Vasoactive Intestinal Peptide (VIP) and Pituitary Adenylate Cyclase-Activation Polypeptide (PACAP) Protect Mice from Lethal Endotoxemia Through the Inhibition of TNF-α and IL-6[1]

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The neuropeptides vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) down-regulate cytokine production. Because human septic shock involves excessive cytokine production, the effect of VIP/PACAP was investigated in a high endotoxemia murine model. Both peptides protect against endotoxin-induced lethality and prevent septic shock-associated histopathological alterations. VIP/PACAP reduce serum and peritoneal TNF-α and IL-6, suggesting that the protective effect is exerted by inhibiting the production of endogenous TNF-α/IL-6. Consistent with this mechanism, VIP does not protect against septic shock induced by exogenous TNF-α. The immunomodulatory role of VIP in vivo is supported by the appearance of high levels of VIP in serum and peritoneal fluid following LPS administration. Thus, the neuropeptides VIP/PACAP protect from the lethal effect of high endotoxemia, presumably by down-regulating TNF-α and IL-6 production, and may offer an alternative in the treatment of human septic shock syndrome. ***The Journal of Immunology*****, 1999, 162: 1200–1205.**

The majority of human septic shocks, which are systemic responses to severe bacterial infections resulting in high mortality, are caused by Gram-negative bacterial endotoxins (1–3). Indeed, LPS administration in experimental animals leads to pathophysiological changes similar to the human septic shock syndrome. The toxic effects of endotoxin are exerted through the generation of endogenous proinflammatory cytokines. High levels of circulating TNF-α, IL-1, IL-6, IL-12, and IFN-γ were reported during endotoxemia (4, 5), and administration of anticytokine Abs or knock-out of TNFR (p55) or IFN-γR greatly diminished or abrogated mortality in endotoxic models (6–9). Although up-regulation of endogenous anti-inflammatory cytokines such as IL-10 and IL-1Ra also occurs in septic shock (10), the proinflammatory cytokine cycle appears to be beyond control during intense endotoxemia. However, administration of exogenous anti-inflammatory cytokines such as IL-10 and IL-13 protects against lethality in endotoxic models (11–14).

Neuropeptides with immunomodulatory properties represent another group of endogenous factors that mediate various immune responses, including cytokine production. Some neuropeptides/hormones, such as the growth hormone, somatostatin, procalcitonin, calcitonin gene related peptide (CGRP),[3] and vasoactive intestinal peptide (VIP), but not substance P, have been reported to increase in patients with septic shock or in septic shock animal models (15–22). A common trait of the neuropeptides/hormones whose levels increase in septic shock is their anti-inflammatory activity (23–28). Our long-term interest lies in the immunomodulatory role of VIP and of the structurally related neuropeptide, the pituitary adenylate cyclase-activating polypeptide (PACAP). VIP is present in various lymphoid organs (29, 30), and immune cells express specific receptors for VIP/PACAP (31–41). VIP/PACAP downregulate the production of several proinflammatory cytokines (42, 43). In addition, elevated VIP levels were reported in septic shock patients and in some endotoxic animal models (21, 22). This finding suggests that VIP, and possibly PACAP, are secreted following endotoxin stimulation to counterbalance the generation of proinflammatory cytokines, but become ineffective in conditions of excessive cytokine production. However, the administration of exogenous VIP/PACAP during septic shock might control the proinflammatory cytokine network. Here we investigate the effect of VIP/PACAP in a high-dose endotoxin murine model. To our knowledge, this is the first report of an immunomodulatory neuropeptide that exerts a protective effect in septic shock.

Materials and Methods

Experimentally induced endotoxic shock

Eight-week-old male BALB/c mice (Iffa Credo, L'Arbresle, France) were injected i.p. with different concentrations (25–600 μg) of LPS (from *Salmonella enteridis*; Sigma, St. Louis, MO), and survival was monitored over the next 4–7 days. Various doses of VIP or PACAP-38 (Novabiochem, Laufelfingen, Switzerland) were administered i.p. either concurrently with or following injection of LPS. Control animals received only medium. Anti-murine TNF-α mAb (1 mg) (PharMingen, San Diego, CA) was administered i.p. 6 h before LPS to serve as a positive control for survival. Lethal shock was induced in some animals by injecting TNF-α (50 and 25 μg) (PharMingen) i.p. All survival studies were conducted in a blind and

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[3] Abbreviations used in this paper: CGRP, calcitonin gene related peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide.

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random fashion. All endotoxin-treated mice appeared acutely ill, displaying lethargy, ruffled fur, and diarrhea. The animals that succumbed to the effect of LPS treatment were necropsied immediately, and all VIP/PACAP-treated survivors were killed for necroscopy at the end of the experimental period. Histopathological examination was performed on lung, small and large intestine, and kidney, and spleen-fixed with Bouin solution; sections were stained with haematoxylin-eosin or with Masson's haemalum and picroindigocarmine using standard techniques.

Mice receiving LPS (400 μg) concurrently with either medium or VIP/PACAP-38 (5 nmol) were sacrificed after various time points. Blood was removed through cardiac puncture, and peritoneal exudate was obtained as described previously (43). The blood samples were allowed to clot for 1 h at room temperature; serum was obtained and kept frozen until TNF-α, IL-6, and VIP ELISA analysis. The peritoneal suspension was centrifuged for 5 min at 1800 $\times$ *g*, and cell free supernatant was harvested and assayed for cytokine and VIP ELISA. The peritoneal cells were subjected to Northern blot analysis as described below.

Cytokine and neuropeptide quantitation: TNF-α, IL-6, and VIP

TNF-α and IL-6 levels present in serum and peritoneal fluid were determined using commercially available murine-specific sandwich ELISAs (PharMingen). Serum and peritoneal fluid VIP levels were measured using a competitive ELISA. The mAb CURE.V55 (44) (2.5 μg/ml; 100 μl/well in 96-well plates), kindly provided by Dr. H. C. Wong (University of California, Los Angeles, School of Medicine), was used for detection of VIP. Biotinylated VIP (Peninsula, Belmont, CA) (0.5 ng/ml) was used as a competitor. VIP (Novabiochem) was used as a standard in the concentration range of 20–2000 pg/ml. Bound biotinylated VIP was detected by using the avidin-peroxidase system (Sigma). The mAb CURE.V55 was shown to react specifically with VIP by RIA (44), and does not cross-react with secretin, glucagon, PACAP-27, PACAP-38, and the VIP_{1-12} and VIP_{10-28} fragments in the ELISA described above (45).

Quantitation of TNF-α and IL-6 mRNA

Northern blot analyses were performed according to standard methods. Total RNA was isolated from peritoneal cells using the Ultraspec RNA Isolation System (Biotecx Laboratories, Houston, TX) according to the manufacturer's instructions. Twenty micrograms of total RNA from each sample were electrophoresed on 1.2% agarose-formaldehyde gel, transferred to nylon membranes, and cross-linked using UV light. Membranes were hybridized with specific probes for TNF-α (5′-TTGACCTCAGCGCTGAGTTGGTCCCC CTTCTAGCTGGAAGACT-3′) and IL-6 (5′-CAAGAAGGCAACTGGAT GAAGTCCTCTTGCAGAGAAGGAACTTCAT-3′) that were designed from the murine TNF-α and IL-6 cDNA published sequences (46, 47). The probe for the murine 18S RNA, as a quantity control for RNA, was an oligonucleotide (5′-CCAATTACAGGGCCTCGAAAGAGTCCTCTA-3′) derived from the published sequence. Oligonucleotides were 3′-labeled with digoxigenin-dUTP/dATP mix using terminal transferase, and hybridization and detection of chemoluminiscent signal were performed using a commercially available kit (Boehringer Mannhein, Mannhein, Germany) according to the manufacturer's instructions.

Statistical analysis

All values are given as means $\pm$ SD. Survival curves were analyzed by the Kaplan-Meier method, and the log rank test was generated to test the homogeneity between treatment groups. Serum and peritoneal cytokines and VIP levels in different experimental groups were analyzed for statistical significance using the nonparametric Wilcoxon rank sum test. A value of $p < 0.05$ was considered to represent a significant difference.

Results and Discussion

VIP/PACAP protect from LPS-induced lethality

BALB/c mice were injected simultaneously with a lethal dose of LPS and VIP or PACAP-38, and survival was monitored. Both VIP and PACAP-38 protect against the lethal effect of LPS as analyzed by the Kaplan-Meier method, and the protective effect is similar to that of anti-TNF-α Abs used as control (Fig. 1*A*). The effect of VIP/PACAP was dose-dependent, with doses as low as 1 nmol being partially protective (Fig. 1*B*). The protective effect of VIP occurred over a large range of LPS concentrations, and VIP shifted the LD_{50} from 100 to 327 μg LPS (Fig. 1*C*). Animals injected with VIP had a survival rate of ~60%. However, even for the nonsurvivors, VIP almost doubled the time until death (Fig. 1*C*, *inset*). The production of proinflammatory cytokines occurs in a rapid sequence starting with TNF-α, which reaches a maximum at 2 h after LPS administration (11, 14). Kinetic studies established that VIP/PACAP exert a full protective effect when given up to 30 min after LPS, with no protection for VIP/PACAP administration 4 h after shock induction (Fig. 1*D*). Since elevated VIP levels correlate with endotoxic shock in humans, we determined the VIP concentration in the peritoneal fluid and serum of endotoxic mice. The VIP levels in the peritoneum and serum increased 10- and 6-fold, respectively, at 8–12 h following shock induction (LPS 400 μg); much lower, but still statistically significant increases were observed in mice injected with a non lethal dose of LPS (25 μg) (Fig. 1*E*). These results suggest that VIP production and/or release occurs in response to LPS stimulation, possibly mediated by cytokines. For example, an augmented LPS-induced release of CGRP from tracheal nerves has been shown to be mediated through IL-1β and TNF-α (48). In addition, since the VIP gene promoter contains a cytokine-responsive element (49), cytokines produced following LPS stimulation could induce VIP gene expression in vivo.

VIP/PACAP prevent the histopathological alterations associated with endotoxic shock

The histopathological alterations associated with endotoxic shock include disseminated intravascular coagulation, leukocyte infiltration and inflammation in various organs, mesenteric ischemia, and acute tubular necrosis in the kidneys. Postmortem examinations were conducted in LPS-injected mice treated with and without VIP/PACAP. Control animals (LPS) suffered from generalized intravascular coagulation with multiple organ failure as evidenced by severe congestion, hemorrhage, hyperemia, fibrin deposits, edema, thrombosis, and massive accumulation of leukocytes in lungs and the intestinal tract (Fig. 2, *a* and *d*). We also observed severe congestion of the medullar sinusoids in the spleen, and segmental ischemia of the bowel with regions of hemorrhage or necrosis, and an infarcted caecum. No pathological changes were observed in the surviving animals (injected with LPS plus VIP) (Fig. 2, *b* and *e*), which presented a similar histology to mice injected with medium alone (Fig. 2, *c* and *f*). Similar results were obtained for the animals treated with PACAP-38 (results not shown).

VIP/PACAP inhibit endogenous TNF-α and IL-6 production

Among the proinflammatory cytokines involved in endotoxic shock, TNF-α appears to play a central role. When macrophages are stimulated with LPS, they secrete high quantities of TNF-α (50). In vivo, although no circulating TNF-α can be detected in healthy individuals, significant concentrations of TNF-α appear in conditions of septic shock (4). In support of the central role of TNF-α, injection of purified recombinant human TNF-α in rats induced shock, tissue damage, and death (51). During endotoxic shock, TNF-α precedes proinflammatory cytokines such as IL-6 and IFN-γ, and anti-TNF-α Abs reduce both IFN-γ and IL-6 levels in endotoxic animal models (52, 53). This finding suggests that IL-6 and IFN-γ are located downstream from TNF-α in the cytokine cascade involved in septic shock, and that their production is dependent on TNF-α. However, this conclusion is still debatable, at least for IL-6. Recently, a specific inhibitor for TNF-α processing was shown to reduce TNF-α, but not IL-6, levels following lethal endotoxin challenge (54). Also, in humans with septic shock, IL-6 appears to be a better predictor for survival, since IL-6, but not TNF-α, shows significantly higher plasma levels in the nonsurvivor group (55).

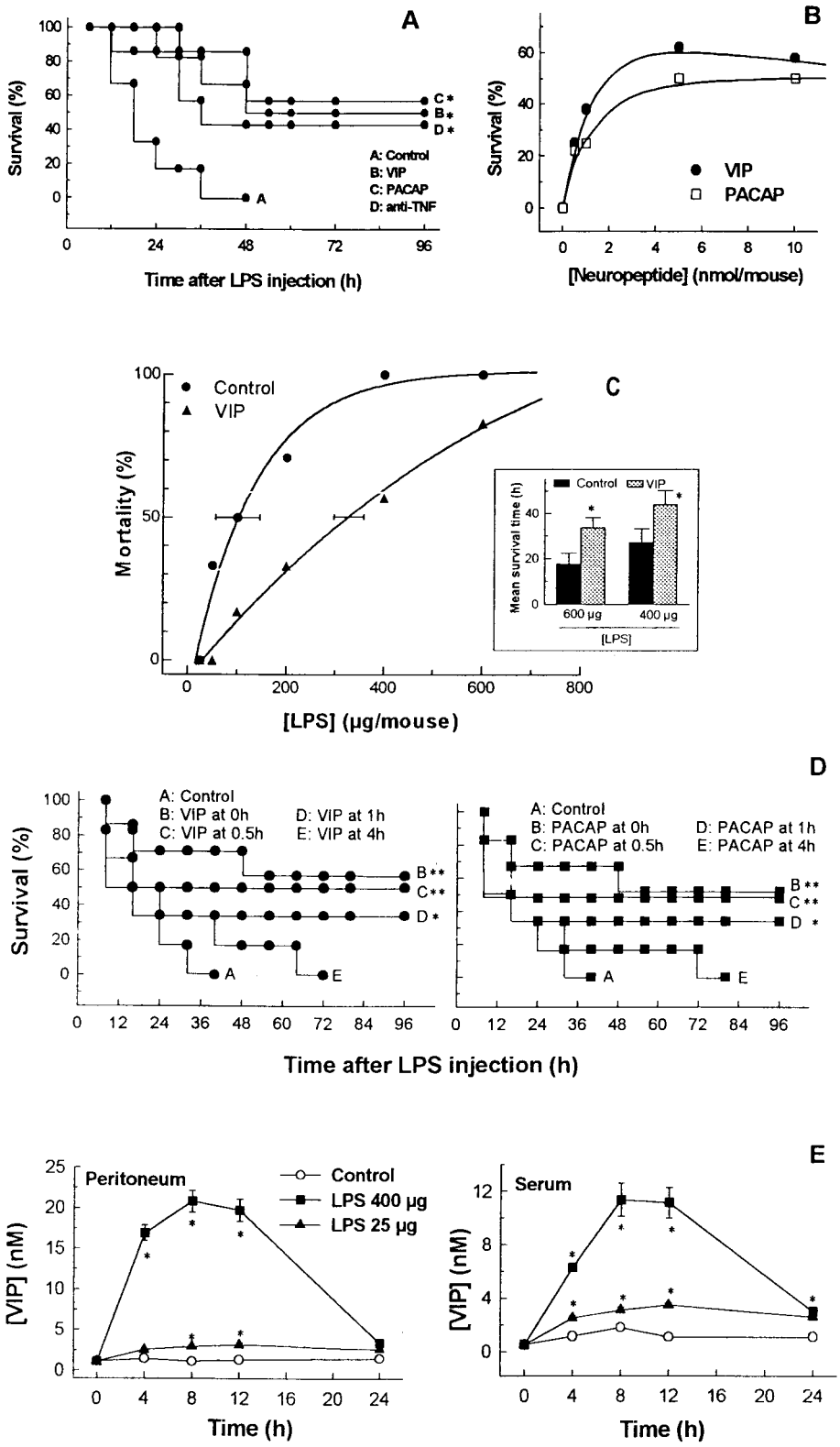

FIGURE 1. *A–D*, VIP and PACAP-38 protect mice from LPS-induced lethality. *A*, BALB/c mice (12 per group) were injected i.p. with 400 μg LPS (control), LPS and VIP or PACAP-38 (5 nmol), or with anti-TNF-α Abs (1 mg) followed by LPS 6 h later. Survival was monitored over the next 96 h, and the survival curves were analyzed by the Kaplan-Meier method. Similar results were obtained in three identical independent experiments. The log-rank test was used for homogeneity between treatment groups (*, $p < 0.001$). *B*, BALB/c mice (six per group) were injected i.p. with 400 μg LPS and various concentrations of VIP or PACAP-38 (from 0 to 10 nmol/animal). Survival was monitored over a 96-h period. Similar results were obtained in four identical independent experiments. *C*, BALB/c mice (8–12 per group) were injected i.p. with various concentrations of LPS in the absence or presence of 5 nmol VIP. Survival curves were used to calculate LD_{50}. Horizontal bars indicate the 95% confidence limits of LD50 determinations. *Inset*, At the end of 96 h, the average survival time was calculated for nonsurvivors in both the LPS (600 and 400 μg/animal) and LPS plus VIP (5 nmol/animal) treated groups. Similar results were obtained in three identical independent experiments. *, $p < 0.001$ with respect to control mice without VIP treatment as determined

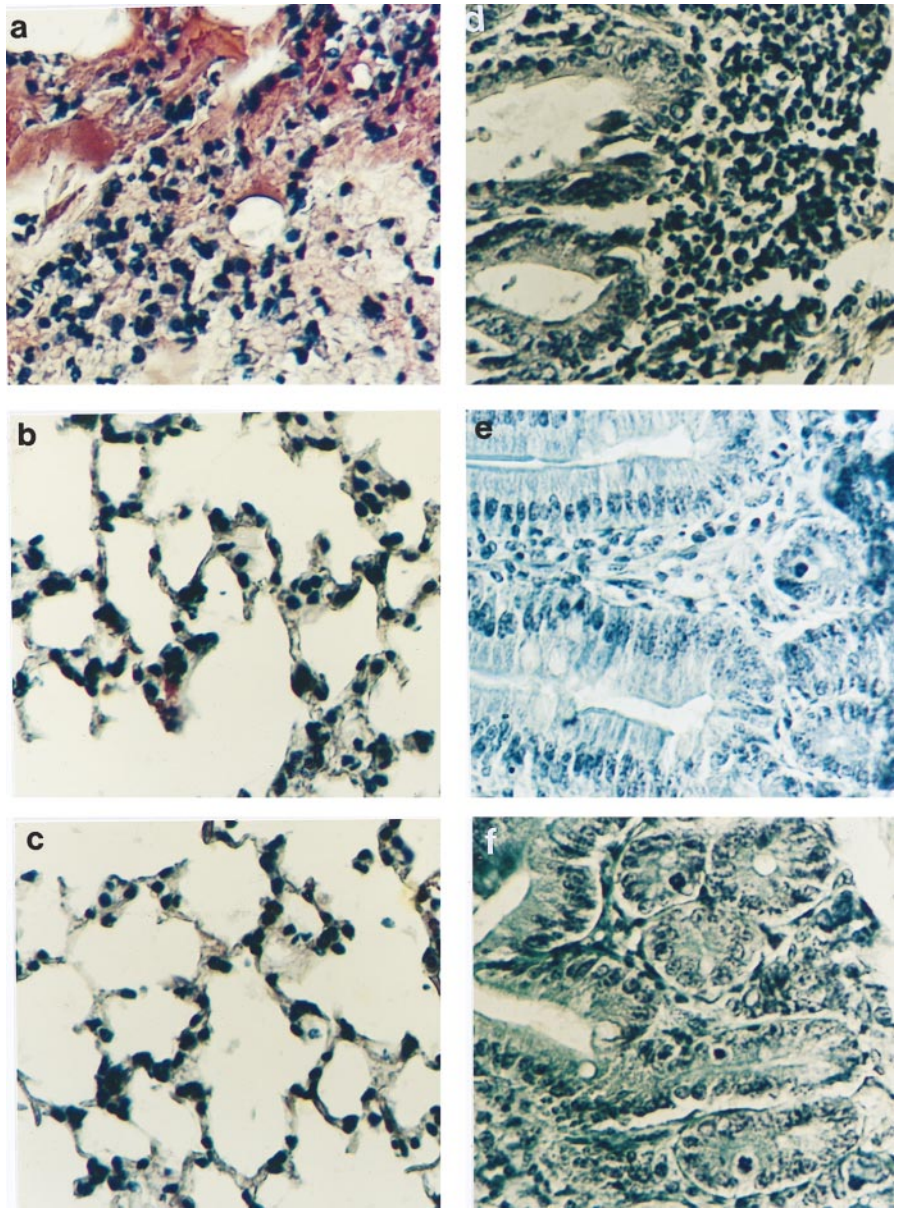

FIGURE 2. Histopathological analysis of tissue sections from LPS-injected mice treated with and without VIP. Mice were injected i.p. with 400 μg LPS in the absence (*a* and *d*) or presence of 5 nmol VIP (*b* and *e*). Control animals were injected with RPMI 1640 medium (*c* and *f*). Lung and large intestine sections were obtained and stained with hematoxylin-eosin (*a–c*) or with Masson's haemalum and picroindigocarmine (*d–f*), respectively. Magnification factor of sections is approximately $\times$150.

Based on these considerations, we investigated the effect of VIP and PACAP on TNF-α and IL-6 production. Both VIP and PACAP reduced by $\sim$50% the levels of secreted TNF-α in serum and peritoneal fluid (Fig. 3*A*). Similar reductions were observed for serum and peritoneal IL-6 (56–57%) (Fig. 3*A*). Northern blots confirmed that VIP/PACAP significantly reduce the steady-state mRNA levels for both TNF-α and IL-6 in peritoneal exudate cells (Fig. 3*B*). If VIP/PACAP protect against the lethal effect of endotoxin through down-regulating the expression of proinflammatory cytokines such as TNF-α and IL-6, the neuropeptides should not protect against death caused by direct cytokine administration. Indeed, VIP/PACAP did not affect the survival of mice injected with lethal doses of TNF-α (Fig. 3*C*).

The findings presented in this study indicate that the neuropeptides VIP and PACAP protect from high-dose endotoxin shock. This protective effect appears to be associated with decreased expression of two proinflammatory cytokines, TNF-α and IL-6, which were previously shown to mediate the deleterious effects of endotoxin. The effect of VIP/PACAP on the expression of TNF-α and IL-6 is in agreement with previous studies regarding the inhibitory effect of VIP/PACAP on cytokine production. High levels of VIP in both serum and peritoneal fluid are induced following the

FIGURE 1. *A–D, Continued* by the Wilcoxon rank sum test. *D*, BALB/c mice (six per group) were injected i.p. with LPS (400 μg) and VIP or PACAP-38 (5 nmol) at times 0, 30 min, 90 min and 4 h after LPS administration. Survival was monitored over the next 7 days. **, $p < 0.001$, and *, $p < 0.01$ in comparison to control mice as determined by the log rank test. *E*, LPS induces VIP secretion in vivo. BALB/c mice were injected i.p. with LPS (25 and 400 μg) or with RPMI 1640 medium (control). At times 0, 4, 8, 12 and 24 h peritoneal fluid and serum were obtained. Three animals were used for each time point. VIP concentration was determined through a competitive ELISA assay using the monoclonal anti-VIP Ab (clone CURE.V55) (44) as a capture Ab. Similar results were obtained in four identical independent experiments. *, $p < 0.001$ with respect to control mice without LPS treatment as determined by the Wilcoxon rank sum test.

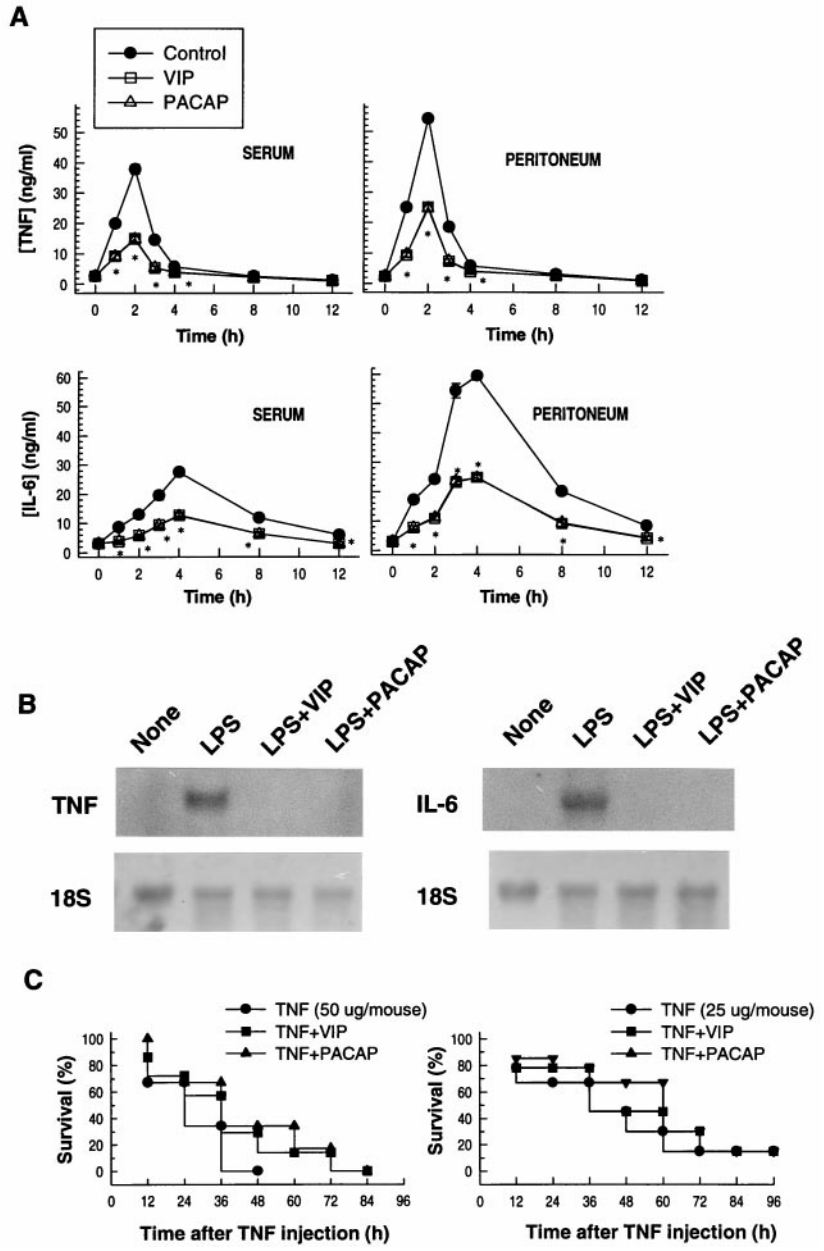

FIGURE 3. *A* and *B*, VIP and PACAP-38 inhibit TNF-α and IL-6 production in vivo. *A*, BALB/c mice were injected i.p. with LPS (400 μg) (control) or with LPS and VIP or PACAP-38 (5 nmol). Groups of six animals were used for each time point. Serum and peritoneal fluid were harvested at various time points (from 0 to 12 h), and the levels of TNF-α and IL-6 protein were determined by ELISA. $*$, $p < 0.001$ with respect to control mice without the VIP/PACAP treatment, as determined by the Wilcoxon rank sum test. *B*, BALB/c mice (groups of three) were injected with RPMI 1640 medium, LPS (400 μg), or LPS plus VIP or PACAP-38 (5 nmol). Peritoneal cells were harvested at 1 h (for TNF-α) and 2 h (for IL-6), and total RNA was prepared and subjected to Northern blot analysis using specific murine TNF-α and IL-6 probes. A representative experiment of three is shown. *C*, VIP/PACAP-38 do not protect from TNF-α-induced lethality. BALB/c mice (five to six per group) were injected i.p. with TNF-α (50 or 25 μg) with or without VIP or PACAP-38 (5 nmol). Survival was monitored over a 96-h period. Similar results were obtained in four identical independent experiments.

lethal endotoxic challenge and were previously reported in patients with septic shock. However, LPS-induced endogenous VIP levels are two to three orders of magnitude lower than the concentrations of protective exogenous VIP. We propose that during a normal immune response, the timely production and/or release of VIP and possibly PACAP within the lymphoid organs following antigenic stimulation serves to down-regulate the ongoing immune response, mostly through modulation of cytokine expression. During septic shock, however, due to severe septicemia leading to an overstimulation of the immune system, the effect of checkpoint molecules such as IL-10, IL-13, VIP, and PACAP is overwhelmed by the proinflammatory cytokine network. However, based on the protective effect of VIP/PACAP in the high-dose endotoxic animal model, the exogenous administration of these anti-inflammatory neuropeptides could offer an alternative to existing treatments for septic shock syndrome.

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