Asthma is increasing in prevalence worldwide and represents a significant economic burden. Despite advances in defining the inflammatory and immunological components of asthma, there is relatively little understanding of the cellular and molecular mechanisms underlying the structural changes seen in the asthmatic lung (airway remodelling). These changes, which include epithelial goblet cell metaplasia and hyperplasia, collagen deposition and thickening of the subepithelial lamina reticularis, increased matrix deposition throughout the airway wall, smooth muscle hyperplasia and hypertrophy, microvascular and neurite proliferation, are consistently observed in ongoing disease as well as asthmatic airways post mortem. In adult asthma the bronchial epithelium shows high expression of the epidermal growth factor receptor (EGFR) and the cyclin dependent kinase inhibitor, p21\textsuperscript{waf}, linked to ongoing stress and injury.

Methods: To determine if these are early markers of disease, sections of bronchial specimens obtained post mortem or by bronchoscopy from non-asthmatic (n = 7), moderate (n = 7), or severe (n = 9) asthmatic children aged 5–15 years were examined immunohistochemically. All severe and one moderately asthmatic children were receiving inhaled corticosteroids.

Results: The lamina reticularis of the asthmatic biopsy sections was found to be thicker (p = 0.01) than normal with increased deposition of collagen III (p = 0.007); submucosal eosinophil numbers did not differ between groups. As in adults, there was an asthma-related increase in epithelial EGFR (p < 0.002) but there was no evidence of proliferation, with Ki67 being reduced (p = 0.001) and p21\textsuperscript{waf} increased (p < 0.004). The thickness of the lamina reticularis was significantly correlated with epithelial EGFR (rho = 0.77, p = 0.001).

Conclusions: These data provide evidence that, in asthmatic children, the epithelium is stressed or injured without significant eosinophilic inflammation. This change in the epithelial phenotype is associated with collagen deposition in the lamina reticularis, suggesting that the epithelial mesenchymal trophic unit is active early in, and may contribute to, the pathogenesis of asthma.
epithelial injury as we and others have observed in adult asthma.15 The purpose of the present study was to examine the bronchial epithelium of normal and asthmatic children for the presence of markers of epithelial stress and repair and to relate these to markers of Th2-mediated inflammation (eosinophils) and remodelling (collagen deposition). Specifically, this involved detection of (1) EGFR whose expression in adult asthma increases according to disease severity, is insensitive to corticosteroids, and is evident throughout the epithelium indicating widespread stress or damage; (2) the cyclin/cyclin dependent kinase (CDK) inhibitor p21<sup>wa</sup>f whose expression is increased in adult asthma, particularly at the severe end of the disease spectrum<sup>14</sup> and also indicating ongoing stress or injury; and (3) the proliferation marker Ki67 to assess the extent of epithelial restitution.

**METHODS**

**Subjects**

Twenty three asthmatic children aged 5–15 years were enrolled into the study. The diagnosis of asthma and its severity were made according to the GINA guidelines<sup>22</sup> and the Russian National Program for the management and prevention of asthma in childhood.<sup>23</sup> Written informed consent was obtained from the children’s parents and the protocol was approved by the ethics committee of the Chelyabinsk City Hospital and the Russian Medical Association. The primary purpose of the procedure was for clinical investigation to examine the airway pathology in order to guide management for the individual. Reasons for investigation included suspicion of an undetected bacterial or fungal infection, foreign body aspiration, gastro-oesophageal reflux, or to exclude congenital malformations (for example, tracheomalacia and bronchomalacia) with a view to establishing a clear diagnosis and treatment strategy. In general, approx 3–4% of asthmatic children who undergo bronchoscopy at the Chelyabinsk City Hospital have an associated diagnosis and 1–2% an alternative diagnosis. There were no changes in medication before bronchoscopy and, at the time of study, the children were classified as being in a stable phase of their disease.

The asthmatic biopsy specimens were compared with bronchial tissue obtained from seven non-asthmatic children with no history of atopy (allergic rhinitis, conjunctivitis or eczema) who had met their death in road traffic accidents. These post mortem samples were acquired with approval from the ethics committee of the Chelyabinsk City Hospital and the Russian Medical Association and written informed consent from the parents of the dead child. Samples were obtained within 2 hours of death and were obtained by whole airway section from the carinae between the right middle and lower lobes, as for the asthmatic biopsies. They were transferred into 10% formalin and processed in an identical fashion to the asthmatic biopsies.

**Bronchoscopy**

Rigid bronchoscopy was undertaken under general anaesthesia using a Friedel (Berlin, Germany) rigid bronchoscope. At this time, fibreoptic bronchoscopy was not available at the Chelyabinsk Hospital. The procedure was conducted in accordance with safety criteria laid down in the recent guidelines described for fibreoptic bronchoscopy.<sup>24</sup> A single mucosal biopsy specimen was taken from the carinae between the right middle and lower lobes. The biopsy specimen was carefully removed and transferred to 10% formalin fixative for processing into paraffin.

| Table 1 Characteristics of children studied |
|---------------------------------|-----------------|-----------------|-----------------|
|                                 | Controls        | Moderate asthma | Severe asthma   |
| Mean [range] age (years)       | 10.3 (5–15)     | 9.2 (5–15)      | 9.9 (7–13)      |
| Mean [range] duration of asthma (years) | 4.1 (0.2–11) | 4.3 (0.3–11) | 4.9 (0.3–11) |
| Sex (M:F)                       | 6:1             | 4:3             | 4:5             |
| Treatment                       |                 |                 |                 |
| Inhaled corticosteroids         |                 |                 |                 |
| Budesonide                      | –               | 1               | 9               |
| Sodium cromoglicate             | –               | 400 µg          | 800–1000 µg     |
| Long acting β<sub>2</sub> agonists |                 |                 |                 |
| Sustained release theophylline  |                 |                 |                 |
| Mean (SD) FEV<sub>1</sub> (% predicted) | NA*             | 77.3 (22.2)    | 58.4 (17.3)     |
| Mean (SD) PEF (% predicted)     |                 | 79.4 (21.1)     | 59.2 (9.9)      |

FEV<sub>1</sub>, forced expiratory volume in 1 second; PEF, peak expiratory flow.

*These children had no previous history of asthma or atopy.
Immunohistochemistry
All embedded tissue samples were initially cut and stained using the haematoxylin and eosin method to assess suitability for immunohistochemistry. To be included for immunohistochemical staining, samples had to have an analysable submucosal area of at least 0.42 mm\(^2\) and epithelial length of 0.8 mm.\(^2\) Of the samples initially analysed, all seven normal tissue samples, seven of nine moderate asthma biopsies and nine of 14 severe asthma biopsies were suitable. The clinical characteristics of each group are shown in table 1.

Five micron sections were cut and stained immunohistochemically using the streptavidin biotin peroxidase technique with the following antibodies: a sheep anti-EGFR polyclonal antibody (an IgG fraction of immune serum raised against affinity purified EGFRs), mouse anti-p21\(^{\text{waf}}\) (Santa Cruz, CA, USA), mouse anti-Ki67 (MIB-1, Dako, Ely, UK), mouse anti-collagen III (Chemicon, Harrow, UK), and mouse anti-eosinophil cationic protein (EG2, Pharmacia Upjohn, Milton Keynes, UK) which recognises eosinophils in formalin fixed tissue, irrespective of activation state.\(^2\) We elected to use Ki67 as a marker of proliferation as other markers such as proliferating cell nuclear antigen have been shown to be expressed in DNA repair. In contrast, the Ki67 (MIB-1) antibody has been shown to correlate with semi-conservative DNA synthesis rather than excision repair DNA synthesis.\(^2\) For the antibodies used, their specificity and lack of cross reactivity were demonstrated in our previous studies;\(^1\) isotype matched antibody controls were also included in each staining run and were negative. Immunostaining for EGFR, p21, and Ki67 was quantified by computer assisted image analysis (KS400, Image Associates, Thame, UK) as described in the supplement available online at the Thorax website (http://www.thoraxjnl.com/supplemental).

### Statistical analysis
Data were not normally distributed and are presented as medians and interquartile ranges. Statistical analyses were

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Global results of immunohistochemical analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (n = 6 or 7)</td>
</tr>
<tr>
<td>Lamina reticularis (μm)</td>
<td>2.60 (2.46–2.65)</td>
</tr>
<tr>
<td>Submucosal collagen III (%)</td>
<td>32.2 (18.0–36.7)</td>
</tr>
<tr>
<td>Submucosal EG2 +ve (cells/mm(^2))</td>
<td>16.9 (13.7–75.5)</td>
</tr>
<tr>
<td>EGFR (% intact epithelium)</td>
<td>47.4 (46.4–49.7)</td>
</tr>
<tr>
<td>p21 (% intact epithelium)</td>
<td>23.7 (16.8–41.0)</td>
</tr>
<tr>
<td>MIB-1 (% intact epithelium)</td>
<td>26.3 (17.9–27.7)</td>
</tr>
</tbody>
</table>

Data are shown as median (interquartile range).
*Analysis of variance using the Kruskal-Wallis rank test.

Figure 2  Typical pattern of immunostaining showing epithelial EGFR (A–C), Ki67 (D–F), and p21\(^{\text{waf}}\) (G–I) expression in bronchial mucosa of a normal subject (A, D, G), a subject with moderate asthma (B, E, H), and one with severe asthma (C, F, I). Note also the relative thickness of the lamina reticularis in these tissue sections.
As also reported previously, there were no significant differences in tissue processing (biopsy versus whole asthmatic and control cases, respectively). From differences in staining for interstitial collagen III (fig 1, top panel); this was accompanied by a significant increase in staining for interstitial collagen III (fig 1, bottom panel). The measured thickness of the lamina reticularis was thicker than normal (table 2 and fig 3), extending into the columnar as well as the basal cells. Immunohistochemical analysis of EGFR expression in the airway mucosa from non-asthmatic children revealed positive epithelial staining which was associated with cell membranes and confined to the basal cells of the epithelium and lateral junctions between the columnar cells; no staining was evident on the luminal surface of the epithelium (fig 2A), as we have observed in normal adult bronchial epithelium. Bronchial epithelium from children with moderate asthma showed a similar pattern of staining, but the amount of staining was increased and occasional goblet cells also showed intense immunostaining (fig 2B). In severe asthma EGFR immunostaining was both more intense and occurred throughout the epithelium, as we have reported in adult severe asthma. The increased staining in the columnar epithelial cells was frequently associated with goblet cells (fig 2C) and showed intense staining of the whole cell. Epithelial immunostaining increased with disease severity (fig 3), extending into the columnar as well as the basal cells and was positively correlated with subepithelial collagen III deposition (rho = 0.47, p < 0.003) and the thickness of the lamina reticularis (rho = 0.77, p < 0.001; fig 4), as we have also reported in adult disease. There were no significant correlations between eosinophil numbers and either EGFR expression or the thickness of the lamina reticularis.

To explore the relevance of EGFR upregulation we further analysed expression of the proliferation marker Ki67 (fig 2D–F) and the cell cycle inhibitor p21waf (fig 2G–I). Immunostaining for Ki67 was mostly restricted to the basal cells and was significantly decreased in the asthmatic bronchial epithelium compared with normal controls (p < 0.003; fig 5 top panel). In contrast, p21waf expression was markedly and significantly increased in asthma children with moderate asthma, and 414 (19.7–105.8) for severely asthmatic children (table 2).

RESULTS
Clinically indicated bronchoscopies in children have been performed at the Chelyabinsk City Clinical Hospital since 1967. Rigid bronchoscopy was undertaken under general anaesthesia to ensure that this invasive procedure could be completed successfully with minimal discomfort to the child; the procedure was performed expeditiously with careful monitoring (oxygen saturation and electrocardiogram, non-invasive blood pressure). The bronchoscopy was found to be safe and well tolerated; no complications were encountered.

The lamina reticularis in the bronchial biopsy specimens of the asthmatic children was thicker than normal (table 2 and fig 3). The measured thickness of the lamina reticularis in the asthmatic and control cases was similar to previous reports, suggesting that the difference between the asthmatic and control cases was similar to previous reports. As also reported previously, there were no significant differences in eosinophil numbers between any of the groups.

The median (IQR) EG2+ eosinophils/mm² of submucosa were 16.9 (13.7–75.5) for normal children, 30.9 (14.7–71.6) for children with moderate asthma, and 41.4 (19.7–105.8) for severely asthmatic children (table 2). The correlation between eosinophil numbers and either EGFR expression or the thickness of the lamina reticularis was markedly and significantly increased in asthma

![Figure 3](image-url) Quantitative analysis of epithelial EGFR immunostaining as determined with computer aided image analysis.

![Figure 4](image-url) Correlation between epithelial EGFR expression and the thickness of the lamina reticularis. Data were analysed using Spearman’s rank correlation.

![Figure 5](image-url) Quantitative analysis of Ki67 and p21waf immunostaining in intact bronchial epithelium.
(p = 0.003; fig 5 bottom panel), with staining of the columnar and basal cell layers. There were significant negative correlations between Ki67 and p21\textsuperscript{wafl} (r = 0.65, p = 0.007) and subepithelial collagen III expression (r = 0.66, p = 0.001). In contrast, there was a positive correlation between EGFR and p21\textsuperscript{wafl} (r = 0.58, p = 0.009). There were no significant correlations between eosinophil numbers and either p21\textsuperscript{wafl} or Ki67.

**DISCUSSION**

A number of non-invasive approaches can be used to study components of the asthmatic phenotype in children, but at present we do not know how these relate to bronchial histology, particularly to airway remodelling. While recognising that obtaining biopsy specimens from children at bronchoscopy is a very serious procedure, sampling can be performed safely under general anaesthetic and was found to be safe, well tolerated, and free of complications, as previously reported.\textsuperscript{29–31} In accordance with ethical guidelines, all children who entered the study were undergoing bronchoscopy for clinical investigation, and only where it was deemed safe was parental consent obtained to take an additional biopsy for research purposes. While a single biopsy specimen may not give a true representation of the whole airway, studies in adults\textsuperscript{24} and children\textsuperscript{28} suggest that variability between subjects is considerably greater than variability between biopsies taken from the same subject at any one time.

The use of bronchoscopy to obtain airway biopsies in children with significant lung disease has recently been reviewed.\textsuperscript{35} At present it is only ethical to undertake such a procedure if it is indicated for a clinical purpose to establish a clear diagnosis and to exclude causes of wheezing other than asthma. Renal and liver biopsies, used to establish clear diagnosis and to exclude causes of wheezing other than asthma, may have a past history of eosinophilic inflammation in these asthmatic children. In the Childhood Asthma Management Program (CAMP) in which children aged 5–11 years were treated for 4–6 years with the inhaled corticosteroid budesonide, there was no long term effect of anterior wall injury on the lamina reticularis in children.\textsuperscript{36} This lack of proliferation may represent an important lesion that merits further detailed investigation. Recognising the growth effects of corticosteroids in vivo\textsuperscript{37} and in vitro,\textsuperscript{38} an obvious possibility that needs to be further explored is the impact of treatment on the bronchial epithelium.

While atopy is a strong risk factor for asthma, we have highlighted the importance of locally acting tissue susceptibility factors that occur in parallel with Th2 inflammation to give rise to the development of chronic asthma.\textsuperscript{39} According to this model, susceptibility to tissue injury is a major factor that contributes to persistent inflammation and airways remodelling and this interacts with Th2 cytokines to augment the responses. In support of such a concept, we have previously shown that asthmatic epithelial cells are more susceptible to oxidant-induced apoptosis\textsuperscript{39} and that oxidant stress leads to high cytoplasmic expression of p21\textsuperscript{wafl}.\textsuperscript{21} The high level of cytoplasmic p21\textsuperscript{wafl} in paediatric asthma is strongly suggestive of a stressed epithelium and, by extrapolation, may reflect susceptibility to oxidants as in adult disease. Such a phenotypic abnormality would explain epidemiological data linking air pollution and diets low in antioxidants with asthma exacerbations.\textsuperscript{40}

Although it has been postulated that airway “remodelling” is a long term consequence of chronic airway inflammation, our data showing excess deposition of interstitial collagen in the lamina reticularis indicate that these changes occur relatively early in life. Furthermore, our data are consistent with four previous reports\textsuperscript{9–11 28} which showed no requirement for concurrent eosinophilic inflammation for thickening of the subepithelial reticular basement membrane. This suggests a dissociation between at least some of the inflammatory and “remodelling” events in asthma, although we cannot completely exclude the possibility that there may have been a past history of eosinophilic inflammation in these asthmatic children. In the Childhood Asthma Management Program (CAMP) in which children aged 5–11 years were treated for 4–6 years with the inhaled corticosteroid budesonide, there was no long term effect of anterior wall injury on the lamina reticularis in children.\textsuperscript{36} This lack of proliferation may represent an important lesion that merits further detailed investigation. Recognising the growth effects of corticosteroids in vivo\textsuperscript{37} and in vitro,\textsuperscript{38} an obvious possibility that needs to be further explored is the impact of treatment on the bronchial epithelium.

While the presence of eosinophils may have been suppressed in children with severe asthma due to their use of corticosteroids, structural changes were apparent in the lamina reticularis of the asthmatic children irrespective of eosinophil numbers. However, it is noteworthy that the thickness of the lamina reticularis was correlated with epithelial EGFR levels, as previously reported in adult asthma.\textsuperscript{15} Since we have found that epithelial EGFR and IL-8 expression in adults with severe asthma are strongly correlated,\textsuperscript{44} it is possible that an EGFR mediated influx of neutrophils contributes to airway dysfunction in paediatric asthma. This proposal is consistent with recent reports showing epithelial disruption and increased neutrophil numbers in relation to symptom severity.\textsuperscript{35} 46

Increased expression of the EGFR is consistently observed in response to epithelial injury, suggesting that epithelial
array of growth factors including FGF, PDGF, ET-1, and VEGF, and this can be augmented by inhibiting EGFR mediated epithelial repair. Consistent with a role for epithelial mesenchymal signalling in paediatric asthma, epithelial EGFR levels were found to correlate closely with the thickness of the lamina reticularis, suggesting that the epithelial mesenchymal trophic unit is active early in asthma.

In conclusion, our data lend support to the view that the early pathological changes in asthma are linked to changes in the local tissue microenvironment related to epithelial stress and injury. This change in the epithelial phenotype is associated with increased collagen deposition in the lamina reticularis, suggesting that the epithelial mesenchymal trophic unit is active early in asthma— pre-modelling — and may contribute to the pathogenesis of asthma.

Further details of the immunohistochemical analysis are provided in the online supplement available at the Thorax website (http://www.thoraxjnl.com/ supplemental).

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The authors have no competing interests to declare.

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Epithelial stress and structural remodelling in childhood asthma

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Thorax 2005 60: 389-394
doi: 10.1136/thx.2004.030262

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