

Expression of epithelial markers in nocturnal asthma

Monica Kraft, MD,^a Ilja Striz, MD, PhD,^b George Georges, MD,^a Takeshi Umino, MD,^c Keichi Takigawa, MD,^c Stephen Rennard, MD,^c and Richard J. Martin, MD^a

Denver, Colo, Prague, Czech Republic, and Omaha, Neb

Background: Although the airway epithelium participates in inflammation and repair, the circadian expression of epithelial cell markers involved in these processes has not been investigated.

Objective: We sought to determine whether expression of CD51 (vitronectin and fibronectin receptor), CD54 (intercellular adhesion molecule-1), HLA-DR (activation marker), CD29 (β1 integrin), CD49b (collagen receptor), and CD11b (complement receptor) exhibit a circadian rhythm in asthma.

Methods: Eleven patients with nocturnal asthma (NA), 9 subjects with nonnocturnal asthma (NNA), and 10 control subjects underwent bronchoscopy at 4 PM and 4 AM in a random order 1 week apart, with brushing of the proximal and distal airways. The percentage of cells staining for a particular marker was determined.

Results: At 4 PM, HLA-DR in the proximal airways and CD54 in the distal airways was significantly greater in control subjects as compared with asthmatic subjects (HLA-DR, control subjects: 10.0% [range, 5.0% to 21.0%]; NNA: 8.0% [range, 4.0% to 14.5%] NA: 3.5% [range, 2.0% to 6.0%], $P = .01$; CD54, control subjects: 17.0% [range, 8.0% to 25.0%], NNA: 8.0% [range, 5.3% to 11.5%], NA: 7.0% [range, 4.0% to 15.0%], $P = .03$). At 4 AM, CD51 in the distal airways was significantly greater in patients with NA as compared with patients with NNA and control subjects (control subjects, 23.0% [range, 13.8% to 30.5%]; NNA, 32.0% [range, 13.0% to 35.0%]; NA, 40.0% [range, 23.0% to 50.0%], $P = .05$). Expression of CD51 in the distal airways correlated with the degree of airway obstruction ($r = -0.57$, $P = .001$). Control subjects exhibited significant circadian variation in the expression of HLA-DR in the proximal airways and CD54 in the distal airways.

Conclusion: The increased CD51 at night in patients with NA may be related to increased airway inflammation and repair processes in response to injury. The circadian changes in CD54 and HLA-DR in control subjects require further study to determine their significance. (*J Allergy Clin Immunol* 1998;102:376-81.)

Key words: Nocturnal asthma, airway epithelium, circadian rhythm

Abbreviations used

FVC: Forced vital capacity
ICAM-1: Intercellular adhesion molecule-1
NA: Nocturnal asthma
NNA: Nonnocturnal asthma
PEFR: Peak expiratory flow rate

It has become recognized that the airway epithelium not only acts as a protective barrier but also participates in the processes of inflammation and repair.^{1,2} In asthma the epithelium is thought to play a significant role in the recruitment of inflammatory cells in addition to being a target for inflammatory cell mediators and cytokines.³⁻⁵ The role of the epithelium as a target is demonstrated by the epithelial desquamation seen in autopsy studies and in bronchial biopsy specimens from patients with asthma.⁶⁻⁸ However, it is now appreciated that epithelial cells secrete several types of mediators, including eicosanoids, prostaglandins, and GM-CSF, all of which have been shown to play a role in the pathogenesis of asthma.⁹⁻¹¹ The epithelium is also involved in repair processes, with the ability to respond to and produce relevant mediators. It has also become recognized that the epithelium can release mediators that could be involved in a fibrotic process leading to remodeling of the airways.^{1,2} Because we have shown that the inflammatory process increases at night¹² and, in particular, the distal airways,¹³ we hypothesized that epithelial cell markers involved in the inflammatory process, the remodeling process, or both would be increased at night in the airways of subjects with nocturnal asthma (NA).

METHODS

Subjects

Twenty subjects with asthma, as defined by the American Thoracic Society,¹⁴ and 10 control subjects were studied. Of the asthmatic subjects, 11 had NA, defined as an overnight fall in peak expiratory flow rate (PEFR) of 15% or greater in 4 of 7 nights measured at home before study. The remaining 9 asthmatic subjects had nonnocturnal asthma (NNA), defined as 10% or less overnight fall in PEFR in 7 of 7 nights before study. Subjects who experienced falls in their overnight PEFR between 10% and 15% were not included in the study. Subject characteristics are shown in Table I. Exclusion criteria included the use of oral or inhaled steroids, theophylline, cromolyn, or nedocromil within 4 weeks of the study, an upper respiratory infection within 4 weeks of the study, cigarette smoking of 5 or more pack-years and/or any cigarette use within 2 years of study, pregnancy, and significant nonasthma medical problems as determined by the principal investigator. All subjects gave informed consent to this institutional review board–approved study.

From ^aNational Jewish Medical and Research Center and the University of Colorado Health Sciences Center, Denver; ^bthe Department of Immunology, Institute for Clinical and Experimental Medicine, Prague; and ^cthe Division of Pulmonary and Critical Care Medicine, University of Nebraska Medical Center, Omaha.

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Reprint requests: Monica Kraft, MD, National Jewish Medical and Research Center, 1400 Jackson St, J107, Denver, CO 80206.

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TABLE I. Subject characteristics

	NA	NNA	Control subjects	P value
Sex (M/F)	7/4	6/3	6/4	—
Age (yrs)	33.1 ± 2.3	32.7 ± 2.4	27.6 ± 5.7	.86
Medications	β ₂ agonists (11)	β ₂ agonists (9)	None	—
4 PM FEV ₁ (L)	3.01 ± 0.63	3.24 ± 0.71	4.21 ± 0.35	.44
4 PM FEV ₁ (% predicted)	71.3 ± 4.5	85.2 ± 2.9	100.8 ± 4.1	.001*
4 PM FEV ₁ /FVC	0.70 ± 0.02	0.74 ± 0.02	0.87 ± 0.02	.0001†
4 AM FEV ₁ (L)	2.76 ± 0.73	3.21 ± 0.77	4.29 ± 0.38	.17
4 AM FEV ₁ (% predicted)	60.5 ± 4.4	85.2 ± 2.3	101.3 ± 4.1	.0001
4 AM FEV ₁ /FVC	0.67 ± 0.02	0.75 ± 0.02	0.85 ± 0.02	.0001‡
Percent overnight fall§	-23.4 ± 4.3	-3.8 ± 1.9	1.9 ± 0.8	.0001*

*Patients with NA less than patients with NNA and control subjects.

†Patients with NA and NNA less than control subject subjects.

‡Patients with NA less than patients with NNA less than control subjects.

§Bedtime = 4 AM.

Protocol

Subjects underwent 2 bronchoscopies, with brushing of the proximal and distal airways, at 4 PM and 4 AM in a random order 1 week apart. These times were chosen to evaluate potential circadian rhythm changes in the expression of epithelial markers. The location of the brushing was randomized to the right or left lower lobe for the first bronchoscopy followed by brushing in the opposite lobe during the second bronchoscopy. Before the 4 AM bronchoscopies, subjects spent the night in the laboratory and underwent spirometry before bedtime. Before each bronchoscopy, spirometry was performed before and after administration of 0.18 mg of albuterol by means of a metered dose inhaler. Subjects then received 60 mg codeine and 0.6 mg atropine intramuscularly. Xylocaine (4%) was used to anesthetize the upper airway and xylocaine (1%) was applied in 2-mL increments to the laryngeal area, trachea, and orifice of the right lower or left lower lobe bronchi by means of the bronchoscope. Brushing of the distal airways was performed under fluoroscopic guidance with a disposable cytological brush (Mill Rose Laboratories, Mentor, Ohio). The brush was advanced to the pleura and then retracted approximately 2 to 3 cm. The proximal airway brushing was performed under direct visualization from the fourth and fifth generation airways. Seven brushing passes were performed at each site, with a new brush used for each pass. Supplemental oxygen was administered throughout the procedure, and heart rate, blood pressure, and oxygen saturation were monitored. An expiratory chest radiograph was performed after each procedure to rule out the presence of a pneumothorax. Vital signs and pulse oximetry were monitored in each subject for at least 4 hours after the procedure.

Epithelial cell staining

Immunohistochemical analysis for the determination of expression of the epithelial markers listed below were carried out by immunoperoxidase assay as previously described,¹⁵ with minor modifications. Briefly, a cell suspension of 2×10^5 cells/mL were transferred into reaction areas of adhesive slides (BioRad, Hercules, Calif). After attachment, cells were fixed with 0.05% glutaraldehyde. They were then washed with NKH buffer (8 g NaCl, 0.4 g KCl, 1 mL Hepes [pH 7.4] in 1000 mL distilled water) and preincubated with gelatin blocking medium to prevent immunoglobulin binding to the glass surface. Expression of the following markers were studied: CD51 (α_v -integrin; Amac, Westbrook, Me), CD54 (intercellular adhesion molecule [ICAM]-1; Amac), HLA-DR (acti-

vation marker; Ortho Diagnostics, Raritan, NJ), CD29 (β_1 -integrin; Amac), CD49b (collagen receptor; Amac), and CD11b (complement receptor; Amac). These markers were selected because they represent markers potentially active in airway inflammation, airway remodeling, or both. CD11b was evaluated to assess the presence of inflammatory cells in the specimens because it is expressed by monocytes and neutrophils and not by epithelial cells.

The staining procedure included the following steps: (1) 5-minute incubation with each antibody diluted 1:50 in gelatin blocking medium; (2) 5-minute incubation with rabbit anti-mouse immunoglobulin (Dako, Carpinteria, Calif); (3) 5-minute incubation with swine anti-rabbit immunoglobulins (Dako); (4) 5-minute incubation with rabbit peroxidase-anti-peroxidase complex (Dako; all antibodies were diluted at 1:30 in NKH buffer); (5) and 10-minute incubation with diaminobenzidine (Sigma, St. Louis, Mo). Step 1 was omitted with each antibody as the negative control. All incubations were performed at room temperature.

The percentage of cells expressing a particular marker (denoted by the presence of dark brown granular staining of the cell membrane) was determined by counting 300 cells/slide with a phase contrast microscope (Olympus BHS) by 2 independent individuals blinded to the subjects' asthma status. Results were expressed as the percentage of cells stained for a particular marker.

To determine whether the cells were indeed epithelial cells, we evaluated brushing samples from 4 additional asthmatic subjects. Paraformaldehyde-fixed specimens were pretreated with microwave and trypsin. The cytokeratin antibody #C-2931 (Sigma, St. Louis, Mo) was used as the primary antibody at a 1:200 dilution. The secondary antibodies were biotinylated horse anti-mouse IgG and streptavidin horseradish peroxidase used as instructed from the Vectastain ABC kit (Burlingame, Calif). Slides were examined, and at least 300 cells were counted.

Statistical analysis

The physiologic outcome variables included 4 PM FEV₁, 4 AM FEV₁, the ratio of FEV₁ and forced vital capacity (FEV₁/FVC), and overnight percentage fall in FEV₁. The epithelial cell variables included the percentage of cells staining for CD51, CD54, HLA-DR, CD29, CD49b, and CD11b, respectively. The data were analyzed by means of a mixed effects model to determine whether significant differences were present among groups, time, and location.¹⁶ The mixed effects model takes into account repeated measures between subjects. Pairwise comparisons within the mixed effects model were performed by using Tukey's

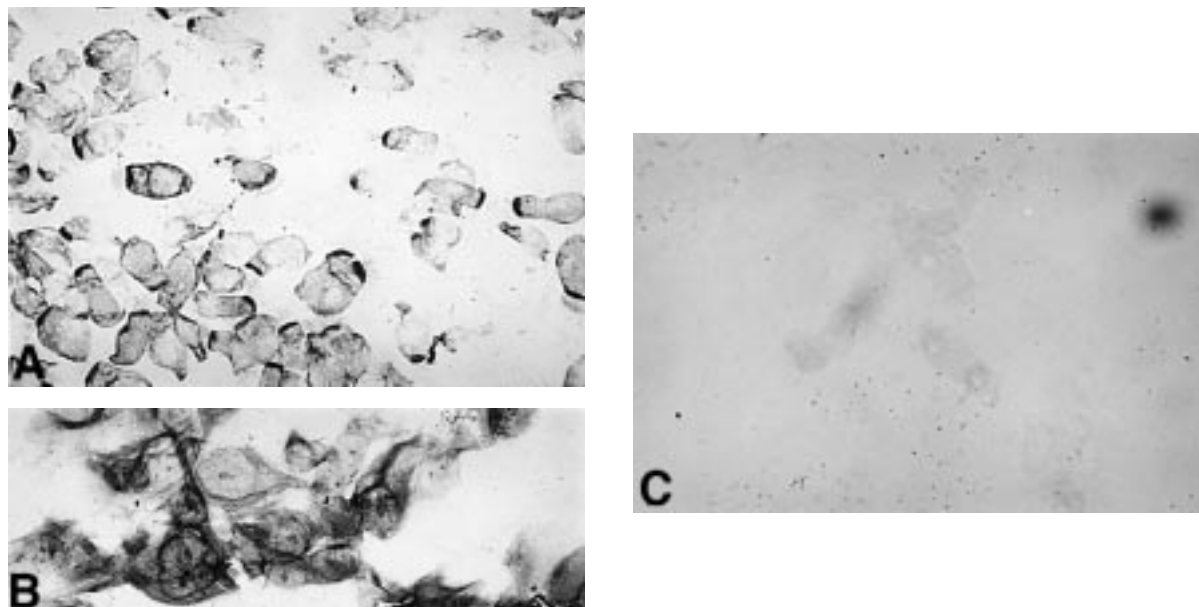


FIG 1. Representative specimen from the proximal airway stained with a pancytokeratin antibody (**A**) along with positive control (human epithelial cell line) (**B**) and negative control (**C**).

multiple comparison procedure.¹⁷ Values are expressed either as medians, with the 25 to 75 interquartile ranges, or means $\pm$ SEM depending on the distribution of the data. A level of significance was defined as a *P* value of 0.05 or less. Reproducibility of counts was assessed by double counting a random sample of biopsy specimens by a second individual also blinded to asthma status and time of day. Inter-rater reliability was tested by means of intraclass correlation.¹⁸ Finally, the sample size reflects a power of 80% to detect a difference of 6%, with a type-I error rate of 5%.¹⁹

RESULTS

Subjects and pulmonary function tests

Subject characteristics, including pulmonary function, are shown in Table I. The mean age and sex distribution were approximately equal between the groups.

Cytokeratin staining and quality control

Analysis of slides stained with a pancytokeratin antibody revealed that greater than 95% of cells were positive (Fig 1). Reproducibility of the epithelial marker counts for quality control analysis revealed a median difference of 6.0% (interquartile range, -2.0% to 14.0%). The intraclass correlation between the counts was 0.80.

Between-group comparisons: Proximal airway markers (4 PM and 4 AM). At 4 PM, HLA-DR was greater in the control subjects as compared with the patients with NA only (control subjects, 10.0% [range, 5.0% to 21.0%]; NNA, 8.0% [range, 4.0% to 14.5%]; NA, 3.5% [range, 2.0% to 6.0%], *P* = .01). There were no significant differences between the groups in the

percentage of proximal airway cells expressing CD51, CD54, CD29, CD49b, and CD11b. At 4 AM, there were no differences between the groups in the percentage of cells expressing any of the markers. The data summarized in Table II and Fig 2 shows examples of a CD29-positive cell and a negative control from the proximal airways.

Between-group comparisons: Distal airway markers (4 PM and 4 AM). At 4 PM, the percentage of cells from the distal airway expressing CD54 was greater in the control subjects as compared with the asthmatic groups (CD54: control subjects, 17.0% [range, 8.0% to 25.0%]; NNA, 8.0% [range, 5.3% to 11.5%], NA, 7.0% [range, 4% to 15%], *P* = .03). There were no differences in expression of CD51, HLA-DR, CD29, CD49b, and CD11b. At 4 AM, the percentage of distal airway cells expressing CD51 was significantly increased in the patients with NA as compared with those with NNA and the control subjects (control subjects, 23.0% [range, 13.8% to 30.5%]; NNA, 32.0% [range, 13.0% to 35.0%]; NA, 40.0% [range, 23.0% to 50.0%], *P* = .05) (Fig 3). There were no differences in expression of CD54, HLA-DR, CD29, CD49b, and CD11b. The data are summarized in Table II.

Within-group comparisons. Within the asthmatic groups, there were no significant differences in the expression of the markers at either time or location. Within the control group, however, there were several differences. Comparing proximal airway markers at 4 PM and 4 AM, the expression of HLA-DR was increased during the day as compared with the night (HLA-DR: 4 PM, 10.0% [range, 5.0% to 21.0%]; 4 AM, 4.5% [range, 4.0% to 6.0%], *P* = .03). Comparing distal

markers in control subjects at 4 PM and 4 AM, CD54 was increased during the day (4 PM, 17.0% [range, 8.0% to 25.0%]; 4 AM, 11.0% [range, 5.8% to 18.8%], $P = .05$).

Comparing proximal and distal markers at 4 AM only within the control group, HLA-DR was significantly increased in the distal airway (proximal, 4.5% [range, 4.0% to 6.0%]; distal, 6.5% [range, 4.0% to 10.0%], $P = .05$). There were no differences in the expression of proximal and distal markers at 4 PM.

Physiologic correlations

There was a significant negative correlation between the degree of obstruction as determined by the FEV₁/FVC ratio and the percentage of cells in the distal airway with the marker CD51 at 4 AM ($r = -0.57$, $P = .001$) (Fig. 4). This relationship was not seen in the proximal airways because the FEV₁/FVC ratio at 4 AM did not significantly correlate with expression of CD51 at 4 AM in the proximal airway CD51 ($r = 0.04$, $P = .85$) (Fig 4).

DISCUSSION

There are 2 very interesting findings in this study. First is the increase in expression of CD51 (α_v -integrin) in the distal airways of the patients with NA at 4 AM, which correlated with the degree of airway obstruction. This observation may indicate that a "repair" process is occurring, ultimately leading to remodeling of the airway. The second observation is the surprising finding that control subjects exhibit marked circadian changes in the expression of ICAM-1 and HLA-DR in the proximal and distal airways.

CD51 corresponds to the α_v -subunit of the integrin receptor. All integrin receptors are composed of one α - and one β -chain and are organized into families according to the specific β -chain present in the heterodimer. In the lungs, CD51 or the α_v -chain may be associated with β_1 as the fibronectin receptor or β_5 as the vitronectin receptor.²⁰

Vitronectin and fibronectin are multifactorial adhesive glycoproteins found in the circulation and in tissue. They are biochemically and immunologically distinct.²¹ Immunohistochemical analysis has revealed that deposited vitronectin and fibronectin may serve as molecular links between elastic fibers and the surrounding proteoglycan/collagen scaffold.²² In addition, fibronectin is also believed to play a role in cellular adhesion, particularly in stabilizing interactions between cell surface components and constituents of the extracellular matrix.²³ These functions have been studied in skin, where fibronectin deposition in wounds precedes collagen deposition.^{24,25} A similar process may occur in the airways, where epithelium is damaged by eosinophil cationic proteins, and increased extracellular matrix formation occurs as a repair response. Macrophages also present during the inflammatory process may secrete transforming growth factor- β , thought to be the most important stimulus for fibroblast production of extracellular matrix. Thus CD51 may be upregulated at night in our patients with NA because of enhanced inflammation in the distal airways,¹³ both as a

TABLE II. Epithelial cell markers*

	4 PM (%)	4 AM (%)
NA		
Proximal airways		
CD51	28.5 (20.8-31.0)	21.0 (17.3-36.5)
CD54	9.0 (7.0-10.0)	8.0 (4.75-10.0)
HLA-DR	3.5 (2.0-6.0)†	6.0 (3.5-10.0)
CD29	90.0 (82.0-92.3)	80.0 (74.0-85.8)
CD49	17.0 (9.0-27.8)	19.0 (10.0-25.8)
CD11b	2.0 (1.8-5.3)	2.5 (1.8-4.5)
Distal airways		
CD51	32.0 (24.0-38.0)	40.0 (23.0-50.0)‡
CD54	7.0 (4.0-15.0)§	6.0 (4.8-10.0)
HLA-DR	4.0 (4.0-10.0)	6.0 (3.5-8.5)
CD29	79.0 (70.0-80.0)	76.0 (69.5-90.5)
CD49b	28.0 (12.0-40.0)	14.5 (9.8-32.5)
CD11b	2.0 (2.0-4.0)	4.5 (1.8-5.3)
NNA		
Proximal airways		
CD51	22.0 (15.0-47.0)	35.0 (11.0-44.5)
CD54	10.0 (6.5-12.5)	10.0 (5.5-15.0)
HLA-DR	8.0 (4.0-14.5)	8.0 (4.0-12.0)
CD29	87.0 (70.5-91.0)	88.0 (75.5-95.5)
CD49b	23.0 (11.0-31.5)	12.0 (7.0-25.5)
CD11b	5.0 (1.5-8.0)	4.0 (2.0-7.0)
Distal airways		
CD51	23.0 (18.0-37.5)	32.0 (13.0-35.0)
CD54	8.0 (5.3-11.5)§	10.0 (5.0-20.0)
HLA-DR	7.0 (5.0-9.5)	8.0 (5.5-12.0)
CD29	70.5 (62.5-85)	90.0 (80.0-95.0)
CD49b	23.0 (11-31.5)	16.0 (12.0-18.5)
CD11b	2.0 (2.0-2.0)	5.0 (2.0-6.5)
Control subjects		
Proximal airways		
CD51	22.0 (20.0-44.0)	27.5 (13.8-42.0)
CD54	15.0 (12.0-19.0)	6.5 (4.0-12.5)
HLA-DR	10.0 (5.0-21.0)† #	4.5 (4.0-6.0)¶
CD29	88.0 (80.0-89.5)	85.0 (73.8-88.5)
CD49b	15.0 (9.0-32.5)	15.0 (11.5-16.5)
CD11b	2.0 (2.0-11.5)	2.0 (2.0-4.0)
Distal airways		
CD51	19.0 (11.0-40.8)	23.0 (13.8-30.5)‡
CD54	17.0 (8.0-25.0)§¶	11.0 (5.8-18.8)¶¶
HLA-DR	5.0 (3.5-12.5)#	6.5 (4.0-10.0)
CD29	85.5 (79.5-94.3)	75.0 (58.0-91.0)
CD49b	27.0 (8.8-37.0)	15.0 (9.5-37.3)
CD11b	4.5 (4.0-6.5)	4.5 (2.0-6.0)

*Values are medians with interquartile ranges.

† $P = .01$ (control subjects > patients with NA).

‡ $P = .05$ (patients with NA > control subjects).

§ $P = .03$ (control subjects > both asthmatic groups).

¶ $P = .03$ (4 PM > 4 AM within control group).

¶¶ $P = .05$ (4 PM > 4 AM within control group).

$P = .05$ (distal airway > proximal within control group).

marker of cell adhesion and as a participant in airway remodeling. If severity of asthma were the main determinant of CD51 expression, we would have expected to see increased CD51 expression during the day and night.

Our results conflict with those of Vignola et al.²⁶ who showed that ICAM-1 and HLA-DR were increased in the epithelium of asthmatic subjects as

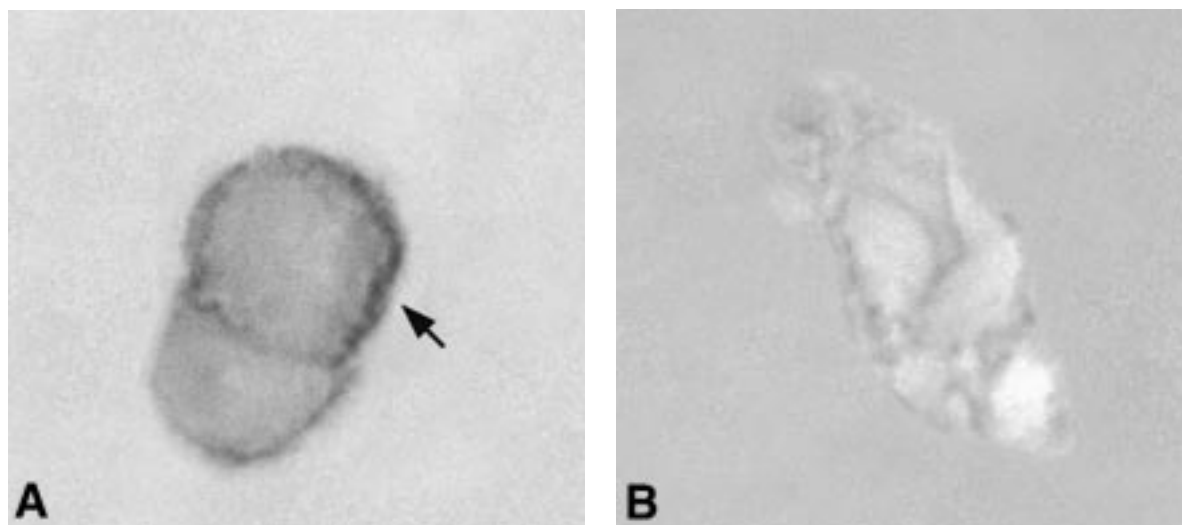


FIG 2. Representative photomicrographs of specimens stained with the markers CD29 (A) and negative control (B).

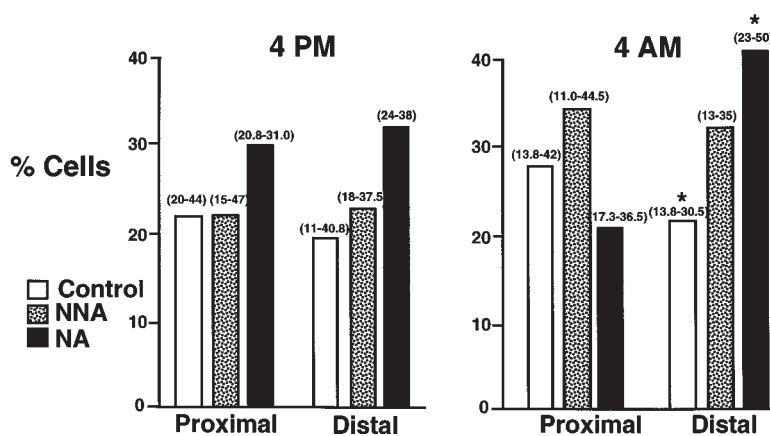


FIG 3. Percentages of cells expressing CD51 from proximal and distal airways at 4 AM and 4 PM in control, NNA, and NA groups. Open bars represent control group, spotted bars represent NNA group, and filled bars represent NA group. Values are expressed as medians with 25 to 75 interquartile range in parentheses above each bar. *P = .05.

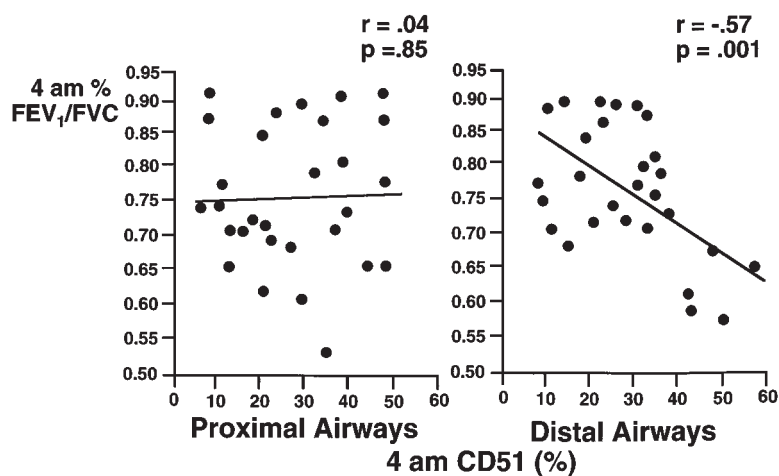


FIG 4. Spearman correlation between FEV₁/FVC ratio and percentage of cells expressing CD51 in distal airways and proximal airways is shown.

compared with control subjects. However, Montefort et al.²⁷ found no difference in ICAM-1 expression in bronchial mucosa between asthmatic and normal subjects. Several possibilities may account for these differences, including the time of day in which the subjects underwent bronchoscopy, processing differences, or loss of cell surface markers by the asthmatic subjects after maximum expression.

In summary, the expression of CD51, the α v-integrin, is increased at night in the airway epithelium of patients with nocturnal asthma. This increased expression may be related to increased inflammation and subsequent repair processes in response to injury. The circadian changes in ICAM-1 and HLA-DR in the control subjects needs further documentation and investigation as to the significance of these changes in this population.

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