Inhaled corticosteroids can modulate the immunopathogenesis of pulmonary sarcoidosis

M.A. Spiteri*, S.P. Newman*, S.W. Clarke*, L.W. Poulter**

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ABSTRACT: We investigated the effect of inhaled corticosteroids on the phenotypes and functional capacity of macrophages obtained by bronchoalveolar lavage from patients with pulmonary sarcoidosis. The results were correlated with clinical status and therapeutic efficacy. Ten symptomatic sarcoid patients (previously untreated) with radiological parenchymal shadowing and abnormal pulmonary function received inhaled budesonide, 800 µg twice daily via a Nebuhaler for 16 weeks. A placebo group included ten healthy volunteers and five sarcoid patients with similar features to the treated group. Drug distribution studies showed that 10% of the inhaled drug was deposited in the alveolar region. All ten treated sarcoid patients had symptomatic relief with no adverse effects. Three of these ten patients had significant resolution of their radiological shadowing. No significant difference in pulmonary function was observed. At the cellular level, a significant decrease in lavage lymphocytosis was seen after 16 weeks, during which time there was a concomitant change in the phenotypic and functional characteristics of the alveolar macrophage population. No similar changes were observed in the placebo group. Our results suggest that inhaled budesonide can modulate the aberrant immunological reactions existing in the lung in pulmonary sarcoidosis, and produce concomitant symptomatic relief with no side effects. It is postulated that this effect may occur through action on the local alveolar macrophage population.


Pulmonary sarcoidosis is a chronic inflammatory disease characterised by a T-cell alveolitis and the formation of granulomata within the lung interstitium [1]. The inflammatory response appears to be driven by aberrant immunological reactions [2]. Sarcoidosis presents with a variable clinical course. In the majority of patients it is self-limiting, resolving spontaneously with or without treatment; yet in 20% of patients the granulomata persist leading to the insidious development of fibrosis, producing significant morbidity and mortality [3].

The lack of any clear prognostic signs leaves the question of who and when to treat, in dispute. Currently only those patients with Stage 2 or 3 disease with persistent abnormal lung function are treated. When therapy is instituted, it consists of systemic corticosteroids in varying doses (20–50 mg prednisolone per day) and regimens producing variable clinical efficacy. These drugs have been shown to control the local inflammatory response in sarcoidosis by decreasing the T-cell CD4/CD8 ratio [4] as well as reducing the lymphocytic production of interleukin-2 [5]. However, as undesirable side-effects invariably occur [6], current therapy may be started too late (or curtailed too soon), with many patients already having developed (or proceeding to develop) irreversible lung damage.

Against this background, there is clearly a need for a therapeutic regimen that is safe enough to commence early in disease, that can be targeted to the lung, and that can be shown (as with systemic steroids) to modulate the underlying immunological dysfunction seen in this disease.

Preliminary studies [7] using inhaled budesonide in a dose of 1600 µg per day have been shown to produce clinical benefit with minimal side-effects in active pulmonary sarcoidosis. As yet it has not been documented whether such therapy is effective in modulating local immune reactivity, nor has it been established that the inhaled mode of administration is effective in actually depositing the drug at the site of the alveolitis.

Studies on both inhaled and systemic steroids have so far only focused on their effect on lavage lymphocytes. However, both the T-cell activation and the granuloma formation occurring in sarcoidosis are mechanisms controlled by macrophage-like cells [8]. Phenotypic and functional aberrations within this macrophage population have been demonstrated in active sarcoidosis [9, 10]. Such alterations reflect changes in clinical status [11]. One might therefore hypothesize that the persistence of granulomata and fibrosis seen in pulmonary sarcoidosis are features determined as much by alveolar...
macrophages as by T-lymphocytes. Thus for inhaled steroids to be properly evaluated as a mean of treating pulmonary sarcoidosis their immuno-regulatory effect on the macrophage population in the lung must be ascertained.

This study was therefore designed to assess the effect of inhaled steroids on the phenotype and functional capacity of alveolar macrophages obtained by bronchoalveolar lavage (BAL) from a homogeneous group of previously untreated sarcoid patients and to correlate the observed local immunological changes with clinical status and thus therapeutic efficacy.

Patients and methods

Subject groups

The study population consisted of 15 patients with at least a 12 month history of biopsy proven sarcoidosis: 10 males and 5 females mean age 38 years; 1 smoker (1 pack per day). None of the patients had received any treatment prior to the study. All 15 patients had unequivocal bilateral parenchymal shadowing (grade 3 chest X-ray) in addition to impaired pulmonary function (FEV1 mean predicted range 55-105%; FVC 51-107%; TLC 51-85%; TLCo 58-80%). A population of 10 healthy volunteers, all nonsmokers, 9 males and 1 female, mean age 22 years, were recruited. All had normal chest X-rays and pulmonary function. None had a past history of lung disease or any viral illness in the two weeks prior to lavage.

Study design

Prior to commencement, all subject groups had a full clinical evaluation with documentation of any presenting symptoms. In addition to chest X-rays, pulmonary function tests and bronchoalveolar lavage (BAL) were performed. Formal written consent was obtained from each. Randomisation into inhaled steroid or placebo group was carried out independently. Ten of the patients with sarcoidosis received budesonide (Pulmicort, Astra Pharmaceuticals) in a dose of 200 J.tgm each) delivered twice daily. Each dose was given as four single puffs (200 J.tgm each) delivered from a pressurised metered dose inhaler via a one-way inhalation valve (Nebuhaler, Astra Pharmaceuticals). Two deep inhalations followed by 10 second breath-holdings were taken after each puff. The remaining 5 sarcoid patients and 10 healthy volunteers received a placebo equivalent in number of puffs and mode of administration. The study period in each group was four months, after which each subject was re-evaluated with chest X-ray, pulmonary function tests and BAL. Immunocytophological analysis and functional capacity studies were carried out on all BAL samples. All chest X-rays were read by two independent radiologists. Subjective improvement in each case was assessed on a visual analogue scale of 1 to 10. Details of any adverse effects during the course of the study were duly noted.

Aerosol deposition analysis

Pressurised aerosol deposition was assessed prior to commencement treatment in the 10 sarcoid patients who received budesonide therapy, by incorporating Teflon particles labelled with technetium 99m into placebo pressurised canisters [12]. The particles had a mass median aerodynamic diameter of 3 μm, which is similar to that of budesonide particles. The percentage of the dose deposited in the lung and oropharynx was assessed by gamma camera, and the twenty-four hour whole lung retention of the insoluble Teflon particles (measured by 5 cm diameter collimated scintillation probes) was taken as a measure of alveolar deposition.

Bronchoalveolar lavage

BAL was performed using a fibreoptic bronchoscope. The right middle lobe was anaesthetised with 2% lidocaine and lavaged with 20 ml aliquots of 0.9% normal saline to a total of 180 ml (corrected to pH 7.4 with NaHCO3). The lavage fluid was gently aspirated after each aliquot and collected into a sterile, siliconised glass bottle maintained at 4°C. All subjects had 20 ml of peripheral blood taken by venepuncture at the same time as the BAL.

Processing of lavage fluid

The lavage fluid was filtered through a coarse gauze and centrifuged at 480 g, 4°C. The cell pellet was resuspended in supplemented RPMI 1640 (containing 1.25% 200mM L-glutamine, 10% heat inactivated foetal calf serum, 100 μg/ml streptomycin and 100 IU/ml penicillin). The cells were then counted in a modified Neubauer haemocytometer and viability assessed by cellular exclusion of trypan blue (>90% in each sample).

Immunocytophological analysis

A portion of the above BAL cell suspension was adjusted to a concentration of 3 x 10⁶ cells·ml⁻¹ and cytocspins prepared using 100 μl aliquots on a Shandon cytocentrifuge. One cytospin from each sample was stained for morphology to facilitate differential cell count. The remainder were air-dried for one hour at room temperature, fixed in 1:1 mixture of chloroform:acetone for 10 minutes, wrapped in plastic film and stored at -20°C until use.

Phenotypically distinct macrophage populations were identified by incubating cytospin preparations with monoclonal antibodies RFD1 and RFD7. RFD1 recognises a unique epitope within the Class II HLA-DR molecule (28-33 kd), which has been shown to be associated with antigen presenting cells [13]. The RFD7 identifies a 77kd antigen, the expression of which appears restricted to acid phosphatase positive mature phagocytic macrophages. Its function is unknown but it
has proved to be a valuable marker of this specific cell type [13]. Individual cell surface antigens were identified by the immunoperoxidase method, which incorporates D-amino benzidine and hydrogen peroxide in Tris hydrogen chloride (pH 7.6, 0.05M) as the developing solution [14]. Co-expression of RDF1 and RDF7 antigens on a single cell was studied by immunofluorescence, incorporating Ig class-specific second layer reagents conjugated with fluorescein isothiocyanate (FITC) or tetraethylrhodamine isothiocyanate (TRITC) [15]. Background staining was identified by comparison with negative control cytopsins on which the McAb was omitted; positive specificity controls with human palatine tonsill were used in each case. Immunoperoxidase staining was read, using a light microscope with high magnification (×600), while a fluorescence microscope was used for reading the double immunofluorescence preparations. At least 150 cells were counted in each cytopsin; and the percentage of positive cells recorded.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBM) were separated on a Ficoll-Hypaque gradient, washed twice in Hanks' balanced salt solution, and then resuspended in supplemented RPMI 1640. The cell count and viability were assessed as above (>95% viability in each case).

Autologous mixed lymphocyte reactions (AMLR)

The BAL and PBM suspensions used in AMLR were each adjusted to a concentration of 1 × 10⁶ cells ml⁻¹. Separate aliquots of these suspensions were initially incubated in the presence of mitomycin C (25 µg ml⁻¹) for 45 minutes at 37°C 5% humidified CO₂ to block cell division; after this the treated cell samples were washed three times in RPMI 1640. AMLRs were then set up for each subject (before and after drug or placebo) to triplicate in microtitre wells using the following cell populations: BAL alone (1 × 10⁶ cells per well); PBM alone (1 × 10⁶ per well); mitomycin treated BAL admixed with PBM (2 × 10⁶ per well); control wells of mitomycin treated BAL and PBM (1 × 10⁶ per well) respectively. These cultures were then incubated at 37°C in 5% humidified CO₂ for four days. After this, each well was pulsed with 2 μCi tritiated thymidine (3HT) (Amersham, 5Ci·mmol⁻¹), and further incubated for 18 hours. The cultures were then harvested using a semi-automatic cell harvester (Titertek-flow, Laborat. Inc, McLean VA); the amount of incorporated radioactivity was measured in a liquid scintillation counter and expressed as average counts per min (CPM) of triplicate cultures. These results were recorded as Stimulation Index (SI) which was defined as the factor by which cpm 3HT of cells is increased over the cpm 3HT of same cells treated with mitomycin. Blank wells with no cells consistently gave readings of <20 cpm.

Statistical analysis

Where relevant, data were analysed using the Students' t test for non-paired data.

Results

Aerosol deposition pattern

In the 10 sarcoid patients who received budesonide therapy, 21.2±2.0 (mean±SEM)% of the dose was deposited in the lungs, of which 9.6±2.1% appeared in the alveoli and 11.6±1.8% on the conducting airways (tracheobronchial zone). The remainder of the dose was either deposited in the oropharynx (11.2±1.8%) or retained in the Nebuhaler and actuator (67.6±2.2%).

Clinical effects

All 10 sarcoid patients receiving inhaled budesonide reported an improvement in their symptoms of cough and dyspnoea (a mean overall improvement of 80% on the visual analogue scale); whereas only one of the patients receiving inhaled placebo had symptomatic relief. There was no significant change in pulmonary function in any group. Three of the 10 patients receiving budesonide showed a significant resolution of their radiological parenchymal shadowing. No such change was seen in any patient in the placebo group. None of the subjects reported any adverse effects; all completed the study period without interruption.

BAL differential cell count

All the patients with sarcoidosis had a greater percentage lymphocyte count in their BAL (40±4.5%) pre-treatment than the normal population (7.1±1.8). Post-treatment, there was a marked decrease in BAL lymphocytosis in those patients receiving budesonide (from 45±1.1% to 25±1.7%). No such change was seen in the other two groups (fig. 1).

BAL immunocytological analysis

At the start of the study all sarcoid patients had higher proportions of macrophages expressing RDF1+, RDF7+ and RDF1+D7+ than the normal volunteers (table 1). After four months, inhaled budesonide produced a reduction in the proportion of RDF1+ cells (from 46.9±11.0% to 21.6±4.0%), with a dramatic decrease in the proportion of macrophages expressing RDF1+D7+ (21.4±3.1% to 8.5±2.0%). No significant effect on the RDF7+ population was seen, nor was any significant change seen in any macrophage subset in the sarcoid group on placebo or the normal volunteers (table 1).
**Table 1.** - Macrophage subsets before and after 16 week study period

<table>
<thead>
<tr>
<th>Subset</th>
<th>Before</th>
<th>After</th>
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<tbody>
<tr>
<td>RFD1+</td>
<td>15.4±4.9</td>
<td>17.5±6.4</td>
</tr>
<tr>
<td>RFD7+</td>
<td>17.3±2.9</td>
<td>26.3±7.0</td>
</tr>
<tr>
<td>D1+D7+</td>
<td>12.5±2.0</td>
<td>9.0±4.3</td>
</tr>
</tbody>
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Normals (n=10)
Sarcoids (Placebo) (n=5)
Sarcoids (Budesonide)

Note: The above represent percentage total number of morphologically identifiable macrophages (mean±sE)
* dose: 800 ugm bd via nebulizer; ** P<0.0001 compared to before treatment.

**Fig. 1.** - The mean percentage of morphologically defined lymphocytes in BAL before (B) and after (A) the four month study period is given for each group investigated. S. placebo = Sarcoid patients receiving placebo; S. steroid = sarcoid patients receiving inhaled budesonide. In all cases standard error of the mean is given in the text.

**Fig. 2.** - The autologous mixed lymphocyte reactivity expressed as mean stimulation index is given for various cell combinations tested before (B) and after (A) 4 months study period. Cells tested were bronchoalveolar lavage cells (BAL); peripheral blood mononuclear cells (PBM); and a mixture of the two (BAL/PBM). Results on each of the three study groups (normals; steroid patients receiving placebo (S. placebo); and sarcoid patients receiving budesonide (S. steroid) are compared. Mean and standard error for each result are given in the text.

**Autologous mixed lymphocyte reactions**

The AMLR experiments in this study were performed on unmanipulated BAL populations. Although it is accepted that this caused varied proportions of macrophages and lymphocytes to be present in different samples, this approach represents the only way in which the in situ capacity of these cells can be ascertained rather than the potential capacity produced by concentrations adjusted in vitro (see discussion). In normal subjects the incorporation of 3HT in peripheral blood mononuclear cells showed an eight-fold increase over mitomycin treated cultures (SI 7.8±0.8). This was markedly higher when compared to the SI of their lavage (0.7±0.2). This PBM reactivity in normals was suppressed by admixture with autologous BAL cells (SI 1.1±0.3). There was no significant change in any of these cell reactions after inhaled placebo (fig. 2).

All sarcoid patients exhibited a reduced reactivity in peripheral blood compared to normal (fig. 2). When PBM was admixed with autologous BAL prior to treatment further reduction in PBM AMLR was seen. After four months of treatment with inhaled budesonide there was a striking increase in PBM reactivity (from SI 1.6±0.1 to 6.6±1.7) as well as BAL reactivity (from SI 0.82±1.0 to
2.9±0.9). In addition, following budesonide, alveolar macrophages were much better stimulators of autologous peripheral blood mononuclear cells than were equivalent populations tested before treatment (from \( \text{SI} 0\pm0.5 \) to \( 3.16\pm1.1 \)).

No similar changes were seen in the sarcoid group receiving placebo except for an increase in PBM stimulation (from \( 3.02\pm0.45 \) to 5.06±0.10, fig. 2).

**Discussion**

Our results show that in the treatment of pulmonary sarcoidosis, inhaled steroids such as budesonide administered via a 750 cm\(^2\) spacer device in a relatively small dose of 800 \( \mu \)g twice daily achieves approximately 10% alveolar deposition and effective symptomatic relief with no adverse effects. Furthermore in just four months, the inhaled therapy was shown capable of modulating the tested features of the aberrant immunological reactions existent in the lung in this disease. Inhaled steroids not only produce a significant decrease in lavage lymphocytes but they concurrently produced a change in the phenotype and functional characteristics of the alveolar macrophage population.

Our study did not attempt to look at the antigen presenting capacity of alveolar macrophages from sarcoid patients or their response to exogenous mitogen as previous workers have done [16, 17]. We know from previous work [10] that consistent phenotypic and functional aberrations are present within the macrophage-like population of sarcoid lavage; in addition, the AMLR in these patients appears to be suppressed both systemically and locally in the lung [10]. Our aim was therefore to assess the way in which inhaled steroids could influence these local cellular reactions (without any manipulation) in sarcoidosis, and to correlate the observations with clinical efficacy.

Following budesonide treatment, there was a striking increase in PBM and BAL AMLR reactivity; in addition the alveolar macrophages from these treated patients were better stimulants of PBM in the AMLR. Such observations might be explained by the change in phenotype witnessed within the alveolar macrophage population subsequent to therapy. Specifically, there was a marked reduction in the proportion of RFD1+ macrophages also expressing RFD7+. As the cell populations in each lavage were not manipulated it is not possible from results presented here to identify the specific subset potentially responsible for these functional changes. However, by investigating the lavage populations as they appear in situ makes these observations directly relevant to the situation in vivo.

Our results therefore offer cogent evidence that within the lung of sarcoid patients studied a situation has developed where aberrations in macrophage-lymphocyte interaction are occurring in situ [10]. Furthermore it is clear that these aberrations can be reversed by inhaled corticosteroids. Whether this is a direct action of steroid on the macrophages or indirect action via effects on lymphocytes is unknown. Subsequent work [18] where macrophage subsets have been isolated and the functional capacity of equivalent concentrations has been compared, do demonstrate that cells with the phenotype RFD1+ RFD7+ can actively suppress mixed lymphocyte reactions. As inhaled budesonide is shown to dramatically reduce the proportion of this population within lavage, it may be by this route that the therapy tested here is effective in reversing aberrations of immunoregulation.

It could be argued that these observations were due not to inhaled budesonide but to the variable clinical course of sarcoidosis, and to the fact that the phenotype and presumably functional capacity of the macrophage population is known to alter during the course of the disease [11]. To limit such variables we recruited a homogenous group of patients for this study in the same stage of their disease in so far as clinical status, pulmonary function and radiological grading were concerned. In addition, the dramatic changes witnessed with inhaled budesonide, were not observed in the placebo group. It may appear from table 1 that the difference in pre-treatment percentage of RFD1+D7+ macrophages between the patient and placebo groups might have biased the results after treatment. This possibility is discounted by the normal group having an even smaller proportion of RFD1+D7+ macrophages before treatment which did not significantly change after the study period.

Interestingly these clinical and immunological changes were not accompanied by any significant improvement in chest X-ray (only 3 out of 10 patients treated showed clearing) and no improvement in lung function. Although in practice it is now recognised that clinical disease activity is not always paralleled by objective assessment, we suggest that perhaps a study period of four months using only relatively small doses of inhaled steroid may be too short to witness a marked improvement in these indices. It also goes without saying that changes at the cellular level would be expected to precede gross changes in pulmonary function.

In an 18 month study [19] on the effect of inhaled budesonide in pulmonary sarcoidosis, Selroos et al demonstrated a significant clinical improvement in symptoms and pulmonary function. Furthermore they observed a decrease in BAL lymphocytosis as well as normalisation of the increased T-cell CD4/CD8 ratio usually seen in active disease. Yet again no significant changes were observed in radiological appearances.

Systemic corticosteroids in varying doses (20–50 mg per prednisolone day) have also been shown to induce a shift in the distribution of T-cell subsets toward a lower CD4/CD8 ratio; a change observed by Crippens et al [4] to be independent of the persistent high lymphocytosis in lavage. Pinson et al [5] showed that in addition systemic steroids suppressed the exaggerated release of interleukin-2 by lung T-lymphocytes with consequent reduction in lung T-cell proliferation and suppression of the lymphocytic inflammation in sarcoidosis. Such action of steroid might also be expected to reduce the release of gamma interferon by T-cells [20]. As expression of the epitope seen by RFD1 monoclonal anti-body is promoted by gamma interferon [21], the effects of inhaled...
steroids on macrophages represented here may result indirectly from their initial action on the lymphocyte population.

Experience with inhaled steroids in pulmonary sarcoidosis is so far scanty. Inhaled therapy has mainly been used in the treatment of airway obstruction. Studies on asthmatic patients and healthy subjects [22] suggest that inhaled steroids produce only a minimal effect on the hypothalamic-pituitary-adrenal function. The dose of 1600 µg/day used in our study is in fact equivalent to 5.0 mg per day oral prednisolone in terms of ability to suppress plasma cortisol level [23]. Treatment with inhaled steroids would thus appear to be much safer than conventional systemic therapy.

We suggest that inhaled steroids have an initial role to play in the treatment of pulmonary sarcoidosis. Their safety would allow early treatment to be instituted in these patients in an effort to abort the potential persistence of granulomata in the lung parenchyma and the development of fibrosis.

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Les corticostéroides en inhalation peuvent moduler l’immunopathogéne de la sarcoïdose pulmonaire. M.A. Spiteri, S.P. Newman, S.W. Clarke, L.W. Poultër. RÉSUMÉ: Cette étude a investigué les effets des corticostéroides en inhalation sur le phenotype et la capacité fonctionnelle des macrophages obtenus par lavage broncho-alvéolaire chez des patients atteints de sarcoïdose pulmonaire. Les résultats sont mis en corrélation avec l’état clinique et l’efficacité thérapeutique. Chez dix patients symptomatiques atteints de sarcoïdose et préalablement non traités, montrant des anomalies radiologiques parenchymateuses et une fonction pulmonaire anormale, le budesonide en inhalation a été administré à la dose de 800 µg deux fois par jour par un Nebuhaler pendant 16 semaines. Un groupe placebo a inclus dix volontaires sains et cinq patients sarcoïdosiques avec des signes similaires à ceux du groupe traité. Les études de distribution médicamenteuse montrent qu’avec le schéma d’administration utilisé, 10% du médicament se déposent dans la région alvéolaire. Les dix patients sarcoïdosiques traités ont eu un soulagement symptomatique sans effets collatéraux. Trois de ces dix patients ont obtenu une résolution significative de leurs opacités radiographiques. On
n'a noté aucune modification significative de la fonction pulmonaire. Au niveau cellulaire, après 16 semaines, on relève une diminution significative de la lymphocytose dans le lavage avec, pendant la même période, une modification concomitante du phénotype et des caractéristiques fonctionnelles de la population de macrophages alvéolaires. Aucune modification similaire n'a été observée dans le groupe placebo. Nos résultats suggèrent que le budesonide en inhalation peut moduler des réactions immunologiques aberrantes qui existent dans le poumon de la sarcoidose pulmonaire, et provoquer un soulagement symptomatique concomitant sans effets collatéraux. On suppose que cet effet pourrait se produire par action sur la population locale de macrophages alvéolaires.