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The Significance of Vasoactive Intestinal Peptide in Immunomodulation

MARIO DELGADO, DAVID POZO, AND DOINA GANEA

Instituto de Parasitologia y Biomedicina, Consejo Superior de Investigaciones Cientificas, Granada, Spain (M.D.); Departamento de Bioquimica Médica y Biologia Molecular, Universidad de Sevilla, Sevilla, Spain (D.P.); and Department of Biological Sciences, Rutgers University, Newark, New Jersey (D.G.)

Address correspondence to: Mario Delgado, Instituto de Parasitologia y Biomedicina "Lopez Neyra," Calle Ventanilla 11, Granada 18001, Spain. E-mail: mdelgado@ipb.csic.es

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Abstract——**First identified by Said and Mutt some 30 years ago, the vasoactive intestinal peptide (VIP) was originally isolated as a vasodilator peptide. Subsequently, its biochemistry was elucidated, and within the 1st decade, their signature features as a neuropeptide became consolidated. It did not take long for these insights to permeate the field of immunology, out of which surprising new attributes for VIP were found in the last years. VIP is rapidly transforming into something more than a mere hormone. In evolving scientifically from a hormone to a novel agent for modifying immune function and possibly a cytokine-like molecule, VIP research has engaged many physiologists, molecular biologists, biochemists, endocrinologists, and pharmacologists and it is a paradigm to explore mutual interactions between neural and neuroendocrine links in health and disease. The aim of this review is firstly to update our knowledge of the cellular and molecular events relevant to VIP function on the immune system and secondly to gather together recent data that support its role as a type 2 cytokine. Recognition of the central functions VIP plays in cellular processes is focusing our attention on this "very important peptide" as exciting new candidates for therapeutic intervention and drug development.**

I. Introduction

Optimal host defense is the result of interactions between the two systems implicated in homeostasis: the neuroendocrine and immune systems. For many years, the neuroendocrine system and the immune system have been viewed as being two autonomous networks functioning to maintain a balance between host and environment. The neuroendocrine system responds to external stimuli such as temperature, pain, and stress, whereas the immune system responds to exposure to bacteria, viruses, and trauma. Within the last 30 years, a direct link between the functions of these two systems has been pointed out by evidence presented in four distinct areas: 1) psychoneuroimmunology, the interaction between stress, behavior, and immune responses to diseases; 2) immunopsychiatry, abnormalities of the immune system that may be associated with mental illness; 3) immunoneurology, the actions of secreted immune factors and immunologically competent cells on the central nervous system (CNS[1]); and 4) neuroimmu-

[1]Abbreviations: CNS, central nervous system; CRF, corticotropin-releasing factor; VIP, vasoactive intestinal peptide; PACAP, pituitary adenylate cyclase-activating polypeptide; CGRP, calcitonin gene-related peptide; αMSH, α-melanocyte-stimulating hormone; PHM, peptide histidine methionine; PHI, peptide histidine isoleucine; bp, base pair(s); VIP-ir, VIP-immunoreactivity; RIA, radioimmunoassay; HPLC, high-pressure liquid chromatography; RT, reverse transcription; PCR, polymerase chain reaction; kb, kilobase(s); Th, T helper; NO, nitric oxide; LPS, lipopolysaccharide; TNFα, tumor necrosis factor α; IL, interleukin; TCR, T cell receptor; PKC, protein kinase C; CRE, cAMP response element; GPCR, G-protein-coupled receptor; GRF, growth hormone releasing factor; PKA, protein kinase A; PLC, phospholipase C; PLD, phospholipase D; AC, adenylyl cyclase; NK, natural killer; IFN, interferon; DC, dendritic cell; NFκB, nuclear factor κ B; RANKL, receptor activator of nuclear factor κ B ligand; GRK, G-protein-coupled receptor kinase; IL-1Ra, interleukin 1 receptor antagonist; TGFβ, transforming growth factor β; IRF-1, interferon regulatory factor 1; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; MEKK1, mitogen-activated protein kinase kinase kinase; MEK4, mitogen-activated protein kinase kinase MKK4; CREB, cyclic-AMP response element binding protein; CBP, CREB-binding protein; TBP, TATA box binding protein; STAT, signal transducer and activator of transcription; GAS, IFNγ-activated site; IP-10, IFNγ-inducible protein-10; iNOS, inducible nitric oxide synthase; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; RANTES, regulated on activation normal T cell expressed and secreted; APC, antigen-presenting cell; NF-AT, nuclear factor of activated T cells; CK, chemokine; SN, substantia nigra; RA, rheumatoid arthritis; TNBS, trinitrobenzene sulfonic acid; PD, Parkinson's disease; SNpc,

nomodulation, the effects of the CNS on immune function (for review, see Weigent and Blalock, 1987). Neuroimmunomodulation has experienced an explosive growth not only in basic research but also expanding to the point that prospective clinical research could become reality. A crucial factor for the functioning of this intimate bidirectional network was the demonstration that the immune and neuroendocrine systems speak a common biochemical language. Indeed, it is becoming increasingly difficult, in the light of recent research, to see clear boundaries between such systems. This implies that: 1) the production of neuroendocrine hormones and neuropeptides by immune cells and of cytokines by neuroendocrine cells, 2) shared receptors on cells of the immune and neuroendocrine systems, 3) the effect of neuroendocrine mediators on immune functions, and 4) the effect of cytokines on the neuroendocrine system (Fig. 1). This raises the question of what can be actually be considered as immune or neuroendocrine. The fact that neurons and endocrine cells possessed similar substances was soon established. The "Rosetta stone" for the deciphering of the mutual chemical language for the neuroendocrine and immune systems was provided by the surprising discovery that lymphocytes are able to produce neuropeptides and hormones that were previously thought to reside exclusively in the nervous and endocrine systems. In 1980, it was shown that macrophages and lymphocytes were able to produce adrenocorticotropin and endorphins (Blalock and Smith, 1980; Smith and Blalock, 1981). To date, immune cells produce around 30 neuroendocrine mediators, including growth hormone (Weigent et al., 1988), prolactin (Montgomery et al., 1987), proenkephalin A (Rosen et al., 1989), somatostatin (Fuller and Verity, 1989; Weinstock et al., 1990), oxytocin-vasopressin (Geenen et al., 1987), atrial natriuretic peptide (Vollmar et al., 1992), substance P (Ho et al., 1997; Lai et al., 1998; Lambrecht et al., 1999), gonadotropin-releasing hormone (Chen et al., 1999), glucocorticoids (Lechner et al., 2000), procalcitonin (Oberhoffer et al., 1999), corticotropin-releasing factor (CRF) (Brouxhon et al., 1998), vasoactive intestinal peptide (VIP; see *Section II.*), pituitary adenylate cyclase-activating polypeptide (PACAP) (Abad et al., 2002), neuropeptide Y (Schwarz et al., 1994), calcitonin gene-related peptide (CGRP) (Singaram et al., 1991), α-melanocyte-stimulating hormone (αMSH) (Lolait et al., 1986; Rajora et al., 1996), and opioid peptides (Przewlocki et al., 1992). Moreover, an additional important fact demonstrated that lymphocytes competent to produce peptidic hormones and neuropeptides possess the biochemical machinery for a regulated secretory pathway as well as the necessary proteases as furin and other convertases to process neuropeptides (Taplits et al., 1988; Decroly et al., 1996). In addition to immune cells, other sources for neuropeptides such as neuropeptide Y, VIP, somatostatin, and galanin are the autonomic noradrenergic and cholinergic innervation, whereas substance P, neurokinin A, and CGRP are released by the sensory innervation present in the lymphoid organs (Felten et al., 1987, 1992; Fink et al., 1988; Nohr and Weihe, 1995; Bellinger et al., 1997). In addition, some of the neuropeptides can be released from the hypothalamic-pituitary axis as hormones or prohormones and arrive in the lymphoid organs via the circulation.

The immunological activity of neuropeptides is mediated through specific receptors. The presence of specific receptors for substance P (Cook et al., 1994; McCormack et al., 1996), somatostatin (Sreedharan et al., 1989; Hiruma et al., 1990), CGRP (McGillis et al., 1991), CRF (Agnello et al., 1998), melanocortin peptides (Catania et al., 1996; Getting et al., 1999), and VIP and related peptides (see *Section III.*) has been reported in immune cells.

The presence and release of neuropeptides within the lymphoid microenvironment and the existence of specific

substantia nigra pars compacta; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine.

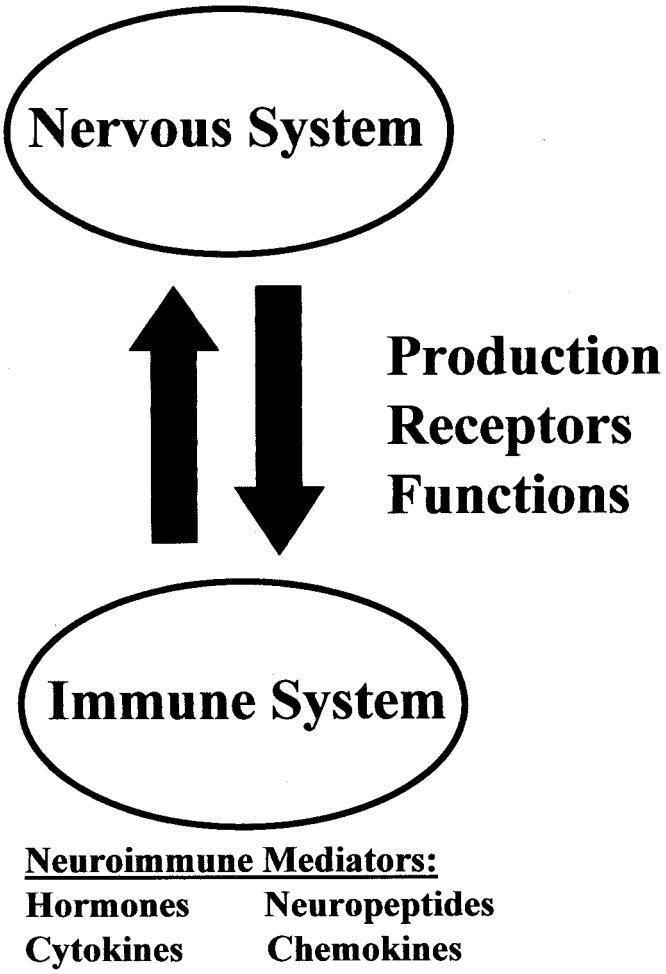

FIG. 1. Bidirectional communication between the nervous and immune systems established through the production of neuroimmune mediators (hormones, cytokines, neuropeptides, and chemokines) and acting on specific receptors expressed by cells of both systems.

neuropeptide receptors on immune cells represent the framework for neuropeptides functioning as mediators of neuroimmune interactions. Although the list of neuropeptides/neurotransmitters affecting immune functions grows at a steady pace (for review, see McCann et al., 1998; Conti et al., 2000; Sternberg et al., 2003), here we review only the effects of VIP, one of the best studied immunoregulatory neuropeptides.

II. Presence of Vasoactive Intestinal Peptide in the Immune System

A. Historical Background: Discovery of Vasoactive Intestinal Peptide

In 1968, based on the observation that patients with severe lung injury or massive pulmonary embolism often developed systemic hypotension and shock, Sami I. Said tried to isolate a vasodilator peptide from lung extracts. Although the lung extract had vasodilator activity (Piper et al., 1970), the isolation of a pure peptide with this property was slow because of tissue paucity. Later, in collaboration with Viktor Mutt, Dr. Said decided to look for the vasodilator peptide in the intestine instead of lung based on the fact that both upper intestine and lung originate from the same embryonic bud, the foregut. The idea proved fruitful, and work on duodenal extracts quickly led to the isolation of a vasodilator, hypotensive peptide (Said and Mutt, 1970), named vasoactive intestinal peptide. Several years later, VIP was identified in the central and peripheral nervous system (Said and Rosenberg, 1976) and has since been recognized as a widely distributed neuropeptide, acting as a neurotransmitter or neuromodulator in many organs and tissues, including heart, lung, thyroid gland, kidney, immune system, urinary tract, and genital organs (Henning and Sawmiller, 2001). In light of this finding, the choice of intestine for the isolation of VIP had clearly been a fortunate one because of the dense enteric plexuses of nerves. The widespread distribution of VIP is correlated with its involvement in a wide variety of biological activities including systemic vasodilation, increased cardiac output, bronchodilation, hyperglycemia, smooth muscle relaxation, promotion of growth, hormonal regulation, analgesia, hyperthermia, neurotrophic effects, learning and behavior, bone metabolism, and some differential effects on secretory processes in the gastrointestinal tract and gastric motility.

B. Vasoactive Intestinal Peptide Gene and Protein Structure

VIP is a 28-amino acid peptide with structural similarities with other gastrointestinal hormones, such as secretin, glucagon, gastric inhibitory peptide, peptide histidine methionine (PHM; in human tissues) or peptide histidine isoleucine (PHI; its counterpart in other mammalian species), growth hormone releasing hormone, helodermin, PACAP (which exists in two amidated forms, PACAP27 and PACAP38, and shows 68% identity with VIP), and CRF (Said and Mutt, 1970, 1988; Said, 1986). The amino acid sequences of the various members of the VIP family peptide are depicted in Fig. 2 (adapted from Said, 1986).

Conformational analysis of VIP by two-dimensional NMR and circular dichroism spectroscopy has show an initial disordered N terminus sequence of eight amino acid residues, probably with two β-turns, followed by two helical segments at residues 7 to 15 and 19 to 27 connected by a region of undefined structure that confers mobility to the peptide molecule (Fry et al., 1989; Theriault et al., 1991; Filizola et al., 1997).

The three-dimensional structure of VIP exhibits substantial similarities with those of other members of the VIP/glucagon family (Braun et al., 1983; Gronenborn et al., 1987; Wray et al., 1993). In particular, both VIP and PACAP27 possess two helices separated by a disordered region, but two residues toward the C terminus shift the position of the first α-helix of PACAP27, and the conformation of the second helix of PACAP27 is closer to a α-helix than that of VIP. These minor conformational differences between PACAP27 and VIP may contribute to the selectivity of the peptides for their receptors (Inooka et al., 1992).

VIP is synthesized from a precursor molecule (prepro-VIP), which also contains PHM/PHI. The 170-amino acid prepro-VIP is metabolized by a signal peptidase in the endoplasmic reticulum to yield the 148-amino acid

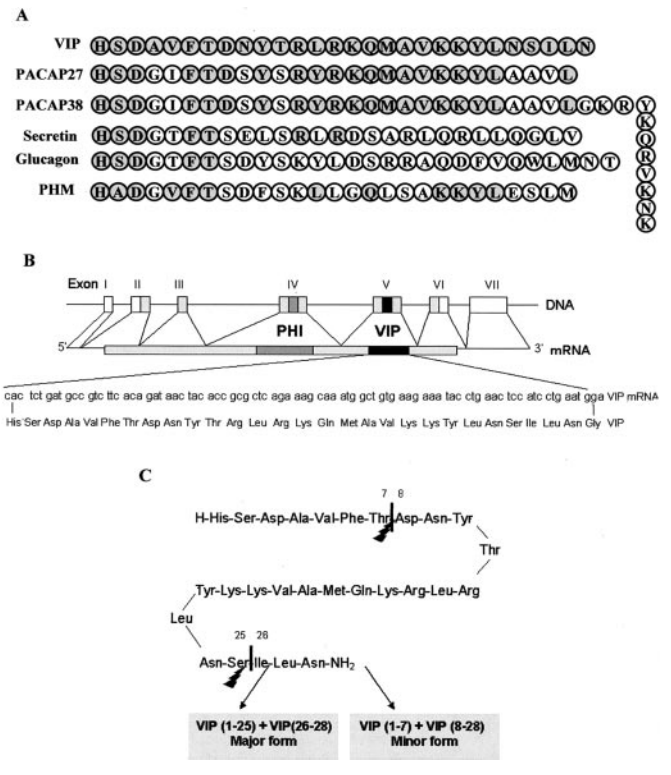

FIG. 2. VIP structure: protein and gene. A, sequence of VIP family peptides. B, VIP gene structure. C, proteolytic generation of VIP fragments.

pro-VIP. Pro-VIP is cleaved by prohormone convertases to VIP-GKR (prepro-$VIP_{125–155}$) and PHM-GKR (prepro-$VIP_{81–110}$) (Bloom et al., 1983). VIP-GKR and PHM-GKR are then cleaved by carboxypeptidase-B-like enzymes to VIP-G and PHM-G (Itoh et al., 1983). The VIP-G and PHM-G can then be metabolized by PAM enzymes to VIP and PHM, which have an amidated C terminus.

The human VIP gene, located on chromosomal region 6q24, contains seven exons, each encoding a distinct functional domain on the protein precursor or its mRNA (Fig. 2). The gene spans 8837 bp. Exon 1 of 165 bp consists of the 5′-untranslated region of the mRNA, exon 2 of 117 bp encodes the signal peptide, exon 3 of 123 bp encodes an N-terminal peptide, exon 4 of 105 bp encodes PHM, exon 5 of 132 bp encodes VIP, exon 6 of 89 bp encodes the C-terminal peptide, and exon 7 of 723 bp consists of the 3′-untranslated region of the mRNA (Bodner et al., 1985; Tsukada et al., 1985; Gozes et al., 1986). The discovery of VIP and PHM or PHI sequences on the same gene, and mRNA suggests that both peptides are cosynthesized in the same tissue. However, one does not always find VIP and PHI in the same cell (Beinfeld et al., 1984); hence, alternative processing of the nuclear precursor RNA could take place. Alternatively, differential protein processing may occur, resulting in cells expressing either VIP or PHI. The fact that all brain cells synthesizing VIP mRNA also contain PHM mRNA (Linder et al., 1987; Card et al., 1988) points out that the differential regulation could be at the protein processing level.

C. Neuronal Vasoactive Intestinal Peptide Sources within Lymphoid Organs

Several investigators have shown by immunohistochemistry the presence of VIPergic nerve fibers in both central (thymus) and peripheral (spleen, lymph nodes, and mucosal-associated lymphoid tissue) lymphoid organs. These VIP-containing nerve terminals establish the anatomical link between the CNS and the immune system.

In the thymus, VIP^+ nerve endings are most abundant in the capsule and extend into the interlobular septa, forming linear arrays. Fine VIPergic nerves exit this plexus to enter in thymic cortex and occasionally get into the medulla (Felten et al., 1985; Bellinger et al., 1996). The origin of these nerve terminals is not well defined. They do not derive from the sympathetic ganglia that provide the noradrenergic innervation to the thymus, because ganglionectomy does not deplete VIP from the thymus.

In spleen, VIP^+ nerve endings are prominent in the red pulp along the venous/trabecular system and course as free fibers in the parenchyma and in the white pulp along the central arterioles and in the periarteriolar lymphatic sheath among T lymphocytes (Bellinger et al., 1996). Although the origin of VIP-containing nerve fibers in spleen is not clear, they may come from the dorsal root ganglion, from vagal input, or from the intrinsic neurons of the gut.

In lymph nodes, the VIP innervation is relatively sparse and depends on their location in the body, being present beneath the capsule particularly near the hilus, and along the vasculature in the internodal regions of the cortex (Fink and Weihe, 1988; Bellinger et al., 1996). VIP^+ fibers occasionally leave the vascular plexus to extend among T cells in the adjacent cortex and along the medullary cords (Bellinger et al., 1990).

Regarding the mucosal-associated lymphoid tissue, an extensive nerve plexus containing various neuropeptides, including VIP, substance P, CGRP, cholecystokinin, and somatostatin, is present in the intestinal lamina propria (Bellinger et al., 1996). VIP^+ fibers course throughout the lamina propria, distributing to the crypts, into the muscularis mucosae, into the core of the villi, and around the margins of the Peyer's patches (Ottaway et al., 1987; Ichikawa et al., 1994). Some VIP^+ enteric fibers traverse the lamina propria to enter into the epithelium. VIPergic nerves in the Peyer's patches course predominantly along lymphatics and high endothelial venules and infrequently in the lymphoid follicle of the patches. The source of the VIP innervation of gut derives largely from intrinsic enteric VIP-containing neurons in the myenteric and submucous ganglionic plexuses and from extrinsic parasympathetic autonomic nerves and sensory fibers (Furness and Costa, 1980). In the bronchus-associated lymphoid tissue, VIP^+ nerves are present in the walls of extra- and intrapulmonary bronchi and bronchioles (Uddman and Sundler, 1979; Dey et al., 1981), with abundant distribution to the smooth muscle layer and around submucosal mucous and serous glands of the airways. Like the gastrointestinal tract, an extensive network of VIPergic fibers courses in the lamina propria, although the anatomical association of VIP-containing nerve fibers with immune effector cells has not been investigated. VIP^+ nerves in the lung probably arise from the vagus nerve and from microganglia present in the walls of the bronchi (Lundberg et al., 1979; Dey et al., 1981).

D. Vasoactive Intestinal Peptide Production by Immune Cells

1. Vasoactive Intestinal Peptide Production by Mast Cells and Granulocytes. The first evidence for the production of VIP by cells of the immune system was the identification of VIP-immunoreactivity (VIP-ir) in rat peritoneal, intestinal, and lung mast cells using radioimmunoassay (RIA) and immunohistochemical studies (Cutz et al., 1978). VIP-related peptides have also been detected in several mast cell lines, mouse bone marrow-derived mast cells, rat basophilic leukemia cells, and mouse peritoneal cell extracts (Wershill et al., 1993). Reversed-phase HPLC molecular characterization showed that the VIP content of mast cell lines differs structurally from naive VIP, mostly corresponding to the

truncated form of VIP_{10-28} with the carboxyl-terminal asparagine as a free acid and in a variable percentage to a mixture of amino-terminally extended VIP (Goetzl et al., 1988; Wershill et al., 1993), apparently derived from a novel prepro-VIP encoded by an alternatively spliced mRNA. Furthermore, mast cell lines appear to be incapable of synthesizing VIP_{1-28}. It is not clear whether multiple forms of VIP-ir molecules are present in normal immune effector cells. If this is the case, differential expression of VIP in specific immune effector cells may add to the complexity of VIP-immune effector cell interaction. The isolation of distinctive fragments of VIP_{1-28} from suspensions of lymphocytes, mast cells, and other leukocytes after incubation in vitro confirmed the involvement of post-translational peptidolysis (Goetzl et al., 1989a) in the structural diversity of some VIPs. Therefore, subpopulations of immune cells may contain different VIP forms, which could preferentially bind to VIP receptor subtypes and have different functions.

In addition to mast cells, VIP-ir has been demonstrated in several types of leukocytes. O'Dorisio et al. (1980) have found VIP (1.1 ng/10^8 cells) in human peripheral blood polymorphonuclear cells, mainly in neutrophils, but not in mononuclear cells, and suggested that VIP production may be regulated under immunopathological conditions, providing the means for the differential diagnosis of certain leukemias. However, others did not confirm these results. For example, several reports have shown much higher amounts of VIP in mononuclear cells than in polymorphonuclear cells (Madden et al., 1981; Lygren et al., 1984). Evidence for the production of VIP in rat basophilic leukemia cells has also been shown by the identification of a specific prepro-VIP/PHI mRNA fragment (Wershill et al., 1993). In addition, Murphy et al. (1981) described substantially lower VIP levels in different types of leukemic cells, and no correlation with leukemic disease states was found. Differences in experimental techniques used for cell isolation or in the specificity of the antibody used in these studies, as well as contamination with other cells or possible degranulation of leukocytes, could explain these discrepancies. Collectively, these studies suggest that VIP is present in both human mononuclear and polymorphonuclear cells. In addition, VIP (72 fmol/10^7 cells) has been found in human eosinophils (Aliakbari et al., 1987). Similarly, Weinstock and Blum (1990) have demonstrated VIP-ir in eosinophils in granulomatous lesions induced by infection with *Schistosomiasis mansoni*.

2. Vasoactive Intestinal Peptide Production by Lymphocytes. After the initial reports describing the presence of VIP (0.2–0.3 pmol/10^8 cells) in mononuclear leukocytes (Madden et al., 1981; Lygren et al., 1984), several studies, using different experimental approach, demonstrated that VIP is synthesized by lymphocytes and secreted in response to different immunological stimuli (Fig. 3). The first evidence was the presence of VIP-ir in thymus, spleen, and lymph node cells using

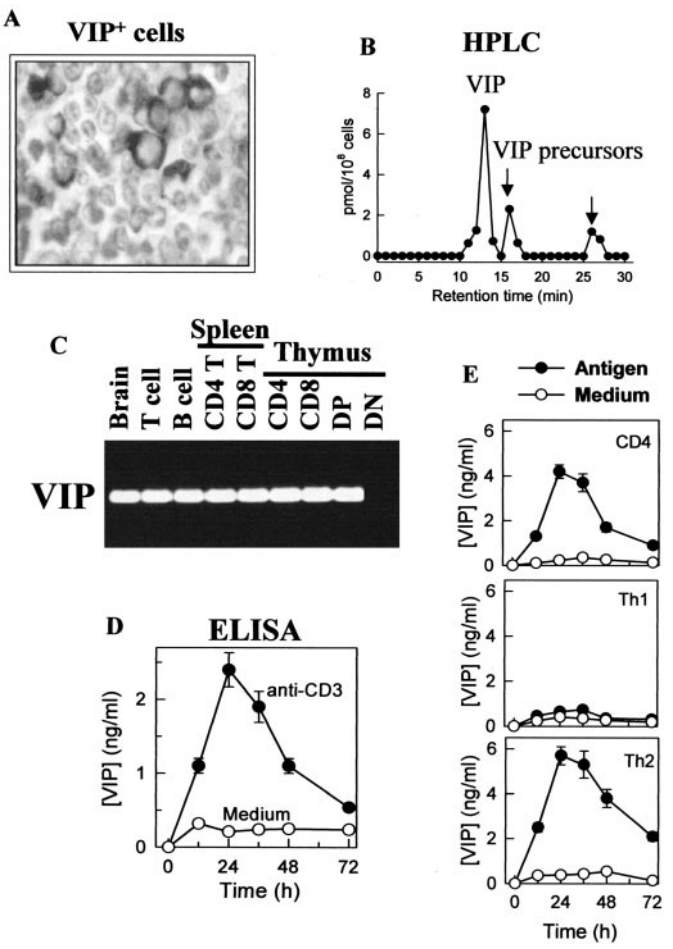

FIG. 3. VIP is synthesized by lymphocytes. A, immunohistochemical analysis of VIP^+ thymocytes. B, HPLC analysis of VIP-ir from murine spleen lymphocytes. The major VIP-ir peak corresponds to naive VIP, and the two minor peaks with higher retention times correspond to VIP precursors (prepro-VIP and proVIP). C, RT-PCR analysis of VIP mRNA in different lymphocyte populations. Brain, positive control. DP, double positive thymocytes. DN, double negative thymocytes. D, VIP is secreted by T cells following TCR stimulation. Determination by a specific enzyme-linked immunosorbent assay. E, VIP is preferentially produced by Th2 $CD4^+$ cells after antigenic stimulation.

immunohistological methods (Gomariz et al., 1990, 1992), where most VIP-ir was strongly identified in cells with lymphocyte appearance. VIP^+ lymphocytes are more numerous in the thymic deep cortex and medulla, in the splenic periarteriolar lymphoid sheaths, and in the lymph node T cell-dependent interfollicular areas and deep cortex. The number of VIP^+ lymphocytes is higher in peripheral organs than in thymus (Leceta et al., 1994). RIAs demonstrated that the VIP amounts in murine lymphoid organs are in the range of those found in mast cells (0.5–2.5 pmol/10^8 lymphocytes) (Gomariz et al., 1992). To exclude the possibilities that the VIP-ir described in these studies really corresponded to nerve terminals in close proximity to lymphocytes or that lymphocytes merely took up VIP from the local microenvironment, biochemical characterization of VIP by HPLC-RIA and light and electron microscopy were performed in isolated lymphoid cell suspensions (Gomariz et al.,

1992; Leceta et al., 1994). HPLC-RIA showed that, although $VIP_{1–28}$ is the predominant molecular form of VIP-ir in isolated lymphocyte extracts, two additional molecular forms with longer retention times, corresponding to higher molecular weight precursors, are also detected (Gomariz et al., 1992), suggesting that VIP could be in fact synthesized by lymphoid cells. VIP occurrence in lymphocytes was definitively confirmed when VIP gene expression was demonstrated by in situ hybridization and reverse transcription (RT) followed by polymerase chain reaction (PCR) in different lymphocyte populations in both central and peripheral lymphoid organs (Gomariz et al., 1993, 1994b). In situ hybridization localized VIP mRNA in lymphocytes of the thymic corticomedullar and medullar regions and in the splenic white pulp (Gomariz et al., 1993). RT-PCR assays demonstrated the presence of mRNA for the precursor of VIP in both CD4 and CD8 T cells as well as B lymphocytes of primary and secondary lymphoid organs (Gomariz et al., 1994b). These findings were confirmed by flow cytometry analysis on double-labeled lymphocytes of thymus, spleen, and lymph nodes (Gomariz et al., 1993; Leceta et al., 1994). Regarding the thymus, VIP gene expression was shown in double positive ($CD4^+CD8^+$) and single positive ($CD4^+CD8^-$ and $CD4^-CD8^+$) but not double negative ($CD4^-CD8^-$) thymocytes subsets (Delgado et al., 1996c). This suggests the possible autocrine involvement of VIP in intrathymic T cell maturation/differentiation. Northern blot analysis of mRNA from spleen lymphocytes showed two transcripts for VIP: a predominant form of 1.0 kb and a less abundant form of approximately 1.7 kb (Leceta et al., 1996). Although the 1.7-kb mRNA is preferentially expressed in most tissues, the 1.0-kb transcript predominates in the anterior pituitary gland (Lara et al., 1994), a product resulting from the utilization of the proximal polyadenylation signal of the prepro-VIP/PHI gene (Chew et al., 1994). This could also be the case in lymphoid cells (Gomariz et al., 1994b). Finally, the development of a specific enzyme-linked immunosorbent assay for VIP demonstrated that lymphocytes not only express mRNA for VIP and accumulate VIP protein in the cytoplasm but also secrete VIP in response to various inflammatory and mitogenic stimuli (Martinez et al., 1999). A recent study indicated that antigen-stimulated Th2, but not Th1, cells synthesize and secrete VIP (Delgado et al., 2001a; see below).

Whether macrophages express VIP is still controversial. We did not find VIP-ir or VIP gene expression in macrophages from lymphoid organs and peritoneal suspensions (Leceta et al., 1994; Delgado et al., 1996d), whereas Metwali et al. (2002) described production of VIP by macrophages. Differences in state of cell activation or macrophage source could explain the discrepancy.

E. Mechanisms for Vasoactive Intestinal Peptide Release in the Immune System

Similar to other neuropeptides, VIP appears to be released in the lymphoid organs from two separate sources, the innervation and immune cells. The challenge remains to identify the exact factors that control neuronal or immune VIP release during an inflammatory reaction and to characterize the molecular mechanisms involved. The nature of the signals leading to neuronal VIP release during an inflammatory reaction is not known. However, a strong candidate is nitric oxide (NO), produced at high levels in inflammatory responses and shown to release VIP from enteric ganglia (Grider and Jin, 1993; Matsuyama et al., 2002). In addition, in a murine model of pulmonary delayed-type hypersensitivity characterized by a massive parenchymal infiltration of T lymphocytes, intratracheal antigenic challenge resulted in an increased secretion of VIP, recruitment of T cells bearing VIP receptors, and up-regulation of VIP receptors on the responding T cells, suggesting prominent antigen-evoked VIP neural immunoregulation (Kaltreider et al., 1997).

Regarding the mechanisms of VIP production by immune cells, initial studies indicated that activation of lymphocytes with mitogenic stimuli such as concanavalin A, with inflammatory stimuli such as lipopolysaccharide (LPS) or cytokines (TNFα, IL-6, and IL-1β), or stimulation through the T cell receptor (TCR) with an anti-TCR antibody leads to VIP expression and secretion (Leceta et al., 1996; Martinez et al., 1999). In addition, agents that increase intracellular cAMP levels or activate protein kinase C (PKC) pathway also induce the production of VIP by lymphocytes (Martinez et al., 1999). These findings are in agreement with the fact that in endocrine and neuroblastoma cells, VIP secretion is positively regulated by cAMP, calcium, PKC, and cytokines, and a cAMP response element (CRE), a phorbol ester-response element, and a cytokine response element have been located in the VIP gene promoter (Sena et al., 1994; Symes et al., 1995, 1997; Jones et al., 2000; Pitts et al., 2001). These mechanisms might also regulate the expression of VIP in immune cells, and inflammatory cytokines or signals that activate these pathways (antigen stimulation, bacterial products or mitogens) might play a role and represent potent inducers of its secretion. Of important physiological significance is the fact that Th2, but not Th1, effector cells express and secrete VIP following specific antigenic stimulation in vitro and in vivo (Delgado and Ganea, 2001a) and that the Th2-derived VIP is functional and promotes Th2 type responses in vivo (Delgado et al., 1999b, 2000b, 2002c; Delgado and Ganea, 2001b; Goetzl et al., 2001; Vassiliou et al., 2001; Voice et al., 2001, 2003). In fact, as we will discuss below, VIP is being seriously considered a type 2 cytokine.

An interesting question that should be addressed in a future is the relative role/relevance of both VIP sources (nerve terminals or immune cells) in immunomodulation. Several reports have suggested that VIP from autonomic nerve terminals in the lymphoid organs is involved in general or systemic aspects of blood circulation such as blood flow and vascular permeability, whereas VIP synthesized by lymphocytes is biologically involved in immune responses. Sympathectomy of thymus and spleen does not significantly alter their VIP contents, suggesting that lymphoid cells produce most VIPs on lymphoid organs (Bellinger et al., 1997). In addition, it has been described that, although mesenteric lymph nodes show few VIP-containing nerves, their VIP content is higher than that of thymus and spleen (Bellinger et al., 1990, 1996). This suggests again that in lymph nodes, VIP is produced mostly by the immune cells. The confirmation of the role of endogenous, T cell-derived VIP, as an important immunomodulator in T helper cell differentiation, has came recently from studies in which elimination of VIP from TCR-stimulated T cells with VIPase IgG resulted in the readjustment of the Th1/Th2 balance (Voice et al., 2003). Several reports indicate that VIP contained in the neuronal compartments is involved in the pathophysiology of several diseases in gut and lung. Loss of VIPergic nerves in these disorders appears to further exacerbate the inflammatory response. Thus, VIP present in nerve endings that distribute to the gut and lung may be important clinically in patients with inflammatory bowel disease (Koch et al., 1987, 1990), inflammation of the intestines associated with infection (Palmer and Greenwood, 1993), and asthma (Ollerenshaw et al., 1989). It has been suggested that liberation of neurotoxic substances from activated granulocytes, such as eosinophils and neutrophils, is responsible for the diminished VIP content during an inflammatory response.

Whether VIP-containing neurons in the CNS affect immune function by modulating neuroendocrine and/or autonomic outflow is not known. However, neurons and nerve fibers containing VIP have been found in CNS regions that influence the immune system, including all regions of the cerebral cortex, limbic forebrain structures (septum, amygdala, hippocampus, and stria terminalis) and hypothalamic areas (paraventricular and periventricular nuclei, arcuate nucleus, and anterior and preoptic areas), and data showing that VIP modulates the hypothalamic-pituitary axis suggest that central VIPergic neurons also influence immune function by interacting with the CNS circuitry that is well documented to play a modulatory role in neural-immune interactions (for review, see Fuxe et al., 1977; Loren et al., 1979).

III. Vasoactive Intestinal Peptide Receptor Expression in the Immune System

A. Introduction and Nomenclature

Recent advances in molecular and cell biology enabling the cloning, expression, and mutagenesis of VIP receptors has prompted an explosion of knowledge in the field, facilitating the discovery of new classes of regulatory proteins and providing a basis and means for manipulating receptor function. VIP binding to specific sites was characterized soon after VIP was first isolated from the small intestine, in liver and fat cell plasma membranes (Bataille et al., 1974), exocrine pancreas (Christophe et al., 1976), intestinal epithelium (Laburthe et al., 1979; Prieto et al., 1979), and human cancer cells (Laburthe et al., 1978). Combined functional information merged from early stages in VIP receptor research showed several common features such as a strong coupling to the activation of adenylyl cyclase and the ability to interact in a species-specific manner with related peptides such as secretin, helodermin, growth hormone-releasing factor, or peptide histidine isoleucine amide (Bataille et al., 1974; Laburthe et al., 1986). Some of these peptides were encoded within the VIP precursor polypeptide mRNA as additional biologically active peptides acting most likely through VIP receptors instead of different and specific sets of receptors. The isolation of the biologically relevant pituitary adenylate cyclase-activating polypeptides PACAP38 and PACAP27 (Miyata et al., 1989), which have a high degree of identity with VIP, led to the concept that VIP receptors interact with both VIP and PACAP as endogenous ligands. The receptors for VIP and PACAP belong to family B or group II of the G-protein-coupled receptors (GPCRs), also called the secretin receptor family (Kolakowski, 1994; Harmar, 2001). So far, three receptors that display high affinity for VIP and PACAP have been cloned and according to the established International Union of Pharmacology nomenclature (Harmar et al., 1998) are named $VPAC_1$ and $VPAC_2$ (high affinity for both VIP and PACAP) and PAC_1 (selective affinity for PACAP).

B. Biochemical, Pharmacological, and Signaling Key Features of Vasoactive Intestinal Peptide Receptors

VPAC receptors belong to the B1 subfamily (Harmar, 2001) together with receptors that respond to structurally related ligands [growth hormone releasing hormone, glucose-dependent insulinotropic polypeptide, glucagon-like peptides (GLP-1 and GLP-2), and glucagon]. These receptors share a common molecular architecture, made of seven transmembrane domains (7TM), three extracellular loops (EC1, EC2, and EC3), three intracellular loops (IC1, IC2, and IC3), a long amino-terminal extracellular domain, and an intracellular carboxyl terminus. As previously reported for other members of the B1 family, the amino-terminal extracellular region, the transmembrane domains, and the first intra-

cellular loop consist of a large number of amino acid residues that are well conserved among VPAC receptors. VPAC receptors have a relatively low extension of *N*-glycosylation sites as cross-linking experiments with ^{125}I-PACAP27 and ^{125}I-VIP to cell membrane preparations revealed (Couvineau et al., 1986; Buscail et al., 1990). PAC_1 binding sites were first characterized in the hypothalamus and the anterior pituitary (Buscail et al., 1990; Gottschall et al., 1990) and showed a high affinity for PACAP38 and PACAP27 with a K_d value around 0.5 nM, whereas the affinity for VIP was more than 500 times lower, with differences among different regions of the central nervous system (Cauvin et al., 1991). In vitro, recombinant rat and human PAC_1 stably expressed in CHO cells recognized PACAP38 and PACAP27 with higher potencies (IC_{50}, 1 nM) than VIP (IC_{50}, 1000 nM) (Ciccarelli et al., 1995). $VPAC_1$ receptors show important species selectivity differences between rat and human, particularly in the recognition of PHI, based in three nonadjacent amino acids within a sequence comprising part of the first extracellular loop and third transmembrane domain (Couvineau et al., 1996; Igarashi et al., 2002). This domain is responsible for the low affinity for PHI (IC_{50}, 1000 nM) of the human $VPAC_1$ receptor. Thus, the order of affinity for rat $VPAC_1$ receptors is as follows VIP (IC_{50}, 1 nM) > PACAP38 = PHI > PACAP27 ≫ GRF (IC_{50}, 80 nM) > secretin (IC_{50}, 300 nM) (Couvineau et al., 1996). Rat and human $VPAC_2$ receptor recognizes VIP with lower affinities (IC_{50}, 3–4 nM), and similar or minor differences in the affinity to PACAP38 have been reported (Lutz et al., 1993; Usdin et al., 1994).

With regard to receptor specificity, natural peptides that are structurally related to VIP have a limited utility: only secretin, which has higher affinity for $VPAC_1$ than for $VPAC_2$, can be relied upon. An important point in establishing the role of VPAC receptors was the development during the past few years of selective agonists and antagonists. The cyclic analog of VIP, Ro 25-1392, is a selective agonist for the human $VPAC_2$ receptor (Xia et al., 1997) and the molecular modeling designed VIP analog, [Ala^{11,22,28}]VIP, is a selective agonist for the human $VPAC_1$ receptor (Nicole et al., 2000). The chimeric VIP/GRF molecule, [K^{15},R^{16},L^{27}]$VIP_{(1-7)}$/$GRF_{(8-27)}$, is a selective agonist for rat $VPAC_1$ receptor (Gourlet et al., 1997b). Ro 25-1553 has been used as a general high $VPAC_2$ preference agonist (Dewit et al., 1998; Delgado et al., 2000a), although cross interaction with the rat PAC_1 receptor has been reported (Gourlet et al., 1997c). Very recently, sequence alignment of PACAP, VIP, [K^{15},R^{16},L^{27}]$VIP_{(1-7)}$/$GRF_{(8-27)}$, Ro 25-1553, and Ro 25-1392 was analyzed to identify $VPAC_2$ selective determinants by saturation recombinant mutagenesis, leading to the production of a high selective and potent $VPAC_2$ agonist as a recombinant peptide (Yung et al., 2003). The strategy followed by the above cited authors overcame important technical issues such as the absence of exogenous amino acids without losing stability and the analysis of a large number of peptides and provides a feasible method of industrial manufacture. A potent general $VPAC_1$ receptor antagonist is the chimeric VIP/GRF derivative, PG 97-269 (Gourlet et al., 1997c), whereas no generally used $VPAC_2$ receptor antagonists have been developed. Recently, the endogenous VIP fragment generated at the surface of lymphocytes by protease activity (Goetzl et al., 1989b), VIP_{4-28}, has been shown in vitro to be a potent agonist for $VPAC_1$ and a potent antagonist for $VPAC_2$ (Summers et al., 2003). The role of this $VPAC_2$ endogenous antagonist could be of physiological importance since fine modulation can be achieved in cells differentially expressing VPAC1 or VPAC2 receptors. The combined use of $PACAP_{6-38}$ as an antagonist for PAC_1 and to a lesser degree for $VPAC_2$ (Gourlet et al., 1995) and of the PAC_1 agonist maxadilan (Moro and Lerner, 1997) has been employed to discriminate PAC_1 receptor-mediated mechanisms (Delgado et al., 1999h; Ganea and Delgado, 2001).

VPAC receptors couple to: 1) stimulation of adenylyl cyclase triggering a protein kinase A (PKA)-cAMP transduction pathway and 2) activation of phospholipase C (PLC) and phospholipase D (PLD) (Delporte et al., 1995; Rawlings and Hezareh, 1996; Van Rampelbergh et al., 1997; Harmar et al., 1998; McCulloch et al., 2000, 2002). VPAC receptors induce responses by activating transduction systems that involve different G-proteins, with Gα as the best characterized in different tissues and cell lines expressing recombinant receptors. Others G proteins that have been shown to be coupled to VPAC receptors belong to Gi/Go and Gq families (Pozo et al., 1997a; Van Rampelbergh et al., 1997; McCulloch et al., 2000; Shreeve et al., 2000; MacKenzie et al., 2001). Also, other VPAC partners different of G proteins have been reported, such as the small G-protein ADP-ribosylation factor (McCulloch et al., 2001) or receptor activity-modifying proteins (Christopoulos et al., 2003), resulting in alterations of receptor phenotype or pharmacological profile (McLatchie et al., 1998).

Genes that encode VPAC receptors have been cloned from frog, fish, chicken, rat, mouse, and human. The $VPAC_1$ gene (*Vpr1*) was originally cloned by cross-hybridization with the secretin receptor from a rat lung cDNA library (Ishihara et al., 1992). The human *Vpr1* gene is located on region p22 of chromosome 3 (Sreedharan et al., 1995). $VPAC_1$ mRNA is particularly abundant in the cerebral cortex and hippocampus, whereas no messenger is detected in the suprachiasmatic nucleus (Usdin et al., 1994). $VPAC_2$ receptors showed a rather complementary distribution, although *Vpr2* mRNA overlapped with *Vpr1* in the hippocampus (Ishihara et al., 1992; Usdin et al., 1994). The $VPAC_2$ gene (*Vpr2*) was cloned from the rat pituitary gland (Lutz et al., 1993) and human SUP-T1 lymphoblasts (Svoboda et al., 1994). The human *Vpr2* gene is located in region q36.3 of

chromosome 7 (Mackay et al., 1996). There are presently no reported isoforms for the *Vpr1* or *Vpr2* genes. The PAC_1 gene (*Adcyap1r*) was first cloned from the AR4-2J rat pancreatic carcinoma cell line (Pisegna and Wank, 1993). PAC_1 mRNA is expressed mainly in the central nervous system and in endocrine glands (Basille et al., 2000; Vaudry et al., 2000). Nine variants resulting from alternative splicing have been described so far. Five alternative isoforms differing in the third intracellular loop have been characterized in rat (Spengler et al., 1993) and human (Pisegna and Wank, 1996). The $PAC_{1\text{-null}}$ is the isoform without cassette insertion, three variants contain one of three 28-amino acid cassettes ($PAC_{1\text{-hip}}$, $PAC_{1\text{-hop1}}$, $PAC_{1\text{-hop2}}$), and an isoform contains a double insert, $PAC_{1\text{-hip-hop1}}$. Despite the conservation of alternative splicing of the *Adcyap1r* gene from rats to humans, there are differences regarding the role of hip and hop coupled to AC and PLC. Rat $PAC_{1\text{-null}}$, $PAC_{1\text{-hop1}}$, $VPAC_1$, and $VPAC_2$ receptors have been reported to stimulate PLD, although less potently than AC (McCulloch et al., 2001). Interestingly, a facilitating role for the rat hop1 cassette in receptor coupling to ADP-ribosylation factor-dependent PLD activation has been reported (McCulloch et al., 2001). The rat isoform $PACAP_{1\text{-TM4}}$ is another variant that differs from the canonical PAC_1 receptor only by discrete sequences located in transmembranes II and IV and the extracellular domain (Chatterjee et al., 1996). $PACAP_{1\text{-TM4}}$ is exclusively expressed in β-cells; it is not coupled to AC, PLC, or PLD but mediates increases $[Ca^{2+}]_I$ by stimulating Ca^{2+} influx via L-type Ca^{+2} channels (Chatterjee et al., 1996). Recent works pointed out that the insulinotropic effect of PACAP involves in part a cAMP but not an inositol phosphate pathway triggered by both PAC_1 and VPAC receptors (Jamen et al., 2000, 2002). There are two additional PAC_1 variants with deletions within the amino-terminal domain, the so-called $PAC_{1\text{-short}}$ and $PAC_{1\text{-very short}}$, the former missing residues 89 to 109, and the latter missing residues 53 to 109 (Pantaloni et al., 1996; Dautzenberg et al., 1999). These variants have altered specificity; $PAC_{1\text{-short}}$ receptor has no selectivity to PACAP compared with VIP, whereas $PAC_{1\text{-very short}}$ exhibits a lower affinity for PACAP.

The mRNA-encoding $PAC_{1\text{-short}}$ receptor is expressed exclusively in pituitary and adrenal glands and hypothalamus (Pantaloni et al., 1996), whereas $PAC_{1\text{-very short}}$ is expressed in the nervous system (Dautzenberg et al., 1999). The last splice variant identified, $PAC_{1\text{–}3a}$, encodes a rat full-length receptor with an extra 24-amino acid cassette between exons III and IV, is coupled to both cAMP and inositol phosphate pathways, and is expressed during the spermatogenic cycle (Daniel et al., 2001).

C. Basis of Vasoactive Intestinal Peptide Signaling in the Immune System

VPAC receptors in the immune system share the same molecular basis of ligand-receptor interaction as in other cells and tissues. We shall focus on the present knowledge regarding various specific aspects of VPAC receptors such as functional expression and regulation, pharmacological tools, and signaling pathways in immunocompetent cells.

1. Functional and Molecular Expression of Vasoactive Intestinal Peptide Receptors in Immune Cells.

a. Vasoactive Intestinal Peptide Binding Sites in Immune Cells. The expression of fully functional VIP receptors in the immune system was first claimed in human peripheral blood lymphocytes in the early 1980s (Guerrero et al., 1981; O'Dorisio et al., 1981) by binding techniques (using ^{125}I-VIP as a ligand) and adenylyl cyclase enzyme measurements. Later on, VIP binding sites were identified in non adherent human peripheral mononuclear cells depleted of B lymphocytes and monocytes (Danek et al., 1983). Binding sites in human peripheral blood lymphocytes were confirmed (Ottaway et al., 1983) and extended to both mouse (Ottaway and Greenberg, 1984) and rat lymphocytes (Calvo et al., 1986b).

Depending on the source, the Scatchard analysis is consistent with a single class of high-affinity binding sites or two classes of binding sites (one of high affinity and low binding sites and the other of low affinity and high binding sites). The K_d value of the single class binding site and the high-affinity binding site is similar. For the high-affinity binding sites, the reported K_d values are 0.05 nM in rat blood mononuclear cells (Calvo et al., 1986b) and 0.24 nM in human blood mononuclear cells (Guerrero et al., 1981), whereas in the case of single class affinity binding sites, K_d values are 0.24 nM (Ottaway et al., 1983) and 0.47 nM (Danek et al., 1983) in human blood mononuclear cells and around 0.2 nM in spleen, Peyer's patches, and mesenteric and subcutaneous lymph node lymphocytes (Ottaway and Greenberg, 1984). Early experiments pointed out a lack of adenylyl cyclase stimulation by VIP in human monocytes (O'Dorisio et al., 1981), so it was reasoned that the low binding sites reported in human mononuclear cells (Guerrero et al., 1981) corresponded to the monocyte component (Danek et al., 1983). Despite that, only high-affinity VIP binding sites are considered to be functionally coupled to the effectors system (Laburthe et al., 2002), and discrepancies may be due to different methods of calculating K_d and B_{max} and to the concentrations of unlabeled ligand used to define specific binding, leading to different numbers of receptor classes rather than actual diverse biologically relevant entities. Later on, specific binding sites of VIP by human blood monocytes were reported, with a K_d value of 0.25 nM for the high-affinity receptor (Wiik et al., 1985).

Early attempts to identify different cells population expressing VIP receptors showed preferential expression of VIP binding sites by mouse T cells throughout the secondary lymphoid organs but not in thymus (Ottaway and Greenberg, 1984; Ottaway, 1987). In humans, al-

though it was initially claimed that B and/or natural killer (NK) cells (Calvo et al., 1986a) could represent the only populations to specifically bind VIP, later experiments proved that $CD4^+$ and $CD8^+$ T cell-enriched suspensions showed high binding capacity (K_d of 0.25 nM in the $CD4^+$-enriched fraction; K_d of 0.42 nM in the $CD8^+$-enriched fraction) (Ottaway et al., 1990). Regarding the human thymus, it has been reported that ^{125}I-VIP binding sites in the cortex and medulla colocalize with anti-$CD3^+$ cells (Reubi et al., 1998; Reubi, 2000). Despite the identification of VIP receptor protein, the subtype of receptors present in human thymus has not been identified. In murine thymocytes, initial studies suggest that murine thymocytes express VIP receptors, although there is no agreement on the number of binding sites due to different experimental procedures. Although thymocytes have relatively few binding sites as compared with purified T cells (Ottaway and Greenberg, 1984; Nguyen et al., 1987), functional receptors were identified on T-cell lymphomas of thymic origin (Robberecht et al., 1989), whereas $VPAC_1$ and $VPAC_2$ mRNA expression has been reported in different sets of mouse native thymocytes and in an immature T-cell line from a spontaneous thymic lymphoma (Pankhaniya et al., 1998). In rat thymocytes, flow cytometry analysis demonstrates a predominant expression of $VPAC_2$ and a more restricted pattern in the expression of $VPAC_1$ (Delgado et al., 1999c). Although it is clear that VIP affects several aspects of thymus biology such as cytokine production, apoptosis, differentiation, and mobility through direct effects on T cells (Ernstrom et al., 1995; Delgado et al., 1996a; Xin et al., 1997; Jiang et al., 1998; Pankhaniya et al., 1998), few data are available concerning the contextual role of epithelial VPAC receptors in thymus (Head et al., 1998; Reubi et al., 1998; Marie et al., 1999).

Human acute lymphocytic and myeloid blasts express functional VIP receptors as well as the transformed preB cell line (Nalm 6) and the T cell line (Molt 4b), whereas the histiocytic line (U937) and the myelocytic line (HL 60) do not appear to express VIP receptors (O'Dorisio et al., 1992). In fact, as we mentioned above, the $VPAC_2$ receptor was cloned from SUP-T1 lymphoblasts (Lutz et al., 1993). In most cases, human transformed cell lines have higher VIP binding affinities and capacities compared with lymphocytes from healthy donors (Beed et al., 1983; Finch et al., 1989; Cheng et al., 1993; Robichon et al., 1993).

The differential expression of specific VIP receptors by lymphocyte subpopulations is a remarkable characteristic. Human intestinal intraepithelial $CD8^+$ T lymphocytes had no high-affinity VIP receptors (Roberts et al., 1991), whereas murine mucosal T cells have a single class of binding sites with a K_d of 9.08 nM (murine intestinal epithelial cell was $K_d = 0.41$ nM) functionally coupled to VIP-stimulated IL-5 release (Blum et al., 1992). These binding data are in close agreement with the $VPAC_1$ and $VPAC_2$ mRNA expression studies (Qian et al., 2001). There are few VPAC receptor binding studies in nontransformed and isolated B cells (Ottaway et al., 1990; Tatsuno et al., 1991) despite the biological effects mediated throughout VPAC receptors (Kimata et al., 1992, 1996; Fujieda et al., 1996; Shimozato and Kincade, 1997). Available data came from transformed B cell lines, i.e., SKW 6.4 B cells with a K_d of 59 nM (Cheng et al., 1993), Raji B cells with a K_d of 0.8 nM (Robichon et al., 1993), Nalm 6 preB cells with a K_d of 12.6 nM, and Dakiki plasma cells with a K_d of 9.1 nM (O'Dorisio et al., 1989, 1992).

NK cells account for 10% to 15% of blood lymphocytes and are found in low numbers in the peripheral lymphoid system. NK cells regulate certain aspects of T and B cell activation and hematopoiesis and defend against certain tumors and intracellular infections. In contrast to cytotoxic T cells, the NK cell-mediated cytotoxicity neither requires previous sensitization nor is MHC restricted. NK cells can be activated to produce cytokines (IL-2, IFN-γ, IFN-α, and TNF-α) that aid in immunomodulation. The first experimental evidence of VIP modulation on natural killer function indicated a decrease in the cytotoxicity (Rola-Pleszczynski et al., 1985). Although later experiments confirmed VIP inhibition (Yiangou et al., 1990; Sirianni et al., 1992), VIP restoration of NK cell activity has also been reported (Azzari et al., 1992; Peruzzi et al., 2000). There are no data available on PACAP modulation on NK cell activity, and in general, the effects of VIP are not completely understood. The binding studies suggest the presence of VPAC high affinity receptors in NK cells, although they have not been fully characterized (Ottaway et al., 1990).

Despite the immunoregulatory properties of PACAP and VIP in isolated neutrophils (Palermo et al., 1996; Kinhult et al., 2002), the presence of receptors is controversial. It has been reported that VIP increases the cAMP levels in human neutrophils (Palermo et al., 1996), although it is claimed to be mediated through nonreceptor mechanisms (Pedrera et al., 1994). In human eosinophils, VIP is able to stimulate migration apparently through $VPAC_1$ receptors (Dunzendorfer et al., 1998). In general, further research will be needed to evaluate the mechanisms of action of VIP in granulocytes, either eosinophils or neutrophils.

Macrophages are key components in the regulation of immune responses as well as being effectors cells of innate immunity. They are also involved in many other physiological processes, such as the development of the hematopoietic system, bone remodeling, and wound healing (Morrissette et al., 1999; Janeway and Medzhitov, 2002). The first report on VIP regulation of macrophage activation (Koff and Dunegan, 1985) clearly foresighted what became one of the main functions of VIP and PACAP as macrophage-deactivating factors (Ganea and Delgado, 2001a,b). Characterization of functional VIP and PACAP binding sites have been reported in both rat and mouse peritoneal macrophages (Segura et

al., 1991; Calvo et al., 1994; Pozo et al., 1997a) and in rat alveolar macrophages (Sakakibara et al., 1994). The analysis of the binding data indicate two classes of binding sites, with the high-affinity site having a K_d between 0.2 and 1.05 nM depending on the cell type. It has been suggested that VIP binding sites on peritoneal macrophages could be considered a marker of the activation state (Segura et al., 1996). Thus, unstimulated or resident macrophages do not posses functional VIP binding sites on the cell surface. Studies regarding the expression of VPAC receptors in murine peritoneal macrophages and the human monocytic cell line THP-1 demonstrated that PAC_1 and $VPAC_1$ mRNA are constitutively expressed, whereas $VPAC_2$ is induced upon activation (Delgado et al., 1999e; Delgado and Ganea, 2001c). VPAC receptors and their immunological effects have been extensively studied, but most of the studies on peritoneal macrophages, including our own, have been performed using casein or thioglycolate-elicited macrophages and classical activation by exposure with LPS (Ganea and Delgado, 2001a,b). This represents a particular kind of activated macrophages, with a role as an effector cell in Th1 cellular immune responses. Activated macrophages are in fact a more heterogeneous group of cells than originally appreciated, with different physiologies and performing distinct immunological functions. Therefore, other types of activated macrophages (Goerdt and Orfanos, 1999; Anderson and Mosser, 2002) should be analyzed in terms of VPAC receptors.

Dendritic cells (DCs) are the best professional antigen-presenting cells with key roles in important regulatory circuits related with immunological disorders. There is no extensive characterization of VIP or PACAP binding sites in DCs, although it has been reported that VIP induces human DCs maturation (Delneste et al., 1999). Functional VPAC receptors coupled to adenylyl cyclase have been observed in murine Langerhans cells (Asahina et al., 1995).

Osteoclasts are multinucleated giant cells originated by fusion of mononuclear cells from hematopoietic precursors closely related to monocytes/macrophages (Teitelbaum, 2000; Boyle et al., 2003). Imbalance osteoclast activation affects bone remodeling which favors resorption, leading to diseases such as osteoporosis or rheumatoid arthritis (Rodan and Martin, 2000). Recent observations have demonstrated a role for VPAC receptors on both osteoclasts and osteoblasts (Lundberg and Lerner, 2002). We focus here on osteoclasts binding sites since osteoblasts are derived from mesenchymal progenitor cells (Harada and Rodan, 2003). Although ^{125}I-VIP and ^{125}I-PACAP38 studies demonstrated higher and more specific binding for PACAP than for VIP in mouse osteoblasts (Lundberg et al., 2001), there are few detailed studies on the VIP binding sites/affinity on osteoclasts (Lehenkari et al., 2000; Lundberg et al., 2000; Charras et al., 2002). The recent insights into the molecular events involved in osteoclast activation (Khosla, 2001; Fox et al., 2003; Itoh et al., 2003; Ritchlin et al., 2003) make it possible to get a better picture of the role of VIP and PACAP in osteoclast biology. Engagement of VPAC receptors appears to be involved in osteoclast differentiation through the down-regulation of receptor activator of nuclear factor κ B (NFκB) and receptor activator of NFκB ligand (RANKL) (Mukohyama et al., 2000). Since IL-3 secreted by TNF-α-activated T lymphocytes inhibits RANKL-induced nuclear translocation of NFκB, blocking RANKL-induced osteoclast differentiation (Khapli et al., 2003), an alternative or overlapping function of VIP could be mediated by its effects on IL-3 (Brenneman et al., 2003).

Mast cells have been implicated in two contrasting types of immune responses. First, they can be activated by engaged IgE receptors to mediate immediate hypersensitivity reactions associated with allergic reactions. Second, through acute activation by microbial products, mast cells produce a series of effector molecules that mediate permeability, inflammation, chemotaxis, and tissue destruction. Recently, from the large number of soluble inflammatory mediators and different cellular effector populations implicated in the onset of arthritis, mast cells have been proven to be the cellular link between soluble factors and inflammatory arthritis (Lee et al., 2002). In general, VIP and PACAP cause histamine release from mast cells (Lowman et al., 1988; Benyon et al., 1989; Mousli et al., 1994; Ottosson and Edvinsson, 1997; Odum et al., 1998), but other effects have been also reported (Tuncel et al., 2000), probably reflecting the functional heterogeneity of mast cells.

The presence of functional VIP receptors on mast cells is uncertain. There are no studies with labeled VIP or PACAP, but recently $VPAC_2$ mRNA has been reported in human skin mast cells (Groneberg et al., 2003), confirming early reports in a murine mastocytoma cell line (Waschek et al., 1995). The relatively high neuropeptide concentrations necessary to induce histamine release, the differences observed between PACAP38 and PACAP27 (with similar affinity for VPAC receptors), and the correlation observed between the ability of basic compounds to trigger mast cell exocytosis and their potency to activate purified G proteins are the basis for a proposed receptor-independent, membrane-assisted process. This is indeed the case for PACAP-mediated effects in rat peritoneal mast cells (Seebeck et al., 1998).

The above-mentioned VPAC receptors in the immune system are functionally regulated within a framework established for others GPCRs (Pitcher et al., 1998; Pierce et al., 2002). VIP interaction with rat immunocompetent cells is a developmentally regulated process, with no significant changes in the K_d values of both high- and low-affinity binding sites, suggesting that postnatal development did not change the kinetics characteristics of the binding sites but increased their number (Pozo et al., 2000). Desensitization is believed to be a

major element of the regulation of functional VPAC receptors, and both agonist-dependent (homologous) desensitization and heterologous desensitization can control receptor activity. Homologous regulation of VPAC receptors has been shown in mononuclear human leukocytes (Wiik, 1988), mouse lymphocytes (Ottaway, 1984), and rat peritoneal macrophages (Pozo et al., 1995). Usually, VIP-induced down-regulation affects the number of high-affinity receptors rather than change receptor affinity. It is worth mentioning that these studies do not discriminate between different VPAC receptors. Heterologous regulation of VPAC receptors have been demonstrated in the BL/VL_3 murine lymphoma cell line (Abello et al., 1989) and human mononuclear leukocytes (Wiik, 1991).

Early reports pointed out a role for internalization in homologous desensitization (Wiik, 1988; Pozo et al., 1995), but little is known about the molecular mechanisms. G- protein-coupled receptor kinases (GRKs) provide a mechanism to desensitize GPCRs by phosphorylation of agonist-dependent receptors, leading to binding to β-arrestins and subsequent internalization via clathrin-coated pits for recycling (Pitcher et al., 1998; Ahn et al., 2003). Recently, endogenously expressed $VPAC_1$ receptors in HEK 293 cells have been shown to be regulated by GRKs 5 and 6 and translocated with β-arrestin complexes (Shetzline et al., 2002). GRK3 has been involved in the homologous regulation of the PAC_1 receptor in the YT-79 cell line (Dautzenberg and Hauger, 2001), and a role in receptor internalization for GRKs, arrestins, and clathrins has been proposed for the $VPAC_2$ receptor (McDonald et al., 1998; McCulloch et al., 2002). New findings such as the interaction of $VPAC_1$ receptor with the receptor activity-modifying protein 2 (Christopoulos et al., 2003) and the recently characterized regulators of G-protein signaling family protein (Hollinger and Hepler, 2002) open novel perspectives to analyze the function of VPAC receptors in the immune system.

b. Vasoactive Intestinal Peptide Receptor mRNA Expression in Immune Cells. The molecular characterization of VPAC receptors genes allowed the study of VPAC expression patterns in the immune system. No alternative splicing variants have been reported so far for *Vpr1* and *Vpr2* transcripts in the immune system. Further studies are required for the PAC_1 receptor, because *Adcyap1r* mRNA is identified usually with primers designed to recognize the N-terminal extracellular domain of the receptor protein, shared by all the receptors variants. Taken together, these results should boost our current incomplete knowledge of other aspects such as promoter polymorphisms or transcriptional regulation by specific activators or repressors.

The expression of genes encoding VPAC receptors (*Vpr1*, *Vpr2*, and *Adcyap1r*) in different cells or tissues of the human and rodent immune system is summarized in Table 1. The localization of VIP and PACAP binding sites revealed a high receptor density in the T cell areas of human lymphoid organs, without identifying the receptors (Reubi, 2000). The first report using a quantitative fluorescence-based kinetic RT-PCR by Lara-Marquez et al. (2001) addressed the question of VPAC receptors gene expression in human purified T-cell subpopulations and monocytes. In general agreement with rodent studies (Gomariz et al., 1994a; Delgado et al., 1996a; Pankhaniya et al., 1998), human *Vpr1* and *Vpr2* transcripts also have a differential distribution and selective regulation within peripheral blood T cell subsets and monocytes. Thus, *Vpr1* is the predominant VPAC receptor expressed on the resting cells, whereas *Vpr2* mRNA was not detectable in monocytes and was present in minute amounts on resting T cells (Lara-Marquez et al., 2001). On the contrary, *Vpr2* transcripts are significantly higher in human $CD4^+$ and $CD8^+$ thymocyte subsets (Lara-Marquez et al., 2000). Peripheral blood $CD8^+$ T cells showed no detectable VIP binding (Lara-Marquez et al., 2001), whereas noticeable VIP binding occurred with $CD4^+$ T cells as previously demonstrated (Ottaway et al., 1990). The fact that *Vpr2* is absent in resting human monocytes that constitutively express *Vpr1* resembles the situation in murine macrophages (Delgado et al., 1998, 1999e), human monocytes (Delgado and Ganea, 2001c), and early binding studies (Segura et al., 1996).

Although the molecular mechanisms remain unknown, *Vpr1* transcription after TCR cross-linking and CD28 costimulation was down-regulated in both human peripheral T cells and thymocytes (Lara-Marquez et al., 2000, 2001). *Vpr2* transcripts are detected in bulk murine thymocytes and murine splenic $CD4^+$ and $CD8^+$ T cells only after TCR engagement and PMA treatment, whereas *Vpr1* mRNA is constitutively expressed (Delgado et al., 1996b). VIP has an antiproliferative effect on T cells through cAMP, and $VPAC_1$ induces higher cAMP levels than $VPAC_2$. Hence, it has been proposed that the selective down-regulation of *Vpr1* after T cell activation could represent a mechanism for restricting bystander activation of T cells (Lara-Marquez et al., 2001).

Vpr1 was reported in single positive ($CD4^+$ and $CD8^+$) and double positive ($CD4^+CD8^+$) rat thymocyte subpopulations but not in the double negative ($CD4^-CD8^-$) subsets (Delgado et al., 1996c). In murine thymocytes, *Vpr2* transcripts have been found to be constitutively expressed in double and single ($CD4^+$ and $CD8^+$) subsets, whereas *Vpr1* is expressed only in the single $CD4^+$ and $CD8^+$ positive subsets (Xin et al., 1997). However, Pankhaniya et al. (1998) reported both *Vpr1* and *Vpr2* expression in single and double ($CD4^+$ and $CD8^+$) thymocytes. *Vpr2* levels were higher than *Vpr1* in $CD4^+CD8^+$ thymocytes, the largest population in the thymus. In agreement with previous reports regarding *Vpr1* expression in rat (Gomariz et al., 1994a) and mouse (Delgado et al., 1996b), splenic $CD4^+$ and $CD8^+$ T cells express *Vpr1* (Pankhaniya et al., 1998). The dis-

TABLE 1
Localization of mRNA of VPAC receptors in immune and immune-related cells

Cell Type	Species	$VPAC_1$	$VPAC_2$	PAC_1	Comments	References
HMC-1 (mast)	Human	+	+	ND	↓ *Vpr2* mRNA after low PMA stimulation	Groneberg et al., 2003
Skin mast cells	Human	ND	+	ND	↓ $VPAC_2$ protein in atopic dermatitis	Groneberg et al., 2003
HC3 (mast)	Mouse	−	+	−		Waschek et al., 1995
Monocytes	Human	+	−	ND	Resting monocytes	Lara-Marquez et al., 2001
Monocytes	Human	ND	+	ND	Resting monocytes	Dewit et al., 1998
THP-1 (monocytic)	Human	+	−(+)	+	*Vpr2* mRNA after LPS stimulation	Delgado and Ganea, 2001b
Raw 264.7 (macrophage)	Mouse	+	−(+)	+	*Vpr2* mRNA after LPS stimulation	Delgado et al., 1998
Peritoneal macrophages	Rat	+	ND	ND		Delgado et al., 1996c
Peritoneal macrophages	Rat	+	ND	+		Pozo et al., 1997a
Osteclasts	Mouse	+	−	+		Ransjo et al., 2000
Bone marrow	Mouse	+	+	+		Ransjo et al., 2000
Osteoblasts	Mouse	−(+)	+	−	*Vpr1* mRNA after differentiation	Lundberg et al., 2001
Osteoblasts	Human	+	−	−		Togari et al., 1997
Alveolar macrophages	Mouse	+	+	ND	↑ *Vpr1* mRNA after lung inflammation	Kaltreider et al., 1997
Alveolar macrophages	Human	ND	+	ND		Groneberg et al., 2001
Granuloma cells	Mouse	+	+	ND	*Vpr2* mRNA only in granuloma T cells	Metwali et al., 1996
Splenic $CD4^+$ T cells	Mouse	ND	−(+)	ND	Inducible after CD3/anti-IL-4 mAb treatment	Metwali et al., 2000
Lymphocytes	Rat	+	ND	ND	Thymus, spleen, and lymph nodes (B and T cells)	Gomariz et al., 1994a
Thymocytes	Rat	+	ND	ND	$CD4^+$ $CD8^+$; $CD4^-$ $CD8^+$; $CD4^+$ $CD8^-$	Delgado et al., 1996b
Lymphocytes	Mouse	+	−	ND	Spleen B and T lymphocytes	Waschek et al., 1995
Thymocytes/splenocytes	Mouse	+	ND	ND	$CD4^+$ and $CD8^+$ T cells; no expression in B cells	Johnson et al., 1996
Thymocytes/splenocytes	Mouse	+	−(+)	ND	*Vpr2* induced after CD3/PMA treatment	Delgado et al., 1996a
Thymocytes/splenocytes	Mouse	+	+	ND	*Vpr2* mediated differentiation into $CD4^+$ $CD8^-$	Pankhaniya et al., 1998
Thymocytes	Human	+	+	ND	*Vpr2* mainly expression	Lara-Marquez et al., 2000
Intestinal T lymphocytes	Mouse	+	+	ND	*Vpr2* only in $CD8^+$ T cells ($CD8\alpha\beta$ and $CD8\alpha\alpha$)	Qian et al., 2001
Peripheral lymphocytes	Human	+	+	ND	↓ *Vpr1* after stimulation; low *Vpr2* levels	Lara-Marquez et al., 2001
Langerhans cells	Mouse	+	+	−		Torii et al., 1997
Microglia	Rat	+	−	+	*Vpr2* expression unresponsive to LPS	Kim et al., 2000
Microglia	Mouse	+	−	+	*Vpr2* expression unresponsive to LPS	Delgado et al., 2002b
Keratinocytes	Human	+	+	−	↑ *Vpr1* upon serum deprivation	Granoth et al., 2001
Keratinocytes	Human	+	−	ND	↑ *Vpr1* by IFNγ, IL-4, TNFα, and VIP	Kakurai et al., 2001
Epithelial cells	Thymus	+	ND	ND		Marie et al., 1999

ND, not determined in the cited reference.

crepancies observed in *Vpr1* and *Vpr2* expression patterns could be attributed to different primers, especially when dealing with negative results. Future experiments using more sensitive techniques are required. The only study reporting a quantitative analysis of *Vpr1* and *Vpr2* transcripts was carried out in human thymocytes (Lara-Marquez et al., 2000) and lends support to the idea that *Vpr2* accounts for the majority of VPAC receptors expressed in thymus (Pankhaniya et al., 1998; Delgado et al., 1999c). Taken together, the expression pattern and the regulated behavior of *Vpr1* and *Vpr2* can explain the role of VIP and PACAP in thymus differentiation and modulation.

Little is known about the molecular mechanisms that control the expression of VPAC genes in the immune system. However, this information is important if we consider that down-regulation of *Vpr1* expression is an optimal requirement for the activation of $CD4^+$ T cells; therefore, *Vpr1* might represent a marker of naive $CD4^+$ T cells. Recently, Dorsam and Goetzl (2002) identified two hemopoietic-specific zinc finger transcription factors, Ikaros 1 and 2, which repress *Vpr1* transcription. Ikaros together with Aiolos plays a critical role in regulating B and T cell development and, interestingly, gene targeting of Ikaros induces hyperproliferation and lymphomas. Members of the Ikaros family of transcription factors are involved in nucleosome remodeling during TCR-mediated activation of T cells (Cortes et al., 1999). Ikaros might drive the constitutive expression of *Vpr1* in resting T cells by a classical transactivating mechanism and silence *Vpr1* in TCR-activated T cells by translocation to heterochromatin. Another possibility to account for the selectively *Vpr1* negative expression in activated T cells is the presence of a repressor protein homologous to the one identified in rats (Pei, 1998).

Qian et al. (2001) have found differential expression of VPAC receptors in the small and large intestine of the mouse. Only *Vpr2* transcripts are selectively expressed in small intestinal $CD8^+$ T cells, whereas *Vpr1* is ubiquitously expressed on the three subsets of intraepithelial and lamina propria T lymphocytes ($CD4^+$, $CD4^-CD8^+$, and $CD4^-CD8^-$) of the small intestine.

Vpr1 is also expressed in intraepithelial and lamina propia T lymphocytes of the large intestine (Qian et al., 2001). The mechanism and the biological role of *Vpr2* expression in small intestinal $CD8^+$ T cells remains to be clarified. Recently, murine intraepithelial $CD8^+$ T cells of the small intestine have been involved in autoimmunity induced by infection or inflammation (Vezys and Lefrancois, 2002). A previous tolerogenic response to cross-presented antigen could lead to an autoagressive response depending on the microenvironment, i.e., inflammatory mediators, with $CD8^+$ T priming. It is tempting to speculate that *Vpr2* expression in $CD8^+$ T cells could be potentially involved in this scenario.

Granulomas, consisting of a central area of macrophages surrounded by activated lymphocytes, have been intensely studied by numerous investigators, since they play a central role in the pathology of schistosomiasis and provide suitable models for the study of inflammatory networks. mRNAs encoding *Vpr1* and *Vpr2* were firstly described in granulomas of mice infected with *S. mansoni* (Metwali et al., 1996). Granuloma $CD4^+$ T cells express both *Vpr1* and *Vpr2* transcripts, whereas T cell-depleted granulomas express exclusively *Vpr2* (Metwali et al., 1996). On the other hand, *Vpr1* mRNA, but not *Vpr2*, is expressed in both murine thymocytes and splenocytes (Metwali et al., 1996). Metwali et al. (1996) were the first suggesting that murine *Vpr2* mRNA expression might be inducible. This was later confirmed by us, showing that although *Vpr1* mRNA is constitutively expressed in murine $CD4^+$ T and $CD8^+$ T cells, *Vpr2* mRNA is induced only after stimulation with anti-CD3 and PMA (Delgado et al., 1996b). B cells isolated from murine splenocytes did not express either *Vpr1* or *Vpr2* in resting or after LPS challenge, suggesting species differences as *Vpr1* mRNA were detected in rat B cells from spleen, thymus, and lymph nodes (Gomariz et al., 1994a).

Metwali and collaborators confirmed and provided new data about the inducible nature of *Vpr2* mRNA in murine splenocytes (Metwali et al., 2000). Thus, *Vpr2* mRNA expression in T cells granulomas and splenocytes is up-regulated after anti-CD3 stimulation only when IL-4 is neutralized in CBA schistosome-infected mice. Using $CD4^+$ Th1 and Th2 cell lines, it was shown that IL-4 regulates *Vpr2* levels in Th2 cell lines. Taken together, it is clear that *Vpr2* mRNA is an inducible gene in T cells, although some strain-related differences exist. For example, normal uninfected CBA mice do not express *Vpr2* mRNA after anti-CD3 stimulation and anti-IL4 blocking antibody, suggesting that the CBA background requires schistosome infection (Metwali et al., 2000), in contrast to BALB/c mice (Delgado et al., 1996b). On the other hand, C57BL/6 mice constitutively express minute amounts of *Vpr2* in both granulomas and splenocytes, although a strong *Vpr2* expression is observed in IL-4 knockout C57BL/6 mice (Metwali et al., 2000).

Vpr1, *Vpr2*, and *Adcyap1r* gene expression has been reported in a wide variety of monocyte macrophage lineage cells. *Vpr1* and *Adcyap1r* transcripts are constitutively expressed in murine peritoneal macrophages (Delgado et al., 1996d, 1998; Pozo et al., 1997a) and in the monocytic THP-1 monocytes (Delgado and Ganea, 2001c, 2003a). In one study, human resting monocytes were reported to express exclusively *Vpr1* transcripts (Lara-Marquez et al., 2001), whereas in another study, they were reported to express *Vpr2* as well (Dewit et al., 1998). It is difficult to ascertain at the present time whether the *Vpr2* gene is inducible by different stimuli. Other data support tissue-specific control of *Vpr2* transcription because human macrophages in the human alveolar lung lumen express *Vpr2* mRNA even in resting conditions (Groneberg et al., 2001), and murine alveolar macrophages do not increase *Vpr2* mRNA expression during inflammation (Kaltreider et al., 1997). In murine osteoclasts, *Vpr1* and *Adcyap1r*, but not *Vpr2* transcripts, have been reported (Ransjo et al., 2000). There are no studies on the possible induction of *Vpr2* mRNA in murine osteoclasts following activation.

Regarding DCs, murine-enriched Langerhans cells and the murine Langerhans cell-like cell line XS52 express *Vpr1* and *Vpr2* but not *Adcyap1r* transcripts (Torii et al., 1997).

Microglial cells mediate inflammatory response in the CNS, being considered the primary immunoeffector cells in brain. The rodent microglia share a common expression pattern with macrophages, expressing both *Vpr1* and *Adcyap1r* transcripts and no *Vpr2* mRNA (Kim et al., 2000b; Delgado et al., 2002b). There are no data available on human microglia or human microglia cell lines.

Reports dealing with VPAC receptors expression patterns in mast cells are still fragmentary. Groneberg et al. (2003) have demonstrated constitutively *Vpr2* mRNA in human skin mast cells. In the human mast cell line HMC-1, *Vpr2* expression was down-regulated after PMA stimulation, which correlates with an impairment in $VPAC_2$ receptor protein in human acute atopic dermatitis samples compared with normal individuals (Groneberg et al., 2003). Although further experimental support is required, these studies suggest a possible protective role for the *Vpr2* gene in atopic dermatitis, because an inverse relationship between cAMP levels and mast cell degranulation has been recognized (Lewis and Austen, 1981; Church et al., 1991). Whether other VPAC receptors could contribute or regulate same of the aspects of skin mast cell biology remains to be clarified.

2. Vasoactive Intestinal Peptide Signaling Pathways in Immune Cells. The number of agonists and antagonists developed before VPAC receptor cloning and widely used to study the role of VIP and PACAP in immunocompetent cells is often confusing and to some extent of limited usefulness. The above (*Section B.*) surveyed a broader view of agonists and antagonists of

VPAC receptors. Several of these analogs were not tested to asses the role of VPAC receptors on immunocompetent cells, although a comprehensive comparative study with those already in use and the newcomers described will be of much value. For these reasons, we shall focus on the actual available collection of analogs used to asses the immunoregulatory involvement of VPAC receptors in rodent and human biological materials both in vitro and in vivo. To elucidate the role of VPAC receptors in the context of their anti-inflammatory effects (Delgado et al., 1998; Dewit et al., 1998; Pozo et al., 2002), the $VPAC_1$ agonist mostly used was the chimeric VIP/GRF molecule, $[K^{15},R^{16},L^{27}]VIP_{(1-7)}/GRF_{(8-27)}$ (Gourlet et al., 1997b), whereas the $VPAC_2$ agonist most often used was Ro 25-1553 (Gourlet et al., 1997c). The PAC_1 agonist employed was maxadilan, a potent vasodilator peptide isolated from salivary gland lysates of the sand fly *Lutzomyia longipalpis*, a vector of leishmaniasis (Moro and Lerner, 1997; Delgado et al., 1998; Soares et al., 1998). Unfortunately, although it was claimed that a modified maxadilan analog could be used as a specific PAC_1 antagonist (Moro et al., 1999), most studies used $PACAP_{6-38}$, despite its weaker antagonist activity toward $VPAC_2$ receptors. The $VPAC_1$ antagonist usually employed was PG 97-269 (Gourlet et al., 1997b). Two lipophilic compounds, the VIP agonist stearyl-norleucine^{17}VIP and the antagonist stearyl-norleucine^{17}neurotensin-VIP hybrid (Gozes et al., 1995; Moody et al., 2002), have been used in immune cells (Delgado et al., 1998; Granoth et al., 2000). The combined used of these analogs could discriminate between pure $VPAC_1$ and $VPAC_2$ effects, whereas the relatively contribution of $VPAC_{1-2}$ and PAC_1 is more difficult to ascertain.

Besides gene characterization, covalent cross-linking of ^{125}I-VIP to human and rodent immunocompetent cell membranes has been carried out. The molecular weight of VPAC receptors in macrophages is particularly well characterized: 52 kDa in rat and mouse peritoneal macrophages (Pozo et al., 1997b) and 73 kDa in rat alveolar macrophages (Sakakibara et al., 1994). The ^{125}I-VIP-receptor complex's apparent molecular mass in rat splenocytes ranges from 52 to 55 kDa (Pozo et al., 2000). Human cell lines such as Molt 4b, Nalm 6, and Dakiki showed ^{125}I-VIP-receptor complexes of 47 kDa (Wood and O'Dorisio, 1985; O'Dorisio et al., 1989). The knowledge of post-translational regulated modifications of endogenous VPAC receptors in immunocompetent cells is not well understood in the absence of suitable antibodies.

The most noticeable and best characterized effect after the engagement of VPAC receptors in immune cells is the accumulation of intracellular cAMP and the activation of PKA. This activation is exerted through stimulation of the αs subunit of Gs proteins. This link was pointed out in early experiments showing guanine nucleotide down-regulation of VIP binding due to an increase of the dissociation rate of peptide bound to the membranes when GTP and its nonhydrolyzable analog guanosine 5′-β,Y-imidotriphosphate were used (Segura et al., 1992). However, guanosine 5′-β,Y-imidotriphosphate acts synergistically with VIP to stimulated AC in membranes from different immunocompetent cells (O'Dorisio et al., 1981). Both events were paradigms of a direct cross talk between VPAC receptors and the AC effector enzyme mediated by stimulatory Gs proteins (Spiegel et al., 1992). The well-recognized PKA signaling pathway for VPAC receptors is not the only existing one. Thus, monocytes and macrophages express functional PAC_1 receptors that bind VIP and PACAP with similar affinity activating a PKC/PLC signaling pathway (Pozo et al., 1997a; Delgado et al., 1998). This PKC/PLC signaling pathway is mediated by a pertussis toxin-insensitive mechanism, although a role for the pertussis toxin-sensitive $\beta\gamma$ complexes in the regulation of the PKC/PLC by VIP and PACAP has been proposed (Pozo et al., 1997a). These features suggest that the PAC_1 receptor expressed in the immune system is to some extent different from its counterpart in the CNS. The binding data seem to indicate that PAC_1 receptor levels might be lower than $VPAC_1$ and $VPAC_2$ (Pozo et al., 1997a). VIP and PACAP use the PKC/PLC pathway in the inhibition of IL-6 in murine peritoneal macrophages (Martinez et al., 1998a). In murine T lymphocytes, in agreement with the gene expression studies, the predominant immunomodulatory roles of VIP and PACAP are mediated by $VPAC_1$ and $VPAC_2$ receptors (Jiang et al., 1998). The use of specific PKA and PKC inhibitors indicate the presence of a cAMP-independent signaling pathway, in parallel to the cAMP-dependent pathway, in murine macrophages (Delgado et al., 1998). The molecular basis for the cAMP-independent pathway remains to be established. VIP was shown to activate both αs and αi3 G proteins in rat alveolar macrophages (Shreeve et al., 2000). Whether the αi3 G protein could account for the cAMP-independent pathway in murine peritoneal macrophages remains to be determined.

IV. Effects of Vasoactive Intestinal Peptide on Innate Immunity

A. Adaptive/Innate Immunity

The basic function of the immune system is to clear "foreign" or "nonself" antigens such as viruses and bacteria from the body. The immune system responds to pathogen invasion with two temporary separate but physically linked responses, mediated by different types of cells. Thus, immunity can be broadly categorized into adaptive and innate immunity. Adaptive immunity is mediated by clonally distributed B and T lymphocytes and is characterized by three distinct features: specificity, diversity, and memory. In contrast, innate immunity, formerly though to be a nonspecific immune response, is considered to be the "first line of defense" and

is characterized by engulfment and digestion of microorganisms and foreign substances by macrophages and leukocytes. However, innate immunity has considerable specificity and is capable of discriminating between pathogens and self, recognizing conserved pathogen-associated molecular patterns (Medzhitov and Janeway, 1997). In addition, the activation of the innate immune response is often a prerequisite for the triggering of adaptive immunity.

Successful elimination of pathogens requires the initiation and participation of both innate and adaptive immunity. However, once the pathogen is eliminated, cells participating in innate and adaptive immunity have to be deactivated or eliminated to reestablish immune homeostasis. Uncontrolled activation of immune system leads to serious consequences for the host, such as tissue destruction, organ failure, even death. In addition to apoptosis of activated immune cells, several endogenous agents, such as anti-inflammatory cytokines, lipid mediators, and glucocorticoids down-regulate the inflammatory process. A large body of recent literature has identified VIP as a potent endogenous anti-inflammatory agent that affects the innate immunity in several ways.

B. Vasoactive Intestinal Peptide Effects on Macrophages as Participants in Innate Immunity

1. Vasoactive Intestinal Peptide Effects on Macrophage Phagocytosis, Adherence, Migration, and Superoxide Ion Production. Macrophages play a crucial role in the fight against pathogens by contributing to both innate and adaptive immunity. Phagocytosis of pathogens is a characteristic of macrophages, leading to their activation of cytokine production and antigen presentation and to the reduction of the pathogen load. The phagocytic process involves several functions such as tissue adherence, mobility, and ingestion and digestion of the ingested material. Different initial studies reported contradictory results regarding the effect of VIP on phagocytosis. Some authors reported an inhibitory effect (Litwin et al., 1992; Ichinose et al., 1994), whereas others observed a stimulatory effect (De la Fuente et al., 1993).

Also, regarding macrophage adherence and mobility, some studies reported that VIP stimulates macrophage adherence and chemotaxis (De la Fuente et al., 1994; Ahmed et al., 1998), whereas others showed an inhibitory action (Litwin et al., 1992; Segura et al., 1993). Similarly, De la Fuente et al. (1993) reported a stimulatory effect of VIP on the digestion capacity analyzed by the production of reactive oxygen species (respiratory burst), whereas other authors reported inhibitory effects (Wiik, 1989; Wiik et al., 1989; Sakakibara et al., 1990; Ishizuka et al., 1992; Kurosawa and Ishizuka, 1993). To explain these opposite effects of VIP on macrophage phagocytic function, differences in cell sources, cell differentiation state, and experimental designs in cell isolation and stimulation were analyzed. We found that a common denominator in these studies is that the effect of VIP on freshly isolated unstimulated/resting macrophages is stimulatory, whereas its effect is opposite on prestimulated macrophages. In addition, the stimulatory actions of VIP (whether phagocytosis, adherence and migration, or reactive oxygen production) appear to be associated with PKC activity, whereas the inhibitory effects appear to be mediated through cAMP. Therefore, the differences in the reported studies could be due to the predominant expression of one or the other of the macrophage VIP receptors, depending on the differentiation stage and the activation state.

2. Vasoactive Intestinal Peptide Effects on Macrophage-Derived Inflammatory Mediators. Macrophages, major participants in innate immunity, contribute to the initiation of the inflammatory response by killing pathogens through phagocytosis, release of cytotoxic oxygen and nitrogen intermediates and TNFα, and release of chemokines and cytokines that attract and activate other immune cells. Following stimulation with microbial products like LPS, macrophages secrete several proinflammatory products, such as TNFα, IL-12, IL-1, IL-6, and NO, followed by secretion of the anti-inflammatory cytokines IL-10 and transforming growth factor β (TGFβ) (Laskin and Pendino, 1995). Despite the beneficial role of proinflammatory factors in host defense, their sustained production can lead to serious pathological conditions (Van Snick, 1990; Vassalli, 1992; Evans, 1995). Therefore, although necessary for the elimination of pathogens, macrophage activation leads to serious deleterious effects in the host if left unchecked. The control of an inflammatory state depends on the local balance between pro- and anti-inflammatory factors. Therefore, in the course of a normal immune response, several inhibitory mechanisms mediated by endogenous "macrophage-deactivating factors" come into play. In addition to the anti-inflammatory cytokines IL-10, IL-1Ra, IL-11, IL-13, and TGFβ, a neuropeptide such as VIP also functions as a macrophage-deactivating factor. VIP inhibits in vitro and in vivo production of the proinflammatory cytokines TNFα, IL-6, IL-12, and of NO, and stimulates the production of the anti-inflammatory cytokines IL-10 and IL-1Ra (Hernanz et al., 1996; Dewit et al., 1998; Martinez et al., 1998a; Xin and Sriram, 1998; Delgado et al., 1999e,f,g,i, 2002a). The different reports have pointed out to macrophages/monocytes as the major cells mediating the anti-inflammatory action of VIP. The use of specific agonists and antagonists for the different receptors has permitted the establishment that $VPAC_1$ is the major receptor involved in the effect of VIP on macrophages. However, the apparent major role of $VPAC_1$ could reflect the balance between $VPAC_1$ and $VPAC_2$ expression. Because $VPAC_2$ is expressed relatively late during macrophage/monocyte activation (12 h), $VPAC_1$ is probably the major receptor type present during early culture period. In fact, in vivo experiments

in an inflammation model using specific agonists and a mouse deficient in PAC_1 have suggested that $VPAC_2$ and PAC_1 are also, although minimally, involved in the effect of VIP in the inflammatory response (Delgado et al., 2000a; Martinez et al., 2002).

3. Molecular Mechanisms Involved in the Anti-Inflammatory Action of Vasoactive Intestinal Peptide. At this point, the obligatory question is: how does VIP regulate such a wide spectrum of inflammatory mediators? The answer to this question can be found in the fact that VIP is capable to regulate several transduction pathways and transcription factors essential for the transcriptional activation of most of these factors, including NFκB, interferon regulatory factor 1 (IRF-1), mitogen-activated protein kinase (MAPK), and CRE.

LPS, an integral component of the outer membranes of Gram-negative bacteria, is one of the major activators of macrophages and can provoke a life-threatening condition called endotoxic shock, characterized by an uncontrolled inflammatory response. A complex of LPS and the serum protein LPS-binding protein initiates signals through membrane-bound CD14 toll-like receptor complex in macrophages/monocytes (Akira et al., 2001). This process results in the activation of two different pathways that involve the Rel family transcription factor NFκB and all three MAPK cascades, i.e., the p38 MAPK, the extracellular signal-regulated kinases, and the c-Jun N-terminal kinase (JNK) (Pyo et al., 1998; Zhang et al., 1998).

Activation of the JNK in response to LPS involves a cascade in which the upstream activator MAPK kinase kinase (MEKK1) phosphorylates and activates MAPK kinases MKK4 (MEK4), which in turn phosphorylates and activates JNK (Sanchez et al., 1994; Lin et al., 1995; Sanghera et al., 1996). Phosphorylation of c-Jun by JNK increases the transactivating properties of this protein (Pulverer et al., 1991; Cobb, 1999). Activation of the MEKK1/MKK4/JNK pathway by LPS is involved in the up-regulation of several proinflammatory factors (Cobb, 1999).

The pleiotropic transcription factor NFκB plays an important role in the transcriptional regulation of all these genes encoding for proinflammatory factors (Baldwin, 1996). NFκB occurs in both homo- and heterodimeric forms. The most common transcriptionally active form is a p50/p65 heterodimer (Karin and Ben-Neriah, 2000). In unstimulated cells, NFκB is localized in the cytosol bound to inhibitory proteins, collectively termed IkB. Cellular stimulation with inflammatory cytokines and bacterial products results in IkB phosphorylation by a specific kinase (IKK), ubiquitination, and proteosomal degradation. This is followed by the rapid translocation of NFκB to the nucleus, where it binds to specific kB elements within promoters. In addition, several studies have shown that the transactivating activity of NFκB requires DNA binding and interaction with coactivators that bridge various transcriptional activators and components of the basal transcriptional machinery. The CREB-binding protein (CBP) is a ubiquitously expressed nuclear coactivator present in limiting amounts (Goldman et al., 1997). A diverse and increasing number of transcription factors and some elements of the basal transcriptional machinery are able to form stable physical complexes with and respond to CBP (Janknecht and Hunter, 1996). CBP functions as an integrator linking various transcription factors to the basal transcriptional apparatus by binding to the basal transcription factor TFIIB, which in turn contacts the TATA box binding protein (TBP) of the transcription factor IID complex in the basal apparatus (Kwok et al., 1994; Kamei et al., 1996; Lee et al., 1996). The interaction of p65 with CBP is essential for NFκB transcriptional activity (Gerritsen et al., 1997; Parry and Mackman, 1997; Zhong et al., 1998), and this interaction can be strengthened by p65 phosphorylation (Kamei et al., 1996; Zhong et al., 1997) or impeded by competition from other CBP-binding factors such as CREB, c-Jun, c-Fos, p53, steroid receptors, c-Myb, and Myo-D (Chrivia et al., 1993; Arias et al., 1994; Bannister and Kouzarides, 1995; Lundblad et al., 1995; Dai et al., 1996; Lee et al., 1996).

In addition to LPS, IFNγ constitutes the most potent macrophage/monocyte-activating factor under inflammatory conditions. Binding of IFNγ to its receptor induces the assembly of an active receptor complex and consequent transphosphorylation of the receptor-associated Janus tyrosine kinases Jak1 and Jak2 (Darnell et al., 1994; Greenlund et al., 1995). The activation of these kinases induces phosphorylation of the cytoplasmic tail of the receptor itself, which lacks intrinsic kinase activity. The cytosolic protein signal transducer and activator of transcription (STAT1) is then recruited to the activated IFNγ-receptor complex and phosphorylated (Darnell et al., 1994). Upon phosphorylation, STAT1 forms homodimers and translocates to the nucleus, where they bind to the IFNγ-activated site (GAS), also termed STAT binding element, found in the promoter of many IFNγ-induced genes including IRF-1 and the adhesion-related ICAM-1 genes (Sims et al., 1993; Caldenhoven et al., 1994; Look et al., 1994; Pine et al., 1994). Many of the regulatory effects of IFNγ in macrophages appear to be mediated by IRF-1 and/or STAT1, which transactivate multiple effector genes including IFNγ-inducible protein-10 (IP-10), CD40, IL-12, and the inducible form of the nitric oxide synthase (iNOS) (Lowenstein et al., 1993; Ohmori and Hamilton, 1993; Chartrain et al., 1994; Kamijo et al., 1994; Martin et al., 1994; Nguyen and Benveniste, 2002; Vanguri and Farber, 1994; Ma et al., 1997).

As was expected from the major involvement of $VPAC_1$, the cAMP-dependent pathway plays a major role in the inhibition of TNFα, IL-12, iNOS, and in the stimulation of IL-10. However, except for IL-10, two pathways, cAMP-dependent and -independent path-

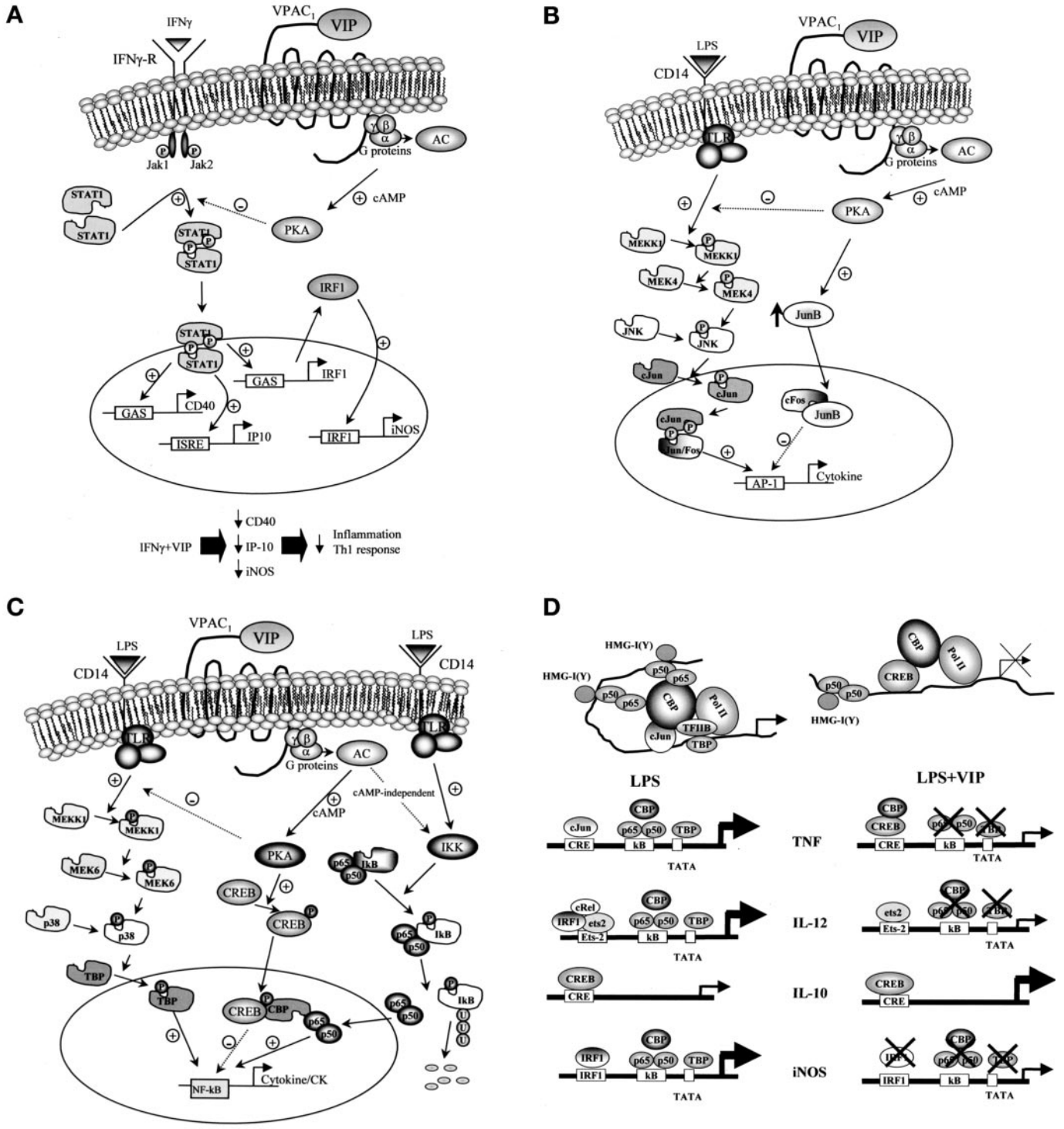

FIG. 4. Molecular mechanisms and transcription factors involved in the anti-inflammatory effect of VIP. A and B, effects on IRF1 and AP-1. Binding of VIP to $VPAC_1$ induces cAMP and exerts several effects. 1) inhibition of IFNγ-induced Jak1/Jak2 phosphorylation, STAT1 phosphorylation, and finally binding to the GAS sequence. As a consequence, IRF-1 transcription and synthesis is inhibited. 2) inhibition of MEKK1 phosphorylation, with subsequent inhibition of MEK4, JNK, and c-Jun phosphorylation. 3) up-regulation of JunB synthesis and nuclear translocation. The composition of the AP-1 complexes changes from the transcriptionally active c-Jun/c-Fos to the transcriptionally inactive JunB/c-Fos. Interrupted arrows indicate inhibitory effects. C, effects on NFκB. Binding of VIP to $VPAC_1$ initiates two transduction pathways, cAMP-dependent and -independent pathways. The cAMP-independent pathway inhibits IKK kinase, stabilizing the IkB inhibitor, and preventing p65 nuclear translocation. The cAMP-dependent pathway affects two separate transduction pathways: 1) phosphorylation of CREB (CREB-P), which due to its high affinity for the coactivator CBP prevents CBP association with p65; and 2) inhibition of MEKK1 phosphorylation and of MEK3/6, p38, and TBP phosphorylation. Nonphosphorylated TBP lacks the ability to bind to the TATA box and to form an active transactivating complex with CBP and NFκB. D, reduction in the amounts of nuclear p65, CBP, and phosphorylated TBP inhibits the formation of the conformationally active transactivating complex required for the transcription of most cytokine and chemokine genes. Interrupted arrows indicate an inhibitory effect. AC, adenylate cyclase; CBP, CREB-binding protein; TBP, TATA-box binding protein, TLR; toll-like receptor.

ways, appear to be involved in the inhibitory effects of VIP. The effects of VIP on both macrophage-derived cytokines and NO are exerted at the transcriptional level through the regulation of several transcription factors (Fig. 4). In the case of TNFα, iNOS, and IL-12p40 inhibition, the cAMP-independent pathway leads to the reduction in NFκB binding. In addition, the cAMP-dependent pathway mediates changes in the CRE-binding complex from ^{high}c-Jun/^{low}CREB to ^{low}c-Jun/^{high}CREB for the TNFα promoter, inhibition of IRF-1 binding for the iNOS and IL-12p40 promoters, and changes in the composition of the AP-1 complexes from c-Jun/c-Fos to JunB/c-Fos with subsequent reduction in AP-1 binding for the TNFα promoter. The VIP-induced increase in IL-10 gene transcription is mediated entirely through an increase in cAMP-dependent CREB binding (Delgado et al., 1998, 1999f,g; Delgado and Ganea, 1999).

Some of the detailed pathways leading from $VPAC_1$ to gene transactivation have been elucidated (Fig. 4). Reduction of IFNγ-induced IRF-1 binding and synthesis is mediated through the inhibition of Jak1/2-STAT1 phosphorylation; although the step(s) between cAMP induction are not known, we eliminated the involvement of the induction of some of the members of the family of the suppressors of cytokine signaling (SOCS 1 and SOCS3) (Delgado and Ganea, 2000a) or of the activation of tyrosine phosphatases that dephosphorylate Jaks and STAT1 (Delgado, 2003a). Changes in the composition of the AP-1 complexes from cJun/cFos to JunB/cFos, with the subsequent decrease in AP-1 binding, are mediated through the cAMP-dependent inhibition of the MEKK1/MEK4/JNK pathway, which leads to a reduction in cJun phosphorylation and a direct, positive effect on JunB synthesis (Delgado and Ganea, 2000b). Replacement of c-Jun with JunB leads to an inactive transcriptional complex for many of the macrophage-derived cytokines. The cAMP-independent pathway stabilizes IkB by inhibiting the kinase activity of IKKα. The stabilized IkB sequesters the p65/p50 complexes in the cytoplasm (Delgado et al., 1998, 1999g; Delgado and Ganea, 1999). This results in a decreased NFκB binding to promoters. The cAMP-dependent pathway phosphorylates CREB, leading to its nuclear translocation and subsequent binding to and sequestration of CBP, which cannot bind to p65 (Delgado and Ganea, 2001c). In the absence of the coactivator CBP, the transcriptional complexes are not fully activated. In addition, the binding of activated CREB to IL-10 promoter activates its transcription (Delgado et al., 1999f). Moreover, by inhibiting the MEKK1/MEK3/6/p38 pathway, VIP reduces the phosphorylation of TBP, resulting in a reduced recruitment of RNA polymerase II (Delgado and Ganea, 2001c).

Of particular interest is the dual effect of VIP on IL-6 production by macrophages, depending on the nature and dose of the inflammatory stimuli (Martinez et al., 1998a,b). VIP inhibits the release of IL-6 from macrophages stimulated with an LPS dose range from 100 pg/ml to 10 μg/ml, whereas it enhances IL-6 secretion in unstimulated macrophages or macrophages stimulated with very low concentrations of endotoxin. Both stimulation and inhibition of IL-6 by VIP are mediated by different receptors and intracellular signals. Although the inhibition of LPS-induced IL-6 production is mediated through PAC_1 binding and PKC activation, stimulation of IL-6 release by VIP is VPAC1/cAMP dependent (Martinez et al., 1998a,b). A recent report has suggested that the inhibitory effect of VIP on IL-6 production could be mediated through the inhibition of LPS binding to its receptor CD14 in macrophages by inducing the shedding of membrane-bound CD14 (Delgado et al., 1999a).

4. Vasoactive Intestinal Peptide Effects on Macrophage-Derived Chemokines. Accumulation of immune cells at the site of pathogen invasion is mediated through inflammatory chemokines and immune cells expressing the appropriate chemokine receptors. The chemokine superfamily consists of low-molecular weight polypeptides that are categorized into four subfamilies, CXC, CC, C, and CX_3C, based on the arrangement of positionally conserved cysteine motifs within the N terminus. The CXC and CC chemokines predominate and, thus, have been the most extensively studied. The inflammatory chemokines are released primarily by cells involved in innate immunity, i.e., macrophages/monocytes, dendritic cells, and microglia. The great interest generated by the discovery of chemokines lies in their specificity; for example, the CXC chemokines IL-8 and macrophage inflammatory protein (MIP)-2 activate and induce the directional migration of neutrophils, whereas CC chemokines, including MIP-1α, MIP-1β, monocyte chemoattractant protein (MCP)-1, and RANTES, are chemotactic for monocytes/macrophages and T cells (Baggiolini et al., 1997; Rollins, 1997; Luster, 1998; Rossi and Zlotnik, 2000). Although several studies have addressed the effects of VIP on lymphocyte adhesion and traffic (Moore, 1984; Moore et al., 1988a,b; Bondesson et al., 1991; Johnston et al., 1994; Delgado et al., 1995; Xia et al., 1996a,b; Schratzberger et al., 1998), the reports are in little agreement beyond the fact that indeed VIP affects lymphocyte traffic. This is probably due to the fact that both chemokine production and expression of adhesion molecules and chemokine receptors depend on the cellular activation and differentiation stage and that VIP might affect all these processes. At the present time, there are few reports regarding the effects of VIP on inflammatory chemokine production and no information as to their effects on chemokine receptors. We reported that VIP inhibits the expression of two CXC chemokines (CXCL1/KC and CXCL2/MIP2) and four CC chemokines (CCL2/MCP1, CCL3/MIP1α, CCL4/MIP1β, and CCL5/RANTES) in LPS-stimulated mouse macrophages (Delgado and Ganea, 2001d; Pozo et al., 2002). Similarly, VIP inhibits endotoxin-induced expression of IL-8 (CXCL1) by human peripheral blood monocytes (Delgado and Ganea, 2003b). The inhibitory effect is medi-

ated by $VPAC_1$ and correlates with a reduction in NFκB binding and transactivating activity (Delgado and Ganea, 2001d, 2003a). The two CXC chemokines CXCL1 and CXCL2 act as specific chemoattractants for neutrophils, whereas the CC chemokines attract monocytes/macrophages and T lymphocytes. Accordingly, VIP treatment of human monocytes resulted in a reduced in vitro chemotactic activity for peripheral blood neutrophils, and the in vivo administration of VIP in a murine model of acute peritonitis led to a significant reduction in the time-dependent recruitment of neutrophils, macrophages, and lymphocytes in the peritoneal cavity (Delgado and Ganea, 2001d, 2003b). The inhibition of cellular recruitment to sites of inflammation may also hamper the possible in situ antigen presentation that may contribute to exacerbation of clinical signs in chronic inflammation and reactivity to these antigens in autoimmune disorders.

The possibility that VIP might modulate the expression of not only chemokines but also chemokine receptors opens new territories in neuroimmunomodulation, since differences in chemokine receptors impose the different migratory patterns on immature versus mature dendritic cells, and of Th1 versus Th2 cells (Sallusto et al., 1998; Sozzani et al., 2000; Zlotnik and Yoshie, 2000). Therefore, VIP might activate participants in the maturation and function of dendritic cells and in the differential development of Th1 and/or Th2 effector cells.

C. Vasoactive Intestinal Peptide Effects on Macrophages as a Link to Adaptive Immunity

Although secondary to dendritic cells, activated macrophages participate in the antigen specific responses acting as antigen-presenting cells (APCs), providing T lymphocytes with costimulatory molecules and a cytokine environment that influences the proliferation and differentiation of the T cells, serving as a link between innate and adaptive immunity. Several signals are required for the activation of naive T lymphocytes. The activation of naive CD4 T lymphocytes requires two signals delivered by APCs, leading to enhanced cytokine production and proliferation. The first signal, which confers specificity, is provided by the interaction of the antigenic peptide/MHC type II complex in the APC with the TCR. The second signal is provided by costimulatory molecules expressed on APCs. Among the accessory molecules, the B7 family appears to be the most potent. The B7 costimulatory pathway involves at least two molecules, B7.1 (CD80) and B7.2 (CD86), both of which can interact with their counter-receptors, CD28 and CTL-4, on T cells (June et al., 1994). In macrophages, B7.1 and B7.2 are expressed only following activation, with B7.2 being induced earlier and at higher levels than B7.1 (Lenschow et al., 1996). VIP is among the endogenous factors that regulate B7 expression in macrophages. Interestingly, this neuropeptide affects B7 expression in resting and activated macrophages in an opposite manner. In resting macrophages, VIP up-regulates B7.2, but not B7.1, expression at mRNA and protein level, both in vivo and in vitro. In contrast, in LPS/IFNγ-activated macrophages, VIP down-regulates both B7.1 and B7.2 expression. The effects of VIP on B7 expression correlate with effects on the stimulatory activity for T cells. The inhibition of B7.1/B7.2 expression in activated macrophages is in perfect agreement with the accepted role of VIP as an endogenous anti-inflammatory agent. Moreover, since VIP affects B7 but not MHC class II expression (Delgado et al., 1999b,j), this neuropeptide could actually contribute to peripheral tolerance by inducing T cell anergy. In contrast, the up-regulation of B7.2 in unstimulated macrophages could represent one of the mechanisms by which VIP supports Th2 differentiation, which is discussed below. A similar dual effect of VIP on B7 expression and costimulatory activity on antigen-specific T cells has been recently obtained with dendritic cells in vitro and in vivo (Ganea et al., 2003).

D. Vasoactive Intestinal Peptide Effects on Hematopoiesis

Immune cells in mammals are generated through hematopoiesis in the bone marrow. During an active immune response, hematopoiesis is enhanced through the effect of immune mediators, particularly cytokines. Once the infectious agent is eliminated, immune cell homeostasis is reestablished, in part through a reduction in hematopoiesis. Although peptidergic innervation has been identified in the bone marrow (Felten, 1993; Tabarowski et al., 1996; Imai et al., 1997), there are few reports on the effects of neuropeptides on hematopoiesis. A recent study has demonstrated that VIP inhibits, by a VPAC1-dependent manner, the proliferation of both granulocytic and erythroid bone marrow progenitors, mostly through a direct effect on the $CD34^+$ bone marrow cells (Rameshwar et al., 2002). This inhibitory effect of VIP on hematopoiesis could contribute to the reestablishment of homeostasis in vivo, supporting the general anti-inflammatory role of VIP.

V. Effects of Vasoactive Intestinal Peptide on Adaptive Immunity

Among the participants in adaptive immunity, T lymphocytes are the major targets for VIP regulation. Both activation of naive T cells and differentiation into effector and memory cells are affected by VIP (Fig. 5).

A. Vasoactive Intestinal Peptide Effects on T Cell Activation

As we have discussed above, the antigen-induced activation of naive $CD4^+$ T cells requires signals provided by APCs, represented primarily by DCs and macrophages in the periphery and microglia in the CNS. One of the mechanisms for the general anti-inflammatory action of VIP could consist of the reduction of the stim-

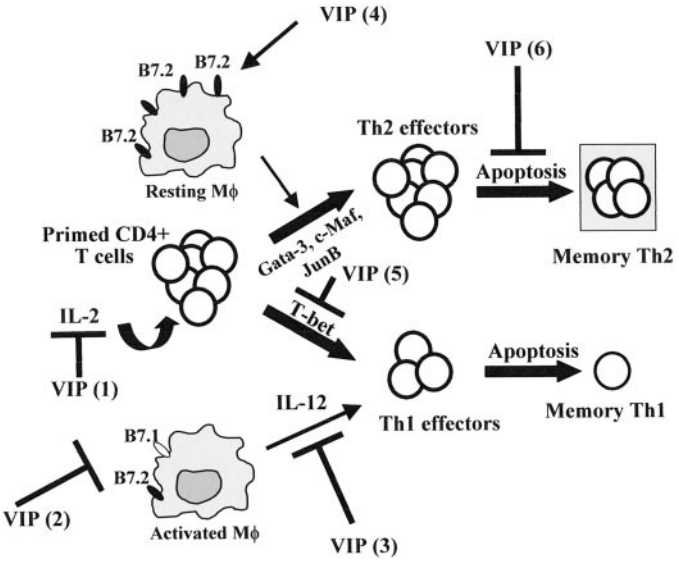

FIG. 5. Effects of VIP on $CD4^+$ T cell activation and differentiation. VIP inhibits IL-2 production and subsequent proliferation of primed T cells (1). VIP reduces the expression of the costimulatory molecules B7.1/B7.2 on activated macrophages and inhibits macrophage-induced activation of antigen-specific T cells (2). VIP reduces IL-12 production from activated macrophages, and inhibits the subsequent differentiation of primed T cells into Th1 effectors (3). VIP induces B7.2 expression on resting macrophages and promotes the subsequent differentiation of primed T cells into Th2 effectors (4). VIP acts directly on $CD4^+$ T cells and biases Th1/Th2 differentiation in favor of Th2 by promoting the expression of Th2 transcription factors (c-Maf, JunB, and GATA-3) and inhibiting the expression of T-bet (the Th1 master transcription factor) (5). VIP inhibits apoptosis of Th2, but not Th1, effectors, promoting the development of Th2 memory cells (6).

ulatory and/or costimulatory activity of APCs. Indeed, VIP down-regulates B7.1/B7.2 expression, both at mRNA and protein level, in LPS/IFNγ-activated macrophages, and the inhibition of B7 expression correlates with a reduction in the stimulation of antigen-specific T cell proliferation (Delgado et al., 1999j). Similarly, VIP inhibits B7.2 and CD40 expression in LPS-stimulated primary microglia and microglial cell lines (Kim et al., 2002). Recently, we focused on the effects of VIP on the expression of stimulatory and costimulatory molecules in DCs. Similar to macrophages, VIP down-regulates the expression of surface B7.1/B7.2, without affecting MHC class II and CD40 expression in LPS-stimulated bone marrow-derived DCs (Ganea et al., 2003). The proliferation of TCR-transgenic $CD4^+$ T cells cultured in vitro in the presence of the specific antigenic peptide and of IFNγ- or LPS-stimulated DCs was inhibited following treatment of DCs by VIP. Similar results were obtained in vivo, upon inoculation of DCs and antigenic peptide into TCR-transgenic mice, followed by purification of lymph node $CD4^+$ T cells and in vitro restimulation. The T cells obtained from animals that received LPS + VIP-treated DCs expressed a lower degree of proliferation (Ganea et al., 2003). The inhibition of the expression of costimulatory molecules in activated macrophages, microglia, and DCs is in agreement with the accepted role of VIP as an endogenous anti-inflammatory agent. Moreover, since VIP affects B7 but not MHC class II expression, the neuropeptide could actually contribute to peripheral tolerance by inducing T cell anergy.

Following interaction with APCs and cognate antigen, activated $CD4^+$ T cells proliferate in the presence of autocrine or paracrine IL-2. VIP has been reported to inhibit IL-2 production and subsequent T cell proliferation (for review, see Ganea, 1996; Gomariz et al., 2001). Both $VPAC_1$ and $VPAC_2$ mediate the inhibitory effect of VIP/PACAP on IL-2 production, primarily through the induction of intracellular cAMP (Wang et al., 1999). VIP inhibits the expression of the IL-2 gene by affecting both nuclear factor of activated T cells (NF-AT) and AP-1 binding (for review, see Ganea, 1966; Wang et al., 2000).

B. Vasoactive Intestinal Peptide Effects on $CD4^+$ T Cell Differentiation

Following antigenic stimulation and several rounds of proliferation, $CD4^+$ T cells differentiate into Th1 and Th2 effectors, with different cytokine profiles and functions. Various factors such as the nature of the APCs, the nature and amount of antigen, the genetic background of the host, and particularly the cytokine microenvironment control the differentiation into Th1 or Th2 effectors (O'Garra, 1998). In addition, endogenous factors such as progesterone and glucocorticoids have been reported to favor Th2 differentiation (Miyaura and Iwata, 2002). Recent studies indicate that neuropeptides, particularly VIP, also favor Th2 differentiation. Macrophages treated in vitro with VIP were shown to gain the ability to induce Th2-type cytokines (IL-4 and IL-5) and inhibit Th1-type cytokines (IFNγ and IL-2) production in primed $CD4^+$ T cells (Delgado et al., 1999b). Similar results were obtained recently with bone marrow-derived dendritic cells (Ganea et al., 2003). In agreement with the in vitro results, administration of VIP to immunized mice results in a reduction in the number of IFNγ-secreting cells and an increase in the number of IL-4 secreting cells (Delgado et al., 1999b). Two recent studies confirmed the prevalence of Th2-type responses in transgenic mice overexpressing the human $VPAC_2$ receptor in $CD4^+$ T cells (Voice et al., 2001) and of Th1-type responses in $VPAC_2$ knockout mice (Goetzl et al., 2001). The studies in mice genetically altered for $VPAC_2$ expression indicate the importance of endogenous VIP for the regulation of Th1/Th2 responses in vivo and the prominent role of the VPAC2 receptor in CD4+ T cell differentiation and/or responses. The role of endogenous, Th2-derived VIP in maintaining the Th2 bias was confirmed recently in studies in which elimination of VIP from TCR-stimulated T cells with VIPase antibodies resulted in the readjustment of the Th1/Th2 balance (Voice et al., 2003). These studies confirm the concept that VIP affects the Th1/Th2 balance in vivo and indicate the prevalent role of $VPAC_2$ in this process.

1. Molecular Mechanisms by which Vasoactive Intestinal Peptide Promotes Th2-Type Immune Response. A number of different, nonexcluding mechanisms could

contribute to VIP bias in favor of Th2 immunity (Fig. 5). The neuropeptides could affect Th1/Th2 generation directly or through effects on APCs or could act on the already generated effectors by preferentially promoting Th2 proliferation and/or survival. VIP was shown to affect APCs in ways that are relevant to Th1/Th2 differentiation. First, VIP inhibits IL-12 production from activated macrophages (Xin and Sriram, 1998; Delgado et al., 1999e). Since IL-12 is a major factor controlling Th1 differentiation, the Th1/Th2 balance will be altered in favor of Th2 in the presence of suboptimal doses of IL-12. Second, the level of costimulatory molecule expression on APCs is more important for Th2, compared with Th1 development (Ranger et al., 1996). VIP has been shown to induce B7.2 expression in resting macrophages and immature DCs and to increase their stimulatory capacity for primed T cells (Delgado et al., 1999b; Ganea et al., 2003). The role of the VIP-induced B7.2 in promoting Th2-type responses is supported by the fact that the capacity of VIP-treated resting macrophages to induce Th2 cytokines in antigen-primed T cells is abolished by neutralizing anti-B7.2 antibodies (Delgado et al., 1999b). VIP could also affect differentiating T cells directly. Indeed, addition of VIP to TCR-transgenic T cells cultured with irradiated APCs and antigenic peptide in the presence or absence of polarizing cytokines (IL-12 for Th1 and IL-4 for Th2 differentiation) leads to increased levels of IL-4 and decreased levels of IFNγ, suggesting that VIP promotes the development of Th2 cells directly by acting on the differentiating $CD4^+$ T cells (Ganea et al., 2003). Whether VIP affects the expression of the master transcriptional factors T-bet and GATA-3, required for Th1 and Th2 differentiation, respectively, remains to be established. Previous studies indicated that VIP induces expression of JunB, one of the transcriptional factors required for Th2 differentiation, in both macrophages and newly stimulated T cells (Delgado and Ganea, 2000b; Wang et al., 2000), and recently, VIP was reported to up-regulate c-Maf, a transcription factor essential for IL-4 expression (Voice and Goetzl, 2003).

Finally, VIP could act on the already generated Th1/Th2 effectors. Both in vitro and in vivo experiments indicate that VIP supports the survival and possibly the proliferation of Th2, but not Th1, effectors (Delgado et al., 2002c; see below). The in vivo-surviving Th2 cells following inoculation with antigen and VIP exhibit memory cell markers (Delgado et al., 2002c). The reasons why VIP and PACAP affect specifically Th2 cell viability are not clear. The answer might reside in differences in VIP receptor density or in the nature of the transcriptional factors affecting anti-apoptotic molecules present in Th1 and Th2 cells. In addition to promoting Th2 survival, VIP also affects Th1/Th2 migration in a differential manner. Recent studies indicate that VIP promotes Th2 and inhibits Th1 migration in vitro and in vivo, through effects on the production of chemokines (Ganea et al., 2003; see below). The preferential recruitment of Th2 effectors could further contribute to the induction of Th2- as opposed to Th1-type immunity by VIP.

C. Effects of Vasoactive Intestinal Peptide on $CD4^+$ and $CD8^+$ T Cell Function

1. T Cell Traffic and Adhesion. Chronic in vivo administration of VIP resulted in a significant inhibition of T cell migration through lymph nodes and Peyer's patches, particularly for $CD4^+$ T cells (Moore et al., 1988a,b; Serizawa et al., 1996; Miura et al., 1997). The effects of VIP on T cell adhesion and chemotaxis could play an important role in respect to traffic. A dual role for VIP in T cell chemotaxis has been reported. In some systems, VIP acts as a T cell chemoattractant (Johnston et al., 1994; Xia et al., 1996a; Schratzberger et al., 1998). However, in T cells expressing solely $VPAC_2$, VIP inhibits cytokine-induced chemotaxis (Xia et al., 1996b). A similar dual effect was observed for dendritic cells. VIP acts as a chemoattractant for immature DCs and inhibits chemotaxis in CCR7-expressing, mature DCs (Dunzendorfer et al., 2001). The significance for the dual effect of VIP on migration of $VPAC_1$ versus $VPAC_2$ regulation during T cell differentiation and DC maturation remains to be established.

2. $CD4^+$ T Cell Function: Production of Cytokines.

a. Th1 Cytokines: Interleukin-2 and Interferon γ. Th1 and Th2 effectors produce different cytokines and have different functions in the immune response. IL-2 and IFNγ are produced by antigen-stimulated Th1 cells and contribute to cell-mediated immunity. A number of studies established that VIP inhibits IL-2 production primarily through a cAMP-dependent pathway (Ottaway, 1987; Boudard and Bastide, 1991; Ganea and Sun, 1993; Sun and Ganea, 1993; Tang et al., 1995, 1996; Jiang et al., 1998). VIP reduces IL-2 gene transcription through inhibition of NF-AT and changes in the composition of the AP-1 complex, from c-Jun/c-Fos to JunB/c-Fos, with subsequent decreases in AP-1 binding and transactivating activity (Tang et al., 1996; Wang et al., 2000). In contrast to IL-2, there is no agreement on the effect of VIP on IFNγ production. Muscettola and Grasso (1990) reported inhibition of IFNγ production by VIP in mitogen-stimulated human peripheral blood mononuclear cells, and Taylor et al. (1994) showed a similar inhibitory effect in murine antigen-primed T cells re-exposed to antigen. In contrast, other studies reported a lack of VIP effect on IFNγ production by naive T cells stimulated through the TCR (Ganea and Sun, 1993; Tang et al., 1995) or an increase in IFNγ production by antigen-specific Th1 cell lines (Jabrane-Ferrar et al., 1999). One reason for the disagreement might be the differential expression of $VPAC_1$ and $VPAC_2$ depending on the differentiation stage of the T cells. However, regardless of the in vitro studies, the in vivo administration of VIP together with LPS results in a definite

reduction in IFNγ in both serum and peritoneal fluid (Delgado et al., 1999e).

b. Th2 Cytokines: Interleukin-4 and Interleukin-5. Initial studies with naive T cells stimulated through the TCR showed that VIP and PACAP inhibit IL-4 production at a posttranscriptional level. The VIP inhibitory effect was mediated through a reduction in IL-2, which led to a destabilization of the newly synthesized IL-4 protein (Wang et al., 1996a). In contrast, no inhibitory effect was observed with established Th2 cell lines cultured in the presence of exogenous IL-2 (D. Ganea, unpublished data). Subsequent studies demonstrated that VIP promotes Th2 differentiation and subsequent IL-4 production (Delgado et al., 1999b, 2002c). Also, $VPAC_2$-overexpressing transgenic mice exhibit higher IL-4 and IL-5 levels in serum, indicative of a prevalent Th2 differentiation (Voice et al., 2001). Therefore, although there is no apparent effect on IL-4 production by already established Th2 cells, VIP promotes Th2 differentiation and subsequently leads to an increase in IL-4 production.

VIP and IL-4 appear to be involved in a complex autoregulatory feedback. On one hand, antigen-stimulated Th2 cells secrete VIP (Delgado and Ganea, 2001a), which then promotes Th2 differentiation and survival, leading to more IL-4 production (Voice et al., 2001; Delgado et al., 2002c). On the other hand, IL-4 inhibits constitutive VIP production by macrophages and reduces the levels of $VPAC_2$ receptors on Th2, but not Th1, cells (Metwali et al., 2000, 2002).

IL-5, a Th2 cytokine, functions as an essential factor for the generation and differentiation of eosinophils. VIP was shown to stimulate IL-5 release from activated splenic and granuloma T cells in a model of murine schistosomiasis (Mathew et al., 1992; Weinstock, 1992). Since VIP promotes Th2 differentiation, it was expected that, similar to IL-4, VIP will induce an increase in IL-5 in vivo; indeed, in $VPAC_2$ transgenic mice, there is an increase in IL-4, IL-5, and eosinophilia (Voice et al., 2001).

c. In Vivo Consequences of the Vasoactive Intestinal Peptide Alteration of the T Helper 1/T Helper 2 Balance. Two recent studies contributed significantly to our understanding of the in vivo effects of VIP. Transgenic mice overexpressing the human $VPAC_2$ receptor in $CD4^+$ T cells show increased blood levels of IgE and IgG as well as eosinophilia, and their $CD4^+$ T cells produce high levels of IL-4 and IL-5. These mice develop more severe cutaneous allergic reactions and have depressed delayed-type hypersensitivity (Voice et al., 2001). In contrast, $VPAC_2$-deficient mice have lower than normal IgE levels, less IL-4, and a 70% decrease in cutaneous anaphylaxis; however, these mice express enhanced delayed-type hypersensitivity (Goetzl et al., 2001). These results position $VPAC_2$ as the functionally predominant VIP receptor in $CD4^+$ T cell differentiation and indicate that $VPAC_2$ plays an essential role in vivo in tilting the Th1/Th2 balance in favor of Th2.

3. Function of Cytotoxic T Cells. Cytotoxic $CD8^+$ T cells kill targets through a major, calcium-dependent, perforin/granzyme-mediated mechanism, with a minor contribution from the calcium-independent, FasL-mediated pathway (Berke, 1995). VIP does not affect the perforin/granzyme-mediated cytotoxicity but inhibits drastically the FasL-mediated lysis of both allogeneic and syngeneic Fas-bearing targets through the inhibition of FasL expression (Delgado and Ganea, 2000c). Since VIP affects only the minor FasL/Fas-mediated lysis in $CD8^+$ cytotoxic T cells, their overall effect on $CD8^+$ T cell cytotoxicity does not have major consequences.

In contrast, $CD4^+$ cytotoxic T cells kill targets through FasL/Fas-mediated interactions (Stalder et al., 1994; Ju et al., 1995), and VIP has a major impact on their cytotoxic function. Cytotoxic $CD4^+$ T cells are activated by APCs in a MHC II-restricted manner, leading to FasL up-regulation. Subsequently, the activated $CD4^+$ T cells lyse both cognate APCs (direct targets) and neighboring Fas-bearing cells (bystander targets) in an antigen-independent and MHC-nonrestricted manner (Wang et al., 1996b; Smyth, 1997). VIP inhibits FasL expression in $CD4^+$ effectors generated in vivo and reduces the cytotoxicity for both direct and bystander targets (Delgado and Ganea, 2001e). Killing of bystander targets results in collateral damage, detrimental particularly in tissues with limited MHC II expression, such as brain. For example, in experimental allergic encephalomyelitis, myelin-specific $CD4^+$ T effectors contribute to the pathology through lysis of antigen-nonspecific targets (Thilenius et al., 1999). Similar mechanisms may be responsible for tissue damage in other autoimmune diseases as well (De Maria and Testi, 1998). In this respect, endogenous agents such as VIP capable to down-regulate FasL expression on T cells may be important in the control of FasL/Fas-mediated lysis of innocent bystanders. This may be particularly beneficial in immune-privileged organs such as brain and the anterior chamber of the eye, where these neuropeptides are abundant.

D. Effects of Vasoactive Intestinal Peptide on the Survival of $CD4^+$ T Effectors

The majority of Th1/Th2 effectors are eliminated through apoptosis following a relatively short period of intense activity. The few surviving effector T cells become antigen-specific memory cells, which differ from naive T cells in terms of homing patterns and activation requirements. The elimination of effector T cells occurs through either active or passive apoptosis, depending on whether the antigen persists or is eliminated.

Although VIP acts as survival factors in the CNS (Uchida et al., 1996; Gressens, 1999; Gozes and Brenneman, 2000; Reglodi et al., 2000; Antonawich and Said, 2002), they were expected to promote T lymphocyte ap-

optosis in the peripheral immune system, based on their general anti-inflammatory function. However, in contrast to the expected outcome, VIP was shown to protect activated $CD4^+$ T cells against active (antigen-induced) cell death both in vitro and in vivo by inhibiting FasL ligand (FasL) expression (Delgado and Ganea, 2000d). FasL expression requires a number of transcription factors, including NFκB and Egr2 and 3. VIP inhibits NFκB binding to the FasL promoter through the stabilization of the IkB inhibitor and subsequent p65 nuclear translocation. In addition, VIP prevents NF-ATp nuclear translocation in a calcineurin-independent manner and inhibits the expression of Egr2 and 3, which is NF-ATp dependent (Delgado and Ganea, 2001f).

At first glance, the protective effect against apoptosis, resulting in a higher number of surviving effector $CD4^+$ T cells, should lead to a more intense inflammatory response, in apparent contradiction to the general anti-inflammatory effect of VIP. However, the effector Th1 and Th2 cells have different immune functions, with Th1 effectors being the major players in acute inflammation, through the mobilization and activation of potent inflammatory cells such as neutrophils and macrophages. Therefore, the preferential development of the Th2-type immunity, with its subsequent reduction in Th1 effectors, would have an anti-inflammatory effect.

VIP might function as specific Th2 survival factors. This would be in agreement with the observed in vivo Th2-type immunity upon administration of exogenous VIP/PACAP (Delgado et al., 1999b) or in transgenic mice overexpressing the $VPAC_2$ receptor (Voice et al., 2001). The preferential effect of VIP on the survival of Th2 effectors in vivo and in vitro was indeed confirmed recently (Delgado et al., 2002c). When thymectomized hosts were reconstituted with Th1 and Th2 cell lines derived from TCR transgenic mice, followed by immunization with the specific antigen and VIP, transgenic $CD4^+$ T cells could be recovered 62 days later only from hosts that received Th2 cells. When the hosts received transgenic $CD4^+$ T cells instead of Th1 or Th2 cell lines, the recovered transgenic T cells exhibited a phenotype typical of memory Th2 cells ($CD44^{hi}$, L-selectin^{lo}, $CD45RB^{lo}$, and IL-4 and IL-5 but not IFNγ or IL-2 producers). The preferential survival of Th2 cells in the presence of VIP was also confirmed in vitro, with VIP/PACAP supporting the survival of Th2, but not Th1, TCR-transgenic cell lines (Delgado et al., 2002c).

E. Vasoactive Intestinal Peptide Favors the Directional Migration of T Helper 2 Cells through Effects on Chemokines

The selective recruitment of effector T cells to an inflammatory site is mediated through chemokines (CKs) and chemokine receptors. Although there is a high degree of redundancy in chemokine function, Th1 and Th2 homing was shown to depend to a large degree to their different response to CKs due to the expression of different chemokine receptors (Bonecchi et al., 1998a; Sallusto et al., 1998). Th1 cells express CXCR3 and CCR5, whereas Th2 cells preferentially express CCR3, CCR4, and CCR8 (Loetscher et al., 1996, 1998; Bonecchi et al., 1998a; Qin et al., 1998). Among CKs, IP-10 (CXCL10), MIG (CXCL9), and I-TAC (CXCL11) bind only to CXCR3, expressed on activated Th1 cells, NK cells, and thymocytes, whereas MDC (CCL22), TARC (CCL17), and TCA-3 (CCL1) bind to CCR4 and/or CCR8, expressed on activated Th2 cells, NK cells, and thymocytes (Zlotnik and Yoshie, 2000). Activated Th1 and Th2 cells were reported to migrate in response to IP-10 (CXCL10) and MDC (CCL22), respectively (Andrew et al., 1998; Bonecchi et al., 1998a, 1998b; Imai et al., 1999). In a recent study VIP was shown to down-regulate CXCL10 and up-regulate CCL22 in spleen cell cultures (Jiang et al., 2002). Similar results were obtained with bone marrow-derived DC (Ganea et al., 2003). VIP induced CCL22 release in unstimulated and stimulated DCs and inhibited CXCL10 release from stimulated DCs. There is a good correlation between the VIP effects on CCL22 and CXCL10 production and chemotaxis for Th2 and Th1 cells, i.e., supernatants from DCs treated with VIP being chemoattractant for Th2, but not Th1, cells (Ganea et al., 2003).

VI. Effects of Vasoactive Intestinal Peptide in Brain Inflammation and Neurodegeneration

Microglia, the ontogenetic and functional equivalents of mononuclear phagocytes in somatic tissues (Dixon et al., 1991), are widely recognized as cells that play a central role in the regulation of immune and inflammatory activities, as well as tissue remodeling in the CNS. Microglia serve as antigen-presenting cells of the CNS control the proliferation of astrocytes, produce cytokines and other soluble factors associated with an immunologic response, and remove tissue debris during development and following brain trauma (Streit et al., 1988; Gonzalez-Scarano and Baltuch, 1999). However, in response to brain injury, infection, or inflammation, microglia readily become activated in a way similar to peripheral tissue macrophages, a process that includes differentiation and probably invasion and proliferation. Activation of microglia is a histopathological hallmark of several neurodegenerative diseases where inflammation plays a major role, such as multiple sclerosis (Matsumoto et al., 1992), Alzheimer's disease (Rogers et al., 1988), Parkinson's disease (McGeer et al., 1988), and AIDS dementia (Dixon et al., 1991; Merrill and Chen, 1991; Spencer and Price, 1992; Dickson et al., 1993). Although the precise mechanism by which microglia mediate neuronal cell injury is not completely understood, it has been proposed that in response to proinflammatory cytokines or antigens such as LPS, activated microglia secrete inflammatory mediators such as NO and proinflammatory cytokines, including TNFα,

IL-6, and IL-1β, which contribute to pathophysiological changes associated with several neuroimmunologic disorders (Lee et al., 1993; Taupin et al., 1993; Lipton et al., 1994; Chao et al., 1995; Liu et al., 1996; Bartholdi and Schwab, 1997; Ding et al., 1997; Peterson et al., 1997; McManus et al., 1998; Streit et al., 1998). Many studies have highlighted the strict link between production of these factors and the nature and intensity of the host inflammatory response in CNS. Secretion of proinflammatory products is followed later by the production of anti-inflammatory cytokines such as IL-10 and TGF-β. Microglia are present in large numbers within the brain, but they are not distributed with a uniform density or morphology in all major divisions of the brain (Lawson et al., 1990), and it has been shown that neurons in various brain regions are differentially susceptible to inflammation-related degeneration (Kim et al., 2000a). Among several regions examined, neurons in the mesencephalic region are more sensitive to bacterial endotoxin-induced neurotoxicity, and this region-specific susceptibility to LPS-induced degeneration is most likely attributable to the abundance of microglia. Since the intensity and duration of an inflammatory process in the CNS depend on the local balance between pro- and anti-inflammatory factors, a number of regulatory molecules termed microglia-deactivating factors have been the focus of considerable research.

In the last 2 years, numerous works have clearly identified VIP as a microglia-deactivating factor. Hence, VIP inhibits in a dose-dependent manner LPS-induced production of TNFα, IL-1β, IL-6, NO, and several proinflammatory chemokines (RANTES, MCP-1, MIP-1α, MIP-1β, MIP-2, and KC) by microglia (Kim et al., 2000b; Delgado et al., 2002b, 2003). At the same time, VIP stimulates the expression of the anti-inflammatory factors IL-10 and IL-1Ra (Delgado et al., 2003). The VIP-mediated inhibition of chemokine production correlates with a decreased chemotactic capacity of activated microglia for macrophages, lymphocytes, and neutrophils (Delgado et al., 2002b). These effects are mainly mediated through $VPAC_1$ and a cAMP/PKA-dependent pathway. Similar to macrophages, the VIP regulation of endotoxin-induced proinflammatory cytokines and chemokines occurs at the transcriptional level and involves several transcriptional factors, such as NFκB, CREB, c-Jun, and CREB binding protein (Delgado, 2002a; Delgado et al., 2002b, 2003), although the blockade of the LPS-induced MEKK1/MEK4/JNK pathway could also be participating (Delgado, 2002b). On the other hand, VIP also inhibits the expression of several genes involved in the inflammatory response of microglial cells induced by one of the most important microglia stimulators, IFNγ, including CD40, IP-10, and iNOS (Kim et al., 2002; Delgado, 2003a). Molecular mechanisms involve the VIP inhibition of IFNγ-induced Jak1-2 and STAT1 phosphorylation and the subsequent binding of activated STAT1 to the GAS motif in the IRF-1 and CD40 promoter and to the IFN-stimulated response element motif of the IP-10 promoter (Delgado, 2003a).

Because VIP has been shown to inhibit endotoxin-induced production of proinflammatory mediators by activated microglia (Delgado et al., 2002b, 2003), this neuropeptide is a plausible candidate for neuroprotection under inflammatory conditions. Indeed, VIP has been shown to prevent bacterial endotoxin-induced neuronal cell loss in vitro in cocultures of mouse embryonic neurons and neonate mesencephalic microglial cells and in primary mixed glia-neuron cultures (Delgado and Ganea, 2003c). This neuroprotective effect was especially relevant when dopaminergic neurons (tyrosine hydroxylase TH^+ neurons) were analyzed in these cultures. VIP-mediated neuroprotection of dopaminergic neurons has important therapeutic perspectives, because the loss of these neurons in substantia nigra is one of the causes of Parkinson's disease (see *Section VII.D.*). LPS-induced neurodegeneration was associated with and probably mediated by an increased microglial activation, characterized by changes in morphology from a ramified resting state to an amoeboid appearance, with an increase in the expression of Mac-1^+ microglial cells, free radical production, and production of the proinflammatory mediators TNFα and NO (Delgado and Ganea, 2003c). Involvement of activated microglia and their derived cytotoxic factors in inflammation-induced neurodegeneration is suggested by the facts that LPS failed to induce cell loss in cultures with neurons alone and that cell-free supernatants from LPS-stimulated microglia cultures induced significant cell death in neurons cultured alone. Treatment with VIP inhibited in a dose-dependent manner all these hallmarks of LPS-induced microglial activation and the production of TNFα and NO, suggesting that the neuroprotective effect of VIP against endotoxin could be mainly mediated through the inhibition of microglia-derived cytotoxic factors.

The neuroprotective effect of VIP under inflammatory conditions has also been described in vivo (Delgado and Ganea, 2003c). LPS injected in the third ventricle (i.c.v.) of adult mouse brains causes a dramatic neuronal cell loss in certain brain areas, especially in the periventricular nucleus, caudate putamen, and substantia nigra (SN). In contrast, other brain areas such as cortex and hippocampus were not susceptible to LPS-induced neurotoxicity. The LPS-induced neurodegeneration is accompanied by massive microglia activation and by an increase in the expression of TNFα, IL-1β, and iNOS mRNA in microglia in the same areas. Local VIP administration (i.c.v. injection) prevented LPS-induced neurodegeneration, microglia activation, and TNFα, IL-1β, and iNOS expression in periventricular nucleus, caudate putamen, and SN. Interestingly, systemic VIP administration was also neuroprotective, although VIP doses 15-fold higher than that local VIP administration were needed to obtain similar results (Delgado and Ga-

nea, 2003c). This is important because it overcomes direct administration in brain. During endotoxemia, caused by a systemic Gram-negative bacterial infections (see *Section VII.A.*), in addition to the clinical manifestations characterized by hyperventilation, hypercoagulation, fever, cachexia, hypotension, and multiple organ failure, certain brain regions, especially the circumventricular organs and the parenchymal structures surrounding them, are seriously affected by endotoxin, expressing massive neurodegeneration. The anti-inflammatory action of VIP in the CNS could contribute to its preventive effect on systemic endotoxemia (Delgado et al., 1999d). The blood-brain barrier, normally present in these areas as tight junctions between endothelial cells, is largely compromised under inflammatory conditions, explaining the diffusion of large molecules (such as VIP) into the perivascular region. In addition, a specific and rapid transport system for VIP and PACAP38 across the intact blood-brain barrier has been recently demonstrated (Somogyvari-Vigh et al., 2000; Dogrukol-Ak et al., 2003). Therefore, adjustments in the dose of systemically administered VIP or analogs could be necessary to maximize the therapeutic effect on brain damage resulting from focal inflammation and brain injury (see *Section VII.*). Another possibility is to increase VIP delivery into the brain. Two strategies have been addressed: 1) enhancing the VIP transport through the brain-blood barrier following systemic administration, and 2) alternative routes of administration. The transcytosis of VIP through the endothelial cells of the brain microvessels into the brain parenchyma can be enhanced by conjugating VIP with a delivery vector such as the monoclonal antibody OX26 against the transferring receptor (a receptor especially abundant in the CNS capillaries) (Bickel et al., 2001). This strategy had physiological consequences, because intracarotid artery perfusion of biotinylated VIP coupled to OX26/avidin resulted in an increased brain blood flow (Wu and Pardridge, 1996). Alternative routes of administration also include intranasal drug delivery, which circumvents the brain-blood barrier and has been demonstrated to be effective for other peptides (reviewed by Gozes, 2001).

VII. Clinical Implications

Due to its pleiotropic and multifunctional characteristics, VIP and VIP analogs have been used or suggested for the development of therapies for several disorders, including impotence, skin disorders, asthma, alterations of circadian rhythms, tumors (glioblastoma, prostate, lung, pancreatic, and breast cancer), heart and lung ischemia/reperfusion injuries, type 2 diabetes, neuronal defects, and learning and memory defects (for review, see Gozes and Furman, 2003). However, we review only the potential therapeutic applications of VIP in immunological disorders. Due to its inhibition of exacerbated inflammatory responses and its ability to shift the immune response toward a Th2-type response, VIP emerges as an attractive therapeutic factor for inflammatory disorders and/or Th1-type autoimmune diseases.

A. Septic Shock

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 (Morrison and Ryan, 1987; Danner et al., 1991). Indeed, the administration of the endotoxin LPS to experimental animals leads to pathological changes similar to the human septic shock syndrome. Our understanding of the pathophysiology of septic shock has increased markedly over the past few years (Parrillo, 1993; Heumann and Glauser, 1994; Baumgartner and Calandra, 1999). Septic shock begins with bacteria overwhelming the host defenses and the release of toxic products that activate plasma factors (complement and clotting molecules) and cells of the immune system (polymorphonuclear cells, monocytes/macrophages, and lymphocytes). Invading bacteria and bacterial products trigger the release of a complex array of host mediators, including cytokines. Normally cytokines are autocrine and paracrine molecules that act locally to control the host response to invading organisms. In fact, by affecting coagulation and leukocyte mobilization and by activating professional phagocytes, cytokines contribute to the control of a local infection. It is generally recognized that the severe pathological consequences of the septic shock syndrome result from a hyperactive and out-of-balance network of endogenous proinflammatory cytokines, including TNFα, IL-12, IL-6, and IFNγ (Van Deuren et al., 1992). The overproduction of inflammatory cytokines generates systemic activation, which affects vascular permeability, cardiac function, and induces metabolic changes that can lead to tissue necrosis and eventually to multiple-organ failure and death.

Since VIP inhibits the production of proinflammatory macrophage-derived cytokines in vivo and in vitro, it was expected to protect against endotoxemia. Indeed, VIP administration protects against high lethal endotoxemia in a murine model for septic shock syndrome (Delgado et al., 1999d). Endotoxemic animals suffer 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, as well as severe congestion of the medullar sinusoids in the spleen and segmental ischemia of the bowel with regions of hemorrhage or necrosis and an infracted caecum. In contrast, VIP-treated individuals did not present any of the histopathological alterations associated with septic shock, such as disseminated intravascular coagulation, leukocyte infiltration and inflammation in various organs, mesenteric ischemia and acute tubular necrosis in the

kidneys (Delgado et al., 1999d). VIP acts presumably by down-regulating the proinflammatory mediators such as TNFα, IFNγ, IL-6, IL-12, and NO and up-regulating production of IL-10 (Delgado et al., 1999d,e,f,g,i,j). In addition, the inhibitory effect of VIP on the production of proinflammatory chemokines is also crucial in its preventive action on endotoxemia (Delgado and Ganea, 2001d), because transmigration of inflammatory cells to target inflamed organs is one of the initial events.

Studies with specific VPAC agonists and the use of a $PAC_{1-/-}$ mouse indicated that $VPAC_1$ is the main mediator involved in the preventive effect of VIP on endotoxemia (Delgado et al., 2000a; Martinez et al., 2002), although $VPAC_2$ and PAC_1 also play a role. $VPAC_1$, and probably $VPAC_2$, are involved in the inhibitory action of VIP in the TNFα-induced cascade, affecting the infiltration of activated inflammatory cells in certain organs, production of cytotoxic inflammatory factors, and the subsequent tissue necrosis (Delgado et al., 2000a). PAC_1 is mainly related to the inhibition of IL-6 production and the subsequent activation of acute phase proteins and disseminated coagulation (Martinez et al., 2002).

The current strategies of adjunctive therapy for human septic shock are mainly derived from observations made in animal models. Promising experimental results prompted large-scale randomized clinical trials with a variety of agents such as anti-endotoxin monoclonal antibodies (Greenman et al., 1991; Ziegler et al., 1991; Bone et al., 1995), glucocorticoids (Bone et al., 1987), or ibuprofen (Bernard et al., 1997) for nonspecific down-regulation of inflammation, antagonists of platelet activating factor (Dhainaut et al., 1994), antagonists of bradykinin (Fein et al., 1997), IL-1 receptor antagonists (Fisher et al., 1994), and monoclonal anti-TNF antibodies (Abraham et al., 1995) or soluble dimeric TNF receptor fusion proteins (Fisher et al., 1996). Unfortunately, despite some promising results during preliminary trials, all the major clinical studies of immunomodulators in sepsis have yielded disappointing results (Bone, 1996; Zeni et al., 1997). Several causes may explain this relative disappointment. In animal models, the cascade of events starting from the initial stimulus and usually ending with the death and the resulting cytokine cascade production generally follows a predictable time course. Thus, experimental protocols designed to block one cytokine cascade or another are relatively straightforward. In contrast, in human septic shock syndrome, the sequence of events is more complex, and the course of the disease generally lasts days rather than hours, as seen in most animal models. The design of a clinical trial is based essentially on experimental models using parenteral injections of LPS/bacteria, in which cytokine blockade is beneficial, not on animal models with focal infections, where such therapy is usually not effective. The organisms and site of infections are diverse; importantly, the patients have a large variety of underlying diseases, which is not the case in animal studies. Interventions in animal models have been successful only when applied early, before the disease is well established, which is the usual in patients.

In view of these findings, what are the possibilities to translate the promising results of VIP observed in animal models to an effective treatment of the human septic shock syndrome? A clinical study is on the way (phase I) in patients with acute respiratory distress syndrome and sepsis with very promising and hopefully results (National Library of Medicine identifier NCT00004494: http://www.ClinicalTrials.gov processed this record on June 24, 2002). Probably, due to its pleiotropic effects inhibiting a wide spectrum of proinflammatory mediators, including mediators which appear later during the inflammatory response, VIP protects from endotoxemia if given 2 hours after endotoxin injection. Therefore, due to a wider therapeutic window, VIP may represent a better biological therapeutic alternative than anti-inflammatory cytokines or anti-TNFα antibodies, which are only effective in the very early states. Furthermore, additional mechanisms could be participating in the therapeutic effect of VIP on endotoxemia. Indeed, other studies (Tuncel and Tore, 1998; Tuncel et al., 2000) suggested that the protective effect of VIP on septic shock syndrome is mediated through the regulation of serum levels of hormones, such as adrenaline and cortisol, which control hemodynamic constants, important events in the pathology of sepsis.

B. Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a common, chronic, and debilitating autoimmune inflammatory disease of unknown etiology that leads to chronic, progressive, and symmetrical inflammation in the joints and subsequent erosive destruction of the cartilage and bone. RA affects approximately 1% of the population, has a disproportionately high incidence in women compared with men (3:1 ratio) and in older individuals, and is a significant cause of disability, chronic ill health, and premature mortality. To find therapeutic alternatives, several strategies have been designed based in the two deleterious aspects of RA, i.e., inflammation and autoimmunity.

The majority of RA treatments were developed with the purpose of decreasing chronic joint inflammation, a multifactorial response dependent upon both regulatory cytokines and proinflammatory chemokines. Therefore, agents that inhibit secretion of inflammatory mediators, especially TNFα, or that can block receptor binding are increasingly being viewed as potential therapeutic agents with increased specificity as compared with traditional drugs (Feldmann et al., 1996).

An alternative therapeutic strategy explored in RA is the alteration of the T cell response, with $CD4^+$ T cells as targets for immunotherapy. The balance of Th1/Th2-type cytokines may play a significant role in the regulation of autoimmune diseases. Although the contribution

of Th1 and Th2 responses in RA is not completely understood, several studies in animal models revealed that the Th1 cytokine profile predominates at the induction and acute phases of the disease, whereas Th2-mediated responses are associated with remission (Mauri et al., 1996; Walmsley et al., 1996; Doncarli et al., 1997; Horsfall et al., 1997; Joosten et al., 1997).

Since a specific causative agent or antigen has not been identified yet, bypassing the potential antigen and targeting the cytokine imbalance might represent a way to control RA.

Because VIP has been clearly identified as a potent anti-inflammatory factor (discussed in *Section IV.*), and some evidence indicates that VIP preferentially induces differentiation toward a Th2 response (Delgado et al., 1999b; Goetzl et al., 2001), VIP has emerged as an attractive candidate for the treatment of arthritis. In fact, two recent reports (Delgado et al., 2001; Williams, 2002) using the animal model of collagen-induced arthritis, which shares a number of common clinical, histologic, and immunologic features with human RA, have demonstrated that treatment of arthritic mice with VIP decreases the frequency, delays the onset, and reduces the severity of the disease. The therapeutic effect of VIP on arthritis is associated with a striking reduction of the two deleterious components of the disease, i.e., the autoimmune and inflammatory response.

VIP reduces the titer of autoreactive anti-collagen antibodies (particularly IgG2a antibodies) in response to a reduction in the collagen-specific T cell response. This is accompanied by a shift from Th1- to Th2-type responses, characterized by a reduction in IFNγ production and an increase in IL-4.

Regarding its effect in joint inflammation, VIP strongly reduces the inflammatory response during arthritis development by down-regulating the production of several proinflammatory agents in the inflamed joints and synovial cells, including TNFα, IL-6, IL-1β, iNOS, IL-12, and IL-18, as well as various chemokines (RANTES, MCP-1, MIP-1α, MIP-1β, and MIP-2) reported to play a role in inflammation and the development of the arthritic responses (Feldmann et al., 1996). In addition, VIP increases the production of the anti-inflammatory cytokines IL-10 and IL-1Ra, which have been reported to ameliorate arthritis symptoms (Feldmann et al., 1996). Chemokines are responsible for the infiltration and activation of various leukocyte populations that are contributing to pannus development and the subsequent pathology of RA. A similar inhibitory action of VIP on the production of inflammatory mediators (TNFα and IL-8) has been described in synovial cells from arthritic patients (Takeba et al., 1999), suggesting a potential application of this neuropeptide in human RA. By inhibiting chemokine production, VIP prevents leukocyte infiltration in the synovium. The capacity of VIP to regulate a wide spectrum of inflammatory mediators may offer an advantage over neutralizing antibodies and receptor antagonists directed against a single cytokine.

VIP prevention of cartilage destruction and bone erosion has to be attributed, at least partially, to its inhibitory effect on the expression and activity of some matrix metalloproteinases, which in addition to proinflammatory cytokines, such as TNFα and IL-1β, have been assigned pivotal roles in the depletion of proteoglycan and collagen observed in the joints, which leads to cartilage and bone erosion in patients with RA (Woessner, 1991).

The rheumatoid synovium shows hyperplasia of fibroblast-like synovial lining cells and is infiltrated with various mononuclear cells, among which macrophages and T lymphocytes predominate. Although the involvement of other cells cannot be ruled out, macrophages and synoviocytes appear to be the VIP target cells.

Of obvious biological significance is the fact that VIP levels, similar to that of other recent described anti-arthritic neuropeptides and hormones, such as CGRP and αMSH (Lipton and Catania, 1997; Takeba et al., 1999), are specifically increased in serum and joints of arthritic mice during the development of the disease (Delgado et al., 2002a), demonstrating that endogenous neuroimmune factors act as natural anti-arthritic agents activated in response to autoimmune/inflammatory conditions to counterbalance the effects of inflammatory mediators. Nevertheless, arthritis-induced endogenous VIP levels are 2 to 3 orders of magnitude lower than the concentrations of protective exogenous VIP. We propose that during a normal immune response, the timely production and release of VIP and other neuroimmune factors within the lymphoid microenvironment following antigenic/inflammatory stimulation serves to down-regulate the ongoing immune/inflammatory response, mostly through modulation of cytokine production. During arthritis, however, due to severe inflammation and overstimulation of the immune system, the effect of checkpoint factors such as VIP, PACAP, CGRP, αMSH, and other anti-inflammatory mediators, including IL-10, IL-1Ra, and IL-13, is overwhelmed by the inflammatory cytokine network. However, the exogenous administration of these anti-inflammatory mediators could offer an alternative to existing treatments for arthritis and other inflammatory/Th1-autoimmune diseases, such as multiple sclerosis, inflammatory bowel diseases, or autoimmune diabetes.

However, mice do not naturally develop RA, and although the results with VIP are encouraging, it requires a leap of faith to extrapolate our findings to the clinic. Also, there are possible side effects to the chronic administration of VIP, including gastrointestinal effects and generalized immunosupression. No such adverse effects were observed short-term in the experimental collagen-induced arthritis model. Extending the use of VIP or VIP analogs to humans will depend on the dosage and way of administration. In addition, VIP gene trans-

fer could be attractive as potential means to deliver consistent, prolonged therapeutic titers of this anti-inflammatory factor with fewer side effects and without the need for repeated administrations.

C. Crohn's Disease

Human inflammatory bowel disease is a worldwide, chronic, idiopathic, inflammatory disease of the distal small intestine and the colon mucosa, clinically characterized by two overlapping phenotypes, i.e., ulcerative colitis and Crohn's disease. Crohn's disease is an incurable autoimmune disease with a prevalence of 0.01% to 0.08% that leads to chronic transmural inflammation resulting in a range of gastrointestinal and extraintestinal symptoms, including abdominal pain, rectal bleeding, diarrhea, weight loss, skin and eye disorders, and delayed growth and sexual maturation in children (Hanauer and Present, 2003). These symptoms can greatly impact the patients' well being, quality of life, and capacity to function. Because Chron's disease is chronic and typically has an onset before 30 years of age, patients generally require lifelong treatment. Although its etiology is unknown, Crohn's disease has been shown to be marked by an exaggerated gut-associated lymphoid tissue-developed immune response, giving rise to a prolonged severe inflamed intestinal mucosa, characterized by uncontrolled production of proinflammatory cytokines and oligoclonal expansion and activation of $CD4^+$ T cells specifically associated with a Th1 response (Blumberg and Strober, 2001).

Therapeutic agents currently used for Crohn's disease, including aminosalicylates, corticosteroids, azathioprine, 6-mercaptopurine, antibiotics, and methotrexate, are not entirely effective, nonspecific, and with multiple adverse side effects. In most cases, surgical resection is the ultimate alternative (Hanauer and Present, 2003). Therefore, the present therapeutic strategy is to find drugs or agents that specifically modulate both components of the disease, i.e., the inflammatory and Th1-driven responses.

In a recent report, Abad et al. (2003) used the established murine model of Crohn's disease induced by intrarectal administration of trinitrobenzene sulfonic acid (TNBS) to demonstrate that treatment with VIP reduced the clinical and histopathologic severity of TNBS-induced colitis, abrogating body weight loss, diarrhea, and intestinal inflammation. The therapeutic effects of VIP occurred in all phases of the disease (early, acute, and chronic) and were associated with the down-regulation of both inflammatory and Th1-driven autoimmune responses. In the early acute phase of bowel inflammation, there is an overlapping of innate and adaptive immune responses, with multiple mediators involved, such as chemokines and cytokines. VIP significantly reduced the inflammatory response by down-regulating the production of different proinflammatory mediators involved in the local and systemic damage, such as TNFα, IL-1β, IL-6, IL-12, MIP-1α, MCP-1, MIP-2, and KC. This broad spectrum of action is related to a decrease in colitis-associated inflammation and the infiltration of neutrophils, macrophages, and $CD4^+$ T cells in the lamina propria. The VIP treatment partially prevented TNBS-induced generation of $CD4^+$ T cells and Th1 responses. In addition, spleen and lamina propria $CD4^+$ T cells from mice treated with VIP preferentially showed a Th2 pattern, with increased production of IL-4 and IL-10 after antigenic stimulation. Novel therapeutic strategies for Crohn's disease include the blockage of some of the proinflammatory and Th1 cytokines (such as TNFα, IL-6, and IL-12) (Fuss et al., 1999; Atreya et al., 2000; Nikolaus et al., 2000; Yamamoto et al., 2000; Neurath et al., 2002). Therefore, the capacity of VIP to regulate a wide spectrum of inflammatory and Th1 mediators in TNBS-induced colitis represents a therapeutic advantage over current treatments directed against a single mediator.

From a therapeutical point of view, it is important to point out that VIP reduced disease severity even when given after the onset of the disease and that VIP dramatically reduced disease recurrence upon administration of a second dose of TNBS. Therefore, VIP could represent a possible multistep therapeutic agent for Crohn's disease.

D. Parkinson's Disease

Parkinson's disease (PD) is a neurodegenerative disorder involving a progressive degeneration of the dopaminergic neurons of the substantia nigra pars compacta (SNpc) and the subsequent loss of their projecting fibers in the striatum (Rodnitzky, 1995). Although current treatments alleviate some of the symptoms, chronic use of these drugs is not effective in dampening the progression of PD and has been associated with debilitating side effects. In addition, the etiology of PD remains unknown, and this has impeded the development of effective therapies. However, insights into the pathogenesis of PD have been achieved experimentally by using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (Bloem et al., 1990; Grunblatt et al., 2000). MPTP produces irreversible clinical, biochemical, and neuropathological effects that mimic those observed in idiopathic PD, including the dramatic neurodegeneration of the nigrostriatal dopaminergic pathway (Bloem et al., 1990; Grunblatt et al., 2000; Przedborski et al., 2000). The meperidine analog is metabolized to 1-methyl-4-phenylpyridinium by the enzyme monoamine oxidase B. 1-Methyl-4-phenylpyridinium is subsequently transported selectively into neurons by the dopamine reuptake system on the dopaminergic terminals and concentrated in neural mitochondria in the SN, where it binds to and inhibits complex I of the electron transport chain, thereby producing the same biochemical defects as those detected in SNpc of PD patients (Przedborski et al., 2001). Several mechanisms, including mitochondrial

dysfunction, oxidative damage, excitotoxicity, and α-synuclein deposition, have been proposed to initiate neuronal damage and subsequent cell death characteristic of this disorder (Schapira et al., 1990; Mizuno et al., 1995; Jenner and Olanow, 1996; Goedert, 2001). In addition to these specific mechanisms, in several neurodegenerative disorders, including PD, Alzheimer's disease, multiple sclerosis, and AIDS dementia, neuronal cell death occurs subsequent to inflammatory responses, mediated at least partially by activated microglia (Kreutzberg, 1996; Gonzalez-Scarano and Baltuch, 1999; Hunot et al., 2001; Vila et al., 2001). Proinflammatory cytokines are known to participate in mitochondrial impairment and oxidative stress; therefore, an inflammatory response may serve as an integral feature of the mechanistic underpinning related to PD pathogenesis. Because VIP has been shown to act as a "microglia-deactivating factor" under inflammatory conditions (Delgado et al., 2002b, 2003) and to prevent inflammation-induced neurodegeneration (Delgado and Ganea, 2003c), this neuropeptide emerges as a plausible candidate for neuroprotection under inflammatory conditions, including PD.

We have recently demonstrated that the stereotaxic administration of VIP into the SNpc in the MPTP mouse model of PD is beneficial, reducing SNpc dopaminergic neuronal degeneration and nigrostriatal nerve fiber loss (Delgado and Ganea, 2003d). VIP prevents MPTP-induced activation of microglia in SNpc and striatum and the expression of the cytotoxic mediators, iNOS, IL-1β, TNFα, and NADPH-oxidase. This effect seems to be mediated through VPAC1 and a PKA-dependent pathway. Based on the correlation between the neuroprotective effect of VIP and its inhibition of MPTP-induced microglial activation, it has been proposed that VIP exerts its neuroprotective activity through its effect as a "microglial-deactivating factor." The involvement of astrocytes in the mediation of VIP, although minimal, cannot be discarded, because VIP slightly reduced MPTP-induced astrogliosis. In addition, although it is unlikely that the reduction of MPTP-induced microglia activation by VIP is secondary to the attenuation of neuronal loss rather than the reverse, a direct action of VIP on neurons cannot be ruled out. Interestingly, systemic administration of VIP (i.p. injection) significantly inhibits MPTP-induced loss of TH immunoreactivity, although it is much less effective, with 15-fold higher doses required for a significant effect, which is 50% less efficient than the cerebral VIP administration. In addition to the dilution factor in a systemic administration, the difference in effectiveness could be due to a diminished entry of VIP into the brain parenchyma though the blood-brain barrier, even in inflammatory conditions, where the blood-brain barrier is slightly compromised.

These findings invite important future directions, including the possible therapeutic role of VIP in brain disorders, such as PD itself, or multiple sclerosis, Alzheimer's diseases, and AIDS dementia, where the inflammatory response plays a major role. Since an inflammatory response is involved in PD, antioxidants and newly developed, nonsteroidal, anti-inflammatory drugs, such as iNOS inhibitors, cyclooxygenase inhibitors, or minocycline (Grunblatt et al., 2000; Wu et al., 2002), have been proposed for treatment. However, although several drugs offered neuroprotection in animal models, there has been little or no success in the clinical treatment of PD. This may indicate that either the animal models do not reflect the events in PD or that neuronal cell death involves a cascade of events, which cannot be prevented by a single neuroprotective drug. Therefore, consideration should be given to multidrug therapy, similar to the approach taken in AIDS and cancer therapy. It is also possible that agents such as VIP, which affect a large spectrum of inflammatory mediators, might be at an advantage compared with other anti-inflammatory agents with a more restricted effect.

E. Brain Trauma

A recent study has demonstrated the potential therapeutic effect of VIP in brain trauma, a pathological condition associated with inflammation (Gonzalez-Scarano and Baltuch, 1999). The increase in the levels of proinflammatory cytokines is a normal and early feature of the CNS response to trauma (Taupin et al., 1993; Bartholdi and Schwab, 1997; Streit et al., 1998). However, it remains controversial as to whether inflammation in the injured CNS serves a beneficial or detrimental purpose (Yong, 1996; Schwartz et al., 1999). Several reports demonstrated that the administration of inflammatory cytokines to injured areas was neuroprotective and/or promoted axonal regeneration (Yong, 1996; Schwartz et al., 1999). It has been reported that blood-derived macrophages modify the properties of CNS white matter near mechanical lesions, converting a nonpermissive state to one promoting axonal growth (David et al., 1990). In contrast, a number of studies have shown the involvement of proinflammatory mediators such as TNFα, IL-1β, and NO in the mediation of neuronal and oligodendroglial death (for review, see Yong, 1996; Gonzalez-Scarano and Baltuch, 1999). Also, treatment with anti-inflammatory agents limits CNS damage and improves recovery after blunt spinal cord trauma (Hall and Braughler, 1982), and there is a positive correlation between the number of macrophages/microglia and the degree of tissue damage (Carlson et al., 1998). It has been proposed that the differences regarding the role of inflammation in CNS injury and recovery could be region or structure specific and/or dependent on the varied stages of neuronal degeneration (Hirschberg et al., 1994; Lawrence et al., 1998). In a model of adult mice stab wound brain trauma in the periventricular area, VIP has been shown to significantly reduce all the pathological hallmarks that follow brain injury, i.e., dramatic neurodegeneration, recruitment of mononuclear phago-

cytes, microglia activation, and increased production of TNFα and IL-1β (Delgado and Ganea, 2003d). The source of inflammatory cytokines in the CNS following trauma remains unresolved. Infiltrating leukocytes are obvious candidates, but the influx of leukocytes in the CNS is delayed in response to an acute insult in comparison with other tissues. Thus, neutrophils do not infiltrate appreciably until about 6 h after lesion, whereas T lymphocytes and monocytes appear 12 to 24 h later (Giulian et al., 1989; Moreno-Flores, 1993; Frank and Wolburg, 1996), suggesting that the CNS mounts an early and intrinsic inflammatory response upon trauma. Microglia has been proposed as the origin of the early increase in CNS inflammatory cytokines following injury (Bartholdi and Schwab, 1997; Streit et al., 1998). It has been proposed that VIP reduces neuronal cell loss in the vicinity of the lesion site through the inhibition of microglial-derived proinflammatory mediators such as TNFα and IL-1β and of chemokines responsible for the inhibition of the influx of blood-derived leukocytes into the parenchyma surrounding the injured region. In addition, although it is unlikely that the reduction of microglia activation by VIP is secondary to the attenuation of neuronal loss rather than the reverse, a direct action of VIP on neurons cannot be ruled out (Delgado and Ganea, 2003c). In addition, neurotrophic effects mediated through astrocytes cannot be excluded. A neuroprotective action of VIP in response to different neuronal insults through the production of neuroprotective factors (i.e., activity-dependent neurotrophic factor) by astrocytes has been previously described (Gressens et al., 1997; Offen et al., 2000).

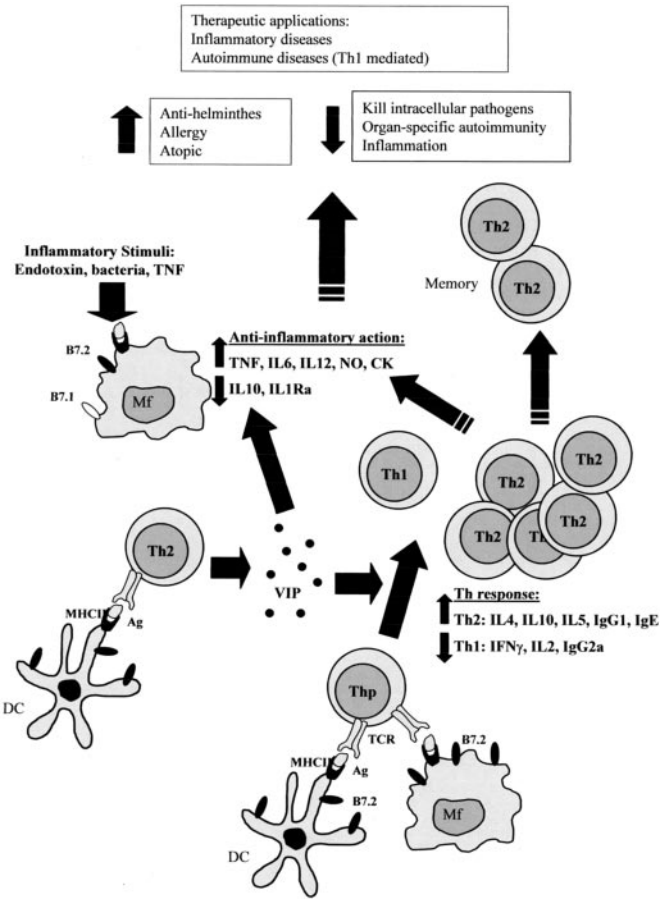

FIG. 6. Is VIP a type 2 cytokine? Keys to be considered: VIP is produced by Th2 cells in response to antigenic stimulation, and VIP induces Th2 generation/functions and decreases Th1 functions. As a consequence, the inflammatory and autoimmune responses are impaired, and the anti-helminthic and atopic responses are increased.

VIII. Conclusions and Perspectives

VIP, released in the lymphoid organs by the innervation and activated immune cells, modulates the function of inflammatory cells through specific receptors, affecting both innate and adaptive immunity. Macrophages and dendritic cells in the periphery and microglia in the CNS are major players in innate immunity and serve as a link to adaptive immunity by functioning as APCs. Activated macrophages, dendritic cells, and microglia decrease the pathogen load through the release of cytotoxic cytokines, oxygen radicals, and nitric oxide, and through the mobilization of additional immune cells in response to inflammatory chemokines. Responding to stimulatory and costimulatory signals delivered by APCs, $CD4^+$ T cells proliferate and differentiate into effector Th1 and Th2 cells. At the conclusion of an immune response, both activated APCs and T cells have to be deactivated and/or eliminated to avoid excessive tissue and organ damage. A number of endogenous factors, particularly anti-inflammatory cytokines, contribute to the down-regulation of the immune response. Neuropeptides, such as VIP and related peptides, have been recently added to the list of endogenous anti-inflammatory molecules (Fig. 6). VIP exerts its anti-inflammatory function in several ways: 1) direct inhibition of proinflammatory cytokine production (TNFα, IL-6, and IL-12) by activated macrophages and microglia; 2) up-regulation of IL-10 production (a potent anti-inflammatory cytokine); 3) inhibition of expression and release of proinflammatory chemokines from activated macrophages and microglia; 4) inhibition of B7.1/B7.2 expression in activated macrophages and dendritic cells, and subsequent inhibition of their stimulatory activity for antigen-specific T cells; and 5) inhibition of Th1 responses (reduction in both the amounts of Th1 cytokines and the number of cytokine-producing Th1 cells). In contrast to these well-defined anti-inflammatory functions, VIP supports the generation and long-term survival of Th2 cells, representing the first described neuropeptides with a possible role in the generation of memory Th2 cells. VIP, produced and secreted by Th2 cells following antigen stimulation, participates in a Th2 autoregulatory loop, where it promotes Th2-type responses through multiple nonexcluding and probably interrelated mechanisms, including direct effects on differentiating $CD4^+$ T cells, indirect effects on APCs, and

modulation of proliferation, survival, and recruitment of already-generated T effectors. From the point of view of a normal physiological role, the fact that VIP promotes the generation and long-term survival of Th2 cells is particularly relevant in view of the concept that immune privilege in organs such as brain and eye is an active process of immune deviation mediated by regulatory T cells generated in the presence of Th2-derived cytokines. From a pathological point of view, these findings open the possibility of using VIP and its analogs in the treatment of inflammatory and autoimmune diseases with a prevalent Th1 background and partially explain the beneficial effect showed by VIP in models of endotoxic shock, Parkinson's disease, brain trauma, rheumatoid arthritis, and Crohn's disease.

Could VIP be considered a type 2 cytokine? The term "cytokine" defines a protein secreted by leukocytes (and other cell types) in response to tissue injury or repair, acting predominantly in a local, paracrine, or autocrine manner, with pleiotropic actions including effects on immune cells and modulation of the inflammatory response. If in addition such a protein is produced by Th2 cells, affects the Th1/Th2 balance, and enhances Th2 and reduces Th1 generation/function, resulting in a generalized anti-inflammatory, antihelminthic, and atopic function, it is included in the group of type 2 cytokines. The findings reviewed here clearly indicate that VIP shares these characteristics. Therefore, if Said and Mutt had discovered VIP in the immune system, instead of the small intestine, VIP would have been considered a type 2 cytokine instead of a neuropeptide (Delgado, 2003b). Although this current "assessment of its potential" is necessarily broad and multidisciplinary, one can anticipate an even greater interface with other disciplines in the future as the full spectrum of VIP's biological action becomes apparent. Although only time will tell whether these advances will translate into new therapeutic approaches, clearly the detailed characterization of VIP effects on the immune system has opened up entirely new areas for basic and clinical investigation.

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