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

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Cytotoxic 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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}, 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 Ca^{2+}-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 Ca^{2+}-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 Ca^{2+}-independent (*i.e.* Fas-based) mechanism than of the Ca^{2+}-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-

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[1] The abbreviations used are: CTL, cytotoxic T-lymphocyte; ICE, interleukin-1β-converting enzyme; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; NK, natural killer.

vated fetal bovine serum (Hyclone 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 resulting PBMCs 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 cytolysis 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, 2×10^7 BJAB target cells were incubated with 200 μCi of $(Na)_2{}^{51}CrO_4$ (ICN) in a 400-μl total volume of Hanks' 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 clamp 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. Backgound 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 ^{51}Cr 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. 1×10^7 BJAB cells were incubated with [*methyl*-^{3}H]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*-^{3}H]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 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 BJAB 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 cytolysis assay based on ^{51}Cr release from target cells. We chose to examine ^{51}Cr release after 24 h because we anticipated that both Ca^{2+}-dependent and Ca^{2+}-independent cytotoxicity would occur at this time point. Two vector-transfected clones (V1 and V4), two CrmA-transfected clones (CrmA 2 and CrmA 3), and two mutant CrmA-expressing clones (mutant CrmA 12 and mutant CrmA 17) were selected that have been previously described and characterized (21, 27). Mutant CrmA carries a Thr → Arg point mutation at codon 291, a critical site in the reactive site loop of CrmA. This mutation abolishes protease-inhibitory capacity without significantly altering tertiary structure, as measured by transverse urea gradient gel electrophoresis (21). This mutation has also been shown to abolish

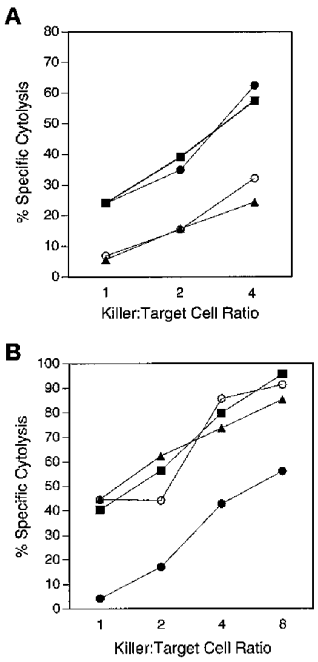

FIG. 1. **Wild-type CrmA, but not mutant CrmA, inhibits CTL-mediated cytolysis.** *A*, clonal lines of BJAB cells stably transfected with either a vector control (clones V1 (■) and V4 (●)) or CrmA expression construct (clones CrmA 2 (▲) and CrmA 3 (○)) were used as target cells in a 24-h PHA-facilitated allogeneic CTL-mediated cytolysis assay based on ^{51}Cr release as described under "Experimental Procedures." *B*, clonal lines of BJAB cells stably transfected with either a vector control (clone V1 (■)), CrmA (clone CrmA 2 (●)), or mutant CrmA (clones mutant CrmA 12 (▲) and mutant CrmA 17 (○)) expression constructs were similarly loaded with ^{51}Cr and analyzed in the 24-h CTL-mediated cytolysis assay. Each of the data points shown in both *A* and *B* represents the mean of samples run in triplicate, and the standard deviations were always less than 5% of the mean. Each experiment was independently repeated at least three times with similar results. Although absolute values of chromium release cannot be directly compared between experiments due to variation arising from differences in blood donors, variation in the degree of protection by CrmA relative to vector or mutant CrmA was less than 5%. 100% chromium release corresponded to values ranging between 4000 and 12,000 cpm, depending upon the individual experiment.

the ability of CrmA to inhibit tumor necrosis factor- and Fas-induced cell death (21).

When examined for susceptibility to CTL-mediated cytolysis, the parental BJAB cell line was killed effectively (data not shown), as were vector transfected cells (Fig. 1*A*, 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. 1*A*, clones CrmA 2 and CrmA 3). When spontaneous background release of ^{51}Cr from BJAB 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 cytolysis 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 CTL-mediated cytolysis required its protease-inhibitory capacity, we examined cell lines that expressed comparable levels of the point mutant of CrmA, that, as mentioned above, lacks protease-inhibitory activity (21). When tested in the same cytotoxicity assay, both lines expressing mutant CrmA were as sensitive to CTL-mediated cytolysis as a vector transfectant, whereas wild-type CrmA was clearly protective (Fig. 1*B*).

Because killing by CTLs is the result of activation of both

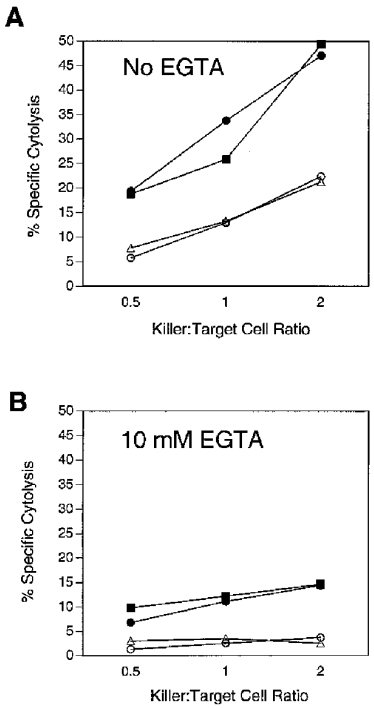

FIG. 2. **CrmA completely blocks the Ca^{2+}-independent component of CTL-mediated killing.** *A*, BJAB cells stably transfected with either vector (clones V1 (■) and V4 (●)) or CrmA (clones CrmA 2 (△) and CrmA 3 (○)) expression constructs were analyzed in the 24-h CTL-mediated cytolysis assay in the absence of EGTA as described under "Experimental Procedures." *B*, the same cell lines were analyzed in the 24-h CTL-mediated cytolysis assay in the presence of 10 mM EGTA and supplemented with 4 mM Mg^{2+}, as described under "Experimental Procedures." Each of the data points shown in both *A* and *B* represents the mean of samples run in triplicate, and the standard deviations were always less than 5% of the mean. Each experiment was independently repeated at least three times with similar results. Although absolute values of chromium release cannot be compared between experiments due to variation arising from differences in blood donors, variation in the degree of protection by CrmA relative to vector was less than 5%. 100% chromium release corresponded to values ranging between 4000 and 12,000 cpm, depending upon the individual experiment.

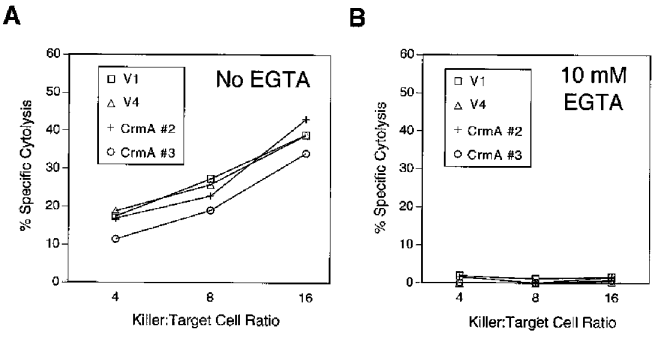

FIG. 3. **CrmA does not block the Ca^{2+}-dependent component of CTL-mediated cytolysis.** *A*, BJAB cells stably transfected with either vector (clones V1 and V4) or CrmA (clones CrmA 2 and CrmA 3) expression constructs were analyzed in a 4-h CTL-mediated cytolysis assay in the absence of EGTA as described under "Experimental Procedures." *B*, the same cell lines were analyzed in a 4-h CTL-mediated cytolysis assay except in the presence of 10 mM EGTA and supplemented with 4 mM Mg^{2+}, as described under "Experimental Procedures." Each of the data points shown in both *A* and *B* represents the mean of samples run in triplicate, and the standard deviations were always less than 5% of the mean. Each experiment was independently repeated at least three times with similar results. Although absolute values of chromium release cannot be compared between experiments due to variation arising from differences in blood donors, variation in the degree of protection by CrmA relative to vector was less than 5%. 100% chromium release corresponded to values ranging between 4000 and 12,000 cpm, depending upon the individual experiment.

granzyme and Fas pathways, it was of interest to determine whether one or both of these was modulated by CrmA, since previous studies indicated that CrmA could potentially inhibit both pathways (26, 27). To investigate this, we took advantage of the Ca^{2+} dependence of granzyme-mediated killing and the Ca^{2+} independence of the Fas pathway. The calcium chelator, EGTA, can be used to block granule exocytosis (30, 31), since calcium is necessary for granzyme release from the CTL. Thus, one can selectively assess Ca^{2+}-independent killing. In the absence of EGTA, CrmA was protective compared with vector controls, but some residual cytotoxicity was present (Fig. 2*A*). In the presence of EGTA, however, CrmA was totally protective (Fig. 2*B*), indicating that CrmA can completely block the Ca^{2+}-independent component of CTL killing. These data suggest that the residual killing observed in the CrmA-expressing lines in the absence of EGTA (Fig. 2*A*) was exclusively due to the Ca^{2+}-dependent granzyme pathway and that at least under the experimental conditions employed, CrmA was ineffective at blocking this pathway.

Studies of Granzyme B-knockout mice (9) have suggested that ^{51}Cr release in the first 4 h after CTL-target interaction is likely to involve the granzyme-based pathway only. We therefore carried out a series of experiments using a 4-h cytolysis assay in place of the 24-h assay employed for the experiments described above. In this 4-h assay there were no significant differences in CTL sensitivity of vector-transfected and CrmA-transfected target cells (Fig. 3*A*). Furthermore, EGTA abolished all cytotoxicity in the 4-h assay (Fig. 3*B*), confirming that the cytolysis at this time point was entirely due to the Ca^{2+}-dependent mechanism and indicating that CrmA therefore does not afford protection from the Ca^{2+}-dependent pathway. We also found that CrmA did not confer any protection from death induced by NK cells (data not shown). This result is consistent with the hypothesis that CrmA does not block the Ca^{2+}-dependent component of cytotoxicity, since NK-mediated cytolysis appears to be entirely accounted for by the degranulation pathway (32).

Whereas the 4-h ^{51}Cr release assay measured only Ca^{2+}-dependent cytolysis, we suspected that assessing DNA fragmentation might be a more sensitive measure of *total* apoptosis at this early time point, as it precedes membrane lysis in the case of Fas-mediated apoptosis. When both vector control and CrmA-transfected lines were examined for induction of DNA fragmentation by CTLs, CrmA-expressing lines showed significantly less DNA fragmentation (Fig. 4), consistent with protection from early DNA fragmentation induced by Fas ligation.

DISCUSSION

Many animal cells undergo apoptosis when exposed to viruses, a response likely to have evolved as a means to halt the production of new viral particles. Not surprisingly, then, the ability to inhibit apoptosis is a recurring theme in viral pathogenesis. Viruses are under tremendous evolutionary pressure to evolve mechanisms that will inhibit apoptotic death of the infected host cell, allowing them time to replicate. The Epstein-Barr (33) and African swine fever viruses (34), for example, encode genes that resemble *bcl-2*, a mammalian gene capable of inhibiting apoptosis. Interestingly, while these genes may offer protection from a multitude of apoptosis-inducing insults, they have not been shown to protect from CTL killing.

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

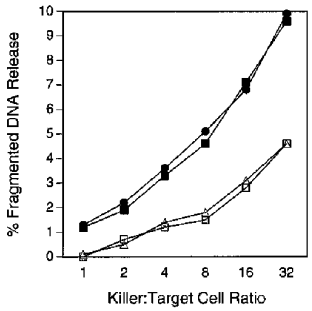

FIG. 4. **CrmA blocks CTL-mediated DNA fragmentation.** BJAB cells stably transfected with either vector (clones V1 (■) and V4 (●)) or CrmA (clones CrmA 2 (△) and CrmA 3 (□)) expression constructs were labeled with [*methyl*-^{3}H]thymidine and DNA fragmentation induced by a 4-h incubation with CTLs 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. Two independent experiments yielded similar results, and the degree of protection by CrmA relative to vector varied by less than 5% between experiments. 100% DNA fragmentation corresponded to absolute values between 400 and 1000 cpm depending upon the individual experiment.

recognition or modification of viral antigens. CrmA represents the first example of a viral protein that blocks CTL-mediated killing by a direct effect on the lytic pathway. Koup (10) has previously suggested, on theoretical grounds, the existence of a class of viral proteins that inhibit the CTL-initiated lethal apoptotic cascade. We now suggest that CrmA may function in such a capacity and thus may be the first member of such a class of viral proteins.

CrmA is a gene product encoded by cowpox virus and although it is not required for *in vitro* replication, it facilitates high titer viral replication *in vivo* (35). It was previously thought that CrmA achieved this by inhibiting ICE, the enzyme responsible for the conversion of interleukin-1β from its inactive precursor to the active mature molecule (12). Such an inhibition would dampen the host inflammatory response to the viral infection. While this may still indeed be the case, the fact that CrmA partially inhibits CTL killing supports the contention that CrmA might allow the virus more time to replicate by attenuating the CTL-based host defense mechanism.

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 apoptosis 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+}-dependent 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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