Resistance to methylprednisolone in cultures of blood mononuclear cells from glucocorticoid-resistant asthmatic patients

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Summary

1. In order to investigate the cellular mechanism of glucocorticoid resistance in chronic asthma, peripheral blood mononuclear cells (MNC) from asthmatic patients were cultured in soft agar.

2. Cells from patients known to be clinically sensitive to glucocorticoid therapy did not differ significantly from those of clinically resistant patients in terms of their immunophenotype or the number of colonies generated by culture in the presence of phytohaemagglutinin.

3. The glucocorticoid methylprednisolone (MP) at low concentration (10 nmol/l) inhibited colony growth from cells of glucocorticoid-sensitive patients, whereas there was much less inhibition of colony growth from resistant patients' cells.

4. In a small prospective study inhibition of colony growth by methylprednisolone in vitro correlated with the subsequently determined sensitivity of the patients' asthma to glucocorticoid therapy.

5. Assessment in vitro of glucocorticoid sensitivity may help to predict which patients may be spared ineffectual glucocorticoid medication. The results raise the possibility that peripheral blood mononuclear cells may respond to glucocorticoid in a similar manner to cells involved in the pathogenesis of asthma.

Key words: asthma, glucocorticoid resistance, methylprednisolone, mononuclear cells, T-cell colony.

Abbreviations: MNC, mononuclear cells; MP, methylprednisolone sodium succinate.

Introduction

Some patients with chronic asthma are resistant to systemic treatment with glucocorticoids, even in high dosage. Measurements of forced expiratory volume in 1 s (FEV1) show striking differences between the response of resistant and sensitive patients to the short-term administration of systemic glucocorticoids, in sharp contrast to the considerable increases recorded in both groups after use of a bronchodilator aerosol [1]. Detailed study of a large group of glucocorticoid-resistant asthmatic patients in Edinburgh has already identified some differences in the aetiology and clinical patterns of asthma between the two groups. It was considered improbable, however, that these differences were central to the phenomenon of glucocorticoid resistance. Only one of a wide range of laboratory investigations discriminated between resistant and sensitive asthmatic patients, the measurement of monocyte complement receptors and their enhancement by casein, a monocyte chemotactic factor [2]. In this paper we show that peripheral blood mononuclear cells (MNC) from glucocorticoid-resistant asthmatic patients are relatively unresponsive to glucocorticoids in vitro and we suggest that this may be used to predict glucocorticoid resistance.
in asthmatic subjects whose clinical response to these drugs has not yet been assessed.

**Patients and methods**

**Patients**

The laboratory studies reported in this paper were performed on venous blood samples obtained from 41 patients with chronic asthma and 15 normal subjects (Table 1).

All 41 asthmatic patients had been shown to respond to the inhalation of an aqueous $\beta_2$-agonist aerosol (salbutamol, 5 mg) by an increase in the FEV$_{1.0}$ of at least 30% from the baseline value of less than 60% of the predicted normal value. This evidence of reversible airflow limitation was regarded as consistent with the diagnosis of asthma. The patients were, however, selected in such a way as to form three separate groups.

**Group 1:** 15 asthmatic patients who were 'glucocorticoid resistant' in that their FEV$_{1.0}$ did not increase by more than 15% after a short course of prednisolone by mouth, even when the dose was increased progressively to at least 60 (and in some cases to 1000) mg/day. Although nine were on regular treatment with a glucocorticoid aerosol, the mean FEV$_{1.0}$ for the whole group was no higher on the day of blood sampling than on earlier clinic visits.

**Group 2:** 15 asthmatic patients who were 'glucocorticoid sensitive', as shown by an increase in FEV$_{1.0}$ of more than 30% after a 7 day course of prednisolone in a dose of only 20 mg/day. Nine of these patients had recently started regular treatment with a glucocorticoid aerosol, and in all 15 the disease was well controlled at the time of blood sampling. This may explain why the mean FEV$_{1.0}$ was significantly higher at that time than at their initial clinic visit.

**Group 3:** 11 'new' asthmatic patients whose response to prednisolone, in terms of FEV$_{1.0}$, had not previously been assessed.

As observed in the original study [1], the glucocorticoid-resistant patients were on average older, had a longer history of asthma, and were more prone to nocturnal wheeze and 'morning dipping' than the sensitive patients. There was no difference between the two groups in respect of atopic status or smoking habits. The more frequent family history of asthma in the resistant patients noted in the original study [1] did not emerge in the smaller numbers included in the present series (Table 1).

The criteria for inclusion of patients in groups 1 and 2 eliminated all asthmatic patients whose FEV$_{1.0}$ response to glucocorticoid was in the 'grey area' between 15 and 30%, with the result that only patients who were unequivocally either 'glucocorticoid resistant' or 'glucocorticoid sensitive' were studied. Although this facilitated correlation of clinical and laboratory findings, it left unexplored the possibility that 'glucocorticoid resistance' and 'glucocorticoid sensitivity' might represent the ends of a continuous spectrum. It was hoped that the inclusion of group 3 would elucidate this point. The 11 sub-

**Table 1. Details of asthmatic patients and controls**

Values are means (±SD), or numbers of patients/total studied, unless otherwise indicated. *P < 0.02 (Mann-Whitney U-test).

<table>
<thead>
<tr>
<th>Group 1 (glucocorticoid-resistant asthmatic patients)</th>
<th>Group 2 (glucocorticoid-sensitive asthmatic patients)</th>
<th>Group 3 (new asthmatic patients)</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>55.7 (±17.0)</td>
<td>58.2 (±12.9)</td>
<td>49.1 (±15.3)</td>
</tr>
<tr>
<td>Duration of asthma (years)</td>
<td>19.3 (±5.3)</td>
<td>14.0 (±11.5)</td>
<td>–</td>
</tr>
<tr>
<td>Atopic</td>
<td>7/15</td>
<td>3/15</td>
<td>–</td>
</tr>
<tr>
<td>Nocturnal wheeze/morning dip</td>
<td>10/15</td>
<td>6/15</td>
<td>–</td>
</tr>
<tr>
<td>Family history of asthma</td>
<td>3/15</td>
<td>3/15</td>
<td>–</td>
</tr>
<tr>
<td>Smokers</td>
<td>3/15</td>
<td>3/15</td>
<td>–</td>
</tr>
<tr>
<td>Total dose of prednisolone in previous 12 months (mg)</td>
<td>205 (range 0-1200)</td>
<td>169 (range 0-700)</td>
<td>–</td>
</tr>
<tr>
<td>On regular treatment with steroid aerosol</td>
<td>9/15</td>
<td>9/15</td>
<td>–</td>
</tr>
<tr>
<td>FEV$_{1.0}$ (Initial clinic attendance) (litres)</td>
<td>1.24 (±0.54)</td>
<td>1.59 (±0.71)</td>
<td>1.33 (±0.29)</td>
</tr>
<tr>
<td>FEV$_{1.0}$ (day of study) (litres)</td>
<td>1.21 (±0.58)</td>
<td>2.48 (±0.79)</td>
<td>1.33 (±0.29)</td>
</tr>
<tr>
<td>Plasma cortisol (day of study) (nmol/l)</td>
<td>506 (±140)</td>
<td>547 (±290)</td>
<td>535 (±150)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>510 (±210)</td>
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</table>
jects in that group were asthmatic patients who had presented with moderately severe airflow obstruction for which a course of oral prednisolone was indicated. Their FEV₁,₀ was measured before, and immediately after, a 7 day course of prednisolone (20 mg/day). Laboratory studies were undertaken in all 11 cases before treatment commenced and again in nine on its completion.

None of the asthmatic patients or the controls had coexisting malignant disease, being treated with cytotoxic or immunosuppressive drugs, or was suffering from any form of intercurrent infection, including oropharyngeal candidiasis. About half of the patients in groups 1 and 2 had previously been treated with oral prednisolone but there was no significant difference between the groups in terms of total dosage. For at least 4 weeks before this study no subject had received glucocorticoid therapy other than beclomethasone dipropionate by inhalation. The dose did not exceed 400 μg daily, which is known not to suppress pituitary-adrenal function [3]. On the morning of blood sampling the mean plasma cortisol was normal in all groups (Table 1).

Analysis of blood samples

Peripheral venous blood (50 ml) was withdrawn at 09.00 hours from each subject into a sterile bottle containing 2 ml of 2% EDTA in phosphate-buffered sodium chloride solution (154 mmol/l). All the laboratory studies were carried out without knowledge of the clinical status of the subjects. Total leucocyte count, differential leucocyte cell count and plasma cortisol were determined on all samples.

MNC were obtained from the peripheral blood by centrifugation over Ficoll-Hypaque [4] and were then washed twice in Hank's balanced salt solution. Subpopulations of T-lymphocytes, monocytes and Ia-positive cells were quantified by means of monoclonal antibodies with the following specificities: OKT3 (peripheral blood T-cells), OKT4 (inducer T-cell subset); OKT8 (cytotoxic/suppressor T-cell subset), OKM1 (peripheral blood monocytes) and Ia231 (MNC bearing Ia antigens, including 90% of B-lymphocytes and monocytes, 20% of null cells, and activated T-lymphocytes). Antibodies in the OK series were obtained from Ortho Diagnostics Ltd.

Cells positive for OKT3, OKT4, OKT8, OKM1 or Ia231 were detected by indirect immunofluorescence using fluorescein isothiocyanatelabelled goat anti-mouse immunoglobulin (Meloy Ltd) as the second antibody. The percentage of cells with membrane fluorescence was calculated from study of 200 cells. For each case a negative control was included: cells to which only second antibody had been added.

Colonial formation from peripheral MNC

MNC from each subject were cultured by using previously described methods [5, 6]. For each culture, 2 x 10⁶ cells were suspended in 40 μl of RPMI 1640 supplemented with 10% heterologous serum and 0.3% agar, and plated on to an underlayer of 0.2 ml of 0.5% agar containing 20 μg of phytohaemagglutinin (Difco) and 10% heterologous AB serum (the same source of serum was used throughout). Methylprednisolone sodium succinate (MP; Upjohn) was added to underlayers to give a final concentration of 10 nmol/l. Control cultures received no MP. All cultures were in 17 mm diameter wells in Multiwell dishes (Linbro) and were incubated at 37°C in a fully humidified atmosphere of 5% CO₂ in air. After 5 days of incubation colonies (defined as aggregates of more than 40 cells) were counted by phase contrast microscopy at x 100 magnification in three central fields in each of three replicate wells. This relatively simple scoring method has been verified by detailed computer-assisted analysis of the size distribution of all cell clusters in the cultures. Detailed immunophenotyping of these colony cells showed them to consist mainly of OKT4 and OKT8 lymphocytes, but most colonies also included a central cell bearing monocyte markers (M. C. Poznansky, A. C. H. Gordon, I. W. B. Grant & A. H. Wyllie, unpublished work).

Results

Analysis of the peripheral blood leucocytes revealed that there were few differences between the known 'glucocorticoid-resistant' asthmatic patients (group 1) and the 'glucocorticoid-sensitive' asthmatic patients (group 2) or between the asthmatic patients and normal control subjects. As expected, the asthmatic patients had an eosinophilia not found in controls. The resistant and sensitive patients did not differ significantly from each other or from control subjects in terms of the proportion of T-cells, T-inducer cells, Ia-positive cells or cells reacting with the OKM1 monocyte monoclonal antibody. There was a slightly higher proportion of T-suppressor/cytotoxic cells in the blood of sensitive asthmatic patients as compared with controls. The mean proportion of T-suppressor/cytotoxic cells in resistant asthmatic patients was less than in sensitive asthmatic patients, but this difference failed to attain significance at the P < 0.05 level (Table 2).
TABLE 2. Immunophenotype of peripheral blood mononuclear cells of asthmatic patients and controls

Mean results ± SD are shown. *P < 0.01 (t-test).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Mean percentage of mononuclear cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 (resistant asthmatic patients)</td>
</tr>
<tr>
<td>OKT3</td>
<td>45.1 (±10.9)</td>
</tr>
<tr>
<td>OKT4</td>
<td>27.2 (±7.1)</td>
</tr>
<tr>
<td>OKT8</td>
<td>17.8 (±5.5)</td>
</tr>
<tr>
<td>Ia231</td>
<td>13.8 (±4.9)</td>
</tr>
<tr>
<td>OKM1</td>
<td>29.0 (±7.4)</td>
</tr>
</tbody>
</table>

TABLE 3. Effect of glucocorticoid on colony formation by peripheral blood mononuclear cells from asthmatic patients and controls

The reduction in colony number with methylprednisolone is significant for controls and sensitive asthmatic patients (P < 0.0001) but not for resistant asthmatic patients. Mean results ± SD are shown.

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Colony number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group 1 (resistant asthmatic patients)</td>
</tr>
<tr>
<td>No glucocorticoid</td>
<td>35.3 (±9.5)</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>31.8 (±12.8)</td>
</tr>
</tbody>
</table>

In the absence of MP, MNC from both controls and asthmatic patients in groups 1 and 2 generated similar numbers of colonies in soft agar after 3-5 days incubation, representing a plating efficiency of approximately 0.05% (Table 3). Incubation during growth with MP at 10 nmol/l significantly inhibited colony numbers developing from the MNC of control subjects and sensitive asthmatic patients. In contrast this concentration of MP had little effect on colony development from the MNC of resistant asthmatic patients (Table 3).

To minimize purely technical variations in the plating efficiency and in order to permit comparison between patients, the number of colonies developing from each patient's MNC in the presence of MP at 10 nmol/l was expressed as a percentage of the number developing in its absence (Fig. 1). It was clear that the responses of individual patients, expressed in this way, fell with very few exceptions into two categories. Colony growth from MNC of glucocorticoid-sensitive patients was strongly inhibited by MP in vitro, whereas growth from MNC of resistant patients was affected little by MP. In all but one of the control subjects colony growth was inhibited by MP to the same extent as that from glucocorticoid-sensitive asthmatic patients.

The clinical sensitivity of the 11 asthmatic patients in group 3 to glucocorticoid was unknown at the outset of the study. In these patients, however, the response to glucocorticoid, as determined by colony inhibition in vitro, was found to correlate closely with the extent to which FEV₁₀ increased during subsequent treatment with oral prednisolone (Fig. 2). This is shown in Fig. 2, where percentage changes in colony number, induced in vitro by MP, are plotted against the percentage change in FEV₁₀ resulting from a 7 day course of oral prednisolone. An identical relationship was evident when the responses in vitro were plotted against the absolute change in FEV₁₀ (data not shown).

The percentage change in colony number, induced by MP in vitro, appeared to be characteristic for each subject. Thus, on re-testing six subjects between 3 weeks and 4 months later, the divergence from the initial value was in every case less than 20% (Fig. 3a). In particular, colony
Fig. 1. Effect of methylprednisolone (MP) on colony formation from mononuclear cells of resistant and sensitive asthmatic patients (groups 1 and 2) and of controls. The number of colonies developing in the presence of methylprednisolone at 10 nmol/l is expressed as a percentage of the number which developed in the absence of methylprednisolone, from cells of the same subject. Each point derives from a different subject. N.S., Not significant.

FIG. 2. Correlation between inhibition of colony growth by methylprednisolone (MP) in vitro and relief of bronchospasm by a course of prednisolone in vivo, in the 11 unselected patients in group 3. The correlation is significant ($P < 0.005$).

Discussion

Previous studies of the characteristics of overtly glucocorticoid-resistant asthmatic patients have revealed few differences from the majority of patients with chronic asthma, who respond well to glucocorticoids [1, 2]. In this paper we show that MNC from such glucocorticoid-resistant patients were significantly more resistant to the effects of MP in vitro than the MNC from clinically sensitive asthmatic patients or normal controls. The criterion adopted was the ability of phytohaemagglutinin-stimulated MNC to grow as colonies in soft agar. This phenomenon is known
to depend upon interactions of monocytes and lymphocytes [6, 7], but as the proportions of monocytes and T-cells in the peripheral blood of the two groups of patients were similar, the observed difference in glucocorticoid responsiveness in vitro cannot be attributed merely to differences in the relative numbers of cell types under study.

MNC from individual patients with clearcut glucocorticoid-resistant or -sensitive asthma (i.e. groups 1 and 2) responded to MP in vitro in a consistent manner on repeated testing. The response bore no relationship to the endogenous plasma cortisol at the time of blood sampling (see Table 1). Further, the response in vitro did not change after a 7 day course of systemic glucocorticoid therapy. Hence it is probable that the MNC of each individual have a characteristic response to glucocorticoid in vitro, uninfluenced by endogenous cortisol or by the administration of exogenous glucocorticoid.

The close correlation between the response to glucocorticoid in vitro and in vivo suggests that the cell types involved in generating colonies in vitro (i.e. monocytes and T-lymphocytes) may be similar to those contributing to the pathogenesis of asthma in these patients. A substantial body of evidence implicates cells of the monocyte lineage in the pathogenesis of asthma [8-10]. There are several ways in which improvement in the clinical condition of asthmatic patients treated with glucocorticoids may depend upon the effects of these drugs on such cells. Of particular relevance to the interpretation of the present results is the well-documented inhibition of production of interleukin 1 by low concentrations of glucocorticoids [11-13]. Interleukin 1 is a monocyte product which mediates mitogen-induced T-cell proliferation in vitro [14], and is known to support T-cell colony formation in soft agar [7]. It is also a pyrogen and promotes the synthesis of inflammatory mediators from arachidonic acid [15, 16]. Other effects of glucocorticoids that may be implicated in their therapeutic action in asthma are modulation of complement receptor expression by monocytes [2] and induction in leucocytes of the phospholipase A2 inhibitor lipomodulin [17], which also suppresses reactions mediated by immunoglobulin E [18]. It thus may be postulated that cells of monocyte lineage within the lungs of glucocorticoid-resistant asthmatic patients might continue to secrete inflammatory mediators, support lymphocyte proliferation and exist in a hyperreactive state with enhanced complement receptor expression, despite the presence of inhibitory concentrations of glucocorticoids.

We embarked on this study on the premise that glucocorticoid resistance in chronic asthma was an all or none phenomenon, which is what prompted us to restrict our investigations in vitro initially to patients whose asthma was readily recognizable on clinical criteria as sensitive or resistant. It would seem, however, from the observations on previously untreated, and thus unselected, asthmatic patients, that sensitivity and resistance may form a continuous spectrum. At one end would be patients whose asthma is overtly resistant to systemic glucocorticoids, even in high dosage. It is important to recognize these patients since they may otherwise be needlessly exposed to hazardous side effects. This study indicates one way in which such patients may be identified.

Although the cellular basis of glucocorticoid resistance remains obscure, our findings suggest that a defect in MNC, and perhaps monocytes in particular, may be responsible. We are currently defining the nature of this cellular defect more precisely. We are also exploring the potential of simpler tests in vitro to identify resistant patients before they are treated with glucocorticoids either for asthma or for other diseases normally responsive to these therapeutic agents.

Acknowledgments

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References

Glucocorticoid resistance in asthma


