

FIGURE 1. Susceptibility of CSF-induced monocyte-derived MΦ and A-MΦ to CSF withdrawal. M-MΦ and GM-MΦ (2.5×10^5 /ml/well) were cultured in medium with or without M-CSF or GM-CSF, and A-MΦ were cultured in medium without GM-CSF. A, cell number and viability of MΦs were assessed using Cetavlon and trypan blue dye as described under "Experimental Procedures." Values are expressed as the means of triplicate cultures \pm S.D. B, electron microscopic features of MΦ cultured in medium with or without CSF for 24 h (magnification $\times 10,000$). C, electrophoresis of low molecular mass DNA from cultured MΦ. MΦ were incubated with M-CSF, GM-CSF, or medium alone for 24 h. Low molecular mass DNA was isolated from the cells, and electrophoresis was

changed from spindle shaped to round and then detached from the dish and floated, with a shrunken cytosol and nucleus and with chromatin condensation and apoptotic vacuoles without microvilli (Fig. 1B). No such morphological change was observed in M-MΦ cultured with M-CSF or in GM-MΦ cultured with or without GM-CSF (Fig. 1B). A typical ladder pattern of internucleosomal DNA cleavage was detected in DNA of M-MΦ cultured for 24 h in the absence of M-CSF, whereas no significant DNA fragmentation was detected in DNA of M-MΦ cultured in the presence of M-CSF or that of GM-MΦ cultured in the presence or absence of GM-CSF (Fig. 1C). Apoptosis and DNA fragmentation of A-MΦ were similar to those of GM-MΦ (data not shown). These findings suggest that M-CSF withdrawal from M-MΦ induces apoptosis but both GM-MΦ and A-MΦ can survive in the absence of CSF.

Different Expression of BCL-2 and BCL-X_L Genes in M-MΦ, GM-MΦ, and A-MΦ and the Opposite Effect of CSF on Expression—BCL-2 family genes play important roles in the apoptosis of many types of cells (10, 16–20). We therefore examined the expression of BCL-2 family genes and the effect of CSF on their expression in M-MΦ and GM-MΦ by RT-PCR. In M-MΦ, the transcript of the BCL-2 gene was stronger than that of the BCL-X_L gene, and M-CSF withdrawal decreased the expression of the BCL-2 gene but induced a slight decrease in the expression of the BCL-X_L gene (Fig. 2A). In contrast to M-MΦ, GM-MΦ expressed strongly the BCL-X_L gene but weakly the BCL-2 gene, and GM-CSF withdrawal had no significant effect on the expression of these two genes (Fig. 2A). In contrast to BCL-2 and BCL-X_L, the transcriptional levels of the MCL-1 and BAX genes were not significantly different between M-MΦ and GM-MΦ and were not affected by CSF deprivation (Fig. 2A). In accordance with the gene expression, the expression of the BCL-2 protein was higher in M-MΦ compared with GM-MΦ and M-CSF withdrawal decreased the expression of BCL-2 protein in M-MΦ (Fig. 2B). Similarly, the expression of the BCL-X_L protein was higher in GM-MΦ than in M-MΦ, but GM-CSF withdrawal had no significant effect on the expression of BCL-X_L protein in GM-MΦ (Fig. 2B).

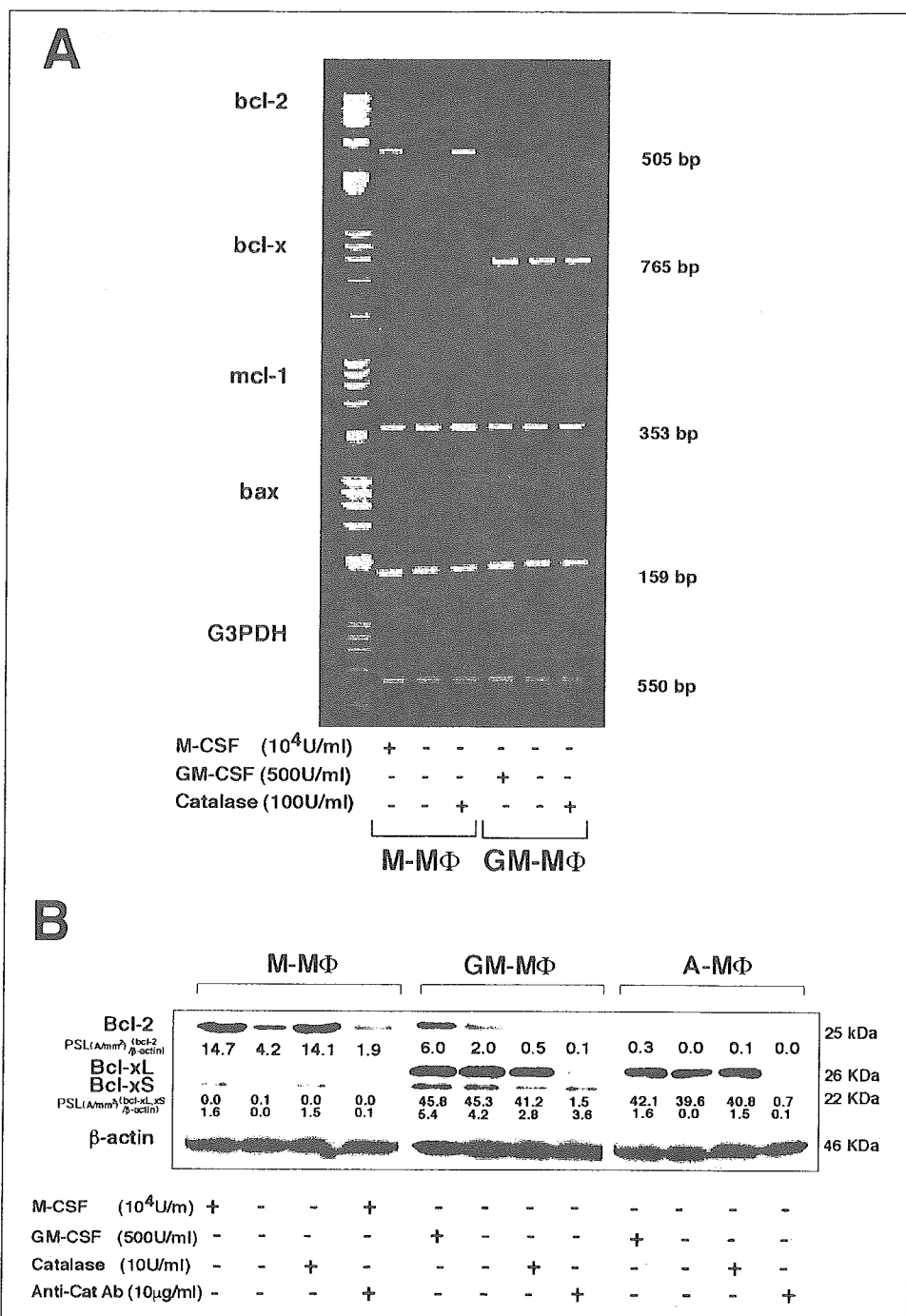
The expression of BCL-2 family proteins in A-MΦ resembles that of GM-MΦ, and the expression was independent of CSF (Fig. 2B). As shown in Figs. 6 and 7, the expression of MCL-1 and BAX proteins in both MΦs was not changed in the presence or absence of CSF, in accordance with the expression patterns of the MCL-1 and BAX genes.

HEC Stimulates the Survival and the Expression of BCL-2 in CSF-withdrawn M-MΦ, and Anti-HEC Antibody Abolishes the Survival-stimulating Activity of CM Obtained from CSF-withdrawn GM-MΦ and A-MΦ—The above findings suggest that GM-MΦ and A-MΦ, but not M-MΦ, can produce factor(s) that maintain their survival in the absence of CSF. To investigate this possibility, the conditioned medium (CM) obtained from these MΦ cultured for 48 h without CSF was used to examine its effect on the survival of M-CSF-withdrawn M-MΦ. CM of GM-MΦ or A-MΦ, but not of M-MΦ, prevented cell death of M-CSF-depleted M-MΦ (Fig. 3A).

A previous study showed that human CEM T cells can survive in serum-free medium supplemented with a low level of extracellular catalase (21). Therefore, we examined whether extracellular catalase prevents apoptosis in M-CSF-withdrawn M-MΦ and stimulates the expression of both BCL-2 gene and protein. Addition of HEC prevented M-CSF-withdrawn M-MΦ from undergoing cell death in a dose-dependent manner, and 10 units/ml of HEC completely rescued the cells

performed as described under "Experimental Procedures." HaeIII-digested DNA fragment size standards were run in lanes 1 and 6. Lane 2, M-MΦ without M-CSF; lane 3, M-MΦ with M-CSF; lane 4, GM-MΦ without GM-CSF; lane 5, GM-MΦ with GM-CSF.

FIGURE 2. Effects of CSF and catalase on the levels of protein and mRNA expression of BCL-2 family genes in monocyte-derived MΦ and A-MΦ. A, RT-PCR analysis of mRNA levels of BCL-2 family genes and the glyceraldehyde-3-phosphate dehydrogenase gene in total RNA preparations (200 ng/lane) from these MΦs at 3 h of cultivation as described under "Experimental Procedures." M-MΦ and GM-MΦ were cultured in medium with or without CSF or supplemented with catalase. B, Western blot analysis of BCL-2, BCL-X_L, or β-actin protein in cell lysates (25 μg/lane) probed using rabbit polyclonal BCL-2 or BCL-X antibody or mouse monoclonal β-actin antibody. M-MΦ were cultured for 48 h in medium with, M-CSF or catalase or with M-CSF supplemented with anti-catalase antibody (Anti-Cat Ab), and GM-MΦ and A-MΦ were cultured in medium with GM-CSF or catalase or supplemented with Anti-Cat Ab. The relative amounts of these proteins in cells were measured using NIH image software, and the expression levels were corrected relative to those of β-actin (photo-stimulating luminescence (PSL), A/mm²).



from apoptosis. Addition of 10 units/ml HEC to GM-CSF-withdrawn GM-MΦ had no such effect (Fig. 3A).

Then we examined the effect of anti-HEC antibody (anti-Cat Ab) on the activity of the CM of GM-MΦ. Pretreatment of the CM with anti-Cat Ab, but not with control IgG, completely abrogated the ability to rescue the cell death of M-CSF-withdrawn M-MΦ (Fig. 3B). Similar results were obtained in experiments using the CM of A-MΦ (data not shown).

Next, we examined the role of catalase in the expression of BCL-2 family genes in M-CSF-withdrawn M-MΦ. Addition of catalase to M-CSF-withdrawn M-MΦ restored the expression of BCL-2 but did not affect the gene expression of BCL-X_L, MCL-1, or BAX (Fig. 2A). Addition of catalase did not significantly change the gene and protein

expression of BCL-2 family genes in GM-CSF-withdrawn GM-MΦ (Fig. 2A). These results suggest that the active molecule in the CM of GM-MΦ and A-MΦ that can rescue the survival of M-CSF-withdrawn M-MΦ via restoration of BCL-2 expression is catalase.

Anti-catalase Antibody Abolished the Expression of BCL-2 in M-MΦ Cultured with M-CSF and BCL-X_L in GM-MΦ or A-MΦ Cultured with or without GM-CSF and Induced Apoptosis of These MΦs—The above results indicate that catalase can stimulate the expression of BCL-2 and rescue the survival of M-CSF-withdrawn M-MΦ. Therefore we next examined whether extracellular catalase also plays a critical role in the CSF-dependent survival of M-MΦ and in the survival of GM-MΦ and A-MΦ. Addition of anti-Cat Ab suppressed the expression of BCL-2 protein in M-MΦ cultured in the presence of M-CSF and of BCL-X_L

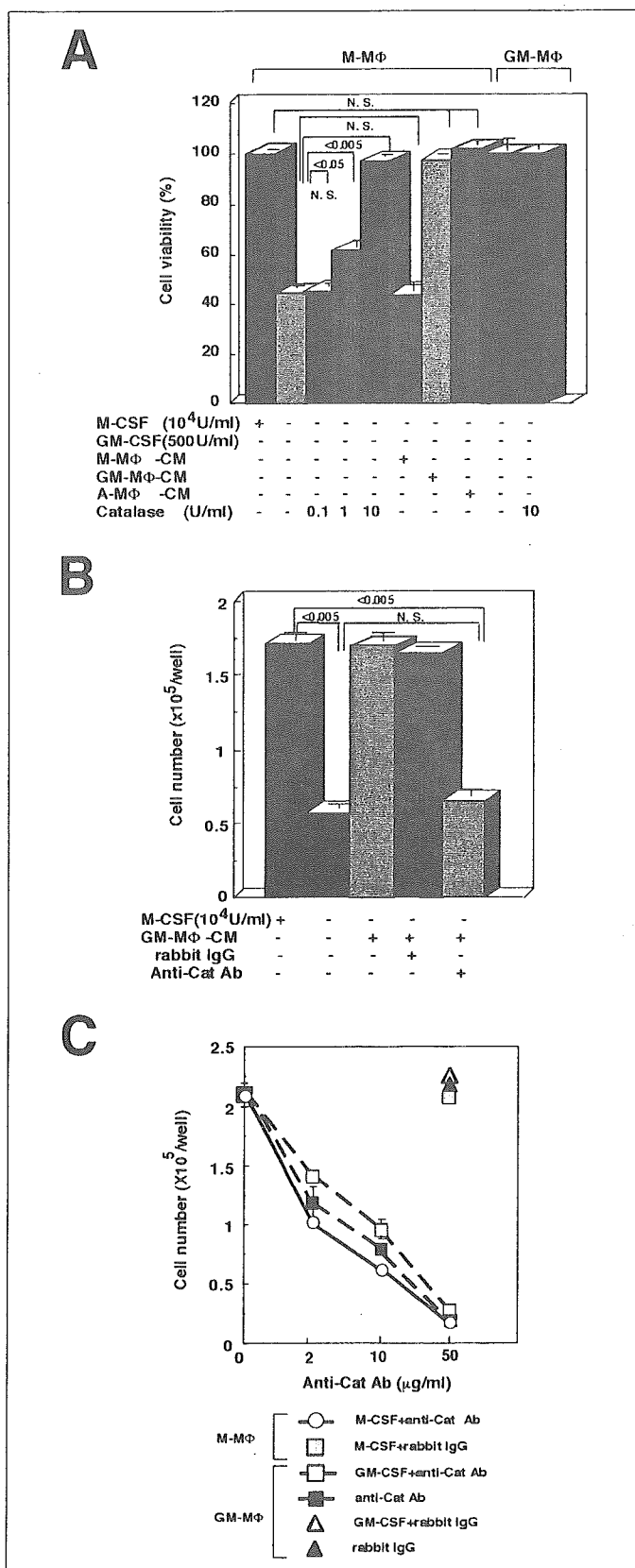


FIGURE 3. Effect of endogenously produced or exogenously added catalase on the survival of M-MΦ and GM-MΦ. A, M-MΦ were cultured for 48 h in the medium with M-CSF, medium containing the indicated concentrations of catalase, or 100% of conditioned medium (CM) obtained from M-MΦ, GM-MΦ, or A-MΦ. GM-MΦ were used as the control. Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D. B, M-MΦ were cultured for 48 h in

protein in both GM-MΦ and A-MΦ cultured without GM-CSF (Fig. 2B). In accordance with these data, addition of anti-Cat Ab induced cell death of M-MΦ cultured with M-CSF and GM-MΦ cultured with or without GM-CSF, and addition of control IgG had no effect on the cell viability of these MΦs (Fig. 3C). The cell death of these MΦ induced by anti-Cat Ab was due to apoptosis, as indicated by terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling staining (data not shown).

GM-MΦ and A-MΦ, but Not M-MΦ, Can Produce Enough Levels of Extracellular Catalase in the Absence of CSF—The above results suggest that GM-MΦ and A-MΦ, but not M-MΦ, can produce extracellular catalase in a CSF-independent manner and that the catalase supports the survival of those MΦs via maintenance of the expression of BCL-2 family genes. To examine this possibility, we measured catalase enzyme activity and catalase protein levels in the CMs of M-MΦ, GM-MΦ, and A-MΦ cultured for 48 h in the presence or absence of CSF. The extracellular catalase activities in the CMs obtained from GM-MΦ and A-MΦ cultured both in the presence or absence of GM-CSF were not significantly different, and the levels were ~4-fold higher (~240 milliunits/ml/well) than that of M-CSF-treated M-MΦ (~60 milliunits/ml/well) (Fig. 4A). In contrast to GM-MΦ or A-MΦ, the extracellular catalase activity in CM of M-MΦ was dependent on CSF and that in CM of M-CSF-withdrawn M-MΦ was significantly lower than that in CM of M-CSF-treated M-MΦ, ~20 milliunits/ml/well (Fig. 4A). Western blot analysis of the extracellular catalase of CMs using anti-HEC antibody showed similar results to the data of the enzyme activity (Fig. 4A).

Next, we examined the cell-associated levels of catalase activity in these MΦs (Fig. 4B). The levels of catalase activity in GM-MΦ and A-MΦ lysates was ~5 units/mg protein both in the presence or absence of CSF, whereas that in M-MΦ in the presence of M-CSF was ~1 unit/mg. The catalase activity in M-CSF-withdrawn M-MΦ lysates (~250 milliunits/mg protein) was ~4-fold lower than that in M-CSF-treated M-MΦ lysates. In agreement with the measurements of enzyme activity, similar results were observed in Western blot analyses (Fig. 4B).

To further confirm the distinction between the regulation of extracellular and cell-associated catalase activity by CSF in M-MΦ versus GM-MΦ, we examined the levels of transcription of the catalase gene in these MΦs cultured with or without CSF (Fig. 4C). The level of the transcript of the catalase gene in M-CSF-withdrawn M-MΦ was ~3-fold lower than that in M-CSF-treated M-MΦ. In contrast, the level of the catalase transcript in GM-CSF-withdrawn GM-MΦ and A-MΦ was similar to that in GM-CSF-treated GM-MΦ and A-MΦ, and the level was 5-fold higher than that in M-CSF-treated M-MΦ.

Thus, the difference of total extracellular plus intracellular catalase activity and the difference in the levels of catalase gene expression between CSF-withdrawn M-MΦ and GM-MΦ reached ~15–20- and ~15-fold, respectively. The results indicate that extracellular catalase activity is regulated at the transcription levels by CSF-dependent M-MΦ but CSF-independent GM-MΦ and A-MΦ.

Thiol Derivatives Act as Additive Effectors That Rescue the Cell Death of MΦ—The above data suggest that extracellular catalase plays a major role in MΦ survival. However, several reports have demonstrated that thiol proteins and thiol compounds such as GSH, adult T cell leukemia-derived factor, and L-cysteine play important roles in the survival of

medium with M-CSF, CM pretreated with Anti-Cat Ab (IgG), or rabbit IgG as a control. Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D. C, M-MΦ and GM-MΦ were cultured for 48 h in medium with or without CSF plus Anti-Cat Ab or rabbit IgG. Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D.

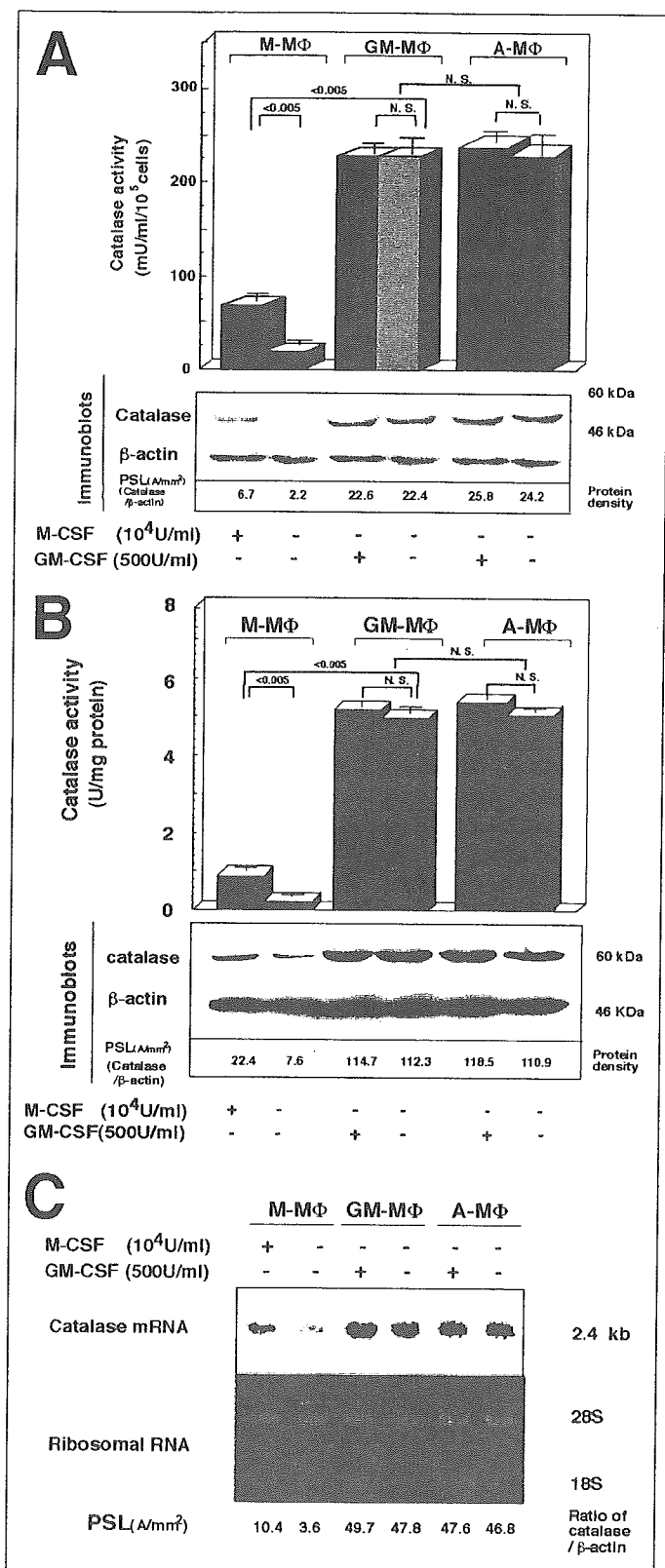


FIGURE 4. Activities, protein levels, and mRNA levels of catalase in monocyte-derived MΦ and A-MΦ. MΦ were cultured with or without CSF for 48 h (A, B) or for 3 h (C). A and B, enzyme activities (upper panel) and protein levels (lower panel) of catalase in the culture medium (25 μl/lane) (A) or cell lysates (25 μg/lane) (B) from MΦ were examined as described under "Experimental Procedures." Western blot analysis of catalase protein in the culture medium or cell lysates of MΦ was performed using anti-catalase antibody. The relative amounts of catalase protein in cells were measured using NIH image software, and the expression levels were corrected relative to those of β-actin (photo-stimulating luminescence, PSL, A/mm²).

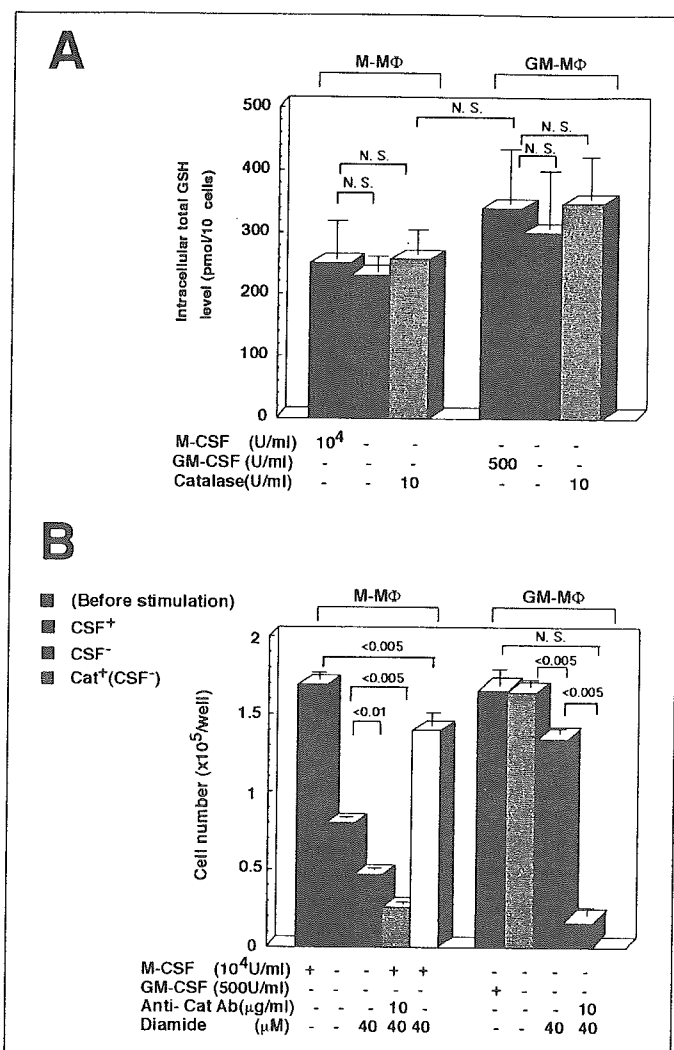


FIGURE 5. Partial effect of GSH on the survival of monocyte-derived MΦ. A, intracellular GSH levels in M-MΦ and GM-MΦ incubated with or without CSF for 24 h were measured as described under "Experimental Procedures." Values are expressed as the means of triplicate cultures ± S.D. B, M-MΦ or GM-MΦ were cultured for 48 h in medium supplemented or not supplemented with CSF, anti-catalase antibody (Anti-Cat Ab), diamide, or the combination of them, and cell number and viability were assessed as described above.

lymphocytes or neuronal cells in the absence of growth factors (22, 23, 24). Thus, thiol derivatives may help MΦ survival. The level of intracellular GSH, however, was almost the same in M-MΦ and GM-MΦ cultured with or without CSF or cultured with catalase (Fig. 5A), and this level (~300 pmol/10⁵ cells) was similar to that in A-MΦ (data not shown). Diamide, which can bind the SH groups of reduced thiols and oxidize them (22), induced cell death of M-MΦ and GM-MΦ, but the levels of cell death were not very high (~20%) and no significant difference was observed between the effects of diamide on these two MΦs (Fig. 5B). 40 μM diamide and 10 μg/ml anti-Cat Ab showed synergistic effects on the cell viability of M-MΦ cultured with M-CSF and GM-MΦ cultured without CSF, causing reduction of their viability to <10% at 48 h (Fig. 5B). These results suggest that, in contrast to that of catalase,

ulating luminescence (PSL, A/mm²). C, mRNA levels of catalase and the β-actin gene were examined in total RNA preparations (10 μg/lane) from these MΦ at 3 h of cultivation by Northern blot analysis. The relative transcript levels of catalase mRNA in cells were measured using NIH image software, and the expression levels were corrected relative to those of β-actin (photo-stimulating luminescence, PSL, A/mm²).

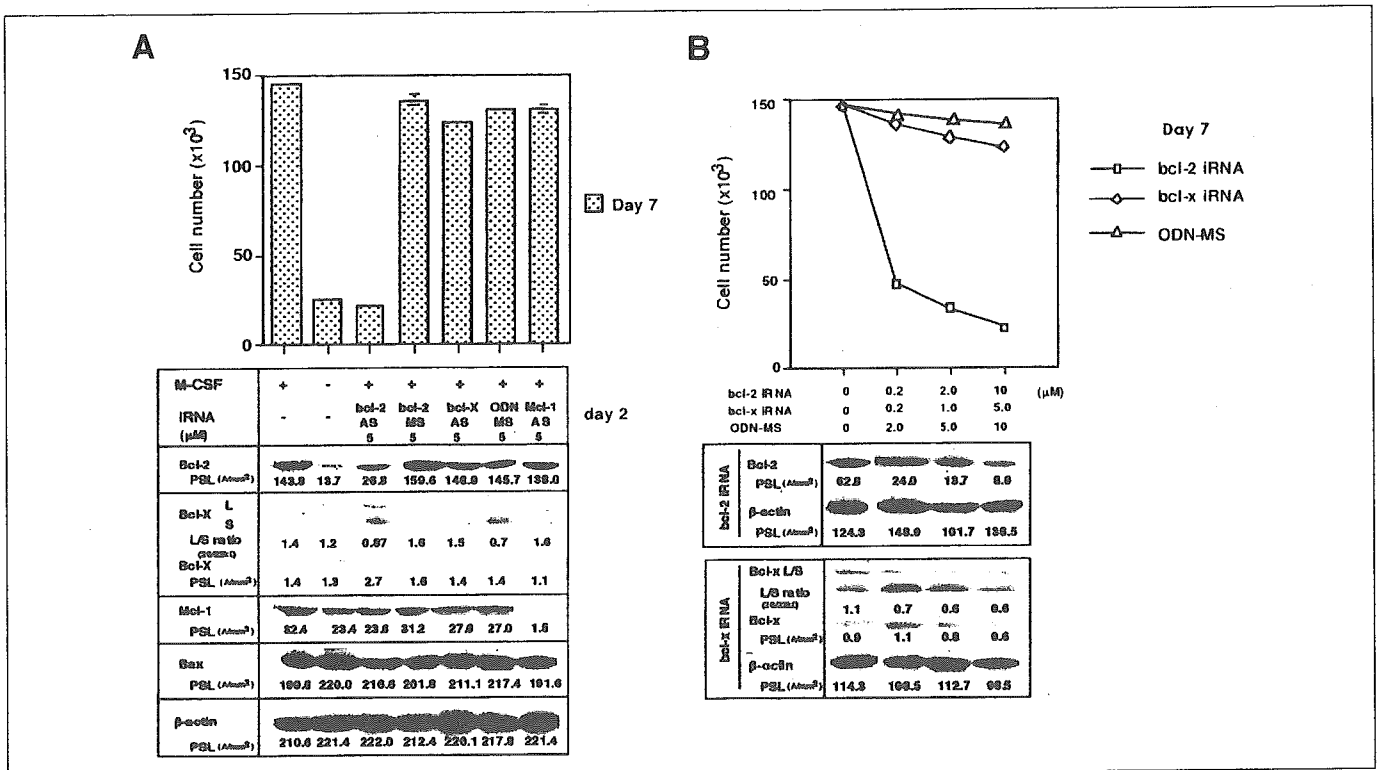


FIGURE 6. BCL-2 AS dominantly induced cell death of M-MΦ. A, cell number and viability of MΦ at 7 days after 5 μM AS treatment (upper panel) and immunoblot analysis at 2 days (lower panel). B, cell number and viability of MΦ at 7 days after the indicated concentrations of AS treatment (upper panel) and immunoblot analysis at 2 days (lower panel). M-MΦ were cultured in medium supplemented with the indicated concentrations of BCL-2 AS (G3139), BCL-2 MS (G4126), 5'-BCL-X AS (BCL-X AS), MCL-AS or control random sequence (oligonucleotide MS). Cell number and viability of MΦ were assessed as described in Fig. 1. Values are expressed as the means of triplicate cultures ± S.D. Western blot analysis of BCL-2, BCL-X_{L/S}, MCL-1, and β-actin proteins in cell lysates of MΦ probed using BCL-2, BCL-X (antibody from Santa Cruz Biotechnology for X_{L/S} band, from Signal Transduction Lab for X_L band only), and β-actin antibody was performed as described in Fig. 4. The relative amounts of these proteins in cells were measured using NIH image software, and the expression levels were shown as photo-stimulating luminescence (PSL).

the levels of thiol derivatives were constant in these MΦs with CSF withdrawal-induced oxidative stress and that thiol derivatives have only a minor effect and cannot support the full survival of MΦ.

BCL-2 AS and BCL-X_L AS Dominantly Induce Cell Death of M-MΦ and GM-MΦ, Respectively—M-MΦ and GM-MΦ were treated with BCL-2 AS targeted to BCL-2 initiation codon (G3139) and 5'-BCL-X AS targeted to the downstream alternative 5'-splice site of exon 2 of the BCL-X gene (12, 13). As controls, these MΦs were treated with BCL-2 MS (G4126, variant G3139), control oligonucleotides with random sequence (oligonucleotide MS), or Lipofectin alone. As shown in Fig. 6, BCL-2 AS (G3139) down-regulated the expression of BCL-2 protein in M-MΦ to 10–20% of that of control cells at 2 days after the oligonucleotide treatment and induced cell death in a dose-dependent manner. The cell viability markedly decreased to ~15% of that of control cells at 7 days of cultivation. In GM-MΦ, however, treatment with G3139 even in the high dose such as 10 μM induced only ~10% cell death, in agreement with the low expression of BCL-2 protein in this MΦ (Fig. 7).

In contrast, treatment of GM-MΦ with 5'-BCL-X AS down-regulated the expression of BCL-X_L protein to 20% of that of control cells treated with oligonucleotide MS at 2 days after the oligonucleotide treatment and induced the cell death in a dose-dependent manner (Fig. 7). The cell viability markedly decreased to ~25% of that of control cells at 7 days of cultivation. As shown in Fig. 6, however, ~90% of the cells are viable in M-MΦ treated with 5 mM 5'-BCL-X AS in agreement with the low expression of BCL-X_L protein.

MCL-1, a main molecule of BCL-2 family protein (14), is expressed in both M-MΦ and GM-MΦ, but down-regulation of this protein by treatment with MCL-1 AS did not stimulate the cell death of either MΦ or

affect the expression levels of BCL-2 in M-MΦ and BCL-X_L in GM-MΦ. These findings suggest that BCL-2 and BCL-X_L expression supported by catalase prevents cell death of M-MΦ and GM-MΦ, respectively, in agreement with the dominant expression levels of gene and protein of BCL-2 in M-MΦ and BCL-X_L in GM-MΦ.

DISCUSSION

The present study showed that extracellular catalase has a novel role in the prevention of apoptosis in human MΦ through the dominant expression of BCL-2 in M-MΦ and BCL-X_L in GM-MΦ and that the regulation of catalase production is CSF-dependent in M-MΦ but CSF-independent in GM-MΦ and A-MΦ. Recently, H₂O₂ has been shown to enhance oxidative damage and apoptosis in C2-ceramide-pretreated HL-60 cells via a mechanism in which C2-ceramide inhibits catalase activity by increasing caspase-3-dependent proteolysis of catalase and down-regulation of catalase mRNA (25). Overexpression of catalase inhibits oxidation-mediated apoptosis through phosphorylated BCL-2, a reduced form of BCL-2 and BAX interaction (20). Thus, constant high activity of extracellular catalase plays an important role in the prevention of ceramide- and caspase-3-induced apoptosis in CSF independent of GM-MΦ and A-MΦ and CSF dependent of M-MΦ (11, 26).

GM-CSF and M-CSF stimulate catalase induction during the differentiation of Mo into MΦ, but GM-CSF alone establishes a CSF-independent autoregulatory system of catalase production in MΦ. We previously showed that human Mo-derived GM-MΦ resembles human A-MΦ in several respects (2, 5–7). In this study, we showed that GM-MΦ and A-MΦ have a similar phenotype of the resistance to apoptosis via CSF-independent expression of catalase and BCL-X_L. Con-

Catalase Inhibits CSF-depleted MΦ Death via BCL-2 and BCL-X_L

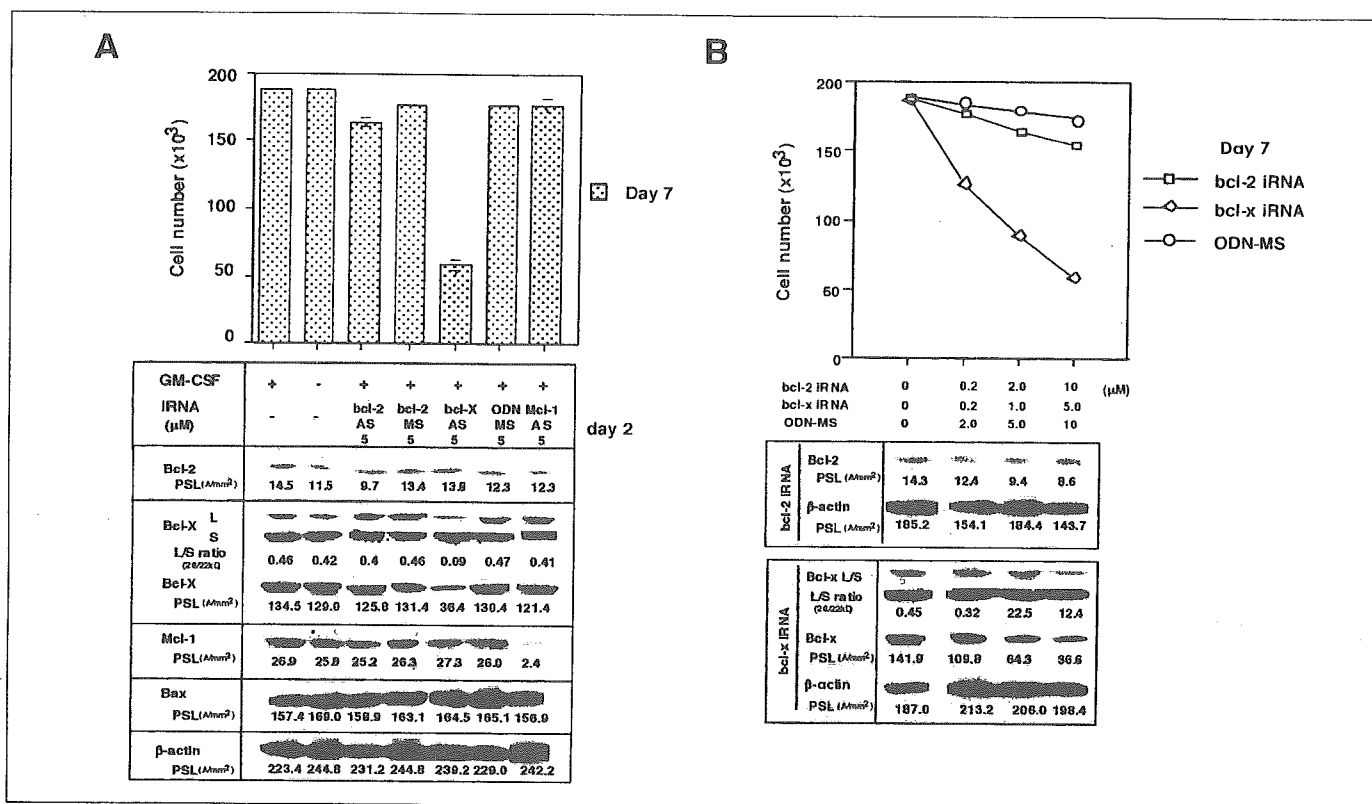


FIGURE 7. BCL-X AS dominantly induced cell death of GM-MΦ. A, cell number and viability of MΦ at 7 days after 5 μM AS treatment (upper panel) and immunoblot analysis at 2 days (lower panel). B, cell number and viability of MΦ at 7 days after the indicated concentrations of AS treatment (upper panel) and immunoblot analysis at 2 days (lower panel). The experimental procedure was performed as described in Fig. 6.

sistent with our present study, A-MΦ from human smokers express higher levels of p21^{CIP1/WAF1} and BCL-X_L, but not BCL-2, and the former two molecules may reduce apoptosis (9). The autoregulatory mechanism of catalase induction in GM-MΦ and A-MΦ is not yet understood but may have a strong correlation with endogenously generated low levels of H₂O₂ (8), because we previously showed that GM-MΦ and A-MΦ, but not M-MΦ, can increase catalase expression at both the protein and mRNA levels when stimulated with H₂O₂ (2). Adequate low levels of H₂O₂ may keep catalase activity constant to prevent CSF deprivation-induced apoptosis in GM-MΦ and A-MΦ.

Thiol derivatives such as GSH, adult T cell leukemia-derived factor, and L-cysteine play an important role in the survival of lymphocytes or neuronal cells in the absence of growth factors (22–24). In our study, however, the activity of thiol derivatives was not significantly different between M-MΦ and GM-MΦ before and after CSF deprivation, and they had only a partial effect on MΦ survival. The reason for the differences between our study and previous studies might have been the differences in the experimental conditions (CSF-withdrawal *versus* serum depletion) or the cell type examined (MΦ *versus* lymphocytes/neuronal cells). Compared with MΦ, lymphocytes or neuronal cells may produce lower levels of catalase so that the thiol derivatives play a dominant role in their survival (21–24).

We demonstrated that catalase regulates apoptosis through the expression of BCL-2 and BCL-X_L in human MΦ used in the present study. In fact, down-regulation of these proteins by RNA interference treatment induced the cell death of the MΦs. Proapoptotic BCL-2 family protein, BAX, can induce apoptosis with permeabilization of mitochondrial membranes and cytochrome *c* release (10, 18, 27). In a recent study, induction of apoptosis of TF-1 cells by GM-CSF withdrawal is shown to be related to down-regulation of the *MCL-1* gene, and over-

expression of *MCL-1* is shown to delay apoptosis (16). In our study, however, BAX and *MCL-1* were not associated with the regulation of apoptosis of MΦ, because both mRNA and protein levels of these genes were not significantly changed in M-MΦ and GM-MΦ after CSF deprivation. Moreover, we showed that down-regulation of the expression of MCL-1 protein by MCL-1 AS treatment did not affect the viability of the MΦs.

Our interesting finding is that BCL-2 and BCL-X_L are differently expressed in MΦ during the Mo differentiation into MΦ in the presence of M-CSF and GM-CSF; the expression of BCL-2 is dominant in M-MΦ and that of BCL-X_L is dominant in GM-MΦ or A-MΦ. In accordance with such different expression patterns, we found by RNA interference experiments that BCL-2 and BCL-X_L play a critical role for the survival of M-MΦ and GM-MΦ, respectively. Similar differential expression of BCL-2 and BCL-X_L is also observed during the selection and maturation of mouse thymocytes toward splenic T cells (17, 19). At present, we do not know the mechanisms that control the different induction of BCL-2 and BCL-X_L in M-MΦ and GM-MΦ or A-MΦ, respectively. The distal promoter region of the BCL-X_L gene responds to very low levels of H₂O₂ at exon 1B-1D in rodent cardiac myocytes, and the expression of BCL-X_L protein is increased by H₂O₂ treatment (28). Thus, GM-MΦ and A-MΦ, which possess high catalase activity even in the absence of CSF, limit the levels of endogenously generated H₂O₂ to low levels that are suitable for BCL-X_L expression.

In conclusion, the present study is the first to reveal that CSF is a critical regulator of extracellular catalase activity that maintains selective expression of BCL-2 family genes and prevents tissue-specific MΦ from apoptosis. These distinct patterns of CSF-induced regulation of catalase activity may greatly contribute to the oxidant stress-induced selection of tissue MΦs suitable for their respective microenvironments.

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Functional heterogeneity of colony-stimulating factor-induced human monocyte-derived macrophages

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Objectives: Macrophages (Mφs) have various functions and play a critical role in host defense and the maintenance of homeostasis. Mφs exist in every tissue in the body, but Mφs from different tissues exhibit a wide range of phenotypes with regard to their morphology, cell surface antigen expression and function, and are called by different names. However, the precise mechanism of the generation of macrophage heterogeneity is not known. In the present study, the authors examined the functional heterogeneity of Mφs generated from human monocytes under the influence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage-CSF (M-CSF).

Methodology: CD14 positive human monocytes (Mos) were incubated with M-CSF and GM-CSF for 6–7 days to stimulate the generation of M-CSF-induced monocyte-derived Mφs (M-Mφs) and GM-CSF-induced monocyte-derived Mφs (GM-Mφs), respectively. The expression of cell surface antigens and several functions such as antigen presenting cell activity, susceptibility to oxidant stress, and the susceptibility to HIV-1 and *mycobacterium tuberculosis* infection were examined.

Results: GM-Mφs and M-Mφs are distinct in their morphology, cell surface antigen expression, and functions examined. The phenotype of GM-Mφs closely resembles that of human Alveolar-Mφs (A-Mφs), indicating that CSF-induced human monocyte-derived Mφs are useful to clarify the molecular mechanism of heterogeneity of human Mφs, and GM-Mφs will become a model of human A-Mφs.

Key words: catalase, colony-stimulating factors, HIV-1, macrophages, *mycobacterium tuberculosis*.

INTRODUCTION

Macrophages (Mφs) have various functions beside phagocytosis such as bactericidal activity, antigen-presentation, tumor cytotoxicity, removal of aged or damaged cells, repair of injured tissue, bone resorption and special lipid metabolism. Mφs produce and secrete a large array of small- and macro-molecules that mediate the recruitment of hematopoietic or other cells, control cell growth, differentiation and function, influence vascular permeability in inflammatory sites and attack target microorganisms. Therefore, Mφs play a critical role in the host defense and the maintenance of homeostasis.

Mφs exist in practically every tissue in the body, but Mφs from different tissues exhibit a wide range of phenotypes with regard to their morphology, cell surface antigen expression and function, and are called by different names. Mφs originate from hematopoietic stem cells in bone marrow, they are predominantly derived from circulating blood monocytes (Mos),¹ which enter various tissues and differentiate into tissue specific Mφ populations² under the influence of the microenvironment. The mechanisms mediating the terminal differentiation and heterogeneity of Mφs, however, are not well known but are of fundamental significance.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage-CSF (M-CSF) are two hematopoietic growth factors that are both implicated in regulating production and function of cells of Mo/Mφ lineage.^{3–5} From the studies of mice, it is clear that GM-CSF regulates the phenotype and functions of Alveolar-Mφs (A-Mφs) critical to surfactant homeostasis and host defense.^{6–8} In contrast, it is clear that

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M-CSF plays an important role in the development, proliferation and differentiation of osteoclasts and certain tissue Mφ populations such as peritoneal Mφs.⁹ Therefore, Mos recruited to the sites where either CSF is dominant might differentiate into a divergent phenotype of Mφs, such as A-Mφs and peritoneal Mφs. In agreement with these studies, the authors' recent studies in human Mos indicate that M-CSF and GM-CSF stimulate the generation of two phenotypically distinct types of Mφs (M-Mφs and GM-Mφs), and the phenotype of GM-Mφs resembles that of human A-Mφs.

METHODS

Cytokines

Recombinant human (rh) GM-CSF (1×10^8 U/mg) and rhM-CSF (1×10^8 U/mg) were provided by Schering-Plough Japan (Osaka, Japan) and Morinaga Milk Industry Co. Ltd. (Tokyo, Japan), respectively.

Preparation of monocyte-derived macrophages

Mos were obtained from PBMC of normal healthy volunteers using a magnetic cell separation system (MACS: Miltenyi Biotec, Bergisch Gladbach, Germany) with anti-CD14 mAb-coated microbeads. CD14⁺ Mos were cultured in RPMI 1640 medium (Nissui Seiyaku Co. Ltd, Tokyo, Japan) supplemented with 10% heat-inactivated fetal calf serum (FCS: Z. L. Bockneck Laboratories Inc., Ontario, Canada) in the presence of the following human recombinant cytokines at optimal concentrations: 5 ng/mL GM-CSF (Schering-Plough Japan, Osaka, Japan) or 50 ng/mL M-CSF (Morinaga Milk Industry Co. Ltd.) at 37°C under a humidified 5% CO₂ atmosphere for 7 days. During the culture, Mos differentiated to Mφs. Human A-Mφs were obtained from healthy volunteers (non-smokers without diseases) by the BAL method. All volunteers gave informed consent to the use of their A-Mφs for part of this study.

Measurement of catalase activity

Intracellular and extracellular catalase activity was measured according to the method described by Aebi.¹⁰

HIV-1 infection and antisense treatment of Hck and C/EBPβ

Macrophage-tropic HIV-1 strains (HIV-1_{BaL}, HIV-1_{JR-FL}) were used. Phosphorothioate-modified antisense oligonucleotides for Hck (AS-Hck: 5'-TTCATCGACC CCATCCTGGC-3') and C/EBPβ (AS-C/EBPβ: 5'-CAG GCGTTCATGAACGCGG-3'), and their corresponding sense oligonucleotides (S-Hck: 5'-GCCAGGATG GGGTCGATGAA-3' and S-C/EBPβ: 5'-CCGCGTTCAT GCAACGCCTG-3'), and their unrelated nonsense oligonucleotides (NS-Hck: 5'-CCATATTTCCCGCTCGC

GTG-3' and NS-C/EBPβ: 5'-CCAGAGAGGGCCCGTGT GGA-3') were synthesized.

Mycobacterium tuberculosis (*mycobacterium tuberculosis*) infection and colony-forming unit assay

Mφs were infected with single-cell suspension of *mycobacterium tuberculosis* H37Rv at MoI of 1–2. Infected Mφs were cultured for 5–7 days in an antibiotics-free culture medium and the number of bacteria in the culture supernatant and within the cells were counted with a colony-forming unit (CFU) assay using Middlebrook 7H10 agar supplemented with OADC. For the determination of the number of intracellular bacteria, Mφs were lysed by the addition of 1 mL of distilled water supplemented with 0.05% Tween 80.

T cell proliferation assay

PBMC (10^5 cells/well) with or without Mφs were stimulated with 10 μg/mL PPD for 5 days in a 96-well microtiter plate at 37°C, and T cell proliferation was determined by the ³H-thymidine incorporation assay.

Assay for IL-10 and γ-interferon activities

Culture supernatants were harvested from Mφs and Mφs plus PBMC stimulated with or without PPD for 24 h and 72 h for the assay of IL-10 and γ-interferon (IF), respectively. The levels of IFN-γ and IL-10 were determined by an ELISA (Endogen).

Statistical analysis

Statistical analysis was performed with the Student's *t*-test.

RESULTS

Monocyte-derived macrophages induced by M-CSF and GM-CSF are different in their morphology and cell surface antigen expression

When blood Mos were cultured in medium alone *in vitro*, Mos died, and CSF such as M-CSF or GM-CSF were necessary for their survival and differentiation into Mφs.¹¹ Mφs induced by M-CSF (M-Mφs) had an elongated and spindle-like morphology, though some small and round cells remained, but Mφs induced by GM-CSF (GM-Mφs) were round and had a fried egg-like morphology. As shown in Figure 1, the expression of cell surface antigens were different between M-Mφs and GM-Mφs.^{12–14} The phenotype of GM-Mφs (fried egg-like shape and c-fms^{low}, CD14^{low}, CD71⁺ and 710F⁺) closely resembled that of human A-Mφs. In contrast, the morphology and the expression of cell surface antigens (c-fms^{high}, CD14^{high}) of M-Mφs resembled that of human peritoneal Mφs or influx Mφs induced by inflammation.

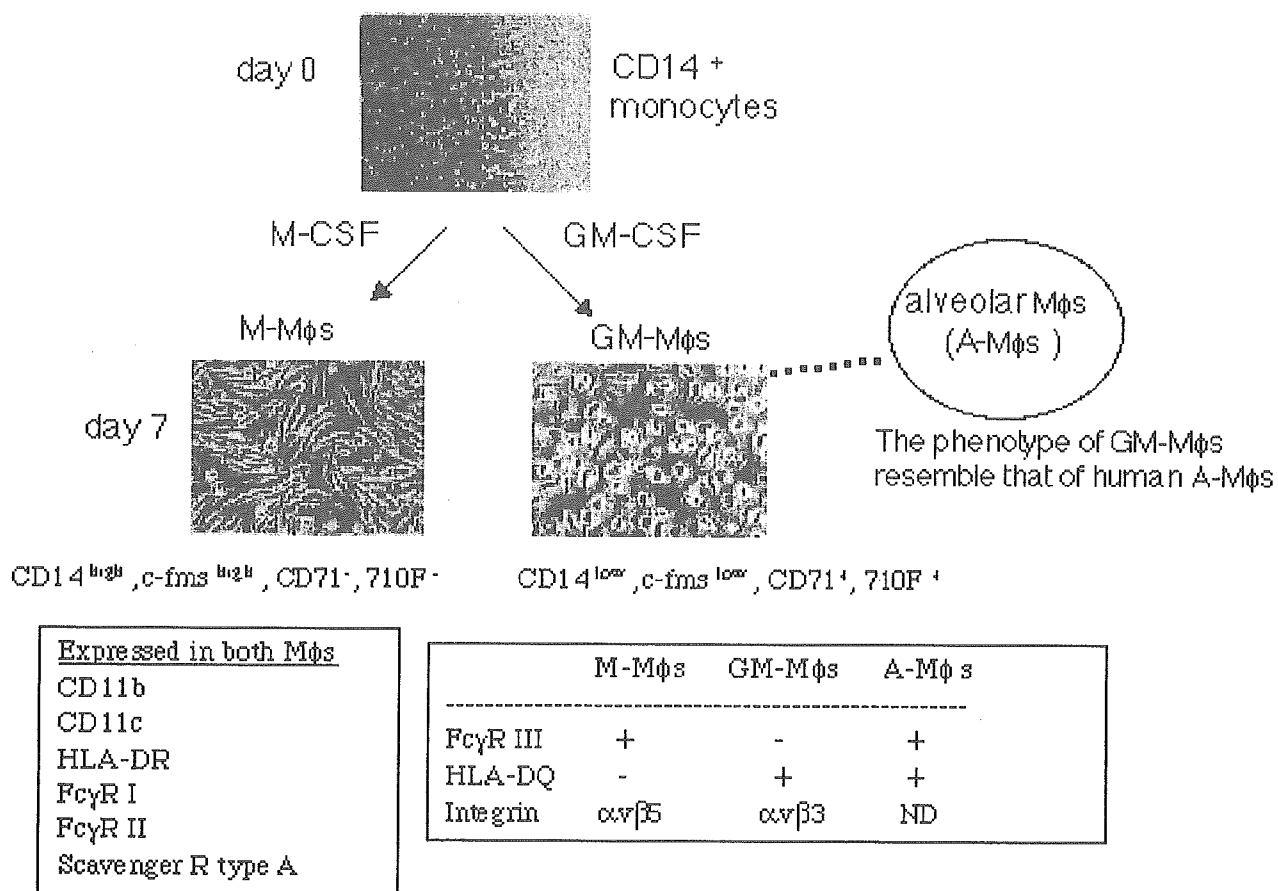


Figure 1 Effects of colony-stimulating factors on the differentiation of monocytes into macrophages.

Monocyte-derived macrophages induced by GM-CSF and M-CSF are different in their capacity for FcγR-mediated phagocytosis

Both M-Mφs and GM-Mφs ingested latex particles, but their capacity for FcγR-mediated phagocytosis was significantly different. More than 98% of M-Mφs phagocytized sensitized erythrocytes (EA), whereas only about 30% of GM-Mφs did.¹³ Both types of Mφ could form rosettes in a similar manner with EA. Therefore, the defect in GM-Mφs may be located at the level of the signal transduction pathway need for ingestion after the binding of EA to FcγR.

Monocyte-derived macrophages induced by GM-CSF and alveolar macrophages but not monocyte-derived macrophages induced by M-CSF are resistant to H₂O₂ via their high basal and inducible levels of catalase activity

Mφs produce and release reactive oxygen species (ROS), such as H₂O₂, O₂⁻, and OH, in response to phagocytosis or stimulation with various agents through activation of a multicomponent NADPH oxidase. ROS are essential for the killing of a wide variety

of microbes. In addition, ROS have been implicated in inflammation and tissue injury. M-Mφs produced a large amount of H₂O₂ compared with GM-Mφs in response to zymosan or heat-killed *Staphylococcus aureus*.¹⁵ GM-Mφs were highly resistant to H₂O₂, and expressed high levels of catalase activity. In contrast, M-Mφs expressed low catalase activity, and were about 50-fold more sensitive to H₂O₂ than GM-Mφs. The antioxidant state of A-Mφs was at a similar level to that of GM-Mφs by assessment of H₂O₂ sensitivity and catalase activity. About 1–10 mmol/L H₂O₂, similar to levels found on exhalation in cases of adult respiratory distress syndrome, did not induce cell death of either GM-Mφs or A-Mφs. GM-Mφs and A-Mφs but not M-Mφs could induce catalase expression in both protein and mRNA levels when stimulated with H₂O₂ or zymosan. These findings indicate that GM-Mφs and A-Mφs but not M-Mφs are strong scavengers of H₂O₂ via a high basal level of catalase activity and a marked ability of catalase induction.

Different susceptibility of monocyte-derived macrophages induced by M-CSF and GM-CSF to macrophage-tropic HIV-1

Recent studies indicate that human Mos/Mφs are a primary target for initial HIV-1 infection and a

major reservoir for latent infection.^{16,17} M-Mφs had a great capacity to produce HIV-1, while GM-Mφs inhibited HIV-1 replication.^{18,19} Similar levels of viral DNA formation were observed in both types of Mφ after HIV-1 infection, indicating that the inhibition of HIV-1 replication in GM-Mφs occurs at post-transcriptional and translational levels but not at the viral entry. M-Mφs expressed a high level of Hck, which can bind HIV-1 Nef and a large isoform of C/EBPβ, and HIV-1 infection increased the expression of Hck but not of C/EBPβ. GM-Mφs expressed a high level of C/EBPβ and a low level of Hck, and HIV-1 infection drastically increased the expression of a short isoform of C/EBPβ, which acts as a dominant negative transcription factor, but decreased that of Hck. Treatment of M-Mφs with antisense oligonucleotide for Hck not only suppressed the expression of Hck, but also stimulated the induction of the short isoform of C/EBPβ and inhibited viral replication. Treatment of GM-Mφs with antisense oligonucleotide for C/EBPβ not only preferentially inhibited the expression of the small isoform of C/EBPβ, but also stimulated the induction of Hck and stimulated the virus production at a high rate. These results suggest that the CSF-induced and HIV-1-mediated distinct regulation of Hck and small isoform of C/EBPβ represent the heterogeneous susceptibility of tissue Mφs to HIV-1 infection. The authors also found that A-Mφs from healthy volunteers dominantly expressed a low level of Hck and small isoform of C/EBPβ and hardly stimulated HIV-1 replication, indicating GM-Mφs resemble A-Mφs in their susceptibility to HIV-1 infection.

Monocyte-derived macrophages induced by M-CSF but not GM-CSF can inhibit the growth of *Mycobacterium tuberculosis* and kill the bacteria

M. tuberculosis is a major human pathogen that resides in the host lung as a facultative intracellular pathogen and is found primarily in A-Mφs. M-Mφs inhibited the growth of *M. tuberculosis* H37Rv and killed the bacteria, while GM-Mφs stimulated the growth. H₂O₂ and NO were not the effector molecules of M-Mφs against *M. tuberculosis*, because addition of catalase or a NO synthesis inhibitor did not significantly change the bacterial killing activity of M-Mφs, and the levels of NO produced by infected M-Mφs and GM-Mφs were very low and no significant difference was observed between them. When Mφs were infected with *M. tuberculosis*, augmented expression of NRAMP1 (SLC11A1) and strong activation of p38MAPK, ERK1/2 and JNK were observed in M-Mφs but not in GM-Mφs. These results indicate that the difference in the activation of MAPK and the augmented expression of NRAMP1 may be related to the distinct susceptibility of M-Mφs and GM-Mφs to *M. tuberculosis*.

Monocyte-derived macrophages induced by M-CSF but not GM-CSF produce IL-10 and inhibit the γ-interferon production by T cells

M-Mφs and GM-Mφs are distinct in their suppressor activity. M-Mφs and GM-Mφs equally suppressed T cell proliferation in PBMC stimulated with PPD, but only M-Mφs suppressed IFN-γ production by T cells in response to PPD.²⁰ M-Mφs but not GM-Mφs released a large amount of IL-10 in response to PPD, and the neutralizing antibody to IL-10 reversed the suppressor activities of M-Mφs on IFN-γ production but not lymphocyte proliferation. Production of IFN-γ by lymphocytes was markedly inhibited by exogenous IL-10, while exogenous IL-10 was not as potent as M-Mφs for suppressing lymphocyte proliferation. Therefore, the mechanism of M-Mφ mediated T cell suppression for IFN-γ production is largely attributed to IL-10. A-Mφs from rodents and humans selectively inhibit T-cell proliferation but permit cytokine secretion, such as interleukin-2 and IFN-γ.²¹ In respect to the lacking of suppressor activity for IFN-γ production, but not for proliferation, by T cells, GM-Mφs resemble A-Mφs.

Down-regulation of IL-10 production by GM-Mφs may be beneficial for host defense mechanisms against bacterial and viral infection to augment Th1 response. In contrast, M-Mφs may play important roles in suppressing lymphocyte responses through potent immunosuppressive activity. Therefore, divergent macrophages play appropriate roles in different situations to maintain homeostasis in the host.

DISCUSSION

The present study shows that GM-CSF and M-CSF stimulate the differentiation of human monocytes into two phenotypically distinct types of macrophages *in vitro*. The phenotype and functions of GM-Mφs resembles that of human A-Mφs, indicating that GM-CSF plays an important role in the differentiation of human A-Mφs. In the serial analysis of gene expression of monocytes, GM-Mφs and M-Mφs identified the specific gene expression in these cells along with the expression of large and overlapping sets of genes.^{22,23} In addition, recent studies indicate the critical role of transcription factor PU.1 in GM-CSF-dependent terminal differentiation of mouse A-Mφs.²⁴ However, the precise molecular mechanisms of the difference of GM-CSF- and M-CSF-dependent differentiation of Mφs are not yet fully known. A model system using CSF-induced human Mo-derived M-Mφs and GM-Mφs may contribute the precise analysis of the molecular mechanisms.

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結核菌薬剤感受性検査のための BACTEC MGIT 960 AST の評価：外部精度管理菌株を用いた研究

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要旨：〔目的〕迅速な結核菌薬剤感受性検査法である BACTEC MGIT 960 結核菌薬剤感受性検査用ミジットシリーズ (Mycobacterium Growth Indicator Tube Antimicrobial Susceptibility Testing: MGIT AST) の精度を調べた。〔材料と方法〕評価には WHO/IUATLD が薬剤感受性検査の外部精度アセスメントに使用している菌株と結核菌 H37Rv を用いた。MGIT AST と Middlebrook 7H10 寒天培地による比率法 (比率法) でイソニアジド (INH), リファンピシリン (RFP), ストレプトマイシン (SM), エタンブトール (EB) に対する感受性を測定した。得られた結果は WHO/IUATLD の Supranational Reference Laboratory Network (SRLN) の結果と比較した。〔結果〕INH と RFP の検査では, SRLN の成績を標準としたとき MGIT AST の感度, 特異性, 一致率, 再現性のいずれも 100% であった。SM の検査について, MGIT AST の SRLN の結果との一致率は 97.9%, EB の検査は 91.5% であり, 主要 4 薬剤について 90% 以上の一貫性を示した。〔結論〕今回の結果から MGIT AST は WHO/IUATLD が目標として掲げた薬剤感受性検査の精度, すなわち検査の感度, 特異性, 再現性および一致率を満足させる迅速な検査であると考えられる。

キーワード：MGIT AST, 薬剤感受性検査, 結核菌, WHO/IUATLD

はじめに

薬剤感受性検査の結果は患者の治療のみならず薬剤耐性結核菌の出現にも影響を与えることから, 検査の精度管理は重要である。薬剤感受性検査は抗酸菌の検査の中で最も精度管理の難しい検査である。世界保健機関 (World Health Organization: WHO) と国際結核肺疾患予防連合 (International Union Against Tuberculosis and Lung Disease: IUATLD) は世界的規模で薬剤耐性結核のサーベイランスを実施するにあたり, 世界で 20 数カ所の研究所または大学の研究室を Supranational Reference Laboratory (SRL) に選び, 薬剤感受性検査の精度管理研究をスタートさせた¹⁾。WHO/IUATLD のコーディネーターから SRL に薬剤耐性菌を含む結核菌を送付し, SRL のネットワーク (SRLN) で標準化し, それらの菌株をサーベイランス実施国の精度管理に用いている。わが国でも日本結核病学会抗酸菌検査法検討委員会が上記の菌株を用

いて医療機関や検査センターを対象に薬剤感受性検査の外部精度アセスメントを実施しており²⁾³⁾, その結果手技の習熟や使用する方法による特性を把握することなど, 改めて精度管理の重要性が明らかになった。

検査の所要日数の長短は患者の治療や管理に影響することから, 時宜にかなった検査結果の報告は微生物検査室に課せられた重要な責務である。わが国で現在用いている小川培地による結核菌の薬剤感受性検査は結果を得るまでに約 1 カ月を要することから, より短時間で結果が得られる検査が望まれている。米国の Centers for Disease Control and Prevention (CDC) から出された検査の所要日数についての提案では, 検体入手後結核菌の薬剤感受性検査の結果を 30 日以内に臨床医に報告することとしており⁴⁾⁵⁾, これは固形培地を用いた従来法で達成することは不可能である。液体培地を用いる BACTEC 460 TB や MGIT AST システムの迅速性と有用性に関する報告は数多くみられる^{6)~12)}。米国やヨーロッパ諸国では液

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体培地を用いる薬剤感受性検査が推奨されており、わが国でも今後普及していくものと思われる。

以前にわれわれは臨床分離株を用い MGIT AST と小川法の成績を比較した結果を報告した¹⁰⁾。今回 WHO/IUATLD が薬剤感受性検査の外部精度アセスメントに使用している菌株を用い、MGIT AST の精度を再評価したので報告する。

検査材料と方法

(1) 菌株

WHO/IUATLD のコーディネーターから分与された結核菌 50 株と結核菌 H37Rv (ATCC 27294) を評価に用いた。これらの結核菌は WHO/IUATLD の SRLN で標準化された菌株である。

(2) 接種菌液の調製

被検菌を 2% 小川培地に接種後 37°C で約 4 週間培養した。培地表面の集落を満遍なくかきとり 5 mL の Middlebrook 7H9 培地に懸濁後 37°C で静置培養した。毎日 1 回攪拌し、培養液の濁度が McFarland #0.5 を超えるまで培養した。濁度計と滅菌蒸留水を用い、培養液から McFarland #0.5 濁度の菌液を調整した。

(3) MGIT AST

BACTEC MGIT 960 結核菌薬剤感受性検査用ミジットシリーズの添付文書に従い、結核菌薬剤感受性用 MGIT チューブに専用サプリメントと薬剤を添加した。検査に用いた薬剤の濃度は、INH: 0.1 $\mu\text{g}/\text{mL}$, RFP: 1.0 $\mu\text{g}/\text{mL}$, SM: 1.0 $\mu\text{g}/\text{mL}$, EB: 5.0 $\mu\text{g}/\text{mL}$ であった。McFarland #0.5 に調整した菌液を滅菌蒸留水で 5 倍希釈し、薬剤添加 MGIT チューブへの接種菌液とした。コントロール用 MGIT チューブへの接種には、接種菌液を滅菌蒸留水でさらに 100 倍希釈したものをを用いた。菌液を各 MGIT チューブに 0.5 mL 接種後、直ちに BACTEC MGIT 960 全自動抗酸菌培養装置 (MGIT 960) で培養を開始した。MGIT AST は、培養開始 4 日目より 13 日目までの間にコントロール用 MGIT チューブの菌発育を示す蛍光強度が一定値を越えた時点で薬剤添加 MGIT チューブの蛍光強度を測定し、個々の薬剤に対する被検菌の感受性を判定するシステムである。

(4) Middlebrook 7H10 培地による比率法

National Committee for Clinical Laboratory Standards (現 CLSI: Clinical and Laboratory Standards Institute) M24-A に記載されている方法に従い Middlebrook 7H10 寒天培地を調製した¹³⁾。検査に用いた薬剤の濃度は、INH: 0.2 $\mu\text{g}/\text{mL}$, RFP: 1.0 $\mu\text{g}/\text{mL}$, SM: 2.0 $\mu\text{g}/\text{mL}$, EB: 5.0 $\mu\text{g}/\text{mL}$ であった。McFarland #0.5 に調整した菌液を滅菌蒸留水で 100 倍希釈し、薬剤添加 Middlebrook 7H10 寒天培地への接種菌液とした。コントロール用 Middlebrook

7H10 寒天培地への接種には、接種菌液を滅菌蒸留水でさらに 100 倍希釈したものをを用いた。菌液を各培地に塗布後 5%CO₂ 条件下 37°C で培養し、3 週目に判定した。

(5) 評価方法

MGIT AST および Middlebrook 7H10 寒天培地による比率法 (比率法) の結果について、WHO/IUATLD の SRLN の結果を標準として感度、特異性、一致率、耐性的中率 (PV-R)、感受性的中率 (PV-S) を計算し評価した¹⁾。感度は SRLN の結果が耐性のものを正しく耐性と判定した割合、特異性は SRLN の結果が感受性のものを正しく感受性と判定した割合、一致率は SRL の結果がそれぞれの薬剤について耐性、もしくは感受性と判定したものを同様に判定した割合である。PV-R は耐性と判定したとき、その判定が正解である確率、PV-S は感受性と判定したときの正解率である。なお、今回評価に用いた菌株の中で SRLN 内の一致率が 70% 以下の菌株は RFP の検査で 6 株、SM で 3 株、EB で 3 株あり、これらはそれぞれの薬剤についての精度計算から除外した。

MGIT AST の再現性は、被検菌の中から無作為に選んだ 6 菌株を用い、MGIT AST の 4 薬剤について 1 日 3 回、3 日間繰り返し (1 菌株、1 薬剤につき計 9 回の検査) 測定し、SRLN の結果との一致率で評価した。

結果

(1) MGIT AST の再現性試験

無作為に選んだ 6 株を用い MGIT AST システムの再現性を調べた。計 212 検査を行い、全体の一致率は 95.3% であった (Table 1)。INH と RFP の試験の一致率は 100% であったが、SM と EB の試験で SRLN の結果と一部異なる成績がみられた。特に EB の試験で一致率は 90% 以下であった。

(2) 外部精度アセスメント株を用いた MGIT AST システムの評価

WHO/IUATLD の SRLN で精度アセスメントに使用している 50 株の臨床分離結核菌および結核菌標準株 H37Rv を用い MGIT AST の精度を評価した。同時に CLSI が標準法としている Middlebrook 7H10 寒天培地を用いる比率法で検査を行い比較した。

INH 感受性検査では、MGIT AST の結果は SRLN の結果とすべて一致した (Table 2)。感度、特異性、一致率、PV-R、PV-S のいずれも 100% であった (Table 3)。比率法の結果は MGIT AST の成績と同様に SRLN の結果といずれも 100% 一致した。

使用した 50 株のうち、6 株は RFP の感受性検査で SRLN 内の一致率が 70% 以下であったため RFP の精度計算から除外し、44 株の結果で評価した。RFP 感受性検査では INH の検査と同様に MGIT AST の結果は SRLN の

Table 1 Reproducibility testing of the BACTEC MGIT 960 AST system

Drug	No. of results	No. of results agreeing with SRLN	Agreement (%)
Isoniazid	53	53	100
Rifampin	53	53	100
Streptomycin	53	49	92.5
Ethambutol	53	47	88.7
Total	212	202	95.3

Reproducibility was assessed with six strains of *M. tuberculosis* in triplicate from three separately prepared inocula (i.e., nine replicate per strain).

Table 2 Comparison of drug susceptibility test results

Drug	No. of isolates with the following results					
	SRLN	R	R	R	S	S
	MGIT	R	R	S	S	S
Isoniazid		32	0	0	0	18
Rifampin		18	0	0	0	26
Streptomycin		20	1	1	0	25
Ethambutol		15	0	4	2	26

SRLN: Referee results of the WHO/IUATLD Supranational Reference Laboratory Network

MGIT: BACTEC MGIT 960 AST system

7H10: Proportion method on Middlebrook 7H10 agar

Table 3 Comparison of the results with the BACTEC MGIT 960 AST system or proportion method on Middlebrook 7H10 agar with the results of the WHO SRL Network

	Sensitivity	Specificity	PV-R	PV-S	Agreement
BACTEC MGIT AST					
Isoniazid	100	100	100	100	100
Rifampin	100	100	100	100	100
Streptomycin	95.5	100	100	96.2	97.9
Ethambutol	78.9	100	100	87.5	91.5
Proportion method					
Isoniazid	100	100	100	100	100
Rifampin	100	100	100	100	100
Streptomycin	95.5	100	100	96.2	97.9
Ethambutol	100	92.9	90.5	100	95.7

Sensitivity: Ability to detect true resistance

Specificity: Ability to detect true susceptibility

PV-R: Predictive value for resistance

PV-S: Predictive value for susceptibility

結果と完全に一致した。感度、特異性、一致率、PV-R、PV-Sはすべて100%であった。比率法の結果もMGIT ASTとまったく同様であり、いずれも100%であった。

SMに対する感受性検査でSRLNの結果と不一致となった菌株はMGIT ASTと比率法でそれぞれ1株ずつ認められた。それらのうち1株はSRLNの結果が耐性をMGIT ASTで感受性として、別の株はSRLNで耐性と判定した株を比率法で感受性と判定していた (Table 2)。Table 3に示したようにSM検査におけるMGIT ASTおよび比率法のSRLNの結果との一致率は97.9%であった。

EBに対する感受性検査でSRLNの成績と不一致の結

果を示した菌株はMGIT ASTで4株、比率法で2株認められた。MGIT ASTで不一致であった4株はいずれもSRLNで耐性と判定したものを感受性と判定していた。一方比率法で不一致の結果を示した2株ともSRLNで感受性と判定した菌株であり比率法で耐性と判定していた。MGIT ASTのSRLNの結果との一致率は91.5%、比率法の一一致率は95.7%であった。

MGIT AST検査用チューブに菌接種後感受性の検査結果が得られるまでに要した日数は6日～13日の範囲であり、中央値は7日であった。一方比率法の所要日数は3週間であった。

考 察

WHO/IUATLDはINHとRFPの感受性検査の感度、特異性、再現性を95%以上に保つこと、SM、EBを加えた主要4薬剤について全体の一致率を90%以上に保つことを目標として掲げている。今回の実験で、MGIT ASTの結果はINHとRFPについてはすべての菌株でSRLNの結果と一致したことから感度、特異性、一致率、PV-R、PV-Sのすべてが100%であった (Table 3)。またINHおよびRFPの再現性試験の結果も100%であった。さらに、SMの一致率は97.9%、EBの一致率は91.5%であり、4薬剤の一致率は90%以上であった。これらの成績は、MGIT ASTがWHO/IUATLDの掲げた精度目標を満足させる検査であることを示している。なおWHO/IUATLDの検査精度アセスメントでは、SRLNの大多数が報告した結果をその菌株のゴールドスタンダードとして精度を計算しており¹⁾、SRLN内の一致率が70%以下の菌株はその結果をゴールドスタンダードとすることが不適當であると考え精度計算から除外している¹⁵⁾。今回の研究では、SRLNの考えに従い一致率が70%以下の菌株を精度計算から除外した。

SRLNの成績と不一致の結果を示した株がMGIT ASTによるEBの検査で4株、SMの検査で1株みられた (Table 2)。これらの菌株の比率法による検査結果はSRLNと一致していた。EBの検査で不一致の結果を示した4株のうち3株はSRLN内でも80%以下 (78.3%, 78.9%, 78.9%) の低い一致率であり、一部の株では検査法や検査条件により異なる結果が出ることを示された。残りの1株はSRLN内のEB検査で95.5%と高い一致率を示した株である。この株についてはMGIT ASTで検査を数回繰り返したが同じ結果であり、不一致の理由はわからない。SMの検査で不一致の結果を示した1株は、SRLN内での一致率は94.7%と高く、この株についても検査を繰り返したが同じ結果であり不一致の理由は不明である。

1994年からSRLN内で年1回薬剤感受性検査の精度試験を実施している。INHとRFPの検査精度は試験開始以来比較的良好であったが、SMとEBについては開始初期にはSRLN内の一致率が低かった¹¹⁾¹⁴⁾¹⁵⁾。特にEBは再現性を得ることが難しい薬剤であると考えられている^{7)~10)16)17)}。RobertsらはMiddlebrook 7H10寒天培地を用いる比率法をゴールドスタンダードとして比較したときBACTEC 460 TBによるEB検査の感度は66%を超えなかったと報告している¹⁸⁾。今回のMGIT ASTによるEB検査の感度は78.9%であり、他の3薬剤についての検査の感度と比べ明らかに低い値であった。一方、Middlebrook 7H10寒天培地による比率法の感度は100%で

あった。EBの検査に用いている薬剤の濃度はMGIT ASTもMiddlebrook 7H10による比率法も同じ5 μ g/mLであり、MGIT ASTの比較的低い感度は検査に用いた薬剤の濃度とは関係なく検査法の違いに起因しているものと考えられる。EBの検査で不一致の株はすべてSRLNで耐性と判定した株をMGIT ASTで感受性と判定しており、MGIT ASTでは感受性に判定される傾向がみられた。

MGIT ASTは迅速に結果が得られる検査として知られている^{7)~12)}。今回の実験で菌接種後測定が終了するまでの所要日数は6日~13日の範囲 (中央値:7日) であり、高い迅速性が再確認された。検査材料の入手後MGITによる結核菌の検出までに要する平均日数は約2週間である^{19)~23)}。液体培養で陽性を示した培養液の少量を結核菌群特異抗原であるMPB64を検出するキャピリアTBのサンプルウエルに添加し15分静置することで迅速に結核菌群を鑑別できる²⁴⁾²⁵⁾。同定結果が結核菌群であれば陽性MGITチューブの培養液から直接MGIT ASTで薬剤感受性を検査できる。このように、一連の検査に液体培地に基づくMGITシステムを用いることにより、分離、同定、薬剤感受性のすべての検査結果を30日以内に担当医に報告することとする目標を達成することが可能である。

結核の診断をより早期に行うことは患者の治療のみならず、感染拡大の防止、予防、ひいてはわが国の結核罹患率の減少に大きく影響する。迅速かつ精度の高い方法を組み合わせることで早期の診断、治療に有用な検査情報を提供することは微生物検査室の大きな責務である。現在入院期間の短縮が求められており、迅速に薬剤感受性を測定できるMGIT ASTシステムは、今後の結核の診断治療に非常に有用であると考えられる。

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Original Article

BACTEC MGIT 960 SYSTEM FOR DRUG SUSCEPTIBILITY TESTING OF
MYCOBACTERIUM TUBERCULOSIS: A STUDY USING
EXTERNAL QUALITY ASSESSMENT STRAINS¹Ikuo KOBAYASHI, ¹Chiyoji ABE, and ²Satoshi MITARAI

Abstract [Objective] To evaluate the performance of the BACTEC MGIT 960 system for drug susceptibility testing (MGIT AST) of *Mycobacterium tuberculosis* to isoniazid, rifampin, streptomycin and ethambutol.

[Design] Fifty external quality assessment strains of *M. tuberculosis* provided by the Coordinating Centers of WHO/IUATLD were tested by BACTEC MGIT 960 system, and the results were compared with the referee results of the WHO/IUATLD Supranational Reference Laboratory Network (SRLN).

[Results and conclusion] Overall concordance rates of the results obtained by MGIT AST and the referee results of the SRLN were 97.3% for four first-line drugs. Agreement rates were particularly high for isoniazid, rifampin, and streptomycin (agreement rate of over 97%), but somewhat lower for ethambutol, which relates to a lower sensitivity of MGIT AST. Turnaround times from inoculation to drug susceptibility results ranged from 6 to 13 days for the MGIT AST system with a median time of 7 days; this contrasted with three weeks for the proportion method using Middlebrook 7H10 agar,

indicating that MGIT AST system has the potential to consistently meet with the turnaround time guidelines suggested by the Centers for Disease Control and Prevention of the United States. These results demonstrate that the fully automated BACTEC MGIT 960 AST system is useful for the rapid diagnosis of drug resistant tuberculosis.

Key words: MGIT AST, Susceptibility test, *Mycobacterium tuberculosis*, WHO/IUATLD

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CASE REPORT

Incipient stage of pulmonary Langerhans-cell histiocytosis complicated with pulmonary tuberculosis was examined by high-resolution computed tomography

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KEYWORDS

Pulmonary Langerhans-cell histiocytosis; High-resolution computed tomography; Tuberculosis

Summary A 29-year-old man with a smoking history of 20 pack years was diagnosed with tuberculosis because *Mycobacterium tuberculosis* was detected in his gastric juice. His chest radiograph showed multiple nodular opacities on left upper lobe. He was treated with anti-tuberculosis drugs in our hospital. Six months after the therapy, he began to complain of dry cough and shortness of breath on exertion. Chest radiograph showed reticular opacities in both upper and middle lung fields. Multiple thick- and thin-walled bizarre-shaped cysts and ill-defined centrilobular opacities were seen mainly in upper and mid-lungs on high-resolution computed tomography (HRCT). He was diagnosed with pulmonary Langerhans-cell histiocytosis (PLCH) by transbronchial lung biopsy specimens from right upper lobe. HRCT findings before the treatment of tuberculosis were retrospectively examined. Several small thin-walled centrilobular cysts were scattered in the right upper and middle lung fields without apparent nodular lesions. We must consider the possibility of PLCH when these findings are detected on HRCT.

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Introduction

Pulmonary Langerhans-cell histiocytosis (PLCH), previously referred to as pulmonary histiocytosis X or eosinophilic granuloma, is a disease of unknown etiology and pathologically characterized by

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granulomas containing a large number of Langerhans cells localized in the lungs.¹ Although progression of the disease has been examined by high-resolution computed tomography (HRCT), the incipient stage of the disease is hard to examine because it insidiously progresses and patients with PLCH are asymptomatic up to 25%.¹ We could examine HRCT findings in a very early stage in this case.

Case report

A 29-year-old man with a smoking history of 20 pack years visited our hospital because of general fatigue. His chest radiograph showed multiple nodular opacities in the left upper lung field. By HRCT examination (Fig. 1a), large nodular lesions were surrounded with multiple small centrilobular nodules occurring in clusters and appearance of

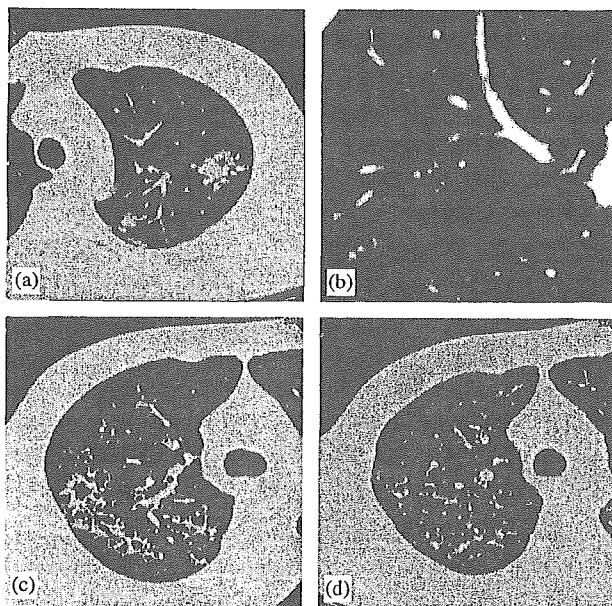


Figure 1 Radiological findings before treatment of tuberculosis (a and b) and after the onset of pulmonary Langerhans-cell histiocytosis (PLCH) (c and d). Before treatment of tuberculosis, high-resolution computed tomography (HRCT) of the left upper lobe showed large nodular lesions surrounded by multiple small centrilobular nodules occurring in clusters and appearance of tree-in-bud (a). Several small thin-walled centrilobular cysts were scattered in the right upper and middle lung field, although apparent nodular lesions suggesting PLCH were not detected (b). After the onset of PLCH, multiple thick- and thin-walled cysts and ill-defined centrilobular opacities were seen mainly in the upper and mid-lungs on HRCT (c). Thickness of the cysts was lessened and apparent centrilobular nodules decreased on HRCT after the cessation of smoking (d).

tree-in-bud. *Mycobacterium tuberculosis* was detected by culturing his gastric juice, and he was diagnosed with pulmonary tuberculosis. Administration of anti-tuberculosis drugs improved his general fatigue and nodular lesions on the chest radiograph decreased. Six months after the therapy, he began to complain of dry cough and shortness of breath on exertion. He visited our hospital again, because of the worsening of symptoms and occurrence of weight loss.

The chest radiograph on admission showed reticular opacities in both the upper and middle lung fields and the costophrenic angles were spared. Multiple thick- and thin-walled cysts and ill-defined centrilobular opacities were seen mainly in the upper and mid-lungs on HRCT (Fig. 1c). Some cysts showed bizarre shapes and branching appearance. The intervening lung parenchyma appears normal and without evidence of fibrosis or septal thickening. The well-defined nodular lesion supposed to be old tuberculosis was detected in the left upper lobe.

Cryptococcus antigen was not detected in his serum. Pathogenic organisms, including *Pneumocystis carinii*, or malignant cells were not detected in bronchoalveolar lavage (BAL) fluid performed in right B⁵. Transbronchial lung biopsy specimens from the right upper lobe showed bronchiolocentric nodular lesions, including proliferation of Langerhans cells with delicate and folded nuclei, and scattered eosinophils. He was diagnosed with PLCH.

HRCT findings before the treatment of tuberculosis were retrospectively examined. Although apparent nodular lesions suggesting PLCH were not detected, several small thin-walled centrilobular cysts were scattered in the right upper and middle lung field (Fig. 1b). These findings were suggested to arise from PLCH. We instructed him to abstain from smoking because PLCH is associated with smoking. Two months after that his shortness of breath and dry cough almost completely disappeared and thickness of the cysts was lessened and apparent centrilobular nodules decreased on HRCT (Fig. 1d).

Discussion

PLCH has been extensively investigated with HRCT.²⁻⁴ Cysts and nodules are present in 80% and 50-80% of patients, respectively,⁵ and the combination of nodules along with cysts predominantly in the upper and middle lung fields is a typical finding of PLCH.^{1,5} The early stage of PLCH is characterized by ill-defined nodules, smaller than 5 mm and