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In vitro production of B cell growth factor and B cell differentiation factor by peripheral blood mononuclear cells and bronchoalveolar lavage T lymphocytes from patients with idiopathic pulmonary fibrosis

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SUMMARY

The activation of B lymphocytes and formation of immune complexes have been suggested to play an important role in the pathogenesis of idiopathic pulmonary fibrosis (IPF). To investigate the mechanisms of activation of B lymphocytes, we studied the production of B cell growth factor (BCGF) and B cell differentiation factor (BCDF) in patients with IPF and those with interstitial pneumonia associated with collagen vascular diseases (IP-CVD), in comparison with healthy controls. Culture supernatants of peripheral blood mononuclear cells from patients with IPF induced more IgM and IgA production by B lymphocytes than those from healthy controls, indicating a higher production of BCDF in the patients. Culture supernatants of T lymphocytes obtained from bronchoalveolar lavage fluids (BALF) of patients with IPF induced higher proliferation of B lymphocytes than those from healthy controls, indicating a higher production of BCGF. An increase in production of BCGF and BCDF was not observed in patients with IP-CVD. In the light of these results, it was suggested that there may be an imbalance in T lymphocyte subsets that release lymphokines like BCGF and BCDF in patients with IPF, and that the subsets may differ between blood and BALF. It remains to be elucidated whether the activation of B lymphocytes depending on T lymphocytes determines the development of disease in IPF.

Keywords idiopathic pulmonary fibrosis B cell differentiating factor B cell growth factor B lymphocytes T lymphocytes

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is a disorder of unknown aetiology, but some immunological mechanisms are thought to be involved in the pathogenic process (Crystal *et al.*, 1981). This is indicated by an increased prevalence of positive rheumatoid factor, positive anti-nuclear antibody, and high level of serum gammaglobulin without association of collagen vascular diseases (Turner-Warwick, Burrows & Johnson, 1980). In an earlier study, we reported a significant increase in the number of immunoglobulin-secreting lymphocytes in blood and bronchoalveolar lavage fluid (BALF); this strongly suggested that B lymphocytes were activated in the blood and lungs in patients with IPF (Izumi, Fujimura & Oshima, 1983). Some studies have shown that circulating and BALF immune complexes are present in patients with IPF (Dreisin *et al.*, 1978; Haslam *et al.*, 1979). It is thought that these immune complexes stimulate the alveolar macrophages to release neutrophil chemotactic factor (NCF), which causes accumulation and activation of neutrophils within the lungs (Gadek *et al.*, 1980; Hunninghake *et al.*, 1981). Activated macrophages and neutrophils release various mediators of inflammation. From such findings, the inflammatory process in IPF was defined as a neutrophil alveolitis, which precedes the following fibrotic process (Crystal *et al.*, 1981).

We investigated the mechanisms involved in the induction of B lymphocyte activation, which would result in fibrosis of the lungs in patients with IPF. With *in vitro* systems for antibody synthesis, it has been shown that antibody responses could be maintained with soluble factors in a system free of T lymphocytes. These factors, designated B cell growth factor (BCGF) (Muraguchi & Fauci, 1982) and B cell differentiation factor (BCDF) (Hirano, Teranishi & Onoue, 1984), are considered to be secreted by T lymphocytes. We suspected that *in vivo* activation of B lymphocytes and an increase of immunoglobulin secretion would depend on such lymphokines as BCGF and BCDF. In fact, we found the elevated production of BCDF by blood T lymphocytes and BCGF by BALF T lymphocytes in patients with IPF.

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Table 1. Characteristics of patients with idiopathic pulmonary fibrosis

Patient no.	Sex	Age	Total protein (g/dl)	Gamma globulin (%)	IgG (mg/dl)	IgA (mg/dl)	IgM (mg/dl)	RAHA	Anti-nuclear antibody	Anti-DNA antibody	Immune complex (μg/ml)
1	F	62	7·3	21·1	1179	777	118	–	–	–	<1·5
2	F	55	7·1	24·4	1610	422	172	–	–	–	<1·5
3	F	65	5·9	12·6	2087	288	237	–	–	–	ND
4	M	65	8·2	23·0	2470	417	84	–	+	–	ND
5	M	62	7·8	29·3	2479	371	54	–	–	–	<1·5
6	M	54	8·5	40·1	3465	592	275	+	+	–	<1·5
7	M	45	7·8	12·7	1360	380	63	–	–	–	<1·5
8	F	64	6·9	15·7	1703	357	185	–	–	–	ND
9	M	57	7·4	14·2	1484	270	112	–	–	–	<1·5
10	M	44	7·4	14·1	1440	392	196	–	–	–	<1·5
Healthy controls upper limit			7·5	18·7	1440	300	190	–	–	–	3·0

RAHA, rheumatoid arthritis haemagglutination test; ND, not done.

MATERIALS AND METHODS

Study population

Ten patients with IPF, eight patients with interstitial pneumonia associated with collagen vascular diseases (IP-CVD), and 12 healthy controls were involved in this study. All were non-smokers at the time of study. All cases of IPF were diagnosed as usual interstitial pneumonia (UIP) (Carrington *et al.*, 1978) based on open lung biopsy. Patients with IP-CVD were six cases with rheumatoid arthritis (RA), one case with systemic lupus erythematosus (SLE), and one case with dermatomyositis. None of the subjects had any signs or symptoms of infection. None of them was treated with corticosteroids. Laboratory findings are shown in Table 1.

Reagents

Purified phytohaemagglutinin (PHA) was obtained from Wellcome Diagnostics (Dartford, UK). The CD3 monoclonal antibody and the CD11 monoclonal antibody were obtained from Ortho Diagnostic Systems, (Raritan, NJ). *Staphylococcus aureus* Cowan I (SAC) was obtained from the Enzyme Center (Malden, MA). Standard BCGF was obtained from Cytokine Technology International (Buffalo, NY) and was a purified product of PHA-stimulated human blood T lymphocytes. Standard BCDF was a product of PHA-stimulated human T lymphocytes obtained from three healthy volunteers in our laboratory.

Separation of blood mononuclear cells and bronchoalveolar lavage fluid cells

Peripheral blood mononuclear cells (PBMC) were separated by standard Ficoll–Hypaque gradient centrifugation (Böyum, 1968). This population contained 40–50% T lymphocytes as determined by staining with anti-CD3 monoclonal antibody.

Bronchoalveolar lavage (BAL) was performed with a routine technique in our laboratory (Nagai *et al.*, 1985). Briefly, after local anaesthesia, the fibre-optic bronchoscope was wedged into a distal bronchus in the right middle lobe. A total of 300 ml, 0·9% sterile saline was used, divided in six aliquots of 50 ml each. Recovered BAL fluid (BALF) cells were filtered through gauze and centrifuged twice at 300 ***g*** for 10 min at 4°C, and then suspended in Eagle's MEM. The T lymphocyte fraction was separated by rosette formation with neuraminidase-treated sheep erythrocytes (Greaves & Brown, 1974). This fraction contained 90% T lymphocytes as determined by staining with anti-CD3 monoclonal antibody.

Preparation of supernatants containing BCGF and BCDF

Culture supernatants containing BCGF and BCDF were prepared as previously described by Muraguchi & Fauci (1982). Briefly, PBMC and BALF T lymphocytes from the subjects were cultured at 37°C at a density of $1·0 \times 10^6$ cells/ml in RPMI 1640 (Nissui, Tokyo, Japan) supplemented with 1% heat-inactivated fetal calf serum (FCS) (M.A. Bioproducts, Walkersville, MD), 5×10^{-5} M 2-mercaptoethanol, and purified PHA at a final concentration of 2 μg/ml. The culture was harvested after 3 days of incubation, and the cells were removed by centrifugation at 1500 rev/min for 5 min at 4°C. The supernatant was stored at −20°C for later use.

Concentrations of interleukin-1 (IL-1), interleukin-2 (IL-2), and interferon-gamma (IFN-γ) in the supernatants were measured for the groups of IPF, IP-CVD, and healthy controls as follows. The concentration of IL-1 was determined by radioimmunoassay (Cistron IL-1β (^{125}I) RIA kit, Cistron biotechnology, Pine Brook, NJ), that of IL-2 by ELISA (Intertest 2, Human interleukin-2 ELISA, Genzyme Corporation, Boston, MA), and that of IFN-γ by radioimmunoassay (Centocor Gamma Interferon Radioimmunoassay, Centocor, Malvern, PA).

Separation of B lymphocytes

PBMC from a healthy volunteer were separated by standard Ficoll–Hypaque gradient centrifugation. B lymphocyte-enriched populations were obtained by depletion of T lymphocytes and monocytes. T lymphocyte depletion was accomplished by rosetting the T lymphocytes with neuraminidase-treated sheep erythrocytes followed by Ficoll–Hypaque centrifugation, and remaining T lymphocytes were killed by treatment with anti-CD3 monoclonal antibody and guinea pig complement. To

deplete monocytes, the T lymphocyte-depleted cell suspension in 5 ml RPMI 1640 supplemented with 10% FCS was placed on plastic dishes for 2 h at 37°C. Monocytes were removed by adsorption onto the dishes.

The B lymphocyte-enriched population thus prepared contained 60–80% of cells positive in surface immunoglobulin as determined by staining with a fluorescein-conjugated $F(ab')_2$ fragment of goat anti-human IgG (Tago, Burlingame, CA), 20% monocytes as determined by staining with anti-CD11 monoclonal antibody and less than 1% T lymphocytes as determined by staining with anti-CD3 monoclonal antibody.

Assay for BCGF activity

B lymphocytes from a healthy volunteer were cultured at a cell density of 1×10^5/0·2 ml in RPMI 1640 supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, plus 0·05 ml of BCGF-containing supernatant in 96-well, flat-bottomed plates (Falcon 3072, Becton Dickinson, Oxnard, CA). Cultures were incubated in 100% humidity and in 5% CO_2 at 37°C for 72 h. The cultures were pulsed with 0·5 μCi of ^{3}H-thymidine during the last 20 h of incubation and harvested. BCGF activity was measured by determining the incorporation of ^{3}H-thymidine.

Assay for BCDF activity

B lymphocytes from a healthy volunteer were cultured at a cell density of 1×10^5/0·2 ml in RPMI 1640 supplemented with 10% FCS, 5×10^{-5} M 2-mercaptoethanol, plus 0·05 ml of BCDF-containing supernatant in 96-well, flat-bottomed plates (Falcon 3072, Becton Dickinson). Cultures were incubated in 100% humidity and in 5% CO_2 at 37°C for 5 days, and then the number of immunoglobulin-secreting cells was measured by a reverse haemolytic plaque assay, using *Staphylococcus* protein A-coated sheep erythrocytes, rabbit anti-human IgM, IgA, and IgG antibodies (Igakuseibutugaku Kenkyusyo, Nagoya, Japan), and complement from guinea pig serum (Gronowicz, Coutinho & Melchers, 1976).

Statistical analysis

Experimental results were analysed for their statistical significance by Student's *t*-test.

RESULTS

Definition of optimal conditions for the assays using standard BCGF and BCDF

Proliferation of SAC-stimulated B lymphocytes in the presence or absence of BCGF. B lymphocytes from a healthy volunteer, at a cell density of 1×10^5/well, were cultured with various concentrations of SAC for 3 days, since higher proliferation of B lymphocytes was reported when preactivated by SAC (Muraguchi & Fauci, 1982). Then, B lymphocytes were transferred to another microplate, and the cultures were continued with or without standard BCGF for 3 days. Proliferation of B lymphocytes is shown in Fig. 1. Incorporation of ^{3}H-thymidine was higher in the presence of BCGF. That was highest when cultured without prior activation by SAC, in contrast with the previous report.

The kinetics of BCGF-induced B lymphocyte proliferation. To study the kinetics of proliferation, B lymphocytes from a healthy volunteer were cultured for various periods of time

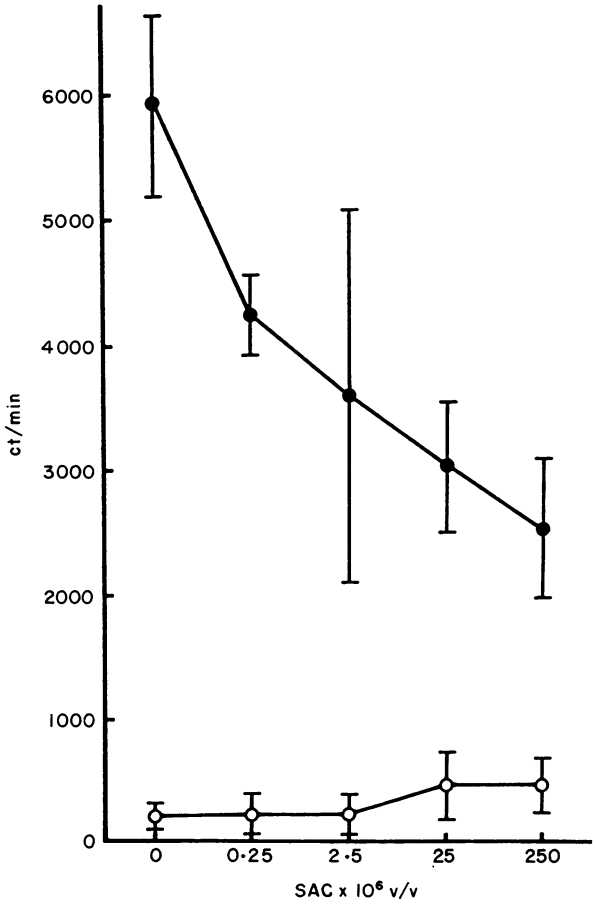

Fig. 1 Proliferative response of B lymphocytes to *Staphylococcus aureus* Cowan I (SAC) and B cell growth factor (BCGF). After B lymphocytes had been activated by SAC for 3 days, they were cultured in the presence (●) or absence (○) of BCGF for 3 days (mean ± s.e.m.).

without activation by SAC, and incorporation of ^{3}H-thymidine was measured. The relation between the level of proliferative response of B lymphocytes and the concentration of standard BCGF in the culture (from 1/2 to 1/100 dilution of standard BCGF) was also studied. Maximal proliferation occurred on day 3 with the highest concentration (1/2 dilution) of BCGF (data not shown).

The kinetics of BCDF-induced B lymphocytes differentiation. B lymphocytes from a healthy volunteer, at a cell density of 1×10^5/well, were cultured with standard BCDF for various periods of time (from 1 to 7 days). The sum of the number of IgM-, IgA-, and IgG-secreting cells was maximal after 5 days of culturing (data not shown). To study the relation between the differentiation of B lymphocytes and the concentration of BCDF, B lymphocytes were cultured with various concentration of BCDF (from 1/2 to 1/100 dilution of standard BCDF) for 5 days. The sum of the number of cells secreting IgM, IgA, and IgG increased, depending on the concentration of BCDF (data not shown).

Results of studies using culture supernatants

BCGF activity in culture supernatants of blood cells from healthy controls, patients with IPF, and IP-CVD. B lymphocytes from a healthy volunteer were cultured with the supernatants

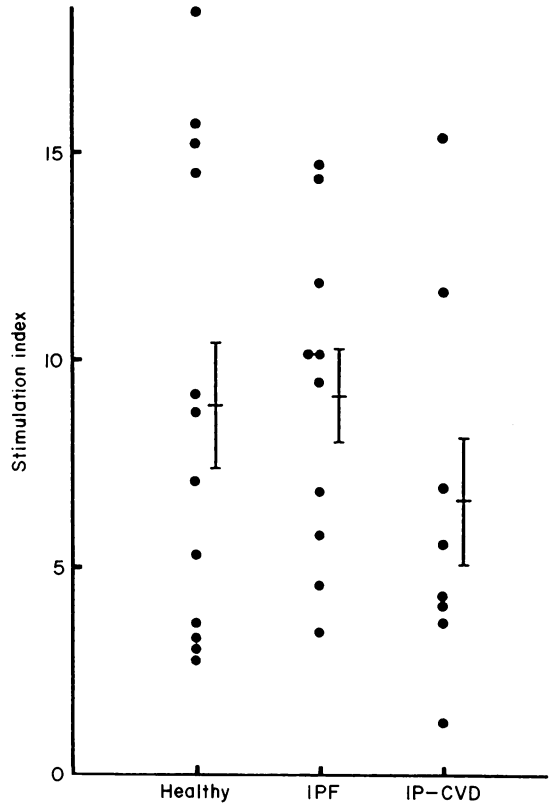

Fig. 2. B cell growth factor (BCGF) activity in culture supernatants of blood cells from healthy controls, patients with idiopathic pulmonary fibrosis (IPF), and interstitial pneumonia associated with collagen vascular disease (IP-CVD). B lymphocytes were cultured with BCGF-containing supernatants at a cell density of 1×10^5/well, and ^{3}H-thymidine incorporation was measured on day 3. The differences among the groups were not significant (mean ± s.e.m.)

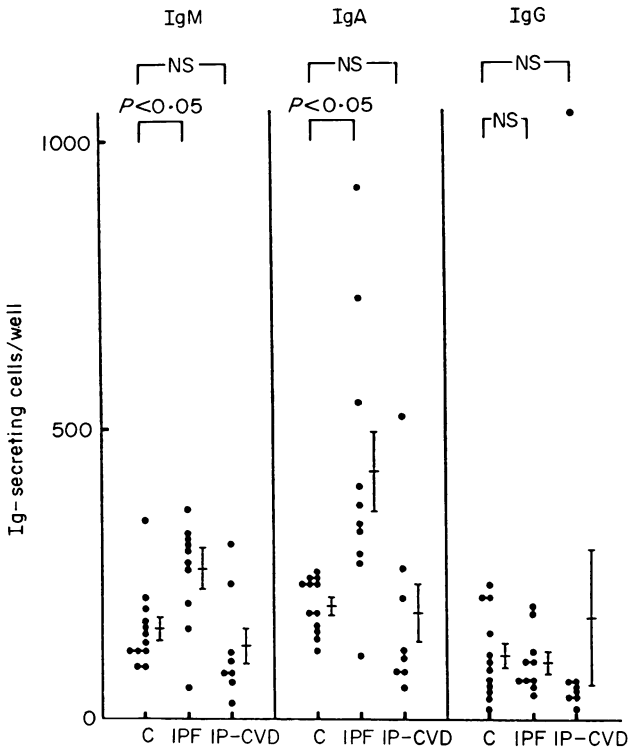

Fig. 3. B cell differentiation factor (BCDF) activity in culture supernatants of blood cells from healthy controls (C), patients with idiopathic pulmonary fibrosis (IPF), and interstitial pneumonia associated with collagen vascular disease (IP-CVD). B lymphocytes were cultured with BCDF-containing supernatants at a cell density of 1×10^5/well, and immunoglobulin-secreting cells were detected after 5 days of culture. The number of IgM- and IgA-secreting cells significantly increased in patients with IPF ($P < 0{\cdot}05$) (mean ± s.e.m.). NS, not significant.

(1/2 dilution) from the subjects for 3 days. The BCGF activity was expressed as stimulation index (SI), obtained from the following formula: SI = ^{3}H-thymidine uptake with BCGF/^{3}H-thymidine uptake without BCGF (ct/min, mean ± s.e.m.). The BCGF activity was 8·9 ± 1·5 in healthy controls, 9·1 ± 1·1 in patients with IPF, and 6·6 ± 1·5 in patients with IP-CVD. There were no significant differences among the groups (Fig. 2).

BCDF activity in culture supernatants of blood cells from healthy controls, patients with IPF, and IP-CVD. B lymphocytes from a healthy volunteer were cultured with the supernatants (1/2 dilution) from the subjects for 5 days. The BCDF activity was expressed as the number of immunoglobulin-secreting cells for each immunoglobulin class (mean ± s.e.m.) (Fig. 3). The number of IgM-secreting cells/well was 158 ± 19 in healthy controls, 253 ± 27 in patients with IPF, and 126 ± 30 in patients with IP-CVD. The number of IgA-secreting cells was 196 ± 13 in healthy controls, 425 ± 70 in patients with IPF, and 180 ± 51 in patients with IP-CVD. The number of IgG-secreting cells was 111 ± 20 in healthy controls, 100 ± 15 in patients with IPF, 175 ± 118 in patients with IP-CVD. The increase in the number of IgM- and IgA-secreting cells in patients with IPF compared with other groups was significant ($P < 0{\cdot}05$).

BCGF activity in culture supernatants of BALF cells. B lymphocytes were cultured with the supernatants of BALF cells from healthy controls and from patients with IPF for 3 days. The BCGF activity expressed as SI (mean ± s.e.m.) was 13·1 ± 1·9 in healthy controls and 20·1 ± 2·0 in patients with IPF. BCGF activity was significantly elevated in patients with IPF ($P < 0{\cdot}05$) (Fig. 4).

BCDF activity in culture supernatants of BALF cells. B lymphocytes were cultured with the supernatants of BALF cells from healthy controls and patients with IPF for 5 days. The BCDF activity was expressed as the number of immunoglobulin-secreting cells for each immunoglobulin class (mean ± s.e.m.) (Fig. 5). The number of IgM-secreting cells was 82 ± 47 in healthy controls and 14 ± 2 in patients with IPF. The number of IgA-secreting cells was 27 ± 14 in healthy controls and 13 ± 3 in patients with IPF. The number of IgG-secreting cells was 34 ± 25 in healthy controls and 6 ± 2 in patients with IPF. There were no significant differences between the groups.

The patients with IP-CVD could not be studied because the recovered volume of BALF was too small to obtain enough T lymphocytes, and because the BAL procedure easily induced diffuse mucosal bleeding in them.

Concentrations of IL-1, IL-2 and IFN-γ in culture supernatants. The concentrations of these factors (mean ± s.e.m.) were examined since some studies have shown that they affect the proliferation and differentiation of B lymphocytes (Pike & Nossal, 1985; Nakagawa *et al.*, 1985). The concentration of IL-1

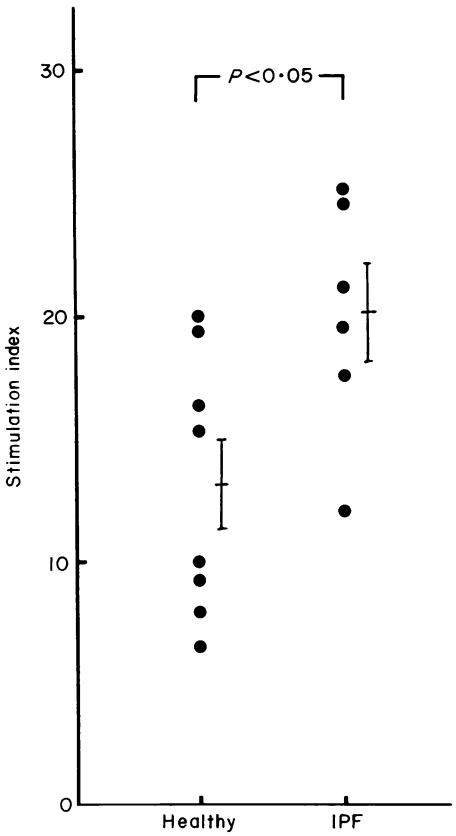

Fig. 4. B cell growth factor (BCGF) activity in culture supernatants of bronchoalveolar lavage fluid cells from healthy controls and patients with idiopathic pulmonary fibrosis (IPF). BCGF activity expressed as stimulation index significantly increased in patients with IPF ($P < 0{\cdot}05$). (mean ± s.e.m.).

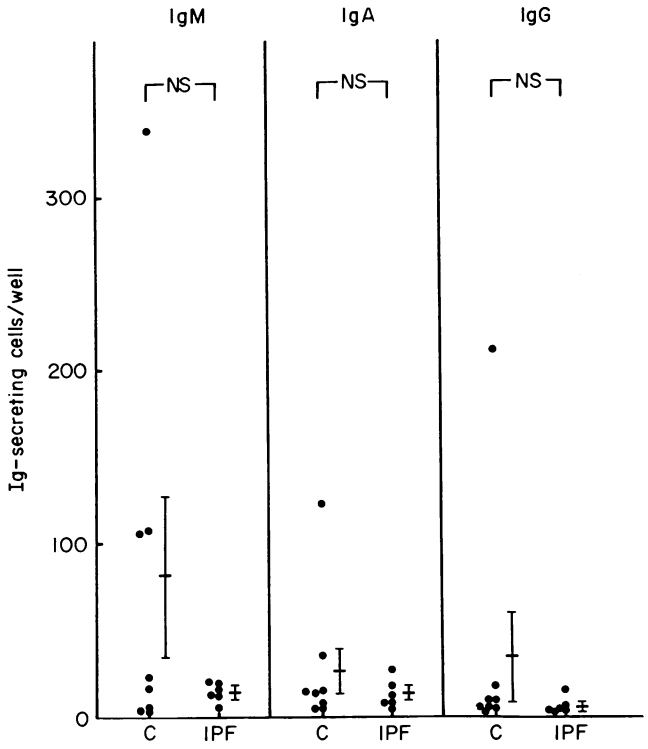

Fig. 5. B cell differentiation factor (BCDF) activity in culture supernatants of bronchoalveolar lavage fluid cells from healthy controls (C) and patients with idiopathic pulmonary fibrosis (IPF). The differences of numbers of immunoglobulin-secreting cells between the groups were not significant (mean ± s.e.m.).

in the culture supernatants of blood cells was 8·8 ± 0·6 ng/ml in healthy controls, 7·5 ± 0·8 ng/ml in patients with IPF, and 2·6 ± 0·5 ng/ml in patients with IP-CVD. The difference between healthy controls and patients with IPF was not significant. The concentration of IL-1 was significantly lower in patients with IP-CVD. The concentration of IL-1 of culture supernatants of BALF cells was 1·4 ± 0·6 ng/ml in healthy controls, and 1·0 ± 0·9 ng/ml in patients with IPF. The difference was not significant. We could not detect IL-2 activity in the supernatants of both blood cells and BALF cells in any of the groups. The concentration of IFN-γ in the culture supernatants of blood cells was 172 ± 34 U/ml in healthy controls, 252 ± 81 U/ml in patients with IPF and 175 ± 90 U/ml in patients with IP-CVD. The differences among the groups were not significant. The concentration of IFN-γ in the culture supernatants of BALF cells was 387 ± 111 U/ml in healthy controls, and 159 ± 38 U/ml in patients with IPF. The difference between the groups was not significant.

We could not find levels of IL-1, IL-2 and IFN-γ of the order known to affect proliferation and differentiation of B lymphocytes in the supernatants.

DISCUSSION

Soluble factors like BCGF and BCDF have been shown to be involved in the activation of B lymphocytes. Recent studies have indicated that the production of BCGF and BCDF is increased in patients with SLE (Martinez-Cordero, Alcocer-Varela & Alarcon-Segovia, 1986). We investigated the production of BCGF and BCDF in blood cells and BALF cells of patients with IPF. We used the assay systems established by Muraguchi & Fauci (1982) and Hirano *et al.* (1984) with slight modifications. Although Muraguchi & Fauci (1982) pre-activated the B lymphocytes by either SAC or anti-IgM, it is controversial whether the pre-activation enhances the proliferation and differentiation of B lymphocytes (Rabin, Ohara & Paul, 1985; Mayer, 1986). In our studies, the synergistic effect of SAC and BCGF could not be demonstrated. B lymphocytes in peripheral blood might have already been pre-activated *in vivo*.

We reported previously that the number of immunoglobulin-secreting cells increased in BALF and blood of patients with IPF (Izumi *et al.*, 1983). It is known that an increased number of plasma cells can be found histologically in the examination of open lung specimens of patients with IPF (Flint & Colby, 1987; Kitaichi, 1989). It is difficult to determine whether the activation of B lymphocytes causes the pathogenic process in IPF or only reflects the disease activity; Crystal *et al.* (1981, 1984) suggested that it represents an important step committing the formation of alveolitis in IPF. Unknown antigenic agents might enter the lungs via the airway or blood stream, and immune complexes could be formed by the binding of locally or systemically produced antibodies to these antigens. In the lungs, immune complexes might activate alveolar macrophages, which release a neutrophil chemotactic factor (NCF) and induce accumulation

and activation of neutrophils (Hunninghake *et al.*, 1981). Activated macrophages and neutrophils, which are the main constituents of the alveolitis, would release various mediators of inflammation and induce a significant lung injury that causes fibrosis.

There has been a controversy about the question where immune complexes are formed (Daniele *et al.*, 1981). Considering that many of the antigens, such as smoke, micro-organisms, and chemical stimulants are inhaled through the airway, and the lungs are lymphoid organs that can synthesize their own antibodies, there is one possibility that the inhaled antigens would encounter these antibodies and form immune complexes in the lungs.

Extrinsic inhaled antigens might be a source of immune complexes formed in the lungs, although specific antibodies would be produced systemically. Altered or exposed lung antigens might be recognized in certain circumstances as foreign materials, and immune complexes could be formed from the binding of locally or systemically produced antibodies to them (Turner-Warwick & Haslam, 1971). Alternatively, immune complexes might be formed systemically and become deposited in the lungs via the bloodstream. Our data showed the possibility that unknown inhaled antigens activate alveolar macrophages which then stimulate T lymphocytes and induce the production of BCGF in the lungs. Through this process, local production of antibodies and local formation of immune complexes might occur. However, our data also showed the enhanced production of BCDF by blood T lymphocytes. It is feasible that T lymphocytes enter the blood stream and systemically produce BCDF after they are stimulated. There is also a possibility that T lymphocytes are systemically stimulated to produce BCDF. In fact, some evidence, such as a detection of rheumatoid factor and an anti-nuclear antibody in blood, and an increased level of immunoglobulins in blood, have suggested that IPF might represent a pulmonary manifestation of a systemic disease mediated through the immune system.

We could not detect the increased production of BCGF and BCDF by blood T lymphocytes in patients with IP-CVD. It has been reported that human B lymphocytes stimulated by aggregated IgG can produce a factor which suppresses the pokeweed mitogen-induced plaque-forming cell response (Pisko *et al.*, 1986). This factor is thought to play a role in suppressing the over-production of antibodies in the human body. It is not known whether increased levels of this suppressive factor are present in the culture supernatants obtained from patients with IP-CVD. Our patients with IP-CVD showed over-production of immunoglobulin, indicating that their B cells are activated. One report on patients with SLE has shown that the production of BCGF and BCDF does not increase although the response of B lymphocytes to BCGF and BCDF is elevated (Hirose *et al.*, 1985). Our IP-CVD group included only one case with SLE, but if this was true in other collagen vascular diseases, our results suggest that production of BCGF and BCDF is enhanced in patients with IPF, but response of B lymphocytes is elevated in patients with IP-CVD. These functional differences may contribute to the differences in therapeutic responses to corticosteroids and the prognosis between IPF and IP-CVD, in spite of the similarity of the histological changes in the alveolar septum.

T lymphocytes from patients with IPF produce increased levels of BCGF and BCDF when stimulated *in vitro*. The increased production of BCDF by blood T cells and of BCGF by BALF T cells might reflect a difference of T lymphocytes subsets, which are considered to play a key role in the pathogenic process of immune-mediated inflammatory disorders like IPF.

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