

A Novel Effect for Annexin 1-Derived Peptide Ac2-26: Reduction of Allergic Inflammation in the Rat

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Received November 11, 2004; accepted March 18, 2005

ABSTRACT

Previous investigations have provided evidence that the N-terminal peptide of annexin 1 (peptide Ac2-26) has the capacity of reproducing the anti-inflammatory actions of the full-length protein in many systems. In the current study, we report the effectiveness of the peptide Ac2-26 as an antiallergic tool in a model of rat pleurisy and provide indication for some of the mechanisms involved. In rats inflamed by injection of ovalbumin into the pleural cavity 14 days postsensitization, peptide Ac2-26 (50–200 $\mu\text{g}/\text{cavity}$) inhibited mast cell degranulation, plasma protein leakage, and the accumulation of both neutrophils and eosinophils. Treatment with either peptide Ac2-26 (200 $\mu\text{g}/\text{cavity}$) or dexamethasone (1 mg/kg i.p.) inhibited ovalbumin-induced eotaxin release in the pleural effluents. In

vitro, peptide Ac2-26 inhibited ovalbumin-evoked histamine release from subcutaneous tissue fragments obtained from sensitized rats (33–66 μM) and interleukin-13-evoked eotaxin generation from cultured rat mesothelial cells (16–33 μM) but not eosinophil chemotaxis. This work demonstrates that the annexin 1 mimetic peptide Ac2-26 prevents allergen-evoked eosinophilic inflammatory response in rats. Combined analysis of the in vivo and in vitro experiments presented herein suggests that the blockade of secretion of pivotal mediators for the allergic response, such as histamine and eotaxin, could be responsible for the inhibitory actions displayed by peptide Ac2-26.

A marked feature of the acute phase of the allergic inflammatory response is the activation of mast cells, resulting in the release of preformed and inducible proinflammatory mediators (Busse, 1998; Lemanske and Busse, 2003). Increased vascular permeability to plasma proteins is one of the major outcomes of the allergic mast cell activation, which is also believed to promote leukocyte recruitment via direct or indirect release of cytokines and chemokines (Metcalfe et al.,

1997). An interesting aspect of the pathogenesis of allergic diseases is the accumulation of large numbers of tissue eosinophils. There is substantial evidence in the literature that eosinophil-derived mediators are strongly associated with the tissue damage observed in these diseases (Gleich, 2000). It is therefore presumed from these findings that drugs that can prevent mast cell activation and/or infiltration of eosinophils into inflamed tissue may have therapeutic potential to control allergic disorders such as asthma (Gleich, 2000).

Anti-inflammatory therapy with glucocorticoid agents is currently the most effective pharmacological approach for severe asthma, but its applicability is limited by the wide range of undesirable side effects associated with steroid therapy (Adamko et al., 2003). At present, it is understood that the glucocorticoid inhibitory effects are due to modulation of gene transcription. Binding of the hormone to its receptor is followed by translocation of the steroid-receptor complex, dimerization, and ligation of the dimer to the so-called “glu-

This study was supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro, and Programa de Desenvolvimento Tecnológico em Insumos para Saúde. C.B.M. and B.L.D. are supported by a Programa Especial de Estímulo à Fixação de Doutores/CNPq Grant; M.P. is a senior fellow of the Arthritis Research Campaign (Chesterfield, Derbyshire, UK); R.J.F. is a Principal Research Fellow of the Wellcome Trust (London, UK); and P.J.J. is supported by the National Asthma Campaign (London, UK).

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Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.104.080473.

ABBREVIATIONS: IL, interleukin; PAF, platelet-activating factor; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcriptase; PCR, polymerase chain reaction; bp, base pair(s); DNP, dinitrophenol.

cocorticoid-responsive elements" in the nuclear DNA, thus activating or inhibiting the transcription mechanism (Barnes, 1996). The suppressive effects of glucocorticoid agents on the transcription of a number of genes for inflammatory mediators, including interleukin (IL)-3, IL-4, IL-5, granulocyte-macrophage colony-stimulating factor receptor, and various chemokines, are well established (Barnes, 1996). However, glucocorticoids can induce the production of annexin 1, an anti-inflammatory protein of the superfamily of annexins, which has been implicated as an endogenous mediator of the glucocorticoid effect in many circumstances (Rovietto et al., 2002). Annexin 1 (previously termed lipocortin 1) was originally described as an inhibitor of lipid mediator formation via blockade of phospholipase A₂ activity, thereby modulating many biological processes, including cellular growth and differentiation, central nervous system response to cytokines, neuroendocrine secretion, and tissue neutrophil accumulation (Flower and Rothwell, 1994; Hannon et al., 2003).

Previous studies have emphasized that the N-terminal peptide of annexin 1 (peptide Ac2-26) can reproduce the anti-inflammatory actions of the full-length protein. This peptide of 25 amino acids inhibits tissue recruitment of neutrophils caused by different stimuli in vivo (Perretti et al., 1993). Peptide Ac2-26 can also reproduce the anti-inflammatory effect of annexin 1 in other processes, including hyperalgesia (Ferreira et al., 1997) and tissue injury caused by artery occlusion and reperfusion shock (Cuzzocrea et al., 1997). The hypothesis that the annexin 1 fragment might be able to affect allergen-induced eosinophil recruitment was previously investigated in a mouse skin model (Teixeira et al., 1998). The study clearly demonstrated that the inhibitory action of dexamethasone on eosinophil trafficking in cutaneous inflammatory reactions in the mouse was neither impaired by annexin 1-immune-neutralization nor mimicked by the annexin 1 N-terminal peptide Ac2-26, leading to the suggestion that the antieosinophilotactic effect of glucocorticoids was annexin 1-independent.

Recent research has shown a direct interaction of annexin 1 and its peptides with the specific seven transmembrane G protein-coupled lipoxin A₄ receptor (Perretti et al., 2002). Furthermore, stable analogs of lipoxin A₄ can block allergic airway inflammation, as shown by decreased eosinophil influx and release of mediators such as IL-4, IL-5, IL-13, and eotaxin, both in the mouse (Levy et al., 2002) and rat (Bandeira-Melo et al., 2000a). The current study was undertaken to evaluate the effect of the annexin 1 peptide derivative Ac2-26 on cell recruitment and protein extravasation triggered by antigen challenge in a rat model of allergic inflammation.

Materials and Methods

Animals. Wistar rats of both sexes weighing 150 to 200 g and guinea pigs of both sexes weighing 250 to 300 g were obtained from the Oswaldo Cruz Foundation (Rio de Janeiro, Brazil) breeding unit. The animals were housed in groups of five animals and maintained on a 12-h light/dark cycle with water and food ad libitum until use. The Ethics Committee for Care and Use of Animals of the Oswaldo Cruz Foundation approved the experimental protocols employed in this study (License 0085-02).

Ovalbumin-Evoked Pleurisy in Actively Sensitized Rats. Active sensitization was achieved by s.c. injection (0.2 ml) of a mixture containing 50 μ g of ovalbumin and 5 mg of aluminum hydroxide

in 0.9% NaCl solution (saline). Ovalbumin dissolved in sterile saline was administered intrapleurally (i.p.) (12 μ g/cavity) 14 days post-sensitization using a 27.5-gauge needle adjusted to 3 mm in length. All i.p. injections were performed under inhalation anesthesia and in a final volume of 100 μ l. All solutions were always made fresh immediately before use. At distinct postchallenge time points, the rats were killed under CO₂ atmosphere, and the pleural cavity was rinsed with 3 ml of heparinized saline (10 IU/ml). The pleural effluent was collected, and its volume was measured with a graduated syringe and then used for cellular and mediator analysis.

Measurement of Pleural Leukocytes and Protein. Total leukocyte counts on samples of pleural effluent were determined in a Coulter Counter ZM (Beckman Coulter, Inc., Fullerton, CA) after red blood cell lysis using Zapoglobin (Beckman Coulter, Inc.). Differential analysis was performed under an oil immersion objective on cytocentrifuged smears stained with May-Grünwald-Giemsa dye. For the mast cell enumeration, pleural effluent samples collected 4 h postantigen challenge were diluted in toluidine blue dye solution (Mota and Peixoto, 1966) and evaluated in a Neubauer chamber using a light microscope. The effluent was then centrifuged at 1300g for 10 min, and the protein content of the supernatant was quantified in a spectrophotometer (540 nm) by the Biuret technique (Gornall et al., 1949).

Drug Treatments. To study the effect of human annexin 1 N terminus on allergic pleurisy, the peptide Ac2-26 (50–200 μ g/cavity) was injected intrapleurally 5 min before allergen challenge. In control groups, the peptide was replaced by the scrambled peptide Ac2-12 (200 μ g/cavity) (La et al., 2001) or 0.9% saline. Alternatively, 1 mg/kg dexamethasone was injected intraperitoneally 1 h before challenge.

Quantification of Eotaxin by Enzyme-Linked Immunosorbent Assay. Eotaxin in rat pleural lavage fluid was measured by a murine eotaxin enzyme-linked immunosorbent assay as reported previously (Bandeira-Melo et al., 2000a). Rat eotaxin (97.3% sequence identity with murine eotaxin) (Williams et al., 1998) cross-reacts in this assay, but there was no detectable cross-reaction with any other chemokine tested, including human and guinea pig eotaxin.

Anaphylactic Histamine Release from Rat Subcutaneous Tissue Fragments in Vitro. Actively sensitized rats were killed under CO₂ atmosphere, and fragments from dorsal subcutaneous tissue (~1 mg) were removed and placed in 48-well plates containing Hanks' balanced salt solution (HBSS) with Ca²⁺ and Mg²⁺ in a final volume of 700 μ l. The fragments were exposed to 300 μ g/ml ovalbumin for 1 h, and then the plates were centrifuged at 150g for 10 min; the samples were collected and added to 0.8 N perchloric acid (1:2 dilution) as reported previously (de Oliveira Barreto et al., 2003). To test the potential effect of Ac2-26 (16–66 μ M) on ovalbumin-evoked histamine release, the tissue system was pretreated with the target substance or its vehicle at 37°C for 1 h. After centrifugation at 170g for 10 min, the supernatant was collected and stored at –20°C for histamine quantification as reported previously (Shore et al., 1959). Briefly, this method consists of sample dilution in 0.1 N HCl followed by 0.8 N NaOH and further addition of the substrate *o*-phthaldialdehyde. After a 4-min incubation, the reaction was stopped with 3N HCl, and the fluorescence was measured in a Shimadzu RF1501 spectrofluorophotometer (Shimadzu, Kyoto, Japan) (excitation, 360 nm; emission, 450 nm). After collecting the medium, tissue fragments were dried in incubators at 40°C for 15 min, and the results were expressed as the amount of histamine released (nanograms) per milligram of dried tissue.

Eosinophil Chemotaxis. Eosinophils were isolated from the peritoneal cavity of normal rats using Percoll density gradient as previously reported (Martins et al., 1989). Eosinophil suspensions of 85 to 95% purity and 96% viability, as attested by the trypan dye exclusion test, were used throughout. Migration experiments were performed using 48-well microchemotaxis chamber (Neuro Probe, Inc., Gaithersburg, MD) and Toyo cellulose nitrate filters (3- μ m

pore) according to the technique described previously (Richards and McCullough, 1984).

To test the potential direct effect of peptide Ac2-26 on eosinophils, cells were preincubated with the peptide or its vehicle at 37°C for 1 h; then eosinophil suspension (2×10^5 cells; 50 μ l) were placed in the upper compartment of the chamber, whereas the lower compartment was loaded with 1 μ M murine eotaxin, 1 μ M platelet-activating factor (PAF), and their vehicle [RPMI medium, pH 7.2, containing 0.1% bovine serum albumin (BSA)]. The chemotaxis chamber was then incubated for 2 h at 37°C in a 5% CO₂/95% O₂ atmosphere. The filter was fixed and stained as described previously (Richards and McCullough, 1984). Eosinophils migrated at 40 μ m from the upper surface of the filter were counted in 15 consecutive high-power fields under an immersion objective (Martins et al., 1989).

Rat Peritoneal Mesothelial Cell Isolation and Cultures. Mesothelial cells were obtained from Wistar rats as described by Yang et al. (2004). Briefly, rats were killed in a CO₂ atmosphere and injected in the abdominal cavity with 25 ml of phosphate-buffered saline containing 0.25% trypsin and 0.02% EDTA-Na₂. Within 2 h, peritoneal fluid was removed under sterile conditions and centrifuged for 10 min at 1500 rpm. Pelleted cells were then washed with HBSS, suspended in DMEM/F-12 medium supplemented with 20% (v/v) fetal calf serum, placed into 25-cm² tissue culture flasks, and incubated at 37°C in a humidified 5% CO₂ atmosphere for 24 h. After removing nonadherent cells with HBSS washing, the adherent population was incubated (5% CO₂) at 37°C in fresh culture medium to reach confluence in 3 to 5 days. Mesothelial cells were passaged by dissociating the monolayer with 0.25% trypsin/0.02% EDTA-Na₂ and reseeded in 48-well plates (5×10^4 cells/well). Subconfluent mesothelial cells were washed with phosphate-buffered saline pretreated with either Ac2-26 (16–66 μ M) or dexamethasone (1–10 μ M) in the presence of fetal calf serum-free DMEM/F-12 medium for 1 h. Cultured cells were then stimulated with 2 ng/ml hIL-13 for 72 h at 37°C in a humidified 5% CO₂ incubator. The culture medium was collected and centrifuged at 2000g for 10 min at 4°C, and the supernatant was stored at –20°C until assayed for eotaxin.

RT-PCR Conditions. RNA was extracted from 10⁷ resting mesothelial cells in 1 ml of TRIzol according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Purified total RNA (1 μ g) was used as a template for first-strand cDNA synthesis using SuperScript II reverse transcriptase (Invitrogen). PCR was performed using 2 μ l of reverse transcription reaction (equivalent to 100 ng of starting RNA) as template, 200 μ M dNTPs, 20 nM of each primer, and 2 U of *Taq* polymerase (Amersham Biosciences Inc., Piscataway, NJ) in a 50- μ l reaction volume according to the enzyme manufacturer's instructions. The sequences of the primers (Alpha DNA, Montreal, QC, Canada) and the sizes of the amplified products were as follows: rat β -actin, 5'-TGACCCAGATCATGTTTGAGAC-3' and 5'-GGATTCCATACCCAGGAAGGA-3' (458 bp); and rat ALXR, 5'-CCGTCCATTAAGAGTCCCTTAC-3' and 5'-TCATATTGCTTTTATATCA-ATGTT (343 bp). PCR conditions were as follows: a denaturing step at 94°C for 2 min; followed by 45 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 60 s; followed by a final extension step at 72°C for 7 min. A total of 20 μ l of each PCR product was resolved on a 2% agarose gel and visualized with ethidium bromide. Primer sequences for rat β -actin were based on GenBank sequence accession number BC063166. Primer sequences for rat ALXR were the same as published previously (Chiang et al., 2003).

Materials. Peptide Ac2-26 (acetyl-AMVSEFLKQAWIENEEQEYV-VQTVK, MW 3,050) and the control scrambled peptide Ac2-12 (acetyl-SVEQKMWALFA, MW 1,424) were prepared by the Advance Biotechnology Center (The Charing Cross and Westminster Medical School, London, UK) by using solid-phase stepwise synthesis. Purity was more than 90% as assessed by high-performance liquid chromatography and capillary electrophoresis (data supplied by manufacturer). Dinitrophenylated albumin conjugate (DNP-BSA) was purchased from Calbiochem (San Diego, CA); murine immunoglobulin E anti-DNP monoclonal antibody was kindly provided by Dr. A. Provoust-Danon (Unité d'Immuno-Allergie, Institut Pasteur, Paris, France); PAF (1-*O*-hexadecyl-2-acetyl-*sn*-glyceryl-3-phosphorylcholine) was purchased from Bachem (Bubendorf, Switzerland). Histamine, rmIL-5, HEPES, Penicillin G, streptomycin sulfate, HBSS, BSA, *o*-phthalal-

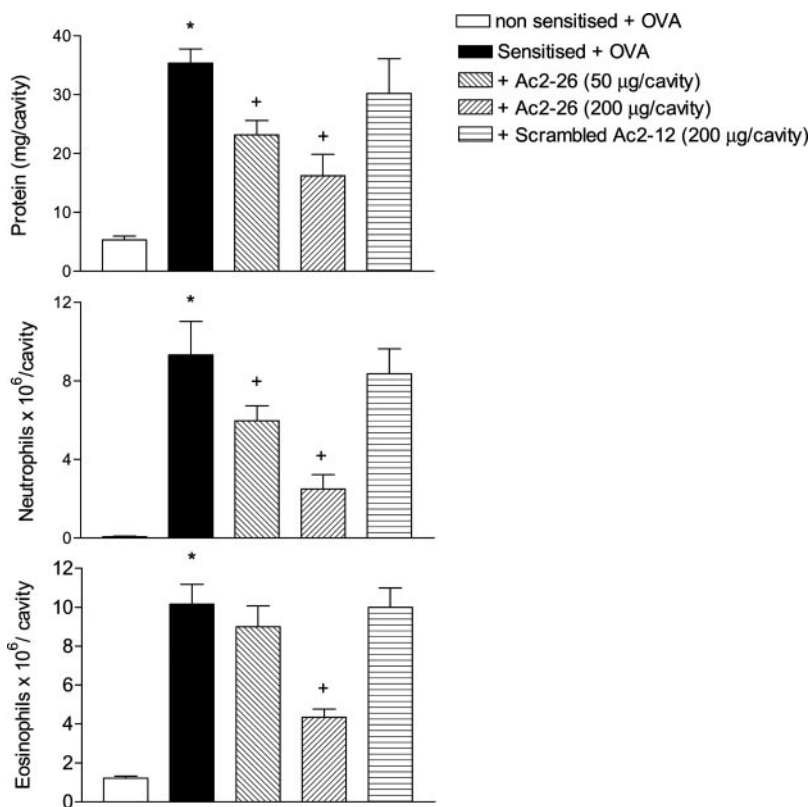


Fig. 1. Effect of peptide Ac2-26 (50 and 200 μ g/cavity i.p.) or scrambled peptide Ac2-12 (200 μ g/cavity i.p.) on pleural protein leakage (top) and neutrophil accumulation (middle) noted 4 h postchallenge, and eosinophil accumulation noted 12 postchallenge (bottom). All rats were administered with allergen (ovalbumin, 12 μ g/cavity i.p.). Results were expressed as means $\pm$ S.E.M. from at least five animals. *, $P < 0.001$ compared with nonsensitized group. +, $P < 0.01$ compared with allergen-challenged sensitized group.

dehyde, ovalbumin, RPMI 1640, thimerosal, Tween 20, and toluidine blue dye were obtained from Sigma Chemical Co. (Poole, Dorset, UK). Dexamethasone sodium phosphate was purchased from David Bull Laboratories (Warwick, UK). DMEM Ham's/F-12 medium was purchased from Invitrogen. Polyclonal anti-murine eotaxin antibody and biotinylated goat anti-murine eotaxin were purchased from R&D Systems (Minneapolis, MN). Recombinant murine eotaxin and recombinant human IL-13 were purchased from PeproTech (Rocky Hill, NJ). Neutravidin-horseradish peroxidase conjugate was purchased from Pierce (Chester, UK). K-blue substrate was purchased from Neogen (Lexington, KY).

Statistical Analysis. All data are presented as means $\pm$ S.E.M. Statistical analysis involving two groups was done with Student's *t* test, whereas analysis of variance and Newman-Keuls-Student's test were used to compare more than two groups. *P* values of 0.05 or less (two-tail test) were considered significant.

Results

Effect of Peptide Ac2-26 on Allergen-Evoked Pleurisy. Ovalbumin pleural challenge (12 μ g/cavity) in sensitized rats provoked an intense inflammatory reaction characterized by intense plasma leakage and leukocyte infiltration (Fig. 1). Analogous to our previous studies (Silva et al., 2001), this cellular infiltration consists markedly of neutrophils (Fig. 1, middle) in the early phase and eosinophils (Fig. 1, bottom) in the late phase of the process. Local treatment with peptide Ac2-26 (50–200 μ g/cavity) dose-dependently inhibited plasma leakage (Fig. 1, top) as well as neutrophil (Fig. 1, middle) and eosinophil accumulation triggered by allergen provocation (Fig. 1, bottom). In contrast, treatment with $\approx$ 4-fold excess of the negative control peptide (scrambled peptide Ac2-12, 200 μ g/cavity) failed to alter these phenomena (Fig. 1). As shown in Table 1, the increased number of pleural mononuclear cells remained unchanged after peptide Ac2-26 treatment or control peptide.

Effect of Peptide Ac2-26 on Allergen-Evoked Mast Cell Degranulation and Histamine Release. In line with previous reports (Lima et al., 1991), the intrapleural challenge of sensitized rats with ovalbumin led to a significant reduction in the number of intact mast cells recovered from the pleural cavity 4 h postchallenge. As illustrated in Table 2, pretreatment with the peptide Ac2-26, but not with the negative control peptide, significantly increased the number of intact mast cells recovered in the pleural effluent following allergen injection. Furthermore, in vitro exposure to peptide Ac2-26 (16–66 μ M) also attenuated ovalbumin-evoked histamine release (Fig. 2), confirming the protective effect of peptide Ac2-26 on mast cell activation.

TABLE 1

Effect of annexin 1-derived peptide Ac2-26 on antigen-induced mononuclear cell infiltration into the pleural cavity of sensitized rats. Local pretreatment with Ac2-26 or scrambled Ac2-12 (200 μ g/cavity) was performed 5 min before allergen challenge. All groups were challenged with ovalbumin (12 μ g/cavity). The cell analysis was performed 4 and 12 h postallergen challenge. Each value represents the mean $\pm$ S.E.M. from at least five animals.

Condition	Treatment	Mononuclear Cells $\times 10^6$ /cavity	
		4 h postchallenge	12 h postchallenge
Nonsensitized		7.4 $\pm$ 0.4	8.0 $\pm$ 0.5
Sensitized		12.9 $\pm$ 1.4*	22.1 $\pm$ 2.6*
Sensitized	Ac2-26	9.7 $\pm$ 1.2	18.3 $\pm$ 2.7
Sensitized	Scrambled	13.9 $\pm$ 1.4*	29.0 $\pm$ 4.3*

* Significantly different from the nonsensitized group (*P* < 0.01).

TABLE 2

Effect of annexin 1-derived peptide Ac2-26 on antigen-induced mast cell degranulation in actively sensitized rats

Local pretreatment with Ac2-26 or scrambled Ac2-12 (200 μ g/cavity) was performed 5 min before allergen challenge. All groups were challenged with ovalbumin (12 μ g/cavity). The mast cell analysis was performed 4 h postallergen challenge. Each value represents the mean $\pm$ S.E.M. from at least five animals.

Condition	Treatment	Intact Mast Cell $\times 10^3$ /cavity
Nonsensitized		679.7 $\pm$ 55.1
Sensitized		4.2 $\pm$ 4.7*
Sensitized	Ac2-26	247.5 $\pm$ 77.8 ⁺
Sensitized	Scrambled Ac2-12	3.8 $\pm$ 1.8*

* Significantly different from the nonsensitized group (*P* < 0.01).

⁺ Significantly different from the sensitized untreated group (*P* < 0.01).

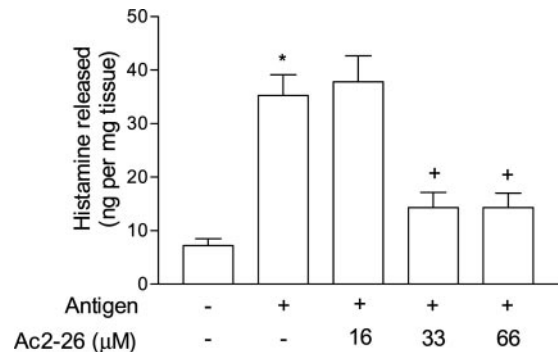


Fig. 2. Effect of peptide Ac2-26 (16–66 μ M) on ovalbumin-evoked histamine release from subcutaneous tissue fragments recovered from actively sensitized rats. Results were expressed as means $\pm$ S.E.M. from at least four fragments. *, *P* < 0.001 compared with the sham-challenged group of fragments; +, *P* < 0.001 compared with the ovalbumin-challenged and Ac2-26 untreated group of fragments.

Peptide Ac2-26 Inhibits Eotaxin Generation in Vitro and in Vivo. According to previous studies (Silva et al., 2001), allergen-evoked eosinophil accumulation in actively sensitized rats is preceded by eotaxin generation and inhibited by anti-eotaxin antibody. We found that local treatment with either dexamethasone or peptide Ac2-26 abolished eotaxin generation, as measured in the pleural effluent 6-h postovalbumin challenge (Fig. 3, left). Since mesothelial cells are recognized as a major source of eotaxin (Katayama et al., 2002), we first checked the expression of ALXR on this cell type. RT-PCR analysis showed that primary rat mesothelial cells express ALXR message (Fig. 4) and therefore could be a potential target for the annexin 1 peptide. We then examined the effect of peptide Ac2-26 on eotaxin release from IL-13-stimulated primary rat mesothelial cells. The data in Fig. 3 (right) showed that peptide Ac2-26 inhibited IL-13-evoked eotaxin production by cultured mesothelial cells in a concentration-dependent manner (16–66 μ M). As shown in the same figure, dexamethasone (1–10 μ M) also abolished eotaxin generation in this system.

Lack of Effect of Ac2-26 on Eosinophil Chemotaxis in Vitro. To determine whether the peptide Ac2-26 inhibited directly eosinophil recruitment, we investigated its potential effect on eosinophil chemotaxis caused by either eotaxin or PAF using the Boyden chamber system. As indicated in Table 3, peptide Ac2-26 (3.3–66 μ M) failed to alter eosinophil migration in vitro.

Discussion

In this study, we investigated the effect of the annexin 1 pharmacophore peptide Ac2-26 on the allergic reaction pro-

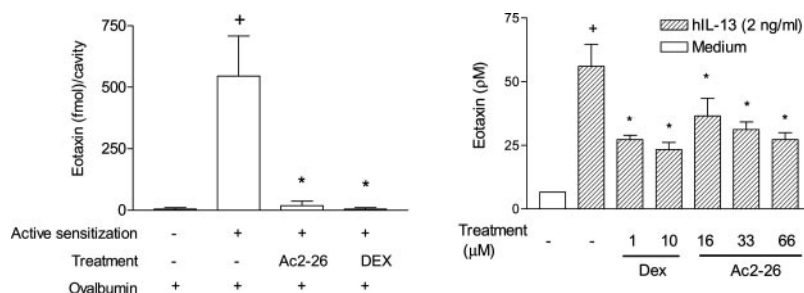


Fig. 3. Peptide Ac2-26 inhibits eotaxin generation in vitro and in vivo. Left, in vivo effect of treatment with dexamethasone (1 mg/kg i.p.) or peptide Ac2-26 (200 μg/cavity i.pl.) on eotaxin generated in the pleural effluent 6-h postallergen challenge. Results are expressed as means ± S.E.M. from at least six animals. Right, in vitro effect of treatment with either peptide Ac2-26 (16–66 μM) or dexamethasone (1 and 10 μM) on IL-13-evoked eotaxin generation from rat mesothelial cells. Results are expressed as means ± S.E.M. from at least four distinct cell donors. *, $P < 0.001$ compared with the negative control group; +, $P < 0.001$ compared with the positive control group.

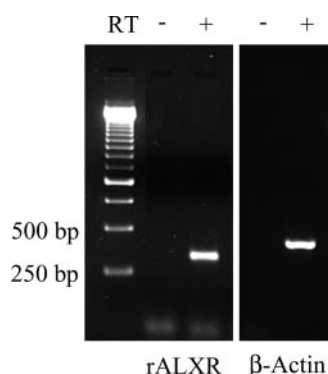


Fig. 4. RT-PCR analysis of ALXR expression by rat mesothelial cells. RNA isolated from cultured rat mesothelial cells was reverse-transcribed (+RT) and used as a template for PCR using rat ALXR or β-actin-specific primers. PCR reactions using RT reaction where reverse transcriptase was omitted (–RT) were used as controls. A 250-bp ladder (Invitrogen) was used to size the PCR products; the 250- and 500-bp bands are indicated.

TABLE 3

Lack of effect of the annexin1-derived peptide Ac2-26 on human eotaxin- and PAF-induced eosinophil chemotaxis in vitro

Eosinophils were preincubated with Ac2-26 (33 and 66 μM) for 1 h at 37°C before stimulation with either murine eotaxin (0.1 μM) or PAF (1 μM) for 2 h. Results are presented as mean ± S.E.M. from four to six experiments.

Ac2-26	Eosinophils per 15 High-Powered Fields		
	Vehicle	Eotaxin	PAF
0 μM	4.2 ± 1.3	15.7 ± 3.6*	91.6 ± 6.9*
33 μM	5.0 ± 1.2	11.0 ± 3.5*	86.1 ± 12.0*
66 μM	4.5 ± 0.7	14.3 ± 4.5*	138.0 ± 9.2*

* $P < 0.01$ compared with nontreated vehicle-stimulated eosinophils.

voked by ovalbumin challenge in the pleural cavity of actively sensitized rats. Our results demonstrate that mast cell degranulation, protein exudation, and leukocyte influx, including eosinophils, were significantly inhibited by the local administration of the annexin 1 fragment. The peptide Ac2-26 failed to alter eosinophil migration in vitro but clearly prevented allergen-evoked eotaxin generation in vivo. Allergen-evoked histamine release from rat subcutaneous tissue was also clearly sensitive to the treatment. Combined analysis of the in vitro and in vivo experiments suggests that the step sensitive to the peptide Ac2-26 might be the generation of pivotal mediators for the allergic response.

Annexin 1 is a steroid-inducible protein that has been claimed to mediate a variety of anti-inflammatory properties of glucocorticoids (Roviezzo et al., 2002; Hannon et al., 2003).

A number of studies have provided evidence for the involvement of this protein in the regulation by glucocorticoids of neutrophil migration (Perretti and Flower, 1993), inflammatory edema (Cirino et al., 1989), ischemic damage (Cuzzocrea et al., 1997), pain (Ferreira et al., 1997), and fever (Davidson et al., 1991). Nevertheless, it is clear that annexin 1 modulation is not the only mechanism of glucocorticoid action that can involve a variety of mechanisms, including direct and indirect genomic routes and other annexin 1-independent signaling effects (Hannon et al., 2003).

Recently, the peptide Ac2-26 and annexin 1 have been shown to activate the lipoxin A₄ receptor, suggesting that annexin 1 peptide derivatives and lipoxin A₄ may act in concert at this G protein-coupled receptor to inhibit formation of mediators of inflammation and leukocyte recruitment (Perretti, 2003). Interestingly, the annexin 1/lipoxin A₄ receptor is also expressed in rats (Chiang et al., 2003), and several parameters of rat allergic pleurisy, including edema, eotaxin formation, and eosinophil accumulation, were inhibited by lipoxin A₄ stable analogs (Bandeira-Melo et al., 2000a,b). Of note, recent studies have positioned lipoxins as strong candidates for novel antiallergic therapies (Levy and Serhan, 2003). For these reasons, we have tested the putative influence of annexin 1 mimetics in a rat model of allergic inflammation.

For some time now, we have explored a model in which ovalbumin-sensitized rats react to ovalbumin intrapleural challenge with a rapid plasma leakage and edema that peaks from 1 to 4 h postchallenge. These changes are followed by neutrophil influx, peaking from 4 to 8 h, and mononuclear cell and eosinophil influx, first noted at 8 h but peaking from 12 to 24 h postallergen challenge (Lima et al., 1997). All these events were clearly sensitive to the systemic treatment with dexamethasone, whereas histamine and 5-hydroxytryptamine receptor antagonists inhibited plasma leakage without altering leukocyte infiltration (Martins et al., 1993). In our first set of experiments, we have shown that the local administration of peptide Ac2-26 reduced plasma leakage and neutrophil and eosinophil emigration into the rat pleural space, leaving intact the mononuclear cell enrichment caused by allergen challenge. These latter data differ from the glucocorticoid wide-ranging effects and could indicate a certain degree of selectivity in the actions of peptide Ac2-26. Thus, we next set out to investigate the Ac2-26 peptide-driven inhibitory mechanisms of both vascular and cellular changes caused by allergen challenge.

A hypothetical mechanism that could account for the inhi-

bition of allergic inflammation by annexin 1-derived peptide is the down-regulation of resident mast cell activity. Mast cells play an important role in allergic inflammation by releasing preformed and inducible proinflammatory mediators (Lemanske and Busse, 2003). With particular reference to allergen-induced pleurisy in rats, activation of mast cells seems to be a primary event that may lead to both plasma leakage and leukocyte infiltration (Diaz et al., 1996; Lima et al., 1996). It is noteworthy that annexin 1 can be up-regulated by distinct stimuli in rat mast cells (Tasaka et al., 1994; Oliani et al., 2000) and correlated with down-regulation of compound 48/80-induced histamine release (Tasaka et al., 1994). In our study, local treatment with the peptide Ac2-26 attenuated ovalbumin-evoked mast cell degranulation in the rat pleural space, a finding that was also substantiated in vitro settings. In fact, active concentrations of peptide Ac2-26 abrogated ovalbumin-evoked histamine release from subcutaneous tissue fragments collected from sensitized rats. Overall, these findings support the interpretation that the mast cell may be a relevant target for the antiallergic effect produced by the peptide Ac2-26 and agree with the previous original study in which human recombinant annexin 1 inhibited compound 48/80-induced edema in the rat paw (Cirino et al., 1989). The sensitivity of the rat mast cell to annexin 1 may also be the basis for the different findings reported in murine models, where dexamethasone inhibitory effects were neither modified by annexin 1-immune neutralization (Das et al., 1997; Teixeira et al., 1998) nor mimicked by the annexin 1 N-terminal peptide Ac2-26 (Teixeira et al., 1998). One can speculate that mouse mast cells are either less sensitive to annexin 1 or that the annexin 1/lipoxin A₄ receptor may be differently expressed on mast cells across species. Future studies will address this point. Of note, lipoxin A₄ analogs did not inhibit allergen-evoked pleural mast cell degranulation in rats (Bandeira-Melo et al., 2000a). To explain such discrepancies, one can speculate that annexin 1-derived peptide and lipoxin A₄ analogs activate ALXR expressed on rat pleural mast cells in a different fashion, coupling distinct signaling pathways as has been observed for other G protein-coupled receptors (Kenakin, 2004). On the other hand, one of these ALXR ligands could activate an alternative receptor in parallel (formyl peptide receptor or formyl peptide receptor-like-2, for example) (Ernst et al., 2004), interfering with ALXR-driven signaling in mast cells.

In addition to the blockade of histamine release by pleural mast cells, a reduced production of other mediators critical to allergic inflammation could represent a complementary mechanism of the annexin 1-derived peptide-driven effects. Likewise, what was observed following treatment with lipoxin A₄ stable analogs (Bandeira-Melo et al., 2000a), administration of either peptide Ac2-26 or dexamethasone clearly abolished allergen-evoked eotaxin generation in parallel with abrogation of eosinophil recruitment. This chemokine plays a crucial role in the mechanism of allergen-evoked pleural eosinophil accumulation in rats (Silva et al., 2001), supporting the interpretation that the antieotaxin effect of the peptide Ac2-26 may also be important in bringing about its antiallergic properties. While trying to identify the source of eotaxin and putative target for the peptide Ac2-26 in this model, we found that rIL-13 is a positive stimulus for the induction of eotaxin in a primary culture of rat mesothelial cells, and this system is also sensitive to both peptide Ac2-26

and dexamethasone. Furthermore, primary rat mesothelial cells express message for ALXR, reinforcing its potential role as target for peptide Ac2-26 inhibition. Therefore, in the current model of allergic pleurisy, it is not unlikely that these agents are acting on pleural mesothelial cells to prevent eotaxin generation.

A final potential mechanism responsible for the antieosinophilic effect of annexin 1-derivative peptide could be the impairment of eosinophil locomotory functions; however, in vitro experiments revealed that peptide Ac2-26 failed to impair eotaxin- and PAF-induced eosinophil chemotaxis. This finding is in line with the inability of the annexin 1 fragment to affect mouse eosinophil migration in response to intradermal application of directly acting eosinophil chemokines such as chemokine macrophage inflammatory protein-1 α (Teixeira et al., 1998). Of note, the refractoriness presented by eosinophils to peptide Ac2-26 is also observed for lipoxin A₄ stable analogs (Bandeira-Melo et al., 2000a), indicating that either activation of the annexin 1/lipoxin A₄ receptor may not regulate intracellular signaling events of eosinophil motility or even that rat eosinophils do not express such a receptor.

In conclusion, we demonstrate that local administration of the peptide Ac2-26 attenuates allergen-evoked eosinophilic inflammation in the rat. The inhibitory mechanism of plasma leakage and eosinophil enrichment seems to be closely associated with inhibition of histamine release from mast cells and local eotaxin generation, respectively. We believe that our results will stimulate further work for the development of annexin 1-derivatives as an innovative form of therapy for allergic dysfunction.

Acknowledgments

We thank Ana Lucia de Aguiar Pires and Juliane Pereira da Silva for skillful technical assistance.

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