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Decreased expression of antioxidant enzymes and increased expression of chemokines in COPD lung

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Abstract

The involvement of inflammation in the pathogenesis of chronic obstructive pulmonary disease (COPD) has been investigated using samples from relatively central airways such as airway biopsies, but there have been fewer studies in the peripheral lung, which is thought to be the main site of the disease process. To determine the molecules that relate to the mechanisms underlying the pathogenesis of COPD, we evaluated the mRNA expression of inflammatory cytokines, chemokines, oxidant enzymes, antioxidant enzymes, proteinases and antiproteinases in peripheral lung tissues from 33 COPD and non-COPD subjects who were undergoing lung resection for lung cancer using an RT-PCR technique.

Among the 42 studied candidate genes, the expressions of mRNA for catalase, glutathion *S*-transferase P1 (GSTP1), glutathion *S*-transferase M1 (GSTM1), microsomal epoxide hydrolase (mEPHX) and tissue inhibitor of metalloproteinase 2 (TIMP2) were significantly decreased in COPD lung tissues compared with those in non-COPD tissues, and most of these decreases were significantly correlated with the degree of airflow limitation. On the other hand, the expressions of mRNA for interleukin 1β (IL-1β), interleukin 8 (IL-8), growth-related oncogene-α (Gro-α) and monocyte chemotactic protein-1 (MCP-1) were significantly increased in COPD lungs. Most of these changes were also associated with cigarette smoking.

These data suggest that an impairment of protective mechanisms against oxidants and xenobiotics, in addition to the upregulation of CXC- and CC-chemokines, may be associated with cigarette smoking and involved in the inflammatory process of COPD.

Keywords: Catalase; Glutathion *S*-transferase P1; Glutathion *S*-transferase M1; Microsomal epoxide hydrolase; Tissue inhibitor of metalloproteinase 2; mRNA; RT-PCR

1. Introduction

Chronic obstructive pulmonary disease (COPD) is a disease characterized by airway inflammation and progressive airflow limitation that is not fully reversible. The morbidity and mortality of the disease have increased in recent years and it is a serious public health problem in many countries throughout the world [1]. Abnormal inflammatory responses of the lungs against noxious gases and particles, such as cigarette smoke, are thought to cause small airway disease, namely obstructive bronchiolitis, and parenchymal destruction, leading to the pathophysiologic changes of COPD such as airflow limitation [2]. Although the precise mechanisms of these processes have not been fully clarified, several mechanisms have been suggested to contribute to the disease process.

The cellular inflammatory response in COPD is characterized by increases in neutrophils, macrophages and CD8-positive T lymphocytes in the lungs [3]. Inflammatory mediators from these cells and epithelial cells contribute to interactions among these cells and cause the

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pathophysiological changes of COPD including mucus hypersecretion, fibrosis, and parenchymal destruction [4]. Oxidative stress, which may result from excessive oxidants and/or impaired antioxidant activities, is thought to be one of the major causes of inflammation and injury in diseased lungs. Oxidative stress arises during the inflammatory process in the lung and also from the environment. Both endogenous reactive oxygen species released from inflammatory cells such as neutrophils and macrophages and oxidant compounds in cigarette smoke or air pollution cause injuries to lung tissues [5]. These harmful molecules are eliminated by antioxidant enzyme activities in normal lungs [6]. In COPD lungs, these protective mechanisms seem to be impaired. Another mechanism in the COPD pathogenesis is an imbalance of proteinase and antiproteinase. α_1-antitrypsin has been shown to be involved in the structural changes in COPD, and a number of proteinases and antiproteinases have been reported to play roles in the inflammatory process in COPD [7].

The involvement of inflammation, oxidative stress and a proteinase/antiproteinase imbalance in the pathogenesis of COPD has been investigated using samples from COPD subjects. Many studies employing samples from relatively central airways such as airway biopsies and induced sputum have revealed an alteration in the formation of inflammatory mediators, oxidant, antioxidant, proteinase and antiproteinase in COPD airways. However, there have been fewer studies in the peripheral lung, which is thought to be the main site of the disease process.

In this study, we evaluated the expressions of 42 genes for inflammatory cytokines, chemokines, oxidant enzymes, antioxidant enzymes, proteinases and antiproteinases in peripheral lung tissues from COPD and non-COPD subjects. We found decreased mRNA expressions for catalase, glutathion *S*-transferase P1 (GSTP1), glutathion *S*-transferase M1 (GSTM1), microsomal epoxide hydrolase (mEPHX) and tissue inhibitor of metalloproteinase 2 (TIMP2) and increased expressions for interleukin 1β (IL-1β), interleukin 8 (IL-8), growth-related oncogene-α (Gro-α) and monocyte chemotactic protein-1 (MCP-1) in the COPD lung. Most of these changes were associated with the degree of airflow limitation and cigarette smoking.

2. Materials and methods

2.1. Subjects

Thirty-three patients with or without COPD who were undergoing lung resection for lung cancer took part in the study after giving written informed consent. This study was approved by the Tohoku University Committee on Clinical Investigations and by the Ethics Review Board of Miyagi Prefectural Cancer Center. According to the presence or absence of COPD and a history of smoking, the subjects were divided into three groups: 10 non-COPD subjects who never smoked, 9 non-COPD smokers and 14 COPD subjects. All patients with COPD satisfied the Global Initiative for Chronic Obstructive Lung Disease guidelines [2] and were diagnosed as having pulmonary emphysema through computed tomography. The smokers were divided into current smokers and ex-smokers, defined as those who had quit smoking for at least 3 months prior to the study. The clinical characteristics of the study subjects are shown in Table 1. All patients were stable and had no respiratory tract infection during the month preceding the study. Forced vital capacity (FVC), forced expiratory volume in 1 s (FEV_1), maximum flow rate at 50% of vital capacity divided by measured body height (V_{50}/HT) and maximum flow rate at 25% of vital capacity divided by measured body height (V_{25}/HT) were measured using a dry rolling-seal spirometer (FUDAC-70; Fukuda Denshi Co., Ltd., Tokyo, Japan) in the week before surgery.

2.2. Lung tissue collection

Peripheral lung tissue was obtained from the subpleural parenchyma of the lobe resected at surgery, avoiding areas involved by tumor. The tissue specimen (size 5 × 5 × 10 mm) was immediately immersed in Isogen (Nippon Gene Co., Ltd., Tokyo, Japan) and kept at −80 °C until RT-PCR analysis.

2.3. Isolation of total RNA and real-time quantitative PCR

Total RNA from each sample was extracted in guanidine isothiocyanate and phenol (Isogen) according to the manufacturer's instructions. Total RNA (500 ng) was reverse transcribed with random hexamer and MultiScribe Reverse Transcriptase using TaqMan Gold RT-PCR Kit (Applied Biosystems, Foster City, CA). cDNA samples corresponding to 8 ng of total RNA were measured by real-time quantitative PCR using Applied Biosystems prism 7900HT Sequence Detection System according to the manufacturer's instructions. The primers and probes were

Table 1
Characteristics of study subjects

	Non-COPD		COPD
	Never smoked	Smokers	
Number	10	9	14
Male/Female	2/8	5/4	12/2
Age (year)	66.6±2.5	57.1±5.3	72.9±1.5††
Smoking status (ex/current)	—	5/4	11/3
Smoking history (pack-year)	0	21.6±6.7**	51.6±6.0**
%FVC (%)	122.3±8.3	107.3±3.0	89.6±4.5**
%FEV_1 (%)	135.6±9.7	111.7±3.2	78.5±6.7**
FEV_1/FVC% (%)	79.8±1.4	77.2±1.0	58.6±3.0**

The data are expressed as mean±SEM.
**$P<0.01$ compared with the non-COPD never-smoked group (Mann–Whitney *U*-test).
††$P<0.01$ compared with the non-COPD smoker group (Mann–Whitney *U*-test).

obtained from TaqMan Gene Expression Assays or Pre-Developed TaqMan Assay Reagents (Applied Biosystems, Foster City, CA) or designed for each of the genes according to the Primer Express 2.0 program provided by Applied Biosystems (Table 2). Reporter dyes and quencher dyes for all designed probes were 6-carboxyfluorescein (FAM) and 6-carboxytetramethylrhodamine (TAMRA), respectively.

The amount of target message in each sample was estimated from a threshold cycle number (CT), which is inversely correlated with the number in its initial mRNA level and used to determine gene expression. The CT-values were generated by the ABI PRISM 7900HT SDS software version 2.0. The CT value of each gene was normalized using the formula $\Delta CT = (CT \text{ of each gene}) - (CT \text{ of } \beta\text{-actin})$. The expression levels of each gene per β-actin were calculated according to the formula $2^{-(\Delta CT)}$.

2.4. Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis was carried out using nonparametric analysis of variance (Kruskal–Wallis test) to evaluate variance among the three groups. If a significant variance was found, an unpaired two-group test (Mann–Whitney *U*-test) was used to determine significant differences between individual groups. For analysis within group correlations, Spearman's rank correlation test was used. Values of $P<0.05$ were considered statistically significant.

3. Results

3.1. mRNA expressions of the 42 studied candidate genes

Table 3 shows all the data of the mRNA amounts observed in this study. We found some significant differences between COPD and non-COPD subjects. The expressions of mRNA for catalase, GSTP1, mEPHX, GSTM1 and TIMP2 were significantly decreased in COPD lung tissues compared with those in non-COPD tissues. On the other hand, the mRNA expressions for IL-1β, IL-8, Gro-α and MCP-1 were significantly increased in COPD compared with non-COPD. None of the other studied genes showed significant changes in mRNA expression in COPD versus non-COPD subjects.

3.2. Decreased expression of enzymes protective against oxidants and xenobiotics

The expressions of mRNA for catalase, GSTP1 and mEPHX were significantly decreased in COPD lung tissues compared with those in non-COPD tissues (Fig. 1). The expression of mRNA for catalase was significantly decreased in lung tissues from COPD subjects (0.165 ± 0.013) compared with those from non-COPD smokers (0.233 ± 0.026, $P = 0.023$) and those who never smoked (0.251 ± 0.025, $P = 0.003$). The GSTP1 mRNA expression was significantly decreased in lung tissues from COPD subjects (0.449 ± 0.027) compared with those from non-COPD smokers (0.554 ± 0.028, $P = 0.023$) and those who never smoked (0.647 ± 0.069, $P = 0.019$). The mEPHX mRNA expression was significantly decreased in lung tissues from COPD subjects (0.153 ± 0.014) compared with those from non-COPD smokers (0.223 ± 0.014, $P = 0.003$) and those who never smoked (0.228 ± 0.028, $P = 0.022$). The expression of mRNA for GSTM1 was significantly increased in lung tissues from non-COPD smokers (0.229 ± 0.044) compared with those from non-COPD subjects who never smoked (0.136 ± 0.033, $P = 0.034$) and those from COPD subjects (0.126 ± 0.019, $P = 0.014$) (Fig. 2).

3.3. Decreased expression of antiproteinase

The expression of mRNA for TIMP2 was significantly increased in lung tissues from non-COPD smokers (1.135 ± 0.101) compared with those from non-COPD subjects who never smoked (0.873 ± 0.166, $P = 0.014$) and those from COPD subjects (0.870 ± 0.110, $P = 0.038$) (Fig. 2).

3.4. Increased expression of inflammatory cytokines and chemokines

The expressions of mRNA for IL-1β (0.203 ± 0.058 vs. 0.051 ± 0.021, $P = 0.004$), IL-8 (0.618 ± 0.188 vs. 0.071 ± 0.036, $P = 0.003$), Gro-α (0.015 ± 0.04 vs. 0.002 ± 0.001, $P = 0.005$) and MCP-1 (1.300 ± 0.316 vs. 0.286 ± 0.120, $P = 0.002$) were significantly increased in lungs from COPD subjects compared with those from subjects who never smoked. The differences in the expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 between in COPD subjects and in non-COPD smokers were not statistically significant (Fig. 3).

3.5. Correlations between gene expressions and pulmonary function and cigarette smoking habit

The expressions of mRNA for catalase, GSTP1 and mEPHX were significantly correlated with the parameters of pulmonary function: % of predicted values of forced vital capacity (%FVC), % of predicted values of forced expiratory volume in one second (%FEV_1), FEV_1/FVC%, V_{50}/HT and V_{25}/HT (Fig. 4 and Table 4). The mRNA expressions for these enzymes were negatively correlated with cigarette smoking history assessed as pack-year (Table 4).

In contrast, the expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 were negatively correlated with V_{50}/HT and were associated with cigarette smoking history (Table 4). The expressions of IL-8 and Gro-α were also negatively correlated with FEV_1/FVC% (Table 4).

When analyzed in two groups of non-COPD smokers and COPD subjects, the expression of mRNA for TIMP2

Table 2
Real-time quantitative PCR primers and probes

Gene	Accession no.	Sequence or assay ID	
XDH	NM_000379	Hs00166010_m1	
HMOX1	NM_002133	Hs00157965_m1	
GPX3	genbank:NM_002084NM_002084	TCCCTGCAACCAATTTGGA	Forward primer: 593–613
		CCATTGACATCCCCTTTCTCA	Reverse Primer: 723–703
		TCCTTCCTACCCTCAAGTATGTCCGACCA	TaqMan probe: 642–670
GSTM1	NM_000561	AGGACTTCATCTCCCGCTTTG	Forward primer: 599–619
		CCATCTTTGAGAACACAGGTCTTG	Reverse Primer: 691–668
		TCTGCCTACATGAAGTCCAGCCGCTT	TaqMan probe: 637–662
GSTP1	NM_000852	CCTCCGCTGCAAATACATCTC	Forward primer: 326–346
		CAGTGCCTTCACATAGTCATCCTT	Reverse Primer: 398–375
		CTCATCTACACCAACTATGAGGCGGG	TaqMan probe: 348–373
GSTT1	NM_000853	CATAAGGTGATGTTCCCTGTGTTC	Forward primer: 346–369
		GGAGATGTGAGGACCAGTAAGGA	Reverse Primer: 492–470
		TCGAGGACAAGTTCCTCCAGAACAAGGC	TaqMan probe: 440–467
SOD1	NM_000454	AAAACACGGTGGGCCAAA	Forward primer: 210–227
		ACATCGGCCACACCATCTTT	Reverse Primer: 293–274
		CCCAAGTCTCCAACATGCCTCTCTTCA	TaqMan probe: 257–231
SOD2	NM_000636	Hs00167309_m1	
SOD3	NM_003102	Hs00162090_m1	
Catalase	NM_001752	Hs00156308_m1	
mEPHX	NM_000120	Hs00164458_m1	
MMP1	NM_002421	Hs00233958_m1	
MMP2	NM_004530	Hs00234422_m1	
MMP9	NM_004994	Hs00234579_m1	
MMP12	NM_002426	Hs00159178_m1	
ELA2	NM_001972	Hs00357734_m1	
TIMP1	NM_003254	Hs00171558_m1	
TIMP2	NM_003255	Hs00234278_m1	
SERPINA1	NM_000295	Hs00165475_m1	
SLPI	NM_003064	Hs00268206_m1	
IL-13	NM_002188	H327046T	
TNF-α	NM_000594	H327055T	
TNF-α R	NM_001065	GCTTCAGAAAACCACCTCAGACA	Forward primer: 552–574
		ATGCCGGTACTGGTTCTTCCT	Reverse Primer: 683–663
		TCAGCTGCTCCAAATGCCGAAAGG	TaqMan probe: 580–603
IL-1β	NM_000576	4327035T	
IFN-α	NM_000619	4327052T	
IL-17	NM_002190	4327048T	
GM-CSF	NM_000758	4327057T	
IL-10	NM_000572	4327043T	
Gro-α	NM_001511	TTCTGAGGAGCCTGCAACATG	Forward primer: 754–774
		TCCCCTGCCTTCACAATGAT	Reverse Primer: 842–823
		CGGATCCAAGCAAATGGCCAATGA	TaqMan probe: 798–821
IL-8	NM_000584	CGGAAGGAACCATCTCACTGT	Forward primer: 73–93
		ATCAGGAAGGCTGCCAAGAGA	Reverse Primer: 145–125
		GTAAACATGACTTCCAAGCTGGCCGTG	TaqMan probe: 96–122
BLT1	NM_000752	CATCTGGGTGTTGTCCTTTCTG	Forward primer: 2137–2158
		GACAGCCTCGAAGATTAGATGGA	Reverse Primer: 2278–2256
		TGCCCTGGAAAACGAACATGAGCC	TaqMan probe: 2193–2216
BLT2	NM_019839	ACCTTCTCATCGGGCATCAC	Forward primer: 1624–1643
		GAAGTCTTCCAGCTCAGCAGTGT	Reverse Primer: 1767–1745
		CCACCTGTAGGCCCAGAAGGATGT	TaqMan probe: 1692–1715
VEGF	NM_003376	Hs00173626_m1	
MUC5AC	AJ298317	TACTCCACAGACTGCACCAACTG	Forward primer: 1263–1285
		CGTGTATTGCTTCCCGTCAA	Reverse Primer: 1391–1372
		TGTGCTTGGAGGTGCCCACTTCTCAA	TaqMan probe: 1343–1368
MUC5B	Z72496	ACCGACCACAGAGCTGGAGA	Forward primer: 863–882
		ATGTCAGTCCTTCTGAGAGGGTG	Reverse Primer: 1005–983
		TTCTCAACGCCGCAGCCTACGAGT	TaqMan probe: 912–935
MUC8	U14383	CGGCCATCATTCCTTTTTACTG	Forward primer: 1226–1247
		AACCCACATGACCATCAACTGA	Reverse Primer: 1315–1294
		CTGTGTGAATCCACCGCTAGAAACCCA	TaqMan probe: 1263–1289
CTSL	NM_145918	Hs00377632_m1	

Table 2 (*continued*)

Gene	Accession no.	Sequence or assay ID
MCP-1	NM_002982	4329524T
TGF-β	NM_000660	4327054T
EGF	NM_001963	Hs00153181_m1
IL-4	NM_000589	4327038T
IL-5	NM_000879	4327039T

Numbers refer to oligonucleotide position contained within the published cDNA.

was significantly correlated with V_{50}/HT ($r_s = 0.469$, $P = 0.028$), but not with other parameters including %FEV_1 ($r_s = 0.376$, $P = 0.077$) nor cigarette smoking history ($r_s = -0.342$, $P = 0.106$). The expression of mRNA for GSTM1 had no significant relationship with parameters for airflow limitation (data not shown) and cigarette smoking history ($r_s = -0.393$, $P = 0.065$).

In smokers with and without COPD, there was no association between the mRNA expressions for these molecules and duration since smoking cessation (data not shown).

4. Discussion

In this study, we observed decreased expressions of mRNA for catalase, GSTP1, GSTM1, mEPHX and TIMP2 and increased expressions of mRNA for IL-1β, IL-8, Gro-α and MCP-1 in peripheral lungs from patients with COPD. Most of these changes in mRNA expressions were associated with the degree of airflow limitation and with the cigarette smoking habit.

Although the precise mechanisms of the pathogenesis of COPD have not been fully elucidated, it is thought that the inflammatory responses of the lungs induced by noxious gases and particles, such as oxidants and xenobiotics, play a major role in the onset and progression of the disease. An impairment of protective mechanisms of the lungs against such harmful molecules would also contribute to the disease process.

Oxidative stress and imbalances in the host defense mechanisms appear to be among the causes of COPD. Reactive oxygen/nitrogen species have been suggested to be involved in the pathophysiology of COPD through several studies analyzing airway samples such as induced sputum [8,9]. It also has been reported that antioxidant enzyme activities are altered in COPD. In the present study, the mRNA expression for catalase, a catalyzing enzyme for the clearance of hydrogen peroxide, was decreased in the peripheral lungs from COPD subjects. It has been reported that catalase activity is decreased in circulating red blood cells in patients with COPD [10], while the level of catalase is often increased in those from smokers, probably due to upregulation as an adaptive response against oxidative stress by cigarette smoking [11]. Recently, Ning and colleagues have reported the decreased gene expression for catalase in surgically obtained lung tissue from patients with COPD, using a serial analysis of gene expression [12]. Our results are consistent with their result and further extend the findings by showing that the decrease in catalase mRNA expression in the peripheral lung tissue was correlated to the degree of airflow limitation in COPD patients. These results suggest that impaired protective activity of catalase against oxidative stress may lead to enhanced pathophysiological changes in COPD.

Other protective mechanisms against toxic substrates involve xenobiotic-metabolyzing enzymes such as GSTP1, GSTM1 and mEPHX, the mRNA expressions of which were decreased in COPD patients in the present study. GSTP1 and GSTM1 are members of the glutathione *S*-transferase family known as antioxidative xenobiotic enzymes. GSTP1 and GSTM1 are expressed in alveoli, alveolar macrophages and respiratory bronchioli in the peripheral lung [13], and play an important role in the detoxification of xenobiotics which are contained in cigarette smoke and occupational and environmental pollutants. GSTP1 has been reported to exert a protective effect against cigarette smoke extract in human lung fibroblasts in vitro [14]. mEPHX is strongly expressed in bronchial epithelial cells in the lung [15], and is involved in the first-pass metabolism of highly reactive epoxide intermediates. Several genetic researches also have suggested that polymorphisms of GSTP1, GSTM1 and mEPHX genotypes may associate with a susceptibility to COPD or the severity of the disease in some races including Japanese [16–18].

Also, members of the TIMP family exert inhibitory activities against matrix metalloproteinases the involvement of which in the disease process of COPD has been suggested [19]. Recently, it has been reported that polymorphisms of the TIMP2 gene are associated with COPD susceptibility in a Japanese population suggesting a relationship between the decreased activity of TIMP2 and the pathogenesis of the disease [20]. It has been also reported that TIMP2 mRNA expression is decreased in the lung tissue from severer COPD subjects [12]. Our data are in line with these reports.

In the present study, decreased mRNA expressions for catalase, GSTP1 and mEPHX were evident in the peripheral lung tissue in COPD. The degree of the decrease in the mRNA expressions for these antioxidant and xenobiotic enzymes significantly correlated with the degree of airflow limitation, %FVC, %FEV_1, FEV_1/FVC%, V_{50}/HT

Table 3
mRNA expressions of the 42 studied candidate genes

	Non-COPD		COPD
	Never smoked	Smokers	
Genes that are significantly downregulated in COPD versus non-COPD			
Catalase	0.251±0.025	0.233±0.026	0.165±0.013**,†
GSTP1	0.647±0.069	0.554±0.028	0.449±0.027*,†
mEPHX	0.228±0.028	0.223±0.014	0.153±0.014*,††
GSTM1	0.136±0.033	0.229±0.044*	0.126±0.019†
TIMP2	0.873±0.166	1.135±0.101*	0.870±0.110†
Genes that are significantly upregulated in COPD versus non-COPD			
IL-1β	0.051±0.021	0.105±0.037	0.203±0.058**
IL-8	0.071±0.036	0.237±0.093	0.618±0.188**
Gro-α	0.002±0.001	0.009±0.006	0.015±0.005**
MCP-1	0.286±0.120	0.739±0.255	1.299±0.316**
Genes where no significant change is observed in COPD versus non-COPD			
XDH	0.001±0.000	0.001±0.000	0.001±0.000
HMOX1	0.133±0.020	0.211±0.040	0.333±0.083
GPX3	0.736±0.178	1.030±0.117	0.831±0.206
GSTT1	0.013±0.006	0.022±0.012	0.023±0.008
SOD1	0.058±0.008	0.047±0.010	0.045±0.007
SOD2	0.384±0.090	0.517±0.122	0.660±0.139
SOD3	0.221±0.018	0.234±0.013	0.187±0.013
MMP1	0.001±0.001	0.002±0.002	0.004±0.002
MMP2	0.649±0.113	0.867±0.161	0.816±0.091
MMP9	0.009±0.002	0.016±0.010	0.012±0.004
MMP12	0.002±0.001	0.002±0.001	0.002±0.001
ELA2	0.001±0.000	0.001±0.000	0.001±0.000
TIMP1	0.545±0.148	0.766±0.240	0.931±0.178
SERPINA1	0.529±0.062	0.685±0.084	0.804±0.085
SLPI	0.524±0.173	0.354±0.089	0.293±0.050
IL-13	0.001±0.001	0.001±0.001	0.003±0.001
TNF-α	0.019±0.003	0.034±0.005	0.034±0.006
TNF-α R	0.114±0.013	0.146±0.011	0.131±0.011
IFN-γ	0.001±0.001	0.002±0.001	0.001±0.000
IL-17	0.000±0.000	0.000±0.000	0.000±0.000
GM-CSF	0.000±0.000	0.000±0.000	0.000±0.000
IL-10	0.004±0.001	0.007±0.002	0.006±0.001
BLT1	0.004±0.000	0.004±0.000	0.003±0.000
BLT2	0.001±0.000	0.002±0.000	0.001±0.000
VEGF	0.412±0.053	0.491±0.039	0.397±0.047
MUC5AC	0.011±0.010	0.004±0.004	0.000±0.000
MUC5B	0.001±0.001	0.001±0.001	0.001±0.000
MUC8	0.001±0.000	0.001±0.000	0.001±0.000
CTSL	0.118±0.019	0.099±0.014	0.134±0.018
TGF-β	0.121±0.013	0.161±0.012*	0.159±0.012
EGF	0.000±0.000	0.000±0.000	0.001±0.000
IL-4	0.000±0.000	0.000±0.000	0.000±0.000
IL-5	0.000±0.000	0.000±0.000	0.001±0.000

The data are expressed as mean±SEM.
*$P<0.05$, **$P<0.01$ compared with the non-COPD never-smoked group (Mann–Whitney U-test).
†$P<0.05$, ††$P<0.01$ compared with the non-COPD smoker group (Mann–Whitney U-test).

and V_{25}/HT. These data suggest that impairments in the activities of these enzymes protective against oxidants and xenobiotics could contribute to the pathophysiological changes in COPD. Since reactive xenobiotics also inhibit antiproteinases and increase proteinase secretion from neutrophils, a combination of oxidative stress and an imbalance of proteinase/antiproteinases may aggravate the airway inflammation and tissue disruption in COPD. On the other hand, the expressions of mRNA for GSTM1 and TIMP2 were significantly increased in lung tissues from non-COPD smokers compared with those from non-COPD subjects who never smoked, but were not increased in lung tissues from COPD subjects. These changes may imply that GSTM1 and TIMP2 could be upregulated due to cigarette smoking as adaptive responses and that these protective mechanisms could be impaired in COPD lungs leading to the development of the disease.

Decreased mRNA expression levels of catalase, GSTP1 and mEPHX, but not GSTM1 and TIMP2, were associated with cigarette smoking history assessed as the amount of cigarettes smoked (pack-years). The relationships between cigarette smoke exposure and the expressions of these enzymes are uncertain. However, some studies have suggested that cigarette smoke exposure may decrease the activities or expressions of catalase and GSTP1. The catalase activity in erythrocytes was decreased in cigarette smokers compared with non-smokers, and was slowly increased in smokers after smoking cessation [21]. The activity of GST in lung tissue was reduced by cigarette smoking [22]. In another study, ongoing cigarette smoke exposure correlated with depressed levels of GSTP1 mRNA expression in buccal cells [23]. Also, cigarette smoke exposure to a previously tobacco-naïve subject induced a transient decline of GSTP1 mRNA expression in such cells [23]. As for mEPHX, however, its activity and mRNA expression have been reported so far to be not affected in rat tissues including lungs [24] or enhanced in the lungs of patients with lung cancer [22] by cigarette smoke exposure. In those studies, the changes of mEPHX activity might have resulted from the relatively short-term effect of cigarette smoke exposure. The negative correlation between the mEPHX mRNA expression in the peripheral lung tissues and cigarette smoking in the present study may suggest a long-term effect of cigarette smoke exposure on the mEPHX expression in the lungs. The mechanisms of the decreases in the mRNA expressions for catalase, GSTP1 and mEPHX in the peripheral lung tissue by the effects of cigarette smoke are unknown. Further investigation that addresses this issue may increase our understanding about the cigarette smoke-induced processes by which the protective mechanisms against toxic substrates in the lungs become impaired.

In the present study, we found elevated expression levels of mRNA for several inflammatory cytokines and chemokines, IL-1β, IL-8, Gro-α and MCP-1, in the lungs from COPD subjects. The cellular inflammatory response in COPD is dominated by neutrophils, macrophages and CD8-positive T lymphocytes in the lungs [3]. It has been reported that increased activities of inflammatory cytokines and chemokines related to these inflammatory cells are associated with COPD and cigarette smoke exposure

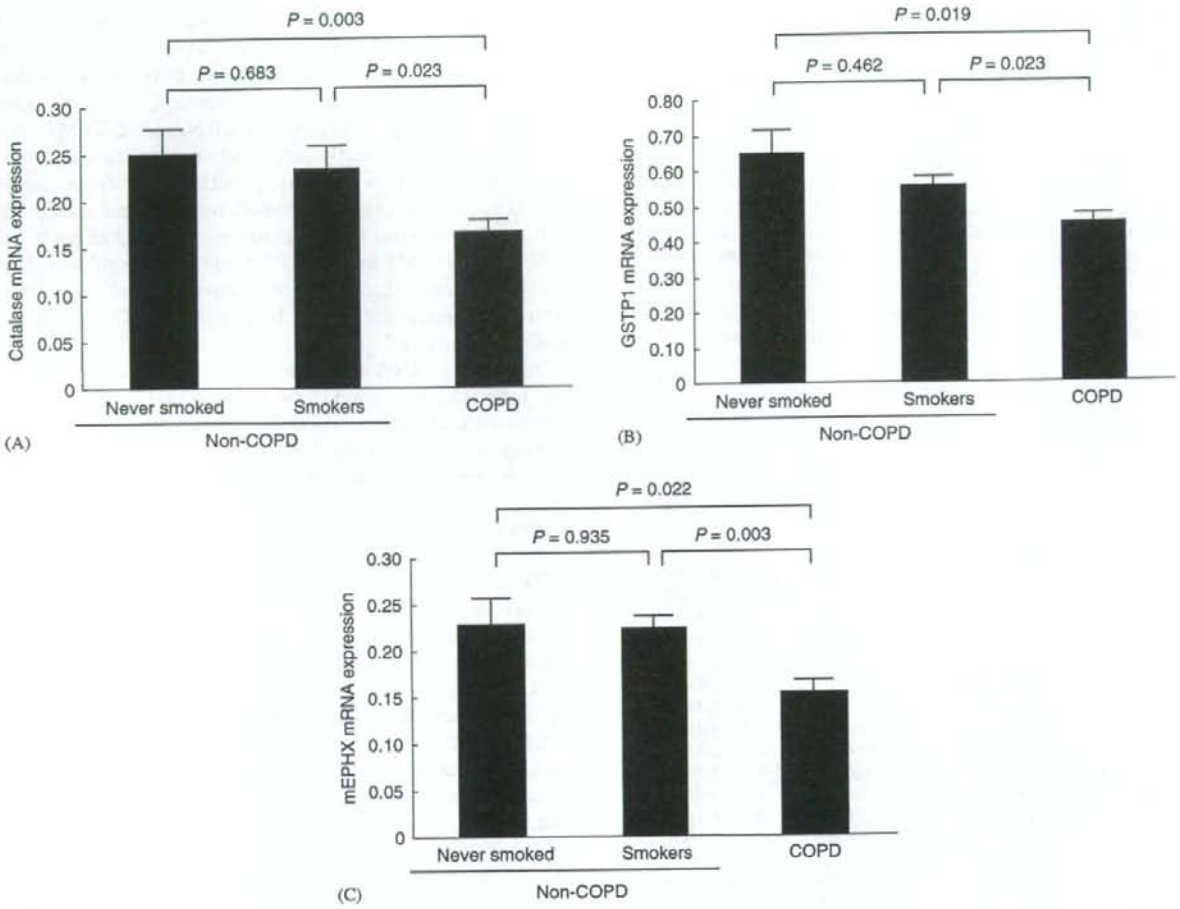

Fig. 1. mRNA expression for catalase (plate A), GSTP1 (plate B) and mEPHX (plate C) in lung tissues from those who never smoked, non-COPD smokers and COPD subjects. Data are shown as mean ± SEM. *P* values compared between two groups (Mann–Whitney *U*-test).

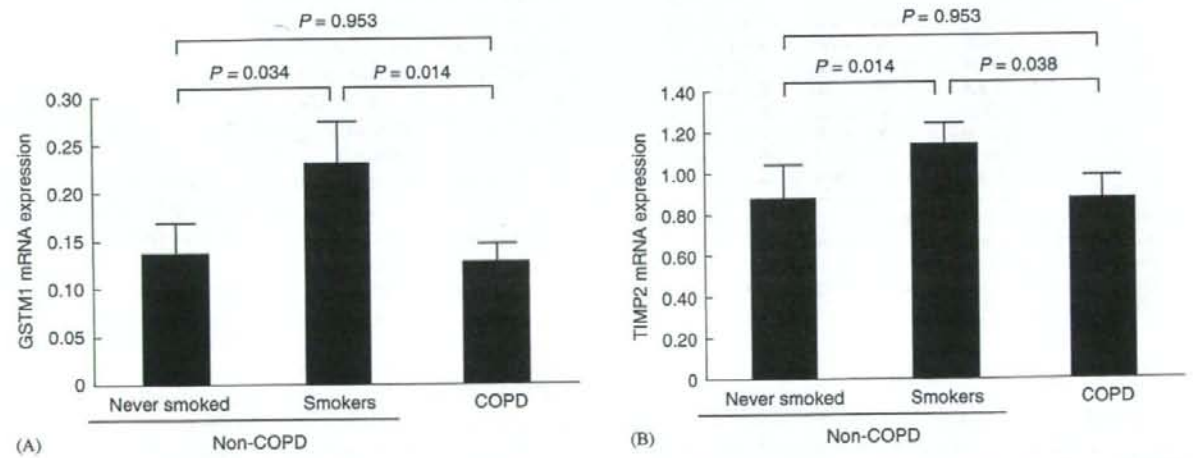

Fig. 2. mRNA expression for GSTM1 (plate A) and TIMP2 (plate B) in lung tissues from those who never smoked, non-COPD smokers and COPD subjects. Data are shown as mean ± SEM. *P* values compared between two groups (Mann–Whitney *U*-test).

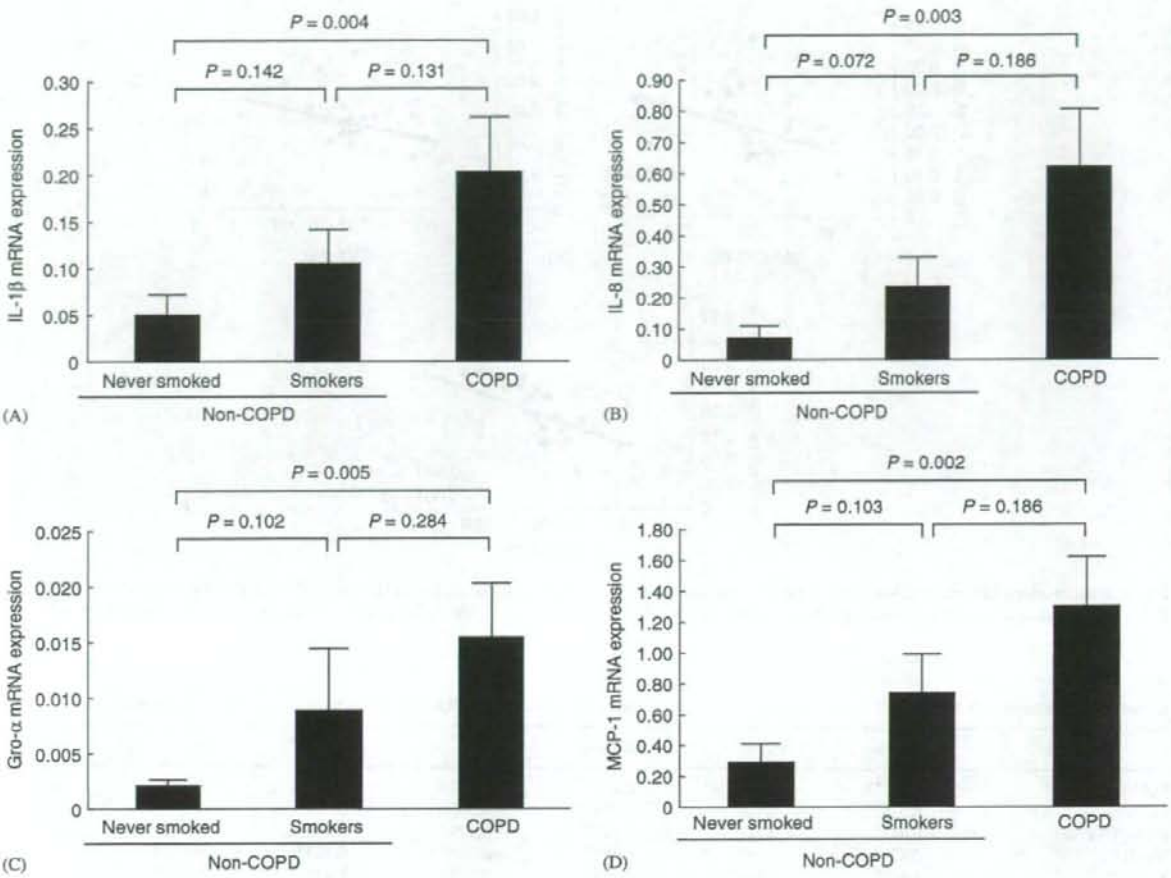

Fig. 3. mRNA expression for IL-1β (plate A), IL-8 (plate B), Gro-α (plate C) and MCP-1 (plate D) in lung tissues from those who never smoked, non-COPD smokers and COPD subjects. Data are shown as mean $\pm$SEM. P values compared between two groups (Mann–Whitney U-test).

[25–30]. Our results are consistent with these previous reports suggesting the involvement of these cytokines in the disease process of COPD.

Although the present study revealed significant changes in the mRNA expressions for several molecules in the whole tissue of the peripheral lungs of COPD subjects, the exact cellular sites of the altered mRNA expressions for individual genes are unknown. As for the chemokines, an analysis of lung tissue using an in situ hybridization technique has revealed higher mRNA and protein expression levels for IL-8 and MCP-1 in bronchiolar epithelium in COPD subjects compared to those in smokers without COPD [31]. Recently, Fuke et al. [32] have reported that the expressions of mRNA for IL-8 and MCP-1 are elevated in bronchiolar epithelial cells but not in alveolar macrophages by means of a laser-capture microdissection technique. These results suggest that the increased expression levels of IL-8 and MCP-1 in the peripheral lung tissue found in the present study may be due to the increased expression in epithelial cells.

The limitations of this study are lack of sex and age matching among the groups and lack of smoking history matching between the groups who had cigarette smoking histories. Cigarette smoking history assessed as pack-years positively or negatively correlated with the mRNA expressions for some enzymes and cytokines mRNA expressions. The data suggest that cigarette smoke exposure affects the disease process of COPD, but do not provide an answer for the question of why only some smokers develop COPD. Recently, two studies reported the altered expressions of a number of genes in epithelial cells from smokers [33] and in lung tissues from COPD subjects [12]. Including the present study, three studies have analyzed gene expressions in the lungs of COPD subjects or smokers, but the profiles of the subjects differed in terms of the disease severity and smoking status among the study groups. The collection of data acquired from a large population having differences in disease severity and smoking profiles is needed to further elucidate the mechanisms of disease onset and progression.

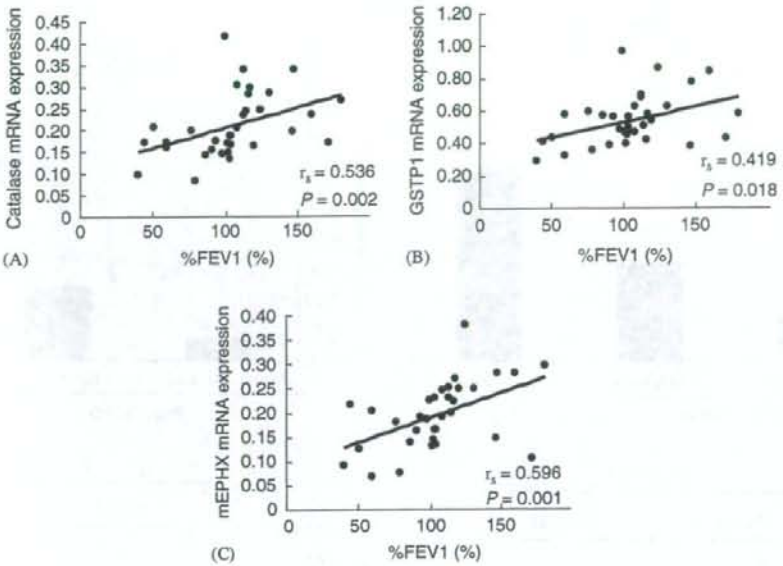

Fig. 4. Relationships between mRNA expressions for catalase (plate A), GSTP1 (plate B) and mEPHX (plate C) and the degree of airflow limitation. Relationship was analysed by Spearman's rank correlation test. A correlation coefficient of Spearman's rank correlation test (r_s) and P-value are indicated.

Table 4
Correlation coefficients of Spearman's rank correlation test (r_s) for relationships between mRNA expression and lung function, smoking history

	Catalase	GSTP1	mEPHX	IL-1β	IL-8	Gro-α	MCP-1
%FVC	0.457**	0.368*	0.495**	−0.297	−0.320	−0.349*	−0.262
%FEV_1	0.536**	0.419*	0.596**	−0.316	−0.329	−0.342	−0.280
FEV_1/FVC%	0.433*	0.411*	0.423*	−0.317	−0.398*	−0.392*	−0.330
V_{50}/HT	0.420*	0.368*	0.402*	−0.357*	−0.383*	−0.375*	−0.373*
V_{25}/HT	0.432*	0.354*	0.442*	−0.264	−0.328	−0.358*	−0.277
Smoking (pack-year)	−0.518**	−0.514**	−0.399*	0.567**	0.511**	0.573**	0.561**

r_s is a correlation coefficient of Spearman's rank correlation test.
*$P<0.05$.
**$P<0.01$ (Spearman's rank correlation test).

In summary, we found decreased mRNA expressions for catalase, GSTP1, GSTM1, mEPHX and TIMP2 and increased mRNA expressions for IL-1β, IL-8, Gro-α and MCP-1 in peripheral lung tissues from COPD subjects. Most of these changes were associated with the degree of airflow limitation and with cigarette smoking habit. The impairment of protective mechanisms against oxidants and xenobiotics as well as upregulation of CXC- and CC-chemokines seems to be associated with cigarette smoking and to be involved in the pathogenesis of COPD. Interventions targeting these molecules may provide a possible strategy for modifying the disease process of COPD.

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Anti-tuberculosis drug susceptibility testing of *Mycobacterium bovis* BCG Tokyo strain

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SUMMARY

SETTING: The *Mycobacterium bovis* bacille Calmette-Guérin (BCG) vaccine is the only vaccine against tuberculosis (TB), owing to its valuable protective effects and low virulence. However, it can occasionally cause systemic infection in immunocompromised hosts. Isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) are known to be effective anti-tuberculosis drugs and are used for the treatment of BCG infections. Unfortunately, there are few studies of the susceptibility of BCG vaccine strains to these drugs.

OBJECTIVE: To measure the minimum inhibitory concentrations (MICs) of BCG Tokyo vaccine products for anti-tuberculosis drugs and assess vaccine safety in terms of drug susceptibility.

DESIGN: We measured the MIC for one seed and five product lots of BCG Tokyo strain for INH, RMP, SM and EMB using Middlebrook 7H11 agar plates.

RESULTS: The MIC results for INH were 0.06 and 0.125 μg/ml for the product and seed lots, respectively. The MIC results for RMP, SM and EMB were 0.25–0.5, 0.25 and 2–4 μg/ml, respectively.

CONCLUSION: Our results indicate that the BCG Tokyo strain was susceptible to the major anti-tuberculosis drugs and treatable even in cases of severe adverse events, including systemic infection.

KEY WORDS: BCG; minimum inhibitory concentration; drug susceptibility

TUBERCULOSIS (TB) is an infectious disease of international importance that remains a major life-threatening disease worldwide. It is estimated that approximately one third of the world's population is infected with *Mycobacterium tuberculosis*. Every year, approximately 9 million people develop active disease and 1.7 million die of TB.[1]

Bacille Calmette-Guérin (BCG) vaccines are safe, attenuated live bacteria and have been shown to have valuable protective effects against TB. The BCG Tokyo strain is recognised as a low virulence strain among all BCGs,[2] and is widely used in several countries as a vaccine strain. If used properly, it protects against the development of TB and the dissemination of TB bacilli. Few severe complications have been reported.[3] However, systemic BCG infection may occur frequently when it is administered to immunocompromised hosts with congenital or acquired immunodeficiency such as human immunodeficiency virus (HIV) infection.[4,5] BCG is contraindicated in symptomatic HIV diseases. When general BCG infection occurs, patients are treated empirically using anti-tuberculosis drugs because there is limited information about the drug susceptibility of BCG strains. It is therefore very important to evaluate the drug susceptibility of BCG Tokyo strain to ensure the safety of the vaccine.

Isoniazid (INH), rifampicin (RMP), streptomycin (SM) and ethambutol (EMB) are the first-line anti-tuberculosis drugs most commonly used in standard TB treatment regimens. These drugs are currently available even in developing countries. The present study aimed at measuring the minimum inhibitory concentrations (MICs) of these drugs against the BCG Tokyo strain to estimate the effect of clinical treatment in case of infection by the BCG Tokyo strain.

MATERIALS AND METHODS

BCG Tokyo strain

Five lots of vaccine product (number 1003 as 'Lot A', 1960 as 'Lot B', 1036 as 'Lot C', 1061 as 'Lot D', 1998 as 'Lot E') and one seed lot were provided by the Japan BCG Laboratory (Tokyo, Japan) and used in this study. These vaccines were produced by Japan BCG Laboratory for vaccination from the seed lot in 2004. The experiment was carried out in a type II-B

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Minimum inhibitory concentrations

The MICs were measured modifying the proportion method described in M24-A of the Clinical and Laboratory Standards Institute (CLSI, former National Committee for Clinical Laboratory Standards) and in previous reports.[6,7] The following procedure was used: lyophilised BCG Tokyo products were suspended in 1 ml of distilled water and were cultured on Middlebrook 7H10 agar (DIFCO, Becton Dickinson Microbiology Systems, Cockeysville, MD, USA) supplemented with oleic acid, albumin, dextrose and catalase (OADC: BBL Prepared Culture Media, Becton Dickinson) at 37°C until sufficient growth was observed. After harvesting colonies from culture media, each lot strain of BCG Tokyo was dispersed by vortex mixing with glass beads (disperser tube: Nichibi, BCG Laboratory, Tokyo, Japan) and two drops of 10% Tween 80 (LC-MS, Santa Fe, CA, USA). After vortex mixing for 30 s, 1 ml of distilled water was added to each sample and they were vortexed again for 10 s. The supernatant of each bacterial suspension was transferred to 10 ml of Middlebrook 7H9 broth supplemented with albumin, dextrose and catalase (BBL Prepared Culture Media, Becton Dickinson), and the suspension density was adjusted to an optical density (OD) of 0.05 at 530 nm. These culture tubes were incubated at 37°C with daily mixing and OD checking. When the OD reached 0.2, they were used as the original bacterial suspension.

To prepare 10^{-2} dilutions, a 100 µl aliquot was transferred into 10 ml of distilled water. In a similar way, 100 µl of the 10^{-2} dilution was added to 10 ml of distilled water for 10^{-4} dilutions. One hundred microlitres of the 10^{-2} dilution were inoculated onto Middlebrook 7H11 agar plates with anti-tuberculosis drugs at the designated concentrations. Final INH concentrations were 0.03, 0.06, 0.125, 0.5, 1.0 and 2.0 µg/ml. RMP (0.03, 0.06, 0.125, 0.25, 0.5, 1.0, 2.0, 4.0 µg/ml), SM (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 µg/ml) and EMB concentrations (0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16, 32 µg/ml) were adjusted accordingly. The 10^{-2} and 10^{-4} suspensions were inoculated onto Middlebrook 7H11 medium containing no drugs for growth control and 1% proportion measurements. These plates were incubated at 37°C. When the 10^{-2} dilution control showed sufficient growth (>100 visible colonies), the MICs were measured as the lowest concentration of drug that inhibited more than 99% of the bacterial population compared with the number of colonies on drug-containing media and the 10^{-4} growth control. Each test was performed in triplicate.

RESULTS

The MICs of one seed and five product lots were measured in triplicate. The MICs of the anti-tuberculosis drugs varied slightly with the lots tested, but were identical among the triplicate tests. The MICs for all tested drugs are shown in the Table. The MICs of INH were 0.06 µg/ml and the seed lot MIC was 0.125 µg/ml. The MIC in test 3 of lot A was not determined due to contamination. For RMP, the MICs for lots A, B and C were 0.25 µg/ml; those for lots D and E were 0.5 µg/ml. It was considered that the MICs of RMP were between 0.25 and 0.5 µg/ml. For SM, the MICs were determined to be 0.25 µg/ml in all tests. For EMB, the MICs were 4 µg/ml for lots A, B and C, while the MICs for lots D and E were 2 µg/ml. The MIC of EMB was 2–4 µg/ml.

DISCUSSION

The BCG vaccine was developed by Calmette and Guérin in 1921. All BCG vaccines consist of live attenuated *Mycobacterium bovis* bacteria. BCG vaccination is commonly performed on neonates and infants once or twice in middle to high tuberculosis prevalence countries, and more than 100 million children have received BCG in recent years.[8] Its safety is therefore a priority issue.

BCG vaccination may sometimes cause complications as a pathogen. Local adverse effects of BCG vaccination have at times been observed and usually improve spontaneously, although severe complications in immunocompromised patients have been reported. McKenzie et al. reported systemic haematological dis-

Table MIC values of four first-line drugs for the BCG Tokyo strain

	MIC (µg/ml)											
	INH			RMP			SM			EMB		
Samples	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3	Test 1	Test 2	Test 3
Lot A	0.06	0.06	cont	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot B	0.06	0.06	0.06	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot C	0.06	0.06	0.06	0.25	0.25	0.25	0.25	0.25	0.25	4.0	4.0	4.0
Lot D	0.06	0.06	0.06	0.5	0.5	0.5	0.25	0.25	0.25	2.0	2.0	2.0
Lot E	0.06	0.06	0.06	0.5	0.5	0.5	0.25	0.25	0.25	2.0	2.0	2.0
Seed lot	0.125	0.125	0.125	ND	ND	ND	ND	ND	ND	ND	ND	ND

MIC = minimum inhibitory concentration; BCG = bacille Calmette-Guérin; INH = isoniazid; RMP = rifampicin; SM = streptomycin; EMB = ethambutol; cont = contaminated; ND = not done.

semination of BCG in a child with X-linked severe combined immunodeficiency.[9] Puthanakit et al. reported four cases of BCG infection in HIV-positive children receiving BCG vaccinations at birth; the strain was not indicated.[10]

BCG strains have also been utilised for immunotherapy in addition to TB prevention. BCG is injected into the urinary bladder for intravesical instillation therapy in the early stages of bladder carcinoma.[11,12] The BCG Tokyo strain is popular for such adjuvant therapy in Japan,[13] whereas the Connaught strain is popular in other parts of the world. In a recent study, Mugiya et al. described good, complete response rates of 84% with BCG Tokyo (40 mg administered every 6 weeks) against bladder carcinoma in situ.[14] However, adverse reactions can also occur after instillation therapy. Eichel et al. reported INH-resistant BCG cystitis successfully treated with RMP and EMB.[15]

There is at present no recommended treatment regimen for BCG infection. Anti-tuberculosis drugs are the most potent agents for treating BCG infection. Drug susceptibility testing (DST) of BCG strains has been reported using different methods. Durek et al. evaluated the Connaught BCG strain using a BACTEC 460TB system (Becton Dickinson).[16,17] DST was performed for 31 drugs, including INH, RMP, SM, EMB and rifabutin. The BCG Connaught strain was susceptible to all of the anti-tuberculosis drugs except pyrazinamide (PZA) (BCG has natural/intrinsic resistance to PZA) and some other drugs used for general bacterial infections. The BACTEC 460 TB system employs critical drug concentrations of 0.1, 1.0, 2.0 and 2.5 for INH, RMP, SM and EMB, respectively. Rousseau and Dupuis reported the DST for a seed lot of the BCG Montreal strain by using solid Dubos medium.[18] They showed that this strain was sensitive to INH (0.2 μg/ml), RMP (1.0 μg/ml), SM (2.0 μg/ml) and EMB (5.0 μg/ml). These reports are not, however, comparable because of the differences in testing methods. There is no standard method for the DST of BCG; however, they may be equivalent to each other in the concept of detecting 1% resistance in the strain population. The proportion method with Middlebrook 7H11/OADC media, which is commonly used for the DST of *M. tuberculosis*, was used for this study.

The MICs indicated in the present study were lower than the critical concentrations employed in the previous studies, except for EMB with MIC close to the critical concentration of BACTEC. In the previous studies, the MICs of EMB to *M. tuberculosis* vary between 0.5 μg/ml and 2.0 μg/ml,[19,20] in 7H12 BACTEC broth MIC varies between 0.95 and 3.8 μg/ml and on 7H10 agar between 1.9 and 7.5 μg/ml.[21] Heifets proposed possible guidelines for the interpretation of MIC to *M. tuberculosis* determined in Middlebrook 7H12 broth (radiometric), and MIC 4.0 μg/ml of EMB as moderately susceptible.[22] It is possible that the MIC of BCG Tokyo strain for EMB was higher than wild type *M. tuberculosis*. However, these reports show the tendency of lower MIC in liquid media than solid media. The plasma concentration (C_{max}) of EMB reaches 2.0–5.0 μg/ml[23] and EMB generally works in a time-dependent manner. For this reason it is suggested that EMB could be effective. Although BCG and *M. tuberculosis* are different species, these MICs and pharmacokinetic data would support the potentials of EMB for the treatment of BCG infection. It was therefore considered that, like the BCG Montreal and Connaught strains, the BCG Tokyo strain is susceptible to the four major anti-tuberculosis drugs.

Hesseling et al. reported that BCG in an HIV co-infected infant who received a BCG Danish 1331 strain vaccination developed INH and RMP resistance following treatment with INH and RMP.[24] The MICs of the original strain were 0.15 and <0.4 μg/ml for INH and RMP, respectively. However, they had risen above 0.3 and 32 μg/ml after treatment. These results suggest that the strain was already clinically resistant to INH (MIC 0.15 μg/ml for INH), and monotherapy with RMP against BCG resulted in RMP resistance. Su et al. reported two general disseminated cases of the BCG Tokyo vaccine strains.[5] One of them was treated using anti-tuberculosis drugs (INH, RMP, SM and EMB) based on the susceptible DST results, and the patient recovered. Another case died following one month's treatment with INH, RMP and EMB. However, no DST data were shown in the mortality case and the infant seemed to have died from severe combined immunodeficiency. The MICs of the BCG Tokyo strain indicated in this study were considered less than or equivalent to those of the previous cases, so it was estimated that BCG Tokyo could be treated successfully even in severe adverse events such as systemic dissemination.

The reason why BCG strains have different phenotypic characteristics with respect to drug susceptibility is not clear. BCG has lost several regions of difference (RD) compared to *M. bovis* as the ancestral strain. In particular, the RD1 deletion made a significant contribution to the attenuation of BCG.[24-26] RD1 encodes a 6 kDa early secreted antigenic target protein (ESAT-6)[27] and a 10 kDa culture filtrate protein (CFP-10)[28] associated with virulence in *M. tuberculosis* complex. The BCG vaccine therefore has attenuated virulence compared to wild *M. bovis* strains. The loss of virulence apparently occurred through repeated passages.

The BCG strains were originally donated by the Pasteur Institute (Paris, France), and have been subcultured by several tuberculosis institutes around the world (Russia, Brazil, Sweden, Denmark, Japan, etc.) since 1924. The donated BCG strains differ from the original BCG strains due to differences in passage cultivation, culture medium and storage conditions. In 1972, Hesselberg found that a Swedish/Norwegian BCG strain became resistant to INH during the period 1953–1964, which was the reason why the serial sub-

culture system was discontinued and a seed lot system was adopted.[29] However, in 2003, low-grade INH-resistant (MIC >0.5 μg/ml) Danish 1331 strains were reported again to the World Health Organization (WHO). The WHO therefore recognises the necessity of a new quality assurance method for BCG vaccines.[30,31]

The BCG Tokyo strain was obtained from Calmette in the Pasteur Institute in 1924. Passage cultivation of BCG Tokyo strain has been performed strictly according to Calmette's original instructions, while some of the other BCG strain passages were tailored to each institute's needs. The BCG Tokyo 172 strain, which has undergone 172 passages since the Second World War II, has been used as the seed lot for lyophilised BCG Tokyo vaccines. In this study, the BCG Tokyo strain proved to be susceptible to the major anti-tuberculosis drugs; however, the results of this study do not apply to all BCG substrains. It will be necessary to ensure the safety of BCG vaccine by checking susceptibility to other antimicrobial agents.

Acknowledgements

The authors thank Dr G Heinner and Mr B Bell for reading and making suggestions regarding the manuscript.

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RÉSUMÉ

CONTEXTE : Le bacille de Calmette et Guérin (BCG) à base de *Mycobacterium bovis* est un vaccin unique contre la tuberculose (TB) en raison de ses effets protecteurs valables et de sa faible virulence. Toutefois, il peut causer occasionnellement une infection systémique chez les sujets en état d'immunodépression. L'isoniazide (INH), la rifampicine (RMP), la streptomycine (SM) et l'éthambutol (EMB) sont des médicaments antituberculeux reconnus comme efficaces et peuvent être utilisés dans le traitement des infections par le BCG. Il n'y a malheureusement que peu d'études concernant la sensibilité des souches de vaccin BCG à l'égard de ces médicaments.

OBJECTIF : Mesurer les concentrations minimales inhibitrices (CMI) du vaccin BCG Tokyo pour les médicaments antituberculeux et évaluer la sécurité du vaccin en ce qui concerne la sensibilité aux médicaments.

SCHÉMA : Nous avons mesuré les CMI sur plaques d'agar Middlebrook 7H11 pour la souche-mère et pour cinq lots de vaccin de la souche BCG Tokyo à la fois pour l'INH, la RMP, la SM et l'EMB.

RÉSULTATS : Les résultats des CMI pour l'INH ont été respectivement de 0,06 et de 0,125 μg/ml pour la souche-mère et pour les lots de vaccin. Les résultats des CMI pour la RMP, la SM et l'EMB ont été respectivement de 0,25–0,5, 0,25 et 2–4 μg/ml.

CONCLUSION : Nos résultats indiquent que la souche BCG Tokyo est sensible à l'égard des médicaments antituberculeux majeurs qui sont efficaces même en cas d'effets indésirables graves, y compris des infections systémiques.

RESUMEN

MARCO DE REFERENCIA : *Mycobacterium bovis*, el bacilo de Calmette y Guérin (BCG), es la única vacuna contra la tuberculosis (TB), debido a su valioso efecto de protección y a su baja virulencia. Sin embargo, esta vacuna puede causar en ocasiones infecciones generalizadas en individuos inmunodeprimidos. Isoniazida (INH), rifampicina (RMP), estreptomicina (SM) y etambutol (EMB) son medicamentos antituberculosos eficaces y se emplean en el tratamiento de las infecciones por BCG. Desafortunadamente, existen pocos estudios sobre la sensibilidad de la cepa de la vacuna antituberculosa a estos medicamentos.

OBJETIVO : Medir las concentraciones mínimas inhibitorias (CMI) de los medicamentos antituberculosos contra el BCG de Tokio contenido en las vacunas y evaluar su seguridad toxicológica en la concentración de sensibilidad al medicamento.

MÉTODOS : Se midieron las concentraciones inhibitorias mínimas de INH, RMP, SM y EMB para un lote de siembra y cinco lotes de vacuna de la cepa BCG de Tokio usando cultivos en placas de agar con Middlebrook 7H11.

RESULTADOS : La CMI para INH fue 0,06 con los lotes de siembra y 0,125 μg/ml con los lotes de vacuna. La CMI para los lotes de vacuna con RMP fue de 0,25 a 0,5 ; con SM fue 0,25 ; y con EMB fue de 2 a 4 μg/ml.

CONCLUSIÓN : Estos resultados indican que la cepa BCG de Tokio es sensible a los principales medicamentos antituberculosos y que es posible tratar los casos de reacciones adversas graves, incluida la infección generalizada.

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ウイルス感染とバイオディフェンス

－注目される補中益気湯の可能性－

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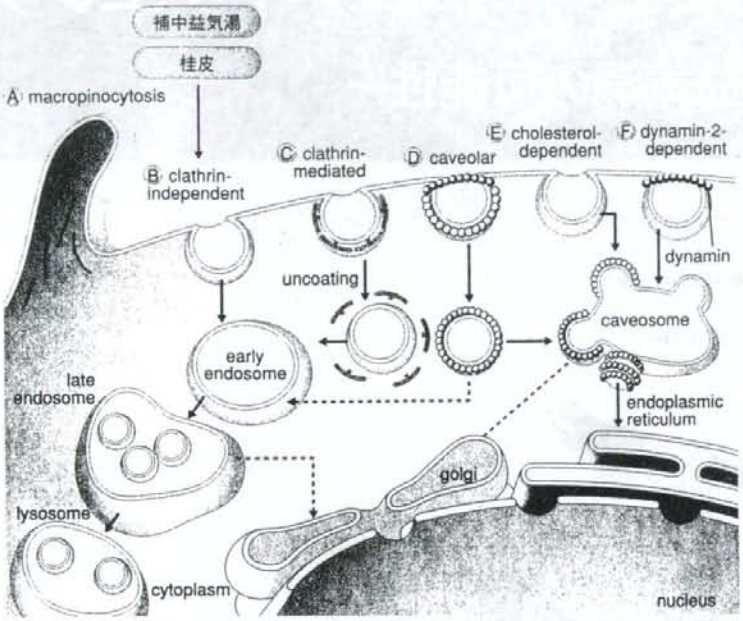

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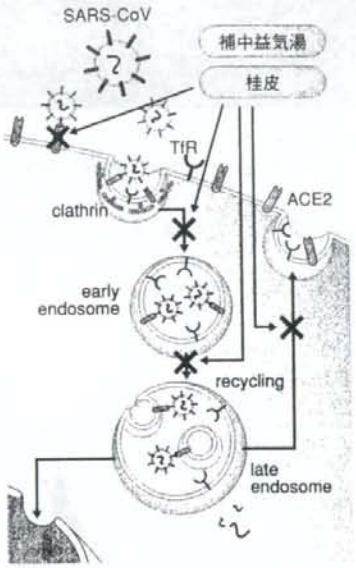

図2　生薬によるエンドサイトーシスの調節の可能性

ルスで最も多いとされるコロナウイルスは主にDの経路で細胞内に侵入すると考えられている。

こうしたウイルスエントリー、すなわちウイルスが細胞内に侵入する経路の解明は、エントリーインヒビターという新しいアプローチの抗ウイルス薬開発には欠かせない。例えば、HIV感染はウイルス表面のスパイク蛋白gp120と標的細胞表面の受容体CD4が結合し、gp120-CD4複合体がさらに細胞表面のケモカイン受容体と結合することでウイルス膜と標的細胞膜との融合が起こる。この融合を、ケモカイン受容体をブロックすることで阻害しようという設計で、いくつかの薬剤が開発段階にあり、そのうち一つはすでに上梓に至っている。ウイルスエントリー経路の詳細な分析は、ウイルスインヒビターのターゲットをより細かく絞り込み、特異的な阻害作用を得ることになる。

服部氏は、これまで一貫してウイルスエントリー阻害による抗ウイルス作用に焦点を当てて、さまざまな成分について精力的な研究を進めてきた。検討した成分には、感染防御作用が指摘される漢方薬の構成生薬や、HIV感染患者に対する臨床効果が報告されているアフリカ原産の植物エキスなども含まれている。また、技術面でもウイルスエントリーを高感度に測定する実験系も確立している。これはルシフェラーゼ遺伝子を組み込んだレポーター遺伝子にウイルスの外膜糖蛋白遺伝子を導入して外膜のスパイク蛋白を発現する疑似ウイルスを使うもので、この実験系ではルシフェラーゼ活性を測定することでウイルスエントリーを詳細に評価することができる。

こうした研究アプローチによって服部氏はこれまでに、漢方薬の補中益気湯や生薬の桂皮などがウイルス感染の抑制効果を示すこと、なかでも桂皮および丁子エキスがHIV/SARS疑似ウイ

ウイルス感染とバイオディフェンス

－注目される補中益気湯の可能性－

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HIV、SARS、鳥インフルエンザ。医学が進歩してもなお、人類はウイルス感染の脅威にさらされ続けている。SARSについては一応の落ち着きをみたが、HIV、そしてインフルエンザのパンデミックに対して、果たして人類は勝利することができるのだろうか。

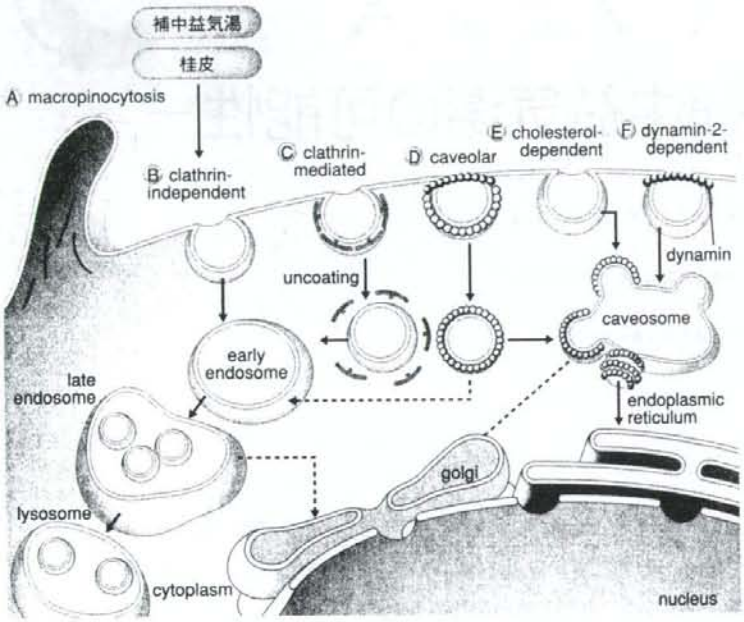
微生物との緊張感ある闘いのなかで、効果的なバイオディフェンス製剤を求めてさまざまなアプローチが試みられている。既存の薬剤や植物成分の見直しもその一つである。そのなかで、漢方薬も有効性が期待されるものとして研究対象になっている。

ここでは、かねてより免疫賦活作用が報告されている補中益気湯（TJ-41）を中心に、バイオディフェンスの視点で漢方薬の位置付けを考える。

今回、東北大学大学院感染症・呼吸器病態学の服部俊夫氏、千葉大学大学院加齢呼吸器病態制御学の巽浩一郎氏にお話を伺い、岡山大学医学部・歯学部附属病院消化管外科の岩垣博巳氏に資料協力をいただいた。

「Open Sesame」－開けゴマ－ ウイルスエントリーの呪文が破られるとき

今年（2006年）、Cell、JAMAに相次いでウイルス学の注目すべき論文が掲載された。Cell掲載の「Virus Entry: Open Sesame」というタイトルを冠した論文[1]は、ウイルス感染経路について最新の知見をレビューしたもので、標的細胞表面にあるウイルス受容体とウイルス膜との結合、ウイルスの刺激による細胞内シグナリング、エンドサイトーシスによるウイルス侵入経路などが明快に解説されている（図1）。ちなみに、インフルエンザウイルスはこの図が示すB、風邪の原因ウイ



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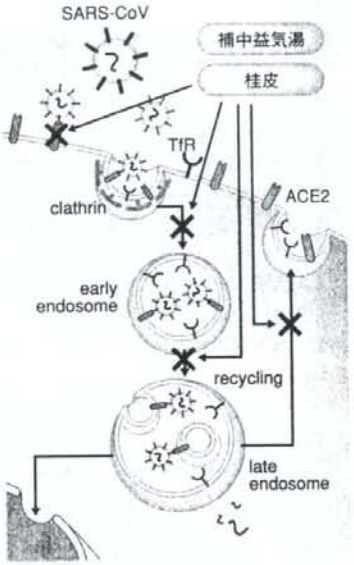

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ルス感染に対し濃度依存性に抑制効果を示すことを明らかにしている[2]。

特に桂皮エキスの抑制効果は大きく、多種類のウイルス感染に対し阻害作用を示すことから、桂皮エキスは「ウイルス感染の共通部分に作用している可能性」が考えられる。そこで服部氏は、桂皮成分がエンドサイトーシスの諸相に抑制的に作用しているとの仮説を立て、より詳細な研究を進めている（図2）。

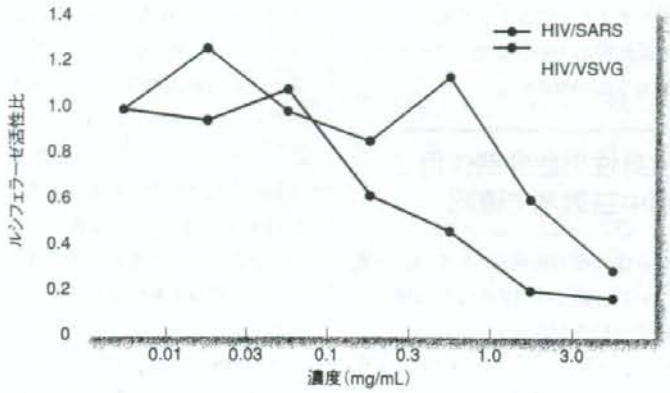

図3　HIV/SARS疑似ウイルスに対する補中益気湯の抑制効果

補中益気湯はHIV/SARSおよびHIV/VSVGを用量依存的に抑制した。（庄 敏・服部俊夫）文献2より引用

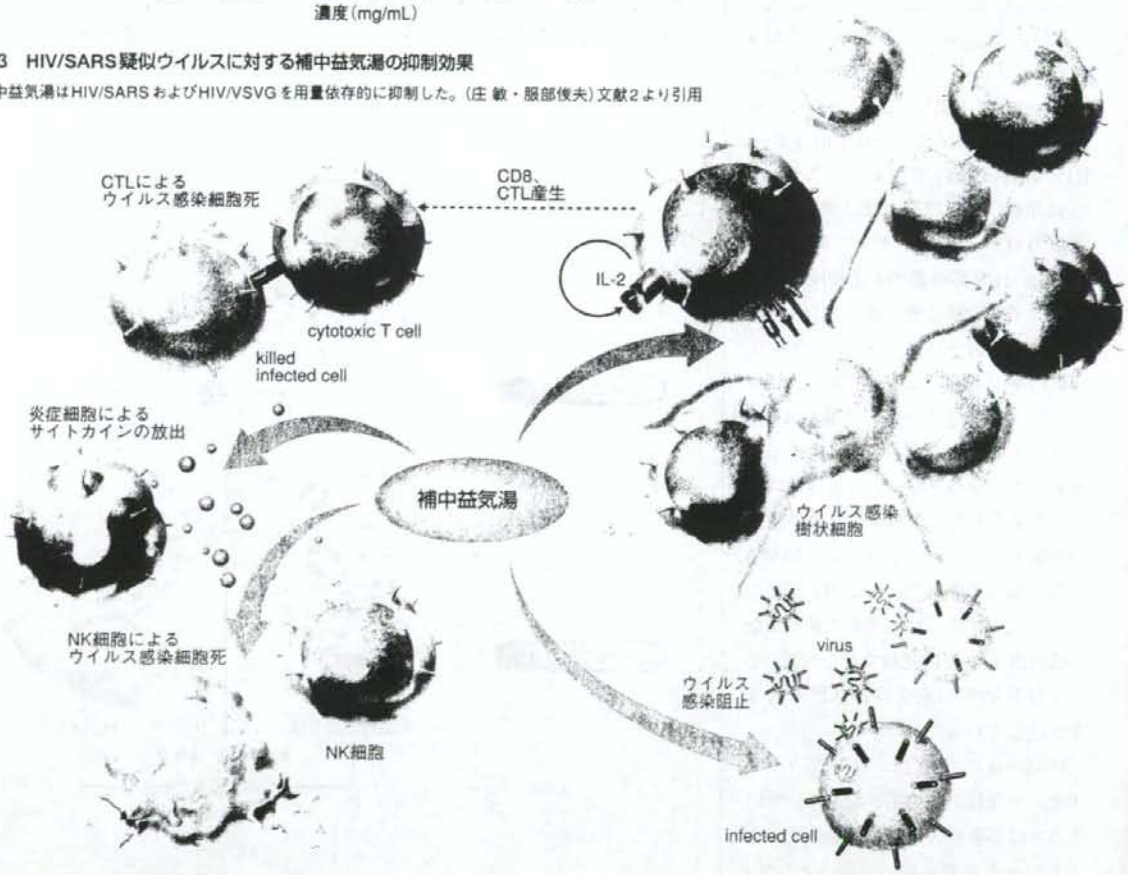

イラスト　補中益気湯の作用機序（監修：服部俊夫）