Inhalation challenge in pigeon breeder's disease: BAL fluid changes after 6 hours


ABSTRACT: The purpose of this study was to determine the cellular events occurring 6 h after bronchial inhalation challenge, in a group of pigeon breeders. Twelve subjects agreed to undergo challenge, either with nebulized pigeon serum (PS) (n=10), or with saline (n=2). The development of characteristic symptoms was used to detect a positive response in combination with monitoring tests (white blood cell count (WBC), body temperature, and spirometry). An initial bronchoalveolar lavage (BAL) was performed before inhalation challenge, and a further BAL on the previously spared right middle lobe subsegment or lingula 6 h after challenge. Paired bronchoalveolar lavage fluid (BALF) samples were analysed for urea, albumin, total and differential WBC, interleukin-1 and interleukin-2.

There was a significant increase in total cells, lymphocytes, T-lymphocytes, and neutrophils; numbers/ml of BALF in responders. A monitoring score reflecting severity of patient response correlated with an increase in all cell types (p<0.05).

In conclusion, responders developed both a BALF lymphocytic and neutrophilic "alveolitis" following inhalation challenge, the degree of BALF "alveolitis" correlating with the severity of patient response.

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Extrinsic allergic alveolitis (EAA) generally results from inhalation of organic material by sensitized persons [1], and is characterized by chronic granulomatous inflammation in the distal lung [2]. Because of limited access to human material, animal models of EAA have provided a means of assessing the response to inhaled antigen, as well as providing some insight into the mechanisms involved in human disease.

The earliest pathological lesion observed in the animal model 4–6 h after inhalation of particulate antigen, is an acute inflammatory alveolitis characterized by parenchymal haemorrhage and the accumulation of polymorphs [3]. The production of chronic granulomatous inflammation by inhalation challenge with antigen has been more difficult, requiring the use of particulate matter, or soluble antigen, or soluble antigen attached to particulate carriers [4]. More recently, Peterson et al. [5] produced chronic pulmonary inflammation in immunized rabbits by repeated aerosol exposure to soluble antigen. Development of the inflammatory response was immunospecific, and could not be transferred to normal recipients using large quantities of immune serum, suggesting a cell-mediated reaction.

The available data from human studies suggest that the pathogenesis of EAA involves either immune complex disease, or cell-mediated immunity, or a combination of both these mechanisms [1, 6]. Because of the delay in presentation to hospital, histology associated with acute disease is rare [7, 8]. Following repeated exposure to antigen, the chronic lesion of EAA becomes established, and is characterized by a mononuclear cell infiltrate, interstitial granulomas and fibrosis [9].

Several studies have demonstrated that the acute inflammatory alveolitis is mediated by humoral mechanisms [3, 8, 10]. In contrast, immunological investigations have suggested that the chronic phase was mediated by cellular immune mechanisms [11, 12]. More recently, a number of investigators have inferred that there is an active cellular immune response during acute disease in the human and in the animal model [3, 13].

A number of previous studies have investigated cellular and humoral abnormalities in BALF in subjects with EAA compared to asymptomatics, normals, and patients with other interstitial pulmonary disorders. These have included the presence of a lymphocytic alveolitis [11, 12], changes in T-lymphocyte subsets [10–12], and levels of BALF cytokines and soluble receptors [13].

The purpose of this study was to determine the early cellular events and changes in secretory substances occurring after bronchial inhalation challenge, in a group of symptomatic (responders) and asymptomatic (non-responders) pigeon breeders, and, more specifically, to determine changes in: 1) BALF cell profile; 2) BALF urea and albumin; and 3) BALF levels of interleukin-1 (IL-1) and interleukin-2 (IL-2).
Materials and methods

Study population

Twelve pigeon breeders agreed to undergo inhalation challenge, either with nebulized pigeon serum or saline. Three subjects (JM, BSM, GM) gave no history to suggest episodes of allergic alveolitis, and had normal chest radiographs and lung function tests (table 1). Two further subjects (BW, MJ) were known to have pigeon breeders’ disease (PBD) on the basis of history, restriction of physiological assessment, radiographic and biopsy evidence of interstitial disease, and a lymphocytic alveolitis on a previously performed bronchoalveolar lavage (BAL) (table 1).

The remaining subjects (AW, MST, BH, DW, RO, JB and AM) gave histories to suggest at least three acute episodes of EAA within the last six months, but none within one month prior to the study. Five of these subjects had abnormal chest radiographs, and three had decreased lung volumes with reduced gas transfer (table 1). All subjects studied had been exposed to pigeon antigens for a similar period of time.

Subjects were categorized as "responders or "non-responders", on the basis of inhalation challenge alone. All had been naturally exposed to pigeon antigens in the 24 h prior to admission to hospital.

All aspects of the study were approved by the local Ethics Committee and written informed consent obtained from all subjects.

Bronchoalveolar lavage

Segmental BAL was carried out according to a standard protocol using an Olympus BF IT10 fiberoptic bronchoscope. All subjects were premedicated with 0.6 mg atropine i.m., lorazepam 1 mg orally, and 1-2 ml of a fentanyl/droperidol mixture i.v. (fentanyl 50 μg·ml⁻¹, droperidol 2.5 μg·ml⁻¹). Topical anaesthesia of the pharyngeal mucosa and the vocal cords was achieved with an amethocaine lozenge (60 mg given 30 min before the procedure), 1% xylocaine spray, and 2-4 ml of 4% lignocaine squirted above the vocal cords under direct vision. The bronchoscope was passed via the mouth and, following general inspection of the bronchial tree, was wedged in the medial subsegment of the right middle lobe, as return of cells and fluid is greatest from this location [14]. Buffered lung lavage solution at 37°C (pH 7.4, 0.672% w/v sodium chloride, 0.0186% w/v potassium chloride and 0.25% w/v sodium bicarbonate) was introduced into the lungs in 30 ml aliquots. The fluid was gently aspirated by hand suction, following instillation at 90 and then 180 ml. In all cases, between 25-60% of administered fluid was recovered. The procedure was carried out with constant cardiac monitoring, ear-lobe oximetry, and with an intravenous line in position. Supplemental oxygen was given via nasal prongs at 4 l·min⁻¹ in all cases, both during and for 2 h after the bronchoscopy.

Bronchial inhalation challenge was performed within one hour of the initial BAL and followed 6 h later by a second BAL. Neuroleptanalgesia was not used on this occasion. Arterial blood gases were taken from subjects on air, half an hour before the second lavage. In all cases the oxygen tension was greater than 75 mmHg (10 kPa). Subjects were premedicated locally with 2 ml of 2% lignocaine above the vocal cords, and boluses of 1 ml of 1% lignocaine, if needed, within the bronchial tree. The bronchoscope was wedged in the previously spared subsegment of the right middle lobe, unless the orifice of the right middle lobe was obviously inflamed and oedematous. This occurred in only one case (BH), and then the lingula was employed. In all subjects, a limited lavage procedure, using a volume of 120 ml of buffered saline, was introduced in 30 ml aliquots, and gently aspirated following instillation of 120 ml. Throughout the procedure, oxygen was delivered via nasal prongs and the oxygen saturation, as measured by ear oximeter, was kept above 90%.

Bronchial inhalation challenge

Following an initial BAL, subjects were challenged one hour after BAL with either pigeon serum (AW, RO, DW, JB, MS, RO, BSM, JM, GM, AM), or saline (BW, MJ). Those challenged with saline already had clinically proven PBD and, as such, provided negative disease controls where a lack of response was predicted.

Bronchial inhalation challenge was carried out according to a modified version of the protocol established by Perry and Hutchcroft [15]. Pooled pigeon serum, which had previously been filtered, sterilized and heated to 56°C for 30 min, was used as the test extract. Concentrations of 1/100 pigeon serum were made up in normal saline. Test extracts were delivered via a Hudson nebulizer, driven by oxygen at 8-10 l·min⁻¹. Before each challenge, skin prick testing was carried out in order to exclude a severe immediate hypersensitivity response [16]. Exposure times were 30, 60 and 120 s, with 10 min intervals between, during which the forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) were measured. When there was no clinical reaction, inhalation of test material was continued to complete a total of 10 min of full antigen exposure. Test solutions were delivered via a mask, in which the reservoir had been taped over to prevent subjects from identifying whether they had inhaled pigeon serum or saline. Thus, challenges were administered in a blind-manner to the subject. All inhalation tests were carried out in a single cubic, which could be well ventilated at the end of each procedure.

Monitoring tests

Following inhalation challenge, patients were visited regularly throughout the next 24-36 h, or until any positive response subsided. Respiratory and/or systemic symptoms that developed, as well as new clinical
Table 1. - Clinical data and results of monitoring tests in responders and non-responders following inhalation challenge

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age yrs</th>
<th>Mean exposure yrs</th>
<th>Pigeon Serum precipitins</th>
<th>Lung function</th>
<th>CXR</th>
<th>Temp °C</th>
<th>Peripheral neutrophils x10^3-mm^3</th>
<th>Peripheral lymphocytes x10^3-mm^3</th>
<th>%FVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>R AW**</td>
<td>48</td>
<td>14</td>
<td>30</td>
<td>++</td>
<td>Normal</td>
<td>Normal</td>
<td>+2.0</td>
<td>+4.72</td>
<td>+230</td>
</tr>
<tr>
<td>R JB*</td>
<td>47</td>
<td>3</td>
<td>37</td>
<td>-</td>
<td>Restriction Tlco 50%</td>
<td>Basal infiltrate</td>
<td>+1.3</td>
<td>+5.48</td>
<td>-620</td>
</tr>
<tr>
<td>R AM</td>
<td>51</td>
<td>2</td>
<td>30</td>
<td>+++</td>
<td>Normal</td>
<td>Mid/lower zone infiltrate</td>
<td>+0.7</td>
<td>+4.12</td>
<td>-630</td>
</tr>
<tr>
<td>R BH</td>
<td>42</td>
<td>10</td>
<td>40</td>
<td>++</td>
<td>Normal</td>
<td>Normal</td>
<td>+1.5</td>
<td>+4.12</td>
<td>-20</td>
</tr>
<tr>
<td>R RO</td>
<td>40</td>
<td>30</td>
<td>40</td>
<td>+++</td>
<td>Restriction Tlco &lt;50%</td>
<td>Mid/lower zone infiltrate</td>
<td>+2.3</td>
<td>+11.24</td>
<td>-530</td>
</tr>
<tr>
<td>R DW</td>
<td>54</td>
<td>8</td>
<td>50</td>
<td>+++</td>
<td>Restriction Tlco 55%</td>
<td>Mid/lower zone infiltrate</td>
<td>+2.2</td>
<td>+3.13</td>
<td>-60</td>
</tr>
<tr>
<td>R MST*</td>
<td>48</td>
<td>20</td>
<td>40</td>
<td>++</td>
<td>Normal</td>
<td>Basal infiltrate</td>
<td>+0.9</td>
<td>+1.37</td>
<td>-120</td>
</tr>
<tr>
<td>NR BW</td>
<td>40</td>
<td>19</td>
<td>50</td>
<td>+++</td>
<td>Nodular shading both lungs</td>
<td>+0.5</td>
<td>-0.10</td>
<td>+100</td>
<td>-10</td>
</tr>
<tr>
<td>NR MJ</td>
<td>37</td>
<td>5</td>
<td>40</td>
<td>++</td>
<td>Nodular shading lower zones</td>
<td>+0.6</td>
<td>+0.30</td>
<td>+70</td>
<td>-5</td>
</tr>
<tr>
<td>NR BSM</td>
<td>42</td>
<td>10</td>
<td>20</td>
<td>+++</td>
<td>Normal</td>
<td>Normal</td>
<td>+0.2</td>
<td>+0.20</td>
<td>+50</td>
</tr>
<tr>
<td>NR JM</td>
<td>45</td>
<td>6</td>
<td>30</td>
<td>+</td>
<td>Normal</td>
<td>Normal</td>
<td>+0.5</td>
<td>+0.60</td>
<td>+50</td>
</tr>
<tr>
<td>NR GM</td>
<td>42</td>
<td>4</td>
<td>20</td>
<td>-</td>
<td>Normal</td>
<td>Normal</td>
<td>+0.3</td>
<td>-0.06</td>
<td>+20</td>
</tr>
</tbody>
</table>

Monitoring test results represent the maximum change following challenge. Precipitating antibody against pigeon serum and droppings represented as strong (+++) and moderate (++) and weak (+). R: responders; NR: non-responders; Tlco: predicted diffusing capacity for carbon monoxide; *: smokers; **: ex-smoker; CXR: chest radiographic appearances before inhalation challenge; FVC: forced vital capacity.
findings, were recorded. Pulse, temperature and blood pressure readings were taken immediately before challenge, then hourly for 12 h, and six hourly for a further 24 h. Measurement of FEV₁ and FVC were carried out using a dry Vitalograph spirometer at similar intervals of time. Repeat BAL employing saline warmed to 37°C has not been found to alter pulmonary function tests significantly and, therefore, spirometry would seem to be a valid monitoring test following bronchial inhalation challenge [17]. Recording of symptoms and signs were documented by an investigator, who was unaware of the test extract used in each patient.

Venous blood was drawn immediately before challenge and then at 6, 12 and 24 h. Total and differential leucocyte counts were measured on a Coulter counter.

Subjects were classified as responders to bronchial inhalation challenge when at least four of the following were recorded: 1) development of "flu-like" symptoms 6-12 h following challenge; 2) an increase in absolute numbers of peripheral neutrophils >2,500 mm⁻³; 3) a fall in peripheral lymphocytes >500 mm⁻³; 4) an increase in body temperature 0.6°C; and 5) a fall in FVC of 20% or more (table 1). Non-responders did not experience symptoms or have more than one of the above abnormalities present (table 1). Similar monitoring tests have been shown to validate a positive alveolar response [18].

**Cellular analysis**

Lavage fluid was immediately filtered through a single layer of sterile surgical gauze to remove excess mucus, and was centrifuged (400 x g, at 4°C for 5 min) to separate cellular and protein components. The supernatant was decanted and stored at -70°C in a number of sterile vials for subsequent protein analysis. The cell pellet was washed with phosphate buffered saline (pH 7.4), was resuspended in 2 ml of PBS. The cells were analysed for total numbers, using a haemocytometer and phase contrast microscopy. Differential cell count was performed in a freshly prepared smear following staining with Jenner-Giemsa stain. At least 300 cells were counted in all cases by the random field method.

In order to estimate T-lymphocyte subsets, BALF cell pellet was spun down over ficoll-hypaque gradient at 1,350 x g for 20 min at 4°C. The interface layer was aspirated and washed with 10 ml PBS. The leucocytes were resuspended in PBS with 5% bovine serum albumin (BSA), and cell numbers determined with a haemocytometer. Cell density was then adjusted to 2 x 10⁶ cells/ml with BSA. Two hundred microlitres of the cell suspension were incubated with 5 ml of monoclonal antibodies (OKT3, OKT4, OKT8 Ortho Diagnostics Ltd) at 4°C for 30 min. Cells were then washed twice with PBS/BSA to remove excess monoclonal antibodies, and incubated at 4°C for 30 min with 100 μl of fluoroscinc isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin G (IgG) (Miles Laboratories Ltd). Cells were again washed twice with PBS/BSA and 300 cells counted in all cases. Processing of cells was normally completed within 4 h of removal from the lung.

**Measurement of soluble factors in BALF**

**Urea assay.** Urea estimation in BALF was performed using a modification of a commercially available assay system (Sigma Diagnostics, Poole, Dorset, UK). The principal of the assay was based on a urease Berthelot reaction. Briefly, urea is hydrolysed by urease to ammonia, which reacts with alkaline hypochlorite and phenol in the presence of sodium nitroprusside, to form indophenol blue, and can be measured spectrophotometrically between 500-650 nm. The normal procedure was modified to enable the assay to be carried out in microtitre plates, and the resulting colour measured using a Titertek Multiscan MC plate reader at 620 nm as described previously [19]. The volume of recovered epithelial lining fluid (ELF) was, thus, derived from the following formula:

\[
\text{ELF} = \frac{\text{BALF volume}}{\text{serum urea}} \times \text{serum urea}
\]

**Albumin assay.** A sandwich, double antibody, enzyme-linked immunosorbtant assay (ELISA) was employed using microtitre plates as described previously [24]. Briefly, each well on the plate (Nunc Immunoplate F) was sensitized with "capture" antibody (200 μl of 1/2,000 sheep anti-human serum albumin (HSA), Sigma, Poole, UK, in 0.05 M carbonate-bicarbonate buffer pH 9.5) at 4°C for 16 h. Serial dilutions of BALF were incubated in sensitized wells (35 min at room temperature (RT)). Bound albumin was detected using rabbit anti-HSA (200 μl of 1/1000, 35 min RT) followed by alkaline phosphatase conjugated sheep anti-rabbit IgG (200 μl 1/100, 35 min RT).

Enzyme function was determined using p-nitrophenol phosphate (Sigma 104, 200 μl at 1 g₂³ in 10% diethanolamine pH 9.8). Released p-nitrophenol was determined at 405 nm.

BALF albumin was determined from the intercept of the linear part of the BALF albumin curve with a reference plot derived from human serum with a known albumin concentration (SPS-01 batch 880, PRU, Sheffield, UK).

**IL-1 and IL-2 assays.** IL-1 and IL-2 were determined in BALF using a modified ELISA as described previously by Reynolds et al. [13]. In brief, microtitre plates (Flow Laboratories, High Wycombe, UK) were coated, overnight at 4°C, with 200 μl of 1 in 10 dilution of BALF or IL-1 and IL-2 standards in 0.05 M carbonate/bicarbonate buffer pH 9.5. Wells were post-coated with 200 μl of 5% BSA in the pH 9.5 buffer (30 min at RT). For IL-1, rabbit anti-IL-1 (Cistron Biotechnology) 100 μl at 1/1000 was added and incubated for 1 h at RT. For IL-2, rabbit anti-IL-2 (Collaborative Research) 200 μl at 1/1000 were added and incubated for 1 h at RT. Bound IL-1 or IL-2 were assayed by adding 100 μl of 1/1000 sheep anti-rabbit IgG conjugated to alkaline phosphatase (Sigma Ltd) (1 h at RT) and enzyme activity determined with 100 μl
INHALATION CHALLENGE IN PIGEON BREEDERS’ DISEASE

of p-nitropheono l phosphate (Sigma 104) at 1 g·f1 in 10% diethanolamine, pH 9.8. Reactions were stopped by adding 50 μl of 5 M sodium hydroxide, and colour development measured at 405 nm.

Inhalation challenge score

Following inhalation challenge, a score was given for the maximum change in each physiological measurement: absolute body temperature, and in reported symptoms (table 2).

Statistics

The Mann Whitney U-test was used to compare maximum change in cell types following bronchial inhalation challenge. Changes in soluble factors were determined by employing the Wilcoxon signed rank test. Correlation of monitoring scores with change in cell numbers was analysed using the Spearman rank correlation coefficient.

Results

Inhalation challenge

Seven patients challenged with pigeon serum (table 1) responded with a positive reaction. Those patients known to have PBD before the start of the study (BW and MJ), who were given nebulized saline, had a negative response. The remaining subjects (JM, BS, GM) failed to respond to 1 in 100 nebulized pigeon serum (table 1). Maximum changes occurred in peripheral neutrophil counts at 6 h, returning to normal within 24 h, while maximum changes in body temperature and spirometry were measured at 7-8 h.

Bronchoalveolar lavage fluid

Cell profile. Changes in absolute numbers of cells in responders and non-responders following inhalation challenge are depicted in table 3. There was a significant increase in

Table 2. - Monitoring scores allotted according to symptoms reported, body temperature and changes in physiological variables as shown

<table>
<thead>
<tr>
<th>Score</th>
<th>Symptom</th>
<th>Body temp °C</th>
<th>Peripheral Neutrophils per mm³</th>
<th>Peripheral Lymphocytes per mm³</th>
<th>FVC % fall</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>&gt;37.0</td>
<td>&gt;2000</td>
<td>&gt;500</td>
<td>&gt;15</td>
</tr>
<tr>
<td>1</td>
<td>Presence of at least one of the following: Dyspnoea, Myalgia, Arthralgia, Chills, Headaches</td>
<td>&gt;37.9</td>
<td>&gt;3000</td>
<td>&gt;600</td>
<td>&gt;25</td>
</tr>
<tr>
<td>2</td>
<td>&gt;37.6</td>
<td>&gt;2500</td>
<td>&gt;550</td>
<td>&gt;20</td>
<td></td>
</tr>
<tr>
<td>3*</td>
<td>&gt;37.9</td>
<td>&gt;3000</td>
<td>&gt;600</td>
<td>&gt;25</td>
<td></td>
</tr>
<tr>
<td>4*</td>
<td>&gt;38.1</td>
<td>&gt;3500</td>
<td>&gt;650</td>
<td>&gt;30</td>
<td></td>
</tr>
<tr>
<td>5*</td>
<td>&gt;38.4</td>
<td>&gt;4000</td>
<td>&gt;700</td>
<td>&gt;35</td>
<td></td>
</tr>
</tbody>
</table>

*: Higher scores reflect increasing clinical severity.

Table 3. - Change in BALF cell profile·m⁻³ in responders and non-responders before and after inhalation challenge

<table>
<thead>
<tr>
<th>Subject</th>
<th>Total cells x10⁶</th>
<th>Macrophages x10⁶</th>
<th>Lymphocytes x10³</th>
<th>T-lymphocytes x10³</th>
<th>Neutrophils x10⁴</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>R AW</td>
<td>1.19</td>
<td>1.89</td>
<td>0.61</td>
<td>0.67</td>
<td>1.10</td>
</tr>
<tr>
<td>R JB</td>
<td>1.61</td>
<td>1.99</td>
<td>3.50</td>
<td>2.00</td>
<td>1.09</td>
</tr>
<tr>
<td>R AM</td>
<td>1.45</td>
<td>2.05</td>
<td>1.46</td>
<td>0.57</td>
<td>1.29</td>
</tr>
<tr>
<td>R BH</td>
<td>1.13</td>
<td>2.04</td>
<td>4.37</td>
<td>1.81</td>
<td>0.63</td>
</tr>
<tr>
<td>R RO</td>
<td>0.70</td>
<td>4.89</td>
<td>2.00</td>
<td>1.17</td>
<td>0.38</td>
</tr>
<tr>
<td>R DW</td>
<td>1.37</td>
<td>1.93</td>
<td>2.48</td>
<td>0.59</td>
<td>1.09</td>
</tr>
<tr>
<td>R MST</td>
<td>0.25</td>
<td>0.71</td>
<td>0.58</td>
<td>1.08</td>
<td>0.12</td>
</tr>
<tr>
<td>Mean</td>
<td>1.1</td>
<td>2.22</td>
<td>2.14</td>
<td>1.20</td>
<td>0.81</td>
</tr>
<tr>
<td>(±sd)</td>
<td>(0.47)</td>
<td>(1.27)</td>
<td>(1.42)</td>
<td>(0.62)</td>
<td>(0.44)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>p</th>
<th>&lt;0.05</th>
<th>&gt;0.1</th>
<th>&lt;0.003</th>
<th>&lt;0.02</th>
<th>&lt;0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>RW</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R AW</td>
<td>1.93</td>
<td>1.39</td>
<td>3.32</td>
<td>9.62</td>
<td>1.56</td>
</tr>
<tr>
<td>R JB</td>
<td>1.80</td>
<td>1.38</td>
<td>2.13</td>
<td>1.18</td>
<td>1.56</td>
</tr>
<tr>
<td>R AM</td>
<td>1.34</td>
<td>1.74</td>
<td>1.42</td>
<td>0.79</td>
<td>1.14</td>
</tr>
<tr>
<td>R BH</td>
<td>1.24</td>
<td>1.45</td>
<td>6.54</td>
<td>7.55</td>
<td>0.56</td>
</tr>
<tr>
<td>R RO</td>
<td>1.34</td>
<td>1.18</td>
<td>12.30</td>
<td>11.00</td>
<td>0.11</td>
</tr>
<tr>
<td>Mean</td>
<td>1.53</td>
<td>1.43</td>
<td>5.14</td>
<td>6.03</td>
<td>0.99</td>
</tr>
<tr>
<td>(±sd)</td>
<td>(0.31)</td>
<td>(0.20)</td>
<td>(4.46)</td>
<td>(4.77)</td>
<td>(0.64)</td>
</tr>
</tbody>
</table>

p: responders; NR: non-responders; BALF: bronchoalveolar lavage fluid; ns: nonsignificant.
total cells ($p<0.05$), lymphocytes ($p<0.003$), T-lymphocytes ($p<0.02$), and neutrophils ($p<0.01$); numbers·ml$^{-1}$ in patients experiencing a positive alveolar response but not in non-responders. No change was observed in BAL macrophages·ml$^{-1}$ in either group ($p=0.14$, NS). Using a monitoring score which reflected the severity of patient response (table 2), there was a positive correlation between total cells ($r_s=0.85; p<0.001$), T-lymphocytes ($r_s=0.76; p<0.01$), lymphocytes ($r_s=0.67; p<0.02$) and neutrophils ($r_s=0.55; p<0.05$) (table 4). Thus, physiological response to inhalation challenge with specific antigen was associated with an increased number of both T-lymphocytes and neutrophils in BAL. Lymphocyte subset analysis showed no difference between patient groups following inhalation challenge (fig. 1).

When cell groups were expressed as a percentage of the total cells, the responders showed a significant rise in neutrophils after challenge (before $4.43\pm3.16$ after $17.18\pm23.59$, $p<0.01$) but not in macrophages (before $20.78\pm10.38$ after $6.71\pm4.42$), lymphocytes (before $69.52\pm16.45$ after $75.96\pm25.06$), or T-lymphocytes (before $36.91\pm16.7$ after $53.59\pm19.05$). The non-responders showed no significant changes in any cell groups as a % of total cells after inhalation challenge (neutrophils $2.58\pm0.85$ to $2.81\pm2.08$, macrophages $36.8\pm31.55$ to $33.05\pm34.86$, lymphocytes $61.19\pm30.5$ to $60.14\pm31.14$ and T-lymphocytes $37.13\pm18.63$ to $38.41\pm21.36$).

### Urea and albumin levels
Following inhalation challenge in responders, there was an increase both in albumin (p<0.02) (fig. 2), and in urea levels (p<0.05) (fig. 2). In non-responders, albumin levels changed but not significantly, an increase in urea was recorded during the second lavage procedure (p<0.05).

### Soluble immune proteins
There was no significant difference in levels of IL-1 and IL-2 expressed in terms of millilitres of ELF, micrograms of albumin, or millilitres of BALF (for responders IL-1 p<0.2, IL-2 p<0.1, non-responders IL-1 p<0.1, IL-2 p<1.0) (figs 3 and 4) in either group following inhalation challenge. Levels of interleukin-1 and interleukin-2 are not detectable in serum. There was no relationship between CD4/CD8 ratios and IL-1 ($r_s=0.28$ data not shown) or IL-2 values ($r_s=0.43$ data not shown) in BALF of responders.

### Sequelae
All positive responders were free of symptoms by 24 h. One patient, although symptom free (BH), had clinical evidence of right middle lobe collapse, confirmed on chest radiograph. This was due to oedema and the patient quickly responded to a short course of oral prednisolone.
INHALATION CHALLENGE IN PIGEON BREEDERS' DISEASE

Fig. 2. — BALF albumin and urea are both significantly increased in responders after inhalation challenge, BALF urea is also increased in non-responders. For abbreviations see legend to figure 1.

Fig. 3. — Illustration of the variability of BALF IL-1 and IL-2 after inhalation challenge, when represented in terms of urea (ml ELF) and μg of albumin both in responders and non-responders. IL-1: interleukin 1; IL-2: interleukin 2; ELF: epithelial lining fluid. For further abbreviations see legend to figure 1.
Discussion

The finding of increased lymphocytes, T-lymphocytes and neutrophils recovered in BALF, following a positive bronchial inhalation challenge at 6 h does not entirely support histological results in some animal models [3, 5]. However, BERNADO et al. [10] demonstrated a lymphocyte response to challenge within 24 h of acute inflammatory changes in guinea-pigs. Indeed, some workers have observed an excess of pulmonary lymphocytes early after challenge tests [20].

While many animal models provide information on the response of the lung to a single challenge with aerosolized antigen, patients associated with aetiological agents, e.g. pigeon breeders, are exposed on a chronic basis.

A T-lymphocytosis may reflect the chronic pattern of exposure to antigen, which was observed in all responders, rather than a single exposure, which was employed in some animal models.

Another variable contributing to differences observed between animal and human models may be the considerable disparity in the inflammatory response on histological sections, compared with that reported in BALF, when studying patients with interstitial lung disease [21]. The results of the present study are at variance with those of the two previous studies involving BALF findings in pigeon breeders following inhalation challenge [22, 23]. A significant increase in BALF neutrophils was noted by both groups of workers, in symptomatic pigeon breeders compared with asymptomatics and controls, 24-48 h after exposure to pigeon serum. Failure of these workers to demonstrate an early increase in absolute numbers of lymphocytes may have been influenced by an absence of exposure to pigeon antigens for 1-6 weeks before these studies began.

At present, it is not known whether increased proportions of T-lymphocytes recovered in lavage are derived solely from cells initially present within the lung, or comprised of cells from both lung and peripheral blood. A mononuclear infiltrate within the lung could result from antigen-induced local proliferation of lymphocytes, inducing T-lymphocytes, already present within the lung. Alternatively, blood T-lymphocytes may be attracted to the lung by the presence of specific antigen within the lung, or by the release of various lymphokines after stimulation of T-lymphocytes with antigen. This study provides some supporting evidence for both hypotheses. Large numbers of lymphocytes were present in the BALF before inhalation challenge and, clearly, some of these local lymphocytes have the potential to become activated and release lymphokines [13]. In support of the latter hypothesis is the finding of peripheral lymphopenia in responders, both in this study and in that by HENDRICK et al. [18]. Low circulating lymphocyte counts have also been noted in patients with other forms of pulmonary hypersensitivity [24].

This is the first study to demonstrate a polymorphonuclear alveolitis 6 h following specific antigenic challenge in the human model. This influx of cells is unlikely to have arisen simply due to a second lavage, as previously reported in animal models undergoing sequential BALs [25, 26], as there was no increase in neutrophils in the non-responder group. VON ESSEN et al. [17] recently reported a significant increase in BAL alveolar neutrophils in normals following a second BAL at 7 h, when performed on the same right middle lobe subsegment. These workers suggested that to exclude the

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Fig. 4. - IL-1 and IL-2 per ml of BALF before and after inhalation challenge in responders and non-responders. For abbreviations see legends to tables 1 and 3.
effect of BAL on neutrophil counts, repeat bronchoscopy should be performed no earlier than 72 h after the initial procedure. However, they failed to show a statistically significant increase in alveolar cells in previously spared subsegments. In further support of our work, JARJOUR and CALHOUN [26], performing sequential bronchoscopy with BAL on different lobes in patients with asthma, failed to show neutrophil influx into the lung.

As with other effector cells, the mechanisms by which neutrophils are attracted to the lung in the acute stage of EAA is unclear. They may be sequestered to the lung following the generation of chemotactic factors, resulting from activation of the complement cascade by immune complexes deposited in the lung parenchyma [27]. In further support of this concept, injection of a purified CS-derived chemotactic factor for polymorphs, results in a homomoragic alveolitis, similar to that seen in acute EAA [28]. Alternatively, polymorphs may be attracted to the lung in the acute stage by the release of a neutrophil chemotactic factor from alveolar macrophages that have ingested particulate antigen [25].

Following an influx of activated bronchoalveolar immune effector cells in responders, it would seem reasonable to predict that macrophages and lymphocytes might be releasing IL-1 and IL-2 into the alveolar lining secretions. In this study, there was a trend towards a fall in levels of immune control proteins in BALF following a positive inhalation challenge. TRENITI et al. [29] found spontaneous cytotoxic activity in BAL lymphocytes from five patients with farmer's lung, against 51Cr-labelled target cells. Despite this spontaneous killer cell activity, IL-2 could not be detected or quantitated in BALF. To explain low levels of BALF IL-2 despite evidence of an active cell mediated response, REYNOLDS [30] suggested that either IL-2 was quantitatively utilized and no reasonable excess could be measured or, alternatively, suppressor cells may be modulating T-helper cell activity. However, in our study, no correlation was demonstrated between bronchoalveolar CD4/CD8 ratios and IL-2 levels.

One reason for the unexpected fall in cytokine levels may be the lowered responsiveness of BAL T-cells. Such a possibility has been considered by YAMASA [AL et al. [31], who demonstrated reduced lymphoproliferative responsiveness to mitogens of BAL lymphocytes from patients with summer-type hypersensitivity pneumonitis. The more probable explanation for the fall in levels of control immune proteins was the significant increase in secretory substances, i.e. urea or albumin, which accompanied a second BAL. In the case of albumin, this was probably secondary to an increase in vascular permeability following a positive response. An increase in urea may have occurred due to carry-over of "urea-rich" BALF from one subsegment to another during BAL procedures, or secondary to inflammatory change around the right middle lobe orifice, which is an inevitable consequence of a second lavage procedure. Interestingly, in the only patient to undergo repeat BAL on the lingula, urea levels remained unchanged.

In summary, BAL was carried out safely in 12 pigeon breeders, 6 h after bronchial inhalation challenge with nebulized pigeon serum or saline. In those who responded to challenge with an episode of EAA, an early increase in total cells, lymphocytes and neutrophils occurred. Increased number of bronchoalveolar lymphocytes may have been influenced by timing of the last antigenic exposure, duration of symptoms and lymphocyte migration from peripheral blood in response to lymphokine release. However, the BALF "alveolitis" did correlate with the physiological insult following inhalation challenge, which was represented as a monitoring score.

Failure to demonstrate an increase in soluble immune proteins despite an influx of inflammatory cells is likely to have been strongly influenced by a rise in urea and albumin into the alveolar secretions between the two lavage procedures.

References


14. Pingleton AK, Harrison GF, Stechschulte DJ, et al. -


