CrmA, a Poxvirus-encoded Serpin, Inhibits Cytotoxic T-lymphocyte-mediated Apoptosis*

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From the Department of Pathology and ‡‡Established Investigator of the American Heart Association. To allow virally infected cells to combat immune surveillance.

Cytoxic T-lymphocytes (CTLs), by virtue of their ability to recognize and induce apoptotic death of virus-infected cells, comprise a major antiviral defense mechanism. The induction of apoptosis by CTLs can be completely accounted for by two mechanisms: (i) a Ca²⁺-dependent component that involves the exocytotic release of serine proteases known as granzymes from CTL granules and their subsequent insertion into the target cell to induce apoptosis and (ii) a Ca²⁺-independent component that involves the activation of Fas, a receptor on the target cell membrane that triggers apoptosis. Although viruses have evolved several indirect mechanisms for evading the CTL response, direct inhibition of the apoptotic cascade has never been described. We now show for the first time that the cowpox virus protein CrmA, a protease inhibitor of the serpin family, is capable of inhibiting CTL-mediated cytolysis. The inhibitory effect is largely the result of blockade of the Ca²⁺-independent (i.e. Fas-mediated) component of CTL killing. CrmA thus represents the first example of a viral gene product capable of directly blocking CTL-mediated cell death.

Cytotoxic T-lymphocytes (CTLs)1 represent a major antiviral mechanism, as they kill virally infected cells by inducing apoptosis (1). Recent studies have demonstrated that killing by CTLs is completely accounted for by two mechanisms: (i) the insertion of serine proteases known as granzymes, and in particular the Asp-specific protease Granzyme B, into the target cell (2, 3), which potently activates the cell’s endogenous death pathway and (ii) activation of the Fas antigen, an apoptosis-inducing receptor on the target cell surface (2–7). The granzyme pathway requires extracellular Ca²⁺, as it involves the exocytotic release of granzymes, along with the pore-forming protein perforin, from granules in the CTL (reviewed in Ref. 8). The Fas pathway, on the other hand, is Ca²⁺-independent (7), requiring only the interaction between Fas ligand expressed on the CTL surface and Fas present on the target cell membrane. Experimental verification of the existence of these two pathways comes from analysis of mice carrying germline inactivation of the Granzyme B gene (9), from knockouts of perforin (2, 3) (which is necessary for the insertion of granzymes into the target cells), and from mice carrying inactivating mutations in either the Fas ligand or Fas genes (2, 3).

Many viruses have evolved indirect mechanisms to evade the host CTL response to infection (10, 11). These include altering expression of cell surface major histocompatibility proteins and altering the expression of specific viral antigens to avoid CTL recognition (10). Until now, however, no example of a viral evasive mechanism that blocks CTL killing by directly interfering with the apoptotic cascade has been described (10). CrmA is a cowpox virus gene product that was originally identified as a serpin that inhibits the interleukin-1β-converting enzyme (ICE) (12, 13), which proteolytically processes pro-interleukin-1β to yield mature, active interleukin-1β (14, 15). ICE, like Granzyme B, is an Asp-specific protease (14, 15), and CrmA, as a member of the serpin family of protease inhibitors, inhibits ICE by forming an active site-directed complex (12, 13). With the discovery that ICE and ICE-like proteases may function in apoptosis (16–22) it seemed plausible that CrmA might have anti-apoptotic activity in addition to its ability to inhibit the processing of interleukin-1β. This was confirmed in a variety of model systems, including nerve growth factor withdrawal-23, serum withdrawal-18, tumor necrosis factor-, and Fas-induced apoptosis (21, 24, 25).

In particular, the finding that CrmA can block Fas-induced apoptosis (21) suggested to us that it might function as an inhibitor of CTL-mediated killing, since Fas is one of the two effector pathways utilized in this process. Furthermore, a recent report demonstrated that, at least in vitro, Granzyme B, an essential component of the Ca²⁺-dependent cytolysis pathway, is inhibited by CrmA (26), suggesting that CrmA may be capable of attenuating both CTL killing pathways. We directly tested this hypothesis in a CTL-mediated cytotoxicity assay using target cells stably transfected either with a vector control, with a CrmA expression construct, or with an inactive point mutant CrmA expression construct. We show here that CrmA functions to inhibit CTL killing and that it is a more potent inhibitor of the Ca²⁺-independent (i.e. Fas-based) mechanism than of the Ca²⁺-dependent (i.e. granzyme-based) component of CTL killing. These studies identify CrmA as the first example of a viral gene product that directly blocks the lethal apoptotic cascade induced by CTLs and suggest that CrmA may function to allow virally infected cells to combat immune surveillance.

EXPERIMENTAL PROCEDURES

Cell Culture—BJAB cells stably transfected with either vector, CrmA, or CrmA mutant expression constructs (21, 27) were maintained in RPMI 1640 medium supplemented with 10% heat-inacti-
vated fetal bovine serum (Hydene Laboratories), l-glutamine, penicillin/streptomycin, nonessential amino acids, and 3 mg/ml G418 sulfate (Life Technologies, Inc.).

Isolation of Human Peripheral Blood Mononuclear Cells (PBMCs)—20–50 ml of heparinized human whole blood was obtained from healthy donors and erythrocyte-depleted over Ficoll-Hypaque gradients. The resultinPBMCs were stimulated with PHA-P at 10 μg/ml for 3 days at 37 °C in RPMI supplemented with 10% fetal bovine serum prior to use in cytology and DNA fragmentation assays. CD4+ and CD8+ lymphocytes constituted approximately 40 and 30%, respectively, of the small lymphocyte pool in stimulated PBMCs as determined by flow cytometry. Subset depletion experiments confirmed that CTL activity at both 4- and 24-h time points was mediated almost entirely by CD8+ T-lymphocytes (data not shown). For measurement of natural killer (NK) cell activity, human PBMCs were used immediately after isolation without additional stimulation.

Cytotoxicity Assays—PHA-facilitated allogeneic CTL assays using PHA-stimulated human PBMCs were carried out as described previously (28) with minor modifications. Briefly, following PBMC stimulation, 2 × 10^5 BJ AB target cells were incubated with 200 μCi of (Na)_2CrO_4 (ICN) in a 400-μl total volume of Hank's balanced salt solution, 0.2% bovine serum albumin for 2 h at 37 °C. Target cells were then washed and plated in round-bottom 96-well plates at 10,000 cells/well. Human PBMCs were plated at various killer to target ratios, ranging from 0.25:1 to 32:1, and PHA-P was added to a final concentration of 10 μg/ml and a total volume of 200 μl. In some experiments, EGTA and MgCl_2 were added at 10 and 4 mm, respectively, to damp intracellular calcium levels. At 4 and 24 h time points the plates were centrifuged, and 30-μl aliquots were harvested and spotted onto glass fiber filters. Samples were analyzed using a β scintillation counter. Specific cytotoxicity (%) was calculated as [(sample cpm − spontaneous release cpm)/(total release cpm − spontaneous release cpm)] × 100. Background release of chromium was typically 5−15% at the 4-h time point and 20−30% at the 24-h time point. The addition of EGTA/MgCl_2 did not by itself affect cell viability as measured by both background chromium release and morphologic examination of cells at both time points. There was no significant difference in background 51Cr release between vector and CrmA-transfected lines (data not shown).

NK cell cytotoxicity assays were carried out as described above except that the cells were not stimulated and there was no PHA present during the assay.

DNA Fragmentation Assay—Assay of CTL-induced target cell DNA fragmentation was carried out as described previously (29) with modifications. Human PBMCs were prepared as described above, 1 × 10^5 BJ AB cells were incubated with [methyl-3H]thymidine (2 μCi/ml) in 10 ml of RPMI with 10% fetal bovine serum for 24 h. Cells were then transferred to radiolabel-free RPMI/fetal bovine serum and incubated for an additional 24 h to deplete cytoplasmic [methyl-3H]thymidine pools. Cells were then plated as described above. After 4 h, 100 μl of 95% ethanol was added to each well, the contents were mixed, and the plates were then incubated for an additional hour. Ethanol addition caused the release of fragmented DNA while high molecular weight chromatin remained in the cells. The plates were then centrifuged, harvested, and analyzed as described above.

RESULTS

To determine whether CrmA can function to inhibit the lethal cascade induced upon interaction of the CTL with its target, we utilized BJ AB cells (a Fas-expressing human B-cell line) stably transfected with either vector control, CrmA, or inactive point mutant CrmA expression constructs (21, 27) as target cells in a 24-h PHA-facilitated allogeneic CTL-mediated cytosis assay based on 51Cr release as described under “Experimental Procedures.” B, clonal lines of BJ AB cells stably transfected with either a vector control (clone V1 (■) and CrmA 3 (□)), or mutant CrmA (clones mutant CrmA 12 (A) and mutant CrmA 17 (C)) expression constructs were similarly loaded with 51Cr and analyzed in the 24-h CTL-mediated cytosis assay. Each of the data points shown in both A and B repre¬

![Fig. 1. Wild-type CrmA, but not mutant CrmA, inhibits CTL-mediated cytosis.](http://www.jbc.org/)

When examined for susceptibility to CTL-induced cytolysis, the parental BJ AB cell line was killed effectively (data not shown), as were vector transfected cells (Fig. 1A, clones V1 and V4), in a dose-dependent fashion over a range of killer:target cell ratios. Lines expressing CrmA, however, were significantly protected from CTL-mediated killing (Fig. 1A, clones CrmA 2 and CrmA 3). When spontaneous background release of 51Cr from BJ AB lines incubated for 24 h without the addition of CTLs was assessed, there was no significant difference between vector and CrmA lines (data not shown). Thus, the difference in susceptibility to killing between vector and CrmA-expressing lines in the CTL-mediated cytosis assays is not simply due to a decreased intrinsic propensity of the CrmA lines to undergo spontaneous lysis.

To determine whether the ability of CrmA to inhibit tumor necrosis factor- and Fas-induced cell death (21), the ability of CrmA to inhibit tumor necrosis factor- and Fas-induced cell death (21),

Because killing by CTLs is the result of activation of both...
The importance of CTL-mediated killing in the host-virus interaction is suggested by the diversity of viral mechanisms that have evolved to combat T cell-mediated lysis. Prior studies of viral anti-CTL defenses have identified mechanisms of evasion of recognition by CTLs (reviewed in Ref. 10) by such means as down-regulation of host surface molecules required for CTL recognition by CTLs (reviewed in Ref. 10) by such means as down-regulation of host surface molecules required for CTL recognition by CTLs (reviewed in Ref. 10) by such means as down-regulation of host surface molecules required for CTL recognition by CTLs (reviewed in Ref. 10) by such means as down-regulation of host surface molecules required for CTL recognition by CTLs (reviewed in Ref. 10).
Fig. 4. CrmA blocks CTL-mediated DNA fragmentation. BJAB cells stably transfected with either vector (clones V1 (●) and V4 (○)) or CrmA (clones CrmA 2 (□) and CrmA 3 (□)) expression constructs were labeled with [methyl-^3H]thymidine and DNA fragmentation induced by a 4-h incubation with CTLs as described under “Experimental Procedures.” Each data point represents the mean of samples run in triplicate, and the standard deviations were always less than 5% of the mean. 

Our findings suggest that the Ca\(^{2+}\)-independent component of the CTL mechanism is targeted by CrmA. These data are consistent with the previous finding that Fas-induced apoptotic signals triggered by agonist monoclonal antibodies is blocked by expression of CrmA (27). The lack of blockade of the granzyme-based pathway is of importance, since Granzyme B is inhibited by CrmA in vitro (26) and since, based on gene knockout studies in mice, Granzyme B has been shown to play a nonredundant role in CTL killing (9). The inability of CrmA to protect from Ca\(^{2+}\)-independent killing might be explained by the finding that CrmA is by 2 orders of magnitude a poorer inhibitor of Granzyme B than it is of ICE (26). Thus, it is possible that higher levels of CrmA expression than those achieved in the present studies may be needed to detect effects on Granzyme B-mediated apoptosis. Since ICE and ICE-like molecules appear to play a role in Fas-mediated killing, this may explain the greater propensity of CrmA to inhibit the Ca\(^{2+}\)-independent component of the apoptotic mechanism. The fact that CrmA does block Ca\(^{2+}\)-independent CTL killing, however, raises the possibility that the Fas-based pathway may play a greater role in eliminating virus-infected cells than has been previously appreciated, since cowpox virus appears to have evolved a mechanism to combat it.

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