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Immunoglobulin and antibody levels in bronchoalveolar lavage fluid from symptomatic and asymptomatic pigeon breeders

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SUMMARY

Twenty-one symptomatic subjects with pigeon breeders' lung (PBL) and 10 asymptomatic pigeon breeders, with a similar exposure to pigeon antigens, underwent bronchoalveolar lavage. Total IgG, IgM and IgA in lavage fluid were determined as were specific antibody levels against antigens in pigeon serum and droppings. Results were converted to levels in epithelial lining fluid (ELF) using lavage and serum urea ratios. It was found that symptomatics represent a group that is hyperreactive to pigeon antigens compared with the asymptomatic group with significantly higher IgG, IgM, IgA levels as well as specific antibody levels against pigeon serum and droppings. Paired serum and ELF samples from 12 symptomatic subjects showed significantly elevated IgG, IgM and IgA levels in ELF compared with serum when values were expressed in terms of albumin. This strongly supports the concept of local production of immunoglobulins within the lung after inhaling immunogens as opposed to their diffusion from the vasculature. Results for IgA indicate that any putative protective role for this immunoglobulin is not valid in relation to the prevention of extrinsic allergic alveolitis. Analysis of smoking habits, lung immunoglobulins and response to inhalation challenge confirm the negative influence of smoking on total and functional lung immunoglobulins; however, levels in the ELF of ex-smokers suggest that the effect of smoking is not permanent. Smoking did not prevent responses to inhalation challenge.

Keywords BALF immunoglobulins antibodies pigeon breeders smoking

INTRODUCTION

The human lung contains considerable lymphoid tissue, with lymphoid aggregates within the bronchial lamina propria, in large bronchi near bifurcations and beneath epithelial cells in the respiratory bronchioles (Racz *et al.*, 1977), all potentially capable of secreting immunoglobulin into the epithelial lining fluid (ELF). Diffusion from vascular compartments represents another source of immunoglobulins in ELF (Hunninghake *et al.*, 1979). Cells producing IgG and IgA occur in approximately equal abundance in the normal human bronchial mucosa (Martinez-Tello, Braun & Blanc, 1968), whereas there are few IgM secreting cells (Brandtzaeg, Fjellanger & Gjeruldsen, 1967). This is reflected in the concentrations of the respective immunoglobulins in bronchoalveolar lavage fluid (BALF) where 10–20% protein in BALF is IgG, approximately 10% is IgA (Bell *et al.*, 1981) and IgM is absent or detectable only in trace amounts (Reynolds & Newball, 1974; Mandel *et al.*, 1976; Warr *et al.*, 1977; Low, Davies & Giancola, 1978; Weinberger *et al.*, 1978; Valeyre *et al.*, 1982).

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However, when the lung has been subjected to immune stimulation, as in extrinsic allergic alveolitis (EAA), increased IgG, IgM and IgA may be seen in ELF although the site of immunoglobulin synthesis still cannot be defined since antibodies from all three classes are also present in the sera from cases from EAA (Patterson *et al.*, 1979; McSharry *et al.*, 1984). It is possible that both pulmonary and extrapulmonary lymphoid tissue contribute since as the amount of particulate antigen deposited in the lung is increased, so the site of potential antibody production 'overspills' from the lung and hilar lymph nodes to other lymphoid tissue, e.g. spleen (Hunninghake & Fauci, 1977). Functionally ELF antibodies could participate in a tissue damaging type III, Arthus reaction (Arthus, 1903) but the evidence for such a reaction mediating EAA episodes is not conclusive: thus, lung biopsies from patients with farmer's lung disease show little evidence of a type III reaction (Reyes *et al.*, 1982); absence of antibody against offending antigens in some cases of EAA (Sennekamp *et al.*, 1981); presence of antibody in exposed asymptomatic pigeon breeders (Fink, Teb & Barboriak, 1969; Moore *et al.*, 1975; Boren *et al.*, 1977); lack of correlation between complement activation by antigen and response to inhalation challenge by the same antigen in farmer's lung (Edwards & Davies, 1981); and unchanging serum comple-

ment and antibody levels during induced episodes of EAA in farmer's lung and humidifier fever (Edwards & Davies, 1981; Edwards & Cockcroft, 1981).

However, the timing of biopsies after the last exposure to dust greatly influences the histological pattern observed (Edwards, Wagner & Seal, 1976) and previous challenge tests have used serum components to follow the course of reaction.

Immunoglobulins in the lung ELF could well influence events after challenge more than serum components, particularly IgA antibody against inhaled antigens, since it is possible that elevated levels of IgA could account for the lack of reactivity in subjects exposed to but not reacting against inhaled antigens capable of inducing EAA.

Such a division between symptomatic and asymptomatic subjects is well documented in PBL (Reed, Sosman & Barbee, 1965), a disease in the EAA group caused by inhaling pigeon antigens where the soluble nature of the provoking antigens allows antibody classes to be quantified.

Thus two groups of pigeon keepers, symptomatic and asymptomatic, exposed to pigeon antigens for equivalent lengths of time were examined to determine: (i) whether symptomatics represent a subject group hyperreactive to inhaled antigens; (ii) if IgA has a protective role against inhaled antigens in terms of EAA induction; and (iii) the role of the lung in the production of antibodies to inhaled immunogens.

MATERIALS AND METHODS

Symptomatic pigeon breeders

Twenty-one male subjects, median age 47 years (range 16–70 years) who had a history consistent with PBL and who responded to inhalation challenge under controlled conditions were termed symptomatic subjects for this study. They had been exposed to pigeons for a median of 10 years (range 2–30 years), four were smokers, five ex-smokers. All had moderate or strong precipitins to pigeon serum (PS) and pigeon droppings by gel diffusion. Precipitins were graded as positive (one easily observable precipitin line on double gel diffusion), moderate (2–4 faint lines or 1–2 solid lines), or strong (three or more discrete precipitin lines).

Asymptomatic pigeon breeders

Ten male asymptomatic subjects, median age 40 years (range 29–49 years) with a median exposure of 10·5 years (range 3–30 years), did not report symptoms suggestive of PBL and failed to respond to inhalation challenge. All had normal chest radiographs, four were current smokers and precipitins to PS and pigeon droppings were observed in five of the subjects.

Control subjects

Eight age-matched normal subjects, all male, median age 46 years (range 30–65 years) were included in the study. None had kept birds previously. All had normal chest radiographs and pulmonary function tests. Three were current smokers and none had precipitins to PS or pigeon droppings.

All participating subjects gave written informed consent and the study was approved by the local Ethical Committee.

Inhalation challenge

Subjects were hospitalized overnight; thus, the minimum guaranteed absence of exposure to pigeon antigens was 20 h. However, none had experienced symptoms for at least 1 week before challenge, hence were not in the refractory period observed after a positive inhalation challenge (Williams, 1963).

Before challenge they were skin prick tested with PS at 1/100. No subject produced an immediate or late (4–8 h) skin reaction. Challenge testing was made on subjects with nebulized PS starting at 1/1000 and increasing to 1/100 and 1/10, where appropriate, on consecutive days. Positive response was defined objectively as any two of the following: (i) increase in temperature $>0{\cdot}5°C$ above resting; (ii) increase in circulating polymorphonuclear leucocytes $>2500/mm^3$; (iii) fall in forced expiratory volume in 1 s (FEV_1) and/or forced ventilatory capacity (FVC) $>15\%$. These tests were carried out at regular intervals up to 36 h post-challenge.

Nineteen of the 21 subjects had all three objective criteria. One subject did not have a raised temperature and one had a predominantly obstructive pulmonary function defect 6 h following challenge. Twenty had a fall in FVC $>15\%$ and the mean maximal falls in FEV_1 and FVC were 21% and 24% respectively, suggesting a predominantly restrictive abnormality in lung function.

That this was not an asthmatic type reaction was evidenced by:

1. No subject was atopic nor had an immediate skin test reaction to PS.
2. Ausculatory signs of asthma, e.g. wheezing, were not observed.
3. Lung function tests were consistent with EAA in 20/21 subjects.
4. Pyrexia was observed in 20/21 subjects.
5. Leucocytosis was observed in 21/21 subjects.
6. Peak response time was 6–13 h post-challenge.

All subjects likened their symptoms to those experienced after exposure to dust in the pigeon loft. Our objective evidence of an EAA response adequately fulfils the requirements as outlined by Pepys & Hutchcroft (1975) and Hendrick *et al.* (1980).

Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was undertaken before inhalation challenge. Subjects were premidicated with 0·6 mg atropine i.m., 1 mg lorazepam orally and 1–2 ml fentanyl/droperidol IV (50 μg fentanyl and 2·5 mg droperidol per millilitre). Vocal chords were anaesthetized with an amethocaine lozenge (60 mg) and 4 ml lignocaine (4%). The Olympus BFIT10 fibre optic bronchoscope was wedged in the right middle lobe or lingula and buffered lavage fluid (0·67% NaCl, 0·019% KCl, 0·25% $NaHCO_3$, pH 7·4) at 37°C introduced via the bronchoscope. Three aliquots of 30 ml were instilled then gently aspirated. A further three aliquots of 30 ml were instilled and then aspirated. In all cases the dwell time of the lavage fluid was less than 4 min to minimize urea seepage from the circulation (Marcy *et al.*, 1987). Between 30% and 60% fluid was recovered in all cases.

BALF processing

BALF was filtered through a single layer of sterile gauze and centrifuged at 500 ***g*** for 5 min. Supernatants were stored at $-70°C$ in aliquots.

Epithelial lining fluid

The actual volume of fluid recovered from the lung, i.e. ELF, was calculated from serum and ELF urea levels using the formula

$$\text{Vol. ELF} = \frac{\text{BALF urea} \times \text{vol. BALF}}{\text{Serum urea}}$$

(Rennard *et al.*, 1986). Urea was determined by the microtitre plate modification of the Berthelot urease reaction (Jones *et al.*, 1990).

IgG, IgM and IgA in BALF by ELISA

Serial four-fold dilutions of BALF were made in saline/azide (1% NaCl, 0·1% NaN_3) and used to sensitize wells in microtitre plates (Immunoplate F; Nunc, Roskilde, Denmark). To 50 μl of each dilution (in triplicate) 150 μl $NaHCO_3/Na_2CO_3$ buffer (0·067 M, pH 9·5) were added and plates left at 4 C overnight. After washing (×3) with PBSNTB (phosphate-buffered saline (PBS) plus NaN_3, 0·02%; Tween 20, 0·05% v/v; bovine serum albumin (BSA) 0·1%) bound immunoglobulins of G, M and A classes were detected with 200 μl 1/1000 alkaline phosphatase-conjugated anti-IgG, IgM or IgA (heavy chain specific antisera; Sigma Chemical Co., St Louis, MO) 45 min room temperature (RT). After washing (×3) with PBSNTB, 200 μl p-nitrophenyl phosphate (1 g/*l* in 10% diethanolamine, pH 9·8) was added and liberated p-nitrophenol determined at 405 nm using a MR950 Minireader (Dynatech, Plochingen, Germany). Reaction times were approximately 45 min and development stopped with 50 μl 5 mol/*l* NaOH.

Each plate used had a standard positive BALF (a pool from 5 symptomatic BALFs) at five dilutions and results were expressed as a per cent standard positive. IgG, IgM, and IgA levels in the standard positive were determined in comparison with reference levels of IgG, IgM and IgA in a standard serum sample (SPS-01 batch 881, Sheffield PRU, Sheffield, UK) using the ELISA technique described and converted to μg/ml BALF or mg/ml ELF by serum/BALF urea ratios as described. The standard positive BALF had IgG 123 ± 15 μg/ml, IgM 14·6 ± 1·7 μg/ml and IgA 51·4 ± 7·6 μg/ml (mean and s.d. for four separate determinations carried out over the period of study).

Anti PS and pigeon droppings activity in BALF

Optimal concentration of PS and pigeon droppings for plate sensitization. PS was made up in dilutions from 1/500 to 1/5000 and pigeon droppings from 10 μg/ml to 1000 μg/ml in saline/azide. Plates were sensitized with 50 μl each dilution per well plus 150 μl of 0·06 M $NaHCO_3/Na_2CO_3$, pH 9·5 overnight, at 4°C. After washing (×3) with PBSNTB, 200 μl of the standard positive BALF were added in dilution from 1/10 to 1/100 and incubated at 45 min RT. After washing (×3) with PBSNTB, bound IgG, IgM or IgA was then determined as described. Plots of OD 405 nm *versus* dilutions of PS or pigeon droppings showed optimal sensitization for PS at 1/5000, and for pigeon droppings at 100 μg/ml; thus 50 μl each of these dilutions plus 150 μl 0·067 M $NaHCO_3$, pH 9·5, were used to sensitize wells for anti-PS and anti-pigeon dropping determinations.

Anti PS and anti-pigeon dropping immunoglobulins in BALF

Wells sensitized with PS or pigeon droppings were incubated with 200 μl serial four-fold dilutions of each BALF. Bound immunoglobulin of each class was detected as described with separate plates for each immunoglobulin. Results were then converted to their appropriate ELF values. The use of five four-fold dilutions of BALF allowed a curve for each BALF antibody to be drawn. Each curve was sigmoidal and was compared with the standard positive BALF curve. Only points on the linear part of these curves were used to obtain the antibody levels relative to the standard.

Competitive binding by immunoglobulins

To assess whether IgG antibody competitively bound PS and pigeon dropping epitopes leading to an underestimation of IgA and IgM, eight BALFs were incubated with formalinized *Staphylococcus aureus* cells (Sigma) absorbing IgG with cell bound protein A. The volume of *S. aureus* suspension used was in excess of ×10 that required to absorb IgG according to the manufacturer's instructions. BALF and cells were incubated at 37 C for 1 h and cells removed by centrifugation at 10000 ***g*** for 10 min. Supernatants were diluted and assayed for anti-PS and anti-pigeon dropping immunoglobulins as above. Results were compared with the same BALF incubated with an aliquot of PBS.

Intraplate and interplate variability

Immunoglobulin levels were determined as described with four sets of standard BALF dilutions and four sets of SPS-01 dilutions for each immunoglobulin class. This provided 16 values for IgG, IgM and IgA per plate. Six plates were used.

Anti-PS and anti-pigeon dropping levels were determined as described using one dilution (1/100) BALF standard in each of 40 wells on one plate.

The variability was expressed as the coefficient of variation (CV), i.e. s.d./mean. For the six plates studied the mean intraplate CVs were: IgG, lower range (250 ng/ml) 0·07 (range 0·01–0·11), upper range (1500 ng/ml) 0·08 (range 0·03–0·13); IgM, lower range (500 ng/ml) 0·06 (range 0·04–0·08), upper range (2000 ng/ml) 0·06 (range 0·04–0·08); IgA, lower range (450 ng/ml) 0·09 (range 0·05–0·13), upper range (2000 ng/ml) 0·08 (range 0·04–0·15). The interplate CVs were: IgG, lower range 0·08, upper range 0·13; IgM, lower range 0·10, upper range 0·05; IgA, lower range 0·10, upper range 0·09. For anti-PS and anti-pigeon dropping IgG, IgM and IgA, all CVs were $< 0·07$.

Contribution of BALF alkaline phosphatase to results

Serial dilutions of each BALF incubated with p-nitrophenyl phosphate indicated detectable levels of this enzyme in all samples. The contribution of this endogenous enzyme to the readings was determined by repeating immunoglobulin levels and antibody levels as described, omitting the alkaline phosphatase conjugated antiserum step.

These experiments indicated a contribution of less than 1% of BALF alkaline phosphatase to the activity using conjugated antisera.

Serum levels of IgG, IgM and IgA

Levels of each immunoglobulin were determined by rate nephelometry using the same standard serum sample (SPS-01) as reference.

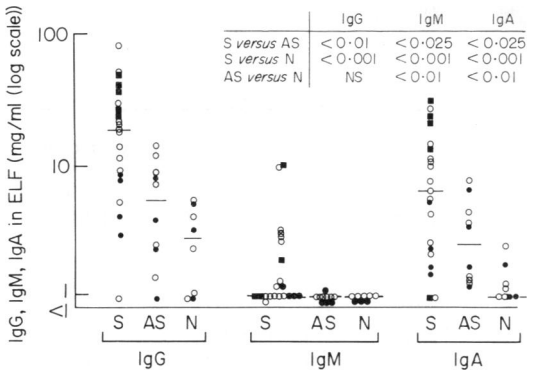

Fig. 1. Total IgG, IgM and IgA in ELF of S (symptomatic; $n=21$), AS (asymptomatic; $n=10$) and N (normal; $n=8$) subjects expressed in mg/ml. Groups divided into ○ (non-smokers), ● (smokers) and ■ (ex-smokers). Bars represent median values for the group. Differences in levels expressed as P values (right inset). NS, not significant.

Statistical analysis

Intergroup comparisons of total and specific immunoglobulins were made using the Mann–Whitney U-test. Correlations were obtained by Spearman's rank testing.

RESULTS

Total immunoglobulins

Total immunoglobulin results in BALF varied considerably, particularly within the symptomatic group (Fig. 1), producing median values of 18·5 mg/ml ELF for IgG (mean 23·4, range 3–81); 0·42 mg/ml ELF for IgM (mean 1·8, range 0·1–0·96) and 5·4 mg/ml ELF for IgA (mean 9·2, range 0–27). For asymptomatics, immunoglobulin levels were: IgG, median 5·5 mg/ml ELF (mean 6·1, range 0·4–14·2); IgM, median 0·26 mg/ml ELF (mean 0·36, range 0·1–1·03); IgA, median 2·5 mg/ml ELF (mean 3·2, range 1·2–7·6). For normals, immunoglobulin levels were: IgG, median 2·7 mg/ml ELF (mean 2·7, range 0·6–5·2); IgM, median 0·06 mg/ml ELF (mean 0·09, range 0–0·3); IgA, median 0·9 mg/ml ELF (mean 1·1, range 0·4–2·4). Levels were significantly higher in symptomatic compared with asymptomatic and normal subjects ($P<0·025$ all comparisons) and between asymptomatic and normal groups for IgM and IgA ($P<0·01$). Patients with the chronic form of PBL had elevated IgG compared with the acute form ($P<0·03$).

When results are expressed as the amount of immunoglobulin and antibody recovered from the lung (Table 1) significant differences are observed between all three groups for total IgG, IgM and IgA.

Analysis of the effects of smoking on the avian-exposed group, i.e. symptomatics and asymptomatics, showed a significant reduction in ELF IgG in smokers ($P<0·001$) and IgA ($P<0·05$), whereas IgM differences were almost at significance ($P=0·057$). Results were: IgG, smokers median 3·95 mg/ml ELF (range 0·4–8·6), non-smokers median 17·5 mg/ml ELF (range 0·2–81·2); IgM, smokers median 0·24 mg/ml ELF (range 0·11–1·11), non-smokers median 0·46 mg/ml ELF (range 0·1–9·7); IgA, smokers median 1·98 mg/ml ELF (range 1·2–6·3), non-smokers median 6·3 mg/ml ELF (range 0–31). Results are summarized in Table 2 as non-smoking, smoking and ex-smoking groups. Paired serum and BALF samples were available for 12 symptomatic subjects. No correlation between the levels of the three immunoglobulins in serum and ELF was observed; neither was there a significant difference in IgG and IgM levels, although ELF IgA was higher than serum IgA (Table 3). Levels of albumin were significantly different, the mean for sera was $45·8 \pm 1·7$ mg/ml (range 44–48) and for ELF $8·8 \pm 7·8$ mg/ml (range 2–17·2) ($P<0·001$). When serum and ELF immunoglobulin levels were expressed in terms of albumin present, all three immunoglobulin levels were elevated in ELF compared with serum (Table 3).

Anti-PS and anti-pigeon dropping in BALF

Since the symptomatics and asymptomatics had been exposed to loft materials before hospitalization it is possible that some antibody determinations are an underestimate due to ELF antibody bound to inhaled pigeon antigens. Attempts to detect pigeon antigen in BALF using rabbit and sheep anti-pigeon serum-droppings in an ELISA system were unsuccessful. Possibly elimination of such complexes by alveolar macrophages had occurred before lavage; alternatively, available epitopes had been blocked by ELF antibody. Antibodies of all three classes against PS and pigeon droppings were detected in BALF of 20/21 symptomatic and 7/10 asymptomatic pigeon breeders. The exception for PBL was one subject with no detectable IgA in serum and ELF. In the asymptomatic group, one subject lacked IgG anti-pigeon dropping, one lacked IgA and IgM anti-PS and the third subject exhibited only IgA anti-PS at $>1\%$ standard control positive ELF. In the normal subject group no anti-PS IgG and IgM were present in ELF at $>1\%$ standard positive, whereas low levels of IgA anti-PS and pigeon dropping were detected in six subjects (all $<10\%$ standard positive) and four subjects had IgM anti-pigeon dropping (all $<5\%$ standard positive) (Figs 2 and 3). These represent statistically significant differences for symptomatic *versus* asymptomatic groups of $P<0·01$ for anti-PS and pigeon dropping (IgG, IgM and IgA). For symptomatics *versus* normal groups all antibody levels are significantly elevated at $P<0·001$ (Figs 2 and 3). For asymptomatic groups *versus* normals IgG, IgM and IgA anti-PS and pigeon dropping levels were elevated ($P<0·01$) except for IgA, anti-pigeon dropping (Figs 2 and 3).

Expressed as total recovered anti-PS or anti-pigeon dropping, significant differences were seen between symptomatics and the other groups, whereas asymptomatics had elevated anti-PS IgG and anti-pigeon dropping immunoglobulins compared with normals (Table 1).

Competitive binding by immunoglobulins

IgG anti-PS and anti-pigeon dropping was reduced to $<2\%$ in 6/8 BALFs, 15% in 1 BALF and 25% in 1 BALF. After removal of IgG, levels of IgA and IgM anti-PS and anti-pigeon dropping were not found to be increased in any of the BALFs, suggesting competitive binding by IgG antibody had not occurred.

DISCUSSION

Central to our calculations of immunoglobulin levels in ELF is the use of urea as a marker. The European Task Group report on BAL state that 'since urea can diffuse across the membrane its use as a marker strongly depends on the time lag between instillation and sampling of the BAL fluid ("dwelling time") and

Table 1. Total immunoglobulins and antibody recovered

Subject no.	Volume instilled (ml)	Volume recovered (ml)	Return (%)	Immunoglobulins IgG (mg)	Immunoglobulins IgM (mg)	Immunoglobulins IgA (mg)	Anti-PS IgG	Anti-PS IgM	Anti-PS IgA	Anti-pigeon dropping IgG	Anti-pigeon dropping IgM	Anti-pigeon dropping IgA
Symptomatic												
1	180	63	35	11·4	0·1	6·4	83	5	204	93	5	171
2	180	81	45	26·9	2·1	9·3	304	81	453	213	119	324
3	180	60	33	13·5	0·3	4·6	288	24	24	441	18	43
4	180	100	55	8·4	6·7	7·8	112	272	228	216	480	204
5	180	63	35	12·0	3·7	9·6	605	166	353	242	162	234
6	180	67	37	1·7	0·1	1·3	35	7	14	83	7	13
7	180	31	17	0·6	1·7	0·6	12	6	14	10	7	15
8	180	81	45	5·4	0·3	2·7	262	8	36	282	21	89
9	180	118	65	30·7	0·9	0	755	62	0	1548	35	0
10	180	81	45	28·8	10·2	25·3	1023	1425	466	440	518	661
11	180	76	42	1·2	0·04	1·1	8	1	21	26	1	76
12	180	63	35	4·8	1·3	1·9	11	19	7	8	32	5
13	180	55	30	0·5	0·07	0·7	2	2	4	2	2	5
14	180	56	31	4·8	0·2	3·5	4	2	8	22	3	9
15	180	67	37	9·8	1·4	0	169	25	0	238	29	0
16	180	60	33	2·4	0·1	1·4	51	15	47	149	13	31
17	180	32	18	0·5	0·04	0·6	5	3	25	5	3	18
18	180	45	25	3·8	0·02	1·9	216	34	106	162	15	45
19	180	100	55	10·4	0·5	13·4	160	51	352	400	28	364
20	180	75	42	13·6	0·3	8·2	264	19	172	270	12	165
21	180	42	23	15·5	0·03	2·4	14	34	63	49	24	34
Median		67	35	8·4	0·3	2·4	112	19	36	162	18	43
Asymptomatic												
22	180	21	12	0·1	0·01	0·05	0·3	0·2	0·8	<0·2	0·4	0·3
23	180	92	51	1·1	0·05	0·99	4·6	<0·9	<0·9	16·0	4·6	0·9
24	180	56	31	4·1	0·09	1·46	9·5	3·4	3·9	2·7	1·1	7·8
25	180	65	36	1·2	0·21	0·65	3·6	5·2	2·3	3·2	7·8	3·9
26	180	95	53	2·3	0·29	1·80	8·5	12·3	1·9	3·8	9·5	9·5
27	180	130	72	2·7	0·19	2·60	28·0	1·3	110·0	12·0	1·3	107·0
28	180	43	24	1·2	0·04	0·62	13·0	0·6	5·6	15·0	0·4	4·7
29	180	63	35	3·9	0·05	1·57	31·0	12·0	28·0	26·0	13·0	25·0
30	180	50	28	0·8	0·05	0·42	<0·5	<0·5	1·0	<0·5	<0·5	<0·5
31	180	67	37	2·9	0·23	0·54	17·0	1·3	0·7	19·0	13·0	4·0
Median		64	35	1·75	0·07	0·82	9·0	1·3	2·1	7·9	2·9	4·3
Normal												
32	180	73	40	0·4	0	0·03	<0·3	<0·8	0·7	0·7	<0·8	0·7
33	180	65	36	0·9	0	0·07	<0·3	<0·7	<0·7	<0·7	<0·7	<0·7
34	180	35	19	0·2	0	0·03	<0·2	<0·4	1·0	<0·4	1·0	0·9
35	180	69	38	2·1	0·01	0·16	<0·3	<0·7	6·2	2·1	2·7	3·4
36	180	65	36	1·2	0·02	0·08	<0·3	<0·7	0·6	<0·7	<0·7	0·6
37	180	55	30	0·4	0·01	0·02	<0·3	<0·6	1·6	<0·6	0·5	0·5
38	180	64	35	1·2	0·01	0·11	<0·3	<0·7	1·9	0·6	1·2	1·9
39	180	68	38	0·4	0·01	0·05	<0·3	<0·7	4·1	<0·7	<0·7	1·0
Median		65	36	0·65	0·01	0·06	<0·3	<0·7	1·3	0·7	0·75	0·8
Significance *P* values												
S* vs AS†		NS	NS	<0·05	<0·01	<0·025	<0·01	<0·001	<0·01	<0·001	<0·001	<0·001
S vs N‡		NS	NS	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001	<0·001
AS vs N		NS	NS	<0·05	<0·001	<0·001	<0·001	NS	NS	<0·05	<0·01	NS

Immunoglobulins IgG, IgM and IgA in lavage fluid from symptomatic pigeon breeders ($n=21$), asymptomatic pigeon breeders ($n=10$) and normal subjects ($n=8$). Volume of lavage fluid instilled was 180 ml. Immunoglobulins are expressed as total immunoglobulins (in mg) recovered. Anti-pigeon serum and anti-pigeon droppings activity expressed as antibody activity (% standard positive) × volume of recovered lavage fluid.

Significance of results is shown underneath as *P* value (Mann–Whitney *U*-test). NS, not significant.

* S, Symptomatic. † AS, Asymptomatic. ‡ N, Normal.

Table 2. Effect of smoking on lung immunoglobulins

	Immunoglobulins			Anti-PS			Anti-pigeon dropping		
	IgG	IgM	IgA	IgG	IgM	IgA	IgG	IgM	IgA
Non-Sm*	11·8	0·4	4·8	107	11	41	108	11	46
	1·4–81	0·1–10	1–27	4–1070	1–258	1–526	4–803	1–456	1–367
Ex-Sm†	40	1·2	23·2	224	80	390	283	44	414
	25–49	0·4–9·7	13–31	55–657	12–915	248–549	189–626	10–333	130–587
Sm‡	3·9	0·2	2·0	8	7	7	7	8	11
	0·4–9	0·1–1	1–63	0–33	0–29	0–147	0–97	0–22	0–105
Non-Sm vs Ex-Sm	<0·01	NS	<0·01	NS	<0·05	<0·01	<0·03	NS	<0·01
Non-Sm vs Sm	=0·01	NS	NS	<0·01	NS	<0·03	<0·01	NS	<0·03
Ex-Sm vs Sm	=0·001	<0·01	<0·01	<0·01	<0·01	<0·01	<0·01	<0·03	<0·01

Results on avian exposed subjects (symptomatic and asymptomatic). Non-smokers ($n=18$); ex-smokers ($n=5$); smokers ($n=8$). For analysis of IgA results one subject with no serum IgA was omitted.

Immunoglobulins expressed as the median in mg/ml ELF with the range beneath. Anti-PS and anti-pigeon dropping calculated as a per cent control lavage positive ELF. Results expressed as median with range beneath.

Lower part of table is the significance of the results as *P* values. NS, not significant.

* Non-Sm, Non-smokers. † Ex-Sm, Ex-smokers. ‡ Sm, smokers.

Table 3. Serum *versus* ELF levels of IgG, IgM and IgA in PBL

	IgG	IgM	IgA	IgG/albumin	IgM/albumin	IgA/albumin
Serum	17·9	1·08	3·37	0·37	0·02	0·07
	10–25	0·6–4·2	0–3·8	0·2–0·5	0·01–0·09	0–0·09
ELF	21·7	0·43	9·3	4·4	0·07	1·21
	4–91	0·1–9·7	0–27	0·4–11·9	0·01–4·9	0–5·7
P values	NS	NS	<0·05	<0·005	<0·005	<0·001

Values are the median in mg/ml with range beneath, for the 12 paired samples of serum and ELF from symptomatics. On right-hand side, values are expressed in comparison with albumin and given as median with range beneath.

Significance of values between serum and ELF shown on bottom line as *P* values. NS, not significant.

requires that BAL be accomplished as quickly as possible' (European Society of Pneumology Task Group on BAL, 1989). In view of this recommendation all our dwell times were less than 4 min (Marcy *et al.*, 1987). A comparison between analyses for total recovered immunoglobulins/antibodies (Table 1) and corresponding ELF concentrations (Figs 1, 2 and 3) provides good evidence of the usefulness of adopting urea as a marker.

Previous work has shown the predominant immunoglobulin in the lower respiratory tract of normal subjects to be IgG, representing 10–20% total protein, with IgA representing approximately 10% (Bell *et al.*, 1981) and IgM absent or present in trace amounts. Our mean ELF values and ranges for the eight normal subjects were IgG, 2·75 mg/ml, range 0·6–5·2; IgM, 0·09 mg/ml, range 0–0·3, and IgA, 1·1 mg/ml, range 0·4–2·4 mg/ml ELF, which corresponds to the order of concentrations for the three immunoglobulins previously reported but does not confirm the relatively high levels for IgA (Bell *et al.*, 1981). The differences may be attributed to assay techniques but confidence in our ELISA system is based on the ability to use BALF directly (or in dilution) without further modification.

The finding of IgM against PS and pigeon droppings in BALF of subjects exposed continuously to pigeon antigen is somewhat contrary to the concept of IgM levels being absent in a secondary immune response. However, we consider it to be a specific anti-PS and anti-pigeon dropping antibody since (i) it was present at >5% standard only in ELF of subjects exposed to pigeon antigens, and (ii) it was absent (<5% standard) in ELF of normal subjects and BALFs from 24 sarcoidosis and 15 idiopathic pulmonary fibrosis subjects tested at the same time (not included in this report). It is unlikely to be non-specific binding since it is not present in sarcoidosis ELF despite levels of IgM equivalent to those found in ELF of asymptomatics. Possibly the use of alpha chain specific antiserum to detect ELF IgA (mainly in dimeric form) and comparisons with serum IgA (mainly monomeric) underestimates ELF IgA, but the use of secretory piece antiserum to detect dimeric IgA was not undertaken due to the presence of free secretory piece in BALF (Merril *et al.*, 1980). Blocking of IgA directed against PS and pigeon dropping epitopes by IgG was not observed (see Results), suggesting IgA and IgG are directed against different epitopes.

When ELF levels of immunoglobulins in PBL are compared

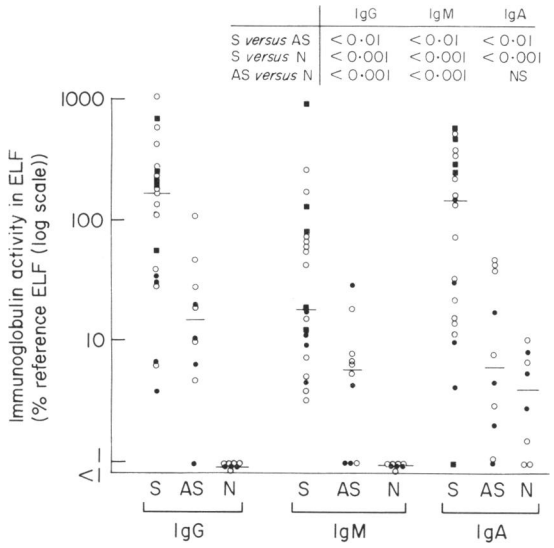

Fig. 2. Anti-pigeon serum levels of IgG, IgM and IgA classes in ELF of S (symptomatic; $n=21$), AS (asymptomatic; $n=10$) and N (normal; $n=8$) groups expressed as a percentage of the standard ELF pool. Bars represent median values for the group. Divisions into smokers, non-smokers, ex-smokers and statistical analysis as Fig. 1, NS, not significant.

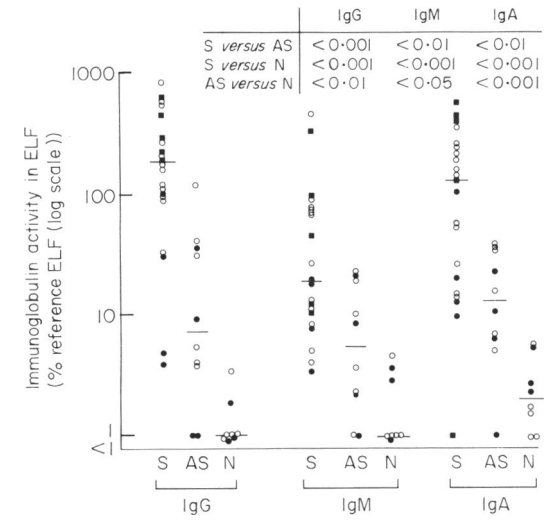

Fig. 3. Anti-pigeon dropping levels of IgG, IgM and IgA classes. Rest as Fig. 2.

with levels from normal subjects, all three immunoglobulins are significantly elevated. Two factors may account for this: increased pulmonary vascular permeability, and/or local production of immunoglobulins. Our previous work has shown higher levels of albumin in ELF of PBL compared with normal subjects (Jones *et al.*, 1990) indicating an increase in pulmonary capillary permeability in PBL and diffusion from the vasculature. However, our current work shows that this alone cannot account for the elevated immunoglobulins in PBL ELF since, first, PBL/normal ELF ratios for each immunoglobulin are far higher than PBL/normal albumin ratios, and second, in PBL levels of each immunoglobulin expressed per milligram albumin are significantly higher in ELF compared with serum (IgG × 11, IgA × 18, IgM × 2·3—derived from Table 2).

This clearly indicates local production of immunoglobulin to have occurred although the presence of serum precipitins and lymphocytes that undergo transformation to PBL antigens also shows systemic immune components in EAA. Any interchange between plasma and ELF immunoglobulins cannot be determined from our study, although immunoglobulin ratios in ELF of PBL suggest pulmonary plasma cells to produce relatively higher ratios of IgG and IgA than those cells responsible for serum immunoglobulins, i.e. if the levels of IgM in serum and ELF are assigned as unity then comparable IgG and IgA levels in ELF are × 3 and × 7 respectively the levels in serum.

Whatever the origins of the immunoglobulins in ELF, their functional role superficially favours their participation in a type III reaction as mediating the disease since titres of the complement activating antibodies IgG and IgM are significantly elevated in ELF of symptomatic compared with asymptomatic breeders ($P<0·01$). However, the hypothesis does not hold if it is based on the assumption that all symptomatics differ from all asymptomatics by having elevated IgG and IgM levels against avian antigens. Figures 2 and 3 show there to be an overlap where some asymptomatics have elevated anti-PS and anti-pigeon dropping IgG and IgM compared with some symptomatics. Five subjects who responded to inhaled PS had lower IgG and/or IgM anti-PS than five asymptomatic subjects. Despite these differences a type III response may still be tenable if there were a role for IgA combining with inhaled antigen and blocking the binding of IgG and IgM. However, Figs 1, 2 and 3 show significantly elevated IgA in symptomatic subjects either as total IgA or anti-PS and anti-pigeon dropping, compared with asymptomatics.

These findings directly contradict any suggestions that IgA has a protective role in EAA and that the unresponsiveness of asymptomatics to challenge is due to elevated IgA antibody. There is however a tendency for smoking to confer some 'protection' in subjects exposed to EAA antigens expressed as reduced serum antibody and lower overall prevalence of EAA in smokers (Boyd *et al.*, 1977; Warren, 1977), in contrast to the effects of smoking on serum IgE levels to inhaled allergens (Zetterstrom *et al.*, 1981).

Our study shows that smoking has little effect on immunoglobulin levels in normal subjects, also observed by Low *et al.* (1978). When immune stimulus occurs the negative effect of smoking is seen as significantly lower levels of IgG and IgA in the ELF of avian-exposed smoking subjects and is reflected in diminished IgG anti-PS and anti-pigeon dropping titres (Figs 1, 2 and 3; Table 1). In contrast, ex-smokers represent a group generally hyperactive to the inhaled antigens with elevated IgG and IgG anti-PS and anti-pigeon dropping antibodies in ELF compared with smokers. Despite its negative effect on immune response, smoking did not prevent response to inhalation challenge.

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