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Measurement of interleukin-6 in bronchoalveolar lavage fluid by radioimmunoassay: differences between patients with interstitial lung disease and control subjects

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SUMMARY

Bronchoalveolar lavage fluid (BALF) from subjects with a variety of interstitial lung diseases (active sarcoidosis, pigeon breeders' disease (PBD), asymptomatic pigeon breeders, patients with idiopathic pulmonary fibrosis) and from control subjects were assayed for interleukin-6 (IL-6) using a novel radioimmunoassay system. IL-6 was detectable in BALF from all groups, with disease groups showing significantly increased IL-6 levels compared with controls ($P < 0{\cdot}01$ in all cases). When these results were standardized, using urea to compensate for dilution effects in the BALF, only the asymptomatic pigeon breeders had significantly higher IL-6 levels than the controls ($P < 0{\cdot}025$), with all other groups showing no difference. When albumin was used for standardization, both the PBD group ($P < 0{\cdot}001$) and the sarcoidosis patients ($P < 0{\cdot}01$) had considerably lower levels of IL-6 than the control subjects. Using either albumin or urea for standardization, the PBD patients had significantly lower levels of IL-6 than do their asymptomatic counterparts ($P < 0{\cdot}001$ in both cases). This is contrasted by the finding of greatly elevated levels of IgG in the BALF of the PBD patients compared with asymptomatics ($P < 0{\cdot}001$). There was, however, no relation between IL-6 and IgG in any patient group, although the PBD patients had the lowest IL-6 and highest IgG as a group. These findings may suggest a mechanism by which asymptomatic subjects remain free from clinical complaints.

Keywords interleukin-6 bronchoalveolar lavage radioimmunoassay IgG

INTRODUCTION

Interleukin-6 (IL-6) is a 26-kD inducible protein produced by a variety of cell types; it was first described as being derived from fibroblastoid cells stimulated with cyclohexamide or virus (Billian, 1987) and has been given several names, including monocyte-derived human B cell growth factor; interferon-$\beta 2$; B cell stimulating factor (Tosato *et al.*, 1988); plastocytoma growth factor (Nordan, Pumphrey & Ruchikoff, 1987); interleukin hybridoma/plastocytoma-1 (van Damme *et al.*, 1987); and hepatocyte-stimulating factor (Gauldie *et al.*, 1987). IL-6 production has been demonstrated in different cell types, such as activated monocytes or macrophages, endothelial cells, fibroblasts and activated T cells, and has been shown to act on a variety of target cells, including T lymphocytes, B lymphocytes, fibroblasts, myeloid progenitors and hepatocytes (Gauldie *et al.*, 1987; Wong & Clark, 1988; Wong *et al.*, 1989; Walther, May & Sehgal, 1988).

IL-6 has been shown to act by interaction with a specific saturable receptor (Taga *et al.*, 1987). The properties ascribed to IL-6 are varied, as is suggested by the variety of cells capable of its production and of its target cells. IL-6 has weak anti-viral activity (Sehgal & Sagar, 1980), hybridoma/plastocytoma growth factor activity (van Snick *et al.*, 1986; Nordan *et al.*, 1987; van Damme *et al.*, 1987), lymphoblastoid growth factor activity in Epstein–Barr virus-transformed B cells (Tosato *et al.*, 1988) and induction of B cell differentiation and stimulation of IgG secretion (Hirano *et al.*, 1986). In addition, IL-6 has been shown to act on murine thymocytes to induce the differentitation of cytotoxic T lymphocytes in the presence of interleukin-2 (IL-2) (Takai *et al.*, 1988) and indirectly supports the proliferation of concanavalin A (Con A) or T cell receptor antibody-stimulated T cells *in vitro* by acting as a second signal for IL-2 production and thereby promoting IL-2-dependent proliferation of T lymphocytes (Garman *et al.*, 1987).

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These functions of IL-6 suggest it may have a role in interstitial lung disease, especially where elevation of other lymphokines such as interleukin-1 (IL-1) and IL-2 have been demonstrated, together with the presence of activated immune effector cells (Crystal *et al.*, 1981; Costabel *et al.*,1985; Reynolds *et al.*, 1989).

The aim of this study was to determine IL-6 levels in bronchoalveolar lavage fluid (BALF) of patients with interstitial lung disease, and to compare these levels with controls. Total IgG was determined in a number of BALF samples and this was correlated with IL-6 across patient groups and also within individual groups. Four groups were studied: sarcoidosis; pigeon breeders' disease (PBD); asymptomatic pigeon breeders; and idiopathic pulmonary fibrosis (IPF); a control group was also studied. IL-6 levels in BALF were standardized by means of urea in serum and BALF to give concentrations of IL-6 per ml of epithelial lining fluid (ELF) and also by reference to albumin, since controversy exists as to which of these substances provides the most suitable reference marker (Rennard *et al.*, 1986; Marcy *et al.*, 1987).

SUBJECTS AND METHODS

Subject groups

Lavage fluids from 57 subjects were investigated; none of these subjects were taking inhaled or systemic steroids, and none had an acute respiratory disease for at least 1 month prior to the time of investigation.

These were: (i) nine men and two women referred with non-specific respiratory symptoms. All investigations, including chest radiograph and bronchoscopy were normal. Two were current smokers. Bronchoscopy was indicated by haemoptysis in six, localized wheeze in two and persistent cough in three subjects. This was the control group;

(ii) eight men and two women with biopsy-proven sarcoidosis with no history of beryllium exposure or fungal disease; all were considered to have active disease on the basis of raised serum angiotensin-converting enzyme (SACE) together with a high intensity lymphocytic alveolitis (more than 28% T lymphocytes) (Crystal *et al.*, 1981). Two were current smokers;

(iii) 15 male pigeon breeders who gave a history of at least three episodes of extrinsic allergic alveolitis (EAA) in the 6 months prior to lavage. All gave a positive response to inhalation challenge with nebulized pigeon serum at 1/10 to 1/100 dilution, and all had circulating precipitating antibodies to pigeon antigens. One was currently smoking and three were ex-smokers;

(iv) eight male pigeon breeders with no history of extrinsic allergic alveolitis, normal chest radiograph, no functional lung impairment and who gave a negative reaction to challenge with pigeon serum. Two were current smokers. Bronchoscopy was performed in order to exclude EEA; and

(v) 13 men with biopsy proven diffuse IPF. Three were current smokers.

All patients gave informed written consent and the study was approved by the local Ethical Committee. Bronchoscopy was performed to confirm original diagnosis unless otherwise stated.

Bronchoalveolar lavage

Bronchoalveolar lavage was performed using 180 ml of bicarbonate-buffered saline, pH 7·4, at 37°C. The right middle lobe was the lavage site chosen for each subject in the study. A lavage volume of more than 120 ml was chosen to ensure adequate alveolar sampling (Kelly *et al.*, 1987), all lavage procedures were completed within 4 min in order to reduce passive diffusion of urea (Marcy *et al.*, 1987). The lavage fluid was filtered through a single layer of sterile gauze to remove gross contamination. The cellular component was removed by centrifugation at 400 ***g*** for 10 min and supernatants were stored at −70°C within 90 min of sample collection prior to assay (up to 6 months).

IL-6 radioimmunoassay

To 100 μl of standard or unknown in 12 × 75-mm polypropylene tubes were added 100 μl of rabbit anti-human recombinant IL-6 (Amersham) diluted in borate assay buffer, pH 7·4, with a final dilution of 1/19000 in the tube. Standards were human recombinant IL-6 (Amersham) and the standard range was 0·5–128 fmol/tube. After incubation at room temperature for 4 h, 100 μl of buffer containing 10 nCi of iodinated recombinant human IL-6 (Amersham) were added, followed by a further incubation overnight (16–24 h) at room temperature. IL-6 was iodinated with Bolton & Hunter reagent to a specific activity of 1200–1600 Ci/mmol. Separation of bound and free ligand was achieved by adding 250 μl of Amerlex-M™ donkey anti-rabbit serum coated onto magnetic polymer particles, followed by magnetic separation. Following decantation the pellet containing the bound fraction was counted in a gamma counter.

The antisera used in this assay did not cross-react with IL-1a (NIBSC, Potts Bar, UK), IL-1, IL-2, IL-4, IL-5 and TNF (Amersham). Specific binding of 38% was obtained with non-specific binding (NSB) of 4·3% and an ED_{50} of 2·7 fmol/tube. The sensitivity, defined as the dose needed to reduce binding by 2 × s.d. from B_0 was 0·4 fmol/tube. All assays were performed in quadruplicate. The within-assay coefficient of variation (CV) was 2·2 ± 0·08 fmol/tube 3·6% CV, $n=20$; and between-assay variability was 2·9 ± 0·35 fmol/tube 12·7% CV, $n=9$.

All IL-6 results from BALF are quoted as fmol/ml ELF and are also given as fmol/mg albumin.

In addition to the BALF samples, 12 matched serum samples were assayed for IL-6 (six from the PBD group and six from the asymptomatic pigeon breeders). This was done in order to determine whether there was any significant leakage of IL-6 into the alveolar space from the peripheral circulation.

IgG and albumin estimations

IgG and albumin in BALF were determined using a double antibody (sandwich) ELISA, and compared with a pooled BALF sample. Final concentrations of IgG and albumin were obtained by reference to a known serum standard.

Urea assay and ELF estimation

Urea concentration was determined using a modified urease Berthelot reaction in microtitre plates (Jones *et al.*, 1990). ELF volume was then calculated using the formula:

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

Statistical analysis

Descriptive statistics are quoted as medians and interquartile ranges. Overall variation was determined using the Kruskal–Wallis analysis of variance; intergroup comparisons were made

Table 1. Interleukin-6 (IL-6) levels (medians and interquartile ranges) before and after standardization with urea or albumin

Subject group	IL-6 (fmol/ml BALF)	IL-6 (fmol/ml ELF)	IL-6 (fmol/mg albumin)
Controls ($n=11$)	5·74 4·4–6·04	901·5 723–1732	512·5 259–958
Sarcoidosis ($n=10$)	6·56 5·91–7·12	1239 1129–1503	180* 89–324
Pigeon breeders' disease ($n=15$)	7·36* 5·64–8·45	879 456–1087	88·4† 71·4–133
Asymptomatic pigeon breeders ($n=8$)	7·24* 6·89–7·49	1784* 1053–2791	325 173–468
Idiopathic pulmonary fibrosis ($n=15$)	6·91* 5·91–8·05	950 906–1344	365 185–795

BALF, bronchoalveolar lavage fluid; ELF, epithelial lining fluid.
*$P<0·01$; †$P<0·001$ compared with control group.

Table 3. IgG levels (medians and interquartile ranges) before and after standardization with urea or albumin

Subject group	IgG (μg/ml BALF)	IgG (μg/ml ELF)	IgG (μg/ml albumin)
Controls ($n=11$)	2·1 1·5–4·2	394 265–1111	189 95·2–545
Sarcoidosis ($n=10$)	6·0* 3·6–12	1593* 1207–2400	171·5 110–205
Pigeon breeders' disease ($n=15$)	68·4* 34–146	4899* 3088–8669	711† 429–1624
Asymptomatic pigeon breeders ($n=8$)	5·4 2·8–8·3	985·5‡ 594–1584	190·5 146–236
Idiopathic pulmonary fibrosis ($n=15$)	13·5† 9–29	1605* 1272–2697	883† 471–1370

BALF, bronchoaveolar lavage fluid; ELF, epithelial lining fluid.
*$P<0·001$; †$P<0·01$; ‡$P<0·05$, with control group.

Table 2. Variation in epithelial lining fluid (ELF) volume albumin concentration and bronchoalveolar lavage fluid (BALF) return between subject groups (medians and interquartile ranges)

Subject group	ELF (vol., ml)	Albumin (μg/ml)	BALF (vol., ml)
Controls ($n=11$)	0·385 0·242–0·414	11·2 3·85–22·2	68·0 64–73
Sarcoidosis ($n=10$)	0·364 0·273–0·450	41·85† 18·2–65·2	80·0 63–82
Pigeon breeders' disease ($n=15$)	0·571* 0·287–1·042	75·5** 63·2–117	66·0 54–80
Asymptomatic pigeon breeders ($n=8$)	0·319 0·186–0·413	26·45 16·1–36·75	59·5 46·5–78·5
Idiopathic pulmonary fibrosis ($n=15$)	0·517 0·300–0·826	16·2 7·3–33·5	60·0 56–90

*$P<0·025$; †$P<0·001$, compared with control group.

using the Mann–Whitney U-test; and correlations were performed using the Spearman rank correlation coefficient.

RESULTS

When IL-6 levels were measured directly in BALF, they were significantly higher in all groups, except the sarcoidosis patients, compared with controls, (Kruskal value 13·6, $P<0·01$) with all other groups showing elevated IL-6 ($P<0·01$ Mann–Whitney *U*-test in all cases). However, these increases over normal levels were negated when IL-6 concentrations were standardized in terms of IL-6/ml of ELF using urea (Table 1) (Kruskal value 10·89, $P<0·05$) with only the asymptomatic pigeon breeders with raised IL-6 compared with the control group; all other patient groups showed no significant difference. When albumin was used for standardization (Table 1) (Kruskal value 23·556, $P<0·001$), two patient groups showed a marked reduction in IL-6 (sarcoidosis $P<0·01$, and PBD $P<0·001$). These findings reflect the fact that both ELF volume and albumin were significantly raised in PBD (Table 2) when compared with controls ($P<0·025$ and $P<0·001$, respectively). Albumin was also higher in the sarcoidosis patients ($P<0·001$) with other patient groups showing no significant difference when compared with the controls. ELF volume shows some variation within patient groups when compared with controls (Kruskal value 10·08, $P<0·05$) but with only the PBD patients having ELF volumes higher than controls (Table 2).

The patients with PBD had lower levels of IL-6 than did their asymptomatic counterparts, using either urea or albumin as method of standardisation ($P<0·025$ and $P<0·001$, respectively). There was no significant difference in concentrations of IL-6/ml BALF between these two groups.

Total IgG levels in BALF were highest in the PBD group and these were significantly increased above control values ($P<0·001$). The sarcoidosis and IPF patients also had elevated levels of IgG/ml BALF ($P<0·001$ and $P<0·01$, respectively) (Table 3).

If expressed in terms of urea, all patient groups have significantly raised levels of IgG ($P<0·001$ for all groups, except the asymptomatics $P<0·05$). When expressed as μg/mg of albumin, then only the PBD and IPF patients ($P<0·01$ in both cases) still show raised levels of IgG (Table 3).

There was no correlation between BALF IgG and IL-6 in any single group or when all groups were considered together.

IL-6 was detected in only six of the 12 sera investigated this was in the range of 3–5 fmol/ml with the other samples below the limit of detection. Both serum albumin and urea in these subjects were within normal ranges (albumin $40{\cdot}5 \pm 4{\cdot}15$ g/l, urea $328 \pm 23{\cdot}5$ μg/ml).

DISCUSSION

The effects of standardization of IL-6 levels using albumin or urea tend to negate or reverse the increase in IL-6 seen in all patient groups when compared with the control subjects. In the case of albumin, this can be explained by its marked increase in the disease groups, particularly the patients with sarcoidosis and with PBD ($P < 0{\cdot}001$ in both cases). These two groups both showed significantly lower levels of IL-6/mg albumin when compared with the control subjects (sarcoidosis $P < 0{\cdot}01$; PBD $P < 0{\cdot}001$).

Urea as used to calculated ELF volume gave significantly higher values only in the PBD group, but when IL-6 was expressed in terms of ELF volume only the asymptomatic group showed a difference when compared with the control group. However, in the case of asymptomatic pigeon breeders, this was in terms of significantly increased IL-6.

Variation in albumin and urea in BALF have been reported previously (Jones *et al.*, 1990) as have the limitations of both these substances to accurately reflect dilution effects in BALF (Rennard *et al.*, 1986; Marcy *et al.*, 1987). In the absence of any reliable alternative, however, we felt it necessary to quote values using both.

The absence of a significant correlation between IL-6 and IgG in this study conflicts with results of *in vitro* studies which have demonstrated a relation between IL-6 and IgG production (Hirano *et al.*, 1986). This lack of a correlation was true even of those patients where IgG was most markedly elevated, the PBD group, although these patients did have the lowest levels of IL-6 when expressed in terms of albumin or urea (not significantly so in the latter). Patients with PBD have also been shown to exhibit raised serum IgG (Patterson *et al.*, 1976) and this feature has also long been recognized in sarcoidosis (Buckley & Dorsey, 1970). The absence of a demonstrable link between IL-6 and IgG production may be attributable to a number of factors. Taga *et al.* (1987) have demonstrated that resting B cells have few receptors for IL-6 but when activated these may increase by up to 3-fold. Thus, significant amounts of IL-6 may be undetected in those patients with elevated IgG levels, due to increased binding to cell surface receptors, thereby making it unavailable for assay detection. Alternatively, IgG itself may exhibit a down-regulatory effect on IL-6 production by a negative feedback mechanism, as yet unidentified. A further possibility is that cell-free soluble receptors for IL-6 may be present in lavage fluid again binding IL-6 and rendering it undetectable by radioimmunoassay. Such receptors for IL-2 have been identified and have been reported in both BALF and serum (Wood, Symons & Duff, 1988, Lawrence *et al.*, 1988; Reynolds *et al.*, 1989). Other studies have identified inhibitors for both IL-1 (Arend *et al.*, 1989) and IL-2 (Emery *et al.*, 1988), and a similar antagonist may operate to control IL-6 secretion. A deficiency in the production of mediators to down-regulate IL-6 production may be associated with the increased levels in asymptomatic pigeon breeders, who, like their symptomatic cohorts, exhibit a lymphocytic alveolitis (Costabel *et al.*, 1984). This increase may also be due to reduced binding to B cell receptors in these subjects. It could be due to the absence of as yet unidentified co-factors required for IL-6-mediated stimulation of B cells, thus reducing the expression of such receptors by these cells. This may explain why such patients remain asymptomatic despite continued exposure to pigeon antigens, and may also suggest a mechanism by which a proportion of such individuals may go on to develop overt clinical disease.

The levels of IL-6 seen in serum in relation to albumin and urea are considerably lower than those seen in BALF. This suggests that IL-6 is produced on a local level and there is no significant contribution from IL-6 present in the peripheral circulation.

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