4例すべてが冠動脈に動脈瘤ができており，血栓閉塞で亡くなっていました．研究班は，これは容易な病気ではないと身にしみて感じたわけです．

実は，それよりも以前，1961年と1965年に日赤でも2例，剖検例がありました．

佐地　その症例も動脈瘤と血栓性閉塞ですか．

川崎　そうです．病理の田中昇先生は，その2例ともPN(periarteritis nodosa)と診断しました．IPN(infantile periarteritis nodosa：乳児結節性動脈周囲炎)とは診断していません．当時はアメリカでIPNという概念ができつつあるときで，病理の先生もわれわれ臨床も，IPNという概念を持っていませんでした．Kussmaul-Maierタイプの成人型，古典的なPNという概念しかなかったのです．田中先生はPNでは病理学的におかしいとは思っていたけれども，IPNという概念がなかったので，PNと病理診断したわけです．

そこで，田中先生は治癒した50例と剖検例2例を同じカテゴリーに入れると主張されたけれども，私は予後良好の50例と死亡例の2例を一緒にすることはできずに，原著では50例だけを扱ったのです．田中先生は，一緒にしなかったことを怒っておられましたが，私には当時予後良好な50例をPNとして扱うことはできませんでした．

その後，1972年に都立墨東病院で5歳の男の子が，治癒した半年後に心筋梗塞を起こして救急車で日赤に運ばれてきました．そこで東京女子医科大学の草川三治先生のところで大動脈造影を行ったところ，左側に大きな瘤が証明されました．元気になった患者で冠動脈瘤が発見されたのは，それが最初だと思います．それ以後，同様の報告が次々に現れました．

1973年の厚生省研究班会議で，久留米大学の加藤裕久先生が冠動脈瘤症例の一部において動脈瘤が退縮するという最初の報告をしました(日本醫事新報 1974；2605：37)．そこで病理解剖と臨床が結びつき，われわれの中にIPNの概念が出てきたのです．その後，さらに心エコー検査によって日常診療で冠動脈瘤の診断が可能になりました．川崎病が血管炎症候群の一つとして認知されるようになったのです．

佐地　1970年に始まった厚生省の研究班は現在も続いているわけですが，今年は，厚生労働省の研究費が不採択になりました．非常に残念です．

川崎　来年も調査は行いますが，それ以後の継続が困難になってしまいます．世界的にも貴重なデータですので，ぜひ継続していきたいのですが．

佐地　加倉井氏はいまどちらにいらっしゃいますか．

川崎　加倉井氏は，しばらくして公衆衛生局長のとき胃癌で亡くなられました．

佐地　では，もう一度意欲を持って厚生労働省に行ってスピーチをする人を見つけないとだめですね．

（次号に続く）

Murine Model of Kawasaki Disease Induced by Mannoprotein-β-Glucan Complex, CAWS, Obtained from *Candida albicans*

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SUMMARY: Intraperitoneal administration of CAWS (water-soluble extracellular polysaccharide fraction obtained from the culture supernatant of *Candida albicans*) to mice induces coronary arteritis similar to Kawasaki disease. We analyzed differences in the production of cytokines involved in the occurrence of coronary arteritis among mouse strains, C3H/HeN, C57BL/6, DBA/2 and CBA/J. The incidence of arteritis was 100% in C57BL/6, C3H/HeN and DBA/2 mice, but only 10% in CBA/J mice. The coronary arteritis observed in DBA/2 mice was the most serious, with several mice expiring during the observation period. The CAWS-sensitive strains revealed increased levels of IL-6 and IFN-γ during the course of a specific response to CAWS by spleen cells. In contrast, IL-10 levels were observed to increase markedly in CAWS-resistant CBA/J mice, but not the CAWS-sensitive strains. However, TNF-α levels were more elevated only in DBA/2 mice. The difference in disease development and cytokine production strongly suggests that the genetic background of the immune response to CAWS contributes to the occurrence of coronary arteritis.

Candida albicans is a clinically important fungus and is known to cause disseminated candidiasis and candidemia in immunocompromised hosts. Analyses have long been conducted on the coagulation reaction of limulus blood cell components with microbial cell components, and the presence of the factor C initiated cascade that reacts with bacterial endotoxins and the factor G initiated cascade that reacts with β-1,3-glucans is known. The factor G cascade is being used for the diagnosis of mycotic contamination and mycotic infections. As was previously mentioned, although patients with deep mycoses have been clearly demonstrated to release β-glucans into the blood, these are present in extremely small amounts, and the overall structure of the factor G activating substance present in the blood is unknown. Although the factor G activating substance has the potential to exhibit various biological activities, this is also unknown. The metabolism of the active components released into the blood from a local site of infection is also unknown. In order to clarify these matters, we conducted research using *C. albicans*.

Biochemical properties of CAWS (1,2): We first cultured *C. albicans* in a completely synthetic medium in order to obtain water-soluble limulus factor G activating substance that is released from the cells, and obtained a water-soluble polysaccharide fraction released into the culture supernatant (*C. albicans* water-soluble fraction: CAWS), which is thought to be similar to the β-1,3-D-glucan actually present in patient blood. CAWS demonstrated a positive reaction to the G-test as expected. It reacted to the G-test as low as 100 ng/mL. The yield of CAWS was approximately 80 mg/L: the polysaccharide content was 70%, the protein content was 10%, the primary component sugars were mannose and glucose (M/G ratio = 6.3 ± 1.3 from *C. albicans* IFO 1385 derived CAWS), and CAWS also reacted with factor serum to the cell wall mannan. According to the results of NMR analysis, CAWS was surmised to have a mannoprotein and a β-1,6-glucan portion, which are the main components of *C. albicans* cell wall. Moreover, fractionation using concanavalin A agarose resulted in separation into column-bound and pass-through fractions, with the column-bound fraction also exhibiting reactivity to the G-test. On the basis of these findings, CAWS was strongly suggested to be a compound that contains mannoprotein, β-1,6-glucan and β-1,3-glucan.

Arteritis induced by CAWS and predicted mechanism (3-6): Kawasaki disease (KD) is a pediatric disease accompanied by acute fever, and its underlying cause remains unknown to date. This disease results in occasionally fatal sequelae such as the formation of aneurysms in the coronary arteries. Although the current standard treatment regimen consists of administration of large doses of globulin preparations, this approach is not always satisfactory. Murata et al. conducted an analysis on children with KD and found that *C. albicans* extract (CADS) isolated from the stool specimens of the patients induced coronary arteritis in mice that resembled KD. During the course of joint research, we found that administration of CAWS according to the standard protocol induced a similar coronary arteritis in mice. Moreover, when additional experiments were conducted on different strains, the resulting coronary arteritis was more pronounced in C3H, DBA/2 and C57Bl mice, and less pronounced in CBA/j mice. Although these differences among strains were similar to the differences among strains observed with CADS by Murata et al., the sensitivity of the DBA/2 mice was different. Moreover, more than half of the DBA/2 mice died during the observation period, suggesting the possibility of a strong manifestation of heart disease (Fig. 1). On the basis of these findings, CAWS-induced coronary arteritis is considered to be a good model for the pathology of arteritis as well as the development of treatment methods.

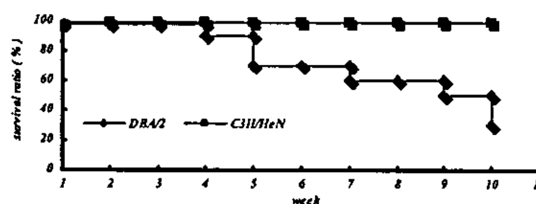

Fig. 1. Survival ratio of CAWS administered mice.
CAWS was administered to DBA/2 and C3H/HeN strains of mice and survival was monitored for 10 weeks.

We therefore attempted to determine the immunological mechanism underlying the arteritis. In strains in which arteritis occurred prominently, splenomegaly occurred frequently and the numbers of neutrophils and macrophages increased. In addition, when spleen cells were prepared immediately after administration of CAWS and cultured in vitro, myeloperoxidase was observed to be released into the supernatant even in the absence of stimulation. In addition, MPO-ANCA levels in the blood were also elevated. On the basis of these findings, neutrophils present in the spleen were suggested to be maintained in an activated state. In addition, spleen cells were re-stimulated with CAWS and cytokine production was compared. The production of cytokines such as IL-6 and IFN-γ was higher in strains in which arteritis was induced. On the other hand, IL-10 production was higher in strain CBA/j that exhibited a low level of induction of arteritis.

Reactivity of DBA/2 mice to fungal glycans (7,8): As mentioned above, DBA/2 is the most sensitive strain to CAWS-induced arteritis, not only from the view point of histology, but also survival. I feel it very close to sudden death of KD-patients carrying aneurysms in the coronary arteries. DBA/2 is a widely used inbred strain that is valuable in a wide number of research areas including cardiovascular

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biology, neurobiology, and sensorineural research, and is known to show a low susceptibility to developing atherosclerotic aortic lesions following 14 weeks on an atherogenic diet. It is of note that the mechanism of CAWS-induced arteritis might not be related to those of atherosclerosis. Thus we planned to analyze the reactivity of DBA/2 mice to fungal glycans and found that DBA/2 contained anti-β-glucan antibody in sera without any active immunization with fungi. In addition, spleen cells of DBA/2 mice strongly reacted with fungal β-glucans to release various cytokines such as IFN-γ, TNF-α, GM-CSF, and IL-12, and the key cytokine was GM-CSF. Characterization of DBA/2 mice for fungal glycan reactivity is still going to concrete the unique property.

CONCLUSIONS

We have discussed the structure and activity of CAWS. Although this research initially started out by focusing on its significance as a means for diagnosing deep mycoses in animal models, since CAWS exhibits various activities in human and mouse, it is clearly a component that provides several extremely interesting topics for future research, such as shock model, endogenous septicemia model and arteritis model. These models are valuable for use as animal models for the treatment of refractory diseases.

CAWS is a compound consisting of mannoprotein, β-1,6-glucan, and β-1,3-glucan portions. In the body, it is metabolized after expressing its activity by means of multiple receptors, such as mannose receptor, mannan-binding protein, complement components, complement receptors, and dectin-1. We previously reported that there are no enzymes in the body that selectively metabolize β-glucans, and that β-glucans are microbial cell components that tend to accumulate in the body. Thus, their basic kinetics in the body differs from that of cellular components having a decomposition system, such as chitin and peptidoglycans. CAWS was found to be mainly deposited in liver. Further analysis must be conducted to determine what types of receptors are used and how CAWS is eliminated from the body.

Study of CAWS is still on the first stage. It is hoped that CAWS will be able to contribute to the elucidation of KD and related diseases, and to develop new therapeutic strategies.

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DNA array analysis of altered gene expression in human leukocytes stimulated with soluble and particulate forms of *Candida* cell wall β-glucan

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Abstract

We previously reported that 1,3-β-D-glucan derived from *Candida albicans*, a pathogenic fungus was obtained by oxidation of the cell wall with sodium hypochlorite (NaClO). It could be solubilized by treatment with dimethylsulfoxide (DMSO). We found that OX-CA and CSBG showed significantly different levels of activity toward leukocytes. Here, we have used cDNA microarrays to analyze the mRNA expression of 1176 genes in PBMCs stimulated with *Candida* cell wall glucan and considered the difference in the activation mechanism of OX-CA and CSBG. Total mRNA showed a significant change for 147 out of 1176 arrayed genes on stimulation with OX-CA and CSBG for 4 h. Among those genes, 62 were common, 26 were OX-CA-specific and 59 were CSBG-specific. Many of these up-regulated genes encode effectors with well-characterized proinflammatory properties. The expression of genes related with signal transduction differed in the particulate and soluble glucans derived from *C. albicans* having exactly the same primary structure. This fact suggested that each glucan induced specific biological activity through a different activation mechanism. This study using cDNA microarrays to analyze a broad spectrum of mRNA expression provides information on the biological activity of *Candida* cell wall glucan as a potential pathogenic factor.

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Keywords: β-Glucan; Leukocyte; Solubility; Microarray

1. Introduction

Due to the intensive therapy being administered to those with cancer, organ transplants, and HIV infection, the number of immunocompromised hosts is increasing, and so too cases of invasive deep mycoses. *Candida* spp. are responsible for most of these mycoses, as well as *Aspergillus* spp. To date, many researchers have studied the pathogenetic factors in various pathogenic fungi [1–4] but comparisons to bacterial endotoxin or exotoxin have not been made and much remains to be clarified.

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β-Glucan is the major component of cell wall, accounting for approximately 50% of the cell wall mass. Also, plasma (1 → 3)-β-D-glucan was detected in invasive fungal infection or fungal febrile episodes, and the limulus G-test with factor G, a horseshoe crab coagulation enzyme, was used in the laboratory diagnosis of such cases [5]. Therefore, it is possible that (1 → 3)-β-D-glucan plays a major role in fungal infection.

(1 → 3)-β-D-Glucan is widely distributed in microbes especially fungi. We and others strongly suggested that (1 → 3)-β-D-glucan has a variety of biological activities, dependent on its physical state, such as solubility in water, molecular mass, degree of branching and conformation [6,7]. The biological activity of soluble and particulate forms of β-D-glucan differs considerably [8–13]. Also in our previous study, the particulate forms showed stronger activity than soluble forms in terms of the synthesis of TNF-α and H_2O_2 by macrophages, activation of an alternative pathway of complement, enhanced vascular permeability, and arachidonic acid production, but conversely, showed less INF-γ production in serum and antitumor activity.

We have demonstrated the fate of these β-glucans in vivo, and found that intravenously administered *Candida* spp. was distributed in the liver and spleen [14]. It was postulated that the deposited insoluble cell well β-glucan is gradually solubilized through oxidative degradation by phagocytes, which utilize nonspecific oxidation reactions involving O_2^-, H_2O_2 and hypochlorous acid. The other cell well component, mannan, is water-soluble and thus would be metabolized faster. Considering these oxidation steps, treatment with sodium hypochlorite (NaClO) yielded particles containing only β-glucan, NaClO-oxidized *Candida* cells (OX-CA). Based on this concept, we have recently demonstrated that the cell wall β-glucan of *C. albicans* could be solubilized by NaClO, followed by dimethylsulfoxide (DMSO) (NaClO–DMSO method) [15]. Under optimum conditions, the treatment with DMSO yielded a water-soluble fraction, *Candida* solubilized β-glucan (CSBG), almost quantitatively.

The 1,6-β-glucan segments influenced physical properties such as the stability of the conformation and biological activities, in the primary structure of the soluble and particulate forms, to a greatly different point. Therefore, we were unable to compare the biological activity in terms of solubility alone. But, using the NaClO–DMSO method, we demonstrated that the solubility of the glucans was related to the biological activity.

The cDNA expression array technique can differentially detect the mRNA levels of a large number of genes in cells in response to a given stimulus at one

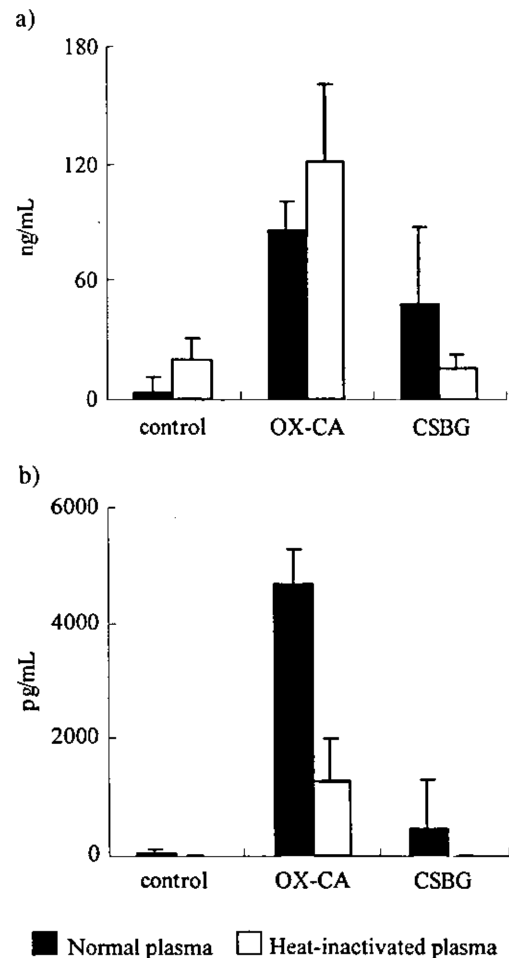

Fig. 1. Activation of leukocytes by OX-CA and CSBG. (a) IL-8 production, (b) TNF-α production by human PBMCs. PBMCs obtained from the peripheral blood of healthy donors were adjusted to a concentration of 2×10^6 cells/ml in RPMI1640 medium containing 10% normal or heat-inactivated autologous plasma and cultured with OX-CA particles or CSBG (0.5%) (100 μg/ml) for 12 h in a 5% CO_2 incubator. Subsequently, the culture supernatants were collected, and IL-8 and TNF-α was measured as described in Materials.

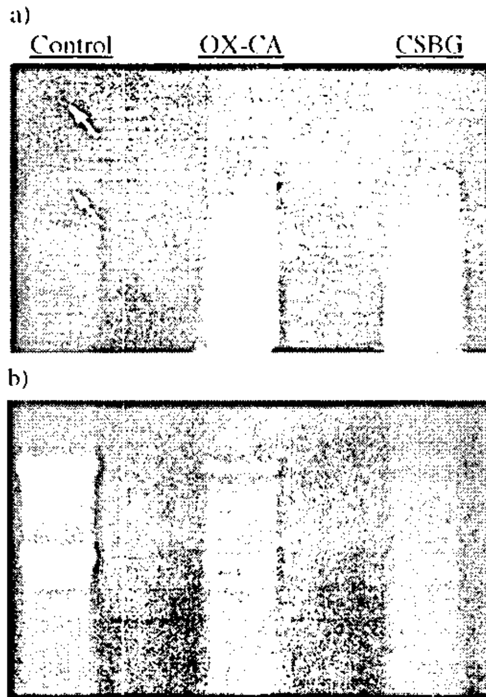

Fig. 2. The quality of the isolated RNA stimulated with *Candida* cell wall glucan. The quality of the isolated RNA was assessed by gel electrophoresis and ethidium bromide staining.

time, thus providing a useful tool to identify broad spectral changes in gene expression [16,17]. Here, we have used cDNA microarrays to analyze the mRNA expression of 1176 genes in PBMCs stimulated with *Candida* cell wall glucan and considered the difference in the activation mechanism of OX-CA and CSBG.

2. Experimental procedures

2.1. Materials

Candida albicans IFO 1385 purchased from the Institute for Fermentation (Osaka, Japan) was maintained on Sabouraud agar (Difco, USA) at 25 °C and transferred once every 3 months. Sodium hypochlorite solution and sodium hydroxide were purchased from Wako (Japan), distilled water (DIW) was from Otsuka (Tokyo, Japan).

2.2. Media

A C-limiting medium originally described by Van Hal et al. [17] was used to grow all strains of yeast unless otherwise stated. The medium contained (per l):

Table 1
Identification of OX-CA-changed genes in human PBMCs by cDNA array[a]

Gene/protein name	GenBank accession number	Relative mRNA expression ratio[b]	
		OX-CA	CSBG
Up-regulated			
MEKK3	U78876	4.9	–
MAPKK 6	U39657	8.2	–
c-myb	M15024	6.7	–
PTP-1B	M31724	2.3	–
Neurogranin	Y09689	1.9	–
NF-kappaB	M58603	2.2	1.6
TRRAP	AF076974	4.1	–
EGR1	X52541	1.8	1.4
Tuberin	X75621	1.8	–
MIP1-beta	J04130	16.0	–
IL-5	X04688	8.2	–
Inhibin beta A subunit	J03634	4.9	–
G-protein-coupled receptor HM74	D10923	9.1	–
ICAM-1	J03132	2.1	1.6
Integrin alpha 5	M14648	6.6	–
Organic cation transporter 1	U77086	6.6	–
APAF1	AF013263	4.1	–
PMS1 protein homolog 2	U13696	2.1	1.6
GADD45 beta	Z71621	8.2	
ARD-1	U14575	1.9	1.4
Ubiquitin	M26880	1.8	1.6
Down-regulated			
RHO12	L25080	2.9	–
SAS	U01160	4.3	–
GABP-beta2	D13318	2.0	–
MCP1	M24545	1.7	–
Zyxin-2	X94991	2.4	1.5
ProT-alpha	M26708	3.2	–
RPS19	M81757	2.1	–
Zyxin-2	X94991	2.4	1.5

[a] cDNA microarrays were analyzed in Materials, and gene signals were normalized to the housekeeping gene.
[b] Ratio of the normalized OD gene signal from *Candida* glucan-stimulated cells to the normalized OD gene signal from unstimulated control cells. mRNA expression was assessed at 4 h stimulation.

Table 2
Identification of CSBG-induced genes in human PBMCs by cDNA array[a]

Gene/protein name	GenBank accession number	Relative mRNA expression ratio[b]	
		OX-CA	CSBG
Up-regulated			
SAS	U01160	–	1.9
TYRO 3	D17517	–	2.3
P55-FGR	M19722	1.7	1.9
lyn	M16038	–	1.9
RAP-1B	X08004	–	1.9
JAK3	U09607	1.5	2.6
MAPKKK11	L32976	1.7	1.8
GN alpha-13	L22075	–	5.8
RAD 1	L24564	–	1.9
PKC inhibiter protein 1	X57346	–	2.3
DLC1	U32944	–	3.4
PP-1A	M63960	–	1.7
Junction plakoglobin	M23410	–	2.6
NF-kappa-B p100 subunit	X61498	–	3.9
NF-ATc	U08015	1.4	1.8
GABP-alpha	D13316		
Adrenoleukodystrophy protein	Z21876	–	2.6
EB1 protein	U24166	–	1.7
TIAM1	U16296	–	2.9
snoN	X15219	–	5.8
HNF4	X76930	–	1.7
Transcription elongation factor SII	M81601	1.6	2.4
DBP	D28468	1.4	1.9
ZNF92	L11672	–	2.4
MCP1	M24545	–	1.8
GCP 2	X78686	–	1.7
VEGF	M32977	–	2.4
CTGF	M92934	–	3.9
OX40L	X79929	–	8.7
C5a receptor	M62505	–	1.8
IL-1R1	M27492	–	2.4
IFN gamma R	A09781	–	1.7
IL-4R-alpha	X52425	–	3.9
ERBB2 receptor	M95667 + M11730	–	2.7
Integrin beta 1	X07979	–	1.9
CD11C antigen	M81695	–	2.9
Caspase-8	U60520	–	2.6
MCL-1	L08246	–	2.5
HSP90A	X07270	1.7	1.8
CDKN1A	U09579	–	3.4
CDK6	X66365	–	1.8
Cyclin A1	U66838	–	2.5
p55CDC	U05340	–	2.3
NEK3	Z29067	–	2.3

Table 2 (continued)

Gene/protein name	GenBank accession number	Relative mRNA expression ratio[b]	
		OX-CA	CSBG
Up-regulated			
SATB1	M97287	–	1.8
Sodium- and chloride-dependent taurine transporter	Z18956	5.8	4.9
CACNLB3	U07139	–	2.3
MMP3	X05232	–	2.6
MMP11	X57766	1.2	2.4
TIMP1	X03124	–	1.7
Ubiquitin-conjugating enzyme E2	M74524	–	1.7
HDC	X54297	1.6	2.7
MAO-A	M68840	–	1.9
Proteasome inhibitor subunit 1	D88378	–	2.3
CRABP2	M68867	–	5.8
HMG-I	M23619	1.2	1.8
PRP	M13667	1.7	1.9
TFIID	U30504	–	7.8
Aquaporin 4	U34846	–	1.9
Aquaporin 9	AB008775	–	2.8
Down-regulated			
GSHPX1	Y00483	1.4	1.8
CTSD	M11233	1.4	2.2

[a] cDNA microarrays were analyzed in Materials, and gene signals were normalized to the housekeeping gene.

[b] Ratio of the normalized OD gene signal from *Candida* glucan-stimulated cells to the normalized OD gene signal from unstimulated control cells. mRNA expression was assessed at 4 h stimulation.

sucrose, 10 g; $(NH_4)_2SO_4$, 2 g; KH_2PO_4, 2 g; $CaCl_2{\cdot}2H_2O$, 0.05 g; $MgSO_4{\cdot}7H_2O$, 0.05 g; $ZnSO_4{\cdot}7H_2O$, 1 mg; $CuSO_4{\cdot}5H_2O$, 1 mg; $FeSO_4{\cdot}7H_2O$, 0.01 g; biotin, 25 μg; final pH, 5.2. Five liters of medium was placed in the glass jar of a microferm fermentor (New Branswick Scientific, USA) and cultured at 27 °C with 5 l/min of aeration and stirring at 400 rpm.

2.3. Preparation of *C. albicans* β-glucan

Briefly, acetone-dried cells of *C. albicans* IFO1385 (2 g) were suspended in 200 ml of 0.1 M NaOH with NaClO having 0.5% of available chlorine for 1 day at 4 °C. After the reaction was completed, the reaction mixture was centrifuged to

Table 3
Identification of *Candida* glucan-changed genes in human PBMCs by cDNA array[a]

Gene/protein name	GenBank accession no.	Relative mRNA expression ratio[b]	
		OX-CA	CSBG
Up-regulated			
Fos-related antigen	X16707	3.3	4.5
Jun proto-oncogene	J04111	2.4	2.5
PRK 1	U33053	3.6	2.7
PLC-delta 1	U09117	3.3	3.1
TNK 1	U43408	4.1	3.8
RAB-7	X93499	2.3	2.5
Transductin beta-1 subunit	M36430	9.3	10.6
PDE 4A	U18087	2.5	1.9
PTPRF	Y00815 + X69398	1.9	1.9
rap 1 GTPase activating protein 1	M64788	2.1	2.5
B-cell specific transcription	M96944	2.1	2.7
Tristetraproline	M92843	3.1	1.7
cAMP-dependent transcription factor	D90209	1.7	3.0
MIP-1 alpha	M23452	24.4	3.7
MIP-2 alpha	X53799	11.6	9.4
IL-8	Y00787	7.0	11.4
TNF-alpha	X01394	32.0	2.4
IL-1 alpha	X02851	5.3	5.4
IL-1 beta	K02770	5.3	7.8
IL-3	M14743	6.0	5.2
IL-6	X04602	2.6	1.6
IL-9	X17543	4.9	12.6
IL-10	M57627	2.7	2.1
TGF-beta	M19154	2.0	1.7
VEGF-related protein	X54936	4.7	3.9
FLT3 ligand	U04806	1.8	1.8
Inhibin alpha subunit precursor	M13981	5.5	4.5
CRF-binding protein	X58022	8.2	21.3
Renin-binding protein	D10232	2.2	1.7
CC chemokine receptor 1	D10925	2.5	2.7
IL-2 R alpha	X01057	2.2	2.4
IL-3 R	M74782	4.9	3.9
IL-5 R	M75914	1.9	1.9
VEGFR 3	D10923	4.1	3.9
ErbB-3 R	M29366	6.5	11.9
U-PAR	U08839	3.4	3.0
RXR-beta	M84820	2.5	2.2
TNFR superfamily member 7	M63928	4.0	2.6
Integrin alpha 7B	X74295	7.5	9.3
Integrin beta 3	J02703	4.1	3.9
alpha 1 catenin	D13866	2.2	2.2
Neurexin III alpha	AF099810	2.1	2.2

Table 3 (*continued*)

Gene/protein name	GenBank accession no.	Relative mRNA expression ratio[b]	
		OX-CA	CSBG
Up-regulated			
MAL	M15800	5.4	7.0
HVEC	AF060231	1.8	2.1
Caspase-3	U13737	1.9	1.9
BCL-2-related protein A1	U29680	3.0	5.4
bax	L22474	5.4	7.3
PRG-1	AF039067	3.5	2.3
DAD1	D15057	1.9	2.9
HSP40	D49547	3.5	4.0
Cathepsin L	X12451	2.1	2.2
GST A1	M25627	2.1	1.9
SERP inhibiter calde B 2	M18082	2.4	4.3
Prothymosin alpha	M80397	2.7	2.4
ID-2	M97796	2.6	2.1
Peptide transporter 1	U13173	3.6	2.0
Down-regulated			
MRP	X70326	4.6	1.9
MRP8	X06234	2.4	1.8
MRP14	X06233	2.3	1.9
PI 1	X02920	2.7	2.0

[a] cDNA microarrays were analyzed in Materials, and gene signals were normalized to the housekeeping gene.

[b] Ratio of the normalized OD gene signal from *Candida* glucan-stimulated cells to the normalized OD gene signal from unstimulated control cells. mRNA expression was assessed at 4 h stimulation.

prepare the soluble and insoluble fractions. The particulate fraction, OX-CA, was dried by washing with ethanol and acetone. OX-CA suspended in DMSO was ultrasonically disrupted, and the resulting supernatant was designated CSBG [15].

2.4. Cell culture

PBMC were obtained from the peripheral blood of healthy donors. The blood was centrifuged (3000 rpm at 4 °C for 10 min) to obtain a leukocyte-rich fraction. The fractions were mixed well with an equal volume of phosphate-buffered saline (PBS) and were centrifuged on 6 ml of HISTOPAQUE (density 1.077; Sigma, USA) in a 15-ml centrifuge tube (Falcon 352196, Becton Dickinson, Lincoln Park, NJ, USA) at 2500 rpm for 25 min at room temperature. After centrifugation, isolated PBMC were washed three times with PBS and the cells

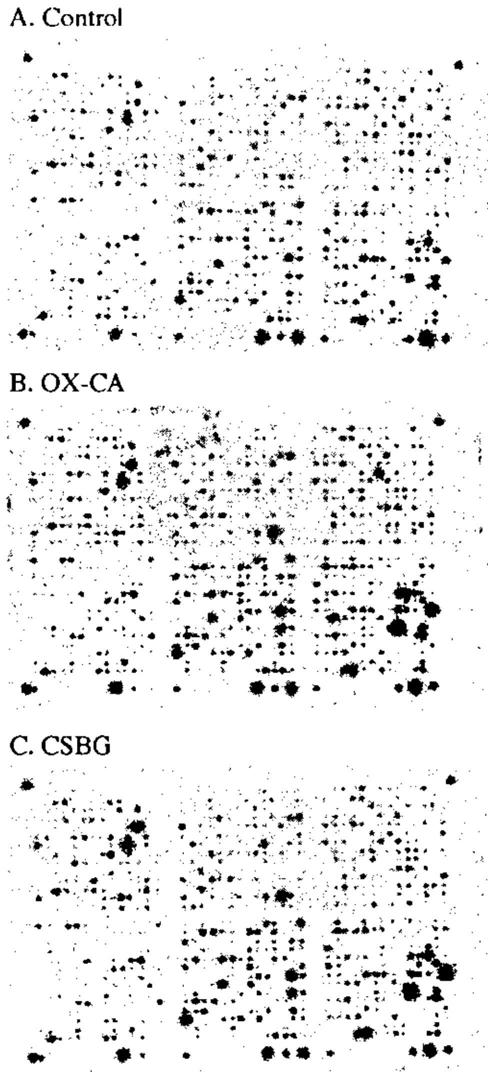

Fig. 3. cDNA microarray analysis of gene expression by PBMCs stimulated with OX-CA and CSBG. Total RNA was extracted from PBMCs (A) unstimulated, or stimulated with 100 μg/ml (B) OX-CA or CSBG for 4 h and subjected to cDNA expression array assay.

(2×10^6/ml) were cultured in polypropylene-tubes (IWAKI GLASS, Japan) in 500 μl of RPMI 1640 medium (Nissui) supplemented with gentamicin sulfate (Sigma) (5 μg/ml) containing 10% heat-inactivated autologous plasma at 37 °C in humidified 5% CO_2 (suspension culture system). The cells were stimulated with OX-CA or CSBG (100 μg/ml) for 4 ~ 12 h.

2.5. ELISA for TNF-α measurements

Immune plates (Nunc 442404, F96 Maxisorp) were coated with capture antibody for anti-human TNF-α monoclonal antibody (Pharmingen, USA) in 0.1 M carbonate buffer (pH 9.6) by incubation at 4 °C overnight. The plates were washed with PBS containing 0.05% Tween 20 (Wako) (PBST) and blocked with PBST containing 10% heat-inactivated fetal calf serum (FCS) at room temperature (RT) for 1 h. They were then washed, incubated with recombinant human TNF-α (Pharmingen) or 100 μl of sample at RT for 3 h, washed with PBST, and treated with biotinylated anti-human TNF-α monoclonal antibody (Pharmingen) and avidin-horseradish peroxidase conjugate, and developed with a TMB substrate system (KPL, Maryland). Color development was stopped with 1 M phosphoric acid and the optical density was measured at 450 nm.

2.6. ELISA for IL-8 measurements

Immune plates (Nunc 442404, F96 Maxisorp) were coated with capture antibody for mouse anti-human IL-8 mAb (Pharmingen) in 5μg/ml of Na_2HPO_4 buffer (pH 9.0) by incubation at 4 °C overnight. The plates were washed with PBST and blocked with BPBST at 37 °C for 40 min. After another wash, the plates were incubated with rh-IL-8 (Pharmingen) or 50μl of sample, obtained by the above procedure, at 37 °C for 40 min. They were washed with PBST and treated with antibody for biotinylated mouse anti-human IL-8 mAb (Pharmingen) in BPBST. Then, each plate was treated with

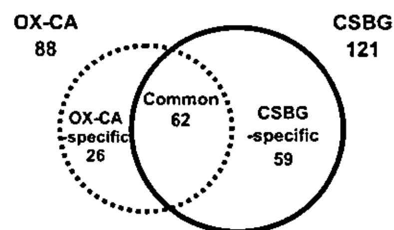

Fig. 4. Influence of solubility on gene expression in human PBMCs stimulated with OX-CA and CSBG. Numbers of genes significantly changed by each stimulation are shown outside of the circle, and stimulant-specific genes or commonly changed genes inside it.

Table 4
Summary of *Candida* glucan-induced genes in human PBMCs by cDNA array

Gene/protein name	Chromosomal location	Relative mRNA expression ratio	
		OX-CA	CSBG
(a)			
IL-8	4q13-q21	++	++
MIP-1α	17q11-q21	+++	+
MIP-1β	17q21-q23	++	−
MIP-2α	4q21	++	++
MCP-1	17q11.2-q21.1	−	+
GCP-2	Chr.4	−	+
TNF-α	6q21.3	+++	+
IL-1α	2q12-q21	+	+
IL-1β	2q13-q21	+	+
IL-10	1	+	+
TGF-β	1q41	+	+
CC chemokine R1	3p21	+	+
G-protein-coupled R HM74	Chr.12	+	−
C5a R	19q13.3-13.4	−	+
IL-1 R1	2q12	−	+
Integrin α 5	Chr.2	+	−
Integrin α 7B	12q13	+	+
Integrin β 1	10q11.2	−	+
Integrin β 2 (CD11c)		−	+
Integrin β 3	17q21.32	+	+
α 1 Catenin	5q31	+	+
ICAM-1	19p13.3-p13.2	+	+/−
(b)			
IL-3	5q23-q31	+	+
IL-5	5q23-q31	+	−
IL-6	7p21-p15	+	+
IL-9	5q31-q-35	+	++
IL-2Rα	10p15-p14	+	+
IL-3R	Xp22.3; Yp13.3	+	+
IL-4Rα	16p11.2-12.1	−	+
IL-5R	3p26-p24	+	+
IFN-γR	13q34	−	+
OX40L	Chr.1	−	+
MAL	2cen-q13	+	+
CD27	12p13	+	+
(c)			
NF-κB p105 subunit	4q24	+	+/−
NF-κB p100 subunit	10q24	−	+
MEKK3	17q24	++	−
MAPKK 6	17q24.3	++	−
Neurogranin	11q24	+	−
Fos-related antigen	11q13	+	+
Jun proto-oncogene	1p32-p31	+	+

Table 4 (*continued*)

Gene/protein name	Chromosomal location	Relative mRNA expression ratio	
		OX-CA	CSBG
(c)			
PRK 1	19p12-p13.1	+	+
PLC-σ 1	3p22-p21.3	+	+
Transductin β1 subunit	1p36.33	+	+
cAMP-dependent transcription factor	22q13.1	+	+
PTP-1B	20q13.1-q13.2	+	−
Rab-7	3q21	+	+
$G\alpha_{13}$	Chr 17	−	+
Tiam-1	21q22.1	−	+
Plakoglobin	17q21	−	+
Rap-1b	12q14	−	+
MLK3	11q13.1-q13.3	+/−	+
NF-AT	15	+/−	+
lyn	8q13	−	+
BASP	9p13	+	+

+, gene expression is up-regulated in response to pathogen; −, gene expression is unchanged; ++ and +++, gene expression is changed at a higher level relative to control (each + denote increased expression by a factor of ∼ 10).

avidin-horseradish peroxidase conjugate (ZYMED Laboratories, USA) and developed with a TMB substrate system (KPL). Color development was stopped with 1 M phosphoric acid and the optical density was measured at 450 nm.

2.7. Analysis of mRNA expression using cDNA arrays

The stimulated cells were collected by centrifugation. Total cellular RNA from cultured cells was extracted and radiolabeled cDNA probe synthesized with the Atlas Pure Total RNA labeling system (Clontech, Palo Alto, CA). The integrity of RNA transcripts was verified by electrophoresis. To determine gene expression, we used the Atlas Human 1.2 (CLONTECH Laboratories), which includes 1176 cDNAs and 9 housekeeping control cDNAs and a negative control immobilized on a nylon membrane. Expression levels of the housekeeping genes were used as standards for normalizing the expression levels of the genes of interest. The cDNA microarray analysis was performed according to the instruction manual (CLONTECH). Results of the gene expression were analyzed and quantified by computer with Atlas image software.

2.8. Cytometric bead array

For cytometric bead array, cell culture supernatant were harvested 12 h post-stimulation. The cell culture supernatant was analyzed using cytometric bead array human inflammation kit (BD Biosciences). The capture beads that covalently coupled to a different capture antibody against one of IL-1β, TNF-α, IL-6, IL-10, IL-12p70, PE detection regent, and standard dilutions or sample add to the assay tube. Each assay tubes were incubated for 3 h at RT and protect from direct exposure to light. After incubation, each assay tubes were washed and analyzed on a FACS Calibur cytofluorometer (Becton Dickinson) and with its software.

3. Results

3.1. Activation of leukocytes by OX-CA and CSBG in vitro

We reported that particulate glucan and soluble glucan showed observably different activities in vitro. By the NaClO–DMSO method, we could prepare both

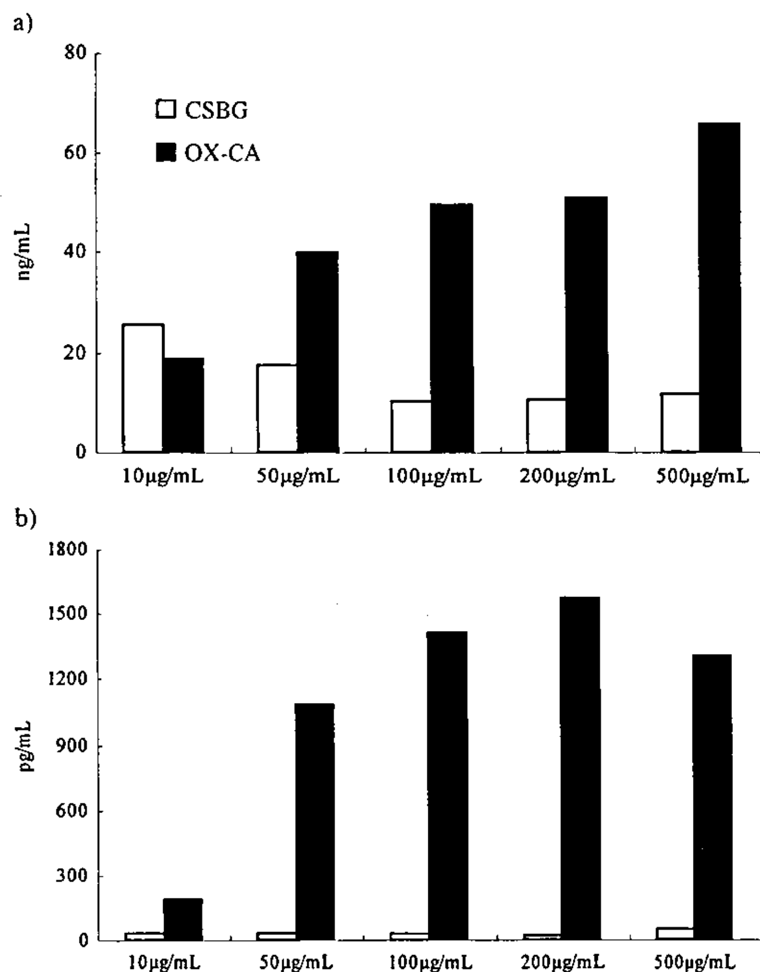

Fig. 5. Dose-dependency of OX-CA and CSBG on IL-8 and TNF-α production in human PBMC. PBMC obtained from the peripheral blood of healthy donors were adjusted to a concentration of 2×10^6 cells/ml in RPMI1640 medium containing 10% normal autologous plasma and cultured with OX-CA and CSBG (0.5%) (the each indicate dose) for 12 h in a 5% CO_2 incubator. Subsequently, the culture supernatants were collected, and IL-8 was measured as described in Materials. (a) IL-8 production, (b) TNF-α production by human PBMCs.

particulate and soluble forms of *Candida* glucan having exactly the same primary structure. Thus, the activation of PBMCs by two different forms was compared in the suspension culture system (Fig. 1). In the presence of heat-inactivated autologous plasma, only OX-CA showed the production of IL-8 and TNF-α. But in the presence of normal autologous plasma, not only OX-CA but CSBG exhibited the activity. These results suggested that CSBG showed the activities through the activation of complement, and OX-CA through a complement-dependent as well as -independent system. These facts suggested that OX-CA and CSBG show the activities through a different activation system.

3.2. The isolation of RNA from PBMC stimulated with Candida cell wall glucan

To study the difference in the activation mechanism of OX-CA and CSBG for mRNA expression in PBMCs, we employed a cDNA microarray approach using the Clontech Atlas microarray. PBMCs were stimulated with *Candida* cell wall glucan under non-adherent conditions with 10% autologous normal plasma. Total cellular RNA from these cells was isolated with the Atlas Total RNA labeling system and hybridized to a nylon membrane array consisting of 1176 genes. Microarray technology is a useful tool for identifying broad spectral changes. But the microarray analysis requires a lot of mRNA, and undecomposed and high quality RNA is indispensable for a reliable result. Also, we checked the quality of the RNA by gel electrophoresis and ethidium bromide staining. For the first time, we have isolated RNA from 2.4×10^7 cells stimulated with each sample for 12 h. Consequently, the RNA is of good quality, but of insufficient quantity, particularly that from PBMCs stimulated with *Candida* cell wall glucan. Then, the second time, we attempted the isolation of mRNA from more cells than previously (12×10^7 cells). The yields were high enough, but the fragmentation of RNA was serious (Fig. 2a,b). It is possible that these phenomena result from the death of PBMCs. The gene expression of apoptotic protein, Caspase-8, Caspase-3 and APAF-1 and anti-apoptotic protein, bax, bcl-2 related protein A1, and DIF-2 was also up-regulated in such PBMCs. This fact suggested that the metabolism of the stimulated cell may have influenced the quantity of RNA. Then, to suppress the fragmentation of RNA, we controlled the culture time and cell numbers and directly added a denaturalized reagent to cultured cells such as guanidine thiocyanate. Finally, we were able to prepare a good quality and quantity of total RNA and hybridized it to the array for non-amplification and reliable analysis.

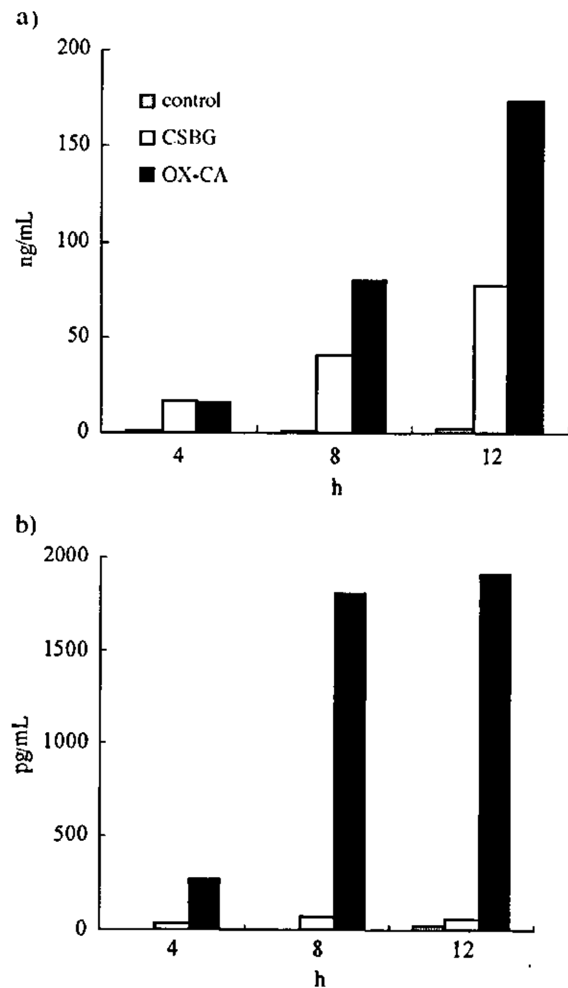

Fig. 6. Kinetics of IL-8 and TNF-α production by human PBMC stimulated with OX-CA or CSBG. PBMCs obtained from the peripheral blood of five healthy donors (A, B, C, D, E) were adjusted to a concentration of 2×10^6 cells/ml in RPMI1640 medium containing 10% normal autologous plasma and cultured with OX-CA particles (0.5%) or CSBG (100 μg/ml) for 12 h in a 5% CO_2 incubator. Subsequently, the culture supernatants were collected, and IL-8 and TNF-α was measured as described in Materials. (a) IL-8 production, (b) TNF-α production by human PBMCs.

3.3. Analysis of PBMC mRNA expression by cDNA microarrays in response to stimulation with OX-CA and CSBG

This array represents 1176 different genes, including those encoding immunological regulatory proteins such as cytokines, chemokines, cellular receptors and apoptosis-related molecules, hormones etc. Fig. 3 shows a microarray hybridized with cDNA taken from PBMCs stimulated with *Candida* cell wall glucan. The total mRNA showed a significant change for 147 out of 1176 arrayed genes stimulated with OX-CA and CSBG for 4 h (Tables 1–3). Among them, common genes numbered 62, OX-CA-specific genes 26 and CSBG-specific genes 59 (Fig. 4). It was suggested that the 62 genes common to both were changed not by physical properties such as solubility but by *Candida* cell wall β-glucan having a primary structure with a branched long 1,6-β-glucan and 1,3-β-glucan segment. The change of gene expression was extensive and the degree varied.

Many of these up-regulated genes common to OX-CA and CSBG encode effectors with well-characterized proinflammatory properties (Table 4a). For example, the expression of TNF-α, IL-1, which contributes to inflammatory symptoms and acute-phase responses such as the activation of leukocytes and enhanced vascular permeability, fever, was up-regulated. Extensive expression was also observed for the chemokines MIP-1α, MIP-1β, MIP-2α, MCP-1, and GCP-2, which selectively recruit other effector cells to site of infection. It was suggested that various cell subsets induced chemotactic activity. It is possible that these peptides induced migration and activation. The expression of adhesion molecules such as the integrin family, ICAM-1, and catenin, which play a considerable role in the

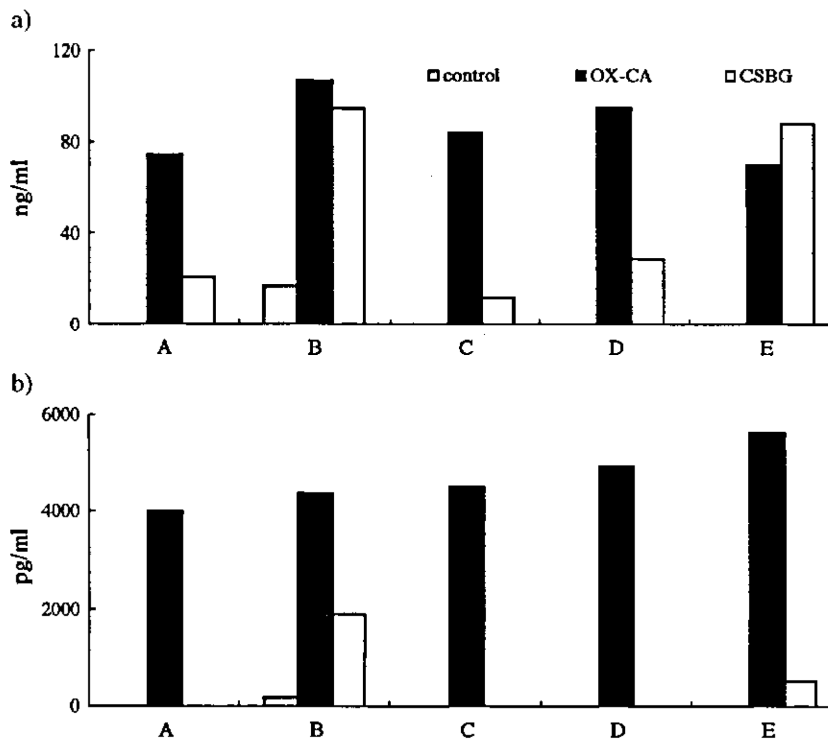

Fig. 7. Individual variations of cytokine production by human PBMC stimulated with OX-CA or CSBG. (a) IL-8 production, (b) TNF-α production by human PBMCs obtained from the peripheral blood of five (A, B, C, D, E) healthy donors were adjusted to a concentration of 2×10^6 cells/ml in RPMI1640 medium containing 10% normal autologous plasma and cultured with OX-CA particles (0.5%) or CSBG (100 μg/ml) for 12 h in a 5% CO_2 incubator. Subsequently, the culture supernatants were collected, and the cytokine measured as described in Materials.

formation of inflammatory pathosis, was also up-regulated. Therefore, those results suggested that *Candida* cell wall β-glucans were related with inflammatory response. On the other hand, a change to an adaptive immune response from an innate immune response was also observed (Table 4b). The gene expression of CD27, which plays an important role in the activation of lymphocytes, and cytokines IL-3, -5, -6, and -9, which are produced by and act on lymphocytes, and those cytokine receptors were up-regulated and showed the activation of the adaptive immune system.

In the changed gene in common, the gene, that was influenced on the mRNA expression levels by the physical properties of *Candida* cell wall glucan, as particle or soluble, existed. In OX-CA stimulation, especially, the gene coding inflammatory cytokine and chemokine, as TNF-α, MIP-1α and MIP-2α highly expressed observably. It supported the results that the MIP-1β, chemokine and further, the expression of leukocyte chemotactic factors, such as leukotriene, receptor, were included in the OX-CA specific changed 26 genes. Also, the expression of the MAP kinase family molecules MAP/ERKK3, MAPKK6, and PTP-1B involved in the signaling through adhesion molecule are specifically up-regulated in OX-CA stimulation. It was thought that those molecules are involved in the OX-CA-specific activation such as TNF-α production.

In CSBG stimulation, the specifically up-regulated expression of the OX40L gene, which act as a costimulatory signal in the activation of T cells and the IL-9 gene compared with OX-CA, suggested that CSBG has a greater ability stronger to activate T cells than OX-CA. Furthermore, the expression of NF-AT involved the downstream of TCR signaling and MLK3 concerned with cell to cell contact, Rho family as Rap-1b, Tiam-1, which regulate cytoskeleton, were strongly up-regulated compared with OX-CA.

These modifications are parallel and support the biological activity. Therefore, the results suggest that *Candida* cell wall glucans, OX-CA and CSBG, induce inflammatory responses and, because the signaling molecules for particulate and soluble forms differed, each glucan showed activity through a different mechanism.

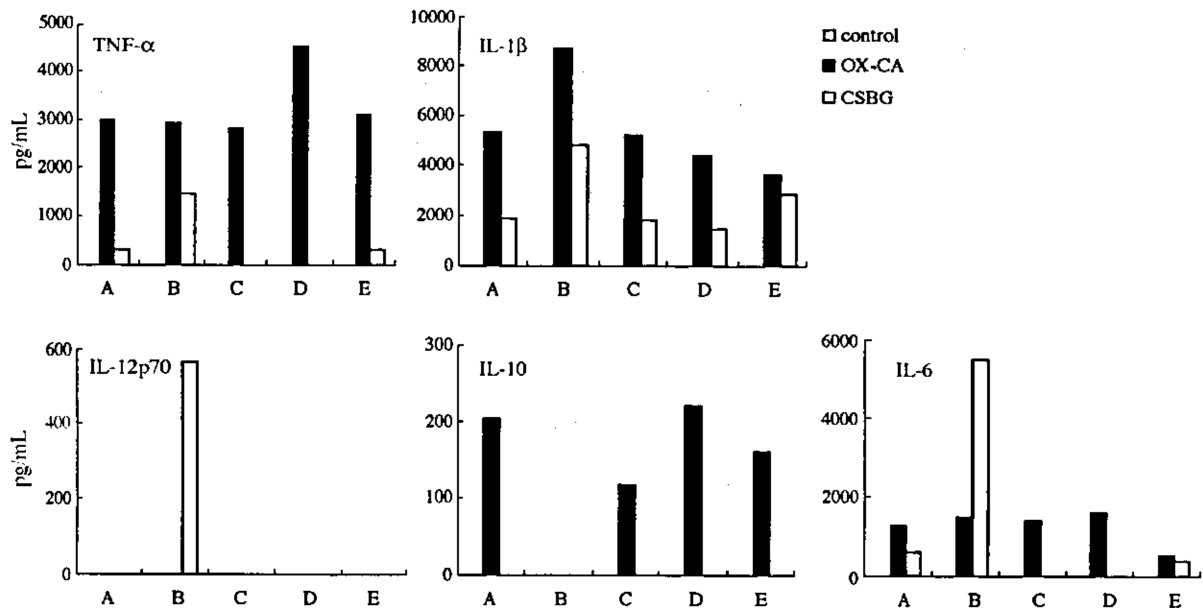

Fig. 8. Cytokine profiles of PBMC stimulated with OX-CA or CSBG. PBMCs obtained from the peripheral blood of five (A, B, C, D, E) healthy donors were adjusted to a concentration of 2×10^6 cells/ml in RPMI1640 medium containing 10% normal autologous plasma and cultured with OX-CA particles (0.5%) or CSBG (100 μg/ml) for 12 h in a 5% CO_2 incubator. Subsequently, the culture supernatants were collected, and the each cytokine was measured by cytometric bead array system as described in Materials.

3.4. *Variation of inflammatory cytokine production by Candida cell wall glucan in five volunteers*

To confirm the data obtained using the cDNA arrays, the cytokine production of IL-8, TNF-α was further analyzed. Because DNA array analysis was conducted by using PBMC from only one healthy donor, we compared these cytokine productions by PBMC stimulated with OX-CA or CSBG in five healthy volunteers.

We refined the dose-dependence and kinetics of OX-CA and CSBG on IL-8 and TNF-α production using one volunteer (Figs. 5 and 6). OX-CA shows the dose-dependence on those cytokine productions. In contrast, CSBG produced less amount than OX-CA in all the dose range tested. The dose–response pattern was in parallel with our previous in vivo and in vitro studies. In kinetics experiment in any time point, OX-CA produced higher concentration on IL-8 and TNF-α production. These results showed that OX-CA induced inflammatory response more strongly than CSBG on protein level, and was reflecting the cDNA array data. In the optimal experience condition obtained herewith, we tested IL-8 and TNF-α production of five volunteers. In all volunteers, OX-CA produced significantly high concentration of TNF-α, on the other hand, CSBG produced slight (Fig. 7). This result was in agreement with the data of cDNA array. In IL-8 production, OX-CA produced higher concentration than CSBG but individual variation existed considerably among volunteers. Furthermore, using cytometric bead array, we examined individual variation of the cytokine production as TNF-α, IL-6, IL-1β, IL-12, IL-10 (Fig. 8). TNF-α production showed the approximately same results as ELISA, and inflammatory cytokines were intensively produced in OX-CA stimulation. Each volunteer showed different profile of the cytokine production, and these results showed that the individual variation of glucan sensitivity existed.

4. Discussion

Because of a large molecular mass and wide diversity, each 1,3-β-glucan has specific chemical and biological properties in addition to a general structural unit and general biological activity. In physical properties and biological activities, the particulate and soluble forms of glucan show significant differences. Then, we prepared particulate and soluble glucans derived from *C. albicans* having exactly the same primary structure by the NaClO–DMSO method and compared the biological activity in terms of solubility alone. We reported that the particles activated leukocytes to a significantly greater extent than the soluble form. But on the immunopharmacological and immunotoxicological activity of CSBG, we have reported the activation of an alternative pathway of complement, enhanced vascular permeability, an adjuvant effect on antibody production, IL-6 synthesis by macrophages in vitro, enhancement of antitumor immunity and so on [18,19]. The solubility and degree of assembly of 1,3-β-D-glucans considerably influences the immunopharmacological activities, and controlling assembly would be essential for estimating the immunopharmacological activities of β-D-glucans. Then, in this study, we used cDNA microarray technology to clarify the difference in the activation mechanism of OX-CA and CSBG. These arrays are composed of 1176 gene-specific cDNA probes, involving cytokines, cellular receptors and other secreted growth-regulatory molecules, immobilized on a solid-phase nylon membrane. Our approach using a cDNA microarray demonstrated that human PBMCs stimulated with OX-CA and CSBG expressed a relatively extensive array of genes (147 out of 1176).

Organisms identify pathogens to its invasion by pattern recognition of the structure of individual components which triggers a biological response through signal transduction. It was reported that β-glucan activated PKC, CaM kinase and signal transduction cascade such as MAPK and NF-κB [20–22], and the activation was important for the expression of biological activities, such as cytokine production. With the cDNA microarray system, a large number of signal transduction pathways was able to be examined at the same time. Although NF-κB which has the recognition sequence of an inflammatory mediator such as cytokine, adhesion molecule, NOS, and COX-2 [23], was activated in both stimulations, NF-κB2 (p100 subunit) was specifically activated in the CSBG stimulation. Besides, the different expression of MAPK cascade genes, which typical signal transduction pathway as well as NF-κB, was observed. The expression of MAP/ERKK3, MAPKK6 in OX-

CA or MLK3 in CSBG stimulation is specifically up-regulated. From these results, it is possible for OX-CA to activate the MAPK family such as ERK, p38, and SAPK compared with CSBG. It was thought that such differences in signal transduction influence biological activities, such as cytokine production.

Many researchers showed that TNF-α production by β-glucan in vitro or vivo is related to solubility [24–26]. TNF-α production was induced only in the case of stimulation by the particulate form or gel form like H_2O_2 production. Also, in this study, the mRNA expression level of TNF-α was 15 times higher in OX-CA stimulation than in CSBG. This result supported previous results. The transcriptional mechanism of the TNF-α gene, which plays a central role in the inflammatory response, has been examined and found to be controlled at multiple levels.

The stability of the TNF-α mRNA was enhanced by p38, and an intensive expression was required for not only NF-kB but multiple signaling accompanied by MAPK such as ERK and JNK/p38. The MAP kinase pathway acts at multiple levels for the full regulation of TNF-α. OX-CA-stimulated monocytes expressed MAPK-related molecules more strongly than CSBG. Therefore, it was suggested that those molecules have an effect on β-glucan-stimulation of TNF-α mRNA expression.

If the difference in signal transduction was due to the molecular weight of β-glucan, it is possible that the difference is borne from signaling accompanied by the cross-linking of the glucan receptor and the transformation of the cytoskeleton. CR3 and CR4, glucan receptors, belong to the integrin family. The formation of desmosomes is important for the signaling and activation of ERK, JNK, and PKC through this receptor [27]. The expression of PTP-1, which interacts with p130Cas, a signaling pathway component through integrin [28], was up-regulated in OX-CA, supporting that this signaling pathway is activated. In addition, it was reported that the ligation of β2 integrin, CR3 and CR4 induced the expression of MIP-1α, MIP-1β mRNA, with an especially intensive expression on OX-CA stimulation [29]. These results suggested that cross-linking of the functional β-glucan receptor(s) would be necessary for such biological activities, and the degree regulated signal transduction and biological activities.

Soluble glucan shows strong activity in INF-γ production in serum and antitumor activity compared with the particulate form. Also, we previously reported that CSBG enhanced antitumor immunity and showed more effective antitumor activity against syngeneic tumors [30]. Therefore, CSBG would be a useful tool in adoptive immunotherapy and gene therapy. In this study, CSBG caused more changes in the expression of gene-related acquired immunity than OX-CA. In CSBG stimulation, the mRNA expression of IL-9 as produced by activated T cells and NF-AT, a downstream signaling molecule of TCR, was more intensive than in OX-CA. Also, the expression of OX40L was specifically up-regulated in CSBG. OX40 acts as a costimulatory signal in the activation of T cells and plays a role in the production of cytokines such as INF-γ, IL-4 and CTL activity [31,32]. These results show lymphocyte activation by CSBG. Besides, it was reported that the expression of OX40L was induced in monocyte-derived dendritic cells (DCs), antigen-presenting cells. Therefore, it was suggested that CSBG induced the differentiation of monocytes to DCs. In addition, the expression of retinoic acid binding protein II (CRABP II) which was induced in human monocyte-derived macrophages [33–35] and MAL which was expressed on the cell membrane during the differentiation of T cells [36], was intensive with CSBG. The results show that CSBG is a powerful activator of differentiation and proliferation. The up-regulation of Rho family molecules, such as $G\alpha_{13}$ and Tiam-1 which may suggest the modulation of cytoskeleton in cell division, and transcription initiation factor IID (TF II D), a general transcription factor which mediates the action of transcriptional activators, may support the activity of CSBG. Furthermore, CSBG induces the specific expression of genes, related to cell to cell contact such as lyn, Rac, MLK3. These results suggested that CSBG was able to activate acquired immunity, such as the antigen presentation, interaction of phagocytes and lymphocytes strongly by the enhancement of interaction between cells.

The expression of genes related with signal transduction was different for the particulate and soluble glucans derived from *C. albicans* having exactly the same primary structure. Therefore, this fact suggested that each glucan induced specific biological activity through different activation mechanisms. Organisms

may regulate the intensity of the biological response by adjusting signal transduction accompanied by the cross-linking of the glucan receptor.

This study was conducted using peripheral leukocytes from only one healthy volunteer. Hence, to confirm the data obtained using the cDNA arrays, the cytokine production of IL-8, TNF-α was further analyzed in five healthy volunteers. In CSBG stimulation, every volunteer produced slight amount of these cytokines than OX-CA stimulation, but significantly produced some kinds of these cytokines with individual variation. As shown in Fig. 1, it was suggested that the IL-8 and TNF-α production was influence of serum protein, as complement and anaphylatoxin. We previously reported that β-glucan was complement-activating substance, and its biological activity was modified by serum component as fibronectin and platelet [37,38]. Also, we recently found out the antibody to *Candida* cell wall glucan in human serum, and reported the individual variation of its titer exists in the certain range. On the other hand, the other researcher reported that Dectin, one of β-glucan receptor, had functional subtypes [39]. The expression degree and subtypes of these receptors may be factor. These factors would influence the immunopharmacological activities of β-glucan in vivo situation. By analysis with many individuals, the activation mechanism would be clarified furthermore.

This study using cDNA microarrays to analyze a broad spectrum of mRNA expression has provided information on the biological activity of *Candida* cell wall glucan as a potential pathogenic factor.

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Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF) Regulates Cytokine Induction by 1,3-β-D-Glucan SCG in DBA/2 Mice *In Vitro*

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ABSTRACT

***Sparassis crispa Fr.* is an edible/medicinal mushroom that recently became cultivable in Japan. SCG is a major 6-branched 1,3-β-D-glucan in *S. crispa* showing antitumor activity. We recently found that the splenocytes from naive DBA/1 and DBA/2 mice strongly react with SCG to produce interferon-γ (IFN-γ). In this study, cytokines induced by SCG were screened and found to be IFN-γ, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-12 (IL-12p70). The addition of recombinant murine GM-CSF (rMuGM-CSF) to spleen cell cultures from various strains of mice synergistically enhanced IFN-γ, TNF-α and IL-12p70 in the presence of SCG. In contrast, neutralizing GM-CSF using anti-GM-CSF monoclonal antibody (mAb) significantly inhibited IFN-γ, TNF-α, and IL-12p70 elicited by SCG. We conclude that GM-CSF is a key molecule for cytokine induction by β-glucan, and GM-CSF induction by SCG is the specific step in DBA/2 mice *in vitro*.**

INTRODUCTION

An immunomodulating substance, biologic response modifier (BRM) or biotherapeutic agent, is important for the treatment of cancer and infectious diseases. β-Glucan is a well-known BRM widely distributed in nature and used as a medicine and food substance. Among β-glucans, 6-branched 1,3-β-glucan is the best characterized. Lentinan from *Lentinus edodes*[(1)] and sonifilan (SPG) from *Schizophyllum commune*[(2)] have been used clinically for cancer therapy in Japan.

Sparassis crispa is a medicinal mushroom that recently became cultivable in Japan. Following preliminary investigations, the β-glucan content of *S. crispa* was found to be 43.6%, as measured by the enzyme method of Japan Food Research Laboratories (Tokyo). Thus, *S. crispa* was determined to be a good source material for preparing antitumor β-glucan with a high yield. In a previous study, *S. crispa* was repeatedly extracted by hot water, cold NaOH, and then hot NaOH, and the structure of the polysaccharide fractions of these extracts was analyzed by chemical and enzymic methodologies. The primary structure of the major polysaccharide fraction was found to be 6-branched 1,3-β-glucan, with one branch approximately every third main chain. These fractions contained 1,6-β-glucan as minor linkages. This molecule shows a strong metachromasy toward aniline blue, similar to other gel-forming 1,3-β-glucans. All fractions showed strong antitumor activity against the solid form of sarcoma 180 in ICR mice, with strong vascular dilation and hemorrhage reaction on administration of a microgram quantity.[(3)]

In our recent study, we used *S. crispa* for several cancer patients in combination with lymphocyte transplantation immunotherapy and obtained a good response.[(4)] To examine the pharmacologic applicability of *S. crispa* β-glucan, SCG was purified from the cold NaOH extracted fraction. SCG enhanced the hematopoietic response in cyclophosphamide-induced leukopenic mice by intraperitoneal (i.p.) and oral (p.o.) routes over a wide range of concentrations.[(5,6)] We recently reported that SCG responded to human peripheral blood mononuclear cells (PBMCs) and induced cytokines.[(7)]

In a previous study, we reported that a gel-forming 6-branched 1,3-β-glucan induced macrophages to produce several mediators, including the inflammatory cytokines inter-

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leukin-1 (IL-1), IL-6, tumor necrosis factor-α (TNF-α), and nitric oxide (NO).[8-11] The activities of β-glucan on other leukocytes and functional β-glucan receptors on such leukocytes are not clear. It was suggested that the cytokine induction by β-glucan from leukocytes derived from normal mice is low. IL-6 production was not induced by SCG in splenocytes derived from ICR mice *in vitro*, whereas splenocytes derived from cyclophosphamide-treated ICR mice respond to SCG and produce IL-6 *in vitro*.[5] Studying cell types responsible for the activation of β-glucan is important because it could lead to identification of β-glucan receptors and to understanding of the mechanisms of β-glucan activity *in vivo*. Cytokines are not induced by SCG in splenocytes from almost all strains of mice, although in splenocytes from naive DBA/2 or DBA/1 mice, high amounts of interferon-γ (IFN-γ) are induced by SCG. The induction of IFN-γ by SCG in DBA/2 mice synergistically requires both soluble factors and cell-cell contact.[12] DBA/2 and DBA/1 mice contain significantly higher titers of antibody to SCG than do other inbred naive mice.[13] This suggests that leukocytes derived from DBA/1 and DBA/2 mice are highly sensitive to SCG *in vivo* and *in vitro*. However, studies on the cause of high sensitivity to SCG in naive DBA/2 and DBA/1 mice have been superficial.

In this study, we investigated the mechanism of IFN-γ production by SCG *in vitro* in splenocytes from DBA/2 mice to determine the reason for sensitivity to SCG in leukocytes from DBA/2 mice.

MATERIALS AND METHODS

Animals

C57BL/6, BALB/c, C3H/HeN, C3H/HeJ, BDF1 (C57BL/6 × DBA/2), and DBA/2 male mice between 6 and 7 weeks of age were purchased from Japan SLC (Shizuoka, Japan). Mice were maintained under specific pathogen-free conditions.

Preparation of SCG

Fruit bodies of *S. crispa* were cultured by Minahealth Co., Ltd. (Saitama, Japan). SCG was prepared as follows. Briefly, air-dried and powdered *S. crispa* was defatted with ethanol (4°C for 2 days) and then extracted with hot water by autoclaving (121°C for 2 h). The resulting residue was extracted with cold alkali (10% NaOH/5% urea at 4°C for 2 days) and hot alkali (10% NaOH/5% urea at 65°C for 1 h). Polysaccharide fractions of alkali extract was collected after extensive dialysis. Cold alkali extract dissolved in 8 M urea was applied to a DEAE Sephedex A25 (Cl^-) column equilibrated with 8 M urea, and the passed-through fraction was collected and extensively dialyzed against tap and distilled water and then lyophilized (elemental analysis CHN = 40.06: 6.77: 0.08). SCG solution was prepared by dissolution in 0.5 N NaOH, followed by immediate dialysis against saline for 3 days. After dialysis, the nondialyzable fractions were autoclaved and frozen until use.

Materials

Tween 20 was purchased from Wako Pure Chemical Co. (Osaka, Japan). Gentamicin sulfate, RPMI 1640 medium, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Fetal bovine serum (FBS) was from Sanko Junyaku Co., Ltd. (Tokyo, Japan), and purified rat antimouse granulocyte-macrophage colony-stimulating factor (GM-CSF) monoclonal antibody (mAb) and recombinant mouse GM-CSF (rMuGM-CSF) were from PharMingen (San Diego, CA).

Preparation of splenocytes for cell culture

Splenocytes were removed from mice. The spleen was teased apart in RPMI 1640 medium, and after centrifugation, the single cell suspension was treated with ACK-lysing buffer (8.29 g/L NH_4Cl, 1 g/L $KHCO_2$, 37.2 mg/L EDTA/$_2$ Na) to lyse red blood cells. After centrifugation, splenocytes were maintained in RPMI 1640 medium supplemented with 50 μg/ml gentamicin sulfate and 10% heat-inactivated FBS. Cells were cultured in 24-well flat-bottomed plates at 5×10^6 cells/well in 1 ml culture medium, and stimulated with saline or SCG (100 μg/ml). Splenocytes were cultured at 37°C for 48 h in a humidified 5% CO_2, 95% air atmosphere.

Cytometric bead array (CBA)

The BD CBA system (PharMingen) uses the sensitivity of amplified fluorescence detection by flow cytometry to measure soluble analytes in a particle-based immunoassay. Each bead in a CBA provides a capture surface for a specific protein and is analogous to an individually coated well in an ELISA plate. The BD CBA capture bead mixture is in suspension to allow for the detection of multiple analytes in a small sample volume. The BD Mouse Th1/Th2 Cytokine CBA Kit can be used to measure IL-2, IL-4, IL-5, IFN-γ, and TNF-α protein levels in a single sample. Five bead populations with distinct fluorescence intensities are coated with capture antibodies specific for IL-2, IL-4, IL-5, IFN-γ, and TNF-α proteins. The five bead populations are mixed together to form the CBA, which is resolved in the FL3 channel of the FACSCalibur flow cytometer. The cytokine capture beads are mixed with phycoerythrin (PE)-conjugated detection antibodies and then incubated with recombinant standards or test samples to form sandwich complexes. Following acquisition of sample data using the flow cytometer, sample results are generated in graphic and tabular form.

ELISA

For IFN-γ, IL-6, and TNF-α, 96-well microtiter ELISA plates were coated with capture antibody for rat antimouse IFN-γ mAb, rat antimouse IL-6 mAb, or rat antimouse TNF-α mAb (all from PharMingen). The plates were washed with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST) and blocked with 0.5% BSA (BPBST). After washing, plates were incubated with rMuIFN-γ, rMuIL-6, rMuTNF-α (all from PharMingen), or 50 μl test sample. The plates were then washed with PBST and treated with antibody for biotinylated rat antimouse IFN-γ mAb, biotinylated rat antimouse IL-6 mAb, or biotinylated rat antimouse TNF-α mAb in BPBST. Subsequently, they were treated with peroxidase-conjugated streptavidine (PharMingen) and developed using a TMB substrate system (KPL, Gaithersburg, MD). Color development was stopped with 1 N phosphoric acid, and the optical density (OD) was measured at 450 nm.