DOI: 10.2169/internalmedicine.47.0402

Table 1. Clinical Features of Patients with *Pneumocystis jiroveci* Pneumonia

	PCP group	Control group		p Value
		Prophylactic TMP-SMX(Y)	Prophylactic TMP-SMX(N)	
Age (yr)	65.1 ± 7.3	64.6 ± 10.1	63.0 ± 12.7	0.85
Sex (F/M)	4 / 3	7 / 8	21 / 21	0.90
Body Mass Index	21.3 ± 12.2	22.3 ± 3.8	21.8 ± 3.82	0.82
Smoking status (N/F/C)	3 / 3 / 1	5 / 6 / 4	18 / 15 / 9	0.95
Diabetes Mellitus (Y/N)	1 / 6	1 / 14	6 / 36	0.74
%VC (%)	67.6 ± 9.0	70.5 ± 23.5	67.6 ± 23.7	0.95
%FEV (%)	79.1 ± 10.9	84.8 ± 26.4	73.2 ± 27.5	0.58
Initial dose of PSL (mg)	55.0 ± 22.2	49.0 ± 11.4	40.1 ± 12.3	0.009
Pulse therapy (Y/N)	3 / 4	6 / 9	8 / 34	0.169
Prophylactic TMP-SMX (Y/N)	0 / 7	15 / 15	0 / 27	
Immunosuppressant (Y/N)	3 / 4	7 / 8	7 / 35	0.10

TMP-SMX: sulfamethoxazole-trimethoprim, Smoking status; N: never smoked, F: former smoker, C: current smoker. Differences between three groups were evaluated by either the χ^2 or one-factor ANOVA.

ment of Respiratory Medicine at Nippon Medical School Hospital, and who received more than 0.5 mg/kg of prednisolone with or without immunosuppressants for more than three weeks between July 1997 and September 2003. We evaluated the following clinical parameters: age, sex, underlying disease, body mass index, smoking status, diabetes mellitus, the dose and duration of glucocorticoid therapy, use of immunosuppressants, the white blood cell count before and 4 weeks after the initiation of glucocorticoid therapy, the results of pulmonary function tests before therapy, and the response to prophylaxis with trimethoprim-sulfamethoxazole (TMP-SMX). In the IP patients who developed PCP, we also evaluated the white blood cell count, circulating CD4+ lymphocyte count, and plasma level of beta-D-glucan at the time of diagnosis of PCP.

The diagnostic criteria for PCP were: 1) appropriate findings on high-resolution computed tomography, and 2) detection of *Pneumocystis jiroveci* by microscopic examination or by a positive polymerase chain reaction (PCR) assay of sputum and bronchoalveolar lavage fluid (BALF) samples (5). Sputum and BALF samples were stained with Papanicolaou stain, May-Giemsa stain, and Grocott's stain for microscopic examination.

Differences between pairs of groups were examined for statistical significance with the χ^2 test or one-factor ANOVA. Differences among three groups were examined for statistical significance with repeated measures ANOVA.

Results

The seventy-four patients with interstitial pneumonia comprised 36 men and 38 women with a mean age of 64 years (range: 23-85 years). The underlying diseases were idiopathic pulmonary fibrosis in 20 patients, idiopathic interstitial pneumonias other than idiopathic pulmonary fibrosis in 24 patients, interstitial pneumonia related to collagen vascular disease in 28 patients (5 with rheumatoid arthritis, 2 with systemic lupus erythematosus, 14 with polymyositis/dermatomyositis, 1 with systemic sclerosis, 3 with Sjogren syndrome, and 3 with microscopic polyangitis), pneumoconiosis in 1 patient, and chronic hypersensitivity pneumonia in 1 patient.

Fourteen (18.9%) of the 74 patients developed opportunistic pulmonary infection: 7 developed PCP, 3 had cytomegalovirus pneumonia, 1 developed aspergillus pneumonia, 3 had expanded MRSA pneumonia, and 3 developed sepsis. The ten patients without PCP infection were excluded from this study. We only evaluated the first infection in each patient. All 7 patients showed high-resolution computed tomography findings consistent with PCP. Two patients were positive for pneumocystis on PCR of sputum samples, two had positive findings on microscopic examination of BALF samples, and five were positive for pneumocystis by PCR of BALF samples (including 2 patients who were positive on both examinations).

We compared the 7 patients who developed PCP (PCP group) with the 57 patients who did not develop any opportunistic infection (control group) (Table 1). Age, sex, body mass index, smoking status, results of pulmonary function tests, presence of diabetes mellitus, use of steroid pulse therapy, and use of immunosuppressants did not differ significantly between the two groups. However, the initial dose of glucocorticoids was significantly different between the PCP group and the control group patients with or without TMP-SMX prophylax (trimethoprim 80 mg-sulfamethoxazole 400 mg/day). In the control group, 15 of the 57 patients received prophylactic therapy with TMP-SMX, while none of the patients in the PCP group received TMP-SMX prophylaxis.

The white blood cell count and neutrophil count before glucocorticoid therapy were significantly different between the PCP and control groups. However, the lymphocyte count four weeks after initiation of glucocorticoid therapy was not significantly lower in the PCP group than in the control group (Table 2). Despite this, changes of the lymphocyte count were significantly different among the three groups i.e., the PCP group, control patients with prophylaxis, and

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Table 2. White Blood Cell Subsets in Patients with *Pneumocystis jiroveci* Pneumonia

		PCP group	Control group		p value
			Prophylactic TMP-SMX(Y)	Prophylactic TMP-SMX(N)	
before glucocorticoid therapy	WBC (/ml)	10585 ± 4832	8953 ± 3928	7002 ± 2557	0.0125
	Neutrophils (/ml)	8863 ± 4983	6620 ± 3401	4357 ± 2428	0.0014
	Lymphocytes (/ml)	1668 ± 846	1432 ± 729	1621 ± 688	0.6522
	Monocytes (/ml)	397 ± 163	506 ± 281	427 ± 186	0.4092
4 weeks after starting glucocorticoid therapy	WBC (/ml)	9528 ± 3345	10260± 4077	10125 ± 2466	0.8637
	Neutrophils (/ml)	7941 ± 3475	7354 ± 3601	7090 ± 2397	0.7591
	Lymphocytes (/ml)	1161 ± 583	2225 ± 1769	2371 ± 1384	0.1284
	Monocytes (/ml)	373 ± 218	543 ± 154	502 ± 227	0.2141

Changes of the lymphocyte count among 3 groups: p<0.001

Data from different groups were compared using one-factor ANOVA or repeated measures ANOVA

Table 3. Cases of *Pneumocystis jiroveci* Pneumonia

No.	Age(yr.)	Sex	Diagnosis	Diabetes mellitus	prednisolone				immunosuppressant
					initial daily dose (mg)	cumulative dose (g)	Duration of therapy (d)	Dose at the time of diagnosis of PCP (mg)	
1	63	F	PM/DM	N	60	3.605	63	55	CYA
2	69	M	MPA	N	50 (after pulse therapy)	4.165	49	40	N
3	64	F	PM/DM	N	100 (after pulse therapy)	16.895	175	45	CPA
4	55	F	SSc	N	30	1.530	58	20	N
5	76	F	IIPs(NSIP)	N	40	1.700	38	30	N
6	58	M	IIPs(NSIP)	Y	50	2.730	70	25	N
7	71	M	PM/DM	N	55 (after pulse thrapy)	8.230	45	45	CYA
average	64				55	5.551	71	37	

PM/DM: polymyositis/dermatomyositis, MPA: microscopic polyangitis, SSc: scleroderma, IIPs: idiopathic interstitial pneumonias, NSIP: non-specific interstitial pneumonia, CPA: cyclophosphamide, CYA: cyclosporin A

control patients without prophylaxis (p<0.001 by repeated measures ANOVA).

The 7 patients who developed PCP had a mean age of 64 years (range: 55-76 years) and they consisted of three men and four women. Their underlying diagnoses were nonspecific interstitial pneumonia (n=2), interstitial pneumonia related to polymyositis/dermatomyositis (n=3), interstitial pneumonia related to systemic sclerosis (n=1), and interstitial pneumonia related to microscopic polyangitis (n=1) (Table 3). The initial dose of glucocorticoid (prednisolone) was 30-100 mg/day, the average initial dose of prednisolone was 55 mg/day, and the cumulative dose was 1.530-16.859 g, and the mean cumulative dose was 5.551 g. The duration of glucocorticoid therapy was 38-175 days, with a mean duration of 71 days. The dose of glucocorticoid at the time of diagnosis of PCP was 25-55 mg/day, with a mean dose of 37 mg/day. In all 7 cases, PCP occurred after the acute phase of interstitial pneumonia. Three patients were receiving immunosuppressant therapy at the time of diagnosis of PCP (two patients were receiving cyclosporine at 150 mg/day, and one patient was receiving cyclophosphamide at 50 mg/day).

Table 4. Laboratory Data at the Time of Diagnosis of PCP

No.	WBC (/μl)	Neutrophils (/μl)	Lymphocytes (/μl)	CD4+ lymphocytes (/μl)	β D-glucan (pg/ml)
1	8200	6830	992	ND	1320
2	13500	11475	1957	836	275
3	12300	11574	651	143	ND
4	17500	15050	1487	537	66.7
5	9000	8244	630	273	68.2
6	15000	12600	1950	ND	231
7	13300	12768	266	59	203
Average	12686	11220	1133	370	360

PCP: *Pneumocystis jiroveci* pneumonia, ND: not determined

The PCP group had a lower lymphocyte count (mean: 1,133/μl) and a lower circulating CD4+ lymphocyte count (370/μl) in 5 of 7 patients. However, three patients who had a CD4+ lymphocyte count of greater than 200/μl developed PCP. Six of the seven PCP patients had a high beta-D-glucan level (range: 66.7-1,320 pg/ml, mean: 360 pg/ml). The three patients who had a CD4+ lymphocyte count of greater than 200/μl and developed PCP are presented below

DOI: 10.2169/internalmedicine.47.0402

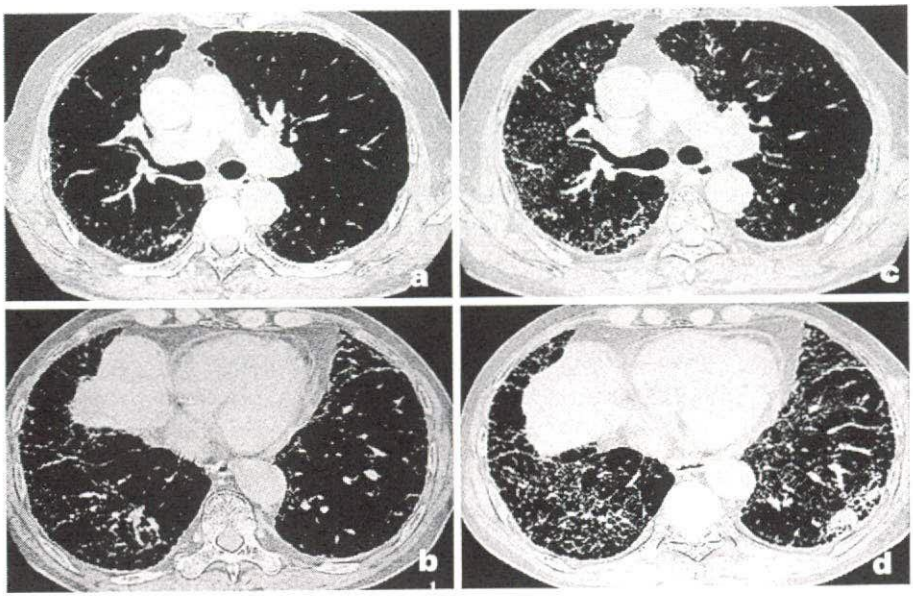

Figure 1. In 'c' and 'd' patchy, bilateral ground-glass opacity is seen, whereas it is absent prior to treatment, 'a' and 'b', respectively.

(Tables 3, 4).

A 69-year-old man (case No. 2) with microscopic polyangitis and usual interstitial pneumonia was treated with steroid pulse therapy (methylprednisolone at 1,000 mg for 3 days) and with prednisolone (50 mg daily). Four weeks after the beginning of therapy, the dose of prednisolone was gradually tapered and the patient showed a good clinical response. Forty-nine days after the start of glucocorticoid therapy (cumulative dose: 4,165 mg), the patient developed a high fever and hypoxia. The white blood cell count was 13,500/μl, lymphocyte count was 1,957/μl, CD4+ lymphocyte count was 836/μl, and beta-D-glucan level was 275 pg/ml. At that time, high-resolution computed tomography revealed patchy bilateral ground glass opacities in addition to honeycomb cysts (Fig. 1). Bronchoalveolar lavage fluid was positive for pneumocystis by PCR. We diagnosed PCP.

A 55-year-old woman (case No. 4) with systemic sclerosis and usual interstitial pneumonia was treated with prednisolone (30 mg/day) and cyclophosphamide (50 mg/day). Four weeks after the beginning of therapy, the dose of prednisolone was gradually tapered and the patient showed a good clinical response. Fifty-eight days after the beginning of glucocorticoid therapy, she was on prednisolone at 20 mg/day and cyclophosphamide at 50 mg/day (cumulative prednisolone dose: 1,530 mg). At this time, the patient developed a high fever and hypoxia. Her white blood cell count was 17,500/μl, lymphocyte count was 1,487/μl, CD4+ lymphocyte count was 537/μl, and beta-D-glucan level was 66.7 pg/ml. High-resolution computed tomography showed patchy bilateral ground-glass opacities in addition to honeycomb cysts. Microscopic examination of BALF revealed *Pneumocystis jiroveci*, and the BALF sample was also positive for pneumocystis on PCR. We diagnosed PCP.

A 76-year-old woman (case No. 5) with idiopathic nonspecific interstitial pneumonia was started on therapy with prednisolone (40 mg/day). Four weeks after the beginning of therapy, the dose was gradually tapered and the patient showed a good response. Thirty-eight days after the beginning of glucocorticoid therapy (on prednisolone at 30 mg/day; cumulative prednisolone dose: 1,700 mg), the patient developed a high fever and hypoxia. Her white blood cell count was 9,000/μl, lymphocyte count was 630/μl, CD4+ lymphocyte count was 273/μl, and beta-D-glucan level was 68.2 pg/ml. The patient developed acute respiratory failure and required mechanical ventilation. At that time, high-resolution computed tomography showed findings consistent with PCP, and her BALF sample was positive for pneumocystis by PCR. We diagnosed PCP.

Discussion

Glucocorticoid therapy is associated with many adverse effects. In particular, opportunistic infection is one of the potentially fatal adverse effects. Stuck et al (4) studied the association between corticosteroid therapy and subsequent infections by pooling data from 71 controlled clinical trials, and found that the rates of both lethal and nonlethal complications were increased in patients who were treated with corticosteroids, with the relative risk being 2.6 and 1.6, respectively. Takabayashi et al (6) reported that 28 (3.1%) of 761 autoimmune disease patients who were treated with glucocorticoid therapy developed pulmonary opportunistic infection and 9 patients (1.2%) developed PCP.

Pneumocystis carinii pneumonia is a major cause of illness and death among persons with an impaired immune system. *Pneumocystis* organisms from different host species have very different DNA sequences. In recognition of its genetic and functional distinctness, the organism that causes human *Pneumocystis* pneumonia has been named *Pneumocystis jiroveci* (7).

In the present study, the rate of opportunistic pulmonary infections among patients with interstitial pneumonia treated with glucocorticoid therapy was 23.0% (17/74), and that of PCP was 9.5% (7/74). The PCP rate was higher than that shown in a previous study (6). One reason for the difference is thought to be improvement of diagnostic methods. Another reason may be that CD4+ lymphocytes are important for host defenses against PCP (8). However, interstitial pneumonia patients who are treated with glucocorticoid therapy not only have CD4+ lymphocyte depletion but are also susceptible to pulmonary infection due to structural abnormalities of the lungs.

In the present patients who developed PCP, the duration of glucocorticoid therapy was 71 days on average and the mean dose of prednisolone at the time of diagnosis was 37 mg/day. These results were similar to those previously reported (6). The initial dose of prednisolone was significantly higher in the PCP group than in the control group, suggesting that a higher dose of glucocorticoids predisposes to this disease. The period of two to three months after the beginning of glucocorticoid therapy is often the stable phase after the acute phase of interstitial pneumonia, and we must be aware of the possibility of PCP.

It was reported that in patients with human immunodeficiency virus (HIV), the circulating CD4+ lymphocyte count was less than 200/μl before 46 of 49 episodes of PCP and that patients with a CD4 count below 200/μl are likely to benefit from pneumocystis prophylaxis (9). Gluck et al (10) studied non-HIV patients receiving immunosuppressive therapy and reported that the CD4+ lymphocyte count was less than 200/μl in all seven patients who developed PCP. They concluded that measuring the CD4+ lymphocyte count was helpful for determining the risk of PCP not only in HIV-positive patients, but also in patients receiving immunosuppressive therapy (10). In the present study, PCP occurred in patients with structural abnormalities of the lungs, but Gluck et al reported on patients with collagen vascular diseases and hematological diseases that are generally not associated with lung tissue distortion. This leads to a critical difference in the risk of PCP. None of the present patients with idiopathic pulmonary fibrosis developed PCP, whereas 5 out of 28 patients who had interstitial pneumonia associated with collagen vascular disease developed PCP. Our collagen vascular disease patients all had nonspecific interstitial pneumonia. Differences of pathological findings were not significantly associated with the risk of PCP in this study (data not shown). Pathological distortion may make the lungs more susceptible to PCP, but the type of damage may be less important. For this reason, PCP can develop even if the lymphocyte count remains over 1,000/μl. We consider that the risk factors for PCP not only include the CD4+ lymphocyte count, but also structural damage to the lungs and changes of the lymphocyte count during treatment with glucocorticoids and/or immunosuppressants. The neutrophil count was increased in the PCP group prior to glucocorticoid therapy, although bacterial infection was not clinically evident at the time of diagnosis of PCP. These results suggest that the development of PCP after the start of glucocorticoid therapy is based on changes that occur prior to therapy (Table 2).

The Japanese Respiratory Society Guidelines for the Management of Hospital-Acquired Pneumonia (11) state that in immunodeficient patients, the risk of opportunistic pulmonary infection (including PCP) increases when the CD4+ lymphocyte count falls below 200/μl. In the present study, at the time of diagnosis of PCP, the white blood cell count was not low (12,686/μl), but the lymphocyte count and CD4+ lymphocyte counts were low (1,133/μl and 370/μl on average, respectively). However, three patients had a CD4+ lymphocyte count of greater than 200/μl at that time. These results suggest that interstitial pneumonia patients who are receiving glucocorticoid therapy may be differently immunocompromised than HIV-positive patients, and that their CD4+ lymphocytes may be dysfunctional because of positive and negative modulation of the expression of several genes involved in inflammatory and immune responses by glucocorticoid therapy (2).

Saito et al (12) evaluated 29 patients with various connective tissue diseases, and reported that the prevalence of PCP was especially high among patients receiving >30 mg/day prednisolone with or without another immunosuppressant. Takabayashi et al (6) also reported that none of the patients receiving <30 mg/day prednisolone developed PCP.

In the present study, none of the seven patients who developed PCP were on prophylactic TMP-SMX therapy. The 15 patients who received prophylactic TMP-SMX therapy did not develop PCP, even though the initial dose of prednisolone was 9 mg/day higher than in patients without TMP-SMX prophylaxis from the control group (Table 1). Thus, interstitial pneumonia patients who are treated with glucocorticoids may benefit from TMP-SMX prophylaxis against PCP. A prophylactic effect of TMP-SMX against PCP was originally reported in cancer patients (13). Sepkowitz et al (14, 15) recommended that prophylaxis might be given to patients who receive glucocorticoid therapy for longer than 4 weeks at a level equivalent to 20 mg of prednisone daily. As a result of this investigation, we recommend that prophylaxis with TMP-SMX should be given if the lymphocyte count prior to corticosteroid therapy is low ($\leq$1,000/μl) and if the lymphocyte count falls (even if it remains over 1,000/μl) after starting corticosteroid therapy in patients with interstitial pneumonia. The present PCP patients received higher corticosteroid doses than the controls, which would have influenced the lymphocyte count.

β-D-glucan is one of the major components of the cyst wall in human *Pneumocystis* infection, and is reported to be detectable in serum from patients with PCP. It serves as a practical serological marker for monitoring the disease during treatment (16, 17). In the present study, all PCP patients who were evaluated showed a high serum β-D-glucan level, so β-D-glucan is thought to be a good serological marker for PCP.

In conclusion, we found that patients who had interstitial

pneumonia treated with glucocorticoids and/or immunosuppressants could benefit from TMP-SMX prophylaxis against PCP. A CD4+ lymphocyte count of greater than 200/μl did not guarantee the prevention of PCP in patients with interstitial pneumonia.

However, a prospective study is needed to confirm the prophylactic effect of TMP-SMX against PCP in interstitial pneumonia patients receiving glucocorticoid/immunosupressant therapy.

Acknowledgement

The authors thank Dr. Seiichi Nakamura, Dr. Yasuhiro Shibuya, and Dr. Takehumi Saito for suitable advice on the evaluation of statistical analysis.

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Respiratory Medicine CME (2008) 1, 75–81

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CME ARTICLE

Novel therapy for idiopathic pulmonary fibrosis —How to evaluate the efficacy?

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KEYWORDS
Idiopathic pulmonary fibrosis;
Interferon-γ;
Pirfenidone;
N-acetylcysteine;
Exercise endurance test;
Progression free survival interval

Summary
As disease concepts have changed, the treatment of idiopathic interstitial pneumonias (IIPs) has become focused on idiopathic pulmonary fibrosis (IPF), which has the most adverse prognosis. Since 1998, large-scale multicenter trials have evolved, and clinical trials using novel therapeutic drugs such as interferon-γ in US and *N*-acetylcysteine in Europe and pirfenidone in Japan, have been organized. Most of these trials were evaluated using 1-year FVC or VC changes as the therapy endpoint, with significant differences obtained for non-progressive stage IPF. However, no significant differences were obtained in the progressive stages for any of the trials, and whether or not they contribute to improvement in life prognosis has been left to the post-market evaluation. A debate has begun in Europe and the US as to the value of assessment evaluation indicator done during activity since constant speed treadmill walking tests were introduced in the pirfenidone clinical trials carried out in Japan. Until now, therapy evaluations for IIPs have been compared using diagnostic imaging, pulmonary function tests, and pathology. In the future, therapeutic efficacy and prognosis will be discussed in terms not only of resting respiratory function evaluation, but also of systemic evaluation using exercise endurance, which is also recognized as an ADL evaluation indicator. In recent years, acute exacerbation of IPF has been the focus of global concern. A joint perspective by ATS/ERS was published, and standardized diagnosis and treatment have been provided. Acute exacerbation is an adverse prognosis factor, as are lung cancer complications, and treatments for improving life prognosis are being explored. This paper presents therapeutic drugs and treatments whose introduction is awaited, and discusses the relationship between indicators of therapy evaluation and disease concepts.

Educational aims:

- To review the therapy for Idiopathic Pulmonary Fibrosis.
- To familiarize the reader with how to Evaluate the Efficacy.
- To look at drug trials which have been carried out in Japan.

Introduction

Idiopathic interstitial pneumonia (IIP) is the generic name for the rare group of diseases constituting interstitial

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1755-0017/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rmedc.2008.06.001

pneumonias of unknown etiology. Currently they are classified into seven different patho-morphologies. In Japan they are recognized as intractable diseases and 4400 cases have been registered (to quality for medical care subsidy, severity of $PaO_2 < 70$ Torr in resting state or $SpO_2 < 90\%$ after 6-min walking).[1] In the US, the number of affected individuals estimated is 83,000. Among these, idiopathic pulmonary fibrosis (IPF) is chronic and progressive, and most resistant to treatment.[2] Therefore, a continuous stream of novel therapeutic drug clinical trials are being carried out in response to the continued demand for IPF therapeutic drug development. Since 1994, when nonspecific interstitial pneumonia (NSIP) was excluded as a separate disease concept,[3] IPF has been seen as a treatment-resistant interstitial pneumonia. Nevertheless, based upon pathologic recognition of the inflammation as pre-existing, comparatively small-scale clinical trials have been developed according to treatment concepts focusing on immune suppression. After reporting hopeful results from interferon-γ clinical trials,[4] several large-scale multicenter trials have been funded. However, due to the variability of the disease trajectory across individuals and the lack of established gold standard of success, the main endpoints for each trial differs and thus efficacy results also differ. Unfortunately, no drug has demonstrated improvement in survival, which is the desired absolute indicator of success. If there is no outcome of improved survival, issues have been raised regarding the evaluation measures of pirfenidone, which has been developed and tested in Japan. In that context, this paper assesses the future prospects of novel drug development, focusing on global IPF clinical trials.

Progression of novel therapies for IPF

Clinical trials of interferon-γ

With regard to interferon-γ, which appeared to great fanfare after small-scale randomized unblinded trial results[4] were reported in 1999, a large-scale randomized double blind multicenter clinical trial was carried out in North America on 330 IPF patients. The results showed no significant difference between those cases and the placebo group when comparing the main intent-to-treat (ITT) endpoint, "progression free survival interval".[5] However, an improved prognosis was suggested in non-advanced cases where $\%VC > 55\%$. Meanwhile, in clinical trials carried out on IPF cases in the progressive stage, respiratory function deterioration was prevented in none of the cases,[6,7] and there were reports of confirmed exacerbated pathology accompanying IFNγ therapy.[8] However, a synthesis of post-analyses of randomized IFNγ therapy results suggests the possibility of its contribution to mortality ratio reduction over the long-term prognosis[9] (Table 1).

Based on these lessons, late phase III clinical trials on non-advanced cases were planned and carried out in Europe and the US, with a trial period of 2 years and target case number of more than 800 IPF patients and "survival ratio" as main endpoint. In particular, the trial design entailed non-progressive cases with $90\% > FVC > 55\%$, and $90\% > DLco/TLC > 35\%$.[10] However, the results published in March 2007 reported it as a "negative study." The main endpoint was changed, but ultimately the efficacy of interferon-γ was rejected.[11]

Table 1 Effect of IFN-g1b treatment in total three study.

Period	Hazard ratio	95% CI	*p*
All	0.418	0.253–0.690	0.0003
1 yr	0.0861	0.0244–0.1478	0.0063
1.5 yr	0.1682	0.1065–0.2299	<0.0001
1.78 yr	0.1939	0.1386–0.2494	<0.0001
2 yr	0.2652	0.1652–0.3652	<0.0001

$n = 390$, reduced mortality. Post-analysis synthesis of interferon-γ therapy trial.[9] The possibility of contribution to lowering the long-term mortality ratio is indicated.

European trial of *N*-acetylcysteine (NAC)

There is anoxidant/antioxidant imbalance in the lungs of IPF patients, and glutathione is low in the bronchoalveolar lavage fluid (BALF).[12] The clinical efficacy of high-dose orally administered NAC for IPF patients was reported from Europe in 1997. Its antioxidant effect on interstitial pneumonia was noted, resulting in a pilot clinical study where NAC was administered orally 600 mg three times a day for 12 weeks.[13] The results showed that plasma total glutathione increased slightly from before to after NAC therapy, while BAL and epithelial lining fluid total glutathione increased demonstrably from before to after NAC therapy. Auscultatory findings confirmed a 22% reduction in crackle intensity and distribution, while breathing difficulty improved by 50%. Results of the respiratory function continue to decline during the pre-treatment observation period, with improvement seen after the 12-week NAC therapy. Based on the above results, it was reported that high-dose NAC oral therapy was an effective adjunctive therapy for IPF patients.

At the 2004 European Respiratory Society (ERS) meeting, a randomized clinical trial with prednisolone (progressive reduction starting from 0.5 mg/kg/day) and azathioprine (2 mg/kg/day) as the intervention arm therapy.

Overall, 184 IPF patients (155 cases available for final analysis) were divided into the NAC (internal dose of 1800 mg/day) and placebo groups and compared. After 12 months, VC and DLco showed less reduction among the intervention group[14] (Figure 1). However, survival was not improved.

In Japan, NAC, as a precursor of glutathione, is used as an inhaled expectorant drug for bronchial asthma and chronic bronchitis, with expectations of preventive effects for pulmonary damage caused by oxidants.

A pilot trial on NAC inhalation therapy for IPF was reported by a Ministry of Health and Welfare study group.[15] Since 2005, physician-led multicenter research (part of "Clinical Research on Landmark Therapy for Idiopathic Interstitial Pneumonias" by a Ministry of Health and Welfare study group) has been carried out as the first publicly financed clinical trial in Japan, and we await the results.

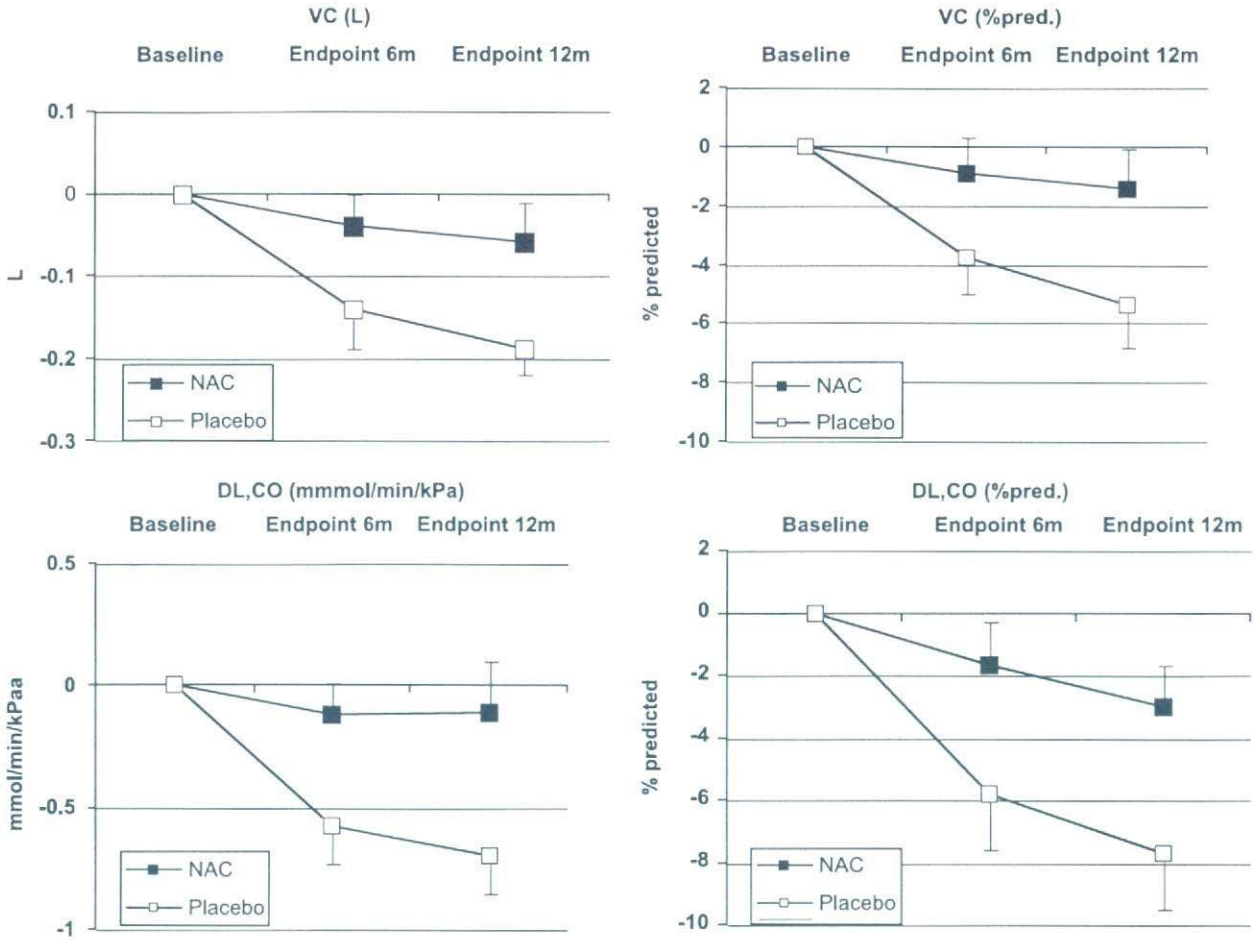

Figure 1 IFIGENIA trial results. Compared to the placebo group, all deterioration indicators (VC, DLco, VC% and DLco%) were alleviated in the NAC therapy group.[14].

The subjects were those with severity degree I or II (PaO_2 more than 70 Torr when resting; SpO_2 more than 90% after 6-min walking test) for the NAC inhalation therapy test.

Clinical trial of pirfenidone

Based on the results of an unblinded open label trial of pirfenidone in the US,[16] a phase II clinical trial was carried out in Japan starting in December 2000.[17] Based on the diagnostic criteria at that time, 107 adults with "chronic idiopathic interstitial pneumonia" (IPF, under current diagnostic criteria) were studied (72 in the pirfenidone group, 35 in the placebo group). This study differed from the American clinical trial in that subjects were not advanced cases of IPF, and those on immunosuppressive drugs or steroids in excess of 10 mg/day PSL equivalent units were excluded. In addition, to more sensitively and critically assess improvements in dyspnea during exertion, a unique evaluation method showing blood oxygen saturation level (minimum SpO_2) improvement after 6 min of constant speed treadmill walking test was employed. The conventional "6-min walking test" is highly effort-dependent. Because walking speed and walking distance change independently as respiratory function improves, it has been difficult to assess this efficacy outcome before and after therapy. However, the constant speed exercise tolerance evaluation should be able to more objectively reflect oxygen consumption for the same task volume. The remaining evaluation indicators analyzed were the same as those in the American clinical trial, but secondary endpoints were added. These were the interstitial pneumonia markers KL-6 and SP-D, often used in Japan, and acute exacerbation incidence,[18] which is frequently recognized in reports from Japan.

After 6 months, the interim analysis reported five cases of acute exacerbation out of 107 (4.5%), and as all cases were skewed toward the placebo group (14.3%), based on the efficacy safety evaluation committee's report, the results were broken 9 months after the start of the trial and analysis was conducted.

For cases in which the main endpoint was completion of the 6-min constant speed walking test, the results showed a significantly higher degree of progression prevention for the pirfenidone group as compared to the placebo group, with better minimum SpO_2 during the 6-min walking test both 6 and 9 months after starting administration. Further, after 6 months the minimum SpO_2 during the 6-min walking test showed significant correlation with the SpO_2 dropping area (Figure 2), with significant improvement shown by the pirfenidone group as compared to the placebo group.

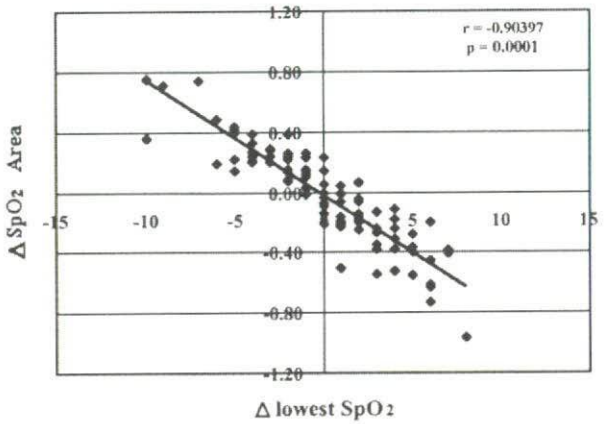

Figure 2 The minimum SpO_2 during the 6-minute walking test showed significant correlation with the SpO_2 dropping area.[17]

With regard to secondary endpoints, in terms of changes in respiratory function test values, VC and TLC deterioration were significantly prevented in the pirfenidone group as compared to the placebo group both 6 and 9 months after starting administration. However, no significant differences were observed between the two groups for PaO_2 changes when resting, and serum KL-6 or SP-D marker value changes. Meanwhile, although image analysis did not show the reduction or disappearance of honeycomb lung, in some cases in the pirfenidone group, the ground-glass appearance of the lung parenchyma was reduced, suggesting that pirfenidone may have an inhibiting effect on the early fibrotic stage. This was also felt to be the reason for the inhibition of exercise endurance decline.

The same as in the clinical trials conduced in the US, adverse events associated with pirfenidone administration included photosensitivity (49.3%) and digestive complaints (lack of appetite: 27.4%, gastric discomfort: 23.3%, nausea: 21.9%). However, all of these were dosage-dependent, and temporary cessation or dose reduction enabled the trial to be continued. Photosensitivity was seen in about half the cases, which highlighted the importance of advising pirfenidone patients to take sufficient care with regard to daylight exposure.

The same as the US clinical trials, the results of the phase II clinical trial carried out in Japan showed that respiratory function deterioration was inhibited, and further the frequency of acute exacerbation was reduced.

Based on these results, a phase III clinical trial was carried out with three groups. A total of 275 patients were randomly assigned to high-dose (1800 mg/day) of pirfenidone group (H), low dose (1200 mg/day) of pirfenidone group (L), and the placebo group (P) (H:L:P = 2:1:2).[19] The main endpoint was VC changes, and a key secondary endpoints progression free survival interval (definition of progression: death or 10% or greater drop in VC). Of 275 patients, 267 were included in the full analysis set (108 assigned to Group H, 55 to Group L, and 104 to Group P). Pirfenidone significantly affected the change in VC; at week 52, the difference in the adjusted mean change in VC from baseline between Group H and P was 0.07 l ($p = 0.0416$). Pirfenidone significantly affected the secondary endpoint of progression free survival: the difference between Groups H and P was statistically significant ($p = 0.0280$, Log-rank test). The most common adverse events reported in patients treated with pirfenidone were photosensitivity in the skin and appetite loss. No significant difference in the serious adverse events was noted among three groups. The phase II and phase III studies demonstrated that pirfenidone therapy stabilized lung function and improved progression free survival in patients with IPF. With the safety and tolerability established, we conclude that pirfenidone therapy is useful for treating patients with IPF.

Beneficial role of PMX hemoperfusion therapy for acute exacerbation of IPF

Acute exacerbation of IPF is a pathology with an extremely adverse prognosis and produces acute respiratory failure during the course of IPF. Activated neutrophilic inflammation damages the lung tissue, pathologically showing diffuse alveolar damage, and the fatality ratio is said to be as high as 80%. In recent years, global attention has focused on acute exacerbation of IPF,[20] with many reports thus far from Japan,[21] suggesting that the frequency differs according to race.[18]

When the authors experimented with polymixicin (PMX) adsorption therapy on IPF-AE patients, for whom improvement was not seen with steroid pulse and immune suppression therapies, improvement was verified for A-aDO_2, and in some cases patients succeeded in coming off the respirator.[22] Originally the PMX column was widely used clinically for the purpose of endotoxin adsorption.[23] After the experience with these cases, PMX adsorption therapy was carried out on five cases of steroid pulse- and immune suppression-resistant acute exacerbation of IIPs. In four cases, success was achieved in the area of respiratory failure, and temporary improvement in A-aDO_2 was verified in two cases.[4] In all of these cases, serum endotoxins were negative, images showed infiltrative shadows with sclerotic images in both lungs as the main and most of those with *P/F* ratio of 300 or less manifested acute respiratory failure. Adsorption of activated neutrophils might be a possible mechanism of the column in the promising therapy for exacerbation of IPF.

Recent trends in IPF clinical studies

As mentioned at the beginning, the world is moving toward large-scale, multicenter (including multiple countries) in IPF clinical studies. Nonetheless, several issues remain to be overcome. The main issues are those of "*diagnostic accuracy*" and "*disease diversity*". IPF is one of the pathological categories of IIPs, but there is a diversity of life expentancy even within the same pathomorphology disease category.

Even actual comparison of UIP and NSIP shows a poor life prognosis for cases with DLco deterioration for each pathomorphology.[24] Also, it is clear that IPF cases with pulmonary hypertension complication have a poorer life prognosis than uncomplicated cases.[25] In either case, the existence of prognostic factors that cannot be determined by pathomorphology alone is suggested.

It is difficult to judge statistical significance without carrying out analysis based on large-scale trials, but variation arises due to sampling because of the rarity. There is also the possibility of change in prognostic factors between the initial and progressive stages.[26] The second issue is *"validity of therapy evaluation indicators."* Complete recovery cannot be expected when the disease progresses, and it can be analogized to the change in brunt of therapy indicators, as when QOL evaluation was introduced as a lung cancer *therapy evaluation indicator.*

Overall, the speed of decline in VC predicted is gradual in the initial stages, but it becomes precipitous in the progressive stages and the chance of acute exacerbation also increases. Therefore, life prognosis also becomes poorer. The results of clinical trials in recent years show that VC and FVC percentages are much used as the best prognostic indicators. However, as can be seen from the results of massive dose NAC therapy, although a significant difference in FVC percentage was found as compared to the placebo group, no improvement in life prognosis was seen. This suggests that FVC percentage is not the only factor that contributes to improvement in survival.

In pirfenidone clinical trials carried out in Japan, it is thought that oxygen saturation minimum SpO_2 during exercise endurance reflects drug efficacy, and plainly indicates the relationship between pulmonary microcirculation and oxygen consumption. As interstitial pneumonia and pulmonary fibrosis advance, the alteration in dynamic structure results in the appearance of a remarkable effect on pulmonary circulation resistance, retaining the possibility of changing prognostic factors. This kind of change in prognostic factors can occur for anyone as the disease advances, but although pulmonary circulation resistance may be an important prognostic factor from the initial stages for some patients (IPF patients with pulmonary hypertension complications), in some IPF patients it never has an effect. Because factors that depend on the host and external factors (environment, smoking, treatment, etc.) are qualified separately, life prognosis is difficult to assess based only on pathological classification and respiratory function indicators in a resting state, and it was seen that differences in minimum SpO_2 during walking resulted in noticeable differences in prognosis.[27,28] In addition, even for the same IPF, a high correlation was seen between pulmonary hypertension and 6-min walking distance and minimum SpO_2, and the mortality ratio was significantly high in cases of pulmonary hypertension complications.[25] Further, life prognosis differed markedly depending on the existence of microthromboembolism (evaluated by increased D-dimer).[29]

These prognostic factors present the necessity of analysis as composite parameters in the future. In the same way as the BODE index was presented for COPD, the authors believe that it is necessary to introduce and develop useful prognosis indicators for IPF.

Prospects for the future

Until now, IIPs depended on pathological diagnosis classification. There is no question that IPF has the worst prognosis. However, the prognosis even for the same IPF is affected by a variety of factors (Figure 3). Currently, VC or FVC percentage is thought to be the most reliable prognosis indicator. However, FVC percentage in interstitial pneumonia complicated by the monogenic disease Hermansky–Pudlak syndrome by no means continues the same type of decrease.[30] The indicators of secondary pulmonary hypertension and pulmonary microcirculation are very much implicated in "exercise endurance," first introduced in pirfenidone clinical trials, and show the possibility of being independent prognostic factors as the disease progresses. Actually, regardless of image findings and lung capacity in a resting state, the prognosis was extremely poor in cases of reduced oxygen saturation while walking. Therefore, there is a need for indicators that provide an overall link between respiratory function and pulmonary circulation and gas exchange, hitherto difficult to evaluate, and prognosis evaluation.

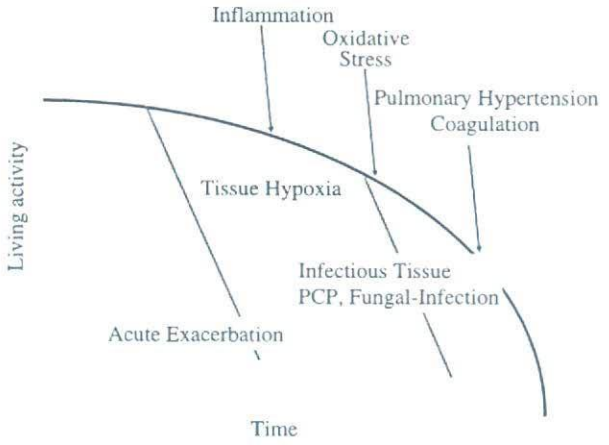

Figure 3 IPF clinical course. Reduction in living activity accelerates as the disease progresses. Precipitous deterioration may also occur in AE-IPF. A variety of prognostic factors are involved, including inflammation, oxidative stress, pulmonary hypertension (PAH), and progression of microvascular coagulation. Composite parameters that can comprehensively evaluate these prognostic factors are required.

In fact, evaluating these indicators, not only at the time of the initial pathological diagnosis, but as they change over time, would appear to be most in line with clinical classification.

The efficacy of interferon-γ was indicated in the first small-scale clinical trials,[4] but the results of a large-scale trial with 330 subjects were judged negative, with no significant statistical difference in the primary endpoint.[5] With the possibility of better therapeutic efficacy in cases of VC% > 55%, a further INSPIRE phase three clinical trial was carried out with 826 IPF subjects having VC% > 55%. Once again no significant difference was obtained (Figure 4). From this series of trial results, it became clear that the expansion to large-scale trials completely overturned all predictions. It is not clear at this point whether or not unknown prognostic factors intervene and dilute the impact of randomization as the scale of trials grows larger. As can be seen from the walking trial analysis by Flaherty et al.[26] the possibility that prognostic factors change with disease stage must be borne in mind. In the progressive stage where

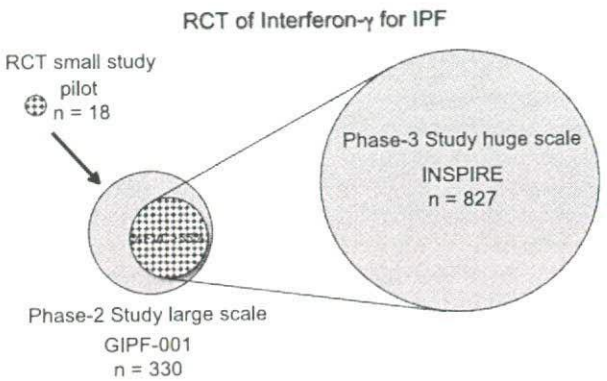

Figure 4 Transition of interferon-γ clinical trials. With the introduction of large-scale trials, significant difference in main endpoint could no longer be obtained. What does this result mean? It means that there is no treatment that can improve the overall IPF prognosis. It is possible that factors influencing the prognosis effected a disequilibrium in allocation in large-scale trials. It is hoped that the analysis of effective cases will lead to the evolution of order-made therapies.

VC percentage dropped, the prognosis can be computed by focusing on 6-min walking distance, but the possibility that multiple indicators may define the prognosis must be taken into consideration in the non-progressive stage. The authors consider that influential prognostic factors should at least be seen as "allocated factors" for clinical trials. Then, while there is room for development of therapeutic drugs relative to each prognostic factor, intervention to prevent progression is important from an early stage.

At the present time in Japan, in addition to arterial blood gas in a resting state, the higher ranked indicator used for IIPs seriousness in Japan is SpO_2 decrease (<90%) during 6-min walking. It would appear necessary to start with a detailed evaluation of prognostic factors, go on to establish validity and reproducibility of indicators, and then progress to evaluate improvement in indicators for promising treatment methods.

Currently, the possibility of IPF life prognosis improvement is anticipated from the endothelin-1 inhibitor (Bosentan) being promoted in Europe and the US, not only through its inhibition of fibrosis progression, but also through its beneficial effect on pulmonary hypertension.

Summary

Drugs for the treatment of interstitial pneumonias continue to be developed and tested yet none has yet proven to be effective in the early stages of the disease. Outcome measures for these studies may be able to use standard imaging and pulmonary function techniques. However, once the disease has moved to the progressive stages, a different type of drug may be required to delay progression rather than prevent initial fibrosis. Outcome measures at that stage may be different and may include survival duration, slower progression in functional decline, and improved or maintained quality of life. These outcomes must be defined a priori so that comparable studies can be designed and results combined in useful meta-analyses.

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Educational questions

Answer the following questions:

1. What factors make it difficult to study drugs for IPF?
   a. Disease has several apparent subtypes
   b. Patients all die before outcomes can be assessed
   c. Pulmonary function results do not change significantly over time
   d. The main outcomes are based on complex imaging studies
   e. Progression is very slow
2. There is a clear and widely agreed upon definition of IPF
   True
   False
3. The 6-min walk test is the only available test for functional outcomes in clinical trials of people with IPF?
   True
   False
4. It is important to develop standardized outcome measures in clinical trials to facilitate:
   a. Better funding opportunities
   b. Meta-analysis opportunities
   c. Opportunities for new equipment to be used
   d. Better collaborative opportunities
   e. 2 and 4

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Granulocyte-Macrophage Colony–Stimulating Factor and Lung Immunity in Pulmonary Alveolar Proteinosis

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The anti–granulocyte-macrophage colony–stimulating factor (GM-CSF) autoantibody is inferred to cause idiopathic pulmonary alveolar proteinosis (iPAP): the antibody neutralizes GM-CSF and thereby impairs differentiation of alveolar macrophages. Administration of GM-CSF improves respiratory function of patients with iPAP, as confirmed in this study using aerosolized GM-CSF. To elucidate its mechanism, we characterized bronchoalveolar lavage fluid and alveolar macrophages obtained from three patients with iPAP who were treated successfully with aerosolized GM-CSF. Cell number, expressions of surface mannose receptor and the transcription factor PU.1, and phagocytic ability of alveolar macrophages were all restored to control levels. With treatment, the neutralizing capacity of GM-CSF activity was reduced markedly, concomitant with the decreasing autoantibody levels. Interestingly, the amount of GM-CSF autoantibody complex also decreased. In one case in which the complex was analyzed, the majority of GM-CSF binding the complex was endogenous protein, suggesting that the complex is removed immediately from the lung after treatment. Our study shows that GM-CSF administration engenders a decrease in the neutralizing capacity against the protein in the lungs. Thereby, it facilitates restoration of the normal function of alveolar macrophages.

Keywords: anti–GM-CSF antibody; bronchoalveolar fluid; GM-CSF; pulmonary alveolar proteinosis

Pulmonary alveolar proteinosis (PAP) is an uncommon lung disease characterized by an accumulation of surfactant that fills terminal airways and alveoli, thereby impairing gas exchange and engendering respiratory insufficiency (1–3). Three clinically and etiologically distinct forms of PAP are acknowledged (congenital, secondary, and idiopathic), but more than 90% of cases are idiopathic (iPAP). In iPAP, respiratory symptoms initiate insidiously, with no precipitating event or illness. Alveolar macrophages from patients with iPAP show impaired chemotactic activity, reduced adhesion to glass, and poor phagocytosis (4). Dysfunction that impairs surfactant clearance of alveolar macrophages is considered responsible for iPAP (2–4).

(Received in original form June 6, 2004; accepted in final form February 21, 2005)

Supported in part by grant H14-trans-014 from the Ministry of Health, Labor, and Welfare of Japan, and grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 171. pp 1142–1149, 2005
Originally Published in Press as DOI: 10.1164/rccm.200406-716OC on February 25, 2005
Internet address: www.atsjournals.org

A serendipitous observation first suggested that abnormalities of GM-CSF signaling may be involved pathogenically in iPAP: mice lacking the hematopoietic growth factor granulocyte-macrophage colony–stimulating factor (GM-CSF) or its receptor develop histologic changes similar to those seen in PAP (5–7). In mice, GM-CSF regulates the terminal differentiation of alveolar macrophages. It is necessary for normal catabolism of surfactant lipids and proteins (8). Genetic abnormalities of GM-CSF or its receptor were reported in a small number of patients with congenital PAP (9), but were not found in iPAP (2). Instead, all patients with iPAP evaluated so far have high titers of neutralizing anti–GM-CSF autoantibody. No more than trace amounts of the antibody were detected in patients with congenital or secondary PAP, other lung diseases, or healthy volunteers (10, 11). Taken together, loss of GM-CSF activity caused by the autoantibody cripples normal functions of alveolar macrophages, thereby reducing surfactant clearance.

Recombinant human GM-CSF is used clinically to stimulate bone marrow recovery in neutropenic patients and after bone marrow transplantation. Several investigators have administered GM-CSF subcutaneously to patients with PAP and have observed varied responses (12–15). A single case report describes a patient who was treated successfully using aerosolized GM-CSF (16). A recent study demonstrated that extrinsic GM-CSF administration restored expression of a transcriptional factor, PU.1, in alveolar macrophages, and thereby improved the maturation of alveolar macrophages in patients with PAP (17, 18). Considering the preexisting autoantibody, which binds GM-CSF with high avidity and specificity (19), it is unlikely that administered GM-CSF can directly stimulate immature alveolar macrophages by binding their GM-CSF receptors.

To investigate mechanisms of action of administered GM-CSF, we observed changes in the function of alveolar macrophages, together with changes in the neutralizing activity against GM-CSF and the amount of autoantibody in bronchoalveolar lavage fluid (BALF) of three patients treated with aerosolized GM-CSF. Results suggested that inhaled GM-CSF reduced the neutralizing capacity of BALF against GM-CSF with decreased concentration of both free autoantibody and the immune complex. Consequently, inhaled extrinsic GM-CSF might condition the alveolar microenvironment in the lung, allowing alveolar macrophages' functional recovery and clearance of proteinaceous materials. Some of the results of this study have been reported previously in the form of an abstract (20).

METHODS

See the online supplement for further details on the methods.

Patients and GM-CSF Administration

The institutional review board approved this study. It was conducted after obtaining written, informed consent from each participant between

December 2000 and October 2002. We treated a series of three individuals with iPAP (one man and two women; age range, 51–57 years) with aerosolized GM-CSF (Table 1). BAL, transbronchial lung biopsy, and anti–GM-CSF antibody in the serum confirmed the iPAP diagnosis. The patients were administered recombinant GM-CSF (125 μg in 2 ml normal saline; Leucomax; Novartis AG, Basel, Switzerland) by aerosol (LC Plus jet nebulizer; PARI Respiratory Equipment, Inc., Starnberg, Germany) twice daily, during alternate weeks for 24 weeks. This schedule of treatment was based on a report by Anderson and coworkers (21). They administered aerosol GM-CSF to seven patients with metastatic lung tumors and found low toxicity. Improvement was defined as 10 mm Hg or greater decrease in the alveolar–arterial oxygen gradient ($A\text{-}aDO_2$).

BAL Procedures

Three 50-ml aliquots of normal saline were instilled and suctioned sequentially from the right middle lobe under bronchoscopy and processed immediately. Cells were stained by modified Giemsa; 400 nucleated cells were counted differentially in cytocentrifuge preparations. Then 200 alveolar macrophages were measured for lengthwise diameter and classified into the following two morphologic groups based on Iyonaga and colleagues (22): (*1*) nonfoamy, monocyte-like cells and (*2*) foamy cells.

Electron Micrograph of Alveolar Macrophages

BALF was incubated in a plastic culture dish at 37°C for 1 hour. After removing nonadherent cells by gentle washing, adherent cells (alveolar macrophages) were fixed and processed for Epon-embedded sections to be observed with a transmission electron microscope.

Phagocytic Activity of Alveolar Macrophages

Alveolar macrophages, isolated as previously described, were suspended in Roswell Park Memorial Institute (RPMI)/10% fetal calf serum and plated in a four-well chamber slide (LabTek Chamber; Nunc, Rosklide, Denmark). After placing at 37°C for 2 hours, cells were incubated with 0.5% phycoerythrin (PE)-labeled latex beads (Sigma-Aldrich Corp., St. Louis, MO) for 30 minutes and fixed in 4% paraformaldehyde at 4°C for 15 minutes. Cells were then stained with a 1:3,000 dilution of Syber green (Dojindo Laboratories, Kumamoto, Japan). The alveolar macrophages that had phagocytosed beads were counted using a confocal laser microscope.

Immunohistochemical Staining

Alveolar macrophages were fixed with 4% paraformaldehyde and stained with antimannose receptor antibody (Beckman Coulter, Inc., Fullerton, CA) and horseradish peroxidase-labeled antimouse IgG antibody (Nichirei Corp., Tokyo, Japan) to examine expression of the mannose binding protein, a maturation marker for macrophages. We examined PU.1 expression by double immunostaining using a rabbit polyclonal anti-PU.1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which was detected using an alkaline phosphatase-labeled antirabbit IgG (Promega Corp., Madison, WI) and a mouse anti-CD68 monoclonal antibody labeled with horseradish peroxidase. Two pathologists independently determined quantification of a ratio of macrophages expressing PU.1. They counted the number of cells stained through a binary decision. Mean values are presented (Figure 6C).

To observe localization of PU.1, alveolar macrophages were immunostained with a rabbit anti-PU.1 polyclonal antibody and PE-labeled antirabbit polyclonal antibody (DakoCytomation, Glostrup, Denmark), counterstained with 1:3,000 dilution of Syber green; they were then examined using confocal laser microscopy.

Quantification of Anti–GM-CSF Autoantibody

Autoantibody concentrations in BALF or in serum were measured using purified autoantibody as a standard (15, 23).

Neutralizing Capacities against GM-CSF in BALF

The GM-CSF bioactivity was quantified using TF-1, a GM-CSF–dependent cell line, as described elsewhere (19).

Detection of GM-CSF in GM-CSF–Autoantibody Immune Complexes

Protein samples obtained from BALF of patients with iPAP and normal control subjects using protein-A sepharose were subjected to ELISA and Western blotting to detect GM-CSF, as described previously (19).

Statistical Analyses

Statistical analyses were performed using StatView version 4 software (SAS Institute, Inc., Cary, NC), using the Mann-Whitney's U test or Kruskal-Wallis rank sum procedures for nonparametric data. Correlation of variables was assessed using the Spearman rank correlation coefficient. We considered $p < 0.05$ to be significant.

RESULTS

Population, Morphology, and Function of Alveolar Macrophages during GM-CSF Treatment

The 24-week course of inhaled GM-CSF therapy showed improved oxygenation of arterial blood with no side effects. All three patients showed a 10 mm Hg decrease or more in $A\text{-}aDO_2$ after treatment (Table 1). Serum levels of surfactant protein-D, lactate dehydrogenase, and carcinoembryonic antigen were also improved (Figure 1; Figures E1 and E2 in the online supplement) (24). Case 1 recurred 20 months after the GM-CSF therapy (*see* Figure 1 and the online supplement for further details). Table 2 summarizes general characteristics of the cells in BALF. Alveolar macrophages increased after a 24-week GM-CSF inhalation ($p < 0.05$), whereas extracellular proteinaceous material and cell debris markedly decreased (Figures 2A–2C and 3A). Although the percentage of macrophages decreased in Case 3 after treatment, the absolute number of macrophages in 1 ml of BALF

TABLE 1. PATIENT PROFILE

	Case 1	Case 2	Case 3†
Age and sex	51-yr female	56-yr male	57-yr female
Smoking	None	Ex-smoker	Smoker‡
Diagnostic procedure	BALF, TBLB, Ab	BALF, TBLB, Ab	BALF, TBLB, Ab
Prior treatment	Left lung lavage	Oxygen treatment immunosuppressants*	Oxygen treatment
$A\text{-}aDo_2$ decrease after GM-CSF inhalation (torr)	17	20	27

Definition of abbreviations: $A\text{-}aDo_2$ = alveolar–arterial oxygen gradient; Ab = serum titer of anti–GM-CSF antibody; BALF = bronchoalveolar lavage fluid; GM-CSF = granulocyte-macrophage colony–stimulating factor; TBLB = transbronchial lung biopsy.

* Case 2 had received prednisone and cyclosporine as the treatment for Wegener's granulomatosis, which was diagnosed 17 months earlier to the onset of idiopathic pulmonary alveolar proteinosis (*see* the online supplement for further details).

† Brief profile and the effects of GM-CSF inhalation on a mucinlike glycoprotein, KL-6, and serum anti–GM-CSF antibody of Case 3 was reported previously (24).

‡ Case 3 had just stopped smoking when pulmonary aveolar proteinosis was diagnosed.

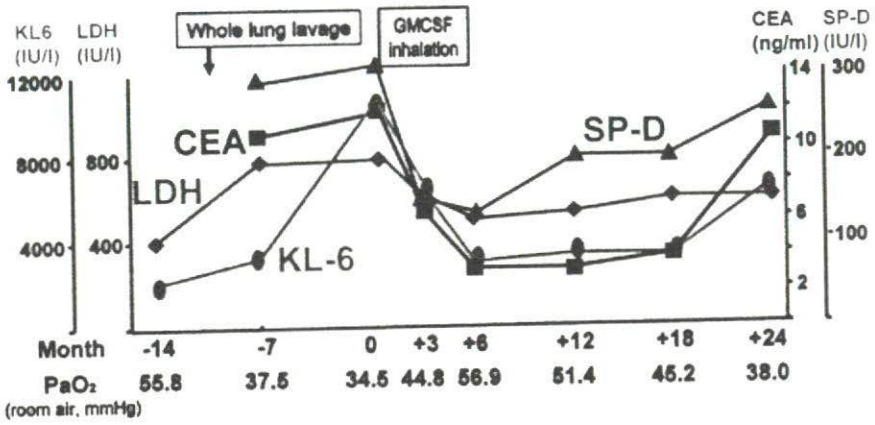

Figure 1. Clinical course of Case 1. Laboratory data for Pa_{O_2} and serum markers for idiopathic pulmonary alveolar proteinosis, including lactate dehydrogenase (LDH), a mucin-like glycoprotein, KL-6, surfactant protein-D (SP-D), and carcinoembryonic antigen (CEA), are presented with clinical information. GM-CSF = granulocyte-macrophage colony–stimulating factor.

increased, for the substantial increase of total BAL cells (Table 1 and Figure 3A). Foamy macrophages decreased after treatment (Figure 3B). Nonfoamy alveolar macrophages, smaller than normal control ($p < 0.01$) before the treatment, were of normal size after GM-CSF treatment (Figure 3C). Alveolar macrophages after the treatment showed mature ultrastructural features with the development of microvilli and clear organelles, compared with those before treatment (Figure 2D).

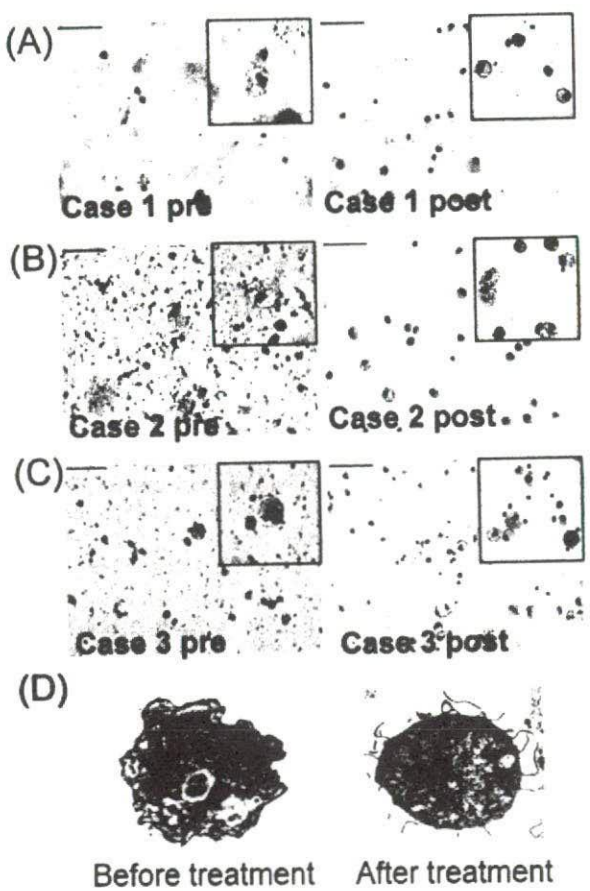

Figure 2. (*A–C*) Wright-Giemsa staining of the cells in bronchoalveolar lavage fluid before (*left*) and after (*right*) the GM-CSF treatment (×200; scale, 40 μm). Insets show higher magnifications of the cells (×400). (*D*) Electron micrographs of the alveolar macrophages of Case 1 before (*left*) and after (*right*) the GM-CSF treatment (×3,000).

We examined alveolar macrophages before and after treatment for changes in phagocytic activity and in the expression of two molecules. Phagocytic activity, as measured using the number of the cells harboring beads, was increased after the treatment (Figure 4). Expression of the mannose receptor, a crucial molecule for macrophages to phagocytose microorganisms, and expression of PU.1, a critical transcription factor regulating differentiation and maturation, were both increased after treatment (Figures 5 and 6). These results suggest that GM-CSF treatment promoted differentiation and restored the normal functions of alveolar macrophages.

Neutralizing Capacity against GM-CSF in BALF Was Reduced after Treatment with a Decreased Level of the Autoantibody

Anti–GM-CSF antibodies in patients with iPAP have a wide range of target epitopes. In addition, the crude amount of the autoantibody may not be correlated with the biological effect or a state of the disease (20). Consequently, to investigate the effect of GM-CSF inhalation on autoantibody levels in the lung, we examined BALF after treatment for the following: (*1*) neutralization capacity, which suppresses biological activities of GM-CSF using a GM-CSF–dependent cell line, and (*2*) the amount of IgG binding to GM-CSF by enzyme immunoassay (EIA) (Table 3). In the three cases of that study, the neutralizing capacity against GM-CSF declined remarkably to normal levels after GM-CSF treatment ($p < 0.05$). Consistently, the amount of the anti–GM-CSF antibody was also markedly decreased after GM-CSF inhalation ($p < 0.05$). The serum titer of the antibody after treatment was approximately 60 to 70% of the titer before the treatment. The neutralizing capacity of GM-CSF in BALF exhibited significant correlation with serum carcinoembryonic antigen ($r = 0.886$, $n = 6$, $p = 0.0476$), serum surfactant protein-D ($r = 0.943$, $n = 6$, $p = 0.035$), and a mucin-like glycoprotein, KL-6 ($r = 0.943$, $n = 6$, $p = 0.035$). It also showed marked correlation with the titer of anti–GM-CSF antibody in BALF ($r = 0.829$, $n = 6$, $p = 0.0639$) and Po_2 ($r = -0.829$, $n = 6$, $p = 0.0639$), but not with the serum titer of anti–GM-CSF antibody ($r = 0.143$, $n = 6$, $p = 0.7494$). Our results suggest that inhalation of GM-CSF restored bioactivity in the lung of patients with iPAP by reduction of neutralizing capacity against GM-CSF with a proportionate reduction in the amount of the autoantibody in BALF.

TABLE 2. BRONCHOALVEOLAR LAVAGE FLUID ANALYSES BEFORE AND AFTER AEROSOLIZED GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR THERAPY

	Case 1		Case 2		Case 3	
	Before	After	Before	After	Before	After
Recovery, % of 150 ml	69	60	53	48	40	70
Cell counts, × 10^4/ml	2.9	15.9	4.0	16.2	7.5	27.1
Macrophages, %	62	76	66	95	47	24
Lymphocytes, %	29	21	32	4	40	76
Neutrophils, %	9	3	2	1	12	0
Eosinophils, %	0	0	0	0	0	0
CD4/8 ratio	2.2	2.2	3.2	1.9	1.7	5.0

GM-CSF–Autoantibody Immune Complex Was Reduced after the Treatment

The effects of inhaled exogenous GM-CSF on reduction of both the neutralizing capacity and titer of the autoantibody suggested that exogenous GM-CSF bound to the free autoantibody and thereby reduced the free autoantibody detected by both ELISA and bioassay. If that occurs, GM-CSF bound to the autoantibody in BALF may increase after treatment. To elucidate this, the concentration of GM-CSF bound or unbound to the autoantibody in BALF was measured and compared before and after treatment. Unexpectedly, concentrations of GM-CSF bound to the autoantibody were reduced consistently to a level below the range of detection after treatment (Table 4). On the other hand, concentrations of GM-CSF that was unbound to the autoantibody in BALF were at levels below the range of detection in any cases before or after treatment, suggesting that GM-CSF in BALF was trapped completely by the autoantibody in the lung of patients with iPAP. To investigate GM-CSF bound to the autoantibody, we performed immunoblotting assay of GM-CSF stripped from the immune complexes in the BALF of Case 1. The assay demonstrated a band of 23 kD corresponding to intrinsic GM-CSF, which was larger than extrinsic GM-CSF of 14.5 kD. Furthermore, the band of extrinsic GM-CSF was not detected in BALF.

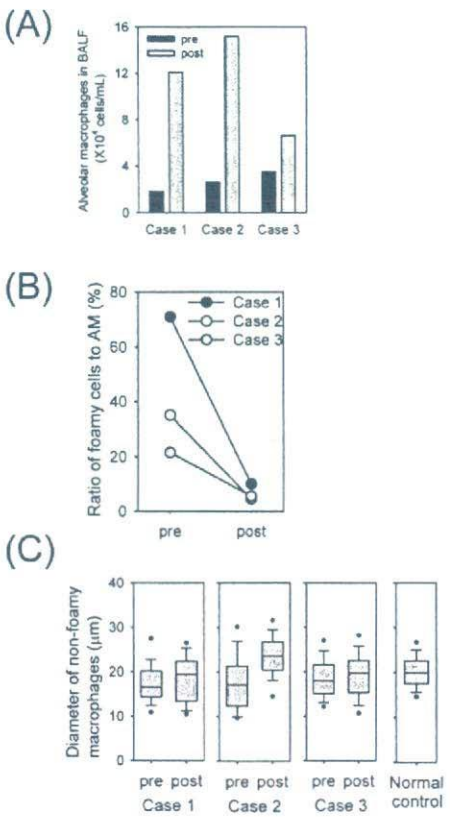

Figure 3. (*A*) Cell counts of alveolar macrophages in bronchoalveolar lavage fluide (BALF) of the patients with pulmonary alveolar proteinosis (PAP) before (*black bars*) and after (*gray bars*) the 24-week GM-CSF inhalation. (*B*) The ratio of foamy macrophages to the total number of macrophages in BALF of the patients with PAP before (pre) and after (post) the 24-week GM-CSF inhalation. (*C*) Diameters of nonfoamy macrophages in BALF of the patients with PAP before and after the 24-week GM-CSF inhalation. *Central bars* show median, *box plots* show 25th and 75th percentiles, *error bars* show 10th and 90th percentiles; *dots* show the minima and the maxima.

DISCUSSION

Alveolar macrophages in the BALF of patients with iPAP in severe cases show defective mature alveolar macrophage functions (25, 26). Surfactant catabolism and host defense immunity regulated by transcription factor PU.1 are typical of such functions (27). Our previous studies suggested that maturation arrest of alveolar macrophages is caused by abundant autoantibody against GM-CSF in the lung (19). Because the therapeutic efficacy of extrinsic GM-CSF on iPAP has been established in clinical trials over the last decade (13–16), it is plausible to hypothesize that administered GM-CSF alters the unclear balance between GM-CSF and the autoantibody in the pulmonary microenvironment.

Several investigators have addressed the mechanism of extrinsic GM-CSF action on the pathologic status of iPAP. Seymour and colleagues (28) reported that patients with iPAP who were treated with 5 μg/kg/day of GM-CSF showed an impaired hematopoietic response to GM-CSF. Schoch and coworkers (15) demonstrated that GM-CSF treatment restored morphology and adhesive function of alveolar macrophages in patients with iPAP. The serum anti–GM-CSF titer has been reported to decrease with improvement of iPAP in patients treated with GM-CSF or plasmapheresis (24, 29). Bonfield and colleagues (18) showed that suppressed expression of PU.1 and macrophage colony–stimulating factor receptor in alveolar macrophages of patients with PAP was changed to upregulation by GM-CSF treatment in both *in vitro* experiments and *in vivo* after subcutaneous injection.

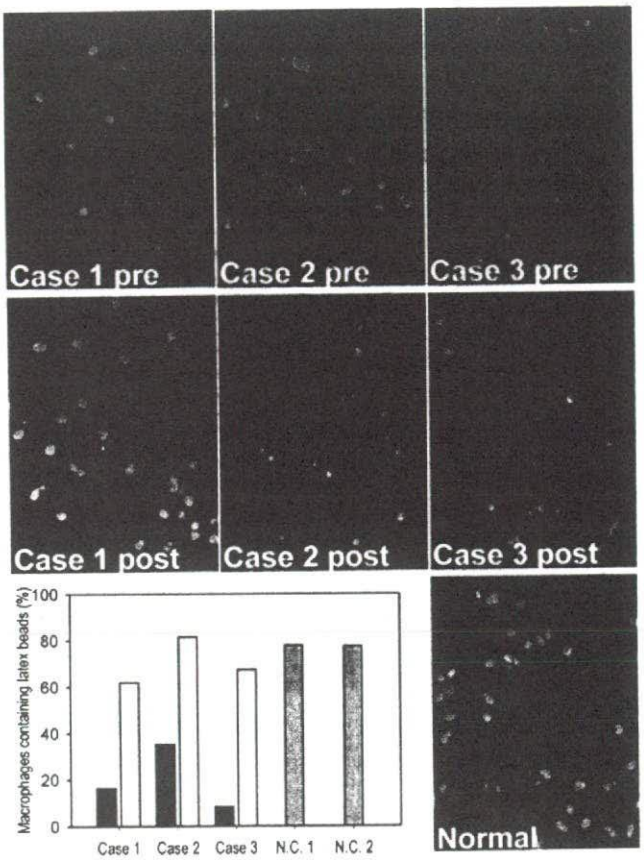

Figure 4. Phagocytosis assay using latex beads. The *upper* and *middle panels* show confocal microscopy images of alveolar macrophages from the patients (*upper panel*: before GM-CSF treatment; *middle panel*: after GM-CSF treatment) incubated with phycoerythrin (PE)-labeled latex beads. The *lower panel* shows confocal microscopy images of alveolar macrophages of a normal control (N.C.) incubated with PE-labeled latex beads, and the ratio of macrophages containing latex beads to total macrophages before (*black bars*) and after (*gray bars*) the GM-CSF inhalation.

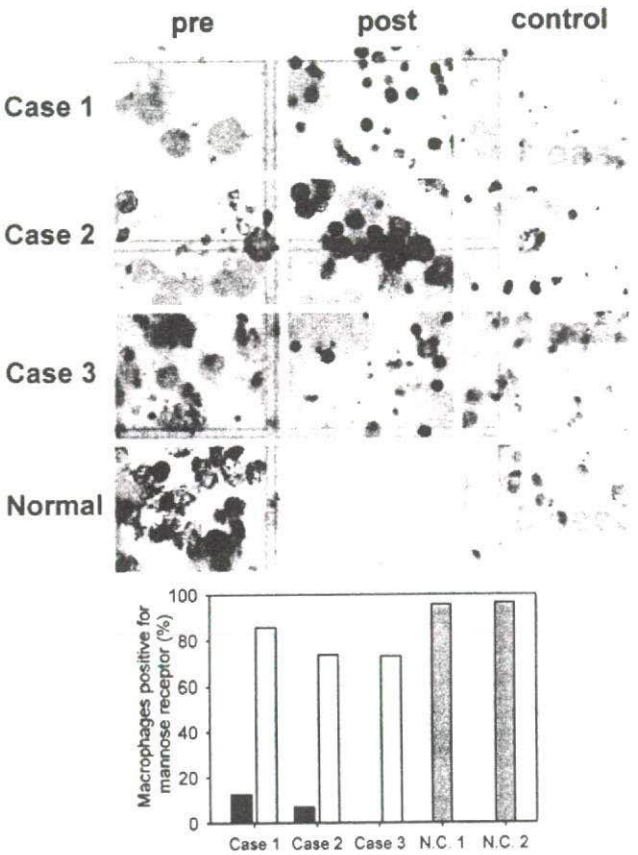

Figure 5. Immunohistochemical staining of alveolar macrophages obtained from patients with PAP and normal control subjects with mannose receptor. The *upper panel* shows alveolar macrophages expressing mannose receptor (*red*) from the patients before treatment (*left*) and after treatment (*middle*), and control staining using murine IgG (*right*). The *lower panel* shows the percentage of macrophages expressing mannose receptor to total macrophages before (*black bars*) and after (*gray bars*) the GM-CSF inhalation.

These studies demonstrated that treatment accelerated maturation of alveolar macrophages, but they did not explore alterations of the pulmonary microenvironment in which alveolar macrophages reside. Consequently, we have conducted analyses that specifically address the following two points: (*1*) estimation of the neutralizing capacity of the BALF against GM-CSF during the treatment and (*2*) determination of the GM-CSF–autoantibody immune complex. We found the following: (*1*) the neutralizing capacities and the levels of autoantibody against GM-CSF were decreased in BALF of patients with iPAP after aerosolized GM-CSF treatment (Table 3), (*2*) the amounts of GM-CSF–autoantibody immune complexes were also decreased in BALF after the treatment (Table 4), and (*3*) GM-CSF bound to the immune complex in BALF was not extrinsic recombinant protein but rather natural glycosylated human protein in Case 1.

Aerosolized recombinant human GM-CSF given to cynomolgus monkeys increased the total number of BAL cells more effectively than intravenous infusion of GM-CSF (30). Aerosolized GM-CSF also improved lung histology, alveolar macrophage differentiation, and surfactant protein B (SP-B) immunostaining to normal levels in GM-CSF–deficient mice (8). These results suggest that inhalation of GM-CSF might be an effective approach to affect alveolar macrophages' proliferation and functional maturation. It is notable that Case 3 demonstrated the increase of lymphocytes in BALF after GM-CSF treatment. The increase of lymphocytes was greater than in other two cases, and it might be associated with smoking cessation (Table 1). BAL lymphocytosis was also observed in a patient with PAP throughout the treatment course of subcutaneous GM-CSF injection, despite clinical improvement (15). The pulmonary infiltrates of lymphocytes in GM-CSF–deficient mice decreased but remained under successful treatment with aerosolized GM-CSF (8). Aerosolized GM-CSF itself increased lymphocytes in BALF of healthy cynomolgus macaques (30). The mechanism of the persistent BAL lymphocytosis during PAP treatment with GM-CSF remains to be elucidated.

The lungs of patients with iPAP contain abundant anti–GM-CSF antibody, and they produce GM-CSF to the comparable extent of normal lung (19). Decreased levels of the anti–GM-CSF antibody and the immune complex in BALF of the post-treatment patients suggested that aerosolized GM-CSF might

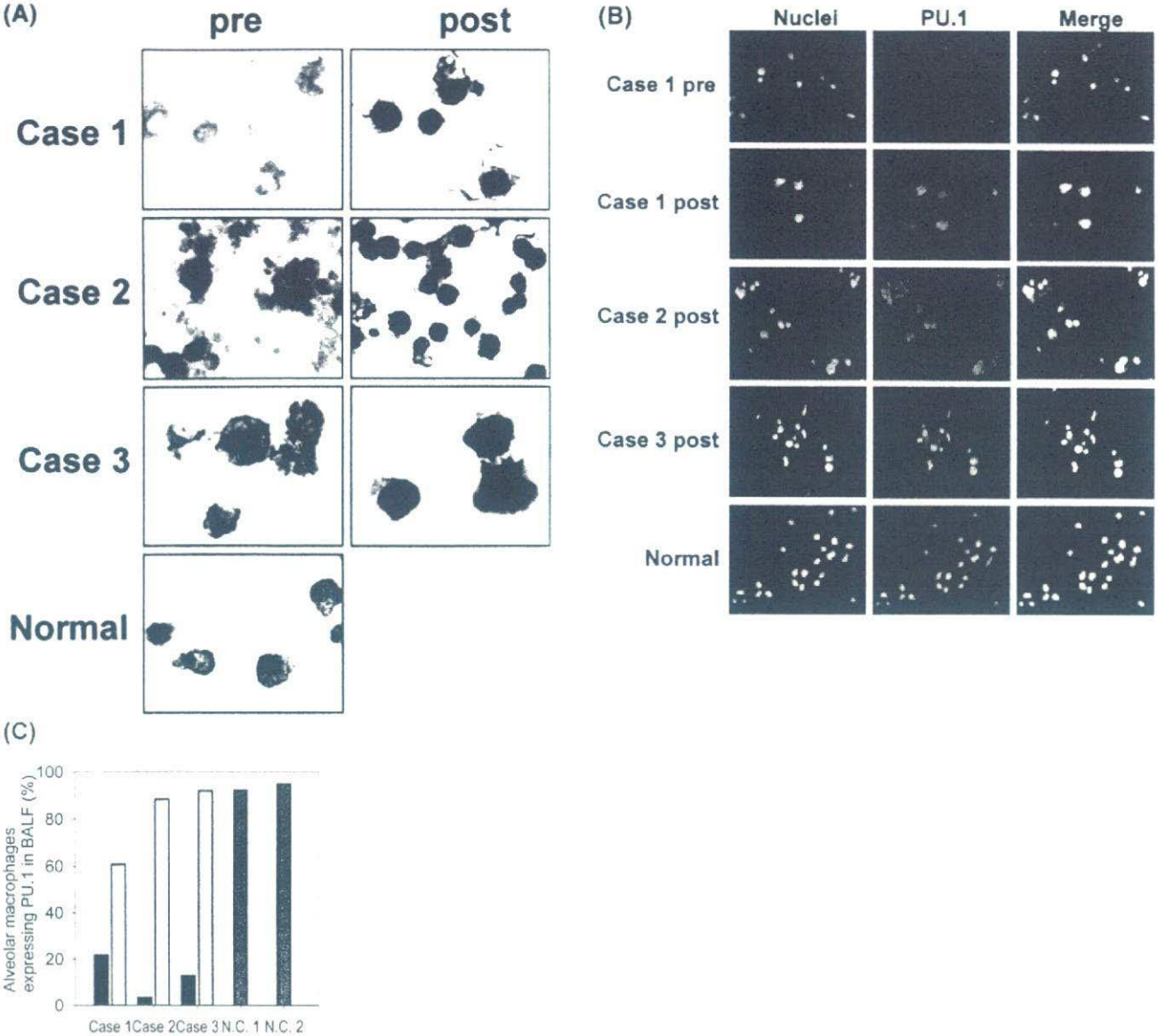

Figure 6. (*A*) Alveolar macrophages expressing PU.1 (*dark blue*) from the patients before (*left*) and after (*right*) treatment and from a normal control subject. (*B*) Confocal microscopy of alveolar macrophages obtained from the patients and stained with syber green (*left panel*: "Nuclei") and PE-labeled anti-PU.1 antibody (*middle panel*: "PU.1"). Merge images are shown in the *right panel*. (C) The percentage ratio of macrophages that were positive for PU.1 to the total macrophages before (*black bars*) and after (*gray bars*) GM-CSF inhalation.

affect the regulatory mechanism of production/disposition of anti–GM-CSF antibody locally or systemically. We infer that the reduced antibody restores bioactivity of intrinsic GM-CSF, engendering an increase of alveolar macrophages. To test the assumption, we attempted to demonstrate the presence of biologically active endogenous GM-CSF in BALF using TF-1, a GM-CSF–dependent cell line. However, neither BALF from normal control subjects nor BALF from the post-treatment patients sustained cell survival; their GM-CSF activities were below the detectable range (data not shown).

It remains unclear why treatment with extrinsic GM-CSF can decrease both the amount and the neutralizing capacity of autoantibody against GM-CSF in BALF of patients with iPAP (14, 15). It is a remarkable finding that the aerosolized GM-CSF therapy decreased the titer and neutralizing capacity of the anti–GM-CSF antibody in BALF during administration of immunosuppressants in Case 2. Further study should address the following: (*1*) the immune complex might modify a profile of T-cell population that regulates the autoantibody production and (*2*) apoptosis of the B cells that produce anti–GM-CSF antibody might be triggered by the immune complex of extrinsic GM-CSF and the autoantibody through Fc receptors, such as inhibitory FcγRIIB, as in the process of negative selection of B cells (31).

The clinical implication of the present study is that quantification of anti–GM-CSF antibody in BALF is useful to predict the response to GM-CSF treatment in each patient. The neutralizing capacity of GM-CSF in BALF is correlated significantly with

TABLE 3. EFFECTS OF GM-CSF INHALATION ON THE ANTIBODY AGAINST GM-CSF AND ITS NEUTRALIZING CAPACITY

	Anti–GM-CSF Ab (μg/ml)		Neutralizing Capacity
	BALF	Serum	(IC50 GM-CSF ng/BALF ml)
Case 1			
Pre	1.38	30.54	4.13
Post	0.10	21.85	0.47
Case 2			
Pre	0.58	57.40	1.33
Post	0.03	33.70	0.32
Case 3			
Pre	5.40	NA	10.27
Post	0.19	NA	0.21

Definition of abbreviations: Ab = serum titer of anti–GM-CSF antibody; BALF = bronchoalveolar lavage fluid; GM-CSF = granulocyte-macrophage colony–stimulating factor; NA = not available.

serum markers including carcinoembryonic antigen, KL-6, and surfactant protein-D. It is also strongly correlated with the titer of anti–GM-CSF antibody in BALF and Po_2. Clinical trials of GM-CSF treatment revealed the existence of patients who showed no improvement in clinical parameters such as Po_2, computed tomographic, and pulmonary function tests (13, 14). Furthermore, these clinical markers often showed delayed response to GM-CSF therapy in some cases. Techniques to evaluate the amount and the neutralizing capacity of anti–GM-CSF antibody in BALF during GM-CSF treatment would be useful tools to enable prediction of the response to GM-CSF treatment.

In conclusion, the present study demonstrated the importance of evaluating microenvironments surrounding macrophages in lungs as well as functions of alveolar macrophages in patients with iPAP. Techniques for detecting the neutralizing capacity and amount of anti–GM-CSF autoantibody in BALF could contribute to optimization of treatment for patients with iPAP.

Conflict of Interest Statement: R.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; E.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.A. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; H.O. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; O.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.U. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.W. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; J.S. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.T. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; I.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.H. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; M.E. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; Y.I. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; K.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript; T.N. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

TABLE 4. CONCENTRATION (pg/ml) OF GRANULOCYTE-MACROPHAGE COLONY–STIMULATING FACTOR BOUND OR UNBOUND TO THE AUTOANTIBODY IN BRONCHOALVEOLAR LAVAGE FLUID

	Before Treatment		After Treatment	
	Bound	Unbound	Bound	Unbound
Case 1	71.3	ND	54.1	ND
Case 2	24.0	ND	ND	ND
Case 3	10.7	ND	ND	ND

Definition of abbreviation: ND = not detected.

The lower detection range of the granulocyte-macrophage colony–stimulating factor enzyme immunoassay kit we used is 2.8 pg/ml.

Acknowledgment: The authors thank Dr. John F. Seymour and Dr. Bruce C. Trapnell for their critical reading of the manuscript.

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