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Mice lacking the VIP gene show airway hyperresponsiveness and airway inflammation, partially reversible by VIP

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Szema, Anthony M., Sayyed A. Hamidi, Sergey Lyubsky, Kathleen G. Dickman, Suni Mathew, Tarek Abdel-Razek, John J. Chen, James A. Waschek, and Sami I. Said. Mice lacking the VIP gene show airway hyperresponsiveness and airway inflammation, partially reversible by VIP. *Am J Physiol Lung Cell Mol Physiol* 291: L880–L886, 2006. First published June 16, 2006; doi:10.1152/ajplung.00499.2005.—The mechanisms leading to asthma, and those guarding against it, are yet to be fully defined. The neuropeptide VIP is a cotransmitter, together with nitric oxide (NO), of airway relaxation, and a modulator of immune and inflammatory responses. NO-storing molecules in the lung were recently shown to modulate airway reactivity and were proposed to have a protective role against the disease. We report here that mice with targeted deletion of the VIP gene spontaneously exhibit airway hyperresponsiveness to the cholinergic agonist methacholine as well as peribronchiolar and perivascular cellular infiltrates and increased levels of inflammatory cytokines in bronchoalveolar lavage fluid. Immunologic sensitization and challenge with ovalbumin generally enhanced the airway hyperresponsiveness and airway inflammation in all mice. Intraperitoneal administration of VIP over a 2-wk period in knockout mice virtually eliminated the airway hyperresponsiveness and reduced the airway inflammation in previously sensitized and challenged mice. The findings suggest that 1) VIP may be an important component of endogenous anti-asthma mechanisms, 2) deficiency of the VIP gene may predispose to asthma pathogenesis, and 3) treatment with VIP or a suitable agonist may offer potentially effective replacement therapy for this disease.

vasoactive intestinal peptide; asthma phenotype; knockout mice

THE PATHOGENESIS OF BRONCHIAL ASTHMA remains incompletely understood. Atopy, cellular and humoral mediators of inflammation, and abnormal neurogenic influences are recognized factors (3). One postulated mechanism is an imbalance between proasthma, e.g., histamine, leukotrienes (11), and potential anti-asthma mediators (1). Among the latter compounds is VIP, a neuropeptide with potent bronchodilator, immunomodulator, and anti-inflammatory properties (8, 31, 39). Together with nitric oxide (NO) and carbon monoxide, VIP is a cotransmitter of the dominant neurogenic relaxant system of airway smooth muscle (31, 33), the natural defense mechanism against airway constriction. VIP also suppresses airway smooth muscle proliferation (25), an important component of airway remodeling in chronic asthma, and thus has biological properties capable of counteracting all major features of the asthmatic response.

Identification of endogenous anti-asthma defenses should provide useful insights into the pathogenesis of the disease and its management. Depletion of *S*-nitrosothiols, key NO-carrying molecules in the lung, was recently found to correlate with increased airway constriction in mice, leading to the conclusion that these compounds form a natural defense against asthma (29). We hypothesized that VIP may provide another such defense mechanism and reasoned that, if this hypothesis is correct, then absence of VIP from the airways should be associated with features commonly identified with the human disease, namely, bronchial hyperresponsiveness and airway inflammation. We report that mice with targeted deletion of the VIP gene do exhibit such hallmarks of asthma. To confirm the interpretation that the absence of the VIP gene was responsible for the asthma-like phenotype, we administered VIP for a 2-wk period, following which the airway hyperresponsiveness was nearly abolished, and the airway inflammation was attenuated. These observations support the conclusions that 1) VIP serves an important physiological role as a modulator of airway constriction and other asthmatic responses, 2) its deficiency may be a causative factor in the pathogenesis of the disease, and 3) agonists of the peptide or its receptors may represent a new, targeted therapeutic approach.

MATERIALS AND METHODS

Animals

VIP knockout (KO) mice, backcrossed to C57BL/6, were prepared as described (5). We bred the mice locally and genotyped them to confirm the absence of the VIP gene (5). We mated homozygous KO males with homozygous KO females or, if necessary, with heterozygous KO females. For genotyping, we extracted DNA from 1-cm-long tail snips using a DNA isolation kit (Qiagen, Valencia, CA). DNA (100 ng) was subjected to PCR using primers to detect both VIP and the neomycin cassette. Control, wild-type (WT) C57BL/6 mice were from Taconic Laboratories (Germantown, NY). All experiments and animal care procedures were approved by the Institutional Animal Care and Use Committee and were conducted according to National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

Airway Responsiveness

To assess bronchial reactivity to a standard airway constrictor, 12 male VIP KO and 9 male control mice were anesthetized with pentobarbital sodium, tracheostomized, and mechanically ventilated

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at a constant tidal volume. After baseline values were established, we delivered methacholine as an aerosol at 1, 10, 10², and 500 mg/ml, using an Aeroneb Nebulizer System for mice (Buxco, Troy, NY), placed inline with the tracheostomy tube. We recorded airway pressure continuously and evaluated the degree of bronchoconstriction by increases in peak airway pressure that, at constant breath volumes, reflected increases in pulmonary resistance. Airway responsiveness was again examined in four KO and five WT mice after sensitization and challenge with ovalbumin, as described below.

Airway Inflammation

Histological evidence. To test for the presence of airway inflammation, a corollary of airway hyperresponsiveness in asthma (42), we subjected the lungs from five male VIP KO and six male WT mice to histological examination by a pathologist who was blinded to the identities of the samples. All abnormalities were graded 0, 1, 2, 3, or 4, based on the intensity and extent of peribronchiolar and perivascular cellular infiltration.

Lung cytokines. As added evidence of possible airway inflammation, we performed bronchoalveolar lavage (BAL) in five knockout and five WT mice. The lungs of each mouse were lavaged three times with 1 ml of PBS, including an EDTA-free protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). The BAL fluid was centrifuged at 400 g for 5 min at 21°C, and the supernatant was analyzed by a quantitative ELISA assay (Pierce Biotechnology, Woburn, MA) for selected inflammatory cytokines, chemokines, and a matrix metalloproteinase (MMP): IL-2, IL-5, IL-6, IL-10, IL-13, thymus activation-regulated chemokine (TARC), osteopontin (OPN), IFN- γ , and MMP-2.

Cell counts. We measured differential cell counts in BAL fluid in cytospin preparations, and by flow cytometry; the latter also permitted analysis of intracellular cytokines (37). After cells were harvested from BAL fluid, they were resuspended in PBS with azide to 1×10^6 /ml after being counted in a Coulter 21 Particle Counter (Hialeah, FL). The cells were then permeabilized with BD Cytotfix/cytoperm and labeled with various monoclonal antibodies according to the manufacturer's recommendations (BD Biosciences, San Diego, CA). Following labeling, the cells were washed and resuspended in 500 μ l of 1% formalin in BSA. Cells were refrigerated until acquisition and analysis with a BD FACSCaliber (Becton-Dickinson, Mountain View, CA). For analysis, the cells were gated on individual fluorescence positivity for CD11c (dendritic cells and macrophages) and CCR3 (eosinophils, mast cells, and T helper 2 cells) and then examined for reactivity to anti-IL-5 and anti-IL-6 antibodies. Neutrophils were gated by CD11c low intensity and CCR3 negativity and confirmed by light-scatter backgating characteristics. CCR3- and CD11c-negative lymphocytes were gated and confirmed by light-scatter backgating. Reactivities to anti-IL-5 and anti-IL-6 were then examined. The production of intracellular cytokines was expressed in "geometric mean fluorescent channels."

Immunologic Sensitization and Challenge

To compare the immunologic airway responses in KO mice with those in control WT mice, we sensitized four KO and five WT mice with ovalbumin (20 μ g, Grade VI, Sigma), emulsified in 2.25 mg of Alum (Pierce), and injected intraperitoneally, on days 0 and 14. On days 21 and 28, we challenged the mice with an aerosol of 100 mg of ovalbumin in 10 ml of PBS, delivered via a DeVilbiss ultrasonic nebulizer over 20 min. On day 29, the mice were tested for airway reactivity and euthanized with pentobarbital sodium, and their lungs removed for histological examination.

Administration of VIP

Nine male VIP KO mice, aged 16–20 wk, received VIP (15 μ g in 0.2 ml of PBS), intraperitoneally, every other day, for 2 wk, for a total

of seven injections, ending the day before testing for airway reactivity and lung cell infiltration. Another group of four male VIP KO mice of a similar age received 0.2 ml of PBS, without VIP, in the same manner and for the same duration. Our choice of the dosage, duration, frequency, and mode of administration of VIP was guided by protocols for related studies by other investigators (7, 22, 34). At the end of this treatment period, we evaluated airway responsiveness to methacholine in the nine mice. In addition, lungs from five of these mice were examined for histological evidence of airway inflammation. Another group of five KO mice sensitized and challenged with ovalbumin were treated with VIP for 2 wk, as above. These mice were then examined for airway responses to methacholine, and their lungs were examined for histological evidence of inflammation.

Immunoreactive VIP Levels in BAL Fluid and Lung Tissue

To examine the degree to which the VIP treatment restored the VIP content in lungs of VIP KO mice, we measured immunoreactive VIP levels (27) in BAL fluid and lung tissue of the treated mice 24 h after completing the VIP treatment. We compared those levels to corresponding values in WT mice under the same conditions, i.e., with or without immunologic sensitization and challenge.

Statistical Analysis

All results were expressed as means $\pm$ SE. Cytokine-chemokine data were analyzed by both parametric (*t*-test) and non-parametric (Mann-Whitney's *U*-test) approaches. Change in peak airway pressure from baseline was modeled using the repeated-measures ANOVA approach, taking into consideration the dependent measures at different concentrations of methacholine in the same mouse. The differences in the peak airway pressure changes among the three groups of mice (WT, VIP KO, and VIP KO+VIP) were further evaluated using post hoc least significant difference (LSD) tests. For histological evidence of lung inflammation, exact tests for contingency tables with ordinal categories were used to compare scores of WT and VIP KO mice. All statistical analyses were done using SAS software, with two-tailed *P* values < 0.05 considered statistically significant.

RESULTS

Airway Hyperresponsiveness

In unsensitized animals, methacholine elicited a greater bronchoconstrictor response in the VIP KO mice than in WT mice (Fig. 1). The airway pressure responses were significantly different among the three groups of mice at the 100- and 500-mg/ml dose levels (*P* = 0.008 and 0.007, respectively). With the use of post hoc LSD tests, the airway pressure in KO mice was significantly higher than in WT mice at the 100- and 500-mg/ml dose levels (*P* = 0.003 and 0.001, respectively).

Airway Inflammation

Histological evidence. Lungs from unsensitized KO mice showed peribronchiolar and perivascular infiltration with lymphocytes and eosinophils, whereas lungs from control WT mice appeared normal (Table 1, Fig. 2, A and B). The degree of cellular infiltration varied among individual KO mice, but at least some infiltration was present in the lungs of three out of five mice. More severe inflammation was associated with prominent eosinophilic infiltration, and occasionally also alveolar edema, but there was no evidence of airway remodeling. By contrast, none of the five WT mice showed any evidence of inflammation. The intensity of inflammatory cell infiltration in lungs from unsensitized, unchallenged VIP KO mice was significantly higher than for WT mice (*P* = 0.028), but the

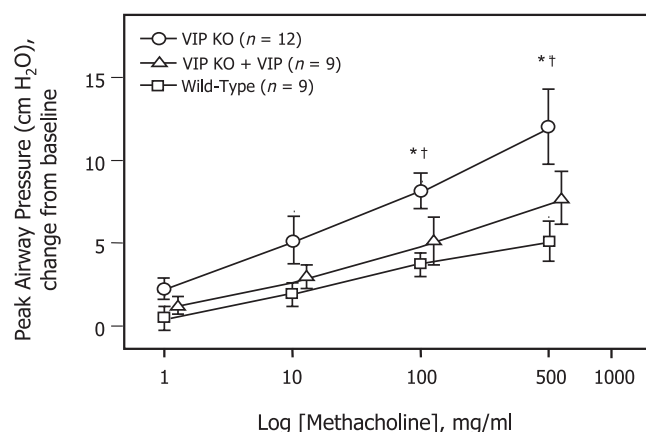


Fig. 1. Airway responses to methacholine challenge in unsensitized VIP knockout (KO) mice were greater than in wild-type (WT) mice or KO mice treated with VIP. Increases in peak airway pressure above baseline values (means $\pm$ SE) are plotted against log methacholine concentration. Pressures at the 100- and 500-mg/ml dose levels of methacholine were higher in KO mice (circles) than in WT control mice (squares, $*P < 0.005$) and in KO mice treated with VIP (triangles, $\dagger P < 0.05$). The latter pressures were not significantly different from those in WT mice.

difference was insignificant between KO and KO+VIP ($P = 0.22$).

Lung cytokines, chemokines, and proteases. Evidence of airway inflammation was supported by analysis of BAL fluid for eight inflammatory cytokines and chemokines and one MMP: IL-2, IL-5, IL-6, IL-10, IL-13, TARC, OPN, IFN- γ , and MMP-2. With the exception of IL-2, mean levels of all cytokines were significantly higher in five KO mice than in five control mice (Table 2).

In unsensitized, unchallenged mice, mean total cell count in BAL fluid was higher in VIP KO mice than in WT mice ($430,000 \pm 250,000$ vs. $95,000 \pm 12,000$, $P = 0.019$ based on *U*-test). The predominant cells in cytospin preparations of BAL

Table 1. Severity of inflammatory cell infiltration in lungs of unsensitized, unchallenged VIP KO and WT mice

Mouse	Peribronchiolar Cell Infiltration
WT (n = 6)	
211	0
215	0
216	0
234	0
235	0
236	0
VIP KO (n = 5)	
217	2
218	3
212	0
237	0
238	2
VIP KO + VIP (n = 5)	
205	4
206	4
209	4
210	1
221	0

Intensity of cell infiltration is indicated by numerals: 0 = none, 1 = mild, 2 = moderate, 3 = severe, 4 = very severe. WT, wild type; KO, knockout.

fluid from WT mice were macrophages and lymphocytes; eosinophils were notably present in BAL fluid from KO mice. With the use of flow cytometry, neutrophils in BAL fluid from KO mice produced more IL-5 than corresponding neutrophils from WT mice (22.7 ± 4 vs. 5.3 ± 5 , $P = 0.067$, approaching significance, based on *t*-test).

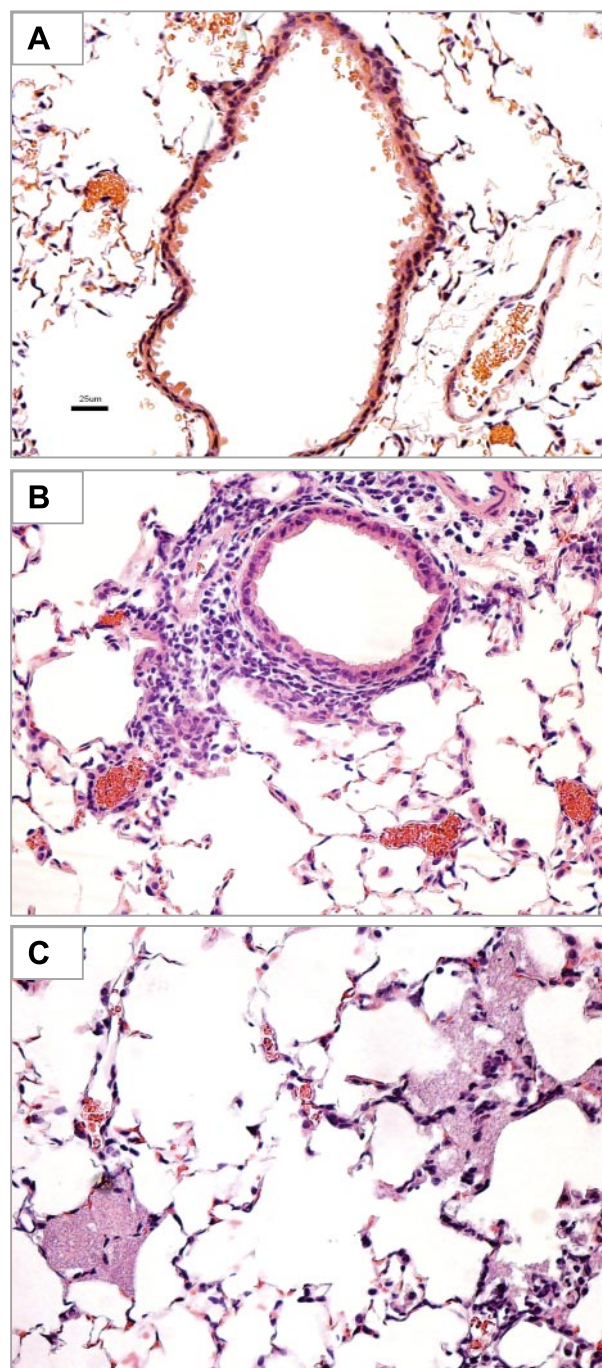


Fig. 2. Histological evidence of airway inflammation in VIP KO mice. Compared with lungs from control WT mice (A), lungs from unsensitized VIP KO mice (B) showed peribronchiolar and perivascular lymphocytic and eosinophilic infiltrates. Following sensitization and challenge with ovalbumin, lungs from some VIP KO mice also showed focal alveolar edema (C). All sections were stained with hematoxylin and eosin. Magnification, $\times 200$.

Table 2. Concentrations of inflammatory cytokines, chemokines, and matrix metalloprotease (pg/ml) in BAL fluid from unsensitized, unchallenged VIP KO and WT mice

	IL-2	IL-5	IL-6	IL-10	IL-13	TARC	IFN- γ	OPN	MMP-2
<i>KO (n = 5)</i>									
M-189	14.9	22.7	102.4	3.2	34.1	107.2	197.8	458723.6	7391.3
M-190	8.1	14.5	30.0	4.6	40.8	230.3	121.8	165631.5	3023.4
M-191	19.7	4.1	26.3	2.0	38.8	20.7	31.7	25502.6	849.6
M-192	21.0	3.1	18.1	1.6	27.4	21.3	21.1	13947.0	334.1
M-193	11.5	9.7	46.9	3.1	27.0	822.7	68.4	414041.1	3780.3
Mean	15.0	10.8	44.8	2.9	33.6	240.4	88.1	215569.2	3075.8
SE	2.4	3.6	15.2	0.5	2.8	150.5	32.6	94285.9	1256.9
<i>WT (n = 5)</i>									
M-194	27.5	5.2	18.5	1.7	36.3	6.3	26.3	11670.6	312.0
M-195	20.2	0.4	8.2	0.4	0.4	6.5	4.7	8526.5	540.9
M-196	20.6	0.4	8.7	1.1	18.6	6.7	11.9	6876.1	327.1
M-197	23.5	0.4	16.5	1.4	12.0	9.6	16.2	7901.1	531.0
M-198	23.0	4.1	21.6	1.4	5.3	6.2	15.9	7709.4	329.6
Mean	23.0	2.1	14.7	1.2	14.5	7.1	15.0	8536.7	408.1
SE	1.3	1.1	2.7	0.2	6.3	0.6	3.5	826.7	52.3
<i>P</i>									
U-test	0.032	0.056	0.032	0.016	0.056	0.008	0.016	0.008	0.032
t-test	0.027	0.072	0.119	0.028	0.034	0.196	0.088	0.093	0.101

TARC, thymus activation-regulated chemokine; OPN, osteopontin; MMP-2, matrix metalloprotease-2; BAL, bronchoalveolar lavage. *P* values are in bold.

Effect of Immunologic Challenge

Airway hyperresponsiveness persisted in VIP KO mice after immunologic sensitization and challenge. The differences in airway pressure among the three groups were significant at the 10-, 100-, and 500-mg/ml dose levels (Fig. 3; $P = 0.029$, 0.013, and <0.001). On the basis of post hoc LSD tests, airway pressures were greater in KO than in WT mice at the 10-mg/ml level ($P = 0.014$), the 100-mg/ml level ($P = 0.007$), and the 500-mg/ml level ($P < 0.001$).

Immunologic sensitization and challenge led to an increase in the degree of lung cellular infiltration in WT mice ($P = 0.04$). Cellular infiltration was also marginally increased in VIP KO mice, relative to unchallenged KO mice ($P = 0.067$). Infiltration was marginally greater in KO compared with WT mice ($P = 0.056$). In some of the KO mice, there were also areas of focal alveolar edema (Table 3, Fig. 2C).

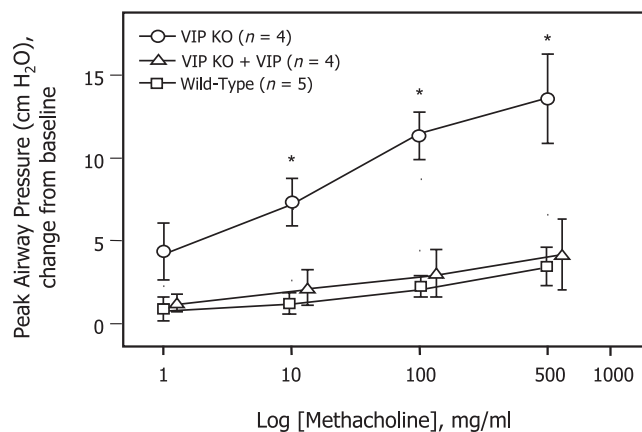


Fig. 3. Airway responses to methacholine in 3 groups of sensitized challenged mice. Treatment of KO mice with VIP (triangles) markedly attenuated the responses relative to those in untreated KO mice (circles, $*P < 0.05$). The responses became statistically no different from those in WT mice (squares).

Judging by cytospin preparations, eosinophils were absent in BAL fluid from unchallenged WT mice but were clearly present after challenge ($P = 0.004$, by Mann-Whitney's test). Eosinophil counts, already present in most unchallenged KO mice, showed a tendency to increase further following challenge ($P = 0.057$). Analysis by flow cytometry showed that, in WT mice, mean neutrophil count in BAL fluid increased from $9\% \pm 2$ to $40\% \pm 11$ ($P = 0.011$) and in VIP KO mice it increased from $7\% \pm 1$ to $32\% \pm 8$ ($P = 0.019$). Both CCR3-positive cells and neutrophils in BAL fluid from WT mice produced more IL-5 in sensitized, challenged mice than

Table 3. Severity of inflammatory cell infiltration in lungs of sensitized and challenged VIP KO and WT mice, with and without VIP treatment

Mouse	Peribronchial Cell Infiltration
<i>WT (n = 5)</i>	
226	4
227	3
231	2
232	1
233	1
<i>VIP KO (n = 4)</i>	
219	4
220	4
222	4
223	3
<i>VIP KO + VIP (n = 5)</i>	
224	3
225	3
228	2
229	0
230	1

Intensity of cell infiltration is indicated by numerals: 0 = none; 1 = mild; 2 = moderate; 3 = severe; 4 = very severe.

in unsensitized mice: 22.2 ± 2.0 vs. 7.3 ± 7.3 for CCR3-positive cells ($P = 0.15$, based on *U*-test) and 24.6 ± 4.0 vs. 5.3 ± 5.3 for neutrophils ($P = 0.035$, based on *t*-test).

CD11c-positive (dendritic) cells in BAL fluid from VIP KO mice produced more IL-5 than dendritic cells in BAL fluid from WT mice: 17.3 fluorescent channels ± 4.5 vs. 4.6 ± 1.1 , $P = 0.12$ based on *t*-test and 0.032 based on *U*-test. Dendritic cells in BAL fluid from VIP KO also produced more IL-6 than dendritic cells in BAL fluid from WT mice, but the difference was not statistically significant (62.0 ± 38.1 vs. 7.8 ± 2.5 , $P = 0.39$ based on *t*-test and 0.095 based on *U*-test).

VIP Replacement

Administration of VIP to a group of VIP KO mice achieved pulmonary levels of the peptide similar to those in WT mice. Analysis of BAL fluid and lung tissue from KO mice treated with VIP for 2 wk showed VIP levels of 20 ± 8.3 pg/ml in BAL fluid ($n = 5$), 0.14 ± 0.07 pg/mg protein in lung tissue ($n = 5$) in unsensitized, unchallenged mice, and 17 ± 12.8 pg/ml ($n = 5$) and 0.14 ± 0.06 pg/mg protein in sensitized, challenged mice. The corresponding values in WT mice (that did not receive VIP) were 12.3 ± 8.7 pg/ml ($n = 6$) and 0.04 ± 0.04 pg/mg protein in lung tissue ($n = 5$) in the absence of sensitization and challenge and 32.7 ± 10.1 pg/ml ($n = 5$) in BAL fluid and 0.12 ± 0.06 pg/mg protein ($n = 5$) in lung tissue, after sensitization and challenge.

VIP replacement therapy markedly attenuated airway hyperresponsiveness in KO mice. In the nine VIP KO mice treated with VIP, methacholine elicited greatly reduced airway pressure responses, which closely resembled those of WT mice (Fig. 3). On the basis of LSD post hoc tests, airway pressures in VIP-treated KO mice were significantly lower than in untreated mice at the 10- ($P = 0.027$), the 100- ($P = 0.011$), and the 500-mg/ml ($P < 0.001$) dose levels. Three of the four KO mice that received only PBS responded like other untreated KO mice.

Compared to untreated or buffer-treated KO mice, most lung sections from VIP-treated KO mice revealed considerably less intense, and more limited, cellular infiltrates (Fig. 4). Cellular infiltration was significantly less severe in KO mice that received VIP treatment compared with those that did not ($P = 0.016$) and was no different than in WT mice ($P = 0.66$, Table 3). Additionally, in contrast to KO mice, neutrophils in BAL fluid from KO mice treated with VIP did not increase after sensitization and challenge ($17\% \pm 7$ vs. $19\% \pm 7$, $P = 0.85$ based on *t*-test). Thus the 2-wk treatment with VIP almost largely corrected the two major asthma-like features observed in untreated KO mice.

DISCUSSION

Our results demonstrate that homozygous VIP KO mice spontaneously express two of the cardinal features of bronchial asthma: airway hyperresponsiveness and airway inflammation, manifested by cellular infiltration with lymphocytes and eosinophils. This infiltration was moderately severe and accompanied by an inflammatory cytokine/chemokine response, consistent with findings in experimental and clinical asthma (2, 9, 23, 41). Lymphocytes, eosinophils, and probably also neutrophils, were present in BAL fluid, especially after immunologic challenge. The presence of neutrophils in BAL fluid under

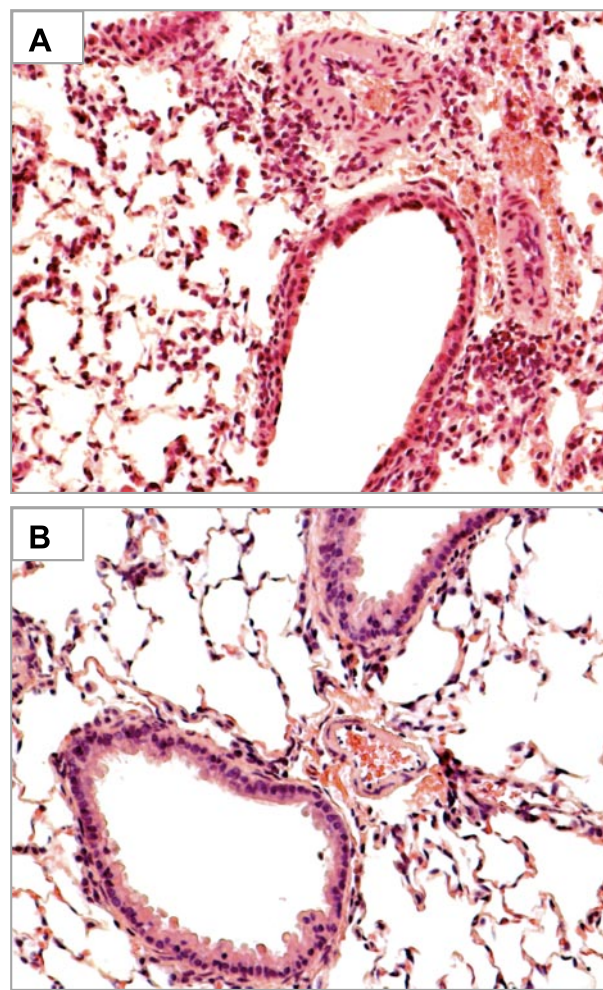


Fig. 4. An illustration of attenuation of airway inflammation by VIP treatment in sensitized, challenged VIP KO mice. Dense inflammatory cell infiltrates in untreated KO mouse (A) contrasts with essentially normal lung field in treated mouse (B).

these conditions suggests that they may have contributed to the inflammatory response. A role for neutrophils in the pathogenesis of asthma, particularly severe forms of the disease, has recently been documented (24a). Airway remodeling, a third feature of the human disease, especially in its chronic stages (1), was not observed in these mice, possibly reflecting a relatively short duration of the disease model.

Despite the traditional view that the asthma phenotype is driven by increased Th2 cytokines, e.g., IL-5 and IL-13, with decreased Th1 cytokines, e.g., IFN- γ , studies of mouse models strongly suggest that simultaneous activation of the Th1 immune response may promote a more severe airway inflammation (4, 20, 30). Possible interactions between IL-13 and IFN- γ , both of which were elevated in our KO mice, are of special interest (14): 1) depending on the antigen, IFN- γ may actually accentuate the inflammatory response; and 2) in a mouse model of airway inflammation induced by mixed T cell responses, blockade of IL-13 partially inhibited airway hyperreactivity but not inflammation (14). The latter observation appears to parallel the greater suppression of airway hyperresponsiveness than of airway inflammation after treatment of KO mice with VIP. The phosphodiesterase inhibitor pentoxi-

fylline, a selective suppressor of Th1 cytokine production, attenuated airway hyperresponsiveness but not airway inflammation in a mouse model of asthma (13).

The possible role of VIP in asthma has long been under investigation, but a clear answer has been lacking. Early after its discovery and isolation, VIP was demonstrated to occur widely in nerve fibers and nerve terminals supplying human and other mammalian airways (10). Soon afterward, it was shown to be a potent relaxant of airway smooth muscle (31) and to act as a cotransmitter of neurogenic airway relaxation (33). Later, its anti-inflammatory and immunomodulator properties were described (7, 15) as well as its ability to inhibit airway smooth muscle proliferation (25). The picture thus emerged of VIP as a compound that is potentially capable of counteracting most major components of the asthmatic phenotype (3, 35). Bolstering this viewpoint, VIP-immunoreactive nerves were reported absent in the airways of a small group of severely asthmatic patients (26), raising the possibility that a deficiency of the neuropeptide might even be causally related to the disease.

Recently, however, a number of immunologic studies have added a different perspective on what may be a complex relationship between VIP and asthma. Findings typical of immediate-type hypersensitivity (elevated blood IgE levels and eosinophil counts) were described in transgenic mice that constitutively and selectively expressed, in CD4 T cells, the normally inducible VPAC2 receptor (38, 40). This is one of three receptors common to VIP and the related pituitary adenylate cyclase-activating peptide that is normally not constitutively expressed (21). Conversely, VPAC2-null mice manifested decreased immediate-type hypersensitivity (17). These and related studies, by themselves, suggested that VIP plays a significant role in shifting the Th1/Th2 balance in favor of the Th2 phenotype (8). There is no real conflict between these observations and the present results. Deletion of the VIP is not equivalent to loss of one of its receptors; the full effects of VIP are mediated by the combined influence of its three receptors, known as VPAC1, VPAC2, and PAC1 (21).

One of the more remarkable findings in this report is that deletion of the VIP gene did not merely predispose the mice to asthma-like features. Many mice spontaneously exhibited airway hyperresponsiveness and airway inflammation, even in the absence of immunologic sensitization and challenge. In this respect, these mice are reminiscent of those with targeted deletion of the T-bet transcription factor (12). A possible link between VIP and T-bet has been considered (28, 38), but none has been established. Not all VIP KO mice expressed the asthmatic phenotype to the same degree, however, and the asthmatic features were more prominent after immunologic sensitization and challenge.

The cause-and-effect relationship between the absence of the VIP gene and the asthma-like phenotype is validated by the marked attenuation of airway hyperresponsiveness and the significant reduction of airway inflammation after a 2-wk administration of the peptide. It is possible that a higher dose of VIP or a longer period of treatment might have resulted in more complete correction of the asthmatic features in KO mice.

Our observations recall the recent report attributing an anti-asthma role for *S*-nitrosothiols, which convey beneficial NO bioactivity (29). As physiological transmitters, VIP and NO act

together synergistically to bring about more effective smooth muscle relaxation through the combined activation, respectively, of adenylate cyclase and guanylate cyclase (32). It should therefore come as no surprise if VIP and NO turned out to be major codefenders against the asthmatic response. A two-pathway protective system with *S*-nitrosothiols acting in conjunction with β -adrenergic agonists has already been proposed (16).

Our results do not answer the question of how the lack of the VIP gene may result in an asthma phenotype or the related question of how VIP protects against asthma. VIP may exert its influence by modulating one or more of the factors involved in asthma pathogenesis, including dendritic and regulatory T cell function (24, 36), Toll-like receptors (6, 18, 19), and various transcription factors.

As well as supporting the role of VIP in defending against asthma, our data shed new light on the genetic determinants of airway hyperresponsiveness and the pathogenesis of human asthma. The added evidence for VIP as a natural anti-asthma compound also suggests that selective agonists of VIP or its receptors may prove particularly effective in the treatment of the disease.

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