewed by Dinarello, 1988). Sambhi et al. (1991) have shown that TNF can induce antiviral mechanisms to which vaccinia virus is susceptible. And recently, poxviruses of the leporipoxvirus genus were shown to encode a secreted protein that resembles the ligand-binding domain of the type I TNF receptor (Smith et al., 1991a, 1991b). Deletion of this gene from myxoma virus greatly diminished the pathogenicity of this virus, suggesting that the secreted viral protein contributes to the virulence of the virus by inhibiting TNF-mediated antiviral responses (Upton et al., 1991). Cowpox virus encodes a similar TNF-binding protein (Hu, Goodwin, Davis, D. J. P., and Smith, unpublished data). The role of this TNF-binding protein in the modification of inflammatory responses to cowpox virus is not known, but it seems probable that it will contribute to this effect. Vaccinia virus (strains WR and Copenhagen) contain two genes similar to the cowpox virus gene encoding the TNF-binding protein, but neither gene appears to be functional (Goebel et al., 1990; S. T. Howard et al., 1991). This is consistent with the proposal that cowpox virus employs cytokine response modifier genes that are lacking or inactivated in most strains of vaccinia virus. In fact, most well-characterized strains of vaccinia virus have been cultured extensively in vitro. Alternatively, they are vaccine strains that have been selected for their attenuated virulence in vivo. Either of these procedures could lead to the isolation of strains lacking functional genes encoding inhibitors of inflammatory responses. The nature and number of virus genes that are required to effect the efficient inhibition of inflammatory responses have yet to be determined.

The results of these studies suggest a model in which cowpox virus rapidly inhibits the synthesis or the release of the cytokines that are normally generated as a consequence of virus infection. The release of these cytokines would normally signal that an infection had begun. The timing of the synthesis of the viral inhibitors is consistent with this model. Viral cytokine response modifiers, such as the product of the *crmA* gene, would be needed at early times in the viral replication cycle when the cytokine responses are being generated. Although larger quantities of viral protein may be synthesized at late times during virus replication, the synthesis of inhibitors at late times may be less important, because by this time, virus-induced inhibition of host protein synthesis may be sufficient to inhibit the generation of cytokine responses. The viral modification of these responses will prevent or delay inflammatory processes, which are processes that both suppress the spread of the infection and stimulate immune defenses against the virus. For this reason, the pathogenicity of a virus may be directly related to the ability of that virus to modify cytokine responses to infection.

The identification of specific inhibitors of cytokine responses should provide valuable tools for the investigation of the contributions of various cytokines to physiological processes. Information on the mechanisms of viral inhibition of inflammatory processes should help further our understanding of the processes involved in acute and chronic inflammation, including those involved in inflammatory diseases such as rheumatoid arthritis. The cowpox virus is capable of swiftly and efficiently inhibiting inflammatory processes. This feat holds out hope that an equally effective form of anti-inflammatory therapy might be designed using the same principles.

Experimental Procedures

Viruses and Cells

The following viruses were used: cowpox virus, Brighton red strain (CPV BR); CPV BR.D1, which is CPV BR containing an insertionally inactivated *crmA* gene (Palumbo et al., 1989); vaccinia virus (VV), WR strain; vaccinia virus VTF7-3, a recombinant virus that expresses the phage T7 RNA polymerase (Fuerst et al., 1986); and VV A479, a recombinant vaccinia virus, containing a gene encoding a GST–CrmA fusion protein. Viruses were cultured either in human 143 cells grown as monolayer cultures in minimal essential medium containing 5% fetal calf serum or in the chorioallantoic membranes of 11-day-old chick embryos as described (Pickup et al., 1984).

VV A479 contains the GST–thrombin cleavage-site linker–*crmA* gene fusion under the control of the phage T7 promoter element, constructed using the insertion vector pTM1 (Moss et al., 1990) as follows. EcoRI linkers were added to the ends of a repaired 1.1 kb NcoI–HaeIII DNA fragment containing the coding region of the *crmA* gene (Pickup et al., 1986). This fragment was inserted into the EcoRI site in the pTM1 vector, generating plasmid p1431. Standard polymerase chain reaction methods were used to generate a DNA copy (flanked by NcoI–BspHI cleavage sites) of the region of the pGEX-2T vector (Smith and Johnson, 1988) encoding the C-terminal region of GST fused to a thrombin-cleavable peptide sequence. This fragment was cleaved at the NcoI and BspHI sites and ligated between the two NcoI sites in p1431 (the site downstream of the region encoding the EMCV RNA leader sequence that promotes cap-independent translation of mRNAs and the repaired NcoI site containing the initiation codon of the *crmA* gene). This generated plasmid p1441, which contains an in-frame fusion of the coding regions of GST, the thrombin cleavage site, and the *crmA* gene. The composition of this construction was verified by nucleotide sequence analysis (Tabor and Richardson, 1990). Standard methods were used to insert this construction into the genome of vaccinia virus (Mackett et al., 1984).

Preparation of Cell Extracts

Monolayers of human 143 cells (3.5×10^6 cells) were infected with virus at a multiplicity of 5. Cells were harvested after 4 hr of incubation at 37°C, washed three times with phosphate-buffered saline, then resuspended in 0.3 ml of buffer containing 20 mM Tris–HCl (pH 8.0), 50 mM NaCl, 1 mM $MgCl_2$. The cells were disrupted by three cycles of freezing and thawing. The suspension was clarified by centrifugation (16,000 × g for 15 min at 4°C in an Eppendorf microfuge). The extracts were desalted as described (Black et al., 1989).

Assay for the Inhibition of Interleukin-1β Converting Enzyme

The cell extracts or protein preparations were assayed for their ability to inhibit the human interleukin-1β converting enzyme as previously described (Black et al., 1989). For this purpose, interleukin-1β converting enzyme was purified about 1000-fold from cultured human cells (THP-1 cell line), using DEAE–Sephacel, hydroxyapatite, blue agarose, and gel filtration chromatography (S. R. K., A. L. Mumma, T. A. G., P. J. Glackin, K. P. Van Ness, C. J. March, and R. A. B., unpublished data). Human pro-interleukin-1β was prepared as previously described (Black et al., 1988). Samples (10 μl) to be tested for inhibitory activity were incubated with about 0.5 ng of interleukin-1β converting enzyme in 10 μl of 10 mM Tris–HCl buffer (pH 8.0) containing 5 mM dithiothreitol and 25% glycerol, for 25 min at 37°C. Then 50 ng of pro-interleukin-1β was added to each reaction, and the incubation was continued for 90 min at 37°C. The pro-interleukin-1β and its derivatives were resolved by polyacrylamide gel electrophoresis (14% gels), transferred to nitrocellulose, and detected using a monoclonal antibody (MAb 16F5) specific for the C-terminal sequence of the mature interleukin-1β, as previously described (Black et al., 1988).

Purification of the CrmA Protein

Human 143 cells (2×10^8 cells) were infected with both VTF7-3 and A479, each at a multiplicity of 5. The cells were harvested 20 hr after infection and resuspended in 2 ml of lysis buffer (20 mM Tris–HCl [pH 8.0], 10 mM NaCl, 1 mM $MgCl_2$). The suspension was frozen, thawed, and centrifuged (10 min at 16,000 × g in an Eppendorf microfuge), and the pellet was extracted with two additional 2 ml aliquots of lysis buffer. This procedure gave a final volume of 6 ml of soluble cell extract, from which residual particulate matter was removed by centrifugation (15,000 × g for 30 min at 4°C, in a Sorvall HB4 rotor). This supernatant was applied to a 1 ml column of glutathione–Sepharose resin (Pharmacia) that had been equilibrated with lysis buffer. The resin was washed with 10 ml of phosphate-buffered saline. The fusion protein was eluted by the addition of 5 ml of elution buffer (20 mM Tris–HCl [pH 8.0], 10 mM NaCl, 5 mM glutathione). This solution was concentrated to a final volume of 0.5 ml, by centrifugation (3,000 × g for 30 min at 4°C, in a Sorvall SS34 rotor) in a centricon 30 microconcentrator (Amicon). The CrmA moiety was cleaved from the fusion protein by digestion with trypsin (bovine pancreas, TPCK-treated, type XIII trypsin obtained from Sigma) at a final concentration of 10 μg/ml (2 hr at 37°C). The trypsin was inactivated by the addition of aprotinin (Trasylol from Boehringer Mannheim) to 30 μg/ml. The solution was applied to a 1 ml DEAE–Sephacel column (Pharmacia) equilibrated with 10 mM Tris–HCl (pH 8.0), 0.1 mM EDTA (buffer A). The column was washed with 5 ml of buffer A and then with 5 ml of buffer A containing 0.1 M NaCl. The CrmA protein was eluted with 3 ml of buffer A containing 0.5 M NaCl and then applied to 1 ml of glutathione–Sepharose resin that had been equilibrated with buffer A containing 0.5 M NaCl. The flowthrough fractions, which contained the CrmA protein moiety, were collected, and then desalted by chromatography through Bio-Rad 10DG columns. Proteins were characterized by polyacrylamide gel electrophoresis as described previously (Patel et al., 1988). Protein concentrations were measured by use of the BCA protein assay (Pierce).

Amino Acid Sequence and Compositional Analysis

Automated Edman degradation was carried out in an Applied Biosystems 477A sequencer with on-line analysis of the phenylthiohydantoins using Applied Biosystems 120A high performance liquid chromatography equipment. Protein samples destined for sequence analysis were desalted into water on a Sephadex G-25 fast desalting column (Pharmacia). Samples were dried in a speed vac (Savant), dissolved in 0.1% trifluoroacetic acid and applied to protein sample-support disks (Porton) employing the modified cycles PI-BGN and PI-1 recommended by Porton Instruments.

Amino acid compositional analysis was carried out in a Beckman 6300 amino acid analyzer. A sample of desalted CrmA protein was dried, redissolved in 6 N HCl, and hydrolyzed for 24 hr at 110°C in vacuo. All residue weights were calculated by integration, with the exception of Pro, Cys, and Trp. The weight of the protein sample was calculated as the sum of the weights of each residue.

Proteinases and Proteinase Substrates

Bovine trypsin, bovine chymotrypsin, pig pancreatic elastase, and papain were obtained from Sigma; human urinary plasminogen activator (u-PA) was from Calbiochem. Human neutrophil elastase and cathepsin G were gifts from James Travis, University of Georgia. Human thrombin was a gift from John Fenton, New York State Department of Health. Human tissue plasminogen activator (t-PA) was a gift from Henry Burger, Burroughs Wellcome, and human plasmin was a gift from Sharon Stack, Duke University. Proteinase substrates were obtained from Sigma or Helena Laboratories, Beaumont, TX.

Proteinase Assays

Papain was dissolved at 1 mg/ml in 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, and activated for 15 min at 23°C in the presence of 5 mM DTT. It was diluted to 1 μg/ml in 50 mM Tris–HCl (pH 8.0), 100 mM NaCl before use. Proteinase activities were assayed by their abilities to hydrolyze the following substrates: trypsin and papain, H-D-Pro-Phe-Arg-*p*-nitroanilide; chymotrypsin and cathepsin G, succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide; pig and human elastases, methoxy-succinyl-Ala-Ala-Pro-Val-*p*-nitroanilide; thrombin, H-D-Phe-pip-Arg-*p*-nitroanilide; plasmin, Val-Leu-Lys-*p*-nitroanilide; t-PA and u-PA, Ile-Pro-Arg-*p*-nitroanilide. Each proteinase (10–200 ng) was mixed with 1–10 μg of CrmA protein in a total volume of 180 μl of 50 mM Tris–HCl (pH 8.0), 100 mM NaCl, containing 0.01% NP-40. Samples were allowed to incubate for 30 min at 37°C in wells of a microtiter plate. Appropriate substrate was added (0.3 mM substrate in a final volume of 200 μl), and residual proteinase activity was measured at 405 nm using a Thermomax plate reader (Molecular Devices) operating in the kinetic mode.

Cleavage of a Peptide Substrate by the IL-1β Converting Enzyme

Synthesis, purification, characterization, and proteolytic cleavage of the synthetic peptide substrate acetyl-Ala-Tyr-Val-His-Asp-Ala-Pro-Val-Arg-Ser-NH_2, which corresponds to Ala-112 to Ser-121 of human pro-interleukin-1β, were done as previously described (Sleath et al., 1990).

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