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Vasoactive intestinal peptide prevents experimental arthritis by downregulating both autoimmune and inflammatory components of the disease

MARIO DELGADO, CATALINA ABAD, CARMEN MARTINEZ, JAVIER LECETA & ROSA P. GOMARIZ

Department Cell Biology, Faculty of Biology, Complutense University, Madrid, Spain
Correspondence should be addressed to M.D.; email: mariodm@eucmos.sim.ucm.es

Rheumatoid arthritis (RA) is a chronic and debilitating autoimmune disease of unknown etiology, characterized by chronic inflammation in the joints and subsequent destruction of the cartilage and bone. We describe here a new strategy for the treatment of arthritis: administration of the neuropeptide vasoactive intestinal peptide (VIP). Treatment with VIP significantly reduced incidence and severity of arthritis in an experimental model, completely abrogating joint swelling and destruction of cartilage and bone. The therapeutic effect of VIP was associated with downregulation of both inflammatory and autoimmune components of the disease. Our data indicate VIP as a viable candidate for the development of treatments for RA.

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Rheumatoid arthritis (RA) is an autoimmune disease of unknown etiology that leads to chronic inflammation in the joints and subsequent destruction of the cartilage and bone. The drugs and agents currently used to treat RA have multiple effects, some of which are undesirable, and in the long term these treatments do not prevent joint damage. To find therapeutic alternatives, several strategies have targeted the hallmark characteristics of RA—inflammation and autoimmunity.

The monocyte/macrophage product TNF-α has a central role in joint inflammation. It induces a pro-inflammatory cytokine cascade involving IL-1, IL-6, GM-CSF and IL-8, as well as several pro-inflammatory chemokines[1]. Agents that inhibit secretion of these cytokines (especially TNF-α) or block their binding to cell-surface receptors are being increasingly considered as potential therapeutic agents that might provide better specificity.

An alternative therapeutic strategy that has been explored for rheumatoid arthritis is the alteration of the T-cell response, in particular, targeting $CD4^+$ T cells. Although the contribution of Th1 and Th2 responses in RA is not completely understood, several studies in animal models revealed that the Th2 response is associated with the remission phase of the disease, whereas the Th1 cytokine profile predominates at the induction and acute phases of the disease [2,3]. These findings point to a pathogenic role for Th1-derived cytokines. In support of this hypothesis, several studies have shown that manipulation of the balance of cytokines produced by Th1- and Th2-cell subsets alters disease outcome[4–6].

Because a specific causative agent or antigen has yet to be identified, bypassing the putative antigen and targeting the cytokine imbalance might represent a viable therapeutic strategy for RA. Vasoactive intestinal peptide (VIP), a neuropeptide present in the lymphoid microenvironment, elicits a broad spectrum of biological functions, including the modulation of innate and adaptive immunity, and shows a predominant anti-inflammatory action[7]. In addition, we have shown that VIP promotes Th2 differentiation and inhibits Th1 responses by regulating macrophage costimulatory signals and probably IL-12/IFN-γ production[8,9]. Therefore, VIP has emerged as one of the more promising candidates for treatment of RA.

Collagen-induced arthritis (CIA) is a murine experimental disease model induced by immunization with type II collagen (CII). Because it shares a number of clinical, histologic and immunologic features with RA (ref. 10), we used the CIA model to study the potential effect of VIP on the pathogenesis of arthritis. Here we show that treatment with VIP has great benefit at the clinical and pathological levels, as the therapeutic effect of VIP was associated with the downregulation of both inflammatory and autoimmune components of the disease.

VIP decreases incidence and severity of CIA

To induce CIA, we immunized DBA/1 mice with bovine CII in complete Freund's adjuvant, then boosted the mice with CII and monitored them for the occurrence of clinical signs of arthritis. After the second immunization, we administered VIP intraperitoneally at different doses daily or on alternate days for two weeks.

Mice treated with VIP showed delayed onset, lower incidence and decreased severity of CIA in comparison with untreated arthritic mice, as assessed by clinical score and paw swelling (Fig. 1*a* and *b*). The therapeutic effect was dose-dependent (Fig. 1*c*). Daily or every other day administration of VIP offered the best protection against disease, although a single administration at the onset of disease was enough to significantly ameliorate the pathologic signs of arthritis. Because we observed few differences between the 5- and 10-nmol doses as well as between daily and alternate-day VIP administrations, all further experiments used the 5-nmol dose on alternate days. After VIP treatment, histopathological analyses of joints showed complete abrogation of CIA-characteristic chronic inflammation of synovial tissue (infiltration of mononuclear cells into the joint cavity and synovial hyperplasia), pannus formation, cartilage destruction and bone erosion (Fig. 1*d* and *e*).

We saw no remission in therapeutic effects two weeks after cessation of VIP administration (day 35; Fig. 1*a* and *c*), indicating that no additional neuropeptide is necessary after a short period of VIP treatment to maintain protection from the disease. Importantly, VIP can prevent and ameliorate already established disease. When administered 12 days after disease onset (day 34, when 85% of the

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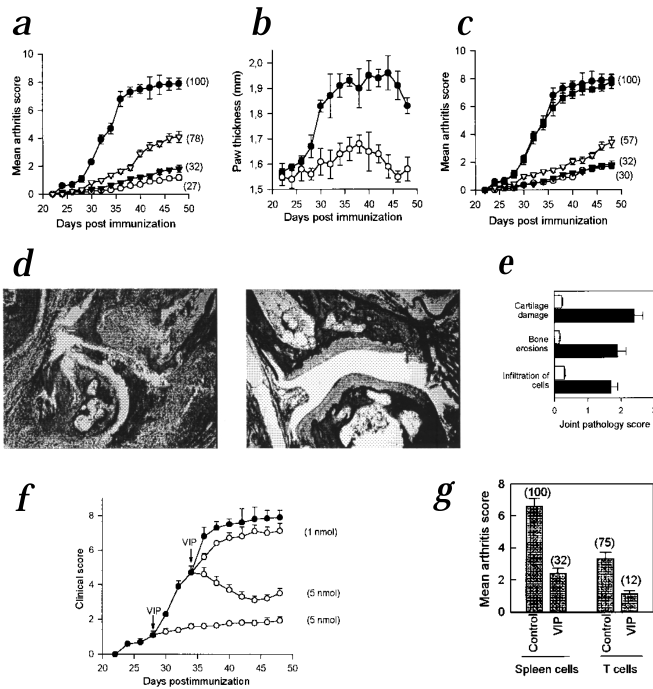

Fig. 1 VIP decreases CIA incidence and severity. ***a***, CIA mice treated with either control or 5 nmol VIP. ●, PBS; ○, VIP daily; ▼, VIP on alternate days for 2 wk; ▽, VIP on days 22 and 24 only. Incidence of arthritis (percentage of diseased mice) in each experimental group is shown in parenthesis. $P < 0.001$ versus control for all treatments after day 30. ***b***, Paw thickness of CIA mice treated with PBS (●) or VIP (5 nmol; ○) on alternate days for 2 wk. $P < 0.001$ versus control after day 30. ***c***, Severity of clinical signs and percentage arthritis incidence (in parenthesis). ●, control; ○, 10 nmol VIP; ▼, 5 nmol VIP; ▽, 1 nmol VIP; ■, 0.1 nmol VIP. $P < 0.001$ versus control for 1 to 10 nmol treatments after day 30. Results in *a–c* are expressed as the mean ± s.d. from 3 separate experiments (8 mice/group/experiment). ***d***, Representative section of joint histopathology on whole paws of control (left) and VIP-treated (5 nmol; right) mice on day 45. ***e***, Scoring of inflammation, cartilage damage and bone erosion of paws shown in *d*. ■, control; □, VIP-treated. Results are the mean ± s.d. from 12 mice/group. ***f***, Clinical score of CIA after VIP treatment. From day 28 or day 34 (arrows), CIA mice were treated with VIP every other day (1 or 5 nmol as indicated). ●, untreated mice; ○, VIP-treated mice. Results are expressed as the mean ± s.d. from 3 separate experiments (8 mice/group/experiment). $P < 0.001$ versus control for both 5 nmol treatments after VIP administration. ***g***, Naive DBA/1 mice were adoptively transferred with whole spleen cells or purified T cells from VIP-treated (5nmol) or control CIA mice. Severity of clinical signs and incidence of arthritis (in parenthesis) were assayed. Results are expressed as the mean ± s.d. from 3 separate experiments (6 mice/group/experiment).

mice had developed arthritis), VIP blocked disease development and led to an improvement in clinical scoring and incidence of arthritis (Fig. 1*f*).

Finally, to confirm the importance of VIP on the modulation of CIA, we performed adoptive cell transfer experiments. Whole spleen cells and T cells from VIP-treated CIA mice caused a lower incidence and severity of arthritis in naive recipient mice compared with cells from control CIA mice (Fig. 1*g*). Therefore, VIP prevents adoptive transfer of arthritis.

VIP regulates Th1/Th2 balance in CIA

We next investigated the mechanisms underlying the decrease in incidence and severity of CIA following VIP treatment. VIP has several immunomodulatory effects including inhibition of T-cell proliferation and regulation of Th1/Th2 balance[7,8,11]. To test whether impaired T-cell functions in VIP-treated mice lead to CIA inhibition, we first tested the effect of VIP treatment on CII-specific, proliferative responses of spleen cells from CIA mice. Whereas spleen cells from control mice proliferated in response to CII, T cells from mice receiving VIP responded to CII to a much lesser extent (Fig. 2*a*). These data indicate that VIP administration during CIA development at least partially inhibits T-cell clonal expansion in response to CII challenge.

Upon antigenic stimulation, naive $CD4^+$ Th cells can differentiate into two distinct types of effector cells, Th1 and Th2, each producing its own set of cytokines and mediating separate functions[12]. As CIA has been identified as a Th1-mediated autoimmune disorder and Th2-derived cytokines ameliorate this disease[2–6], we next investigated whether VIP treatment had any effect on Th1 and Th2 cytokine production in CIA mice. CIA resulted in the development of CII-specific effector T cells that produce high levels of IFN-γ and low levels of IL-4 (Fig. 2*b*). However, treatment with VIP led to the inhibition of IFN-γ, and significant increase in IL-4 production (Fig. 2*b*). To test whether the Th1/Th2 cytokine balance was regulated by the

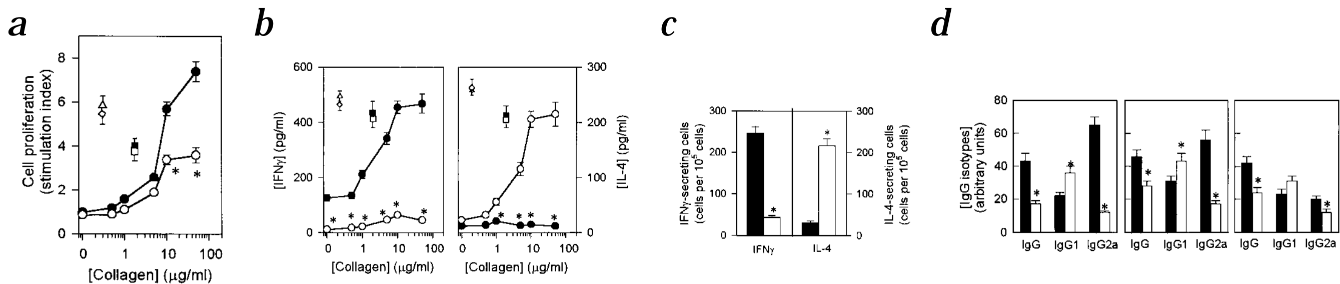

Fig. 2 VIP modulates Th1/Th2 balance in CIA. ***a–c***, VIP inhibits Th1-mediated response in CIA mice. In *a* and *b* spleen cells were stimulated with different concentrations of inactivated CII (*x*-axis). In *a* and *b*, spleen cells treated with PBS (●) or VIP (○) stimulated with anti-CD3 antibodies were used for assessment of nonspecific stimulation: ■, control; □, VIP; and spleen cells from PPD/CFA-immunized CIA mice stimulated with PPD antigen were used to assess antigen-specificity: △, control; ◇, VIP. A pool of 3 nonimmunized DBA/1 spleens was used for assessment of the basal response. No proliferation or cytokine production by T cells was detectable in the presence of an unrelated antigen (OVA, data not shown). *a*, Proliferative response expressed as a stimulation index [(CII-specific proliferation – unstimulated proliferation)/unstimulated proliferation]. *b*, IL-4 and IFN-γ levels in culture supernatants assayed by ELISA. *c*, Number of CII-specific T cells producing IFN-γ or IL-4. Spleen cells were stimulated with 10 μg/ml inactivated CII. ■, control; □, VIP. Results represent the mean ± s.d. from 2 independent experiments (5 mice/group/experiment). ***d***, VIP regulates CII-specific IgG levels. CII-specific IgG, IgG1 and IgG2a levels in serum collected 25 d (left), 35 d (middle) and 45 d (right) after primary immunization with CII. ■, control; □, VIP-treated. Data are represented as the mean ± s.d. using arbitrary units, as analyzed in 3 separate experiments (8 mice/group/experiment). *, $P < 0.001$ versus control.

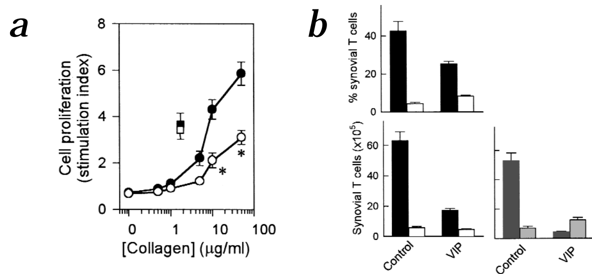

Fig. 3 VIP decreases T helper/suppressor ratio in ongoing CIA. ***a***, Synovial cells were stimulated with different concentrations of inactivated CII and proliferative response was determined and expressed as a stimulation index. ●, control; ○, VIP. Synovial cells stimulated with anti-CD3 antibody were used for assessment of unspecific stimulation: ■, control; □, VIP. ***b***, Synovial cells were counted, stained with antibodies against CD4; (■) or CD8 (□) and IL-4 (■) or IFN-γ (■), and analyzed by flow cytometry. Each bar represents the mean percentages (upper panels) and numbers (lower panels) ± s.d. performed in triplicate.

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generation/differentiation of CII-specific Th1 and Th2 effector cells, we used the ELISPOT assay to assess the frequency of antigen-reactive Th1 and Th2 cells based on secretion of IFN-γ and IL-4, respectively. Mice injected with antigen in the absence of VIP had many IFN-γ–secreting Th1 cells, and very few IL-4–secreting Th2 cells (Fig. 2*c*). In contrast, CIA mice injected with VIP generated few IFN-γ–secreting Th1 cells, but many IL-4–secreting Th2 cells (Fig. 2*c*).

High levels of circulating antibodies directed against CII invariably accompany the development of CIA and seem to be required for disease development[13,14]. Thus, the production of antibodies against CII is a major factor in determining susceptibility to CIA. Th1 and Th2 lymphocytes differentially affect the shift from the T-cell–independent IgM isotypes to IgG isotypes in activated B lymphocytes by inducing a switch to IgG2a/b and IgG1, respectively. Because the development of antigen-specific antibodies requires T-cell help, one mechanism of CIA inhibition by VIP could be due to failure to produce antibodies against CII, particularly autoreactive IgG2a antibodies that have been implicated in the pathogenesis of CIA. We measured the serum levels of total IgG or isotype-specific IgG2a and IgG1 anti-CII antibodies at different times after onset of arthritis. CIA resulted in high levels of CII-specific IgG antibodies, characterized by a high IgG2a:IgG1 ratio (Fig. 2*d*). In contrast, treatment of CIA mice with VIP significantly reduced CII-specific IgG levels, mainly during the first days after disease onset (Fig. 2*d*). This inhibitory effect was accompanied by a reduction in the IgG2a:IgG1 ratio, due to inhibition of CII-specific IgG2a and stimulation of CII-specific IgG1 production (Fig. 2*d*). This IgG isotype composition, together with the decreases in IFN-γ–secreting T cells, indicate that ***in vivo***, VIP attenuates type 1 helper response during development of CIA.

A characteristic of diseased synovium is the presence of high T helper:suppressor ratios as compared with blood and spleen. We therefore investigated whether VIP treatment of ongoing CIA can affect this ratio in synovial cells. VIP-treated mice showed a significantly lower proliferative response of synovial cells to CII as compared with control arthritic mice (Fig. 3*a*). CIA mice showed a T helper (CD4) to T suppressor (CD8) ratio of approximately 11, but VIP treatment reduced this ratio to approximately 4.5 (Fig. 3*b*). The decrease in the CD4:CD8 ratio seems to be due to impaired development of Th cells, rather than to an increase in the number of T suppressor cells, given that T suppressor numbers were not significantly affected, although Th numbers are lower in VIP-treated mice than in controls(Fig. 3*b*). This relative decrease of synovial Th cells mediated by VIP was due to a preferential diminution of IFN-γ– versus IL-4–producing CD4 cells (Fig. 3*b*). VIP probably inhibited the development and/or entry into joints of CII-specific Th1 cells.

VIP downregulates inflammatory response in CIA

The involvement of a wide array of cytokines and chemotactic factors (chemokines) has been reported in inflammation and in the development of arthritic responses[1]. The most relevant site of cytokine production in arthritis is the synovial membrane, and synovial cells from arthritic DBA/1 mice, like those from human RA joints, spontaneously release several pro-inflammatory cytokines and chemokines when cultured ***in vitro***[15–17]. As VIP has been shown to be

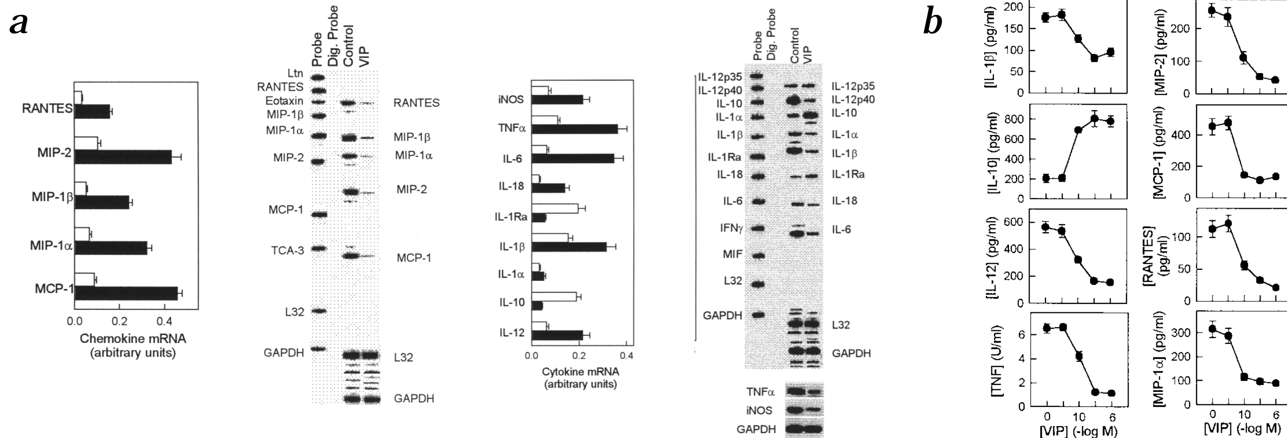

Fig. 4 VIP inhibits inflammatory response in CIA. ***a***, mRNA analysis on hind paws of CIA mice treated with PBS (■) or VIP (□). Blots are representative of 5 similar experiments. Results are expressed in arbitrary densitometric units normalized for the expression of GAPDH in each sample (mean ± s.d. of 5 separate experiments). A paw from an unimmunized mouse was analyzed simultaneously for assessment of the basal response. $P < 0.001$ versus control for all cytokines and chemokines. ***b***, Synovial membrane cells from CII-immunized mice were cultured in different concentrations of VIP or, as a control, medium alone. Supernatants were assessed at different times for cytokine and chemokine production. Results represent the mean ± s.d. $P < 0.001$ versus control in all cases.

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Table 1 Effect of the VIP agonists and related peptides in CIA

	Incidence	Clinical Score	Histology	IFN-γ	IgG1/IgG2	TNF-α	MIP-2
Control	100	7.9 ± 0.6	1.74 ± 0.21	457 ± 51	33/55	0.36 ± 0.04	0.44 ± 0.04
VIP	32*	1.6 ± 0.2*	0.31 ± 0.04*	52 ± 7*	43/18*	0.14 ± 0.02*	0.13 ± 0.02*
PACAP	34*	1.7 ± 0.2*	0.29 ± 0.03*	58 ± 6*	44/19*	0.12 ± 0.02*	0.11 ± 0.03*
VIP_{1-12}	99	7.8 ± 0.6	1.81 ± 0.22	466 ± 42	34/56	0.38 ± 0.03	0.46 ± 0.03
VIP_{10-28}	100	8.2 ± 0.8	1.73 ± 0.24	451 ± 55	33/57	0.34 ± 0.04	0.42 ± 0.04
Secretin	98	7.7 ± 0.7	1.69 ± 0.16	449 ± 36	32/50	0.35 ± 0.02	0.44 ± 0.03
Glucagon	99	8.1 ± 0.5	1.77 ± 0.24	445 ± 43	31/52	0.33 ± 0.04	0.41 ± 0.05
VPAC1	35*	1.8 ± 0.2*	0.36 ± 0.04*	50 ± 6*	40/16*	0.15 ± 0.02*	0.11 ± 0.02*
VPAC2	91	7.2 ± 0.7	1.70 ± 0.17	411 ± 44	31/50	0.34 ± 0.03	0.41 ± 0.04
PAC1	98	7.7 ± 0.6	1.72 ± 0.19	453 ± 42	34/56	0.33 ± 0.04	0.45 ± 0.05

CIA mice were treated i.p. with PBS (control) or 5 nmol of indicated agonist every other day. Incidence of arthritis, severity of clinical signs and infiltration of inflammatory cells in joints were assessed as described in Fig. 1. IFN-γ production by spleen cells and relative levels of anti-CII IgG1/IgG2 antibodies were determined as described in Fig. 2. mRNA expression of TNF-α and MIP-2 in joints was assessed as described in Fig. 4. Data represent mean ± s.d. of 3 independent experiments (8 mice/group/experiment). *, $P < 0.001$ versus controls.

a potent anti-inflammatory factor[7,11], we tested whether VIP could affect the expression and production of these pro-inflammatory agents. Analysis of paws showed that VIP inhibited CIA-induced mRNA expression of the pro-inflammatory factors TNF-α, IL-6, IL-12, IL-18, IL-1β, IL-1α and inducible nitric oxide synthase (iNOS), as well as of several chemokines, such as RANTES, monocyte chemoattractant protein (MCP)-1, macrophage inhibitory protein (MIP)-1α, MIP-1β and MIP-2 (Fig. 4*a*). However, mRNA levels of the anti-inflammatory cytokines IL-10 and IL-1Ra were significantly increased upon treatment of arthritic mice with VIP (Fig. 4*a*). We observed similar results with primary synovial membrane cultures derived from the knees of arthritic mice. *In vitro* treatment of synovial cells with VIP dose-dependently inhibited spontaneous release of IL-1β, TNF-α, IL-12, RANTES, MIP-1α, MCP-1 and MIP-2, and significantly increased production of IL-10 (Fig. 4*b*). Inhibition in chemokine production corroborates VIP inhibition of chemotactic activity of both synovial T cells and macrophages from CIA mice (chemotaxis indices for synovial cells from untreated and VIP-treated CIA mice were, respectively, 274 ± 16 and 74 ± 8 for T cells, and 382 ± 35 and 62 ± 8 for macrophages). Together, these results indicate that treatment with VIP reduces the arthritic inflammatory response by downregulating expression of pro-inflammatory and upregulating expression of anti-inflammatory agents in inflamed joints.

VIP inhibits MMP expression and activity in CIA

In addition to pro-inflammatory cytokines, matrix metalloproteinases (MMPs) have pivotal roles in the depletion of proteoglycan and collagen in the joints, which leads to cartilage and bone erosion in RA patients[18]. We therefore tested whether VIP regulates MMP production by arthritic synovial cells. VIP treatment significantly inhibited mRNA expression of the gelatinase MMP-2 in paws of arthritic mice (Fig. 5*a*). This effect was correlated with a VIP-induced decrease of MMP-2 gelatinase activity in synovial membrane cultures from arthritic mice (Fig. 5*b*). In contrast, MMP-13 and MMP-9 production were not affected by VIP treatment (data not shown). The inhibitory effect of VIP on MMP-2 could be directly related, at least partially, to the VIP-mediated inhibition of cartilage destruction and bone erosion.

VPAC1 receptor specifically mediated VIP effect on CIA

VIP acts through a family of receptors consisting of VPAC1, VPAC2 and PAC1 with VPAC1 being the major mediator of the immunomodulatory activity of VIP (refs. 7,11). To test the specificity of VIP's effect on arthritis and to identify the VIP receptor involved, we tested a newly described VPAC1 agonist[19], Ro25-1553 (a VPAC2 agonist)[20] and maxadilan (a specific PAC1 agonist)[21]. We assessed the effects of these agonists on arthritis clinical score, incidence, Th1/Th2 balance and production of some pro-inflammatory mediators in arthritic mice. Whereas the VPAC1 agonist mimicked VIP effects on all parameters assayed, both the VPAC2 and PAC1 agonists showed weak or no effect on CIA (Table 1). VIP specificity was confirmed as neither secretin and glucagon, two peptides of the VIP family, nor the two fragments VIP_{1-12} and VIP_{10-28}, showed any effect on arthritis clinical score (Table 1). However, pituitary adenylate cyclase-activating peptide (PACAP), a neuropeptide that shows immunological actions similar to VIP, significantly reduced arthritis severity and incidence (Table 1). These results indicate that the effect of VIP on arthritis is mainly mediated through VPAC1. VPAC1 is expressed in both macrophages and T lymphocytes[7,9,11], supporting the involvement of both immune cell types in the therapeutic effect of VIP on arthritis. In agreement with this finding, we have previously demonstrated that VPAC1 is the main mediator in the regulatory effect of VIP on the immune system[7,11].

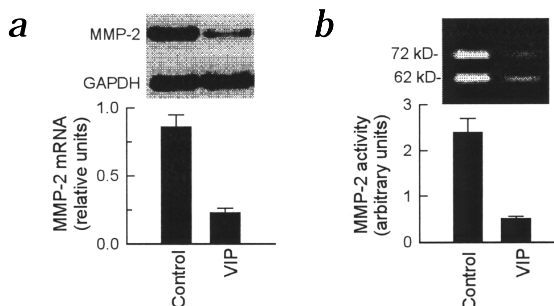

Fig. 5 VIP inhibits MMP-2 expression and activity in CIA. ***a***, CIA mice were treated with PBS (controls) or with VIP every other day. At day 35, MMP-2 mRNA levels in hind paws were determined by northern blot. Results are expressed in arbitrary densitometric units normalized for the expression of GAPDH (mean ± s.d. of 5 experiments). An unimmunized control was analyzed simultaneously to assess basal response. ***b***, Synovial membrane cells from CII-immunized mice at day 30 were cultured in the absence (control) or presence of 1×10^{-8} M VIP. Using gelatin zymography, MMP-2 activity (gelatinase activity) in culture supernatants was estimated. The positions of the 72-kD pro- and 62-kD active forms of MMP-2 are indicated. Results represent the mean ± s.d. of the lysis zones and are expressed as arbitrary units of relative band intensity. $P < 0.001$ versus controls.

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Discussion

In this study we show that the neuropeptide VIP provides a highly effective therapy for CIA, a murine experimental model for RA. Treatment of arthritic mice with VIP decreased the frequency of arthritis, delayed onset, ameliorated symptoms and prevented joint damage. The therapeutic effect of VIP on arthritis is associated with a striking reduction of the two deleterious components of the disease, that is, autoimmune and inflammatory responses.

The balance of Th1/Th2-type cytokines might have a substantial role in the regulation of autoimmune diseases. CIA has been identified as a Th1-mediated autoimmune disease. In contrast, Th2-mediated responses have beneficial effects on the severity and progression of the disease[2–6]. Here we demonstrate that administration of VIP to arthritic mice results in a decreased CII-specific T-cell response by specifically inducing a shift in the Th phenotype from a Th1-toward a Th2-type response. Similar VIP effects on Th1/Th2 balance following antigenic stimulation have been previously described[8]; such effects are associated with the VIP-mediated preferential upregulation of the costimulatory molecule B7.2 against B7.1 on antigen-presenting cells, in particular macrophages[8], as well as the downregulation of macrophage IL-12 production and subsequent IFN-γ secretion by Th1 cells[9]. An additional mechanism might be the preferential VIP-induced prevention of clonal deletion of Th2 against Th1 cells following antigenic stimulation, resulting in the generation of Th2 effectors and memory cells (M.D. *et al.*, manuscript submitted).

Chronic joint inflammation is a multifactorial response dependent upon both regulatory cytokines and pro-inflammatory chemokines. VIP strongly reduces inflammatory response during arthritis development by downregulating the production of several pro-inflammatory agents in inflamed joints and synovial cells, including TNF-α, IL-6, IL-1β, iNOS, IL-12 and IL-18, as well as various chemokines, which have been shown to exacerbate CIA (ref. 1). In addition, VIP increases production of the anti-inflammatory cytokines IL-10 and IL-1Ra, which have been reported to ameliorate arthritic symptoms[1]. Among these pro-inflammatory mediators, TNF-α has recently attracted special attention from researchers, because it is at the pinnacle of a pro-inflammatory cascade[1]. Recent studies using soluble TNF receptor, an antibody against TNF, IL-10 and IL-1Ra indicate that such factors might have improved efficacy over conventional drug therapy in the treatment of arthritis[1,22–25]. Chemokines are responsible of the infiltration and activation of various leukocyte populations in joint tissue, which are contributing factors to pannus development and the subsequent pathology of RA. The inhibitory effect of VIP on chemokine production could at least partly explain the absence of leukocyte infiltration in the synovium of arthritic mice treated with VIP. The capacity of VIP to regulate a wide spectrum of inflammatory mediators might offer a therapeutic advantage over neutralizing antibodies and receptor antagonists directed against a single cytokine.

The rheumatoid synovium shows hyperplasia of fibroblast-like synovial lining cells and is infiltrated with various mononuclear cells, predominantly macrophages and T lymphocytes. VIP is a potent anti-inflammatory factor that inhibits the production of pro-inflammatory factors such as TNF-α, IL-6, IL-12 and nitric oxide[7,11], as well as several chemokines (M.D. *et al.*, manuscript submitted) in activated peritoneal macrophages. Therefore, this finding points at macrophages as a likely target for the anti-inflammatory effect of VIP on arthritis. However, VIP regulation of other synovial cells could also play a role. For example, it has been reported that VIP inhibits proliferation and production of IL-6, IL-8 and MMP-2 by fibroblast-like synovial cells from RA patients[26]. This last finding also strengthens VIP as a possible therapeutic agent for human RA. Therefore, although a direct effect of VIP on T-cell activity is not ruled out, our results indicate that the effect of VIP on CIA is possibly mediated through antigen-presenting cells, mainly macrophages.

In conclusion, VIP has a profound therapeutic effect on CIA through its anti-inflammatory actions and through a specific effect on the Th1 response. We did not observe any adverse effects of the neuropeptide in this murine system. Extending the use of VIP to the human system, however, will depend on the dosage. Although optimal preventive amounts of VIP are much higher than serum and joint VIP levels reached in arthritic mice (data not shown), our data indicate that very low pharmacological doses of VIP are needed to see a significant therapeutic effect, and although VIP administration every day or on alternate days results in the highest preventive effects, a single injection of the peptide at the onset of the disease is enough to exert a significant remission. The ability of delayed administration of VIP to prevent and ameliorate ongoing disease also fulfills an essential prerequisite for an anti-arthritic agent, as treatment is started after the onset of patient arthritis. VIP or its analogs are therefore attractive candidates for the development of treatment of RA and other chronic inflammatory diseases and autoimmune disorders.

Methods

Induction and assessment of CIA. Native bovine type II collagen (CII, Sigma) was dissolved in 0.05 M acetic acid at 4 °C overnight, and emulsified with an equal volume of complete Freund's adjuvant (CFA; Difco, Detroit, Michigan). Male DBA/1J mice (6–10-wk-old, Jackson Labs, Bar Harbor, Maine) were injected s.c. at the base of the tail with 0.15 ml of emulsion containing 200 μg of CII. At 21 d after the primary immunization, mice were boosted i.p. with 200 μg CII in PBS. Mice were analyzed by two independent, blinded examiners every other day and monitored for signs of arthritis onset using two clinical parameters, paw swelling and clinical score. Paw swelling was assessed by measuring the thickness of the affected hind paws with 0–10 mm calipers. Clinical arthritis was assessed by using the following system: grade 0, no swelling; grade 1, slight swelling and erythema; 2, pronounced edema; 3, joint rigidity. Each limb was graded, giving a maximum possible score of 12 per animal. Group comparisons were performed using the χ-square test for disease incidence and unpaired, two-tailed Student's *t*-test for arthritis scores.

Treatment protocols. Treatment with peptides commenced with the secondary immunization, and VIP (Calbiochem, Laufelfingen, Switzerland), PAC1, VPAC1 or VPAC2 agonists were administered i.p. either daily or on alternate days at the specified doses until day 35 after primary immunization. In some cases, VIP was added at different times after disease onset. In each experiment, a control group of mice was injected with PBS alone.

Histological analysis. Mice were killed by cervical dislocation at day 45 after immunization, and the hind paws from 5 to 9 animals were randomly collected by 2 independent experimenters, then fixed with 10% paraformaldehyde, decalcified in 5% formic acid, embedded in paraffin, and 5-μm sections were stained with hematoxilin/eosin/safranin O. Histopathological changes were scored in a blinded manner using the following parameters: Infiltration of cells was scored on a scale of 0–3, based on the amount of inflammatory cells in the synovial cavity (exudate) and synovial tissue (infiltrate). Cartilage destruction was graded on a scale of 0–3, ranging from the appearance of dead chondrocyte (empty lacunae) to complete loss of the articular cartilage. Bone erosions were graded on a scale 0–3, ranging from normal bone appearance to fully eroded cortical bone structure in patella and femur condyle.

***In vitro* T-cell function.** Mice were killed at day 28 after primary immunization. Single spleen-cell suspensions were prepared and cultured at a density of 1×10^6 cells/ml in complete medium, composed of RPMI 1640 (Bio-Whittaker, Verviers, Belgium), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 40 μM β_2-mercaptoethanol, and 10% heat-inactivated FCS (Bio-Whittaker), in the presence or absence of different concentrations of heat-inactivated CII. For proliferation assays, cells were cultured for 4 d, and 1 μCi/well of $[^3H]$TdR was added

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in culture for the last 18 h. Cells were collected and [^{3}H]TdR uptake was measured using a β scintillation counter. For IFN-γ and IL-4 production, cells were cultured for 72 h and supernatants were collected and analyzed for IFN-γ and IL-4 by sandwich ELISA using antibody pairs (PharMingen, San Diego, California) as described[8]. The frequency of CII-specific T cells producing IFN-γ or IL-4 was determined as described[8] after 24-h culture by the enzyme-linked immunospot (ELISPOT) technique according to supplier's protocol (Pharmingen). As a recall antigen control, purified protein derivative (PPD, 30 μg) was injected intradermically in the CII/CFA emulsion, and *in vitro* T-cell function after culture stimulation with 10 μg/ml PPD was assayed as described above.

Synovial cell culture. Mice were killed at day 30 after primary immunization, rear limbs were removed, and the synovium of the knee joints was separated from the bone and cartilage by microscopic dissection. The synovial tissue was then digested and isolated as described for human synovial tissue[27]. The digested tissue was passed through a nylon mesh to form a single-cell suspension, washed extensively and then cultured in 24-well plates at a density of 2×10^6 cells/ml in complete medium in the presence or absence of different concentrations of VIP or VIP agonists. Supernatants were collected after 24 h for chemokine, IL-1β and TNF-α analysis, or 72 h for IL-10 and IL-12 analysis and stored at –20 °C until assay. The levels of immunoreactive IL-12p40, IL-1β, IL-10 and chemokines were measured by sandwich ELISA as described[9,28,29]. The levels of bioactive TNF-α were determined using the WEHI 164 cell-line assay[30].

Adoptive transfer of arthritis. DBA/1 mice were immunized with CII and CFA in the presence or absence of VIP. After 14 d, spleen-cell suspensions were prepared and T cells were isolated as described[8]. Whole spleen cells (5×10^7 cells) or purified T cells (3×10^7 cells) were resuspended in complete medium and injected i.v. into naive DBA/1 mice. Arthritis development was observed in recipient mice as described above.

mRNA analysis. Joints were homogenized with a Tissue Tearor (Biospec Products, Bartlesville, Oklahoma), and total RNA was isolated using the Ultraspec RNA reagent (Biotecx, Houston, Texas) as recommended by the manufacturer. RNase protection assays (RPA) were perfomed on 2.5 to 5 μg of RNA using the Riboquant MultiProbe RNase Protection Assay System (PharMingen) following the manufacturer's instructions. TNF-α, iNOS and MMP-2 mRNA levels were determined by northern-blot analysis according to standard methods[28,29,31]. The membranes were exposed to X-ray films, and signal quantification was performed in a PhosphorImager SI.

Measurement of serum anti-CII antibody levels. Serum samples were collected before immunization and, 25, 35, and 45 d after primary immunization for the detection of anti-CII IgG, IgG1, and IgG2a antibody levels. The level of serum antibodies to CII was measured by ELISA as described[32].

Zymography. Synovial culture supernatants were resolved as zymograph samples in 0.05 M Tris-HCl, pH7.4, 5 mM $CaCl_2$, 1% SDS and 5% glycerol, then subjected to 10% SDS–PAGE into which gelatin (1 mg/ml) had been cross-linked, as described[26,31].

Flow cytometric analysis. Analysis of intracellular cytokines (IL-4 and IFN-γ) and surface antigens (CD3, CD4 and CD8) in synovial cells was performed by flow cytometry as described[33].

Statistical analysis. The Mann-Whitney *U*-test to compare nonparametric data for statistical significance was applied on all clinical results and cell-culture experiments.

Acknowledgments

We thank P. Robberecht for the VPAC1 agonist and antagonist; D. Bolin and A. Welton for the VPAC2 agonist Ro 25-1553; and E. Lerner for the PAC1 agonist maxadilan. This work was supported by grant PM98-0081, and by the postdoctoral fellowships from the Spanish Department of Education and Science (M.D.) and the Autonomic Community of Madrid (C.M.).

RECEIVED 9 AUGUST 2000; ACCEPTED 22 FEBRUARY 2001

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