

Pituitary Adenylate Cyclase-Activating Polypeptide Inhibits Collagen-Induced Arthritis: An Experimental Immunomodulatory Therapy¹

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Rheumatoid arthritis is a chronic, systemic, autoimmune, and inflammatory disorder that affects the synovial lining of the joints. We describe the beneficial effects of the pituitary adenylate cyclase-activating polypeptide (PACAP) in the collagen-induced arthritis experimental murine model being proposed as a novel therapeutic approach in the treatment of rheumatoid arthritis. PACAP greatly decreases arthritis frequency and severity in the studied mice by improving clinical symptoms, ameliorating joint damage, and blocking both the inflammatory and autoimmune mediators which are the main keys of the pathogenesis of this disease. With this study, PACAP emerges as a promising candidate for the treatment of a pathology with a high world incidence but currently no effective treatment. *The Journal of Immunology*, 2001, 167: 3182–3189.

Rheumatoid arthritis (RA)³ is a chronic inflammatory disease of unknown etiology but with autoimmune features characterized by a chronic inflammation of the synovial tissues. The main symptoms of this immune disorder, such as joint pain, redness, warmth, and dystrophy in the most advanced stages, are due to a massive infiltration of immune cells into the synovial membrane and fluid, mainly neutrophils, macrophages, and T cells (1, 2). These cells release an excessive amount of chemokines which recruit larger amounts of cells to the site of inflammation (3), matrix metalloproteinases which destroy the tissues (4), and cytokines produced by highly activated macrophages, fibroblasts (proinflammatory mediators like TNF- α , IL-1, and IL-6) (4, 5), and T cells (mainly IFN- γ) (6) which contribute to generate the joint damage. In this sense, although the role of T cells in the pathogenesis of arthritis is not clear, it has been discovered that there is a polarization of their response to a Th1 one, suggesting that an unbalance of the T response toward a self-Ag could be also important in the development of arthritis (6–9). As a result of all these facts, the synovium, which in normal conditions is a fragile bilayer membrane covering the cartilage and bone in the joint, is transformed into a thick invasive one that destroys its whole structure, creating an instability and deformation of the tissue that explains the stiffness and ankylosis experienced by the patient in the last steps of the disease (10, 11).

Currently, the main treatment used in RA is drug therapy (12). First-line agents are primarily used to relieve the symptoms. Second-line agents (also called disease-modifying agents) appear to have an effect on the underlying process. Because many patients do not respond to the traditional antirheumatic drugs, and some of these treatments show harmful side effects, considerable effort is being focused on developing new drugs. In this sense, the blockade of TNF- α (13) and IL-6 (14) with mAbs seems to be efficient to ameliorate the synovial inflammation in RA, although a long term treatment with these Abs may result in an increased incidence of other autoimmune diseases. Because a specific causative agent or Ag has not been identified yet, bypassing the potential Ag and targeting the cytokine unbalance could represent a solid way to control RA.

Pituitary adenylate cyclase-activating polypeptide (PACAP), a 38-aa neuropeptide belonging to the secretin-glucagon-vasoactive intestinal peptide (VIP) family, exerts important functions in the nervous and immune systems (15). One of the most interesting functions of PACAP in the immune system is the antiinflammatory function, which is exerted by its capacity to decrease proinflammatory cytokines like TNF- α and IL-6 and by the increase of antiinflammatory cytokines like IL-10 (16–24). Thus, by modifying the levels of these cytokines, it has been proved that PACAP prevents death from lethal endotoxemia in mice (25). Also, PACAP can redirect a Th1 cell response to a Th2 response (26). In view of these findings, PACAP appears to be a potential therapeutic compound for RA, regulating the two main causes of the disease, the inflammatory and the autoimmune responses. We have used the collagen II-induced arthritis (CIA) model in DBA/1 mice (27, 28), which resembles many of the clinical, histological, and immunological features of human RA, to study the effect of an exogenous injection of PACAP, expecting a remission of the symptoms of the disorder, by modulating the levels of macrophage and T cell-secreted cytokines. Our results show that PACAP administration is beneficial at both the pathological and the clinical level and is a promising candidate for the treatment of RA.

Materials and Methods

Animals

Male DBA/1J mice 6–10 wk of age were purchased from The Jackson Laboratory (Bar Harbor, ME).

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³ Abbreviations used in this paper: RA, rheumatoid arthritis; PACAP, pituitary adenylate cyclase-activating polypeptide; VIP, vasoactive intestinal peptide; CIA, collagen II-induced arthritis; CII, type II collagen; MMP-2, matrix metalloproteinase 2; IL-1Ra, IL-1R antagonist; iNOS, inducible NO synthase; PPD, purified protein derivative; MCP, monocyte chemoattractant protein; MIP, macrophage-inhibitory protein.

Materials

Native bovine type II collagen (CII), DNase type IV, polymyxin B sulfate, and avidin peroxidase were purchased from Sigma (St. Louis, MO). CFA was obtained from Difco (Detroit, MI). Calipers were obtained from Kroepelin (Schluchtern, Germany). PACAP was purchased from Calbiochem (La Jolla, CA). Ab pairs (capture/biotinylated) for IL-4 and IFN- γ ELISAs (BVD4-1D11/BVD6-24G2, and R4-6A2/XMG1.2 respectively), as well as biotin-conjugated goat anti-mouse IgG, IgG1, or IgG2a, ELISPOT kits, and the Riboquant MultiProbe RNase Protection Assay System were obtained from BD PharMingen (San Diego, CA). Matrix metalloproteinase 2 (MMP-2) standards were obtained from Chemicon International (Temecula, CA). Collagenase A was purchased from Boehringer Mannheim (Mannheim, Germany). RPMI 1640 and FCS for tissue cultures were purchased from BioWhittaker (Verriers, Belgium). The tissue tearer was obtained from Biospec Products (Bartlesville, OK). [γ - 32 P]dATP (3000 Ci/mmol) was purchased from Amersham (Arlington, IL). X-ray films were obtained from Kodak (Rochester, NY), and PhosphorImager SI was from Molecular Dynamics (Sunnyvale, CA).

Induction of CIA

To study the effect of PACAP on arthritis, we used the experimental model of CIA. Briefly, CII, dissolved in 0.05 M acetic acid at 4°C overnight, was emulsified with an equal volume of CFA. Mice were injected intradermally at the base of the tail with 0.15 ml of the emulsion containing 200 μ g CII and then boosted i.p. with 200 μ g CII in PBS 21 days after the primary immunization.

Assessment of CIA

For the analysis of mice, conducted every other day, signs of arthritis onset were monitored using as representative parameters paw swelling and clinical score. The study was conducted in a blinded manner by two independent examiners who determined the level of paw swelling by measuring the thickness of the affected hind paws with 0- to 10-mm calipers. Arthritis symptoms were assessed by using a scoring system (grade 0, no swelling; grade 1, slight swelling and erythema; grade 2, pronounced edema; grade 3, joint rigidity and ankylosis). Each limb was observed and graded with a maximum possible score of 12 per animal. Group comparisons were performed using the χ^2 test for disease incidence and an unpaired, two-tailed Student *t* test for arthritis scores.

Treatment protocols

Treatment with PACAP began when the secondary immunization was performed, and it was administered i.p. at the specified doses either daily or every other day until day 35 after primary immunization. In some cases, PACAP was added at different times in pulses after the onset of the disease. A control consisting in a group of mice injected with PBS alone, was used in each experiment.

Histopathology

Forty-five days after the first immunization, mice were sacrificed by cervical dislocation, and two independent experimenters randomly collected the hind paws from five to nine animals. Paws were fixed with 10% paraformaldehyde, decalcified in 5% formic acid, and embedded in paraffin. Sections (5 μ m) were stained with hematoxylin-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, depending on the amount of inflammatory cells in the synovial cavity (exudate) and synovial tissue (infiltrate). Cartilage destruction was graded on a scale from 0 to 3, from the appearance of dead chondrocytes (empty lacunae) to the complete loss of the joint cartilage. Bone erosion was graded on a scale of 0–3, from a normal appearance to completely eroded cortical bone structure.

mRNA analysis

A tissue tearer was used to homogenize mice joints, and total RNA was isolated with the Ultraspec (Biotecx Laboratories, Houston, TX) RNA reagent as recommended by the manufacturer. The Riboquant MultiProbe RNase Protection Assay System was used on 2.5–5 μ g RNA following the manufacturer's instructions for RNase protection assays. A set of cytokine-chemokine templates and a template for the housekeeping gene *GAPDH* were supplied by each commercial kit. In vitro transcription from these cDNA templates was necessary to synthesize [α - 32 P]UTP-labeled antisense RNA probes, being purified by phenol-chloroform extraction and ethanol precipitation and hybridizing with the RNA samples at 56°C overnight (unhybridized ssRNA was digested by RNase treatment). dsRNA

purified by phenol-chloroform extraction and ethanol precipitation was electrophoresed on a 5% denaturing polyacrylamide gel, which was then dried and exposed to x-ray films. The signal was quantitated in a PhosphorImager SI.

The levels of TNF- α , inducible NO synthase (iNOS), and MMP-2 mRNA were determined by Northern blot analysis according to standard methods. The probes for murine TNF- α , iNOS, and GAPDH were generated by RT-PCR as described previously (20, 21, 29), using oligonucleotides that were end-labeled by a T4 polynucleotide kinase. The membranes were exposed to x-ray films, and the signal was quantitated in a PhosphorImager SI.

Zymography

Synovial culture supernatants were resolved as zymograph samples in 0.05 M Tris-HCl (pH 7.4), 5 mM CaCl₂, 1% SDS, 5% glycerol and subjected to electrophoresis in 10% SDS-polyacrylamide gels into which gelatin (1 mg/ml) had been cross-linked (29, 30). After electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 h. The gelatinolytic reaction was induced by incubating the gels in the reaction buffer (50 mM Tris-HCl (pH 7.6), 0.15 M NaCl, 5 mM CaCl₂, 0.02% Na₃N, 1 mM ZnCl₂) at 37°C for an appropriate time (usually 24 h). Gels were stained and destained with 0.125% Coomassie brilliant blue and 10% acetic acid, 10% methanol, respectively. The stained gels were digitized, and the zones of proteolysis, corresponding to the presence of proteases in the gel, were quantitated using NIH image (National Institutes of Health, Bethesda, MD). Identification of the 72- and 62-kDa forms of MMP-2 was determined by comparison of the migratory position of the band with known MMP-2 standards.

In vitro study of T cell function

Mice were sacrificed at day 28 after primary immunization. Single spleen cell suspensions were prepared and cultured in 96-well flat-bottom microtiter plates at a density of 1×10^6 cells/ml (200 μ l/well) in complete medium in the presence or absence of different concentrations of heat-inactivated CII. For proliferation assays, cells were cultured at 37°C in 5% CO₂ for 4 days, and 1 μ Ci/well [3 H]TdR was added in culture for the last 18 h. Cells were harvested, 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 harvested and analyzed for IFN- γ and IL-4 by sandwich ELISA using Ab pairs as previously described (26). ELISPOT was used after 24 h culture according to the supplier's protocol to determine the frequency of CII-specific T cells producing IFN- γ or IL-4 as previously described (25). Purified protein derivative (PPD, 30 μ g) was injected intradermally in the CII-CFA emulsion as a recall Ag control, and in vitro T cell function after culture stimulation with 10 μ g/ml PPD was assayed as described above.

Culture of synoviocytes

Mice were sacrificed at day 30 after primary immunization, the rear limbs were removed, and the synovial membrane of the knee joints was carefully separated from the bone and cartilage by microscopic dissection. Digestion of the synovial tissue in the presence of 33 μ g/ml polymyxin B sulfate was done with collagenase A (1 mg/ml) and DNase type IV (150 μ g/ml) at 37°C for 20 min, as previously described for human synovial tissue (31). To prepare a single-cell suspension, the digested tissue was passed through a nylon mesh. After being washed extensively, and cells were cultured in 24-well plates at a density of 2×10^6 cells/ml (1 ml/well) in complete medium (RPMI 1640, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, 40 μ M β_2 -mercaptoethanol, and 10% heat-inactivated FCS) with or without PACAP at different concentrations. Supernatants were collected after 24 h for chemokines, IL-1 β , and TNF- α analysis or 72 h for IL-10 and IL-12 analysis and stored at -20°C until cytokine and chemokine determination. Sandwich ELISA was performed to measure the levels of immunoreactive IL-12p40, IL-1 β , IL-10, and chemokines as previously described (20–22). The WEHI 164 cell line assay (32) was used to determine the levels of bioactive TNF- α .

Arthritis by adoptive transfer

DBA/1J mice were immunized with CII and CFA in the presence or absence of PACAP. After 14 days from the immunization, T cells were isolated from the mice spleens as previously described (26). 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.

Measurement of serum anti-CII Ab levels

Serum samples were collected for the detection of anti-CII IgG, IgG1, and IgG2a Ab levels by ELISA 25, 35, and 45 days after primary immunization, as previously described (33). Briefly, ELISA plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 10 µg/ml native bovine CII in PBS and washed with PBS-0.05% Tween 20, and nonspecific protein-binding sites were blocked with 3% BSA-PBS for 2 h at room temperature. After a washing, samples in serial dilutions from 1/100 to 1/10⁵ were added and incubated for 2 h at room temperature. After three washes, biotin-conjugated goat anti-mouse IgG, IgG1, or IgG2a was added and incubated at room temperature for 1 h, followed after extensive washing by avidin-peroxidase, and the plates were developed using ABTS as substrate. The OD was measured with a microplate reader. A standard serum, i.e., mixture of sera from arthritic mice, was added to each plate in serial dilutions, and a standard curve was generated to design arbitrary units of total IgG, IgG1, and IgG2a anti-CII Abs.

Flow cytometric analysis

Flow cytometry was performed for the analysis of intracellular cytokines (IL-4 and IFN-γ) and surface Ags (CD3, CD4, and CD8) in synovial cells as previously described by Kusaba et al. (34).

Statistical analysis

To compare nonparametric data for statistical significance, we applied the Mann-Whitney *U* test on all clinical results and cell culture experiments. The χ^2 test was used to analyze histological data.

Results

Reduced incidence and severity of arthritis after PACAP treatment

To study the effect of PACAP on arthritis, we used the CIA experimental model. DBA/1 mice were immunized with bovine CII in complete adjuvant, boosted with CII, and monitored for the occurrence of clinical signs of arthritis. Several doses of PACAP were given i.p. in two pulses, daily, or on alternate days for 2 wk after the onset of the disease.

The i.p. administration of PACAP resulted in a dose-dependent improvement of the clinical symptoms and paw swelling (Fig. 1), with a direct decreasing effect on the incidence and the clinical score of the disease. Daily or every other day administration of PACAP 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. No remission in the therapeutic effect was observed after 2 wk of cessation of PACAP administration on day 35 (Fig. 1, A and C), suggesting that after a short period of PACAP treatment no additional neuropeptide is necessary to maintain the protection from the disease.

The dose-dependent therapeutic effects of PACAP were confirmed in the homologous CIA model (35), a chronic relapsing arthritis with a clinical pattern that more closely resembles human disease (area under the curve, which reflects overall disease severity during 35 days, was 46.7 in the controls and 43.2 in the 0.5-nmol PACAP group and was reduced to 25.8 and 23.6 in the 5- and 10-nmol PACAP groups; data not shown).

Because few differences were observed between the 5- and 10-nmol doses and between PACAP administration daily and every other day, all additional experiments were conducted at the 5-nmol dose on alternate days.

Histopathological analysis of joints shows that CIA-characterized chronic inflammation of synovial tissue (synovocyte proliferation and leukocyte infiltration), pannus formation, cartilage destruction, and bone erosion were completely abrogated by treatment with PACAP (Fig. 2).

An important aspect in the therapeutic effect of PACAP is the fact that the neuropeptide was able to prevent and ameliorate already established disease, because administration of PACAP 12 days after disease onset (day 34), when most mice had developed

arthritis, blocks development of disease, and even an improvement on clinical score and incidence of arthritis was observed (Fig. 3).

Above all, PACAP beneficial role in arthritis was also tested in a model consisting in the adoptive transfer of whole or T spleen cells from CIA mice to naive nonarthritic mice. Transfer of cells from CIA mice produced high incidence and severity of arthritis in the recipient naive mice. However, when the source of transferred cells was PACAP-treated CIA mice, the level of joint damage was significantly reduced (Fig. 4). Therefore, PACAP prevents adoptive transfer of arthritis.

PACAP effect on the inflammatory component of CIA

The mechanisms responsible for the observed decrease in incidence and severity of CIA following PACAP treatment were next investigated. One of the causes of the joint damage in arthritis is an excessive inflammatory response (1). Because PACAP has been reported to be a potent anti-inflammatory molecule (13–24), we tested whether PACAP was able to affect the expression and production of these proinflammatory agents in arthritic joints. Analysis of paws by RNase protection assay and Northern blot shows

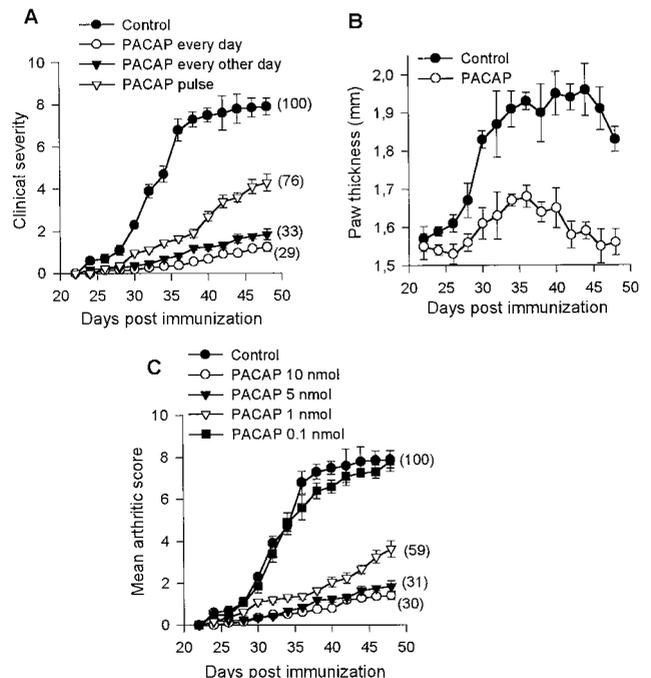


FIGURE 1. PACAP decreases CIA incidence and severity. Mice were immunized with 100 µg bovine CII in CFA on day 1 and boosted on day 21 with 100 µg CII in PBS. **A**, From day 21 after primary immunization, mice were treated i.p. with PBS (controls) or with 5 nmol PACAP daily, every other day for 2 wk, or at day 22 and day 24 only (pulse). Severity of clinical signs was assessed. Incidence of arthritis (percent of diseased mice) in each experimental group is shown in parentheses. Results are expressed as the mean \pm SD from three separate experiments (eight mice/group per experiment); $p < 0.001$ vs control for all treatments after day 30. **B**, From day 21 after primary immunization, mice were treated i.p. with PBS (controls) or with 5 nmol PACAP every other day for 2 wk, and paw thickness was measured. Data are expressed in millimeters and are the mean \pm SD from two separate experiments (eight mice/group per experiment); $p < 0.001$ vs control after day 30. **C**, From day 21 after primary immunization, mice were treated i.p. with PBS (controls) or with different amounts of PACAP every other day for 2 wk. Severity of clinical signs was assessed. Incidence of arthritis (percent of diseased mice) in each experimental group is shown in parentheses. Results are expressed as the mean \pm SD from three separate experiments (eight mice/group per experiment); $p < 0.001$ vs control for 1- to 10-nmol treatments after day 30.

that PACAP inhibited CIA-induced mRNA expression of the proinflammatory factors TNF- α , IL-6, IL-12, iNOS, IL-18, IL-1 β , and IL-1 α , as well as of several chemokines, such as RANTES, monocyte chemoattractant protein (MCP)-1, macrophage-inhibitory protein (MIP)-1 α , MIP-1 β , and MIP-2 (Fig. 5A). However, mRNA levels of the anti-inflammatory cytokines IL-10 and IL-1R antagonist (IL-1Ra) were significantly increased on treatment of arthritic mice with PACAP (Fig. 5A). Similar results were obtained with primary synovial membrane cultures, derived from the knees of arthritic mice. In vitro treatment of synovial cells with PACAP inhibited, in a dose-dependent manner, spontaneous release of IL-1 β , TNF- α , IL-12, RANTES, MIP-1 α , MCP-1, and MIP-2 and significantly increased production of IL-10 (Fig. 5B). Inhibition in chemokine production is correlated by the fact that PACAP inhibits chemotactic activity of both synovial T cells and macrophages from CIA mice (not shown). Together, these results indicate that treatment with PACAP reduces the inflammatory response characteristic of arthritis by down-regulating and up-regulating the expression of proinflammatory and anti-inflammatory agents, respectively, in inflamed joints.

MMP expression and activity is inhibited by PACAP in CIA

MMPs have been assigned pivotal roles in the depletion of proteoglycan and collagen observed in the joints, which leads to the cartilage and bone erosion in patients with RA. We further tested whether PACAP regulates MMP production by arthritic synovial cells. PACAP treatment significantly inhibited mRNA expression of the gelatinase MMP-2 in paws of arthritic mice (Fig. 6A). This

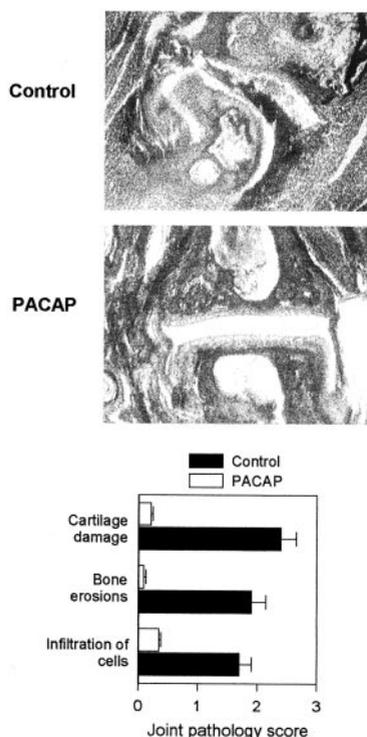


FIGURE 2. PACAP prevents CIA-associated histopathological alterations. Mice were immunized with 100 μ g bovine CII in CFA on day 1 and boosted on day 21 with 100 μ g CII in PBS. From day 21 after primary immunization, mice were treated i.p. with PBS (controls) or with 5 nmol PACAP every other day for 2 wk, and joint histopathology was examined at day 45. Histology was performed on whole paws. A representative section is shown. Scoring of inflammation, cartilage damage, and bone erosion was performed by two independent observers on decoded slides. Results are the mean \pm SD from 12 mice/group.

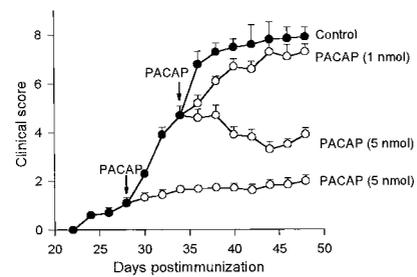


FIGURE 3. PACAP inhibits established CIA. Mice were immunized with 100 μ g bovine CII in CFA on day 1 and boosted on day 21 with 100 μ g CII in PBS. From day 28 and day 34 (arrows), mice were treated i.p. with PACAP (1 or 5 nmol) every other day. Severity of clinical signs in untreated (●) and PACAP-treated (○) mice was assessed. Results are expressed as the mean \pm SD from three separate experiments (eight mice/group per experiment); $p < 0.001$ vs control for both 5-nmol treatments after PACAP administration.

effect was correlated with a PACAP-induced decrease on MMP-2 gelatinase activity on synovial membrane cultures from arthritic mice (Fig. 6B). In contrast, MMP-9 and MMP-13 production was not affected by the treatment with PACAP (not shown). The inhibitory effect of PACAP on MMP-2 could be directly related, at least partially, to the PACAP-mediated inhibition of cartilage destruction and bone erosion.

PACAP inhibits CII-specific T cell proliferation in CIA

Because one of the main characteristics of CIA is hypercellularity, and PACAP has several immunomodulatory effects, including inhibition of T cell proliferation and regulation of Th1-Th2 balance (26, 36), we investigated whether impaired T cell functions in PACAP-treated mice lead to CIA inhibition. We first tested the effect of PACAP treatment in 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 PACAP responded slightly to CII (Fig. 7A). Thus, T cell clonal expansion toward CII is inhibited, at least partially, by PACAP, this being one of the mechanisms that this neuropeptide could be using to abrogate the disease.

PACAP modulates Th1 and Th2 response in CIA

There is evidence that CIA is an autoimmune disorder mediated by Th1 cytokines, whereas the supply of Th2 cytokines has great benefit in the development of severe arthritis (6–9, 37, 38). Because PACAP has been noticed to polarize the Th response to a Th2 response, we next studied the effect of PACAP on T cytokine production in CIA mice by using CII as a specific stimulus.

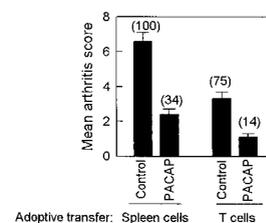


FIGURE 4. PACAP inhibits adoptive transfer of arthritis. Naive DBA/1 mice were adoptively transferred with whole spleen cells (5×10^7 cells) or purified T cells (3×10^7 cells) from CIA mice treated in the presence or absence of PACAP (5 nmol). Severity of clinical signs and incidence of arthritis (numbers in parentheses) were assayed. Results are expressed as the mean \pm SD from three separate experiments (six mice/group/experiment).

As shown in Fig. 7B, CIA resulted in the development of CII-specific effector T cells producing high levels of IFN- γ and low levels of IL-4, showing a Th1 secretion pattern, which is one of the characteristics of the disease. Inversely, cells isolated from PACAP-treated mice produced large amounts of IL-4 and low levels of IFN- γ (Fig. 7B). Therefore, PACAP leads the T cell response to a Th2 response in the CIA model of arthritis. No cytokine production by T cells was detectable in the presence of an unrelated Ag (OVA; data not shown).

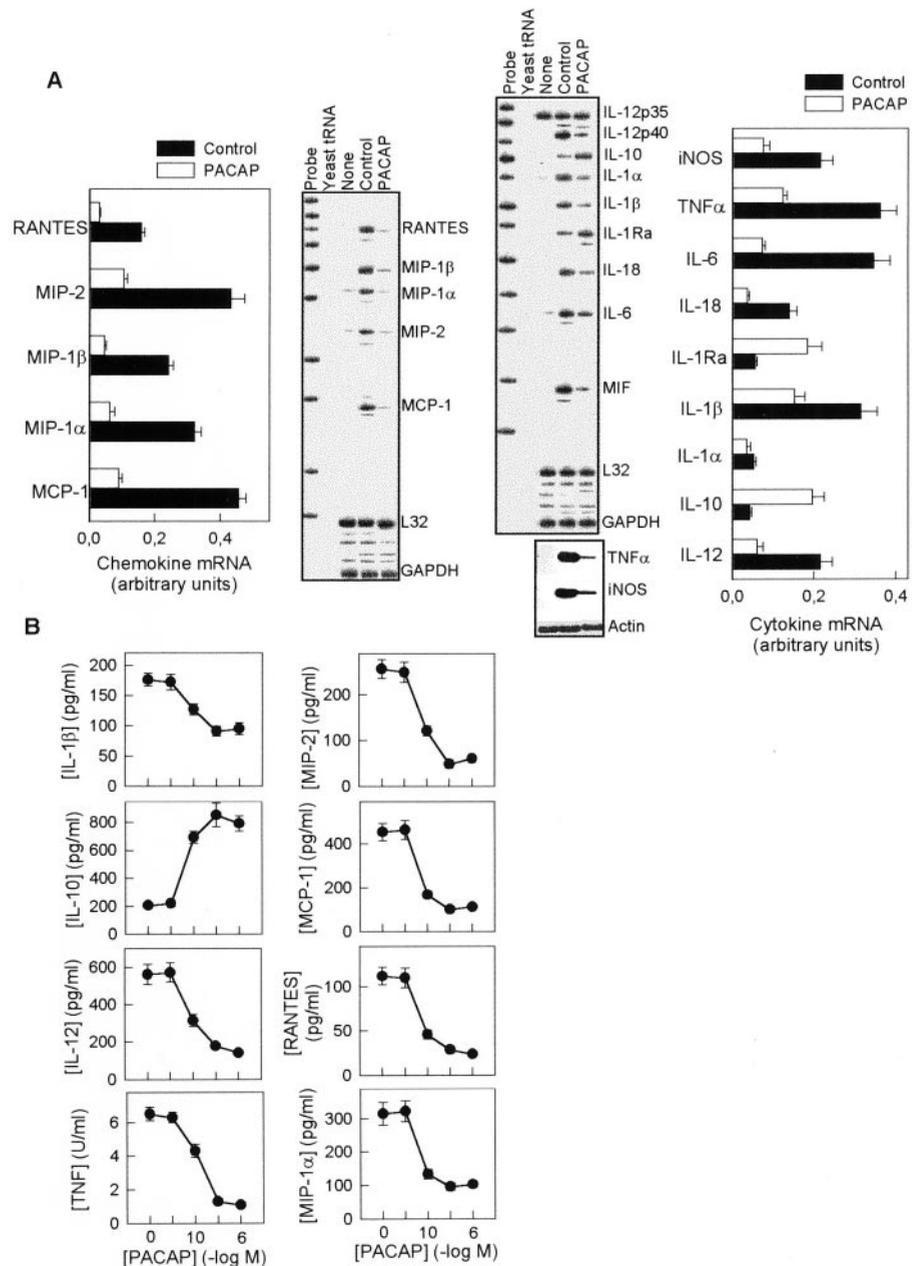
To test whether this control of Th1/Th2 cytokine balance was exerted by regulating the *in vivo* generation/differentiation of CII-specific Th1 and Th2 effector cells, the frequency of Ag-reactive Th1 and Th2 cells was assessed on the basis of IFN- γ - and IL-4-secreting cells, respectively, using ELISPOT assays. Mice injected with the Ag in the absence of PACAP developed many IFN- γ -producing Th1 cells, and very few IL-4-producing Th2 cells (Fig. 7C). In contrast, CIA mice injected with PACAP generated few IFN- γ -specific spots, while developing high numbers of IL-4-secreting Th2 cells (Fig. 7C).

PACAP regulates CII-specific IgG1 and IgG2a Abs in CIA

Production of high levels of circulating Abs against CII is a major factor in determining susceptibility to CIA. Th1 and Th2 lymphocytes differentially affect switching to IgG2a/b and IgG1, respectively. Because the development of Ag-specific Abs requires T cell help, one mechanism of CIA inhibition by PACAP could be due to a failure to produce anti-CII Abs, particularly autoreactive IgG2a Abs that have been involved in the pathogenesis of CIA. The serum levels of total IgG or isotype-specific IgG2a and IgG1 anti-CII Abs were measured at different times after onset of arthritis.

As Th1 and Th2 cytokines modulate Ig isotype switching, we measured the serum levels of specific anti-CII IgG isotypes as another proof of PACAP modulation of the Th1-Th2 response in CIA. This study was performed at different times from the onset of the induced arthritis. CIA resulted in high levels of CII-specific IgG Abs, characterized by a high IgG2a:IgG1 ratio (Fig. 7D). In contrast, treatment of CIA mice with PACAP significantly reduced

FIGURE 5. PACAP inhibits inflammatory response in CIA. Mice were immunized with 100 μ g bovine CII in CFA on day 1 and boosted on day 21 with 100 μ g CII in PBS. A, From day 21, mice were treated i.p. with PBS (controls) or with 5 nmol PACAP every other day. Mice were sacrificed on day 35, hind paws were removed, and mRNA analysis was performed by RNase protection assay or Northern blot (for TNF- α and iNOS). Blots are representative of five similar experiments. Results are expressed in arbitrary densitometric units normalized for the expression of GAPDH in each sample (mean \pm SD of five separate experiments). A paw from an unimmunized mouse was analyzed simultaneously for assessment of the basal response; $p < 0.001$ vs control for all cytokines and chemokines. B, Groups of three CII-immunized mice were sacrificed at day 30 after primary immunization, and synovial membrane cells were isolated from knee joints and pooled for each of five separate experiments. Synovial cells were then cultured in the absence or presence of various concentrations of PACAP. Medium alone was added to control cultures. After different times, supernatants were assessed for cytokine and chemokine production. Results represent the mean \pm SD; $p < 0.001$ vs control for all cytokines and chemokines.



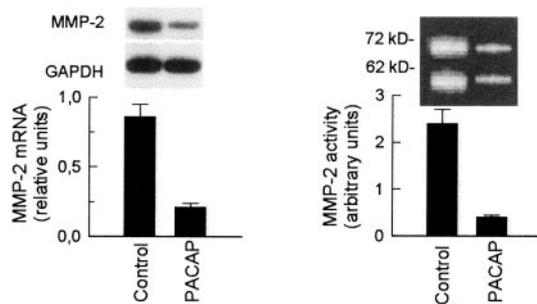


FIGURE 6. PACAP inhibits MMP-2 expression and activity in CIA. Mice were immunized with 100 μg bovine CII in CFA on day 1 and boosted on day 21 with 100 μg CII in PBS. *A*, From day 21, mice were treated i.p. with PBS (controls) or with 5 nmol PACAP every other day. Mice were sacrificed on day 35, hind paws were removed, and MMP-2 mRNA levels were determined by Northern blot. Results are expressed in arbitrary densitometric units normalized for the expression of GAPDH in each sample (mean \pm SD of five separate experiments). A paw from an unimmunized mouse was analyzed simultaneously for assessment of the basal response. *B*, Groups of three CII-immunized mice were sacrificed at day 30 after primary immunization, and synovial membrane cells were isolated from knee joints and pooled for each of five separate experiments. Synovial cells were then cultured in the absence (control) or presence of 10^{-8} M PACAP. With the use of gelatin zymography, MMP-2 activity (gelatinase activity) in culture supernatants was estimated. The positions of the 72-kDa pro- and 62-kDa active forms of MMP-2 are indicated. Results represent the mean \pm SD of the lysis zones and are expressed as arbitrary units of relative band intensity; $p < 0.001$ vs controls.

anti-CII-specific IgG levels, mainly during the first days after disease onset (Fig. 7*D*). This inhibitory effect was accompanied by a reduction in IgG2a:IgG1 ratio, due to inhibition and stimulation of anti-CII IgG2a and IgG1 production, respectively (Fig. 7*D*), another proof of PACAP modulation of the Th1-Th2 response in CIA.

PACAP decreases Th-suppressor ratio in the synovial tissue

Arthritic joints present an elevated Th-suppressor ratio in comparison with the blood and spleen T cell ratios. Therefore, we next investigated whether this ratio in synovial cells can be affected by PACAP treatment of ongoing CIA. As Fig. 8*A* shows, PACAP-treated mice showed a significantly lower CII-specific proliferative response of synoviocytes than that in control arthritic mice. PACAP treatment of CIA mice reduced the Th (CD4)-T suppressor (CD8) ratio from 11 (controls) to ~ 4.4 (Fig. 8*B*, *top*). Because T suppressor numbers were not significantly affected, although Th numbers are lower in PACAP-treated mice than in controls (Fig. 8*B*, *bottom*), the CD4:CD8 ratio decrease seems to be due to impaired development of Th cells, rather than to an increase in the number of T suppressor cells. This relative decrease of synovial Th cells mediated by PACAP was due to a preferential diminution of IFN- γ vs IL-4-producing CD4 cells (Fig. 8*B*), probably by inhibiting development of CII-specific Th1 and/or their entry into joints.

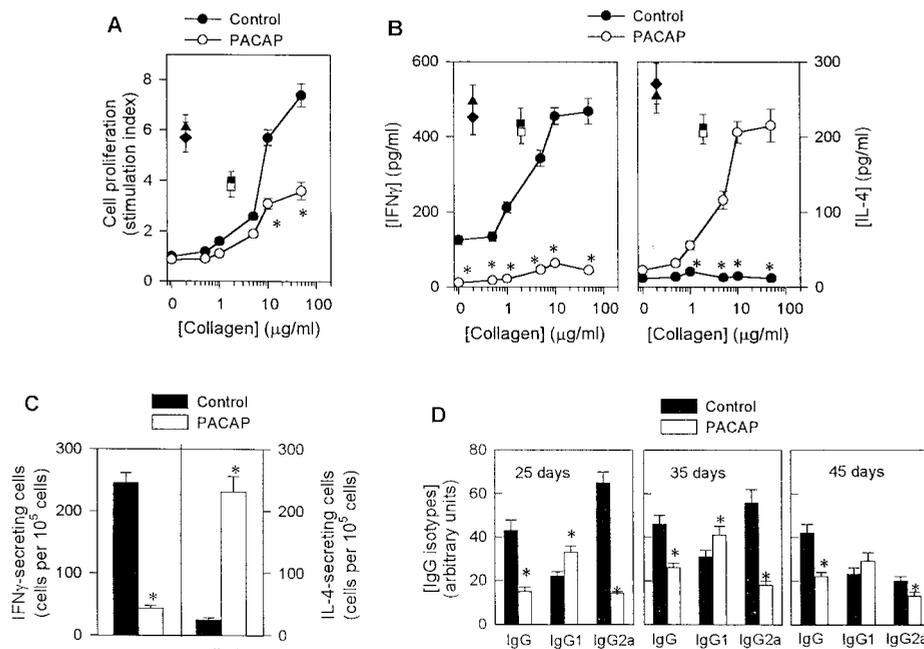


FIGURE 7. PACAP modulates Th1-Th2 balance in CIA. Mice were immunized with 100 μg bovine CII in CFA on day 1 and boosted on day 21 with 100 μg CII in PBS. From day 21, mice were treated i.p. with PBS (controls) or with 5 nmol PACAP every other day. *A–C*, PACAP inhibits *in vitro* Th1-mediated response in CIA. Mice were sacrificed on day 28, and spleen cells were stimulated with different concentrations of inactivated CII (10 $\mu\text{g/ml}$ in *C*). Spleen cells stimulated with 2 $\mu\text{g/ml}$ anti-CD3 Ab were used for assessment of unspecific stimulation (■, control; □, PACAP). Spleen cells from PPD/CFA-immunized CIA mice stimulated with PPD Ag (10 $\mu\text{g/ml}$) were used for assessed Ag specificity (▲, control; ◆, PACAP). A pool of three unimmunized DBA/1 spleens were used for assessment of the basal response. *A*, Proliferative response was determined after 4 days of culture by [^3H]TdR incorporation and was expressed as a stimulation index ((CII-specific proliferation – unstimulated proliferation)/unstimulated proliferation). *B*, After 72 h culture, IL-4 and IFN- γ levels in supernatants were assayed by ELISA. *C*, The number of CII-specific T cells producing IFN- γ or IL-4 was determined after 24-h culture by ELISPOT. Results represent the mean \pm SD from two independent experiments (five mice/group/experiment). *D*, PACAP regulates CII-specific IgG1 and IgG2a levels. Serum samples were collected 25, 35, and 45 days after primary immunization with CII. Anti-CII-specific IgG, IgG1, and IgG2a levels were measured by ELISA. Data are represented as the mean \pm SD using an arbitrary unit, as analyzed in three separate experiments (eight mice/group/experiment). *, $p < 0.001$ vs control.

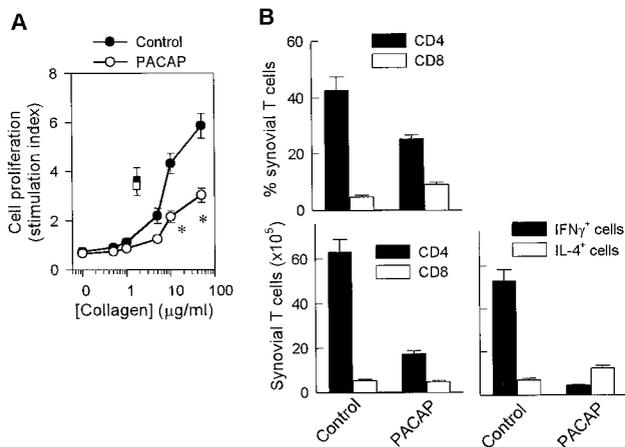


FIGURE 8. PACAP decrease Th-suppressor ratio in ongoing CIA. Mice were immunized with 100 μg bovine CII in CFA on day 1 and boosted on day 21 with 100 μg CII in PBS. From day 28, mice were treated i.p. with PACAP (5 nmol) every other day. Mice were sacrificed at day 35 after primary immunization, and synovial membrane cells were isolated from knee joints and pooled for each of five separate experiments. *A*, Synovial cells were stimulated with different concentrations of inactivated CII (10 $\mu\text{g}/\text{ml}$). Proliferative response was determined after 4 days culture by [^3H]TdR incorporation and was expressed as a stimulation index. Synovial cells stimulated with 2 $\mu\text{g}/\text{ml}$ anti-CD3 Ab were used for assessment of unspecific stimulation (■, control; □, PACAP). *B*, Synovial cells were counted, stained with Abs against CD4 or CD8 and IL-4 or IFN- γ , and analyzed by flow cytometry. Each point represents the mean percentages (*top*) and numbers (*bottom*) \pm SD performed in triplicate.

Discussion

Multiple molecular and histological data shown in this report prove that PACAP administered as an exogenous drug in the CIA mouse model is capable of ameliorating arthritis symptoms by disrupting the molecular autoimmune and inflammatory basis of the disease. Thus, CIA mice treated with this neuropeptide show a disappearance of the inflamed aspect of their paws, and when a microscopic study is performed there is a clear reduction of the invasive tissue, called pannus, which causes the main damage in the structure of the joints in arthritis. All of these observations suggest a potential therapeutic role for PACAP in RA, which should be considered, because most of the treatments for this disorder are not completely successful, and many of them have relevant secondary effects.

The main action of PACAP in relation to this chronic disease is its capacity to modulate the levels of several soluble factors including proinflammatory, anti-inflammatory cytokines, and chemokines, which deregulation triggers an excessive immune response leading to the damage of the joint (15–23). On the one hand, PACAP function is mediated by its effect on the inflammatory component of the disease by down-modulating the cytokines, chemokines, and destructive enzymes that are massively released by the activated fibroblasts and macrophages in the altered synovial tissue (1–5). Thus, this neuropeptide not only acts directly, reducing the levels of proinflammatory mediators like TNF- α , IL-6, IL-1 β , iNOS, and IL18, but also increases the amounts of antiinflammatory cytokines like IL-10 and IL-1Ra. In this sense, the down-regulation of TNF- α is crucial to explain the healing of the affected mice because this cytokine is considered to have a critical role in the development of inflammatory diseases like RA or Crohn's disease in that it promotes the increase of the production of most other proinflammatory mediators (1–5). PACAP also has antirheumatic effects because it reduces the levels of chemo-

kines like RANTES, MIP-1 α , MIP-1 β , MIP-2, and MCP which are responsible for the hypercellularity of the synovial membrane of CIA mice. Other soluble mediators like MMPs (MMP-2), which contribute to destruction of the joint structure by attacking the conjunctive support (10), are also down-regulated by PACAP. The main action of PACAP as an anti-inflammatory factor on all these mediators is carried on macrophages (9–24, 39). Thus, this cellular type might be the main target of the antiinflammatory action of this neuropeptide in the synovial tissue. As a result of the reduction of the levels of all of these harmful soluble factors in RA, there is a clear remission of the chronic inflammation of the joint of the affected mice.

The effect of PACAP is not restricted to the mediators produced by synoviocytes; it also affects the cytokines released by the infiltrated T cells. There is strong evidence that the majority of the T cells in the inflamed tissues in RA as in many other autoimmune diseases show a Th1 cytokine pattern (6–8). In fact, certain factors that promote a Th2 response instead of a Th1 response are beneficial for patients with these disorders. Moreover, the Ig isotype switching that is directed by Th1 or Th2 cytokines in a different way (i.e., IFN- γ and IL-4 induce IgG2a and IgG1 synthesis, respectively) is another marker of this disease that shows high anti-CII IgG2a circulating levels (40). PACAP has been previously shown to induce a Th2 response by stimulating the release of Th2 cytokines and inhibiting the production of Th1 cytokines (22). Moreover, PACAP preferentially induces the expression of costimulatory molecules related to Th2 differentiation (i.e., B7.2) in APC like macrophages after Ag stimulation (26). An additional mechanism could be the preferential PACAP-induced prevention of clonal deletion of Th2 against Th1 cells after antigenic stimulation, resulting in the generation of Th2 effector and memory cells.⁴ The data of the present report regarding these cytokines (reduction of IFN- γ levels and increase of IL-4 in treated mice) and the levels of the different Ig isotypes (reduction of IgG2a but increase of IgG1 in PACAP-treated mice) confirm the Th2-inducing response of PACAP, possibly being one of the factors that contribute to the remission of the disease, blocking the autoimmune component of this disease.

All in all, the effects of PACAP in the CIA model suggest it as a therapeutic agent that could be possibly used in the treatment of the human RA. It has been reported that endogenous neuropeptides and hormones like calcitonin-gene related peptide and melanocyte-stimulating hormone, which are released in the immune microenvironment, have beneficial effects on different arthritic disorders (30, 41). Recently, VIP, a neuropeptide that is structurally related to PACAP and shares its receptors and many of the functions that PACAP exerts in the immune system, has been demonstrated to have an important down-regulative action on arthritis (42). Therefore, as in other inflammatory diseases, all these endogenously produced peptides, probably including PACAP, are increased in the arthritic mice, acting as a natural antiarthritic attempt to reduce the excessive response of the immune system.

The fact that PACAP exerts most of its actions in immune system by increasing intracellular cAMP levels (18–24, 36) suggests that the activation of this pathway by PACAP is the mechanism of action mainly involved in its antiarthritic effect. In this sense, other cAMP-inducing agents, such as rolipram and other type IV phosphodiesterase inhibitors, have been shown to prevent CIA and are currently searched as alternative therapies for arthritis (43–45).

⁴ M. Delgado and D. Ganea. Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide enhance the *in vivo* generation of memory Th2 cells by inhibiting the peripheral deletion of antigen-specific effectors. *Submitted for publication.*

In view of these findings, pharmacological studies to determine the correct dose, route of administration, and possible side effects could be of special interest because arthritis has become a frequent disease in the world population and without many expectations of cure with the current alternatives. The studies performed in mice do not show any harmful secondary effects, supporting the idea that PACAP could be investigated as a drug for the treatment of arthritis. Moreover, although a supply of PACAP in daily or a every other day doses is more successful, a single injection in the beginning or even in the later stages of the illness is efficient enough to prevent its worsening. All of these facts should be taken into account in future studies on the use of PACAP in the treatment of RA. Based on our results, PACAP is a promising candidate for the development of treatment of RA and other chronic inflammatory diseases and autoimmune disorders.

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