

ORIGINAL ARTICLE

β 1-integrins shedding in a guinea-pig model of chronic asthma with remodelled airways

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Summary

Background A hallmark of airway remodelling in asthma is subepithelial fibrosis, but its relation with airway dysfunction is still controversial.

Objective To describe airway functional abnormalities and subepithelial remodelling induced by repetitive antigenic challenges.

Methods Nine inhaled antigenic challenges were applied every 10 days to guinea-pigs sensitized to ovalbumin (OVA). Antigen-induced airway hyperresponsiveness (AI-AHR) to histamine and its immunohistopathological relationship was evaluated at the first, third and ninth OVA challenges.

Results From the first challenge on, OVA induced acute transient bronchoobstruction followed by development of AI-AHR. A progressive rise in baseline Penh (a bronchoobstruction index) and granulocyte airway infiltration was also observed. After the ninth OVA challenge, the amount of extracellular matrix in the subepithelial region (SER) of bronchi and bronchioles was increased. Immunohistochemistry analysis showed that this SER fibrosis was associated to β 1-integrin subunit overexpression, even in acellular areas. Immunoelectronmicroscopy corroborated the location of β 1-integrin in extracellular matrix, essentially in types I and II collagen fibres. Presence of α 1- and α 2-integrin subunits in these areas was also corroborated. AI-AHR was correlated with degree of SER increment, cell infiltration, and β 1-integrin expression.

Conclusion Our data suggested that β 1-integrin shedding produced by repetitive allergen challenges in guinea-pigs was associated with collagen deposition in SER of bronchi and bronchioles, along with inflammatory cells infiltration and AI-AHR development.

Keywords airways, asthma, collagen, fibrosis, guinea-pig, hyperresponsiveness, integrins, remodelling, shedding

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Introduction

Asthma is classically considered as a disease with episodic exacerbations of airflow obstruction and respiratory symptoms [1]. Although airflow obstruction is usually spontaneously resolved or controlled with treatment, some individuals with long-lasting asthma exhibit a residual and permanent airflow limitation [2]. Permanent airway obstruction has been attributed to thickening of the airway wall due to multiple structural changes, collectively denominated airway remodelling, which are

generated by abnormal repair processes induced by inflammatory and non-inflammatory mechanisms [3, 4].

A hallmark of asthma remodelling is increased subepithelial deposition of extracellular matrix components [4, 5]. Interaction between extracellular matrix components and cells occurs mainly through integrins, a family of 24 transmembrane glycoproteins that recognize extracellular matrix components, whereas its intracellular domain interacts with the cytoskeleton. Structurally, integrins are non-covalently bound dimers composed by α - and β -integrin subunits. The β 1-integrin subunit is unique

among the eight known β -subunits due to its capability to associate with at least 12 ($\alpha 1$ – $\alpha 11$, and αV) of 18 α -integrin subunits [6].

Functionally, $\beta 1$ -integrins play a crucial role in adhesion, migration, proliferation, secretion, and differentiation processes in many cells. In airways, $\beta 1$ -integrins are expressed in smooth muscle, fibroblasts, eosinophils, and mononuclear leucocytes. $\beta 1$ -Subunit activation in airway smooth muscle by monomeric type I collagen and fibronectin regulates the differentiation, secretion, and proliferation capacities of myocytes [7]. Moreover, integrin-linked kinase, which interacts with the $\beta 1$ -integrin cytoplasmic domain, can activate and regulate smooth muscle contraction directly by myosin phosphorylation, and indirectly by myosin light-chain phosphatase inhibition [8]. Pre-treatment with antibodies against $\alpha 1\beta 1$ - and $\alpha 4\beta 1$ -integrins was effective in blocking airway hyperresponsiveness in a sheep model of allergic asthma [9]. Hence, involvement of $\beta 1$ -integrins during remodelling and its contribution to airway dysfunction in asthma can be inferred.

The relationship between airway remodelling and airway dysfunction remains controversial [5, 10] and animal models might contribute to a basic understanding of the problem. Guinea-pigs are susceptible to generate pulmonary hypersensitivity to several antigens or sensitizers; thus, they have been used widely as an animal model of allergic asthma [11]. Here, we describe a model of chronic asthma in sensitized guinea-pigs induced by repetitive allergen provocation, in which the relationship between airways remodelling and inflammatory response or functional lung abnormalities was studied.

Methods

We used healthy male guinea-pigs (350–400 g) purchased originally from Harlan México (strain HsdPoc:DH) and reproduced at our institutional laboratory animal facilities. Animals were bred in filtered air-conditioned, 12/12-h light/dark cycles, at $21 \pm 1^\circ\text{C}$ and 50–70% humidity, and fed with sterilized pellets (2040 Harlan Teklad Guinea-Pig Diet) and water *ad libitum*. The protocol was revised and approved by the Scientific and Bioethics Committees of the Instituto Nacional de Enfermedades Respiratorias in Mexico City.

Study design

As described in Fig. 1, after an initial sensitization protocol guinea-pigs were intermittently exposed to the aerosolized allergen (ovalbumin, OVA) for up to 95 days, receiving a total of nine allergenic challenges. Four separate groups of animals were studied at days 8, 15, 35, and 95. At these time-points, development of antigen-induced airway hyperresponsiveness (AI-AHR) was

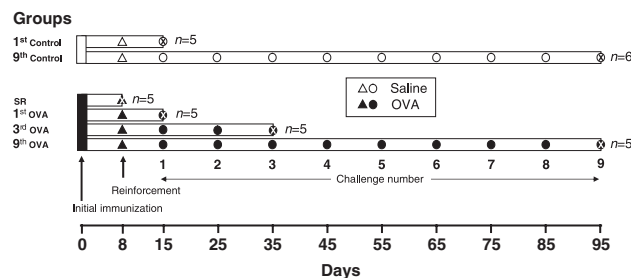


Fig. 1. Experimental design. After initial immunization and reinforcement, guinea-pigs received up to nine antigen challenges. Animals were killed at days 8, 15, 35, and 95 after antigen-induced airway hyperresponsiveness (X) was evaluated by constructing two dose-response curves to aerosolised histamine (before and after an antigenic challenge). OVA, ovalbumin; SR, sensitization reinforcement group.

evaluated in anaesthetised animals by performing dose-response curves to histamine before and after an antigenic challenge. Afterward, bronchoalveolar lavage was carried out to analyse the cell count, and then animals were killed to obtain lung samples. In these latter samples, changes in the amount of collagen in subepithelial region (SER) and the extent of airway smooth muscle layer (ASML) were analysed through light microscopy, and $\beta 1$ -integrin deposition was explored through immunohistochemistry (and also by electron microscopy in the 95-days group). For the purpose of this report, SER was defined as the area comprising between basal edge of epithelial cells and the beginning of ASML, a region that mainly contains basement membrane and lamina propria. Control animals receiving sham manoeuvres with saline solution were studied at days 15 and 95.

Sensitization procedure

On the first day, each guinea-pig received a single intraperitoneal injection of OVA (0.06 mg/mL) with aluminium hydroxide (1 mg/mL) dispersed in saline solution. Sensitization was reinforced 8 days later with OVA aerosols (3 mg/mL saline) delivered during 5 min. Aerosols were produced by an US-1 Bennett nebulizer (flow, 2 mL/min), releasing mixed particles, 44% $< 4 \mu\text{m}$ in size, 38% 4–10 μm , and 18% $> 10 \mu\text{m}$ (multistage liquid impinger, Burkard Manufacturing Co., Rickmansworth, Hertfordshire, UK). From day 15 onward, guinea-pigs were challenged every 10 days with OVA aerosols (1 mg/mL in the first challenge and 0.5 mg/mL in the eight subsequent challenges) during 1 min. Two additional groups of guinea-pigs subjected to one or nine inhalatory challenges with vehicle (saline solution) were used, representing young and mature age-matched controls, respectively. All nine inhalatory challenges were carried out while the guinea-pig was inside a barometric plethysmograph, allowing us to record acute bronchoobstructive response to the antigenic challenge immediately after OVA delivery

was finished, as described in the following section. This guinea-pig model of allergic asthma does not develop a noticeable late airway response. We corroborated that this sensitization procedure induces production of OVA-specific IgG1.

Barometric plethysmography

Whole-body single-chamber plethysmography for freely moving animals (Buxco Electronics Inc., Troy, NY, USA) was used to evaluate the pulmonary function indirectly according to principles described previously [12, 13]. Briefly, pressure inside the plethysmograph was measured through a differential transducer connected to a preamplifier. Because air in lungs is heated and humidified, during the inspiratory phase the volume of air inside the thorax is larger than the volume of air drawn by the animal from the plethysmographic chamber. This larger volume of air inside the thorax produces an increase in the plethysmographic chamber pressure, and the reverse occurs during the expiratory phase. The signal from the chamber was processed with computer-installed software (Buxco Biosystem XA v1.1) to calculate several respiratory parameters, including Penh (enhanced pause). This index was obtained by the following formula [13]:

$$\text{Penh} = ((T_e - R_t)/R_t)(\text{PEP}/\text{PIP})$$

where T_e = expiratory time (s), R_t = relaxation time (s), PEP = peak expiratory pressure (cmH₂O), and PIP = peak inspiratory pressure (cmH₂O). The software was adjusted to only include breaths with a tidal volume of 1 mL or more, with minimal inspiratory time of 0.15 s, maximal inspiratory time of 3 s, and maximal difference between inspiratory and expiratory volumes of 10%. After the guinea-pig was placed inside the plethysmographic chamber, a 5-min baseline Penh recording was initiated 5 min later. One minute after OVA or vehicle administration, Penh was recorded at 5 and 10 min, and every 15 min thereafter. Because Penh was calculated for each breath, adjustments were made to the software to average values from all breaths occurring during 15 s, and then to average these values during the last 5 min of each period.

Antigen-induced airway responsiveness

Airway responsiveness was evaluated in controls and in different experimental groups at day of sensitization reinforcement and at first, third, and ninth OVA challenges by comparing histamine dose-response curves before and after OVA administration. Briefly, increased non-cumulative doses of histamine (0.013–0.32 mg/mL) aerosols were administered after an initial Penh baseline acquisition. Each histamine dose was delivered during 1 min, and the average Penh value during the following 5 min was obtained. The interval between doses was 10 min. The

dose-response curve was stopped when Penh reached three times its baseline level. Once Penh had returned close to the initial baseline value (< 50% increment) [14], OVA challenge was administered. The second curve was performed 3 h after OVA challenge.

Cell counts in bronchoalveolar lavage

One hour after concluding the second histamine curve, and when at least 50% of baseline Penh was reached [14], animals were overdosed with an intraperitoneal injection of pentobarbital sodium (65 mg/kg) and the trachea was cannulated. Using a syringe, 5 mL of saline solution (37 °C) was introduced through the tracheal tube and gently recovered 1 min later. This procedure was repeated and the recovered fluid was mixed with the first bronchoalveolar lavage (BAL) fluid. Total recovered fluid was immediately centrifuged for 10 min at 500 g (4 °C), the cell pellet was resuspended in 1 mL saline solution, and total cells were counted employing the Kimura stain in a Neubauer haemocytometer. Volume was adjusted to obtain 1×10^6 cells/mL and 50 μ L of this mixture was deposited on a slide stainer (7120 Aerospray, Wescor Inc., Logan, UT, USA) to be stained with Romanowsky stain for differential cell counting. Cell counts were expressed as number of cells per millilitre of BAL fluid. Cell viability > 80% was confirmed by Trypan blue exclusion technique in each BAL. All smears were coded, and cells were counted in blind fashion.

Conventional histology and automated morphometry analysis

Left caudal lung lobe was dissected and fixed by manually perfusing 10% neutral-buffered formaldehyde solution via intra-arterial route until lung lobe was exsanguinated. Lung fragments obtained by sagittal cutting were embedded in paraffin, and 4 μ m thick lung sections were stained with haematoxylin-eosin (H/E), picrosirius-red, and Masson trichrome stain.

Surface areas (μm^2) of SER and ASML were determined through the use of automated morphometry (Qwin, Leica Microsystems Imaging Solutions, Cambridge, UK). To determine hyperplasia, total nuclear counts of bronchiolar smooth muscle bands were carried out in H/E sections at $\times 200$ magnification. Finally, number of neutrophils, eosinophils, and mast cells in SER and ASML was also evaluated. All measurements were conducted in six bronchi and six bronchioles chosen at random from each animal, data were adjusted by length of the corresponding basement membrane (except for nuclei count), and their average was considered the final result. Bronchus and bronchiole were identified by presence or absence of cartilage in airway wall, respectively.

Immunohistochemistry

The same paraffin-embedded lung tissue blocks utilized for the morphometry study were used for immunohistochemistry. Sections (3 µm) were deparaffinized (55 °C, 30 min) and rehydrated through submersion in graded alcohols (xylene, 1 : 1 xylene-alcohol, alcohol, and 70% alcohol for 10 min each, and finally rinsed in distilled water). Antigen retrieval was performed with 10 mM citrate buffer, pH 6, for 5 min in a microwave oven. Samples were treated with hydrogen peroxide (3%) to quench endogenous peroxidase, and non-specific sites were later blocked with horse serum (2%). Sections were incubated at 4 °C overnight with an appropriate dilution of the following antibodies: antibody to β 1-integrin (1 : 100, clone M-106 rabbit polyclonal IgG raised against aminoacids 375-480 mapping within an extracellular domain of β 1-integrin of human origin; Santa Cruz Biotechnology, Santa Cruz, CA, USA). A different (monoclonal) antibody to β 1-integrin was also used (1 : 75, clone P4G11 mouse monoclonal IgG1 raised against calcium-dependent epitopes on the human β 1 integrin subunit; Chemicon Int., Temecula, CA, USA). Antibody to α 1-integrin (1 : 75, clone FB12 mouse monoclonal IgG₁ raised against I domain of human α 1-integrin; Chemicon Int.). Antibody to α 2-integrin (1 : 75, clone A-18 goat polyclonal IgG antibody raised against a peptide mapping within an extracellular domain of human α 2-integrin, Santa Cruz Biotechnology). Antibodies to α -actin (1 : 150, anti-human mouse monoclonal actin smooth muscle IgG2a; BioGenex, San Ramon, CA, USA), neurofilament protein (1 : 75, anti-human monoclonal mouse IgG; Dako, Cambridgeshire, UK), or vimentin (1 : 75, clone V9 mouse monoclonal IgG1 κ ; Dako, Glostrup, Denmark). To detect the specific binding of these primary antibodies, an R.T.U. Vectastain Universal Quick Kit was used (Vector Laboratories Inc., Burlingame, CA, USA) in which tissues were incubated sequentially with blocking serum, a pan-specific secondary antibody, and streptavidin/peroxidase complex. Finally, 3-amino-9-ethyl-carbazole (BioGenex) or diaminobenzidine (Vector) was used as chromogen. Sections were counterstained with Mayers haematoxylin. Slides were rinsed twice with 0.1% phosphate-buffered saline and Tween 20 during the whole process. As control for non-specific binding of the secondary antibody, sections from the same lung were processed without the primary antibody. No positive staining was observed in non-specific binding controls. Rabbit IgG (Southernbiotech, Birmingham, AL, USA), goat IgG (Southernbiotech), mouse IgG1 (Clone DD7, Chemicon) and mouse IgG2a (Clone GC270, Chemicon) isotype controls were negative. Immunostaining percentage of the β 1-integrin (M-106) area was determined by an automated image analyser (Qwin, Leica, Milton Keynes, UK) after counting positive and negative areas in five random SER and ASML fields at $\times 200$ magnification.

Electron microscopy

We performed immunoelectronmicroscopy to determine β 1-integrin subcellular localization in lung. Briefly, five animals at ninth OVA challenge were killed, and their lungs were perfused with 4% (v/v) paraformaldehyde dissolved in 0.2 M Sørensen buffer for 4 h at 4 °C. Then, small tissue fragments were dehydrated in graded ethyl alcohols and embedded in LR-White hydrosoluble resin (London Resin Co., Hampshire, UK). Sections (1 µm width) were taken from five different tissue blocks per animal and stained with toluidine blue and checked for bronchioles with widening basement membrane, submucosal, and muscle layers using conventional light microscopy. Selected tissue blocks were trimmed to reduce the selected area from which thin sections, 70–90 nm wide were obtained and placed on nickel grids, incubated overnight at 4 °C with the same specific antibodies to β 1-integrin (M-106 and P4G11), α 1-, and α 2-integrins described in the previous section, and diluted 1/5 in PBS. After extensive washing with PBS, the grids were incubated for 1 h at room temperature with their respective secondary antibodies conjugated to 5 nm gold particles (Sigma) diluted 1/20 in PBS. The grids were contrasted with uranium salts (Electron Microscopy Sciences, Fort Washington, PA, USA) and examined with an M-10 Zeiss electron microscope (Karl Zeiss, Jena, Germany). As negative control, normal rabbit serum was substituted for primary antibody.

Drugs and reagents

OVA (chicken egg albumin) grade II, histamine dihydrochloride, and all stains for microscopy were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Aluminium hydroxide was purchased from J.T. Baker (Phillipsburg, NJ, USA). Pentobarbital sodium was acquired from Pfizer (Toluca, Mexico).

Statistical analysis

Airway responsiveness to histamine was evaluated by means of the provocative dose 200% (PD₂₀₀), i.e. the interpolated histamine dose that caused a threefold increase of basal Penh. Changes in histamine responsiveness induced by antigenic challenge were evaluated by means of paired Student's *t*-test, and also by the PD₂₀₀ ratio, i.e. PD₂₀₀ value observed after OVA challenge divided by PD₂₀₀ value before challenge. Airway structural comparisons between the initial and after several OVA-challenged groups were evaluated by unpaired Student's *t*-test. In multiple comparisons, one-way or repeated measures ANOVA followed by Dunnett's tests were used. Associations between β 1-integrin and lung function, lung structure, or cell counts were assessed through Spearman's correlation coefficient. Statistical significance was set at two-tailed

$P < 0.05$. Data in the text and figures are expressed as mean $\pm$ SE.

Results

After corroborating that the two control groups (young and mature guinea-pigs) were similar with respect to functional and structural analyses, they were evaluated jointly as a single control group.

Chronic allergen administration increased basal Penh

OVA nebulization on the sensitization reinforcement day did not cause noticeable acute Penh modifications. From the first challenge on, OVA induced a transient Penh increment (Fig. 2a), while maximal Penh response to these challenges was not different throughout the study (ANOVA $F = 0.429$, $P = 0.89$, $n = 5$, Fig. 2b). OVA challenges in saline controls did not induce bronchoobstruction ($F = 1.909$, $P = 0.09$, $n = 6$, Fig. 2b).

Baseline Penh values were similar in control animals throughout the study ($F = 2.022$, $P = 0.07$, Fig. 2c). In contrast, OVA-challenged guinea-pigs showed a progressive baseline Penh increase. Thus, after the repeated

measures ANOVA corroborated differences among groups ($F = 3.689$, $P = 0.004$), a *post hoc* analysis demonstrated statistically significant higher Penh values at the sixth ($P < 0.01$) and ninth ($P < 0.01$) OVA challenges in comparison with the first OVA challenge (Fig. 2c). This progressive rise in basal Penh was also corroborated by the straightline regression ascendant slope, which was statistically significant ($P < 0.0004$, data not illustrated).

Antigen-induced airway responsiveness

Basal PD₂₀₀ to aerosolised histamine was not different from PD₂₀₀ obtained after OVA challenge in controls (data not shown) and in the sensitization reinforcement group (Fig. 3). By contrast, enhanced responsiveness to aerosolised histamine during the second dose-response curve, as compared with the basal curve, was observed in the first, third, and ninth OVA challenges. This AI-AHR was evident because guinea-pigs exhibited a significant decrease of histamine PD₂₀₀ after OVA challenge in comparison with basal value ($n = 6$, 5, and 5, respectively, $P < 0.05$; Fig. 3). Delta values for PD₂₀₀ to histamine at the first, third, and ninth OVA challenges were not different (0.0082 ± 0.002 , 0.0183 ± 0.006 , and 0.0142 ± 0.005 mg/mL histamine, respectively, $F = 1.158$, $P = 0.35$).

Cell counts in tissue and BAL

Neutrophils, eosinophils, and mast cells were counted in SER and ASML in bronchi and bronchioles (Fig. 4). In general terms, in bronchial tissues these cells notably increased in SER and ASML only at the last (ninth) OVA challenge ($P < 0.01$ and $P < 0.05$), and the same occurred for mast cells in bronchioles. In contrast, the number of neutrophils and eosinophils increased progressively in bronchioles, counted in either SER or in ASML, reaching statistical significance ($P < 0.05$ and $P < 0.01$) from the first OVA challenge in the majority of groups.

Lymphocyte and macrophage numbers in BAL were not different among groups ($F = 1.474$, $P = 0.24$, and $F = 0.71$, $P = 0.59$, respectively, data not shown). A progressive neutrophil-counts increase ($F = 16.253$, $P < 0.001$) was statistically significant at the first ($P < 0.05$), third ($P < 0.01$) and ninth ($P < 0.01$) OVA challenges in comparison with the control group (Fig. 4). Likewise, eosinophil counts increased from the sensitization reinforcement day ($F = 8.481$, $P < 0.001$), but reached a statistically significant difference in comparison with the control group from the first challenge onward ($P < 0.01$; Fig. 4), and this increase was maintained at the subsequent challenges.

Effect of chronic allergen challenge in airway structure

In airway subepithelial structures, collagen deposit was identified with Masson trichrome staining as continuous

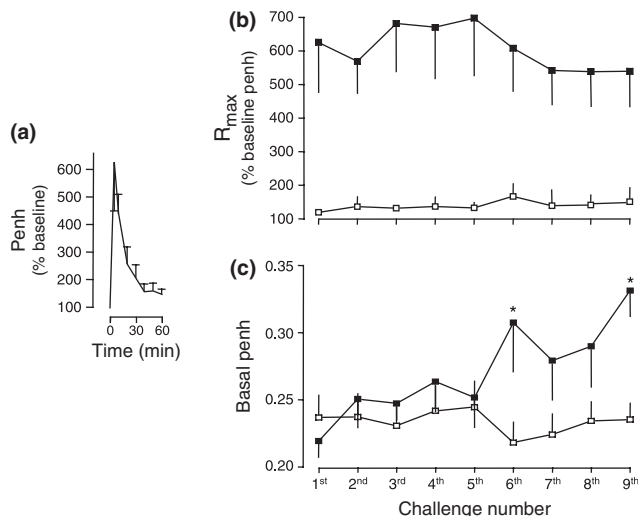


Fig. 2. Ovalbumin challenge induced transient bronchoobstruction in allergic guinea-pigs. (a) Temporal course of average responses during first ovalbumin challenge in sensitized guinea-pigs. (b) Average of maximum bronchoobstructive responses (R_{max}) induced by ovalbumin (closed squares) and saline (open squares) challenges in sensitized guinea-pigs. (c) Progressive increment of baseline bronchoobstruction in allergic guinea-pigs. Values of saline (open squares) and ovalbumin (closed squares) challenged sensitized guinea-pigs were obtained at the beginning of each plethysmographic recording and before drug administration. * $P < 0.05$ compared with their respective first challenge value (repeated measures ANOVA with Dunnett's multiple comparisons test). Symbols represent the means $\pm$ SE of $n = 5$ (closed squares) or $n = 6$ (open squares) animals of each group. Penh, bronchoobstruction index.

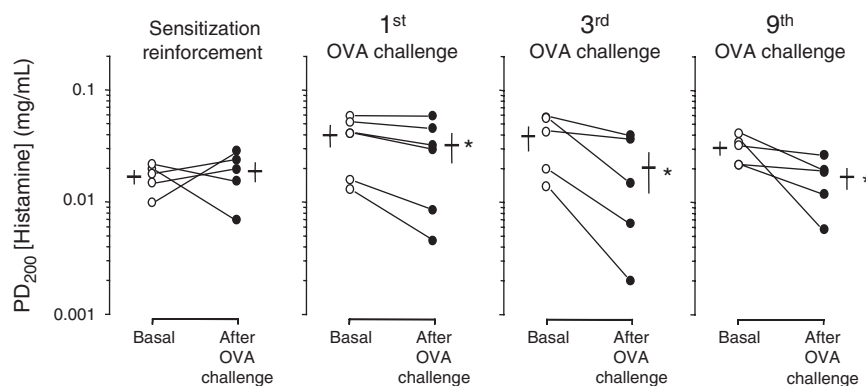


Fig. 3. Airway responsiveness to histamine before and after OVA challenge in sensitized guinea-pigs. Symbols correspond to PD_{200} value of the dose-response curves to histamine before (open circles) and 3 h after OVA challenge (closed circles). Horizontal and vertical lines represent mean and SEM, respectively. * $P < 0.05$ compared with their respective basal value (paired Student's t -test). OVA, ovalbumin challenges.

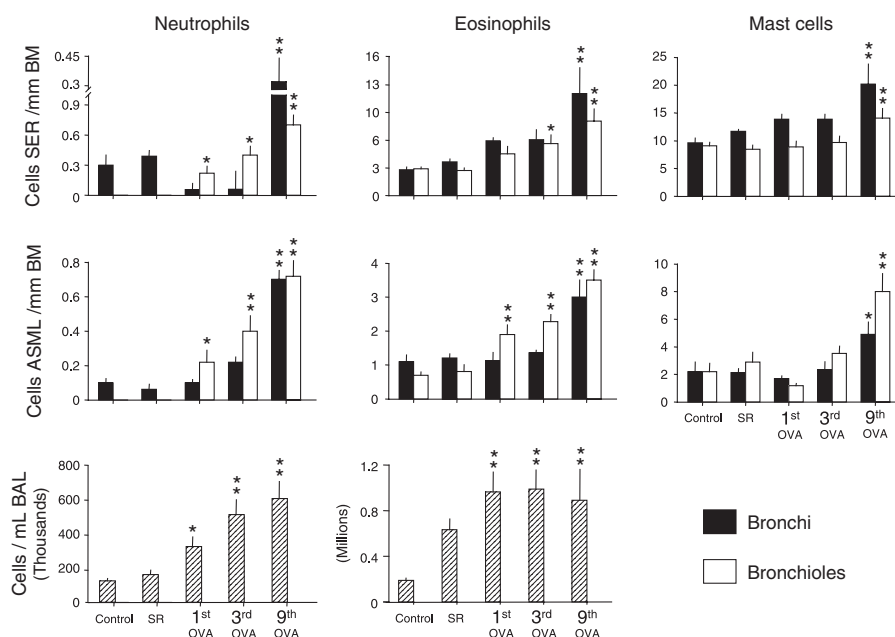


Fig. 4. Differential cell counts in allergic asthma model in guinea-pigs. Number of cells infiltrating subepithelial region (SER) or airway smooth muscle layer (ASML) of bronchi and bronchioles, adjusted by the basement membrane (BM) perimeter, and cells recovered in bronchoalveolar lavage (BAL) fluid (hatched bars) are shown for each group. Bars represent the average $\pm$ SEM of $n = 11$ guinea-pigs in control group and $n = 5$ in each experimental group. * $P < 0.05$ and ** $P < 0.01$ compared with control group (one way ANOVA with Dunnett's multiple comparisons test). SR, sensitization reinforcement group; OVA, ovalbumin challenge.

blue bands (Fig. 5). A clear distinction of smooth muscle layers was easily attained based on their strong red cytoplasmic staining with Masson trichrome. ANOVA tests of automated morphometric analysis achieved statistical significance in the SER proportion in bronchioles ($F = 8.362$, $P < 0.001$) and bronchi ($F = 13.743$, $P < 0.001$). *Post hoc* analyses showed that control group ($n = 11$) was not different from sensitization reinforcement ($n = 5$), first challenge ($n = 5$) and third challenge ($n = 5$) groups, but demonstrated a statistically significant increase of SER in either bronchioles ($P < 0.01$) or bronchi ($P < 0.01$) at the ninth challenge guinea-pigs ($n = 5$, Fig. 5). Relative ASML area was not different in any group, in either bronchioles

($F = 1.649$, $P = 0.19$) or in bronchi ($F = 1.023$, $P = 0.41$, Fig. 5). Total number of smooth muscle nuclei in bronchioles of chronic allergic (ninth challenge) animals and their respective age-matched control group was similar (28 ± 2.6 and 30 ± 2.8 nuclei, respectively; $n = 5$ or 6 per group, data not illustrated).

Relationship between functional and structural/inflammatory changes

Pulmonary function in guinea-pigs evaluated by basal $Penh$ and PD_{200} ratio was associated with some structural or inflammatory changes, as exemplified in Fig. 6.

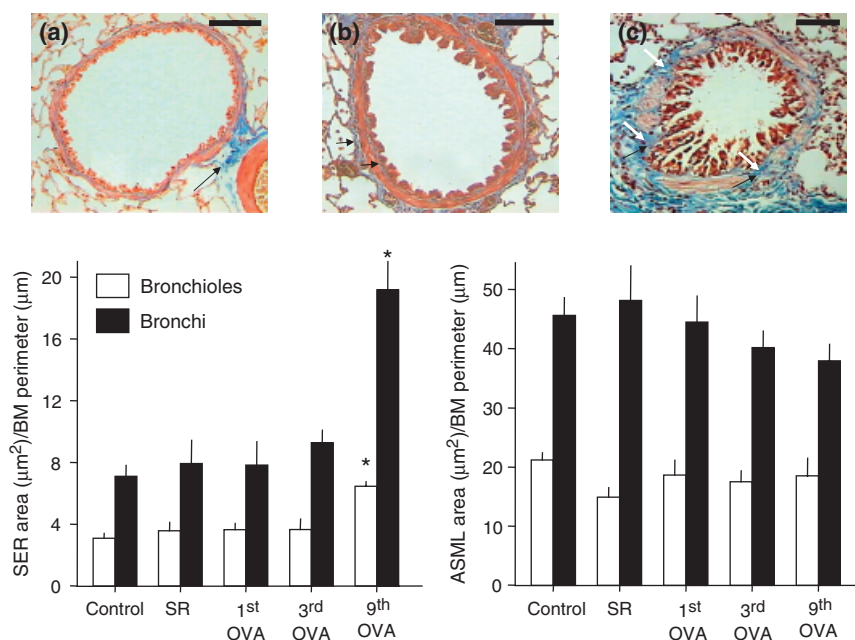


Fig. 5. Representative histological features of airway subepithelial region in a guinea-pig allergic asthma model. (a) Low-power micrograph from the lung of control guinea-pig showing a bronchiole, note the small amount of fibrous tissue in the adventitial layer (arrow). (b) Lung from a sensitized guinea-pig after third OVA challenge, there is slight increase of fibrous tissue (arrows) in the subepithelial and adventitia layers and evident epithelial hyperplasia. (c) Lung section from a sensitized animal after ninth OVA challenge, there is extense fibrosis around the bronchiole which extends among smooth muscle fibers (arrows). (d) Area of subepithelial region (SER) and (e) airway smooth muscle layer (ASML) of bronchi and bronchiole, adjusted by the basement membrane (BM) perimeter, as measured by automated morphometry. Bars and vertical lines are mean ± SE of $n = 11$ in controls and $n = 5$ animals in each experimental group. * $P < 0.01$ (one-way ANOVA with Dunnett's multiple comparisons test). SR, sensitization reinforcement group; OVA, ovalbumin challenge.

Basal Penh was correlated with extent of SER area in bronchi ($r = 0.45$, $P = 0.01$) and also had a tendency to be correlated with extent of SER in bronchioles ($r = 0.27$, $P = 0.14$), but was unrelated with extent of ASML in either bronchi or bronchioles.

In turn, PD₂₀₀ ratio had an inverse correlation with extent of SER area in bronchi ($r = -0.38$, $P = 0.04$) and bronchioles ($r = -0.46$, $P = 0.01$), implying that the greater the SER area, the higher the AI-AHR. However, this association of PD₂₀₀ ratio was not apparent for area of ASML at any airway level.

PD₂₀₀ ratio also possessed an inverse correlation with number of eosinophils infiltrating SER of bronchi ($r = -0.40$, $P = 0.03$) or bronchioles ($r = -0.62$, $P < 0.01$), and this same association was observed for neutrophils in SER of bronchi ($r = -0.46$, $P = 0.01$) and bronchioles ($r = -0.50$, $P = 0.005$). Finally, PD₂₀₀ ratio was also inversely correlated with number of cells recovered in BAL fluid, either eosinophils ($r = -0.56$, $P = 0.001$) or neutrophils ($r = -0.48$, $P = 0.007$).

Integrin immunolocalization

β1-Integrin expression in non-sensitized guinea-pig lungs was observed in the cytoplasm and cell membrane of smooth muscle cells from the blood vessel muscular

wall and airways wall, as well as in fibroblasts from adventitial layer, airway epithelium, basement membrane, lamina propria, alveolar walls, and leucocytes (Figs 7a–c). This β1-integrin distribution pattern was similar in sensitization reinforcement, and first and third OVA-challenged guinea-pig groups. By contrast, in the ninth OVA-challenged group overexpression of this integrin subunit was observed in SER and ASML in both bronchioles and bronchi (Fig. 7d). This increased expression was evident not only because there was a much stronger staining, but also because its distribution was greater ($P < 0.01$; $n = 5$, Figs 7e and f) as compared with control group ($n = 11$) and third OVA-challenged groups ($n = 5$). We corroborated that SER was basically an acellular area, because in addition to the very low number of inflammatory cells (as described previously), only some vimentin- and α-actin-positive cells but no neurofilament protein-expressing cells were observed (data not illustrated).

The immunoelectronmicroscopy study corroborated the presence of β1-integrin immunolabelling in extracellular matrix and cytoplasm of cells such as smooth muscle (Fig. 8a). In bronchiolar SER, β1-integrin immunolabelling was also observed in basement membrane and lamina propria (Fig. 8b). These findings obtained with the polyclonal antibody to β1-integrin were also corroborated by

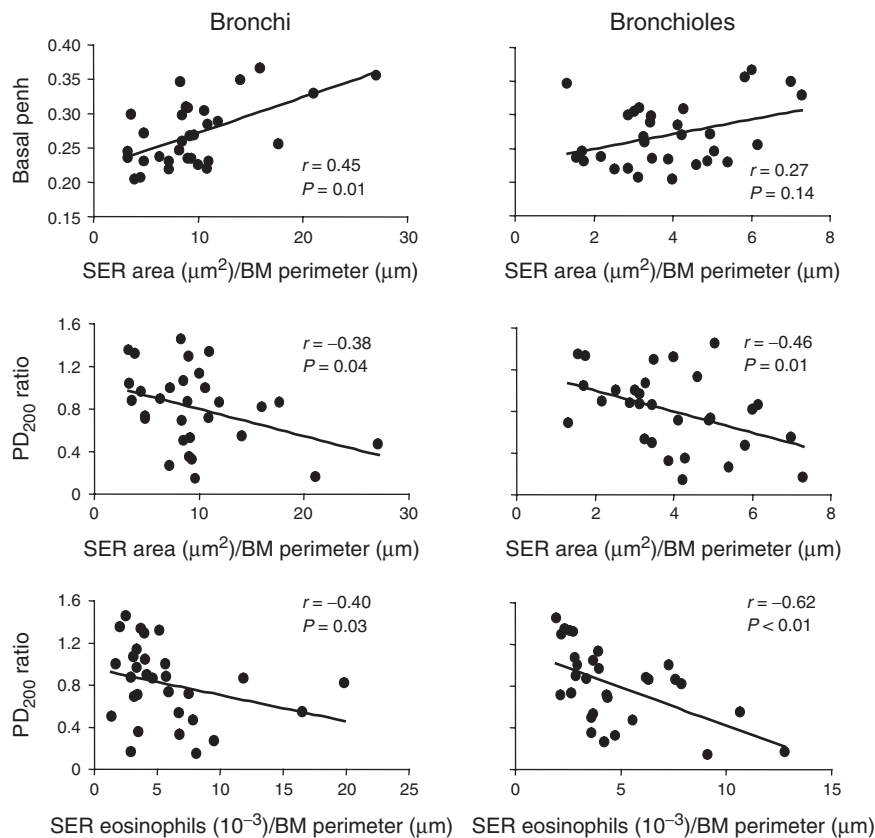


Fig. 6. Relationship between functional and structural/inflammatory changes. Scatter-graphs showing that basal Penh correlated with extent of subepithelial region (SER) of bronchi and bronchioles (upper panels), and that PD₂₀₀ ratio [i.e. change of histamine provocative dose after antigenic challenge, as compared with basal (pre-challenge) value] inversely correlated with extent of SER (middle panels) and with number of infiltrating eosinophils (lower panels). All values in x-axis were adjusted by their respective basement membrane (BM) perimeter. *r*, Spearman correlation coefficient.

employing the monoclonal antibody (Fig. 8c). β 1-integrin immunolabelling was observed in close association with collagen fibres types I and II (Fig. 8d). Similarly, α 1- and α 2-integrins could also be identified by immunoelectron-microscopy in SER acellular areas (Figs 8e and f).

As can be observed in Table 1, the percentage of SER in which β 1-integrin was expressed, either in bronchi or bronchioles, correlated positively with basal Penh and negatively with PD₂₀₀ ratio (implying that the larger the β 1-integrin expression, the higher the AI-AHR developed). Expression of this integrin in ASML of bronchi also presented an inverse correlation with PD₂₀₀ ratio. Similarly, a positive correlation indicated that the larger the extent of SER in bronchi and bronchioles, the greater the expression of β 1-integrin in this region. Such an association was not observed for ASML. Regarding cellularity, in BAL fluid the number of recovered cells was higher as the percentage of β 1-integrin expression in bronchiolar SER increased, although this trend only reached statistical significance for neutrophils. Finally, in general number of mast cells, eosinophils, and neutrophils was higher as the β 1-integrin percentage increased either in SER or ASML of bronchi and bronchioles.

Discussion

The most consistent and fundamental characteristic of asthma is airway hyperresponsiveness to various stimuli. Although the precise mechanism that induces airway hyperresponsiveness remains unknown, it is usually considered that airway inflammation [1] and remodelling are involved [3]. Inflammatory response after an asthma exacerbation event is characterized by a transient influx of neutrophils, followed by eosinophils [15]. In this study, AI-AHR was evident from the first OVA challenge (day 15) and persisted throughout the study (day 95). This heightened responsiveness was coincident with an increased number of inflammatory cells either infiltrating SER and ASML at least at the bronchiolar level, or recovered in BAL fluid (Fig. 4). Moreover, we corroborated that the higher the number of neutrophils or eosinophils in BAL fluid or in airway tissue, the higher the degree of AI-AHR (Fig. 6). Therefore, our study is in agreement with the relevance of inflammation in the development of airway hyperresponsiveness.

Neutrophils and eosinophils are key contributors to airway remodelling [5]. However, our guinea-pig model

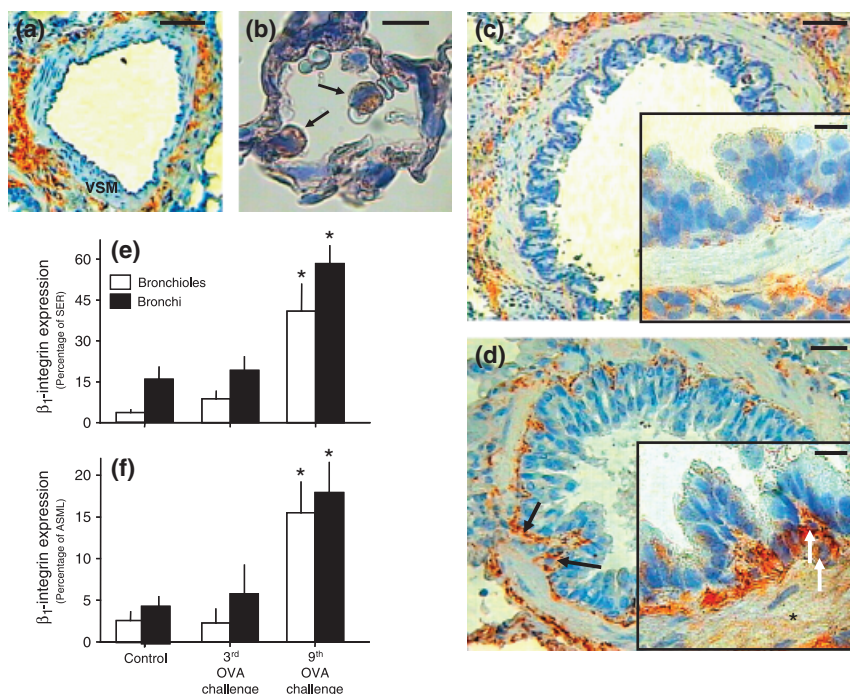


Fig. 7. Determination of β 1-integrin expression in control and allergic guinea-pigs by immunohistochemistry. (a) Section of a pulmonary blood vessel from a control guinea-pig exhibiting β 1-integrin immunostaining in muscle cells and adventitia layers. (b) Some alveolar macrophages also show β 1-integrin immunoreactivity (arrow) in non-sensitized guinea-pigs. (c) Numerous fibroblasts and some smooth muscle cells from the bronchiolar wall show strong β 1-integrin immunoreactivity in the lung of control animals. Inset shows a closer view of bronchial epithelium also exhibiting β 1-integrin immunoreactivity. (d) Strong β 1-integrin immunostaining is localized in the fibrous tissue below the bronchiolar epithelium in the lungs of guinea-pigs after nine OVA challenge (arrows). Inset corresponds to a high-power micrograph demonstrating extracellular β 1-integrin immunostaining in the subepithelial fibrous tissue, as well as in lower intensity in smooth muscle cells (asterisk), inflammatory cells (arrows) and epithelial cells. Scale bars correspond to 500 μ m (a), 100 μ m (b), 30 μ m (d) or 10 μ m (insets). (e) Area of β 1-integrin expression in subepithelial region (SER) and (f) airway smooth muscle layer (ASML) determined by automated morphometry. Bars and vertical lines are mean $\pm$ SEM of $n = 11$ in controls and $n = 5$ animals in each experimental group. * $P < 0.01$ (one-way ANOVA with Dunnett's multiple comparisons test). OVA, ovalbumin, VSM, vascular smooth muscle.

showed that many antigenic challenges were necessary to produce subepithelial structure alteration, indicating that chronic inflammation might be required in airway remodelling.

An important component of airway remodelling comprises subepithelial fibrosis, which causes extracellular matrix enlargement. This phenomenon is commonly observed even in mild childhood asthma [16] and has been linked with disease severity [17]. It has been suggested that a functional consequence of subepithelial-area thickening is amplification of airway smooth muscle contraction by decreasing airway distensibility and allowing smooth muscle adaptation to shorter lengths [5]. In addition, smooth muscle layer thickening could increase muscle capacity to narrow airway lumen [4, 5]. Taken as a group, in our guinea-pig asthma model AI-AHR began early at day 15 (Fig. 3), while SER enlargement appeared much later, at day 95 (Fig. 5). Thus, although at first sight AI-AHR was dissociated from structural changes, individual analysis demonstrated that the larger the bronchial or bronchiolar SER, the greater the AI-AHR (Fig. 6). Thus, the herein proposed guinea-pig model of chronic asthma also displays an association between SER enlargement and AI-AHR.

Chronic allergic guinea-pigs developed a progressive rise in the baseline bronchoobstruction index. In humans, it has been observed that submucosal thickening could contribute to fixed airflow limitation [3, 17]. Thus, in challenged guinea-pigs airway wall thickening could have contributed to the rise of baseline airway obstruction (Fig. 6).

A noteworthy change in chronic asthmatic animals was β 1-subunit integrin overexpression in SER and ASML of bronchi and bronchioles. Moreover, percentage of area in which β 1-integrin was expressed had an inverse correlation with PD₂₀₀ ratio, indicating that the more β 1-integrin expression, the higher the AI-AHR (Table 1). Interestingly enough, this β 1-integrin overexpression was observed in non-cellular areas of airways. These areas include basement membrane and lamina propria, in which diverse cell types are embedded in a large and dynamic extracellular matrix complex. In this compartment, we detected some fibroblasts and inflammatory cells, but according to immunohistochemistry there was also β 1-integrin not associated with such cells. Moreover, the immunoelectron-microscopy study showed that the β 1-integrin subunit was associated with collagen types I and II. It is known that only five integrins (α 1 β 1, α 2 β 1, α 3 β 1, α 10 β 1, and

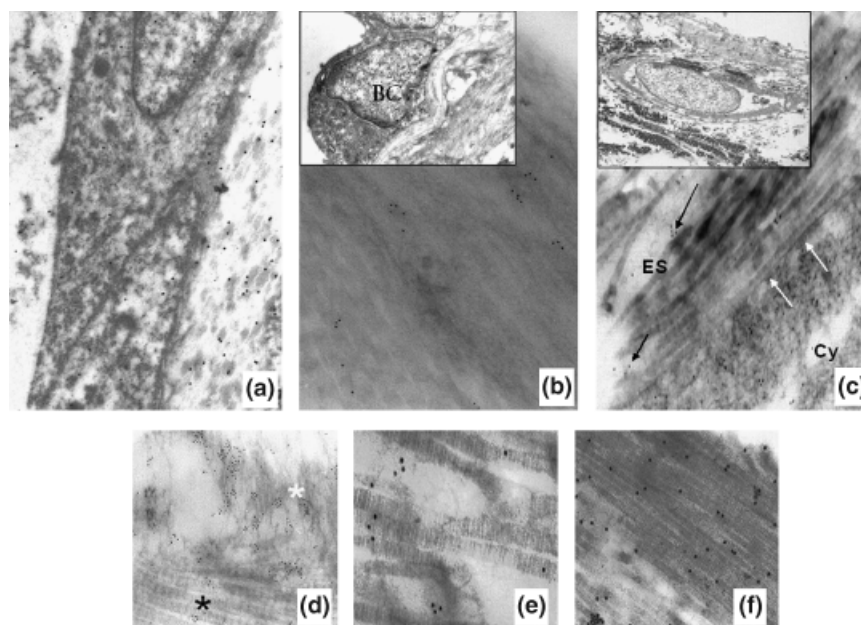


Fig. 8. Representative micrographs of lung integrin localization determined by immunoelectronmicroscopy in sensitized guinea-pigs after nine OVA challenges. Immunoreactivity to β 1-integrin detected by polyclonal antibodies labelled with colloidal gold (black dots) in smooth muscle (a) showing scarce cytoplasmic and membrane labeling for β 1 integrin, while transversal sectioned collagen bundles exhibit strong reactivity after incubation with specific polyclonal antibodies. Bronchiolar ultrastructure showing non-ciliated epithelial cell (BC; insert in b) and wide basement membrane (BM) and lamina propria (b). Similar immunolocalization than figure A is showed when monoclonal antibody against β 1 integrin is used, longitudinal collagen fibers from the extracellular space (ES) around smooth muscle cells (inset) exhibit strong labelling (c). Strong immunolabeling to β 1-integrin was seen in collagen type I (black asterisk) and type II (white asterisk) from subepithelial fibrous tissue (d). Fibrous tissue from the bronchial subepithelium also showed α 1- (e) and α 2-integrin (f) immunoreactivity. Cy, cytoplasm. Immunoelectronmicroscopy magnifications correspond to $\times 12\,000$ (a), $\times 63\,000$ (b and d), $\times 31\,000$ (c), $\times 80\,000$ (e), $\times 50\,000$ (f), $\times 10\,000$ (inset of b) and $\times 6000$ (inset of c).

Table 1. Spearman correlation coefficients of β 1-integrin expression and selected variables

	β 1-integrin expression			
	Bronchi		Bronchioles	
	% SER	% ASML	% SER	% ASML
Lung function				
Basal Penh	0.56**	0.20	0.57**	0.35
PD ₂₀₀ ratio	-0.50**	-0.37*	-0.50**	-0.26
Lung structure				
SER (μm^2 /BM perimeter)	0.47**	-	0.49**	-
ASML (μm^2 /BM perimeter)	-	0.12	-	0.17
Cells in BAL				
Eosinophils (cells/mL)	0.16	0.06	0.34	0.15
Neutrophils (cells/mL)	0.29	0.15	0.39*	0.28
Cells in SER				
Mast cells (cells/BM perimeter)	0.12	-	0.39*	-
Eosinophils (cells/BM perimeter)	0.21	-	0.50**	-
Neutrophils (cells/BM perimeter)	0.28	-	0.47**	-
Cells in ASML				
Mast cells (cells/BM perimeter)	-	0.32	-	0.45*
Eosinophils (cells/BM perimeter)	-	0.38*	-	0.34
Neutrophils (cells/BM perimeter)	-	0.41*	-	0.42*

* $P < 0.05$, ** $P < 0.01$.

ASML, airway smooth muscle layer; BM, basement membrane; PD₂₀₀ ratio, change of PD₂₀₀ after antigenic challenge, as compared with basal (pre-challenge) PD₂₀₀ value; SER, subepithelial region; -, not evaluated.

α 11 β 1) are collagen receptors. The collagen binding site of these integrins is located in the I-domain of the α -integrins subunit [18]. Consequently, the β 1-integrin subunit located in collagens is probably associated with an α -integrin subunit, and we corroborated the presence of α 1- and α 2-integrin subunits in these same areas, both of which should correspond to the extracellular domain. In this respect, it has been described that integrins without transmembrane and cytoplasmic domains can assemble to form stable, soluble heterodimers that preserve the same antigenic and ligand binding activity of the entire transmembrane integrin [19].

Shedding of integrins has been already described in tissues other than those of airways. Shakibaei et al. [20] were the first to describe localization of some integrin subunits (α 1 and α 3) in cartilage matrix of mouse embryo limb buds. More recently, Goldsmith et al. [21] reported the presence of a β 1-integrin fragment in interstitium of mouse heart, and related this finding with an increased attachment of heart myocytes to extracellular matrix. Menon et al. [22] demonstrated that continuous infusion of isoproterenol to mice induced heart β 1-integrin cleavage, and they suggested that metalloproteases were involved in this process. On the other hand, in experimentally-induced skin-blister fluid in human volunteers, Evans et al. [23] found that granulocytes had 72.8% fewer LFA-1 (α L β 2-integrin) than blood granulocytes, suggesting that inflammatory cells lost this adhesion molecule when transmigrating from blood into inflamed tissues. To our knowledge, our study is the first to report the β 1-integrin shedding in an inflammatory model.

Collagen-binding integrins are important regulators of collagen production and degradation, a necessary process to replace old or damaged collagen [24, 25]. In particular, fibroblasts initiate collagen degradation through α 2 β 1-integrin [24], whereas α 1 β 1-integrin is important in collagen fibrils organization and in feedback inhibition of collagen I synthesis [26]. On the other hand, it has been described that receptors that have been shed can modulate cellular responses on acting as competitive membrane-receptors antagonists [27]. Thus, β 1-integrins that have been shed could impede collagen turnover, and this could contribute to collagen-deposit increase to induce subepithelial structure widening. Because extent of airway SER was related with degree of AI-AHR, this might constitute the potential mechanism by which β 1-integrin relates with AI-AHR in our guinea-pig model of chronic allergic asthma.

Recently, it has been observed that β 1-integrin recruitment is involved in active tension of canine tracheal smooth muscle [8]. Hence, it is possible that β 1-integrin overexpression, acting directly on airway smooth muscle, might be an additional factor involved in AI-AHR.

Our study has some potential limitations. Barometric plethysmography remains a controversial method [28], mainly due to lack of certainty concerning what this

dimensionless variable is really measuring. Nevertheless, several authors have demonstrated that Penh is a good surrogate of specific airway resistance (sRaw) in guinea-pigs [29–31]. Moreover, in a previous work [32] we used two plethysmographic methods, the barometric (Penh) and the single-chamber plethysmography for anaesthetized-ventilated animals (R_L), to explore possible differences in route of administration (inhaled vs. intravenous) of a dose–response curve in guinea-pigs. By means of re-analysed data from this work, we corroborated that Penh reasonably reflects R_L changes after increasing doses of either acetylcholine or histamine. Additionally, in recent experiments we found that Penh responses to increasing doses of intravenous acetylcholine (5.6, 18, and 56 μ g/kg) were rather similar between anaesthetized nose-breathing guinea-pigs and tracheostomized guinea-pigs (data not shown). Thus, at least within the context of a dose–response curve in guinea-pigs, it appears that Penh is assessing lower airway responsiveness.

In conclusion, we found that repetitive allergen challenges in guinea-pigs induced strong β 1-integrin expression in extracellular matrix of bronchi and bronchioles, which was associated with increased collagen deposition in SER, in addition to inflammatory cells infiltration and AI-AHR development.

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