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Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Inhibit T Cell-Mediated Cytotoxicity by Inhibiting Fas Ligand Expression

Mario Delgado* and Doina Ganea**

We reported recently that the neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) protect CD4+ T cells against Ag-induced apoptosis by down-regulating the expression of Fas ligand (FasL). Because the cytotoxic activity of CD8+ CTLs is mediated through two mechanisms, which involve the perforin/granzyme and the FasL/Fas pathways, in this study we investigated the effects of VIP/PACAP on the generation and activity of allogeneic CTLs, of CD8+ T1 and T2 effector cells and of alloreactive peritoneal exudate cytotoxic T cells (PEL) generated in vivo. VIP/PACAP did not affect perforin/granzyme-mediated cytotoxicity, perforin gene expression, or granzyme B enzymatic activity, but drastically inhibited FasL/Fas-mediated cytotoxicity against allogeneic or syngeneic Fas-bearing targets. VIP/PACAP inhibit CTL generation, but not the activity of competent CTLs. The inhibition is associated with a profound down-regulation of FasL expression, and these effects are mediated through both VPAC1 and VPAC2 receptors. VIP/PACAP inhibit the FasL/Fas-mediated cytotoxicity of T1 effectors and do not affect T2 cytotoxicity, which is entirely perforin/granzyme mediated. Similar effects were observed in vivo. Both the FasL/Fas-mediated cytotoxicity and FasL expression of cytotoxic allogeneic PELs generated in vivo in the presence of VIP or PACAP were significantly reduced. We conclude that, similar to their effect on CD4+ T cells, the two structurally related neuropeptides inhibit FasL expression in CD8+ cytotoxic T cells and the subsequent lysis of Fas-bearing target cells. The Journal of Immunology, 2000, 165: 114–123.

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splenocytes (1–5 × 10^7) were cultured with 5 × 10^6 irradiated (3000 rad) B6 or BALB/c splenocytes, respectively, in the presence of 10 U/ml IL-2. Live cells harvested over Ficoll (density, 1.077) were cultured (1 × 10^7 cells/well) with periodic (7-day intervals) stimulation with irradiated B6 or BALB/c spleen cells and T cell growth factor (10% Con A rat spleenocyte supernatant). Effector cells for cytotoxicity assays and for mRNA and flow cytometry analysis were harvested on day 5 of the stimulation cycle. VIP and PACAP (10^-8 M) were added simultaneously with CTLDNA generation and with each periodical restimulation. In most experiments CTLD were stimulated before the assay (see below).

T1 and T2 CTL cell lines were generated as previously described (19). B6 CD8<sup>+</sup> cells (>97% CD8<sup>+</sup>) were purified by passage through nylon wool, followed by complement-mediated lysis of CD4<sup>+</sup> and B cells. The purified B6 CD8<sup>+</sup> T cells (1 × 10<sup>6</sup>/ml) were incubated for 5 days with T cell-depleted BALB/c splenocytes (3 × 10<sup>6</sup>/ml) to generate T1, the cells were cultured in the presence of IL-2 (20 U/ml), IL-12 (20 U/ml), and anti-IFN-γ (2 μg/ml) for 5 days; for T2, CD8<sup>+</sup> T cells were cultured in the presence of IL-2 (20 U/ml), IL-4 (40 U/ml), and anti-IFN-γ (80 μg/ml). The cultures were supplemented on day 3 with IL-2 (20 U/ml) and IL-4 (40 U/ml), respectively. T1 and T2 were harvested on day 5 and characterized through cytokine profiles (IFN-γ and IL-5) following restimulation with T cell-depleted BALB/c splenocytes (3 × 10<sup>6</sup>/ml). T1 cells produce high amounts of IFN-γ and no IL-5, whereas T2 cells secrete variable, but small, amounts of IFN-γ and high levels of IL-5 (20).

**Stimulation of CTL**

CTL (5 × 10<sup>6</sup> cells/well) were activated in 96-well plates with immobi-lized anti-CD3 mAbs (2C11, 5 μg/ml) or with 10 ng/ml PMA and 3 μg/ml ionomycin in the presence or the absence of different concentrations of VIP or PACAP and cultured for 6 h at 37°C to allow FasL expression.

**Preparation and culture of peritoneal exudate lymphocytes (PEL)**

PEL were generated as previously described (21). Briefly, C57BL/6 and BALB/c mice were injected i.p. with allogeneic tumor cells L1210-Fas<sup>+</sup> (H-2<sup>d</sup>) or EL4 (H-2<sup>d</sup>; 25 × 10<sup>6</sup> cells/mouse) with or without VIP or PACAP (5 nmol/mouse). Eight to 10 days after the primary allogenein-stimulation or 4–5 days after the secondary stimulation (which was administered 6–9 wk after priming) the mice were sacrificed, and the peritoneal exudate cells were harvested in PBS/0.5% FCS. The crude PEC were depleted of adherent cells on nylon wool. After 1 h, the nonadherent cells were eluted by rinsing the columns with cold PBS/0.5% FCS. The eluted cells (PEL) contained >95% T cells, 80–90% of which were CD8<sup>+</sup>, with about 50% of the PEL forming specific conjugates as described by Berke et al. (21).

**Cytotoxicity assay**

Cytotoxicity was assessed by 51Cr release (18). Activated CTL, PEL, and T1/T2 cells were added to 96-well V-bottom microtiter plate to obtain various E:T cell ratios. The target cells (P815, EL4, L1210-wt, and L1210-Fas<sup>+</sup> cells, 1 × 10<sup>6</sup>/well) were labeled for 2 h at 37°C with 200 μCi of sodium [51Cr]chromate (Amersham, Arlington Heights, IL), and were added at a concentration of 1 × 10<sup>7</sup> cells/well. The plates were centrifuged at 300 × g to promote conjugate formation and were incubated for 6 h at 37°C, followed by the removal of 100-μl supernatant aliquots for measurement in a gamma 8000 counter (Beckman, Fullerton, CA). The percent stimulation with T cell-depleted BALB/c splenocytes (3 × 10<sup>6</sup>/ml) was determined by Western blotting as described by Makrigiannis and Hoskin (26).

**Analysis of functional FasL expression**

The function of FasL, expressed on anti-CD3 stimulated CTL was assessed by the ability of these cells to cause DNA fragmentation in Fas<sup>+</sup> target cells as previously described (24). Briefly, BALB/c anti-H2<sup>b</sup> alloreactive CTL were activated with plate-bound anti-CD3 mAbs for 3 h to allow FasL expression in the presence or the absence of different concentrations of VIP or PACAP. The cells were washed twice and incubated for 8 h with [3H]Tdr-labeling buffer (L1210 target cells or Fas<sup>+</sup> L1210-Fas<sup>+</sup> cells containing 2 × 10<sup>6</sup> cells/well), L1210 and L1210-Fas<sup>+</sup> cells were labeled for 2 h with 5 μCi/ml [3H]Tdr (70 Ci/mmol: Amersham) at 37°C in RPMI/5% FCS. [3H]Tdr labeled unfragmented high m. DNA was harvested on glass filters and counted in a liquid scintillation counter. DNA fragmentation was calculated as follows: % DNA fragmentation = 100 × (cpm control group – cpm experimental group)/cpm control group ± SD.

**Detection of granzyme B activity and perforin protein expression**

Cytosolic proteins were obtained from 5 × 10<sup>6</sup> CTL by treatment with 200 μl of ice-cold lysis buffer (1% Nonidet P-40, 5 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml peptatin, and 10 μg/ml aprotinin) for 30 min. Cell lysates were cleared by centrifugation at 14,000 × g for 6 min, and the supernatants were stored at −20°C. The granzyme B activity was measured by a colorimetric enzyme assay as previously described (25) using the granzyme B-specific synthetic substrate Boc-Ala-Asp thiobenzyl ester. Absorbance of 0.01 at 405 nm was arbitrarily defined as 1 U of esterolytic activity. Detection of perforin in cytosolic proteins was determined by Western blotting as described by Makrigiannis and Hoskin (26).

**Results**

VIP and PACAP inhibit FasL-mediated CTL cytotoxicity

Alloreactive CTL lyse target cells through both perforin/granzyme-dependent and FasL/Fas-dependent mechanisms. Because the perforin/granzyme pathway requires Ca<sup>2+</sup>-dependent degranulation, the only lytic mechanism operating in the presence of FasL...
**FIGURE 1.** VIP and PACAP inhibit the FasL/Fas-mediated cytotoxicity of allogeneic CTL. BALB/c anti-H-2^b (A) and B6 anti-H-2^d (B) allogeneic CTL (5 × 10^4 cells) generated in 5-day MLC as described in Materials and Methods in the absence (control) or the presence of VIP or PACAP (10^-8 M) were activated by incubation in flat-bottom 96-well plates coated with anti-CD3 Ab (5 μg/ml) in the presence or the absence of VIP or PACAP (10^-8 M). After 6-h activation 51Cr-labeled target cells (EL.4, L1210-wt, L1210-Fas^+, or P815) were added at various E:T cell ratios, and the cultures were incubated in the presence or the absence of 4 mM EGTA/3 mM MgCl2 (+EGTA). After 12 h supernatants were harvested to measure 51Cr release. Cytotoxicity was expressed as the mean of triplicate samples, between 18 and 29%. The results were calculated as the mean of triplicate samples.

EGTA is the FasL/Fas-mediated lysis. We generated allogeneic BALB/c anti-H-2^b or B6 anti-H-2^d effector CTL in the presence or the absence of VIP or PACAP, activated them with immobilized anti-CD3 Abs in the presence or the absence of either neuropeptide, and determined the cytotoxicity against syngeneic and allogeneic targets. The anti-CD3-activated BALB/c anti-H-2^b CTL lysed the allogeneic EL.4 cells (H-2^d; Fig. 1A, upper panel), but failed to lyse the syngeneic L1210-wt (H-2^b) or P815 cells (H-2^d; Fig. 1A, lower panels). However, L1210 cells transfected with Fas cDNA (L1210-Fas^+) were efficiently lysed (Fig. 1A, middle panels), in agreement with previous reports that lysis of syngeneic targets is entirely FasL/Fas mediated (18). VIP and PACAP had a poor inhibitory effect in the absence of EGTA, but almost completely inhibited the Ca^{2+}-independent lysis of EL.4 cells (Fig. 1A, upper panels). The two neuropeptides dramatically inhibited the lysis of L1210-Fas^+ cells (Fig. 1A, middle panels). Conversely, B6 anti-H-2^d CTL effectively lysed the allogeneic L1210-wt, L1210-Fas^+, and P815 targets (Fig. 1B, left panels), but not the syngeneic EL.4 targets (Fig. 1B, lower/right panel). In addition, whereas L1210-Fas^+ cells were efficiently killed in the presence of EGTA, L1210-wt and P815 cells, which express very low levels of Fas, required Ca^{2+} for lysis (Fig. 1B). Again, VIP and PACAP showed little, if any, inhibitory effect on the cytotoxicity against L1210-wt and P815, although they decreased the lysis of L1210-Fas^+ cells (Fig. 1B). In addition, both neuropeptides inhibited completely the Ca^{2+}-independent cytotoxicity against L1210-Fas^+ targets (Fig. 1B).

VIP and PACAP inhibit the cytotoxic activity of BALB/c anti-H-2^b CTLs stimulated with anti-CD3 Abs or with a combination of PMA and ionomycin against L1210-Fas^+ targets (Fig. 2). The inhibition is dose-dependent, with maximum effects at 10 nM VIP/PACAP (Fig. 2).

These results suggest that VIP and PACAP inhibit the CTL Ca^{2+}-independent, FasL/Fas-mediated lysis of syngeneic and allogeneic target cells, but do not affect the Ca^{2+}-dependent, perforin/granzyme-mediated cytotoxicity.

The failure of VIP and PACAP to inhibit perforin/granzyme-mediated lysis correlates with their lack of effect on granzyme B activity or perforin expression. In contrast to rapamycin, a known inhibitor of perforin/granzyme-mediated cytotoxicity (26), VIP and PACAP did not affect granzyme B activity or perforin expression in anti-CD3-stimulated CTL (Fig. 3).

Because FasL/Fas-mediated cytotoxicity leads to target cell apoptosis (27), we investigated whether VIP and PACAP inhibit DNA fragmentation in target cells. BALB/c anti-H-2^b effector CTLs were generated and activated by CD3 cross-linking in the presence and the absence of VIP/PACAP. The effector cells were incubated with [3H]TdR-labeled L1210-Fas^+ target cells in the presence of EGTA, and the percentage of DNA fragmentation was determined as a measure of apoptosis. TCR-stimulated CTL induced DNA fragmentation in L1210-Fas^+ cells, but not in L1210-wt cells (Fig. 4A), and VIP/PACAP inhibited DNA fragmentation in a dose-dependent manner (Fig. 4B).

VIP and PACAP affect the generation and/or activation of CTL

VIP and PACAP could affect the generation and/or stimulation of effector CTLs or simply the Fas signaling pathway in target cells. To distinguish between these possibilities, we measured the Ca^{2+}-independent cytolysis of L1210-Fas^+ by BALB/c anti-H-2^b CTLs generated and activated in the absence or the presence of VIP or PACAP. Unstimulated effector CTL exhibit poor cytotoxicity, even toward L1210-Fas^+ cells (Fig. 5A). However, if effector CTL are first activated with immobilized anti-CD3 Abs or with PMA/ionomycin, the cytotoxicity against L1210-Fas^+ target cells is greatly increased (Fig. 5, B–F). Addition of VIP or PACAP during CTL generation (Fig. 5B) or during CTL activation (Fig. 5E) dramatically inhibited lysis, and addition of neuropeptides during both CTL generation and stimulation resulted in a higher inhibitory effect (Fig. 5, C and D). However, addition of VIP or PACAP during the effector phase (the cytotoxicity assay) failed to inhibit cytolytic activity (Fig. 5F). These results suggest that VIP and PACAP affect one or more events during the generation and stimulation of
effector CTL, but not the signaling pathway of the Fas-mediated cytotoxic process.

VIP and PACAP inhibit CTL FasL expression

To determine whether the inhibition of CTL FasL/Fas-mediated cytotoxicity correlates with neuropeptide inhibition of FasL expression, BALB/c anti-H-2b and B6 anti-H-2d effector CTL generated and stimulated with or without VIP/PACAP were analyzed for FasL expression by flow cytometry and Northern blot analysis. Whereas unstimulated CTL express low levels of both protein and mRNA FasL, TCR stimulation through CD3 cross-linking results in a great increase in FasL expression (Fig. 6). Treatment with VIP or PACAP resulted in a significantly reduced expression of FasL expression at both protein and mRNA levels (Fig. 6). The inhibitory effect on FasL expression was dose dependent and specific, because neither VIP nor PACAP affected CD8 expression (Fig. 6). To address the question of whether VIP/PACAP affect already expressed FasL, we stimulated CTL with anti-CD3 and added VIP or PACAP at different times after stimulation. Additions at 0 and 30 min reduced both FasL expression and cytotoxicity, but additions at later times (1, 2, and 4 h) did not have any effect (Fig. 7).

Involvement of VPAC1 and VPAC2 in the inhibitory effect of VIP and PACAP on FasL/Fas-mediated cytotoxicity and FasL expression in CTL

VIP and PACAP act through a family of receptors; VPAC1 and VPAC2 exhibit similar affinities for the two neuropeptides and activate primarily the adenylate cyclase system, whereas PAC1 exhibits a 300- to 1000-fold higher affinity for PACAP and activates both adenylate cyclase and phospholipase C (reviewed in Ref. 28). Although naive CD4+ and CD8+ T cells express both VPAC1 and VPAC2 following anti-CD3 stimulation (29, 30), the expression of VIP/PACAP receptors in effector CTL has not been studied. We investigated first the expression of VPAC1, VPAC2, and PAC1 mRNA by RT-PCR in allogeneic BALB/c anti-H-2b

FIGURE 2. Dose-response effect for the inhibition of cytotoxicity by VIP and PACAP. BALB/c anti-H2b alloreactive CTL were generated in 5-day MLC as described in Materials and Methods in the absence (control) or the presence of different concentrations of VIP or PACAP. CTL (5 × 10^4 cells) were activated by incubation in flat-bottom 96-well plates coated with anti-CD3 Ab (5 μg/ml) or by treatment with 10 ng/ml PMA plus 5 μg/ml ionomycin in the presence or the absence of various concentrations of VIP or PACAP. After 6-h activation, ^51^Cr-labeled target cells (L1210-wt or L1210-Fas−) were added at a 10:1 E:T cell ratio, followed by incubation in the presence of 4 mM EGTA/3 mM MgCl2 (EGTA). After 12 h, supernatants were harvested to measure ^51^Cr release. Cytotoxicity was expressed as specific ^51^Cr release after subtraction of spontaneous ^51^Cr release, which ranged between 11 and 23%. The results were calculated as the mean of triplicate samples.

FIGURE 3. VIP and PACAP do not affect granzyme B activity or perforin expression. BALB/c anti-H2b alloreactive CTL generated in 5-day MLC in the absence (control) or the presence of VIP or PACAP (10^-8 M) were activated with anti-CD3-coated plates (5 μg/ml) in the presence or the absence of VIP or PACAP (10^-8 M) or rapamycin (1 ng/ml). A. Following 48 h of culture, cytosolic lysates were obtained from equal numbers of CTL and added to a colorimetric reaction mixture containing synthetic granzyme B substrate as described in Materials and Methods. Data from a representative experiment (n = 3) are expressed as the mean units of esterolytic activity ± SD. B. Alternatively, after 48-h culture, perforin expression in cytosolic lysates was analyzed by Western blotting. Data are representative of two independent experiments.

FIGURE 4. VIP and PACAP inhibit CTL-induced apoptosis in Fas-bearing target cells. BALB/c anti-H2b alloreactive CTL (5 × 10^4 cells) generated in 5-day MLC in the absence (medium) or the presence of VIP or PACAP (10^-8 M) were activated on plates coated with anti-CD3 mAbs (Fig. 6) and added VIP or PACAP at different concentrations of VIP or PACAP (B) and 10^-8 M (A). Cells were cultured for 3 h to allow FasL expression, harvested, washed twice, and incubated for an additional 8 h with [^3H]Tdr-labeled L1210 (wt) or L1210-Fas− target cells at different E:T cell ratios in A and at a 10:1 E:T cell ratio in B. The percentage of DNA fragmentation was calculated as described in Materials and Methods. Each result is the mean ± SD of three independent experiments performed in duplicate.
CTL. VPAC1- and VPAC2-specific, but not PAC1-specific, fragments were amplified (Fig. 8A). Next, we investigated the effects of VPAC1-, VPAC2-, and PAC1-specific agonists (31–33) on the induction of cytotoxic activity of BALB/c anti-H-2b effector CTL for L1210-Fas<sup>1</sup> cells. Both VPAC1 and VPAC2 agonists, but not the PAC1 agonist, inhibited cytotoxic activity, with a potency similar to that of VIP/PACAP (Fig. 8B). A similar pattern was observed for the effect of VIP/PACAP agonists on FasL expression in CTL (Fig. 8C). These results suggest that the two neuropeptides exert their actions primarily through VPAC1 and VPAC2.

VIP and PACAP inhibit the FasL/Fas-mediated cytotoxicity of alloreactive T<sup>1</sup> CD8<sup>+</sup> T cell effectors

Polarized populations of cytokine-secreting CD8<sup>+</sup> effector T cells (T1 and T2) can be generated in vitro (34–36). CD8<sup>+</sup> T cells from B6 mice were stimulated with BALB/c APCs in the presence of IL-2, IL-12, and anti-IL-4 for T1 polarization or IL-2, IL-4, and anti-IFN-γ for T2 polarization. After 5 days, the H-2<sup>b</sup>-anti-H-2<sup>d</sup> CD8<sup>+</sup> effectors were restimulated and assayed for their ability to secrete representative type 1 (IFN-γ) and type 2 (IL-5) cytokines. Both T1 and T2 populations were >92% CD8<sup>+</sup>, with T1 populations secreting large amounts of IFN-γ and nondetectable amounts of IL-5, and T2 populations secreting substantial amounts of IL-5 and reduced levels of IFN-γ (Fig. 9A). T1 and T2 effectors were assayed for their cytotoxic activity using L1210-wt and L1210-Fas<sup>+</sup> targets in the presence or the absence of EGTA. T1 effectors killed both targets, whereas cytotoxicity against L1210-wt was entirely Ca<sup>2+</sup>-dependent, substantial residual Ca<sup>2+</sup>-independent killing was observed for the L1210-Fas<sup>+</sup> targets (Fig. 9B). As previously described (19), the T2 population displayed no significant Ca<sup>2+</sup>-independent cytotoxicity (Fig. 9B, right panels). Addition of VIP or PACAP during T1 generation resulted in a slight decrease in the lysis of L1210-Fas<sup>+</sup> cells in the absence of EGTA and a complete inhibition of Ca<sup>2+</sup>-independent lysis (Fig. 8B). In contrast, the neuropeptides did not affect the lysis of L1210-wt cells (Fig. 9B). Also, VIP and PACAP failed to inhibit the cytotoxic activity of allogeneic T2 effectors (Fig. 9B). Therefore, VIP and PACAP inhibit only the FasL/Fas-mediated Ca<sup>2+</sup>-independent cytotoxic activity of alloreactive T1 effectors.

To investigate whether the inhibitory effect of VIP/PACAP correlates with reduced FasL expression in T1 effectors, T1 cells were generated in the presence or the absence of VIP or PACAP, and FasL expression was assayed by flow cytometry. Both neuropeptides significantly decreased the expression of FasL protein in alloreactive T1 effectors (Fig. 9C).

VIP and PACAP inhibit the Fas-mediated cytotoxicity of alloreactive PEL

To investigate the in vivo effects of VIP and PACAP on FasL/Fas-mediated cytotoxicity, we used the murine model developed by Berke et al. (21, 37) for in vivo-generated peritoneal exudate cytotoxic CD8<sup>+</sup> T cells (PEL). BALB/c and B6 mice were injected...
with EL-4 and L1210-Fas<sup>+</sup> cells, with or without VIP or PACAP (5 nmol/mouse). Peritoneal exudate cells depleted of adherent cells, consisting of >95% T cells (80–90% CD8<sup>+</sup>), were tested for cytolytic activity against syngeneic and allogeneic targets. The BALB/c anti-EL.4 PEL exhibited potent, specific cytotoxicity toward EL-4 cells, with both Ca<sup>2+</sup>-dependent and -independent components. PEL obtained from mice injected with VIP or PACAP exhibited slightly reduced cytolytic activity toward EL-4 targets in the absence of EGTA, but showed significantly reduced Ca<sup>2+</sup>-independent lysis (Fig. 10A, upper panels). Although BALB/c anti-H-2<sup>d</sup> PEL did not kill syngeneic targets with low Fas expression (L1210-wt and P815; Fig. 10A, middle panels), they lysed L1210-Fas<sup>+</sup> cells through a Ca<sup>2+</sup>-independent mechanism (Fig. 10A, lower panels). PEL obtained from mice injected with VIP or PACAP had a reduced lytic activity toward L1210-Fas<sup>+</sup> targets (Fig. 10A, lower panels).

On the other hand, B6 anti-H-2<sup>d</sup> PEL specifically lysed allogeneic L1210-wt, P815, and L1210-Fas<sup>+</sup> targets, but not syngeneic EL.4 PEL (Fig. 10B). Whereas in vivo administration of VIP and PACAP did not reduce PEL cytotoxicity against low Fas-expressing L1210-wt and P815 cells (Fig. 10B, middle and lower panels), it significantly inhibited lysis of L1210-Fas<sup>+</sup> cells (Fig. 10B, upper panels). Therefore, similar to the in vitro results, the in vivo administration of VIP and PACAP inhibits the Ca<sup>2+</sup>-independent, FasL/Fas-mediated cytotoxicity of effector PEL.

Next we investigated whether the inhibitory effect of VIP and PACAP on PEL cytotoxicity correlates with a reduced FasL expression. BALB/c anti-EL.4 and B6 anti-L1210-Fas<sup>+</sup> effector PEL generated in vivo in the presence or the absence of VIP and PACAP were analyzed for FasL expression by flow cytometry and Northern blots. The freshly isolated PEL, at peak lytic ability following immunization, express high levels of both FasL protein and mRNA without restimulation with cognate target cells. Although...
specific Cr release after subtraction of spontaneous Cr release, which ranged between 16 and 21%. These results were calculated as the mean of three experiments performed in duplicate. B. VIP and PACAP inhibit the Fas-mediated cytotoxicity of T1 cells. CD8+ T1 (left panels) and T2 (right panels) effectors from C57BL/6 mice were generated in the absence (medium) or the presence of VIP or PACAP (10^{-8} M) and assayed for cytolytic activity in a 12-h assay with 51Cr-labeled target cells (L1210-wt or L1210-Fas+). Supernatants were harvested to measure Cr release. Cytotoxicity was expressed as specific Cr release after subtraction of spontaneous Cr release, which ranged between 16 and 21%. These results were calculated as the mean of triplicate samples. C. VIP and PACAP inhibit FasL expression in CD8+ effectors. BALB/c anti-H2b alloreactive CTL (5 x 10^5 cells) generated in 5-day MLC as described in Materials and Methods. BALB/c anti-H2b alloreactive CTL (5 x 10^5 cells) generated in 5-day MLC as described in Materials and Methods. In the absence (medium) of the presence of VPAC1, VPAC2, or PAC1 agonists (10^{-7} M) were activated by incubation in flat-bottom 96-well plates coated with anti-CD3 Abs (5 μg/ml) in the absence (medium) or the presence of 10^{-7} M VPAC1, VPAC2, or PAC1 agonists. After 3-h incubation, 51Cr-labeled target cells (L1210-wt or L1210-Fas+) were added at different E:T cell ratios in the absence or the presence of 4 mM EGTA/3 mM MgCl_2 (+EGTA). After 12 h, supernatants were harvested to measure Cr release. Cytotoxicity was expressed as specific Cr release after subtraction of spontaneous Cr release, which ranged between 9 and 17%. The data are representative of three experiments. C. VIP and PACAP inhibit FasL expression in CD8+ T1 effectors. T1 cells were generated in the absence (medium) or the presence of VIP or PACAP (10^{-8} M). FasL expression was analyzed by flow cytometry. Each result is the mean ± SD of three separated experiments performed in duplicate.

Discussion

Several studies suggested that the FasL/Fas-mediated pathway represents the basis for the Ca\(^{2+}\)-independent CTL lysis reported previously (4, 5, 38–40). Once initiated, the FasL/Fas-mediated lysis is every bit as vigorous as lysis via the degranulation pathway, provided, of course, that the target cell is Fas\(^+\) (41). The FasL/Fas-mediated cytolytic activity is up-regulated in CTL effectors and clones through direct restimulation through the TCR complex (4, 41–43). Such stimulation results in an increase in CTL cytolytic activity and is accompanied by an increase in de novo FasL synthesis and surface expression. The requirement for new protein synthesis explains why the onset of the Ca\(^{2+}\)-independent lysis is delayed with respect to Ca\(^{2+}\)-dependent (degranulation) lysis in cloned CTL (44). In agreement with the reported FasL up-regulation following direct TCR restimulation, our study shows that anti-CD3-activated CTL lyse Fas\(^+\) targets, and that this ability is paralleled by an increase in FasL expression. In addition, the killing of Fas\(^+\) cells by activated CTL is blocked by the anti-Fas mAb Jo2 (results not shown). The expression of FasL in CTLs varies with activation and differentiation stage. The fact that naive and memory CTLs do not express FasL whereas cytotoxic effectors do (45) supports the idea that FasL expression occurs following specific CTL-target cell interaction and TCR triggering (2). This highly controlled FasL expression could explain the target cell specificity for FasL/Fas-mediated CTL cytotoxicity.

In the present study we demonstrate that whereas VIP and PACAP do not affect the Ca\(^{2+}\)-dependent, perforin/granzyme-mediated CTL cytotoxicity, both neuropeptides significantly decrease the Ca\(^{2+}\)-independent, FasL/Fas-mediated killing of Fas\(^+\) target...
cells. VIP/PACAP affect various CTL effectors, including in vitro-generated primary alloreactive CTL and T1 CD8\(^+\) effectors and in vivo-generated PEL effectors.

The inhibitory effect of both neuropeptides appears to be mediated by preventing FasL gene expression. Indeed, VIP and PACAP inhibit both the anti-CD3- and PMA/ionomycin-stimulated cytotoxic activity and FasL expression in primary alloreactive CTLs. The inhibition occurs if the neuropeptides are added during CTL generation and/or stimulation. In contrast, the addition of VIP/PACAP during the effector phase or 1 h after restimulation with anti-CD3 does not affect the cytotoxic activity. This is reminiscent of the lack of effect of VIP/PACAP on the apoptosis of Fas-bearing targets induced by soluble FasL (17) and supports the idea that VIP/PACAP interfere with FasL expression but not with Fas signaling. We reached similar conclusions from the effects of VIP/PACAP on cytolytic activity and FasL expression in the CD8\(^+\) cytotoxic subsets T1/T2. Although both subsets are cytotoxic and exhibit perforin/granzyme-mediated cytotoxicity, only the T1 subset uses the FasL/Fas-mediated pathway (19). VIP/PACAP inhibit the cytotoxic activity of T1, but not T2 cells, and this inhibition correlates with a decrease in T1 FasL expression.

Although the in vivo PEL effectors were reported to express functional FasL without apparent Ag stimulation and in the presence of transcriptional and translational inhibitors (42), our data indicate that administration of VIP/PACAP during the in vivo generation of PEL effectors results in the inhibition of both FasL expression and cytotoxicity against Fas\(^+\) target cells; in contrast, treatment of PEL with either neuropeptide after their isolation from the peritoneal cavity has no effect on FasL expression or cytotoxicity (results not shown). Taken together, these results suggest that VIP and PACAP act both in vivo and in vitro as modulators of the FasL/Fas-mediated cytotoxicity by inhibiting FasL expression in CD8\(^+\) T lymphocytes and T cell hybridomas (17).

Both CD4\(^+\) and CD8\(^+\) naive T cells stimulated in vitro with anti-CD3 mAbs were shown to express VPAC1 and VPAC2, but not PAC1, mRNA (29, 30). Here we report that CTL generated in
response to alloreactive cells express both VPAC1 and VPAC2, but not PAC1, mRNA. The studies presented here are not quantitative. It remains to be established whether VPAC1/VPAC2 levels change during the inductive and effector phases of the cytotoxic response. Our agonist studies suggest that both VPAC1 and VPAC2 mediate the inhibitory effect on FasL-mediated cytotoxicity and expression in CTL. We reported similar results for the inhibition of FasL expression and subsequent clonal deletion of activated CD4+ T cells (17).

Cytokines such as IL-2, RANTES, and IFN-α were shown to up-regulate FasL expression in T cells (46–49). Although it is not known whether VIP/PACAP affect RANTES or IFN-α, the two neuropeptides inhibit IL-2 production (reviewed in Ref. 13). Therefore, the effect of VIP/PACAP on FasL expression might be mediated at least partially through a reduction in IL-2. However, this seems improbable, because we generated and stimulated the alloreactive CTLs in the presence of high levels of exogenous IL-2. However, the involvement of IL-2 in the regulation of FasL expression by VIP/PACAP in vivo cannot be excluded.

Because VIP and PACAP specifically inhibit Fasl/Fas-mediated cytotoxicity without affecting the perforin/granzyme pathway, the physiological relevance of this immunoregulatory process depends on the function of the two cytotoxic pathways. During an allogeneic response, perforin/granzyme lysis represents the major mechanism for the destruction of specific allografts (50). In contrast, Fasl/Fas-mediated lysis is responsible for the observed bystander cytotoxic effect against Fas-bearing syngeneic cells (46). Although several studies describe CD4+ T effector cells as the major players in Fasl/Fas-dependent lysis of syngeneic cells (8, 9, 50, 51), numerous reports, including the present study, demonstrate clearly that CD8+ primary alloreactive CTL, T1 cells, and PEL effectors, CTL clones, and hybridomas express Fasl/Fas-mediated cytotoxicity against allogeneic and syngeneic Fas+ targets (4–6, 19, 38–44, 52). This study demonstrates that VIP/PACAP inhibit CTL cytotoxicity against Fas+ syngeneic cells. In addition, preliminary experiments show that VIP/PACAP suppress the cytotoxicity against Fas+ bystanders by CD4+ T cells responding to specific Ag. Therefore, VIP and PACAP appear to be especially relevant for the control of FasL expression and the subsequent Fasl/Fas-mediated cytotoxicity against bystander syngeneic cells.

Despite the important role of PfasL/Fas-mediated cytotoxicity in immune homeostasis, unrestricted lysis of any Fas+ cell is potentially deleterious and may play a role in the pathology of some inflammatory and autoimmune diseases (reviewed in Ref. 53). Therefore, the existence of factors, such as VIP and PACAP, that control an excessive Fasl/Fas-mediated bystander cytosis by regulating FasL expression, may offer new avenues for an effective therapeutic intervention. On the other hand, it has been proposed that VIP and PACAP might favor the development and/or maintenance of a population of memory CD4+ T cells by inhibiting Ag-induced cell death through the down-regulation of FasL expression (17). Because memory CTL down-regulate FasL expression (45), the question arises whether VIP/PACAP could participate in the establishment of the memory CD8+ T cell population. Further investigation will answer this question.

Previous studies defined VIP and PACAP as endogenous factors that play an important role in the control of immune homeostasis. The VIP/PACAP control of Ag-induced cell death and of cytotoxicity against syngeneic bystander targets through the down-regulation of FasL expression adds a new dimension to their immunomodulatory properties.


