The CD4$^+$ T lymphocyte is a site of steroid resistance in asthma

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Summary

Phytohaemagglutinin (PHA)-induced T-cell proliferation is suppressed completely in steroid-sensitive asthma (SSA) by fluticasone propionate (FP). By contrast, in patients with steroid-resistant asthma (SRA), this proliferative response is only partially attenuated by steroids, which suggests that the T lymphocyte may harbour a key molecular defect in these patients. Both CD4$^+$ and CD8$^+$ T cells may be involved in orchestrating the inflammation underlying asthma. We examined whether CD4$^+$ or CD8$^+$ T cells isolated from SRA and SSA patients are equally susceptible to steroid suppression of PHA-induced proliferation. Complete suppression of CD4$^+$ T-lymphocyte proliferation was seen in both SSA and control subjects at concentrations of $10^{-9}$ M FP. In contrast, proliferation of CD4$^+$ T cells from SRA patients was only partially inhibited, even at $10^{-6}$ M FP. CD8$^+$ responses from SRA, SSA and controls were all similar, with only a partial suppression of proliferation at $10^{-6}$ M FP. Differential suppression by FP of CD4$^+$ T cells has thus been demonstrated between SRA and SSA patients.

Introduction

Corticosteroids are used to treat a wide variety of inflammatory diseases and inhaled glucocorticoids have become the mainstay of modern asthma therapy. Steroids reduce airway inflammation and improve lung function, and are the only drugs which have been shown unequivocally to reduce non-specific bronchial hyper-responsiveness (BHR) in asthma. The molecular mechanisms underlying this process are complex and not fully understood. Steroids exert their effects on inflammatory cells inter alia by regulating the transcription of target genes, either directly or indirectly. However, a subset of asthmatics described as steroid-resistant (SRA), respond poorly to systemic or inhaled corticosteroids, and often require such high doses that the side-effects cause considerable morbidity. Corticosteroid resistance in asthma is not due to abnormal drug absorption or rapid elimination, and a similar lack of responsiveness to glucocorticoids is also observed in patients suffering from other chronic inflammatory disorders such as rheumatoid arthritis and systemic lupus erythematosus. Thus, an understanding of the molecular and cellular mechanisms involved in SRA might identify key anti-inflammatory actions of steroids and hence abnormalities underlying the chronic inflammatory process, in all these conditions.

Bronchial mucosal inflammation underpins asthma pathogenesis. CD4$^+$ T lymphocytes play a pivotal role in the propagation of antigen driven inflammatory responses through the recognition of class II MHC-peptide complexes by the CD3$^+$/T-cell receptor (TCR) complex. This binding leads to lymphocyte activation with secretion of lymphokines which attract and activate eosinophils and mast cells. There is increasing evidence to suggest that activated CD4$^+$ T lymphocytes co-ordinate the inflammatory reaction observed in asthma. Poznansky et al. showed that similar numbers of T-cell colonies were observed when unfractionated PBMCs from SSA and SRA patients were cultured with the T-cell mitogen.
PHA in soft agar, in vitro. However, co-incubation with methylprednisolone (10^-8 M) produced a lower degree of inhibition of colony formation in cells from the patients with SRA. This suggests an impairment of the suppression of T-cell proliferation by glucocorticoids, in these patients.

Corrigan et al.9,15 showed that PHA-induced proliferation of peripheral blood T cells was inhibited by dexamethasone in SSA but not SRA. This resistance in SRA was not absolute but relative, reflecting a shift in the dose-response curve, in that proliferation of T cells from patients with SRA could be inhibited by steroids, but not at concentrations which are difficult to achieve therapeutically. The mechanism(s) by which glucocorticoids inhibit lymphocyte proliferation is not well understood, but could be due to decreased IL-1 and IL-2 production16-18 or to decreased IL-2 receptor expression.19 Subsequent work20 has shown that inhibition of phytohaemagglutinin (PHA)-induced proliferation of peripheral blood T cells from asthmatics, by dexamethasone in vitro, is reproducible over several months. This suggests that the glucocorticoid sensitivity of peripheral blood T cells, from patients with asthma, remains relatively constant over time.

Both CD4^+ and/or CD8^+ T cells might orchestrate the inflammation underlying asthma. The TH2 subset of CD4^+ T cells produces IL-4 and IL-5, which not only switch B-cells to IgE production but also drive CD4^+ T cells towards a TH2 phenotype. In contrast, CD8^+ T cells have recently been shown to switch Peripheral blood mononuclear cells (PBMCs) from IFN-γ production to IL-4 and IL-5 synthesis in the presence of IL-4.21 We investigated the sensitivity of PHA-induced CD4^+ T-cell proliferation22 to the inhibitory effects of fluticasone propionate (FP) in SRA, and SSA, and compared it to that for CD8^+ T cells in the same patients and controls.

**Methods**

**Patient selection**

Fourteen adult asthmatic patients (5 males, 9 females, aged 22–55 years) were studied, of whom six had SSA and eight SRA. All patients had a >15% increase in FEV1, following 200 μg inhaled salbutamol. In addition, the SSA patients had a >15% increase in FEV1, following a 4-week therapeutic trial of inhaled FP at 2000 μg/day. In contrast, the SRA patients had a <15% improvement in FEV1 after this trial, but a >15% increase in FEV1, following 4 weeks of FP at 4000–6000 μg/day. Two SRA patients were also taking 15 mg and 20 mg oral prednisolone/day, respectively. Ethical approval for the study was provided by the Leeds (East) Medical Ethics Committee.

**Non-asthmatic subjects**

A group of five normal, healthy volunteers (aged 21–35 years) with no history of asthma or allergy were included as controls in the study. None of these subjects was taking any medication.

**Materials**

Ficoll was purchased from Nycomed. Sterile 50 ml tubes were purchased from Falcon. M-450 CD4 Dynabeads, M-450 CD8 Dynabeads, M-450 CD8 Detachabead and M-450 CD4 Detachabead were purchased from Dynal. Foetal calf serum (FCS), RPMI-1640 medium and L-glutamine were from Gibco/BRL. MsIgG1-FITC, MsIgG1-RDI, MsIgG1-EC and anti-CD8-EC were obtained from Coulter. Anti-CD3-FITC was a gift from Professor A.W. Boylston, University of Leeds, UK. Anti-CD4-RPE was from Dako. 1 × penicillin/streptomycin was from Sigma. Culture well plates were from Corning. PHA was from Wellcome Diagnostics. FP and recombinant IL-2 were obtained from GlaxoWellcome Pharmaceuticals. [5^-3H] thymidine was purchased from Amersham. Printed filter mats and the cell harvester were from Wallac.

**CD4^+ T-cell isolation**

**Isolation of PBMCs from blood samples**

Peripheral blood mononuclear cells (PBMCs) were isolated23 from whole-blood samples by density gradient centrifugation over Ficoll. Peripheral venous blood was separated into 20 ml aliquots and made up to 35 ml with 1 × phosphate-buffered saline (PBS), pH 7.4. Each 35 ml aliquot was layered over 15 ml Ficoll in a 50 ml sterile tube. After centrifugation at 1200 g for 15 min at 20 °C, PBMCs were removed from the plasma/Ficoll interface, transferred to a fresh sterile 50 ml Falcon tube and made up to 50 ml with 1 × PBS. The PBMCs were pelleted by centrifugation at 2000 g for 10 min at 20 °C; the cells were washed twice with 50 ml 1 × PBS and collected by centrifugation at 450 g for 5 min at 20 °C. During subsequent experimental manipulations cells were maintained at 4 °C.

**Isolation of CD4^+ T cells**

Isolated PBMCs were resuspended in 1 ml of 1 × PBS at 4 °C and Dynabeads M-450 CD4 were added to give a ratio of beads to target cells of 3:1. The cells were incubated with the Dynabeads for 60 min at 4 °C on a rotary mixer. The beads were washed five times with 1 × PBS/2% FCS, placing in a magnetic-particle concentrator (MPC) for 3 min between washes.24
Separation of CD4\(^+\) T cells bound to Dynabeads with Detachabeads

Dynabeads with bound CD4\(^+\) T cells were resuspended in 100 μl of 1× PBS at 20°C in a 1.5 ml microcentrifuge tube and 1 unit of Detachabead M-450 CD4 was added. The tube was placed on a rotary mixer for 60 min at 20°C and the volume made up to 1 ml with 1× PBS. The tube was placed in a MPC for 3 min and the cell suspension transferred to a 15 ml Falcon tube. The beads were washed twice with 1 ml 1× PBS and detached cells were pelleted by centrifugation at 350 g for 5 min at 4°C.

Depletion of CD8\(^+\) T cells

The pelleted cells were resuspended in 1 ml, 1× PBS/2% FCS at 4°C and 40 μl M-450 CD8 Dynabeads added. After incubation at 4°C for 30 min on a rotary mixer, 5 ml 1× PBS/2% FCS was added to the cells. The Falcon tube was placed in a MPC for 3 min and the supernatant removed. The purified CD4\(^+\) T cells were pelleted by centrifugation for 5 min at 4°C, then resuspended in 3 ml 1× PBS.

CD8\(^+\) T-cell isolation

The PBMC suspension depleted of CD4\(^+\) T cells was used in similar fashion to isolate CD8\(^+\) T cells using Dynabeads M-450 CD8 and Detachabead M-450. Any contaminating CD4\(^+\) T cells were removed using Dynabeads M-450 CD4. The resulting purified CD8\(^+\) cell population was resuspended in 2 ml 1× PBS.

Flow cytometry to check the purity of CD4\(^+\) and CD8\(^+\) T-cell preparations

Two 50 μl aliquots of CD4\(^+\) or CD8\(^+\) T cells were removed per sample, to check the purity of each subpopulation of cells. To one 50 μl aliquot, 20 μl each of MsIgG1-FITC, MsIgG1-PE, and MsIgG1-PerCP antibodies were added. To the second aliquot of cells, anti-CD3-FITC, anti-CD4-PE, and anti-CD8-PerCP were added. The cells were stained for 1 h at 4°C in the dark and fixed with 500 μl fixing buffer (1× PBS, 2% FCS, 2% normal human serum, 1% sodium azide, 1% formalin). The purity of cells was then determined by flow cytometry. Only cells >98% pure were used in the study.

Proliferation assay for CD4\(^+\) and CD8\(^+\) T cells

Purified CD4\(^+\) and CD8\(^+\) T cells were resuspended in RPMI-1640 medium, containing L-glutamine, 5% FCS and 1× penicillin/streptomycin, at 1×10\(^6\) and 0.5×10\(^6\) cells/ml, respectively (to account for the differing proportion of CD4\(^+\) and CD8\(^+\) T cells in the T-lymphocyte population). Triplicate 100 μl aliquots of cells were transferred to a sterile flat-bottomed culture well plate for CD4\(^+\) T cells and to a round-bottomed culture well plate for CD8\(^+\) T cells (the lower surface area provided by this latter type of plate made it more suitable for CD8\(^+\) T cells, as 10,000 cells were added per well compared to 100,000 per well of CD4\(^+\) T cells). PHA was added at a final concentration of 0.1 μg/ml (assays at 0.1 and 1 μg/ml PHA produced identical results) and FP was added at a concentration range of 10\(^{-10}\) to 10\(^{-6}\) M. Recombinant IL-2 was included in the CD8\(^+\) culture at 20 U/ml, since culturing without IL-2 did not result in proliferation of these cells. Controls included were medium alone, and cells in the absence of PHA. Culture plates were incubated for 72 h in a humidified atmosphere at 37°C with 5% CO\(_2\). Cell proliferation was measured by the uptake of [5-\(^3\)H] thymidine (0.5 μCi) added for 18 h at the end of the 72-h incubation period. After incubation, cells were harvested onto a printed filter mat using a cell harvester, and the incorporated tritiated thymidine was counted using a beta-spectrometer (Betaplate). The results were expressed as mean corrected counts/min of triplicate cultures.

Statistical analysis

Statistical analysis used the Mann–Whitney U test. A p value of 0.05 was considered significant for each comparison.

Results

Figure 1 shows the degree of inhibition by FP, at varying concentrations (10\(^{-10}\) to 10\(^{-6}\) M), of PHA-induced proliferation of peripheral blood CD4\(^+\) T lymphocytes, isolated from individuals in each of the three clinical groups studied. These results show an almost complete suppression of CD4\(^+\) T-lymphocyte thymidine uptake after PHA-stimulation in SSA and non-asthmatic control subjects at a concentration of 10\(^{-9}\) M FP. Also, the mean IC\(_{50}\) values for SSA and non-asthmatic subjects are lower than that for SRA patients (Table 1). In CD4\(^+\) T cells from SRA patients, there was a reduction of only about 70% at 10\(^{-6}\) M concentration, with no further reduction even at 10\(^{-5}\) M, compared to a reduction of about 95% in SSA patients and non-asthmatics (Table 2).

In contrast, the suppression of thymidine uptake by FP in CD8\(^+\) T cells was identical in the three clinical groups. Absolute suppression of thymidine uptake was most consistent with the pattern seen in the CD4\(^+\) T cells from the SRA group (Figure 2). The IC\(_{50}\) values of the three groups were around
Figure 1. Mean dose-response curves for inhibition by FP of PHA-induced proliferation of CD4$^+$ T lymphocytes isolated from SSA patients ($n=6$) (open squares) or SRA patients ($n=8$) (filled squares) and non-asthmatic controls ($n=5$) (open triangles). The y-axis shows uptake of tritiated thymidine expressed as a percentage of that observed in the absence of FP.

**Table 1** Mean IC$_{30}^*$ values of CD4$^+$ and CD8$^+$ T cells

<table>
<thead>
<tr>
<th>Category</th>
<th>CD4$^+$ Mean IC$_{30}^*$ value (M)</th>
<th>CD8$^+$ Mean IC$_{30}^*$ value (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid-sensitive asthma</td>
<td>$0.6 \times 10^{-10}$ (vs. SR, $p=0.01$)</td>
<td>$4 \times 10^{-10}$</td>
</tr>
<tr>
<td>Steroid-resistant asthma</td>
<td>$2.5 \times 10^{-10}$ (vs. controls, $p=0.01$)</td>
<td>$7.5 \times 10^{-10}$</td>
</tr>
<tr>
<td>Non-asthmatic control</td>
<td>$0.5 \times 10^{-10}$</td>
<td>$7.5 \times 10^{-10}$</td>
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*IC$_{30}^*$ refers to the concentration of FP required to inhibit the percentage proliferation of T cells by 30%.

**Table 2** Proliferation of CD4$^+$ and CD8$^+$ T cells at $10^{-8}$ M FP, expressed as a percentage of that observed in the absence of FP

<table>
<thead>
<tr>
<th>Category</th>
<th>CD4$^+$ Mean % proliferation at $10^{-8}$ M</th>
<th>CD8$^+$ Mean % proliferation at $10^{-8}$ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Steroid-sensitive asthma</td>
<td>6.1 (vs. SR, $p=0.01$)</td>
<td>48.7</td>
</tr>
<tr>
<td>Steroid-resistant asthma</td>
<td>34.4 (vs. controls, $p=0.01$)</td>
<td>54.6</td>
</tr>
<tr>
<td>Non-asthmatic controls</td>
<td>5.0</td>
<td>49.5</td>
</tr>
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$6 \times 10^{-10}$ M FP (Table 1), with a reduction of only 50% in thymidine uptake at $10^{-8}$ M FP (Table 2).

Controls such as medium alone and cells in the absence of PHA (counts of <100) did not show proliferation compared to cells + PHA (counts of 1000s to 10 000s).

**Discussion**

Differences in the response to steroids after PHA-stimulation of peripheral blood T cells, have previously been observed between SRA and SSA. It appears that the primary site for this variability of FP-mediated inhibition of PHA-induced T-cell proliferation in asthmatics, is the CD4$^+$ T lymphocyte, because the responses exhibited by CD8$^+$ T cells remained constant across the various clinical categories studied. Furthermore, under these experimental conditions, CD8$^+$ T cells were relatively unresponsive to steroids. The explanation for this is unclear, although it has been shown that in contrast to CD4$^+$ T cells, CD8$^+$ T lymphocytes show no significant changes in the expression of HLA-DR, CD45RA, CD45RO and CD25 markers of activation, in response to glucocorticoid therapy. Also, the percentages of CD8$^+$ T cells expressing HLA-DR, CD45RA, and CD45RO in PBMCs from asthmatic
patients were not significantly different to those in the controls. Likewise, the percentages of CD25-expressing CD8$^+$ T cells were only marginally elevated.\textsuperscript{25} We therefore conclude that there is differential suppression of the proliferation of CD4$^+$ T cells between SRA and SSA, by FP, while the proliferative responses of CD8$^+$ T cells from both SSA and SRA are relatively resistant to high doses of this drug. This points to the CD4$^+$ T cell as the more important target for steroids in asthma. Although differential steroid suppression of proliferation of T-cell populations, in response to PHA, has been demonstrated previously in asthma, this is the first report demonstrating that the differential suppression occurs in the CD4$^+$ subset, and that this may be deficient in SRA.

PHA induces T-cell proliferation through an ATP-activated ion channel, expressed on the plasma membrane.\textsuperscript{26} Concentrations of PHA which induce an optimal proliferative response induce an increase in cytosolic free calcium [$\text{Ca}^{2+}$] by increasing the calcium influx across the plasma membrane.\textsuperscript{26,27} This increases the production of IL-2.\textsuperscript{27} An increase in [$\text{Ca}^{2+}$] is central to induction of proliferation by PHA and this increase is required for the production but not the action of IL-2.\textsuperscript{27} Low concentrations of PHA also deliver an additional signal independent of the increase in [$\text{Ca}^{2+}$], which induces IL-2 receptor expression, allowing a proliferative response in the presence of exogenous IL-2.\textsuperscript{26,27} The response seen with PHA is likely to be the same as that obtained with anti-CD3 monoclonal antibodies because, like PHA, they act by increasing the free cytosolic [$\text{Ca}^{2+}$].\textsuperscript{28}

The molecular pathways mediating PHA-induced proliferation in CD4$^+$ T cells from SRA patients, appear refractory to the inhibitory effects of FP. In contrast, CD4$^+$ T cells from SSA patients show complete suppression of this induced proliferation, even at low doses of this drug. These results point to the CD4$^+$ T cell as the more important target for steroid therapy in asthma and other inflammatory diseases. They also suggest that alternative therapeutic options should be considered when CD8$^+$ T cells are the major component of the inflammation present in a particular pathological condition.

**Acknowledgements**

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**References**