



CHEST contents

VOLUME 100 / NUMBER 5 / NOVEMBER, 1991

editorials

- 1185 Clinical Diagnosis
James E. Dalen,
- 1187 Dyspnea: Terminology
Melvin R. Pratter,
- 1188 Pseudosepsis: A
Regulatory Element
George M. Matulis,
- 1189 Aerosolized Inhaled
Robert McCabe,
- 1191 When Life Starts
John H. Hansen,

4 Med. 67 88

100
1991

1185-1174
+ Suppl.

Inhibition of

clinical investigations

- 1193 Endobronchial Lavage
Deborah Shure, San Diego
- 1197 Mast Cells in Bronchoalveolar Lavage Fluid and in Transbronchial Biopsy Specimens of Patients with Farmer's Lung Disease
Alberto Pesci, Giuseppina Bartorelli, Dario Olivieri, Parma, Italy
- 1203 Renal Artery Stenosis
Markku Hyöty,
- 1210 Detection of Tuberculosis
John J. Hui,
- 1215 Esmolol
Michael R. ...
- 1219 Urine Protein Excretion
Gerald C. ...
- 1224 Pneumonia
Michael D. ...
- 1229 The Effect of
Omri Inbar,
- 1235 The Effect of
Shinya Kori,
- 1239 Nocturnal Asthma; Beta₂-Adrenoceptors on Peripheral Mononuclear Leukocytes, cAMP- and Cortisol-Plasma Concentrations
Ekkehard Haen; Rainer Hauck; Hans Peter Emslander; Irmgard Langenmayer; Barnhard Liebl; Jochen Schopohl; Jörg Remien; Günter Fruhmann, Munich, Germany
- 1246 Beta-adrenergic Receptors and m-Cholinergic Receptors in Human Lung; Findings following *in Vivo* and *in Vitro* Exposure to the Beta-adrenergic Receptor Agonist, Terbutaline
Michael Böhm; Susanne Gengenbach; Rainer W. Hauck; Ludger Sunder-Plassman; Erland Erdmann, Munich, Germany
- 1254 Duration of Protection by Inhaled Salmeterol in Exercise-induced Asthma
Sandra D. Anderson; L. T. Rodwell; J. Du Toit; I. H. Young, Camperdown, Australia
- 1261 Diagnosis of Tuberculous Pleural Effusion by the Detection of Tuberculostearic Acid in Pleural Aspirates
Wing Wai Yew; Chiu Yeung Chan; Susan Yuk-lin Kwan; Siu Wai Cheung; Gary L. French, Shatin, Hong Kong
- 1264 The Relationship between Pleural Fluid Findings and the Development of Pleural Thickening in Patients with Pleural Tuberculosis
Carmen S. V. Barbas; Alberto Cukier; Carlos R. R. de Varvalho; Joao V. Barbas Filho, Sao Paulo, Brazil; Richard W. Light, Long Beach, California
- 1268 Primary Pulmonary Hypertension in HIV Infection
Rudolf Speich; Rolf Jenni; Milos Opravil; Marlene Pfab; Erich W. Russi, Zurich, Switzerland
- 1272 Bilateral Bronchoalveolar Lavage in the Diagnosis of Opportunistic Pulmonary Infections
Gianfranco Umberto Meduri; Diane E. Stover; Ronald A. Greeno; Thomas Nash; Muhammad B. Zaman, New York
- 1277 Human Alveolar Macrophages of Anergic Patients with Lung Cancer Lack the Responsiveness to Recombinant Interferon Gamma
Hidetaka Kawatsu; Yoshinori Hasegawa; Eishi Takagi; Kaoru Shimokata, Nagoya, Japan
- 1281 Measurement of Sleep-related Breathing Disturbances in Epidemiologic Studies; Assessment of the Validity and Reproducibility of a Portable Monitoring Device
Susan Redline; Tor Tosteson; Mary Ann Boucher; Richard P. Millman
- 1287 Transcutaneous Oxygen Saturation and Carbon Dioxide Tension during Meals in Patients with Chronic Obstructive Pulmonary Disease
Annemie Schols; Rob Mostert; Nicolle Cobben; Peter Soeters; Emiel Wouters, Horn, The Netherlands

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Study

Nocturnal Asthma*

β_2 -Adrenoceptors on Peripheral Mononuclear Leukocytes, cAMP- and Cortisol-Plasma Concentrations

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To evaluate pathophysiologic mechanisms of the predominantly nocturnal complaints in atopic bronchial asthma, the expression and function of β_2 -adrenoceptors on peripheral mononuclear leukocytes (pMNL), the cAMP,—as well as the cortisol—plasma concentrations were studied in eight healthy men and ten so far untreated male asthmatic patients at 4-h intervals for 24 h. No difference was seen in the β_2 -adrenoceptor density (Bmax) on pMNL between healthy and asthmatic men (24-h means \pm SE: 908 \pm 59 sites per cell and 821 \pm 54 sites per cell, respectively). The equilibrium dissociation constant (Kd), however, was significantly higher in the asthmatic patients (24-h mean \pm SE: 8.8 \pm 1.2 pmol/L vs 3.0 \pm 0.2 pmol/L in healthy men, $p < 0.0001$), which is equivalent to a lower affinity of the β_2 -adrenoceptors for the radioligand 125 I-iodocyanopindolol. Bmax showed a statistically significant circadian variation, but Kd did not. The circadian variation in Bmax was reflected in the basal intracellular cyclic adenosine-monophosphate (cAMP) content of the cells investigated. High Kd values (equivalent to low receptor affinities) tended to be associated with small increases of the intracellular cAMP content after *in vitro* stimulation by 10^{-7} mol/L isoprenaline (isoproterenol) (24-h mean \pm SE: 1.4 \pm 0.2 pmol/ 10^6 cells; $r = -0.529$, $p = 0.05$ at $r = -0.549$, $n = 10$). Plasma cAMP concentrations were found to be significantly lower in the asthmatic patients (24-h means \pm SE: 22.9 \pm 1.3 nmol/L vs

29.1 \pm 1.1 nmol/L, $p < 0.0001$). Plasma cortisol concentrations were significantly higher in the asthmatic patients (24-h means \pm SE: 0.500 \pm 0.084 μ mol/L vs 0.319 \pm 0.063 μ mol/L). The results support the hypothesis that a lesion of the β -adrenergic system contributes to the pathophysiology of atopic bronchial asthma. In the patients investigated in this study, such a lesion could be demonstrated in the affinity rather than in the number of β_2 -adrenoceptors expressed on peripheral cells of the immune system (pMNL). According to present-day knowledge of adrenergic effects on pMNL, such an affinity decrease of β_2 -adrenoceptors could account for overshooting immune responses. In association with other factors influencing respiratory function, it could be responsible for the predominantly nocturnal complaints in atopic bronchial asthma. Plasma cortisol concentrations did not appear to be related to the principal cause of "nocturnal asthma," they rather reflected an endogenous defense mechanism to the disease.

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ANOVA = analysis of variance; Bmax = β_2 -adrenoceptor density; cAMP = cyclic adenosine monophosphate; IBMX = isobutyl methylxanthin; 125 I-CYP = 125 I-iodocyanopindolol; Kd = equilibrium dissociation constant; PDE = phosphodiesterase; PEF = peak expiratory flow; pMNL = peripheral mononuclear leukocytes

Bronchial asthma is characterized by refractoriness against β -adrenergic stimuli.¹ Drugs amplifying the sympathetic tone (β -sympathomimetic drugs, methylxanthines) are most effective in this disorder,² so that a disturbance of the expression and/or function of β -adrenoceptors was suggested as an underlying cause.¹ This theory has been the subject of intense investigation; however, it has not been possible to

either reject or substantiate it clearly. We are interested in the circadian variation of asthmatic attacks.³⁻⁶ Circadian variations in the excretion of catecholamines, in plasma adrenaline concentration, and in plasma cyclic adenosine monophosphate (cAMP) concentration have already been described⁷⁻¹⁰ with low values in the early morning matching the time of increased asthmatic symptoms. As well as these variables, the number and affinity of β_2 -adrenoceptors are necessary to fully describe the sympathetic tone and its influence on the bronchial tone. Recently we observed a circadian variation in the β_2 -adrenoceptor density (Bmax) expressed on peripheral mononuclear leukocytes (pMNL) of healthy men.¹¹ A reduced β_2 -adrenoceptor density in asthmatic patients has been assumed^{12,13} but not yet substantiated convincingly. Previous investigations may have been biased by pretreatment of the patients under investigation, by

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circadian variations of the physiologic variables studied, by differences between male and female subjects,¹¹ and by a conceptual error in the analysis of radioligand receptor assays.^{14,15} On the other hand, the circadian variations of plasma cortisol concentrations are also assumed to contribute to nocturnal asthma.^{5,16,17} We therefore studied the expression of β_2 -adrenoceptors on pMNL, the isoprenaline-induced intracellular cAMP production in these cells, and the cAMP as well as the cortisol plasma concentrations of so far untreated asthmatic men for 24 h. As cells of the immune system, pMNL might be directly involved in the pathophysiology of atopic bronchial asthma. Like bronchial tissue, they bear β -adrenoceptors of the β_2 -type.¹⁸ These cells may be simply and repeatedly collected without undue discomfort for the patient. They are therefore widely used in clinical studies on receptor disturbances in bronchial asthma.¹⁹⁻²³

MATERIALS AND METHODS

At 4-h intervals, 20 ml of venous blood was drawn from the antecubital vein of eight healthy men and ten male asthmatic patients. Seven healthy men and two asthmatic patients were hospitalized in the Clinical Research Unit of the Walther-Straub-Institute; one healthy man and three asthmatic patients were hospitalized in the I. Medical Clinic of the Technical University, the other five patients were hospitalized in the I. Medical Clinic Großhadern of the Ludwig-Maximilians-University of Munich. Collection of blood, lung function testing, and handling of blood specimens were always done by the staff of the Walther-Straub-Institute. All laboratory tests were performed in the Walther-Straub-Institute; only plasma cortisol was assayed in Dr. Schopohl's laboratory.

The healthy subjects (22 to 34 years of age) had a negative history and showed no signs of allergic, pulmonary, cardiac, renal, and hepatic disease, nor of any other disease by clinical inspection. Total plasma IgE concentrations were below 120 kU/L. The asthmatic patients were 19 to 33 years of age. They had never been consistently treated for their complaints, which implies that their asthma was of a mild type. Some of them had occasionally used medications, inhaled β -sympathomimetic or vagolytic drugs (less than one or two puffs within three weeks), and/or antihistamines, but usually overcame their symptoms without medication. They were referred from private practices to the participating hospitals for diagnostic check-ups. They complained of breathlessness and coughing predominantly at nighttime. Their respiratory function was monitored over the period under investigation by a computer-based bedside spirometer providing full flow/volume spirometry (Spiropro 3000, Ganshorn Electronic GmbH, Műnnerstadt/FRC) in five patients (patients hospitalized at the I. Medical Clinic of the Technical University and at the Clinical Research Unit of the Walther-Straub-Institute) and by oscillatory airway resistance (Raw, Siregnost FD 5, Siemens AG, Erlangen/FRC) in the other five patients (hospitalized at the I. Medical Clinic Großhadern of the Ludwigs-Maximilians-University); measurements were performed immediately after drawing blood. The atopic nature of their asthma was verified by anamnesis, positive skin tests, demonstration of specific IgE antibodies, and by exposure to the antigen in question. The repeated assessment of respiratory function at 4-h intervals allowed the documentation of bronchial obstruction and its spontaneous reversibility (Fig 1).

The study was approved by the ethical committee of the hospital of the Technical University of Munich and informed consent was

obtained. The subjects were hospitalized for the full length of the investigation. They continued to follow their regular lifestyle, which they had recorded for the week preceding the study. On the average, they slept from 11 PM to 7 AM, and meals were served at 8 AM, 11:30 AM, and 5:30 PM. During the day, the subjects did not stay in bed, but either did some paper work, read, or went for short walks. Blood was drawn after 30 min of rest in the supine position. No drugs were taken.

Directly after venipuncture, intact pMNL were harvested by density centrifugation (using Lympho-paque, Nyegaard & Co, AS, Oslo/Norway). The expression of β_2 -adrenoceptors was studied for each time point in radioreceptor assays as described elsewhere.¹⁵ In brief, pMNL were incubated for 2 h at 37°C with 12 concentrations of (-)-¹²⁵I-iodocyanopindolol (¹²⁵I-CYP, Amersham Buchler, Braunschweig/FRC) in the range of 1.0 to 150.0 pmol/L to determine the number of high affinity binding sites (Bmax) and their equilibrium dissociation constant (Kd). Nonspecific binding was determined in parallel incubations with 10⁻⁵ mol/L (-)-timolol. Pipetting was performed (by a Tecan Robotic Sample Processor Model 5052, dual arm system, Tecan AG Frankfurt/FRC).²⁴ A binding equation for two independent classes of binding sites was fitted to the data, using a nonlinear iteration procedure.^{14,15}

The function of β_2 -adrenoceptors on pMNL was tested in the asthmatic patients by stimulating the intracellular cAMP content with 10⁻⁷ mol/L isoprenaline (isoproterenol) over basal values (incubation with buffer) for 10 min at 37°C. The cells were preincubated for 30 min at room temperature with 10⁻⁴ mol/L isobutylmethylxanthin (IBMX) to inhibit the degradation of intracellularly formed cAMP by the enzyme phosphodiesterase (PDE). The incubation with isoprenaline was stopped by placing the cell suspensions for 5 min in boiling water to rupture the cells. Cell

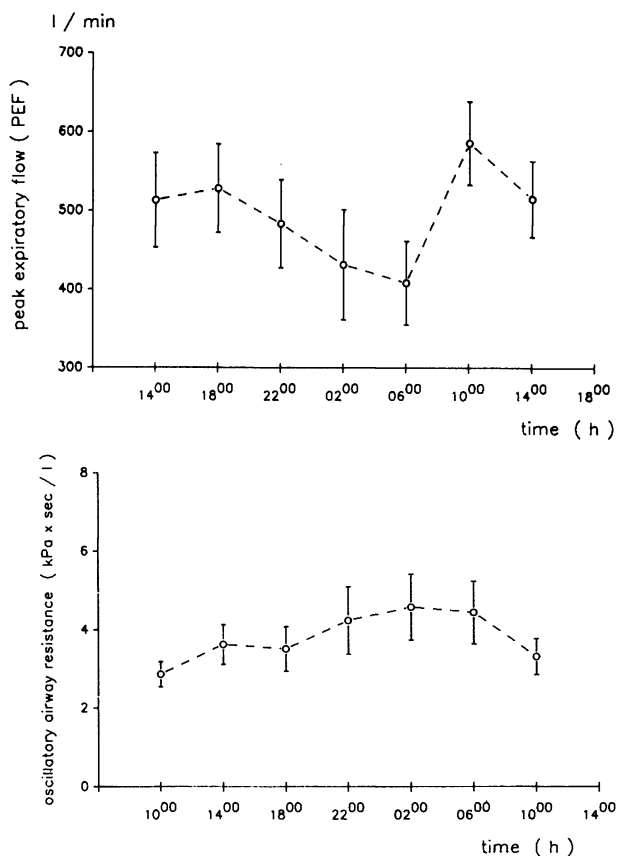


FIGURE 1. Mean circadian variation in peak expiratory flow (top panel) and in oscillatory airway resistance (bottom panel) of five so far untreated male asthmatic patients.

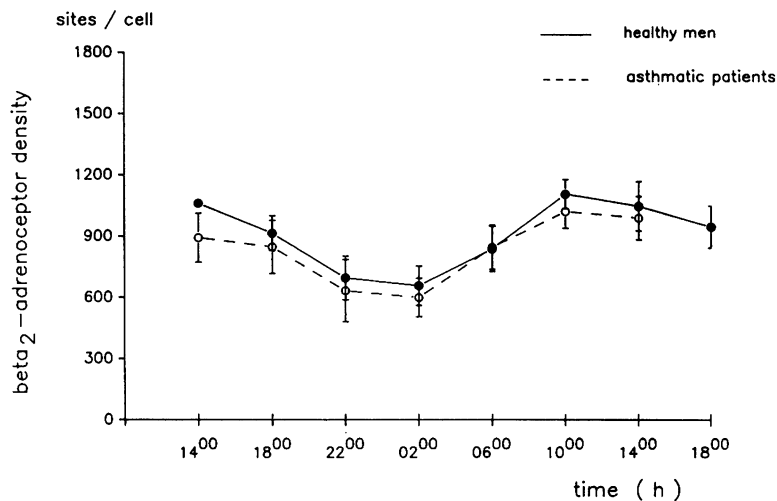


FIGURE 2. Mean circadian variation in the expression of β_2 -adrenoceptors (Bmax) on peripheral mononuclear leukocytes (pMNL) of 10 so far untreated male asthmatic patients, 19 to 33 years of age, and 8 healthy men, 22 to 34 years of age.

particulates were centrifuged for 5 min at 15,000g and the supernatant stored at -20°C until determination of the cAMP content. Intracellular cAMP, plasma cAMP, and plasma cortisol were determined by radioimmunoassay (Amersham Buchler, Braunschweig/FRG and DRG Instruments GmbH, Marburg/FRG, respectively).

Mean values are given with their standard errors (SE). The 24-h mean was defined as the mean of each set of raw data within 24 h, the circadian range as the difference between its maximal and minimal value, and the circadian variation as the time-specified pattern of variation observed over 24 h. Time of day effects were statistically validated by an analysis of variance (ANOVA) with subjects and time of day as components. Correlations between mean values were tested by linear regression analysis. To test for differences between asthmatic patients and healthy men, a Student's *t* test was used. The significance level used was $\alpha = 0.05$.

RESULTS

Respiratory function tests revealed a mild form of asthma in the ten male patients (peak expiratory flow [PEF] 24-h mean \pm SE: 494 ± 21 L/min, Raw 24-h mean \pm SE: 3.8 ± 0.22 kPa \times s/L). All of the asthma patients, however, showed a marked nocturnal dip, even if their respiratory function was in the normal range during daytime. This was apparent in both PEF (17.7 ± 4.8 percent of the 24-h mean at 6 AM, top

panel in Fig 1) and oscillatory airway resistance (18.4 ± 5.9 percent of the 24-h mean at 2 AM, bottom panel in Fig 1). The spontaneous reversibility of the nocturnal dip was demonstrated in each patient.

Statistically significant time of day effects were observed in the circadian variation of the β_2 -adrenoceptor density on pMNL in the healthy controls (ANOVA: $p < 0.001$; 24-h mean \pm SE: 908 ± 59 sites per cell; circadian range: 71.6 to 126.9 percent of the 24-h mean), whereas in the asthmatic patients, these effects were not statistically significant (ANOVA: $p = 0.077$; 24-h mean \pm SE: 821 ± 54 sites per cell; circadian range: 69.3 to 133.7 percent of the 24-h mean; Fig 2). In both groups, β_2 -adrenoceptor density was lowest around midnight. There were no statistically significant differences between healthy and asthmatic subjects in Bmax.

No statistically significant time of day effects were observed in the circadian variation of the apparent equilibrium dissociation constant *Kd* representing the affinity of the β_2 -adrenoceptors for the radioligand ^{125}I -CYP. In the asthmatic patients, however, the affinity was decreased over the whole study period (which

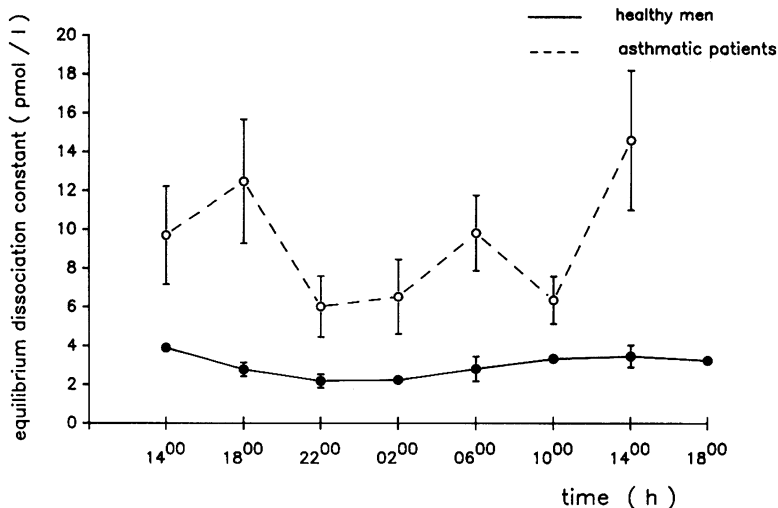


FIGURE 3. Mean circadian variation in the apparent equilibrium dissociation constant (*Kd*) for the radioligand ^{125}I iodocyanopindolol of β_2 -adrenoceptors expressed on peripheral mononuclear leukocytes (pMNL) of 10 so far untreated male asthmatic patients, 19 to 33 years of age, and 8 healthy men, 22 to 34 years of age.

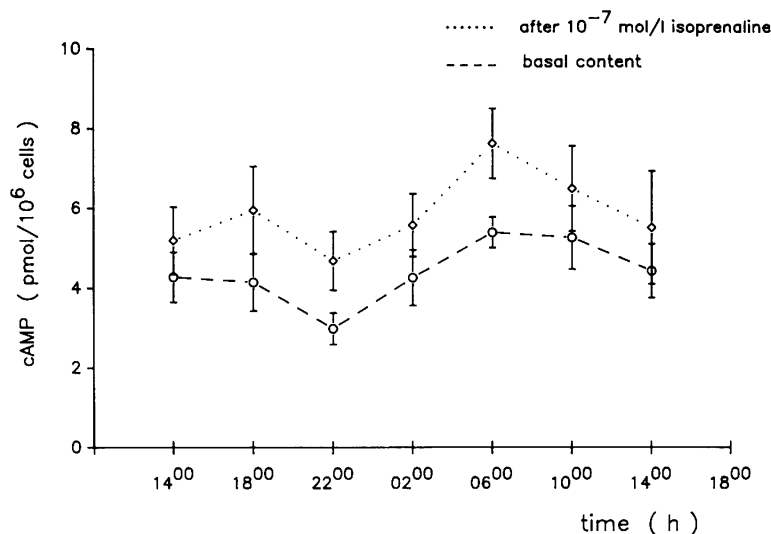


FIGURE 4. Mean circadian variation in the basal cyclic adenosine monophosphate (cAMP) content of peripheral mononuclear leukocytes (pMNL) of 10 so far untreated male asthmatic patients, 19 to 33 years of age, and its stimulation by 10^{-7} mol/L isoprenaline.

is equivalent to an increased Kd value, Fig 3): The 24-h mean \pm SE was 8.8 ± 1.2 pmol/L in the asthmatic patients as compared with 3.0 ± 0.2 pmol/L in the healthy controls ($p < 0.0001$).

The basal intracellular cAMP content of pMNL derived from asthmatic patients reflected the circadian variation in the β_2 -adrenoceptor density expressed on these cells (ANOVA: $p < 0.01$; 24-h mean \pm SE: 4.4 ± 0.3 pmol/ 10^6 cells; circadian range: 70.2 to 131.9 percent of the 24-h mean, Fig 4). After isoprenaline stimulation, the 24-h mean of the intracellular cAMP content increased by 1.4 ± 0.2 pmol/ 10^6 cells (24-h mean \pm SE, circadian range: 63.2 to 154.9 percent of the 24-h mean, Fig 4). Expressed as pmol/ 10^6 cells, stimulation was highest at 6 AM and lowest at 2 PM; expressed as a percentage of the basal value, stimulation was highest at the time of lowest basal value (10 PM: 168.8 percent of basal value) and lowest at the time of highest basal value (2 PM: 120.8 and 118.6 percent of basal value). When the 24-h means were fitted by a linear regression, 28 percent of the total variation could be explained by a negative correlation

between the stimulation of intracellular cAMP and the apparent equilibrium dissociation constant Kd; *ie*, high Kd values (equivalent to low receptor affinities) tended to be associated with small increases of the intracellular cAMP content after *in vitro* stimulation by isoprenaline. The correlation coefficient was just below the significance level ($r = -0.529$; $p = 0.05$ at $r = -0.549$, $n = 10$). The correlation between the stimulation of intracellular cAMP and Bmax was not statistically significant ($r = 0.0996$).

Statistically significant time of day effects could also be demonstrated in the circadian variation of cAMP plasma concentrations in both healthy subjects (ANOVA: $p < 0.05$) and asthmatic patients (ANOVA: $p < 0.05$). The values were significantly lower for the asthmatic patients (24-h mean \pm SE: 22.9 ± 1.3 nmol/L) compared with the healthy controls (24-h mean \pm SE: 29.1 ± 1.1 nmol/L, $p < 0.0001$, Fig 5). The circadian range for this variable was similar in both groups (healthy subjects: 87.1 to 117.5 percent of 24-h mean; asthmatic patients: 86.3 to 108.0 percent of 24-h mean).

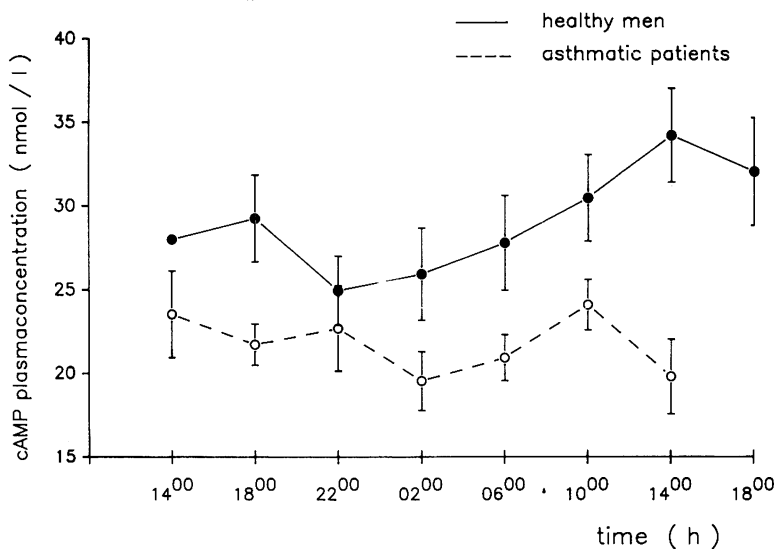


FIGURE 5. Mean circadian variation in the plasma cyclic adenosine monophosphate (cAMP) concentration of 10 so far untreated male asthmatic patients, 19 to 33 years of age, and 8 healthy men, 22 to 34 years of age.

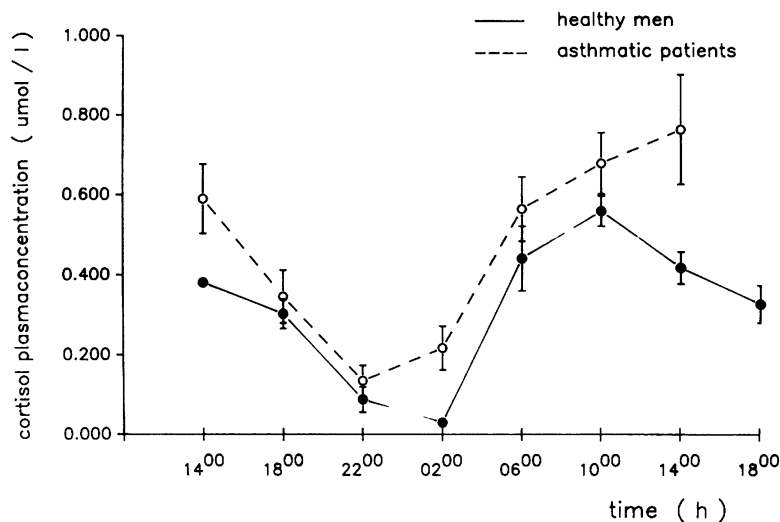


FIGURE 6. Mean circadian variation in the plasma cortisol concentration of 10 so far untreated male asthmatic patients, 19 to 33 years of age, and 8 healthy men, 22 to 34 years of age.

The circadian variation of plasma cortisol concentrations showed statistically significant time of day effects in both healthy men (ANOVA: $p < 0.001$) and asthmatic subjects (ANOVA: $p < 0.001$, Fig 6). The 24-h mean was significantly lower in the healthy controls (mean \pm SE: 0.319 ± 0.063 $\mu\text{mol/L}$) compared with the asthmatic patients (mean \pm SE: 0.500 ± 0.084 $\mu\text{mol/L}$, $p < 0.01$). Plasma cortisol concentrations dropped at night to 0.030 ± 0.014 $\mu\text{mol/L}$ in the healthy controls, but only to 0.135 ± 0.039 $\mu\text{mol/L}$ in the asthmatic patients. The circadian range was 9.6 to 182.9 percent of the 24-h mean in the healthy men, and just 27.6 to 148.2 percent of the 24-h mean in the asthmatic patients.

DISCUSSION

Studies on the pathophysiology of bronchial asthma are often biased by some kind of pretreatment of the patients under investigation. In this study, particular care was taken in the selection of untreated patients. To the best of our knowledge, expression and function of β_2 -adrenoceptors on pMNL were not influenced by any kind of (pre)treatment. These patients are rather rare, at least in academic centers. It has to be pointed out that, since the patients were able to manage their disease without any drugs, their disease was mild. The clinical diagnosis, however, was unequivocally established in each case. The conclusions that follow apply, strictly speaking, just to this group of patients. They suggest an important aspect of the multifunctional pathophysiologic condition of bronchial asthma that may possibly only be observed in untreated subjects. Plasma cortisol was originally determined as a marker for the circadian system. Results demonstrated that the circadian system of each individual in this study was synchronized to the 24-h day.

It has been suggested that bronchial asthma is caused by a malfunction of β_2 -adrenoceptors.¹ Such a malfunction was often assumed to be a reduced

receptor number,^{12,13} whereas the original hypothesis included all kinds of β -adrenoceptor disturbances, even adenylate cyclase insufficiency.¹ In our study, no difference in the number of β_2 -adrenoceptor sites expressed on pMNL could be observed between healthy men and untreated male asthmatic patients of the same age group. We did detect, however, a reduced affinity of these β_2 -adrenoceptors for the radioligand ¹²⁵I-CYP in our asthmatic patients. This indicates a conformational change of the receptor protein, although it does not prove at present, a reduced affinity for the physiologic ligand adrenaline. ¹²⁵I-CYP is a sympathetic antagonist that might bind to a site on the receptor protein different from the agonist binding site. A reduction in the affinity of β_2 -adrenoceptors for ¹²⁵I-CYP without concomitant change in the β_2 -adrenoceptor density has already been reported by Liebl et al²⁵ under the influence of reducing agents. Although the significance of this latter finding for the pathophysiologic condition of bronchial asthma is not clear at present, it demonstrates yet another example of affinity changes in β_2 -adrenoceptors.

In vitro stimulation of intracellular cAMP production is used to test the function of β_2 -adrenoceptors determined by radioreceptor assays.^{26,27} Both experiments are performed using the same pMNL cell suspension. In this study, the increase of intracellular cAMP tended to be negatively correlated to the equilibrium dissociation constant K_d —in other words, the lower the affinity of β_2 -adrenoceptors, the lower (in the same cells) the intracellular increase of cAMP after *in vitro* stimulation by isoprenaline.

Plasma cAMP concentrations in our asthmatic patients were significantly lower than in our healthy controls. According to Holmegaard,²⁸ 50 percent of plasma cAMP results from leakage out of cells expressing β -adrenoceptors; the other 50 percent presumably stem from kidney cells under stimulation by parathormone.²⁸ Plasma cAMP may therefore be used as a

crude, integral marker of the β -adrenergic tone. Although this has been done in several leading publications, to our knowledge, data for healthy subjects and asthmatic patients have been never compared in the same study. Barnes et al⁹ gave data for five asthmatic men whose bronchodilator medication was stopped 48 h before the investigation. According to PEF measurements, the patients were suffering from a more severe bronchial asthma than our patients; their plasma cAMP concentrations were even lower (8 to 16 nmol/L) than plasma cAMP in our patients. Mikuni et al¹⁰ studied 13 healthy male subjects whose cAMP plasma concentrations were lower than in our healthy control group, but still higher than in the asthmatic patients of Barnes et al (13.2 to 19.3 nmol/L).

How could an affinity decrease of β_2 -adrenoceptors throughout 24 h contribute to "nocturnal asthma" in our patients? At night, a fall in plasma adrenaline⁷⁻¹⁰ and cortisol^{5,16,17} concentrations as well as in the number of β_2 -adrenoceptors (this study) diminish physiologically the effectiveness of endogenous bronchodilating mechanisms, whereas the activity of endogenous bronchoconstrictors, such as the parasympathetic system² and/or the histamine release,⁹ is increased. At that time, the affinity decrease of β_2 -adrenoceptors cannot be counterbalanced as efficiently as during the daytime, thus giving rise to asthmatic attacks.

In this study, the expression and function of β_2 -adrenoceptors was studied on white blood cells that may be easily and repeatedly collected without causing undue discomfort to the patient. Many investigations on the possible role of β -adrenoceptors in atopic bronchial asthma used pMNL as a kind of tissue model for bronchial tissue,^{12,13,18-22} since it is impossible to obtain human lung tissue for routine analyses, especially not for repeated investigations on the same individual as in this study. The changes observed on these cells are assumed to occur in the same way on β_2 -adrenoceptors in bronchial tissue. In general, this working hypothesis is not even mentioned explicitly. However, it has not yet been convincingly demonstrated that this approach is actually valid. There are many good arguments in favor of this assumption;²³ however, there are also reports opposed to it.²⁹ It is unlikely that the characteristics of β_2 -adrenoceptors vary under physiologic conditions among receptors expressed in different tissues of the body. Likewise drugs should have the same effects on these receptors as long as they are administered systemically. It is feasible, however, that pathologic mechanisms may affect just one population of β_2 -adrenoceptors.

With regard to this study, such considerations are less important. The reduced affinity of β_2 -adrenoceptors was studied in cells collected from blood. If such

a reduced affinity is biologically significant, it should result in a reduced formation of intracellular cAMP, which in turn, may leak to a lesser extent out of these cells into the surrounding plasma. In this study, both the reduced affinity as well as the reduced cAMP concentration in plasma could be demonstrated in the same individuals; in other words, a reduced sympathetic tone was demonstrated in two physiologic variables directly interrelated, notwithstanding the type of cells investigated. Even if our results simply indicate a reduced sympathetic influence on lymphocytes, they demonstrate that at least pMNL are exposed to a reduced sympathetic tone in asthmatic patients.

Moreover, such an interpretation might be even more challenging. Atopic bronchial asthma is an allergic and inflammatory disease involving immune reactions. The pMNL do take part in these reactions. The impact of the sympathetic system on immune functions exerted by pMNL has been under investigation. A clear-cut concept has not yet emerged, but there has been much speculation, mostly based on investigations of the role of intracellular cAMP levels.³⁰ Available evidence points in general to an inhibition of immune functions by an increasing intracellular cAMP content.³⁰ A reduction in affinity of β_2 -adrenoceptors expressed on pMNL would therefore reduce this inhibiting action leading to overshooting immune reactions: allergy and inflammation.

Decreasing cortisol plasma concentrations are generally discussed as contributing to "nocturnal asthma." However, no comparison has been made so far between healthy subjects and asthmatic patients in the same study. We were unable to trace lower cortisol plasma concentrations at night in our asthmatic patients than in our healthy controls. On the contrary, plasma cortisol concentrations were found to be higher; there was a small, but statistically significant difference in the 24-h mean and in the early morning values. We do interpret the higher cortisol secretion in our asthmatic patients as an endogenous defense reaction against the disease. It has been suggested that the administration of glucocorticoids may upregulate the expression of β -adrenoceptors.^{31,32} The elevated cortisol concentration observed in our asthmatic patients would then be necessary to maintain the β_2 -adrenoceptor density in the range observed in our healthy controls. If this assumption is not correct, then physiologic concentrations of glucocorticoids do not have any effect on the number of β -adrenoceptors. The "upregulation" observed after steroid administration³² could be a misinterpretation of the circadian variation in the expression of β_2 -adrenoceptors on pMNL: Brodde et al³² administered prednisone at 9 AM and determined the steroid effect on the β_2 -adrenoceptor density in the afternoon.

The results of this study support the hypothesis of a β -adrenergic lesion contributing to the pathophysiologic condition of atopic bronchial asthma. In our patients, this lesion could be demonstrated in the affinity rather than in the number of β_2 -adrenoceptors. If this β -adrenergic lesion does not lead to a general reduction in sympathetic tone but rather affects only pMNL, a disturbed modulation of immune responses by the sympathetic system should be considered in atopic bronchial asthma.

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