Cell-mediated immunity in pigeon breeders' lung: the effect of removal from antigen exposure

M.A. Johnson*, A. Nemeth, A. Condez**, S.W. Clarke*, L.W. Poulter**


ABSTRACT: Eighteen precipitin-positive pigeon breeders, thirteen symptomatic (SPB), with extrinsic allergic alveolitis (EAA), and five asymptomatic (APB), without lung disease, underwent bronchoalveolar lavage (BAL). Cytospins were prepared on which differential cell counts were performed. Immunocytological methods, using monoclonal antibodies, were performed to identify lymphocyte and macrophage subsets. Marked abnormalities in cell populations were observed in both groups but with no suggestion of differences between the groups. All subjects had a lymphocytosis in BAL (SPB 45%; APB 29%). These lymphocytes were almost exclusively T-cells. The cluster designation CD4/CD8 ratio was decreased (SPB 0.86; APB 1.13) and a significantly higher proportion of these cells than normal expressed UCHL1 (an antigen associated with the common leucocyte antigen complex) indicating immune commitment. In the macrophage population increased proportions of cells expressing antigens associated with interdigitating cells (RFD1+) and mature macrophages (RFD7+) were also abnormal. When six SPB patients were relavaged after isolation from pigeons for three weeks, there was a significant reduction in the lymphocytosis and in the proportion of UCHL1+ lymphocytes. This was accompanied by reductions in the percentage of macrophages expressing RFD1 and UCHL1. We suggest that EAA in pigeon breeders is associated with a cell-mediated immune response which is down-regulated by isolating patients from exposure to pigeon derived antigens.


Although the pathogenesis of extrinsic allergic alveolitis (EAA) has been considered to be an immune complex mediated tissue injury, current opinion considers that EAA reflects a local cell-mediated immune response [1-3]. Antigen is deposited in the lung and precipitating antibodies are present in the blood [1, 4]. However, in lung biopsy specimens polymorphonuclear cells are not predominant in the tissue reaction [2, 3], complement components are generally not found [2, 5] and vasculitis is not usually observed [2, 3]. Instead the disease is usually manifested by a patchy mononuclear infiltrate of lymphocytes, with few plasma cells and granulomas present in the majority of patients [1-3], all findings which suggest a cell-mediated immune response and not an immune complex mediated reaction.

Bronchoalveolar lavage (BAL) is now the most important technique used to investigate the immunopathology of the lung. In EAA there is almost invariably a striking lymphocytosis [6-8] with increased CD8+ lymphocytes [9-11]. Although most exposed individuals, including those with precipitating antibodies, do not develop EAA, BAL studies have shown that exposed asymptomatic subjects, including some without precipitating antibodies, may also have a lymphocytosis [10, 12]. Current information does little to explain the emergence of lung disease in some individuals but not in others, although it has been demonstrated that in those with disease, T-cells have in vitro suppressor as well as definite cytotoxic function, whilst healthy individuals have only suppressor activity [12]. To date, few investigators have focused on the role of the alveolar macrophage in the pathogenesis of EAA. Antigenic and foreign body material have been found in macrophages and giant cells of granulomas in the lung [4, 14]. HLA/DR antigens, important for effective antigen presentation by macrophages to T-cells, are expressed on most alveolar macrophages in EAA but in similar numbers to normal controls and patients with other interstitial lung diseases [15]. As it is well known that cells of the macrophage family are intermittently involved in the induction as well as the expression of the immune response, a detailed analysis of the macrophage population is warranted.

The aims of this study were to use a panel of
monoclonal antibodies to try to correlate cell membrane markers on both lymphocytes and macrophages with disease activity and clinical indices of EAA, and to determine which cell populations in patients are directly affected by exposure to antigens by repeat BAL after removal from antigenic exposure.

Subjects

Pigeon breeders

Eighteen pigeon breeders with specific precipitating antibodies to pigeon precipitins were selected for investigation. A detailed history was obtained of acute episodes and chronic symptoms of breathlessness and wheeze. A full clinical examination was performed. Pulmonary function measurements including forced expiratory volume in one second (FEV1), and forced vital capacity (FVC) were made with a Vitalograph Spirometer and the total diffusing capacity for carbon dioxide (DLco) by the single-breath technique. Posteroanterior (PA) chest radiographs were obtained.

Of the eighteen subjects, thirteen were symptomatic, with a history of acute episodes in seven patients, and all thirteen had chronic symptoms of breathlessness or wheeze. Six of the thirteen were using inhaled bronchodilators but none were on oral or inhaled steroids. All had impairment (>15% predicted) of either FVC or DLco and eight had abnormal chest radiographic shadowing. This group will subsequently be referred to in the text as SPB (symptomatic pigeon breeders).

Table 1. – Subject details, lung function % predicted (±SD)

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic pigeon breeders</th>
<th>Asymptomatic pigeon breeders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=13</td>
<td>n=5</td>
</tr>
<tr>
<td>Age</td>
<td>35.4±12.6</td>
<td>42.3±12.1</td>
</tr>
<tr>
<td>FEV1</td>
<td>87.7±26.1</td>
<td>100.2±25.6</td>
</tr>
<tr>
<td>FVC</td>
<td>85±19.7</td>
<td>102±19.5</td>
</tr>
<tr>
<td>TLco</td>
<td>77.1±26.1</td>
<td>109±14.7</td>
</tr>
<tr>
<td>Kco</td>
<td>78.3±22.8</td>
<td>103±4.1</td>
</tr>
<tr>
<td>Abnormal CXR</td>
<td>n=6</td>
<td>n=0</td>
</tr>
</tbody>
</table>

FEV1: forced expiratory volume in one second; FVC: forced vital capacity; TLco: total diffusing capacity for carbon monoxide; Kco: carbon monoxide transfer coefficient; CXR: chest X-ray.

Five subjects were asymptomatic with normal lung function and normal chest radiographs and will subsequently be referred to as APB (asymptomatic pigeon breeders). Table 1 gives subject details and table 2 gives details of exposure. All subjects underwent bronchoalveolar lavage (BAL). None had a past history of a viral illness in the preceding four weeks, or reported acute episodes in the three months prior to the study.

Six of the SPB were instructed after initial BAL to have no contact with their pigeons for a three week period. If a member of the household were to have contact with the pigeons, protective clothing was to be worn and to be removed before entering the house of the breeder or having any close contact. In this group BAL was repeated at the end of the three week period.

Table 2. – History of exposure in symptomatic and asymptomatic pigeon breeders

<table>
<thead>
<tr>
<th></th>
<th>Symptomatic pigeon breeders</th>
<th>Asymptomatic pigeon breeders</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=13</td>
<td>n=5</td>
</tr>
<tr>
<td>Number of pigeons</td>
<td>77.6±32</td>
<td>86.4±36</td>
</tr>
<tr>
<td>(1-120)</td>
<td>(120-240)</td>
<td>(240-360)</td>
</tr>
<tr>
<td>Years of exposure</td>
<td>21.5±9.4</td>
<td>16.0±7.6</td>
</tr>
<tr>
<td>(1-40)</td>
<td>(40-80)</td>
<td>(80-120)</td>
</tr>
<tr>
<td>Hours of exposure</td>
<td>2.3±0.8</td>
<td>2.42±0.65</td>
</tr>
<tr>
<td>per day</td>
<td>(1-4)</td>
<td>(1.5-3.0)</td>
</tr>
</tbody>
</table>

Normal controls

These consisted of five male and one female volunteers (age range 22-41 yrs), who underwent fibreoptic bronchoscopy and bronchoalveolar lavage. All volunteers were nonsmokers and none were on any regular medication.

All subjects gave written informed consent. Ethical approval was obtained for all lavage studies.

Bronchoalveolar lavage

This was performed in the lateral basal segment of the lower lobe of the right lung. Aliquots (3 x 60 ml) of sterile normal saline (buffered with sodium bicarbonate (NaHCO3) to pH 7.4), pre-warmed to 37°C, were introduced and the fluid gently aspirated after each aliquot and collected into sterile siliconized glass bottles maintained at 4°C. Mean volumes recovered were 86.6±15.7 ml and 82.3±17.3 ml in SPB and APB groups, respectively. The mean absolute numbers of cells collected were 5.75 x 10⁶/ml and 5.02 x 10⁶/ml in SPB and APB groups, respectively.

Processing of BAL fluid

The total cell count was determined from an aliquot of neat unfiltered BAL fluid in a modified Neubauer haemocytometer. Viability of the cells was assessed in all cases by Trypan blue exclusion and was always greater than 80%. Mucus strands were removed by filtering the fluid through a layer of sterile gauze. The fluid was then centrifuged at 450 g for 10 min and the supernatant decanted from the cell pellet. The cells were then washed twice with Hank's balanced salt solution (HBSS) and the cell suspension adjusted to a concen-
etration of $2-3 \times 10^5 \text{ml}^{-1}$. Cytospin slide preparations were obtained with 50–100 µl aliquots in a Shandon Cytospin II (Shandon Runcorn). The cytospin slides were then air dried for 1–2 h and half were stored. The remainder were fixed in a 1:1 chloroform:acetone mixture for 10 min. They were then air dried again for 1–2 h. All cytospin preparations were stored, wrapped in plastic cling film, at -20°C until use (one week to three months). Differential counts were performed immediately on cytospin preparations stained with May-Grunwald-Giemsa, a total of 300–600 cells being counted.

**Immunocytological analysis**

The panel of monoclonal antibodies used in the study are shown in table 3. The pattern of reactivity to cells within normal tissue has previously been characterized.

**Immunoperoxidase staining**

A standard protocol for the indirect immunoperoxidase method was used [22]. The cytospin preparations were first incubated with each monoclonal antibody for one hour with phosphate buffered saline (PBS) in an optimal dilution. After washing in PBS, the cytospins were incubated for 45 min with peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO immunoglobulin a/s Denmark). After further washing in PBS the preparations were developed for 5–15 min in freshly prepared D-aminobenzidine (DAB) and hydrogen peroxide solution. The cytospins were counter-stained in haemotoxylin for 20–40 s, dehydrated, mounted with cover slips in DPX and examined under a light microscope using an oil-immersion objective. Three hundred cells were counted in successive high power fields and assessed as being either positive or negative (background staining only) for each antibody used, with the result expressed as the percentage positive. Positive controls (tonsil sections) were used with each antibody in all staining sessions. Negative controls (PBS incubation instead of first layer monoclonal antibody) were used for each patient to assess background staining.

**Double immunofluorescence**

A standard technique for the simultaneous identification of two cellular antigens was used [23]. Fifty µl of each relevant monoclonal antibody was mixed and incubated with the cytospin preparations for 1–2 h. After washing in PBS a 50 µl mixture of goat anti-mouse immunoglobulin M tetraethy l rhodamine isothiocyanate (TRITC) and goat anti-mouse immunoglobulin G fluorescein, isothiocyanate (FITC) (both from Southern Technology Associates) was added as a second layer and incubated for 45 min. After PBS washing the cytospin slides were mounted with cover slips in PBS/glycerol and examined immediately with a fluorescence micro-

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Cluster designation</th>
<th>Mol wt Antigen</th>
<th>Source in normal tissue</th>
<th>Specificity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RFDR1</td>
<td>–</td>
<td>28/33 KD</td>
<td>RFHSM</td>
<td>Framework epitope on HLA-DR molecule</td>
<td>[16]</td>
</tr>
<tr>
<td>RFD1</td>
<td>–</td>
<td>28/33 KD</td>
<td>RFHSM</td>
<td>Integridigating</td>
<td>[17]</td>
</tr>
<tr>
<td>RFD7</td>
<td>–</td>
<td>77 KD</td>
<td>RFHSM</td>
<td>Mature macrophages</td>
<td>[17]</td>
</tr>
<tr>
<td>OKT4</td>
<td>CD4</td>
<td>65 KD</td>
<td>ORTHO T-cells</td>
<td>Helper-inducer</td>
<td>[18]</td>
</tr>
<tr>
<td>RFT8</td>
<td>CD8</td>
<td>30–32 KD</td>
<td>RFHSM</td>
<td>Suppressor-</td>
<td>[19]</td>
</tr>
<tr>
<td>RFT2</td>
<td>CD7</td>
<td>40 KD</td>
<td>RFHSM</td>
<td>Pan T</td>
<td>[20]</td>
</tr>
<tr>
<td>UCHL1</td>
<td>–</td>
<td>180 KD</td>
<td>P. Beverley (Middlesex Hospital)</td>
<td>Leucocyte common antigen reactive antibody</td>
<td>[21]</td>
</tr>
</tbody>
</table>

RFHSM: Royal Free Hospital School of Medicine.
scope. Two hundred cells were counted in successive high powered films, first under phase contrast and then using appropriate barrier filters for TRITC and FITC. The number of cells fluorescing either red only, green only, or both were counted and expressed as a percentage of total cells. Positive and negative controls were always performed.

Statistics

Statistical analysis was performed when appropriate using the Wilcoxon Rank-Sum test for non-parametric data.

Results

Differential cell counts

Both the APB and the SPB had markedly raised proportions of lymphocytes in BAL when compared to samples from control studies (table 4). There were no significant differences between the groups (APB 29±18.8%; SPB 43.5±13.7%) (fig. 1). Cell counts did not correlate significantly with any clinical parameter.

Table 4. – Differential cell counts

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>SPB</th>
<th>APB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocytes</td>
<td>5.2±4</td>
<td>43.5 (13.7)</td>
<td>29.0 (18.8)</td>
</tr>
<tr>
<td>Macrophages</td>
<td>91±10.1</td>
<td>44.6 (17.1)</td>
<td>54.0 (25.3)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>2±2.1</td>
<td>10.8 (10.3)</td>
<td>16.8 (24.4)</td>
</tr>
</tbody>
</table>

% total cells (±so), APB and SPB: asymptomatic and symptomatic pigeon breeders, respectively.

Immunocytological analysis

A) Macrophage-like cells. In normal subjects and in both groups of pigeon breeders the majority of cells of the macrophage morphology in the lavage fluid expressed HLA-DR (Normal 88±19.3%; SPB 68.2±27%; APB 70.8±24.3%).

A large proportion of macrophages stained with RFD1 in both pigeon breeder groups with no significant difference between the groups (APB 64.2±24%; SPB 63.5±27.3%). This proportion was significantly higher in both groups than that found in normal subjects (fig. 2).

The proportion of RFD7 positive cells was higher in breeders than in normal subjects (APB 64±24.8%; SPB 43.4±21.3%). This was statistically significant for the APB but not for the SPB but significant when the two groups of breeders were combined (fig. 2).

A substantial proportion of macrophages in pigeon breeders showed positivity with both McAb's RFD1 and RFD7 and this was significantly higher than normal subjects. There was no difference between APB and SPB (APB 37.8±6.8; SPB 35±11.3) (fig. 3).

![Fig. 1. Percentage BAL lymphocytes and CD4/CD8 ratio in pigeon breeders and normal subjects. BAL: bronchoalveolar lavage; APB, SPB: asymptomatic and symptomatic pigeon breeders, respectively.](image)

![Fig. 2. Percentage of total macrophages (a) RFD1 positive; (b) RFD7 positive in pigeon breeders and normal subjects. APB, SPB: asymptomatic and symptomatic pigeon breeders, respectively.](image)

![Fig. 3. Percentage of total macrophages RFD1/RFD7 positive in pigeon breeders and normal subjects. APB, SPB: asymptomatic and symptomatic pigeon breeders, respectively.](image)
The proportion of macrophages expressing UCHL1 was significantly raised in both groups of breeders when compared to the normal control population (APB 49.3±34.3%; SPB 43.7±22.2%) (fig. 4).

**Relationship between immunocytological results and specific indices of clinical disease**

There was no correlation between duration of exposure or value of pulmonary function measurement and expression of any of the markers studied. Proportions of cells expressing RFD1, RFD7, UCHL1, CD7 and CD4/CD8 ratios were equivalent irrespective of whether patients exhibited clinical symptoms of EAA.

**Repeat BAL following removal from antigen exposure**

In the SPB who underwent repeat BAL after three weeks isolation from their pigeons there was a reduction in lymphocytosis but no change in CD4/CD8 ratio. The proportion of lymphocytes expressing UCHL1 also declined. In the macrophage population the number of RFD1 positive macrophages fell as did the number of macrophages expressing UCHL1 (fig. 6). There was no significant change in any of the other measured parameters.

**Discussion**

This study shows that both lavage lymphocytes and macrophage populations from precipitin-positive pigeon breeders are quantitatively and qualitatively abnormal, not only in patients with symptoms of disease but also in a group of asymptomatic breeders. Within the macrophage population significant increases in cells staining with the subset markers RFD1 and RFD7 were observed and the proportion expressing the two markers...
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simultaneously was also elevated. In addition a significantly increased number of macrophages expressed UCHL1.

In the lymphocyte population there was a significant lympholytosis in the lavage, with increased CD8+ T-cells. There were also increased proportions of CD7+ cells and a substantial increase in lymphocytes expressing UCHL1. The latter is considered to be part of the common leucocyte antigen complex and is expressed following mitogenic or antigenic stimulation [21]. It is within this population that the memory cell pool is considered to reside. The CD7 antigen, although present on all T-cells, is expressed in greater concentration on blast cells [20]. Similar changes were present both in patients with symptoms and the asymptomatic breeders investigated. Such a result suggests that other factors, probably environmental, contribute to the exacerbation of symptoms as immunological abnormalities alone do not distinguish symptomatic from asymptomatic patients.

The immunocytochemical findings, however, do add further evidence supporting the current consensus that EAA reflects a local cell-mediated immune response. Specifically few B-cells were identified, there was evidence of lymphocyte stimulation (indicated by increased proportions of CD7+ and UCHL1+ cells) and a concurrent increase of macrophage-like cells expressing epitopes associated with antigen presenting cells (RFD1 expression). The observed macrophage abnormalities are not unique to EAA. Similar increases in macrophages expressing RFD1+ have been reported in both sarcoidosis [24, 25] and cryptogenic fibrosing alveolitis (CFA) [26]. Increased RFD7+ expression has been observed in CFA [26]. In addition, increased proportions of cells expressing both markers to a similar degree have been reported in sarcoidosis with smaller increases having been observed in CFA. The functional significance of double expression RFD1+/RFD7+ is unknown. They may represent transitional forms between RFD7+ and RFD1+ cells. This appears to be unlikely as small numbers appear in normal lavage fluid, where presumably there is no stimulus for change. There is some evidence from functional studies in patients with sarcoidosis to suggest that they may exert a suppressive influence on lymphocytes [27]. We have observed for the first time a high proportion of macrophages expressing UCHL1. In the lymphocyte population this marker is associated with immune commitment [21] and may have a similar implication in the macrophage population.

The observed differences following removal from antigenic exposure may provide important clues for further investigation. We observed substantial decreases in UCHL1+ lymphocytes in isolated SPB. This may represent a preferential loss of these lymphocytes and the possibility that lymphocytes naturally arriving in the lung and not being stimulated following removal from antigenic exposure dilute the primed population. The population of RFD1+ macrophages is also reduced and this may result in reduced lymphocytic stimulation resulting in fewer UCHL1+ lymphocytes. Alternatively, reduced lymphocyte activation may result in fewer lymphokines being released including interferon, the latter being a documented promoter of RFD1 expression [28]. These mechanisms are clearly not mutually exclusive and in combination may act synergistically to down-regulate the immune response. The observation of reduced populations of UCHL1+ macrophages following removal from antigenic exposure cannot be explained at present as the function of these cells is unknown.

Our observation of a reduction in BAL lymphocytes after removal from exposure is in contrast to some previous reports [8], whilst confirming others [7, 29] where a reduction in lymphocytes was reported from 2–12 months after removal from antigen. In the present study this decrease caused no change in the CD4/CD8 ratio. Any differences may be accounted for by the timing of re-investigation. We deliberately chose a short period of isolation from antigenic exposure to increase patient compliance.

In conclusion, our findings suggest lavage is a helpful tool in investigating the disease process in EAA. We have identified clear-cut immunocytochemical abnormalities with a different profile to other interstitial lung diseases which have been investigated to date. It is suggested that the changes observed after removal from antigen are directly affected by exposure and may be helpful in monitoring patient compliance.

References