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Differential Display of messenger RNA Expressed in Bronchoalveolar Lavage Cells in Pulmonary Sarcoidosis Patients

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ABSTRACT

Sarcoidosis is a systemic granulomatous disease of unknown origin. To clarify its pathogenesis, we searched for known or unknown genes which are specifically expressed in sarcoidosis.

Bronchoalveolar lavage (BAL) cells from 18 patients with sarcoidosis and 8 patients with various lung diseases were analyzed by differential display method. mRNA was extracted from BAL cells and reverse transcribed with 12 kinds of anchored primer, which theoretically cover all mRNAs, followed by polymerase chain reaction (PCR) with the anchored primer and a 10-mer arbitrary primer. PCR products were displayed on a polyacrylamide gel and fragments showing characteristic alterations in intensity between sarcoidosis and other patients were extracted, sequenced, and compared against Genbank and EMBL DNA data bases.

One fragment was detected with specifically increased intensity and another disappeared in patients with sarcoidosis. These fragments were likely derived from unknown genes. CD44 and tumor necrosis factor (TNF-α) cDNA sequences were also detected as fragments commonly expressed in sarcoidosis.

The cloned fragments with specifically increased or decreased intensity in sarcoidosis may provide important information on the pathogenesis of sarcoidosis, and the display pattern implies the potential usefulness of this method as a tool for diagnosis of the disease.

Key words: *Sarcoidosis, Bronchoalveolar lavage (BAL) cells, Differential display, mRNA*

Sarcoidosis is a systemic granulomatous disease of unknown origin characterized by enhanced cellular immune processes[6]. While almost all organs can be affected by the disease, the lung is most commonly involved and accounts for the most morbidity and mortality[37].

Many investigators have focused on the etiology and pathogenesis of pulmonary sarcoidosis, and recent studies have demonstrated the increased expression of cytokines such as tumor necrosis factor-α (TNF-α)[2,4,10,15,26,32,33,36,39], interleukin-1β (IL-1β)[26,33,36], interleukin-6 (IL-6)[4,15,33], platelet-derived growth factor-B (PDGF-B) and granulocyte-macrophage colony stimulating factor (GMCSF)[15]. In addition to cytokines, increased expression of LeuCAM leucocyte adhesion molecules (CD11/CD18) on peripheral blood leucocytes[29] and intercellular adhesion molecule-1 (ICAM-1) in serum and BAL fluid in patients with sarcoidosis have been reported[14]. Although these cytokines or adhesion molecules may be involved in the pathogenic mechanism of sarcoidosis, the cause of sarcoidosis still remains unknown. To clarify the pathogenesis of sarcoidosis, it is of use to search further for other genes which play an important role in the pathogenic processes of the disease. We hypothesized that genes specifically expressed in BAL cells in sarcoidosis may be a key to the underlying information in the biological process or pathological changes of the disease. In order to find such genes whose expression is altered in sarcoidosis-specific fashion, we applied differential display (DD) method developed by Liang and Pardee[22]. For the candidate fragments, we determined their DNA sequences and investigated their novelty by computer search against DNA/RNA data bases.

MATERIALS AND METHODS

Patients

BAL cells were obtained from 18 patients with sarcoidosis and 8 with various other lung diseases, including: hypersensitivity pneumonitis,

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miliary tuberculosis, idiopathic pulmonary fibrosis, pneumoconiosis, lupus pneumonitis, and bronchiolitis obliterans with organizing pneumonia (BOOP). All BAL samples from sarcoidosis patients were kindly provided by Dr. M. Ohmichi at Sapporo Hospital of Hokkaido Railway Company, Japan. All sarcoidosis patients were histologically diagnosed, except for case 17, who was clinically diagnosed with ocular manifestations. None of them were treated with glucocorticoids at the time of BAL. Ten were smokers. The 8 patients with various other lung diseases were clinically diagnosed by hematological examination, radiographic finding, BAL findings, and

Table 1. Clinical features and BAL profiles of sarcoidosis patients*

Case	Age (y)	Sex	Smoking**	BAL cells†			
				Total ($\times 10^5$/ml)	AM (%)	Ly (%)	CD4/CD8
1	22	M	S	3.7	91	9	2.1
2	13	M	NS	1.7	59	35	1.0
3	69	M	S	1.8	87	13	3.6
4	17	M	S	2.0	61	34	1.3
5	36	F	NS	1.2	61	38	2.0
6	55	F	NS	2.4	53	47	2.4
7	26	M	S	3.2	35	65	2.9
8	13	F	NS	1.2	69	30	2.4
9	50	F	S	1.8	94	4	0.4
10	25	M	S	2.2	96	4	6.5
11	69	M	NS	0.5	70	28	7.6
12	19	M	S	3.0	83	16	9.7
13	28	M	S	3.4	91	9	4.6
14	28	M	S	2.0	70	29	5.0
15	22	F	NS	3.6	22	77	25.4
16	27	M	S	2.0	81	19	7.9
17	77	F	NS	0.6	61	38	4.2
18	30	F	NS	1.3	85	13	3.2

* No patients were treated with glucocorticoids.
All patients except for case 17 were histologically diagnosed as sarcoidosis
** S=smoker; NS=nonsmoker.
† AM=alveolar macrophage; Ly=lymphocyte

Table 2. Diagnosis and BAL profiles of patients with other pulmonary diseases

Case	Age	Sex	Diagnosis*	Steroid	Smoking†	BAL cells§			
						Total ($\times 10^5$/ml)	AM (%)	Ly (%)	CD4/CD8
1	47	M	HP	+	S	7.5	21	69	1.6
2	64	M	HP	+	NS	8.2	46	46	1.8
3	49	M	MTB	+	S	0.9	52	47	0.2
4	64	M	HP	–	NS	5.2	38	54	0.8
5	68	F	IPF	–	NS	1.1	79	14	0.1
6	55	M	Pneumoconiosis	–	NS	1.2	94	5	ND
7	70	F	BOOP with RA	–	NS	5.0	44	51	3.2
8	54	F	SLE	–	NS	1.3	79	20	0.9

* HP=hypersensitivity pneumonitis; MTB=miliary tuberculosis; IPF=idiopathic pulmonary fibrosis; SLE=lupus pneumonitis;
† S=smoker; NS=nonsmoker.
§ AM=alveolar macrophage; Ly=lymphocyte; ND=not determined.

clinical course. For all patients, BAL was performed for diagnostic purpose by standard procedure with informed consent. The recovered cells were analyzed on total and differential cell counts and on lymphocyte phenotype (CD3, CD4, and CD8). The rest of the cells were then washed with Hanks' balanced salt solution (Nissui, Tokyo, Japan), centrifuged at 400 × *g* for 10 min, and the cell pellet was stored at –80 °C until use. Clinical features and BAL profiles of each subject are summarized in Tables 1 and 2.

RNA Extraction and RT

Poly(A)$^+$ RNA was extracted directly from 1-2 × 10^6 BAL cells using guanidinium thiocyanate solution and oligo (dT)-cellulose spun column (Quick Prep mRNA Purification Kit^{TM}, Pharmacia Biotech, USA). RNA was reverse transcribed into cDNA in a 20 μl reaction mixture containing: 20 pmol of an anchored dT primer (T_{15}AA, T_{15}AG, T_{15}CG, T_{15}GC, T_{15}GT, T_{15}GA, T_{15}AC, T_{15}GG, T_{15}AT, T_{15}CT, T_{15}CC or T_{15}CA, synthesized by Toyobo, Osaka, Japan), 20 μM each deoxynucleotide (Takara, Kyoto, Japan), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 20 units of RNase inhibitor (RNasin^{TM}, Promega, Madison, MI), and 200 units of Molony murine leukemia virus RNase H$^-$ reverse transcriptase (Superscript^{TM}, GIBCO/BRL, Gaithersburg, MD). The RT mixture was incubated at 42°C for 60 min, followed by 95°C for 5 min to inactive the reverse transcriptase.

Table 3. List of primers used for differential display

Primer set	Anchored Primer	Arbitrary Primer
1	T_{15}CA	5′–GGAACCCTTA–3′
2	T_{15}CA	5′–GGTTAGACGC–3′
3	T_{15}AG	5′–TCAGGTGACC–3′
4	T_{15}AG	5′–ACCAAGGGGT–3′
5	T_{15}AA	5′–GAATGATGAG–3′
6	T_{15}AA	5′–ACCGGGGTAT–3′
7	T_{15}CA	5′–GGTTCCATGC–3′
8	T_{15}AG	5′–GAATGATGAG–3′
9	T_{15}AA	5′–GGAACCCTTA–3′
10	T_{15}AG	5′–GGAACCCTTA–3′
11	T_{15}CG	5′–GGAACCCTTA–3′
12	T_{15}GC	5′–GGAACCCTTA–3′
13	T_{15}GT	5′–GGAACCCTTA–3′
14	T_{15}GA	5′–GGAACCCTTA–3′
15	T_{15}AC	5′–GGAACCCTTA–3′
16	T_{15}GG	5′–GGAACCCTTA–3′
17	T_{15}AT	5′–GGAACCCTTA–3′
18	T_{15}CT	5′–GGAACCCTTA–3′
19	T_{15}CC	5′–GGAACCCTTA–3′

PCR amplification and differential display

Differential display was performed as previously described[21,22] with minor modification. We used 19 sets of primers as listed in Table 3. The primer set 1 was designed so that a 330-base pair (bp) fragment of phosphoglycerate kinase (PGK)[24] will be amplified as an internal control. One microliter of the cDNA solution was subjected to PCR in a 20 μl reaction mixture containing 8 pmol of an anchored primer which had been labeled with 74 KBq of γ-^{32}P ATP, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 2 μM each deoxynucleotide, 50 pmol an arbitrary primer and 1 unit Taq DNA Polymerase (Wako, Osaka, Japan). PCR was carried out using a Program Temp Control System PC-700^{TM} (ASTEC, Fukuoka, Japan) for 40 cycles under the following conditions: denaturation at 95°C for 60 s, annealing at 40°C except for primer sets 4, 6, and 8 (35°C) for 90 s, and extension at 72°C for 60 s. Each PCR product was loaded on a 6% denaturing polyacrylamide gel and electrophoresed at 500 V for 16 h, dried, and exposed to Fuji X-ray film for 16 h at –80°C. These procedures were repeated at least twice to confirm the reproducibility of the display pattern. The intensity of the band on the display was measured by charge-coupled device (CCD) imaging system (Densitograph^{TM} AE-6900 MF, ATTO, Tokyo, Japan) and expressed as the percentage of (peak area of the concerned band)/(total area of all detectable bands) to adjust the total mRNA amount. The fragments whose intensity was characteristic in sarcoidosis patients with reproducibility were then excised from the dried gel, and eluted into 100 μl of distilled water.

Sequencing

One microliter of this DNA solution was subjected to PCR using identical primer set used in DD in a 50 μl reaction mixture containing: 20 μM dNTP, 4 pmol of arbitrary primer, 20 pmol of anchored primer, 1 unit of Taq DNA Polymerase. The PCR was carried out under the same conditions as in DD. The amplified PCR products were purified using Ultrafree-C3TK Millipore Column^{TM} by centrifugation and subjected to sequencing using dsDNA cycle Sequencing System Kit^{TM} (GIBCO BRL) with each one of the primers used in PCR according to the manufacturer's recommendation. For each band, DNAs eluted from more than 2 samples were sequenced separately to confirm the reproducibility. The novelty of the sequence was determined by computer search and comparison against Genbank and EMBL data bases (Table 4).

Table 4. Sequence analysis of fragments obtained by differential displays from BAL cells

Fragment number	Primer set	Fragment size(bp)	Sequenced size(bp)	Expression level*	Homology
1	1	330	300	Equivalent	PGK
2	1	217	217	Equivalent	Novel
3	1	≈260	160	Equivalent	Novel
4	5	≈175	135	Equivalent	Novel
5	5	≈210	170	Equivalent	Novel
6	5	≈230	185	Equivalent	Novel
7	5	≈330	230	Equivalent	Novel
8	5	≈200	120	Common	CD44
9	6	285	230	Common	TNF-α
10	6	≈220	172	Increased	Novel
11	7	≈180	146	Decreased	TS**
12	8	≈240	115	Undetectable	Novel

* Equivalent=detectable with comparable intensity among all sarcoidosis and control patients; Common=detectable with comparable intensity among most sarcoidosis patients; Increased=with increased intensity in sarcoidosis compared to other lung diseases; Decreased/Undetectable=with decreased intensity in sarcoidosis compared to other lung diseases.
** 97% homology to the third intron of thymidylate synthase.

Quantification of mRNA of specific genes

When known genes were obtained as bands with characteristic intensity in DD of sarcoidosis patients, we confirmed their expression level by quantitative RT-PCR with specific primers for that cDNA. Since fragments corresponding to CD44 and TNF-α were obtained as commonly expressed genes in sarcoidosis, specific primers for CD44 and β-actin (an internal control) were designed according to the previous reports[18,27] and primers specific for TNF-α were purchased from Perkin Elmer Cetus (Norwalk, CT) for RT-PCR as follows:

CD44 (162 bp)

F: 5′-CCATACCACTCATGGATCTG-3′

R: 5′-GCAATGCAAACTGCAAGAATC-3′

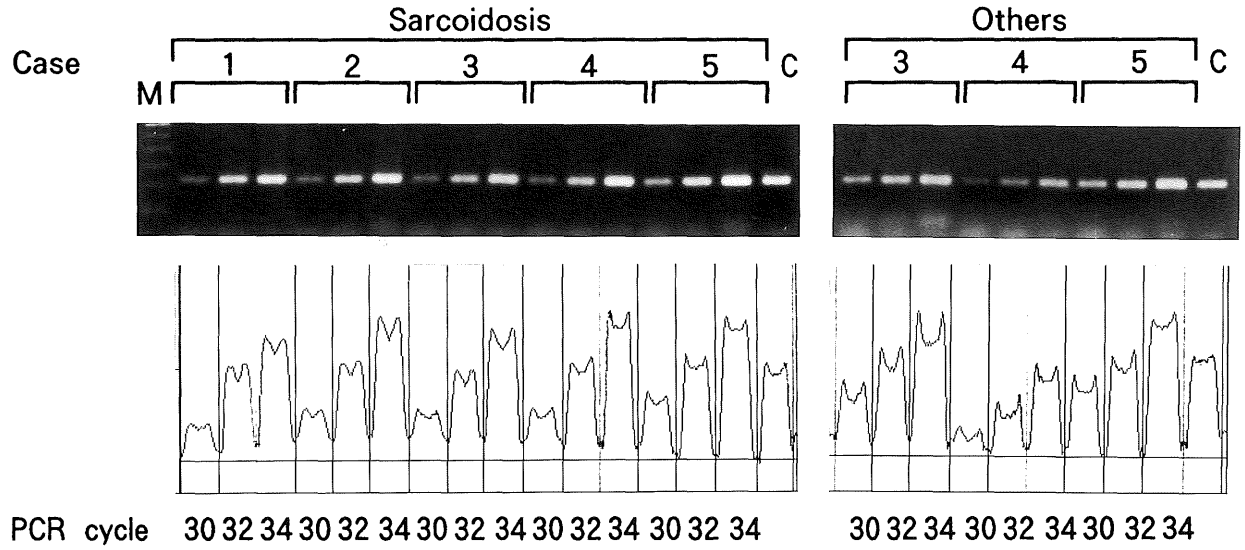

Fig. 1. Quantification of relative amount of β-actin mRNA as an internal control by CCD imaging system. Using specific primers for β-actin cDNA, each sample cDNA was subjected to PCR for 30, 32, and 34 cycles. PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide (upper panel). The intensity of the band luminescence was measured by CCD imaging system (lower panel). It was confirmed that the amplification process was in the exponential phase of amplification from 30 to 34 cycles in all samples.
M=size marker (100 bp DNA ladder); C=control β-actin PCR product to adjust the variance of luminescence between gels.

TNF-α (301 bp)
F: 5′-CAGAGGGAAGAGTTCCCCAG-3′
R: 5′-CCTTGGTCTGGTAGGAGACG-3′
β-actin (218 bp)
F: 5′-AAGAGAGGCATCCTCACCCT-3′
R: 5′-TACATGGCTGGGGTGTTGAA-3′
One microliter of cDNA solution which had been reverse transcribed with an anchored primer $T_{15}AA$ was amplified by PCR in a reaction mixture containing 25 pmol of each primer, 200 μM of each deoxynucleotide, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$ and 1 unit of Taq DNA polymerase (Wako, Osaka, Japan). PCR was carried out under the following conditions: denaturation at 95°C for 60 s, annealing at 58°C (CD44), 60°C (TNF-α), or 63°C (β-actin) for 60 s, and extension at 74°C for 60 s (CD44 and β-actin) or for 90 s (TNF-α). Cycles for amplification were 30–34 for β-actin (Fig. 1) and 40 for CD44 and TNF-α. Aliquots of the PCR reactions (8 μl) were electrophoresed on a 2% agarose gel and stained with ethidium bromide. The intensity of ethidium bromide luminescence for each PCR product was measured by the CCD imaging system as shown in Fig. 1.

Table 5. Relative expression levels of PGK, fragment 10, CD44, and TNF-α estimated by DD or RT-PCR technique

case*	DD**		RT-PCR†	
	PGK	#10	CD44	TNF-α
S 1	7.04	7.10	1.03	1.24
S 2	6.56	8.31	0.78	0.66
S 3	6.89	5.19	0.57	0.28
S 4	6.84	6.76	0.81	0.64
S 5	7.51	9.27	0.51	0.26
S 6	7.43	3.69	0.89	1.10
S 7	7.93	4.82	1.1	1.10
S 8	7.11	9.61	0.8	0.76
S 9	9.19	3.82	0.64	1.06
S10	9.41	4.29	1.58	0.48
S11	7.45	1.91	0.94	1.30
S12	8.74	6.21	0.9	1.45
S13	9.63	5.32	0.93	1.60
S14	7.67	29.2	0.97	1.04
S15	10.62	7.0	0.98	0.86
S16	8.56	15.8	0.85	0.90
S17	7.03	0	1.72	1.30
S18	6.77	4.35	0.95	0.78
C 1	9.17	0	1.45	1.10
C 2	11.59	0	1.77	0.10
C 3	7.45	0.85	0.56	0.28
C 4	9.44	0	0.8	0.02
C 5	6.73	1.73	0.82	0.20
C 6	7.53	2.88	0.54	0.80
C 7	4.39	3.82	5.2	0.80
C 8	7.76	6.33	0.38	0.80

* S1-18=patients with sarcoidosis; C1-8=patients with other lung diseases
** The relative intensities of PGK and #10 fragments in DD analysis were calculated as (peak area of the concerned band)/(total area of all detectable bands)
† The relative intensities of CD44 and TNF-α bands in RT-PCR analysis were calculated as (peak area of the concerned band)/(peak area of β-actin band)
Expression levels of #10 and TNF-α were higher in sarcoidosis patients compared to other lung diseases ($p < 0.01$ and $p < 0.05$, respectively), while no statistical differences were observed between them for PGK and CD44.

Statistical analysis

Statistical analysis was done using software StatView-JII on a Macintosh computer. The non-parametric Mann-Whitney U test was used to compare the quantitative value between patients with sarcoidosis and other lung diseases. A probability value of less than 0.05 was considered as statistically significant.

RESULTS

We analyzed expression levels of various genes in BAL cells from patients with sarcoidosis comparing those with other pulmonary diseases using 19 sets of primers for DD. About 100-150 bands ranging from 100 to 350 bp could be visualized per set of primers for DD. The fragment derived from PGK mRNA, an internal control, was detected using primer set 1 in all samples with comparable intensity except for one case with weak intensity (case 7 in "Others", Fig. 2, upper display). This finding was confirmed by estimating the relative intensity of the band by the ratio of (peak area of PGK band)/(total area of all detectable bands). There was no statistical difference in this value between patients with sarcoidosis and other diseases, indicating the feasibility of this method in comparing the mRNA expression levels between samples (Table 5).

Although the DD patterns of mRNA in BAL cells were generally similar in most subjects, as previously reported using other materials[20,22,34], 2 fragments were found to be specifically and reproducibly altered in intensity in patients with sarcoidosis compared to those with other pulmonary diseases (Fig. 2, #10 in middle and #12 in lower displays, respectively). Fragment 10 (#10) was detected in all histologically diagnosed sarcoidosis patients, while it was not detected in 3 of 8 control samples nor in case 17, who was clinically diagnosed as having sarcoidosis but lacking histological evidence. The ratio of (peak area of #10)/(total area of all detectable bands) in sarcoi-

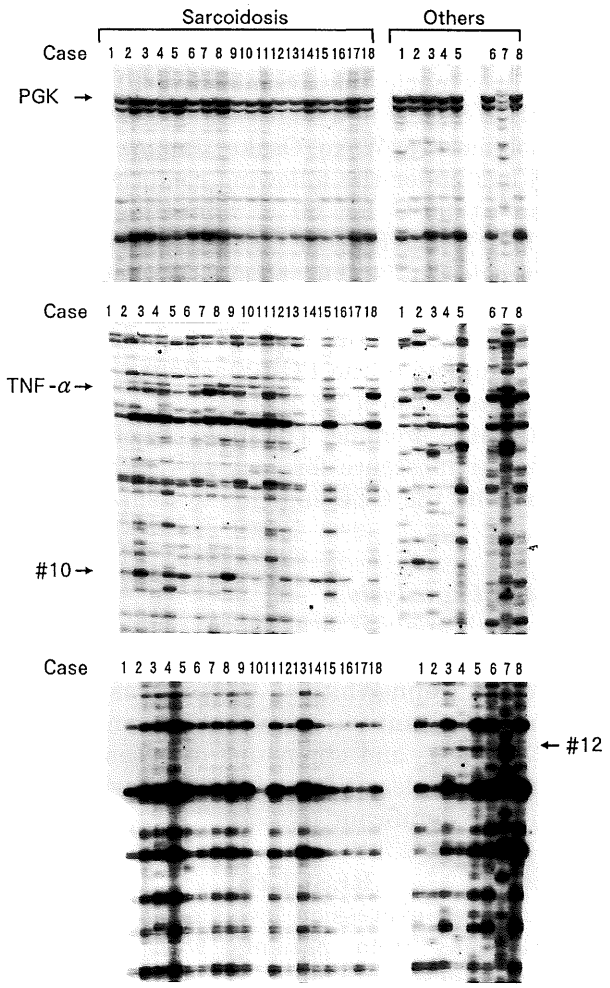

Fig. 2. Differential mRNA display from BAL cells. PGK fragment was detected as an internal control using primer set 1 (upper display). This band was observed in all samples with comparable intensity except for case 7 in "Others" with weak intensity. TNF-α and fragment 10 (#10) were cloned using primer set 6 (middle display), as fragments highly expressed in sarcoidosis patients. Fragment 12 (#12) was cloned using primer set 8 (lower display) as a candidate gene whose expression is repressed in sarcoidosis.

dosis was significantly higher than that in controls ($p < 0.01$, Fig. 3, Table 5). Fragment 12 (#12) was detected in none of the sarcoidosis samples while more than half of control diseases showed this band. Both #10 and #12 fragments did not have significant homology to any human genes deposited in GenBank or EMBL data bases (Fig. 4).

In addition to these fragments, we cloned and sequenced several fragments which were commonly expressed in sarcoidosis patients (Table 4). Most of these fragments, which seemed to be highly expressed in comparable level in sarcoidosis, showed no significant homology to any known human mRNA or DNA sequences deposited in Genbank and EMBL data bases. However 2 fragments, in addition to PGK, were revealed to be identical to known mRNA sequences, i.e. TNF-α[25], and cell surface glycoprotein CD44[11,24]. The sizes of PGK and TNF-α fragments were consistent with their expected sizes based on the mRNA sequences. Although the sequence of fragment 8 is identical to a part of CD44, the poly A site seemed to locate 544 bp upstream of the published reticulocyte CD44 cDNA sequences[11] in all 3 BAL samples sequenced, including 2 sarcoidosis and 1 HP. The sequence of fragment 11 (#11), which was cloned as a fragment of somewhat decreased intensity in sarcoidosis, was found to be 97% homologue to the intron 3 of human thymidylate synthase (TS) gene[17], indicating the possibility of a false-positive band derived from genomic DNA contaminated in mRNA. To confirm that the CD44 and TNF-α genes are commonly expressed in sarcoidosis patients as observed in DD, we performed quantitative RT-PCR analysis using specific primers for CD44, human TNF-α, and human β-actin as an internal control. Expression of both genes was constantly detected in sarcoidosis patients as expected by DD analysis, and the expression level of TNF-α was significantly higher in sarcoidosis patients compared to other pulmonary diseases ($p < 0.05$, Mann Whitney test, Fig. 5). The values of CD44/β-actin and TNF-α/β-actin ratio of each sample are shown in Table 5. In case 7 of "Others", whose PGK band was remarkably weak, the PCR product of β-actin was also less than one tenth of the remaining cases, resulting in the remarkably high value of the CD44/β-actin ratio.

DISCUSSION

This experiment is the first report which compares the mRNA expression levels of various genes, including unknown genes, in sarcoidosis patients and other pulmonary diseases. We applied the DD technique for this purpose and found it sensitive as well as reproducible as previously described[22,34]. In order to detect the specific genes for sarcoidosis, not those whose expression levels are non-specifically modified by inflammation nor by increased number of lymphocytes in BAL cells, we used BAL samples from patients with various other lung diseases instead of using normal subjects as control. Thus, no significant difference was found in differential counts of lymphocytes and macrophages between the sarcoidosis group and the control group (Table 1, 2, Mann-Whitney U Test). The autoradiogram patterns of differentially expressed mRNAs from BAL cells in sarcoidosis and other pulmonary diseases were almost identical, providing a reproducible background over which specific differences could be observed. In this context,

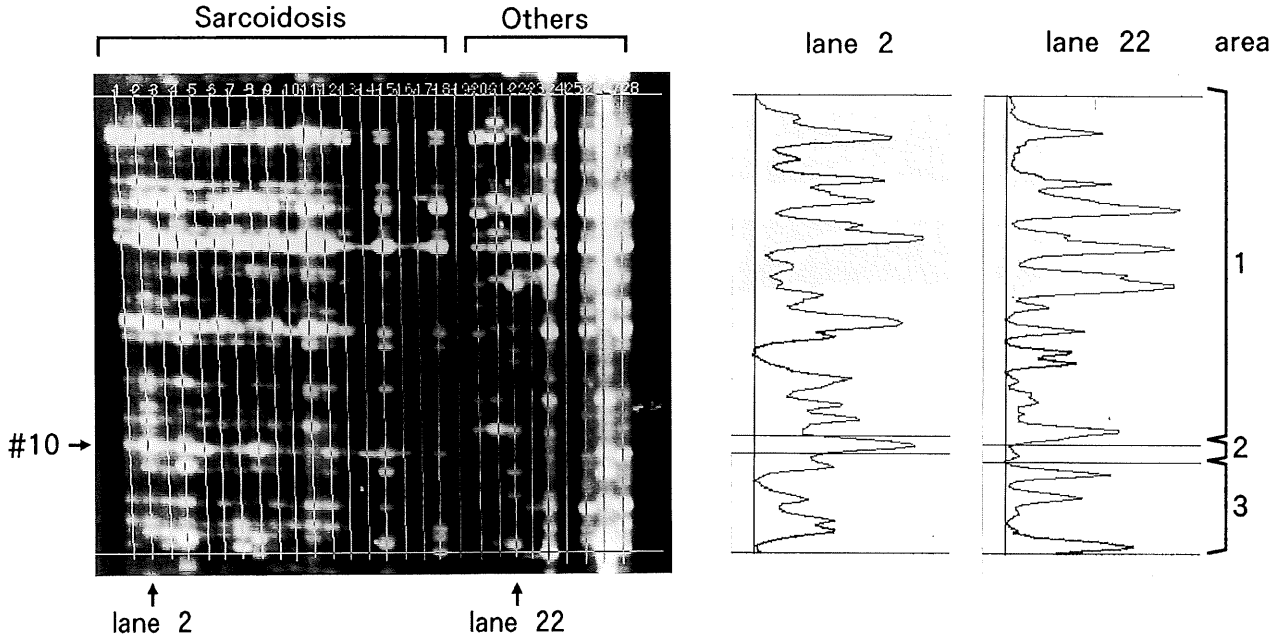

Fig. 3. The display exhibiting fragment 10 (#10) was analyzed by CCD imaging system. The relative intensity of #10 in each sample was calculated by (area 2)/(area 1 + 2 + 3), i.e., (peak area of #10)/(total area of all detectable peaks). The value in lane 2 (case 2 in sarcoidosis) was 8.31, while that in lane 22 (case 3 in others) was 0.85.

Fragment 10 (#10)

ACCGGGGTAT --(~60bp)-- AGGGATAATG AAACTTAGGN GNCAACTGGG
AAGATTGTAA NNNCATAGTA CTCAACCAAT GAGGAACNGG GGGAGGGACT
TGCGTGCTAG GAGATAAATT ACCTGTTGTG ACTGCCGTGG ATGTGCCTGC
TCACCAGACA CCNAATCTTG CAAGACTGTT ATNAANGTCT CA
--(~20bp)-- TTAAAAAAAAAAAAAAA

Fragment 12 (#12)

GAATGATGAG --(~10bp)-- GAGGAAGTAG AGTNNNCAAA AACATTTTGA
AAAGGAGAGA AAGTAATGGC TATTTTGAAA GATTATAGCT GGCTCTGTAA
AACTACTCAG AAAACCCTTT ATGAGATTAG GCAAA --(~10bp)--
CTAAAAAAAAAAAAAAA

Fig. 4. Nucleotide sequences of fragments 10 and 12, whose expression levels were increased or decreased, respectively, in BAL cells from patients with sarcoidosis. Flanking sequences of primer sets are underlined. These sequences showed no significant homology to any known genes deposited to GenBank or EMBL data bases.

case 7 among the patients with other lung diseases whose PGK and β-actin mRNA expression levels were unusually low as housekeeping genes, i.e. as internal controls, should be considered as an unsuitable sample due to possible degradation of mRNA. Thus, we can sort out the unsuitable samples for DD by using internal controls.

Theoretically, 120–240 primer sets are necessary for DD to cover all mRNAs expressed in a cell[22]. We obtained two characteristic fragments, #10 and #12, which are reproducibly increased or decreased in intensity in samples from patients with sarcoidosis using 19 primer sets for DD (Fig. 2), indicating the potential usefulness of this method as a tool for the diagnosis of the disease. Fragment 10 (#10) was detected in all histologically diagnosed sarcoidosis patients, and was not detected in case 17, who was clinically diagnosed as sarcoidosis but had no histological evidence. Although she had uveitis, there were no chest X-ray findings nor was there granulomatous lesion in transbronchial lung biopsy specimens. Thus, fragment 10 is likely to be specific for definite sarcoidosis with granulomatous lesions. Since we already used all 12 possible anchored primers for RT, application of different arbitrary primers in this DD analysis provides the possibility for detection of additional characteristic bands for sarcoidosis.

Among the 12 fragments which we cloned and sequenced, 2 common fragments with comparable intensity in most sarcoidosis cases were revealed to be CD44 and TNF-α. This finding was further confirmed by the quantitative RT-PCR analysis using specific primers for these cDNAs. CD44 is a cell surface adhesion molecule which belongs to the cartilage link protein family, and it has been suggested that it plays a role in a number of important physiological processes including the homing of lymphocytes[16], cell adhesion[31], T cell activation[8,31], and cell migration[19]. To our knowledge, CD44 expression has not been previously reported in the sarcoidosis patients, while

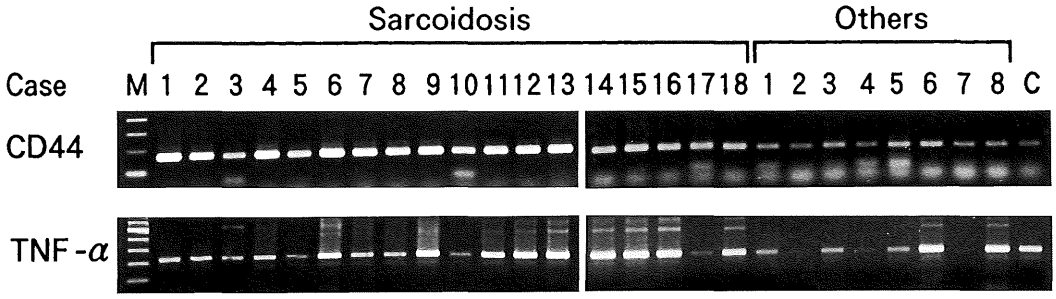

Fig. 5. Quantitative RT-PCR of CD44 (upper panel) and TNF-α (lower panel) mRNAs. Using specific primers for each cDNA, expression of both genes were detected in all sarcoidosis patients. The luminescent intensity of each band was divided by that of β-actin in each sample, and the CD44 or TNF-α/β-actin ratio was considered to represent the relative amount of these mRNAs. The relative amount of TNF-α mRNA in BAL cells from sarcoidosis patients was significantly higher than that from patients with other lung diseases.
M=size marker (100 bp DNA ladder); C=control CD44 or TNF-α PCR products to adjust the variance of luminescence between gels.

some reports showed overexpression of the alternatively-spliced products CD44 gene in neoplastic tissues[23,35]. CD44 is a principal cell surface receptor for hyaluronate[1]. Hyaluronate binding to macrophages and lymphocytes promotes cell aggregation, cytokine release, and T cell activation (reviewed in ref.12). Although the expression of CD44 has also been observed in other lung diseases and is not so specific to sarcoidosis, together with the fact that hyaluronate level in BAL fluid is reportedly elevated in patients with sarcoidosis[3,9], the present finding suggests the possible contribution of hyaluronate and CD44 in the pathogenesis of sarcoidosis. CD44 is expressed in large amounts on macrophages (reviewed in ref.13), and macrophages are immunoinflammatory cells in lung which contribute to inflammation processes in interstitial lung disease[28]. Thus, CD44 may be involved in the pathogenesis of interstitial lung diseases in general. On the other hand, the increased expression of TNF-α in BAL cells from sarcoidosis patients has already been observed by us[15] and other researchers[2,4,10,26,32,33,36,39]. In the present study, a significantly higher level of TNF-α mRNA expression was also found in sarcoidosis compared to other pulmonary diseases by using RT-PCR technique. TNF-α is a cytokine produced mainly by monocytes and macrophages after stimulation with various agents[30]. TNF reportedly increases the binding of lymphocytes to endothelial cells, suggesting that it may cause enhanced migration of lymphocytes from blood into the tissue[5]. TNF-α also has a role in developing a fibrotic process as shown in experimental models[7]. Interestingly, Webb et al reported that engagement of CD44 by a specific monoclonal antibody induced TNF-α and IL-1β release *in vitro*[38].

One clone (#11) was considered to be a false positive band derived from genomic DNA, since its sequence showed 97% homology to the third intron of human thymidylate synthase (TS) gene[17]. However, since apparently 4 nucleotides were different from the reported sequences, the possibility remains that it was not false positive but derived from unknown mRNA with high homology to the third intron of the TS gene. Since the amount of mRNAs obtained from BAL cells is limited, it is difficult to confirm the expression level of mRNA by Northern blotting.

In conclusion, the results reported here show the suitability of the mRNA differential display to analyze the expression of various genes in the BAL cells to elucidate the pathogenesis of sarcoidosis, and potential usefulness of this method as a tool for the diagnosis of the disease. The fact that we could detect CD44 and TNF-α mRNAs, which are really expressed in BAL cells in sarcoidosis patients, by the DD analysis, supports the idea that the 2 fragments of characteristic intensity in sarcoidosis, with no significant homology to known genes, may represent 2 novel genes playing important roles in the pathogenesis of sarcoidosis. Further analysis of these fragments, i.e., cloning the entire cDNA sequences from cDNA library using these fragments as probes, and analysis of their structure and function will elucidate the hypothesis.

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