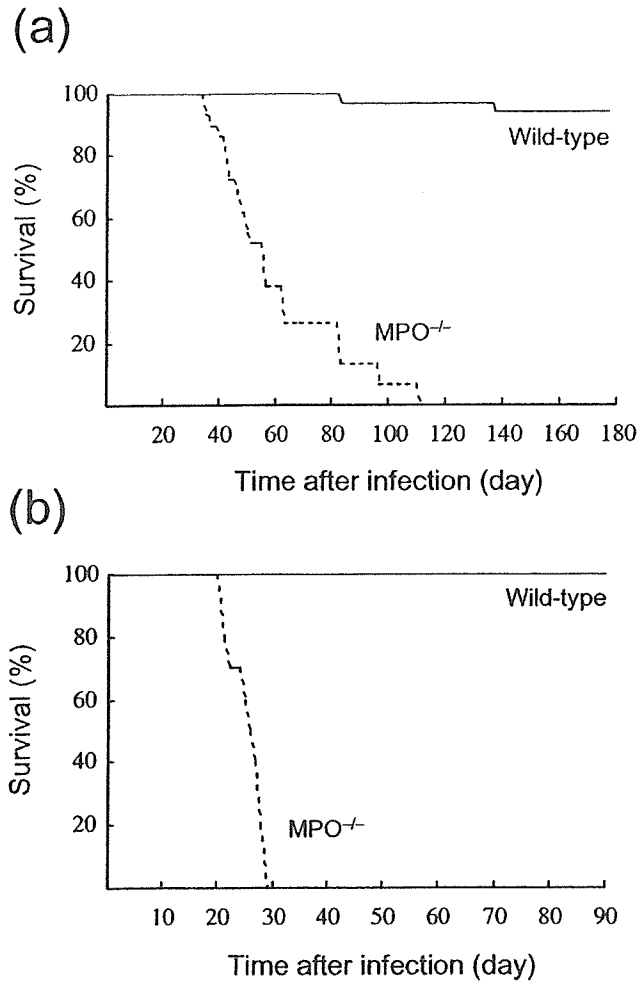

Fig. 1. Survival of wild-type and $MPO^{-/-}$ mice following infection with *C. neoformans*. (a) Wild-type ($n=32$) and $MPO^{-/-}$ ($n=29$) mice were intranasally infected with $3{\cdot}6\times10^6$ c.f.u. *C. neoformans*, and survival was observed over the course of 180 days. (b) Wild-type ($n=10$) and $MPO^{-/-}$ ($n=10$) mice were intravenously infected with $8{\cdot}7\times10^5$ c.f.u. *C. neoformans*. $P<0{\cdot}001$ ($MPO^{-/-}$ versus wild-type mice) for both intranasal and intravenous infections. The data are representative of two independent experiments.

Our previous report also demonstrated that the numbers of *C. neoformans* in the lungs of wild-type and $MPO^{-/-}$ mice 48 h after infection do not differ significantly (Aratani *et al.*, 2000). Taken together, these results strongly suggest that the MPO-dependent oxidative system plays an important role in the *in vivo* host defence against *C. neoformans*, although this system is inefficient in the early period after the infection.

Since Th1 cytokines have been shown to be critical for effective host defences in murine models of cryptococcosis (Decken *et al.*, 1998; Hoag *et al.*, 1997; Kawakami *et al.*, 1996) and there is accumulating evidence that neutrophils play an important role in modulating the balance of Th1 and Th2 responses (Mednick *et al.*, 2003; Tateda *et al.*, 2001), we suspected that the higher fungal burden observed in the infected $MPO^{-/-}$ mice could be caused by a reduction in Th1 cell activity. Interestingly, levels of interleukin (IL)-2, IL-12p70 and interferon (IFN)-γ in the lungs of $MPO^{-/-}$ mice at day 7 of infection were significantly lower than those in infected wild-type mice (Fig. 4), suggesting a role for neutrophil-derived hypohalous acids in driving Th1-type host responses.

Previous studies have shown that the constitutive production of IL-4 in transgenic mice results in an increased susceptibility to infection with *Leishmania major* (Leal *et al.*, 1993), and that resistance to infection occurs when the endogenously synthesized IL-4 is neutralized in the susceptible mice (Chatelain *et al.*, 1992). Consistently, in our study, the level of IL-4 was significantly higher in the $MPO^{-/-}$ mice at day 7 compared to the wild-type controls (Fig. 4), although there was no significant difference between the mice with different genotypes in the levels of IL-5 and IL-10 (data not shown). The levels of these cytokines were not significantly different between the mice with different genotypes before infection and at day 34 of infection (Fig. 4). These results indicate that MPO deficiency affects Th1 and Th2 immune response in the early stage of pulmonary cryptococcus infection.

In murine models, production of IL-1 in the airways is required for full neutrophil migratory responses to LPS or diesel exhaust particles (Ulich *et al.*, 1991; Yang *et al.*, 1997). It is of interest that the lung concentration of IL-1β was significantly higher in the $MPO^{-/-}$ mice than in the wild-type at days 7 and 34 post-infection, and that IL-1α was also significantly higher in the $MPO^{-/-}$ mice at day 34 (Fig. 4), since these data suggest that higher concentrations of these pro-inflammatory cytokines enhanced the pulmonary inflammation of the $MPO^{-/-}$ mice. Overexpression of IL-1β in the lung epithelium leads to pulmonary inflammation, with an increase in the level of keratinocyte-derived cytokine (KC) (Lappalainen *et al.*, 2005). Indeed, in our model, we have observed a time-dependent increase in the level of KC in the lung (Fig. 4). However, there was no difference in the level between the wild-type and $MPO^{-/-}$ mice, suggesting that this chemotactic mediator for neutrophils is not a limiting factor for the higher inflammation observed in the $MPO^{-/-}$ mice. It is well accepted that leukocyte migration from the vasculature occurs by a multistep process, and that this process is dictated by the sequential activation of adhesive proteins and their ligands on both leukocytes and endothelial cells (Wagner & Roth, 2000). Exposure of endothelial cell monolayers or neutrophils *in vitro* to IL-1 causes expression of selectins and integrins (Schleimer & Rutledge, 1986; Scholz *et al.*, 1996). In addition, IL-1 treatment *in vivo* induces intercellular adhesion molecule-1 (ICAM-1) in lung (Komatsu *et al.*, 1997). Taken together, it is possible that the elevated IL-1α/β levels in $MPO^{-/-}$ mice could facilitate the transmigration of circulating neutrophils into tissues.

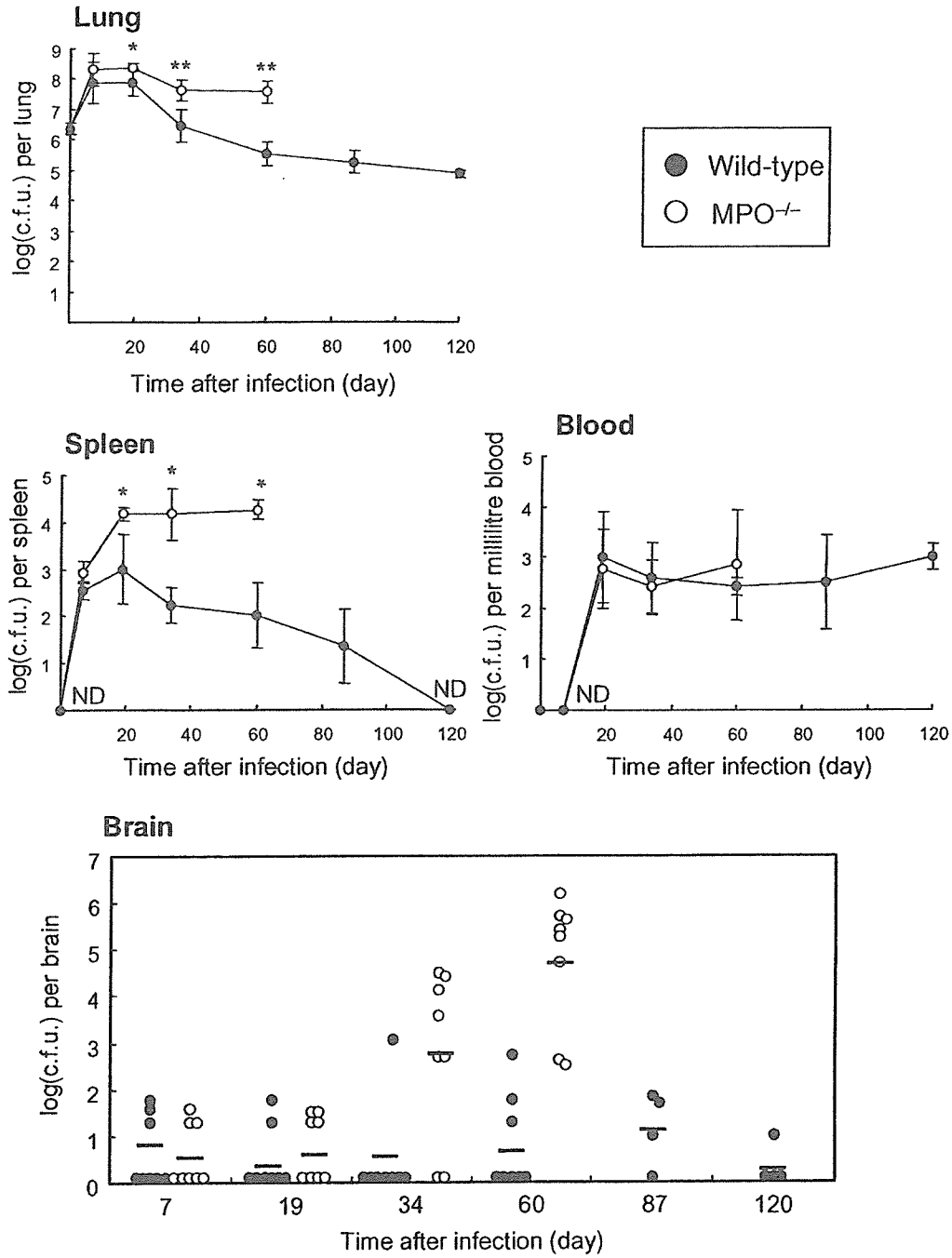

Fig. 2. Culture of *C. neoformans* from various organs after intranasal infection of mice. Wild-type and MPO$^{-/-}$ mice were inoculated intranasally with $3{\cdot}6 \times 10^6$ c.f.u. *C. neoformans* per mouse and analysed on days 0, 7, 19, 34, 60, 87 and 120. Aliquots of homogenized organs were plated on agar plates, and total c.f.u. per organ was determined. Five mice were used for each group. Results represent mean log(c.f.u.) per organ ± SD. ND, not detectable (below 5 c.f.u. per organ). *, $P<0{\cdot}05$; **, $P<0{\cdot}01$ compared to wild-type infected mice. The data are representative of two independent experiments.

The recovery of *C. neoformans* from the brain of infected wild-type and MPO$^{-/-}$ mice was compared (Fig. 2). Whereas cryptococci were nearly undetectable in the brain of wild-type mice at all time points, significant numbers of fungi were found in the MPO$^{-/-}$ mice on days 34 and 60 post-infection. We carried out histological analysis of the brain in five control and five MPO$^{-/-}$ mice on day 60 after infection. One MPO$^{-/-}$ mouse showed a cellular infiltrate including neutrophils in the meningeal area (Fig. 5a, b). Another mouse showed cyst-like cavities in the medulla

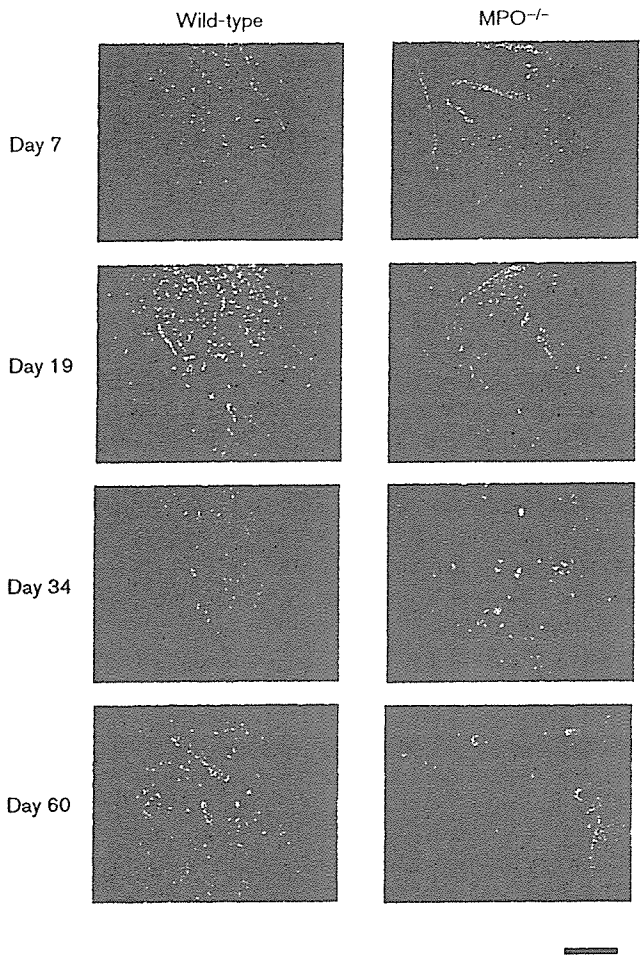

Fig. 3. Lung sections taken from wild-type and $MPO^{-/-}$ mice 7, 19, 34 and 60 days after intranasal challenge with $3{\cdot}6 \times 10^6$ c.f.u. *C. neoformans*. Sections were stained with H&E. Three mice in each experimental group were examined and a representative result is shown. Bar, 1 mm.

oblongata (Fig. 5d), in which no cellular infiltrate was observed. Numerous cryptococci were observed in the meningeal area (Fig. 5c) and within huge cysts (Fig. 5e) in the brain and medulla oblongata. However, no obvious inflammation was found in the brain of three other $MPO^{-/-}$ mice. In contrast, no pathological change was observed in all five of the wild-type mice (Fig. 5f). Since dissemination of the fungi into the cerebrum is often fatal in humans, we considered the possibility that the large fungal burden was the cause of death of the $MPO^{-/-}$ mice. However, this process was unlikely to be the main cause of death, because no wild-type mice infected intravenously ($8{\cdot}7 \times 10^5$ c.f.u.) died, despite the fact that $> 1 \times 10^6$ c.f.u. fungi had invaded the brain at day 20 of infection (data not shown). In the $MPO^{-/-}$ mice given higher doses of *C. neoformans*, the more severe pneumonia as well as the higher lung fungal burden may be the cause of death, since the difference in survival of the mice with different genotypes correlated well with the differences in the levels of lung fungal burden (Figs 1 and 2) and in the severity of pneumonia (Fig. 3).

Of note, the $MPO^{-/-}$ mice showed a dramatic increase in KC production in the brain at day 34 post-infection, which was significantly higher than that in wild-type mice (Fig. 6), suggesting that this chemokine at least might partly contribute to the occasional infiltration of neutrophils into the brain, as observed in Fig. 5. Since *C. neoformans* induces IL-8 production by human microglia (Lipovsky *et al.*, 1998), the higher KC level in $MPO^{-/-}$ mice could result from the higher fungal burden in the brain. In an animal model in which IL-8 was delivered intracerebrally, neutrophils rapidly invaded the blood–brain barrier (Bell *et al.*, 1996). In one of five mice, we observed a slight but consistent accumulation of inflammatory cells, and a subset of these cells could be morphologically identified as neutrophils (Fig. 5). This accumulation of neutrophils suggests that a higher brain fungal burden occasionally stimulates a neutrophil mobilization into the brain across the blood–brain barrier in response to KC. In mice of both genotypes, the brain concentrations of Th1-associated (IL-2, IL-12p70, IFN-γ), Th2-associated (IL-4, IL-5, IL-10) and pro-inflammatory (IL-1α, IL-1β) cytokines did not change during the course of infection (data not shown).

Although the fungal burdens in the spleen were similar in both groups for the first 7 days, those of $MPO^{-/-}$ mice on and after day 19 were significantly higher ($P<0{\cdot}05$) than those of wild-type mice (Fig. 2). However, the blood fungal burden of the mice was equivalent to that in the wild-type (Fig. 2), suggesting that the higher distributions of fungi from the lungs to the spleen were not due to an increased systemic dissemination from the lungs, but rather due to a decreased local resistance in the spleen. To more rigorously examine the role of MPO during systemic infection without an ongoing localized pulmonary infection, an intravenous infection experiment was performed. Wild-type and $MPO^{-/-}$ mice were infected intravenously with $8{\cdot}7 \times 10^5$ viable yeast. To determine fungal dissemination kinetics from the bloodstream, wild-type and $MPO^{-/-}$ mice were analysed on days 1 and 5 post-infection. The fungal burden in the spleen of wild-type mice on day 5 was equivalent to that on day 1. In contrast, the burden in $MPO^{-/-}$ mice on day 5 was significantly higher than that on day 1 (Fig. 7), indicating that the control of fungal growth within the spleen was dependent on MPO production. Since intravenous infection experiments result in a systemic infection of blood-borne fungi in the absence of an ongoing pulmonary infection, the results indicate that MPO was important for host defence against *C. neoformans* not only in the lungs but also in peripheral organs.

C. neoformans is acquired via the respiratory tract and occasionally disseminates to the central nervous system in some immunocompromised patients (Lee *et al.*, 1996). So far, MPO deficiency is not known to be associated with human cryptococcosis. Since mouse neutrophils are devoid

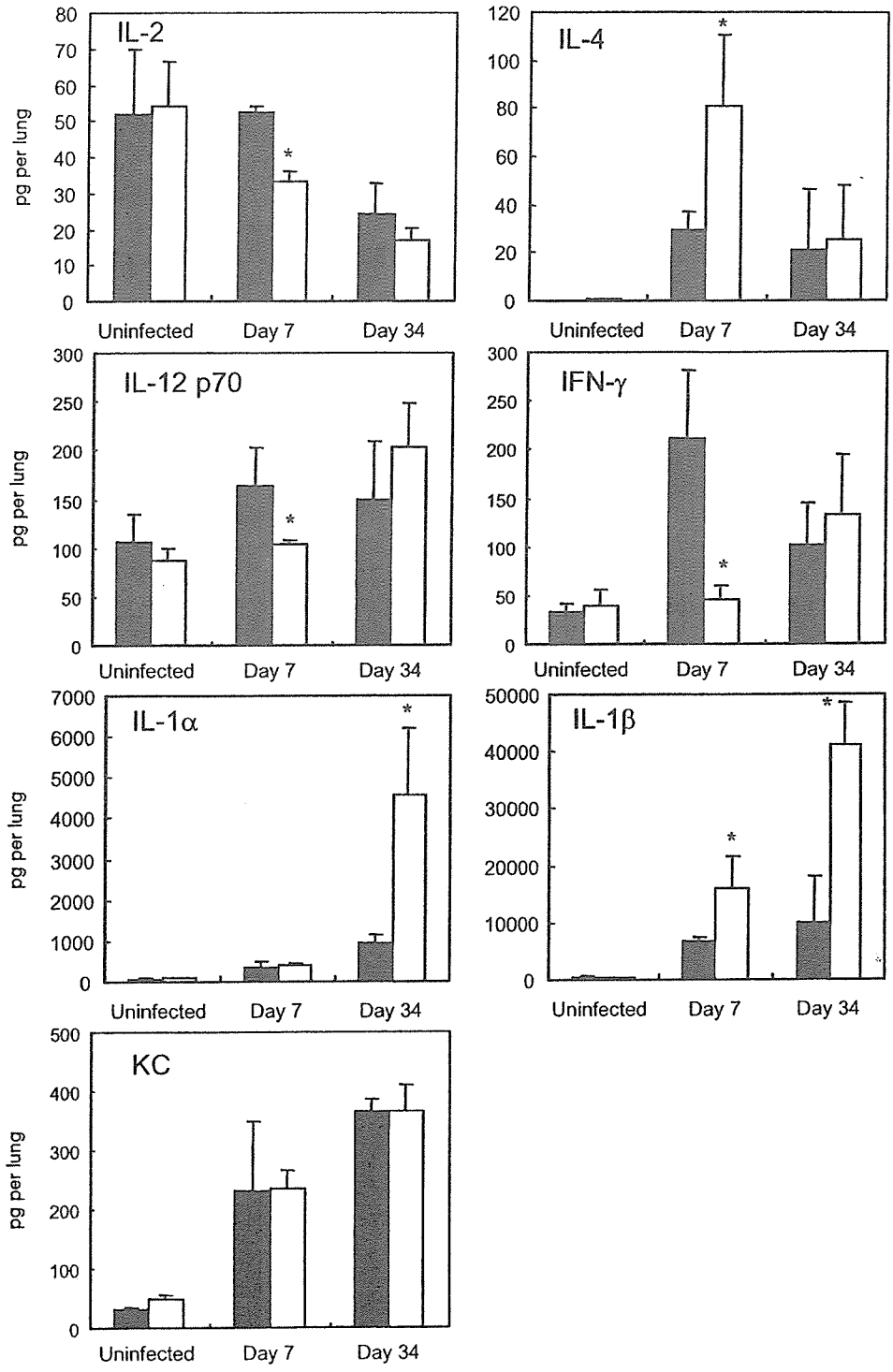

Fig. 4. Cytokine and chemokine levels in the lungs of wild-type and $MPO^{-/-}$ mice after infection with *C. neoformans*. Wild-type (black bars) and $MPO^{-/-}$ mice (open bars) were intranasally infected with $3{\cdot}6 \times 10^6$ c.f.u. *C. neoformans*. Mice were killed before infection, or at 7 or 34 days post-infection, and the lungs were harvested and homogenized. Cytokines and chemokines were measured in triplicate using the Bio-Plex system. Data are expressed as pg cytokine per lung. Results are presented as the mean ± SD of four infected or uninfected mice. *, $P<0{\cdot}05$ compared to wild-type infected mice.

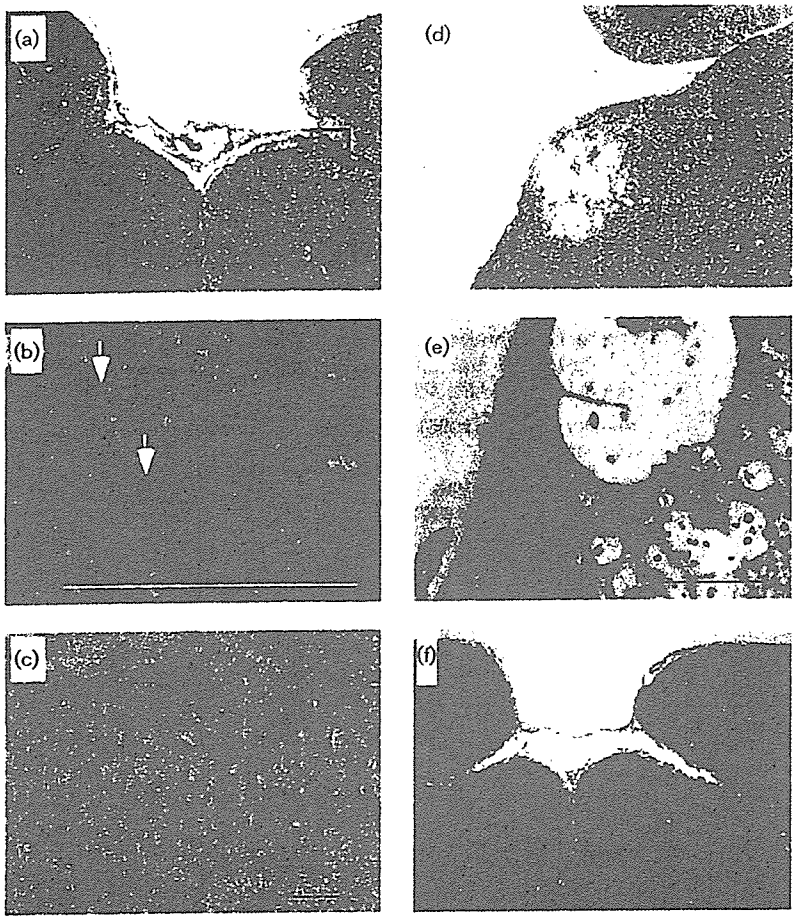

Fig. 5. Histopathology of brain of MPO$^{-/-}$ mice 60 days after *C. neoformans* infection. Brain sections of wild-type (f) and MPO$^{-/-}$ mice (a–e) 60 days after intranasal challenge with $3{\cdot}6 \times 10^6$ c.f.u. are shown. *C. neoformans* were stained with H&E (a, b, d and f) or Grocott methamine silver (c and e). Panel (b) is a higher magnification of the cellular infiltrate observed in the box shown on panel (a). Arrows indicate neutrophils. Bars, 1 mm.

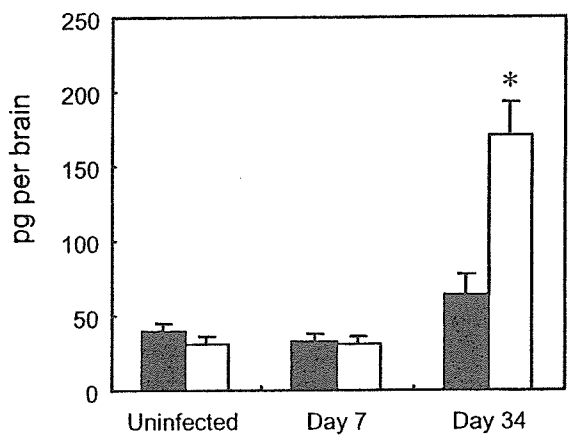

Fig. 6. KC levels in the brains of wild-type and MPO$^{-/-}$ mice after infection with *C. neoformans*. Wild-type (black bars) and MPO$^{-/-}$ mice (open bars) were intranasally infected with $3{\cdot}6 \times 10^6$ c.f.u. *C. neoformans*. Mice were killed before infection, or at 7 or 34 days post-infection. The KC levels of brain homogenates were measured in triplicate using the Bio-Plex system. Data are expressed as pg KC per organ. Results are presented as the mean ± SD of four infected or uninfected mice. *, $P<0{\cdot}05$ compared to wild-type infected mice.

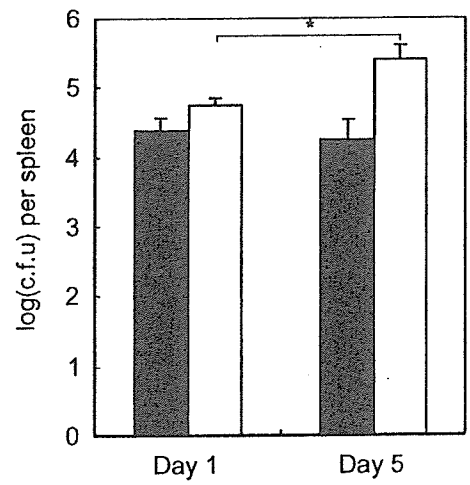

Fig. 7. Cultures from spleen after intravenous infection with *C. neoformans*. Wild-type (black bars) and MPO$^{-/-}$ mice (open bars) were inoculated intravenously with $8{\cdot}7 \times 10^5$ c.f.u. *C. neoformans* per mouse and analysed on days 1 and 5. Aliquots of homogenized organs were plated on agar plates, and total c.f.u. per organ was determined. Five mice were used for each group. Results represent mean log(c.f.u.) per organ ± SD of the means for five animals. *, $P<0{\cdot}05$.

of defensins (Eisenhauer & Lehrer, 1992), prominent non-oxidative contributors to human anticryptococcal defence (Mambula *et al.*, 2000), impaired oxidative systems in mice may permit a more severe infection than in humans. Whereas cell-mediated immunity, non-oxidative systems and reactive nitrogen intermediates (Lovchik *et al.*, 1997) likely contribute to microbial clearance, our data strongly suggest that impairment of the MPO-mediated antimicrobial system could be one of the risk factors for the infection.

ACKNOWLEDGEMENTS

We thank Ayako Onuma for animal care, and William M. Nauseef for helpful comments and critical review of the manuscript. This work was supported by Grants of Research on Health Sciences focusing on Drug Innovation from the Japan Health Sciences Foundation.

REFERENCES

Aratani, Y., Koyama, H., Nyui, S., Suzuki, K., Kura, F. & Maeda, N. (1999). Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. *Infect Immun* 67, 1828–1836.

Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Suzuki, K., Maeda, N. & Koyama, H. (2000). Differential host susceptibility to pulmonary infections with bacteria and fungi in mice deficient in myeloperoxidase. *J Infect Dis* 182, 1276–1279.

Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Suzuki, K., Dinauer, M. C., Maeda, N. & Koyama, H. (2002a). Critical role of myeloperoxidase and nicotinamide adenine dinucleotide phosphate-oxidase in high-burden systemic infection of mice with *Candida albicans*. *J Infect Dis* 185, 1833–1837.

Aratani, Y., Kura, F., Watanabe, H., Akagawa, H., Takano, Y., Suzuki, K., Dinauer, M. C., Maeda, N. & Koyama, H. (2002b). Relative contributions of myeloperoxidase and NADPH-oxidase to the early host defense against pulmonary infections with *Candida albicans* and *Aspergillus fumigatus*. *Med Mycol* 40, 557–563.

Bell, M. D., Taub, D. D. & Perry, V. H. (1996). Overriding the brain's intrinsic resistance to leukocyte recruitment with intraparenchymal injections of recombinant chemokines. *Neuroscience* 74, 283–292.

Buchanan, K. L. & Doyle, H. A. (2000). Requirement for $CD4^+$ T lymphocytes in host resistance against *Cryptococcus neoformans* in the central nervous system of immunized mice. *Infect Immun* 68, 456–462.

Chatelain, R., Varkila, K. & Coffman, R. L. (1992). IL-4 induces a Th2 response in *Leishmania major*-infected mice. *J Immunol* 148, 1182–1187.

Chuck, S. L. & Sande, M. A. (1989). Infections with *Cryptococcus neoformans* in the acquired immunodeficiency syndrome. *N Engl J Med* 321, 794–799.

Decken, K., Kohler, G., Palmer-Lehmann, K., Wunderlin, A., Mattner, F., Magram, J., Gately, M. K. & Alber, G. (1998). Interleukin-12 is essential for a protective Th1 response in mice infected with *Cryptococcus neoformans*. *Infect Immun* 66, 4994–5000.

Diamond, R. D., Clark, R. A. & Haudenschild, C. C. (1980). Damage to *Candida albicans* hyphae and pseudohyphae by the myeloperoxidase system and oxidative products of neutrophil metabolism in vitro. *J Clin Invest* 66, 908–917.

Eisenhauer, P. B. & Lehrer, R. I. (1992). Mouse neutrophils lack defensins. *Infect Immun* 60, 3446–3447.

Graybill, J. R., Bocanegra, R., Lambros, C. & Luther, M. F. (1997). Granulocyte colony stimulating factor therapy of experimental cryptococcal meningitis. *J Med Vet Mycol* 35, 243–247.

Hansson, M., Olsson, I. & Nauseef, W. M. (2006). Biosynthesis, processing, and sorting of human myeloperoxidase. *Arch Biochem Biophys* 445, 214–224.

Hoag, K. A., Lipscomb, M. F., Izzo, A. A. & Street, N. E. (1997). IL-12 and IFN-gamma are required for initiating the protective Th1 response to pulmonary cryptococcosis in resistant C.B-17 mice. *Am J Respir Cell Mol Biol* 17, 733–739.

Kawakami, K., Tohyama, M., Teruya, K., Kudeken, N., Xie, Q. & Saito, A. (1996). Contribution of interferon-gamma in protecting mice during pulmonary and disseminated infection with *Cryptococcus neoformans*. *FEMS Immunol Med Microbiol* 13, 123–130.

Klebanoff, S. J. (2005). Myeloperoxidase: friend and foe. *J Leukoc Biol* 77, 598–625.

Komatsu, S., Flores, S., Gerritsen, M. E., Anderson, D. C. & Granger, D. N. (1997). Differential up-regulation of circulating soluble and endothelial cell intercellular adhesion molecule-1 in mice. *Am J Pathol* 151, 205–214.

Kozel, T. R. (1993). Cryptococcosis. In *Fungal Infections and Immune Responses*, pp. 277–302. Edited by J. W. Murphy, H. Friedman & M. Bendinelli. New York: Plenum.

Lappalainen, U., Whitsett, J. A., Wert, S. E., Tichelaar, J. W. & Bry, K. (2005). Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung. *Am J Respir Cell Mol Biol* 32, 311–318.

Leal, L. M., Moss, D. W., Kuhn, R., Muller, W. & Liew, F. Y. (1993). Interleukin-4 transgenic mice of resistant background are susceptible to *Leishmania major* infection. *Eur J Immunol* 23, 566–569.

Lee, S. C., Dickson, D. W. & Casadevall, A. (1996). Pathology of cryptococcal meningoencephalitis: analysis of 27 patients with pathogenetic implications. *Hum Pathol* 27, 839–847.

Lehrer, R. I. & Cline, M. J. (1969). Leukocyte myeloperoxidase deficiency and disseminated candidiasis: the role of myeloperoxidase in resistance to Candida infection. *J Clin Invest* 48, 1478–1488.

Lipovsky, M. M., Gekker, G., Hu, S., Ehrlich, L. C., Hoepelman, A. I. & Peterson, P. K. (1998). Cryptococcal glucuronoxylomannan induces interleukin (IL)-8 production by human microglia but inhibits neutrophil migration toward IL-8. *J Infect Dis* 177, 260–263.

Lovchik, J., Lipscomb, M. & Lyons, C. R. (1997). Expression of lung inducible nitric oxide synthase protein does not correlate with nitric oxide production in vivo in a pulmonary immune response against *Cryptococcus neoformans*. *J Immunol* 158, 1772–1778.

Mambula, S. S., Simons, E. R., Hastey, R., Selsted, M. E. & Levitz, S. M. (2000). Human neutrophil-mediated nonoxidative antifungal activity against *Cryptococcus neoformans*. *Infect Immun* 68, 6257–6264.

Mednick, A. J., Feldmesser, M., Rivera, J. & Casadevall, A. (2003). Neutropenia alters lung cytokine production in mice and reduces their susceptibility to pulmonary cryptococcosis. *Eur J Immunol* 33, 1744–1753.

Miller, M. F. & Mitchell, T. G. (1991). Killing of *Cryptococcus neoformans* strains by human neutrophils and monocytes. *Infect Immun* 59, 24–28.

Murphy, J. W. (1996). Cell-mediated immunity. In *The Mycota*, vol. 7, pp. 67–97. Edited by J. D. Miller & D. H. Howard. New York: Springer.

Nauseef, W. M. (1998). Insights into myeloperoxidase biosynthesis from its inherited deficiency. *J Mol Med* 76, 661–668.

Nunoi, H., Kohi, F., Kajiwara, H. & Suzuki, K. (2003). Prevalence of inherited myeloperoxidase deficiency in Japan. *Microbiol Immunol* 47, 527–531.

Parry, M. F., Root, R. K., Metcalf, J. A., Delaney, K. K., Kaplow, L. S. & Richar, W. J. (1981). Myeloperoxidase deficiency: prevalence and clinical significance. *Ann Intern Med* 95, 293–301.

Retini, C., Vecchiarelli, A., Monari, C., Tascini, C., Bistoni, F. & Kozel, T. R. (1996). Capsular polysaccharide of *Cryptococcus neoformans* induces proinflammatory cytokine release by human neutrophils. *Infect Immun* 64, 2897–2903.

Schleimer, R. P. & Rutledge, B. K. (1986). Cultured human vascular endothelial cells acquire adhesiveness for neutrophils after stimulation with interleukin 1, endotoxin, and tumor-promoting phorbol diesters. *J Immunol* 136, 649–654.

Scholz, D., Devaux, B., Hirche, A., Potzsch, B., Kropp, B., Schaper, W. & Schaper, J. (1996). Expression of adhesion molecules is specific and time-dependent in cytokine-stimulated endothelial cells in culture. *Cell Tissue Res* 284, 415–423.

Tateda, K., Moore, T. A., Deng, J. C., Newstead, M. W., Zeng, X., Matsukawa, A., Swanson, M. S., Yamaguchi, K. & Standiford, T. J. (2001). Early recruitment of neutrophils determines subsequent T1/T2 host responses in a murine model of *Legionella pneumophila* pneumonia. *J Immunol* 166, 3355–3361.

Ulich, T. R., Watson, L. R., Yin, S. M., Guo, K. Z., Wang, P., Thang, H. & del Castillo, J. (1991). The intratracheal administration of endotoxin and cytokines. I. Characterization of LPS-induced IL-1 and TNF mRNA expression and the LPS-, IL-1-, and TNF-induced inflammatory infiltrate. *Am J Pathol* 138, 1485–1496.

Vecchiarelli, A., Retini, C., Casadevall, A., Monari, C., Pietrella, D. & Kozel, T. R. (1998). Involvement of C3a and C5a in interleukin-8 secretion by human polymorphonuclear cells in response to capsular material of *Cryptococcus neoformans*. *Infect Immun* 66, 4324–4330.

Wagner, J. G. & Roth, R. A. (2000). Neutrophil migration mechanisms, with an emphasis on the pulmonary vasculature. *Pharmacol Rev* 52, 349–374.

Yang, H. M., Ma, J. Y., Castranova, V. & Ma, J. K. (1997). Effects of diesel exhaust particles on the release of interleukin-1 and tumor necrosis factor-alpha from rat alveolar macrophages. *Exp Lung Res* 23, 269–284.

Yuan, R. R., Casadevall, A., Oh, J. & Scharff, M. D. (1997). T cells cooperate with passive antibody to modify *Cryptococcus neoformans* infection in mice. *Proc Natl Acad Sci U S A* 94, 2483–2488.

Gene Expression, Vol. 13, pp. 67–71
Printed in the USA.

1052-2166/06 $90.00 + .00
E-ISSN 1555-3884
www.cognizantcommunication.com

Arginine to Cysteine Mutation (R499C) Found in a Japanese Patient With Complete Myeloperoxidase Deficiency

AMANDA S. PERSAD,* YOSUKE KAMEOKA,† SHUJI KANDA,‡ YOSHIYUKI NIHO,‡ AND KAZUO SUZUKI*

**Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan*
†Division of Genetic Resources, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan
‡Chihaya Hospital, Fukuoka, Japan

Animal models suggest that a deficiency in myeloperoxidase (MPO; EC 1.11.1.7), a lysosomal hemoprotein involved in host defense, may be associated with a decreased level of immunity. A nonsynonymous mutation, resulting in an arginine to cysteine substitution (Arg499Cys or R499C), has been identified in the exon 9 genetic coding region of a Japanese patient with complete MPO deficiency. Genetic analysis revealed that the mRNA of the patient could be correctly transcribed then further translated into a peptide sequence. However, the Western blot analysis confirmed the absence of MPO peptides. An initial screening assay of the patient's blood exhibited an abnormal hematograph, and no MPO activity was detected. To determine if this mutation might be associated with MPO deficiency, DNA samples for 387 controls were examined. Genetic analysis was performed using standard PCR techniques for amplification and sequencing. None of the control samples possessed the R499C substitution. This mutation is in close proximity to a different mutation (G501S) previously found in another Japanese MPO-deficient patient, and the amino acid, H502, which is strongly involved in heme binding, leading to the speculation that heme binding may play a role in complete MPO deficiency.

Key words: Myeloperoxidase (MPO); Myeloperoxidase deficiency; Arg499Cys; R499C

INTRODUCTION

Upon comparison with the United States and Europe, complete myeloperoxidase deficiency [Mendelian inheritance in Man (MIM) #254600] is considered a rare occurrence in the Japanese population with a prevalence of 1.75/100,000 (15). Located in neutrophils and monocytes, myeloperoxidase (MPO) plays a role in host defense against a wide range of organisms. Mouse studies by Aratani et al. (1,2) suggest a link between MPO deficiency and an increased occurrence of infections.

In the biosynthesis of MPO, the translational product is first converted into the enzymatically inactive apoproMPO. Heme binding transforms this protein to an active proMPO form and subsequently mature MPO. It has been postulated that MPO deficiency may be a result of its inability to undergo posttranslational processing (11,12). Though the translational process leaves a margin for the development of MPO deficiency, genetic anomalies in the coding region also contribute to the inability to produce this enzyme.

To date, there have been four allelic mutations related to MPO deficiency. These include: arginine to tryptophan substitution (R569W) (11,12), tyrosine to cysteine conversion (Y173C) (4), methionine to threonine substitution (M251T) (14), and glycine to ser-

Address correspondence to Kazuo Suzuki, Chief, Department of Bioactive Molecules, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. Tel: +81-3-5285-1111, ext. 2329; Fax: +81-3-5285-1160; E-mail: ksuzuki@nih.go.jp

ine conversion (G501S) (13). In addition to nonsynonymous mutations, studies have found enzyme deficiency due to alternative splicing and deletions in the genetic sequence for MPO (6,14).

This study presents a novel nonsynonymous mutation found in a Japanese patient with complete MPO deficiency. This point mutation is in close proximity to the G501S mutation, also identified in the Japanese population, as well as the amino acid histidine, at position 502 (H502), that is involved in heme binding. DNA samples from the indigenous population were analyzed to determine the probable prevalence of the mutation.

MATERIALS AND METHODS

Subjects

A 28-year-old Japanese female presented with complete MPO deficiency as identified by Bayer-Technicon automated hematography (15). Blood from one healthy control was sampled along with the patient for simultaneous measurement of enzymatic activity and superoxide anion production. DNA samples from 387 individuals were utilized in the genetic analysis. These genetic samples included rheumatoid arthritis patients (21%), hepatitis C patients (41%), and healthy volunteers (38%). No information on the activity of MPO or superoxide dismutase was available for these donors. Informed consent was obtained from all subjects. The low prevalence of both complete and partial MPO deficiency was used to justify the unlikelihood of these DNA samples being from MPO-deficient donors.

MPO Activity and Superoxide Production

MPO release and superoxide anion production from neutrophils were assayed as described previously (8). Briefly, neutrophils were prewarmed at 37°C then transferred onto plates containing cytochalsin B (CB) and fMet-Leu-Phe (FMLP) for 10 min. The cell suspension was then centrifuged at $400 \times g$ for 5 min. MPO activities in the supernatant and in the homogenate of the cell pellet were measured spectrophotometrically using 3,3′,5,5′-tetramethylbenzidine as a substrate (16,17). For elucidating superoxide anion production, neutrophils were mixed with ferricytochrome C for 2 min at 37°C prior to the addition of CB and FMLP. Total MPO activity was calculated as the sum of the enzyme activity in supernatants and cell pellet homogenates after treatment with CB and FMLP. MPO release was expressed as the percentage of total enzyme activity (MPO activity in supernatant ÷ total enzyme activity). Superoxide anion production was determined by measuring the increase in absorbance at 550 nm in 1-min intervals using a microplate reader.

TABLE 1
MPO AND SUPEROXIDE DISMUTASE ACTIVITY IN THE PATIENT AND HEALTHY CONTROL

	MPO Activity (Units/10^6 Cells)	Superoxide Anion Production (nmol/10^6 Cells)
Patient	0.9	10.5
Control	5.2	6.8

Western Blot Analysis

Western blot analysis was performed on the patient and the healthy control using the method described previously (13). Briefly, neutrophils were dissolved in a loading buffer, consisting of 50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% sodium dodecyl sulfate (SDS), 0.3 M β-mercaptoethanol, and 0.01% bromophenol blue, and incubated at 98°C for complete reduction. The samples were fractionated by electrophoresis on 8–12% SDS polyacrylamide gel and transferred onto a PVDF membrane (Millipore, Bedford, MA, USA). Antibodies used for immunodetection included 1:2000 anti-human MPO rabbit antibody (Dako, Glostrup, Denmark) at room temperature for 2 h, followed by an incubation with a 1:2000 alkaline phosphatase-conjugated anti-rabbit IgG goat

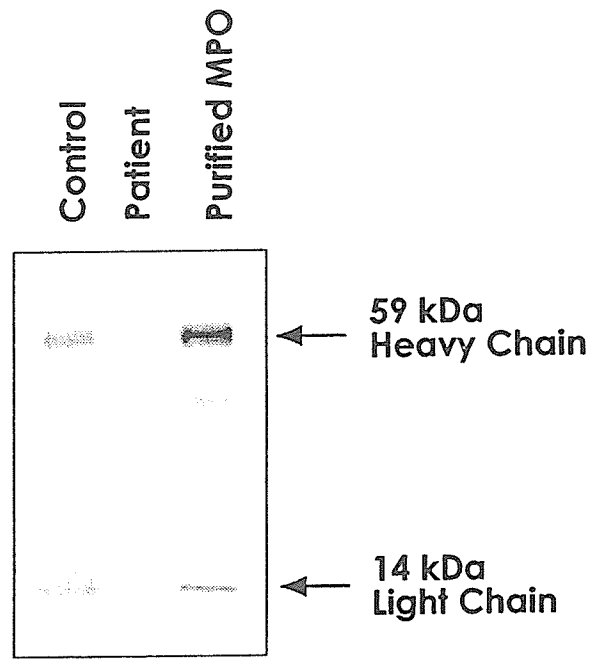

Figure 1. Western blot analysis. Proteins in neutrophils of the patient, normal control, and purified MPO protein were fractionated on 8–12% polyacrylamide gel, transferred on PVDF membrane, and detected by rabbit anti-human MPO antibody. MPO polypeptides are indicated on the right.

antibody (Organon Teknika, Durham, NC, USA) for 2 h. The PVDF membrane was visualized using an AP conjugate substrate kit (Bio-Rad, Hercules, CA, USA).

cDNA and Genomic DNA Analysis

MPO cDNA was acquired from the mRNA of mononuclear cells (MNC) by polymerase chain reaction (PCR) after reverse transcription (RT) reaction with AMV reverse transcriptase, using the specific primer pairs for MPO mRNA as described in Ohashi et al. (13). Genomic fragments were also amplified by PCR. The obtained RT-PCR products were compared with a normal MPO cDNA sequence (7,9,18).

DNA segments obtained were purified with the QIAquick PCR purification system (Qiagen, Hilden, Germany) and served as templates in a PRISM Ready Reaction Dye Terminator Cycle Sequencing procedure utilizing an automatic sequencer (Applied Biosystems, CA, USA). Sequences were then analyzed using Genetyx-win software (Software Development Co., Ltd., Tokyo, Japan).

RESULTS

MPO Activity of Neutrophils

Diminished levels of MPO activity were detected in the patient (i.e., 0.9 units/10^6 cells), which is a level approximately one sixth that observed in the healthy control (Table 1). The patient's elevated superoxide dismutase activity, compared to the healthy control, was reflective of MPO deficiency.

Western Blot Analysis

Western blot analysis of the neutrophils from the patient and the healthy control was performed alongside purified MPO, using anti-human MPO antibody. While the healthy control and purified MPO clearly showed 59 kDa of heavy chain and 14 kDa of light subunit, MPO peptides could not be confirmed in the patient (Fig. 1).

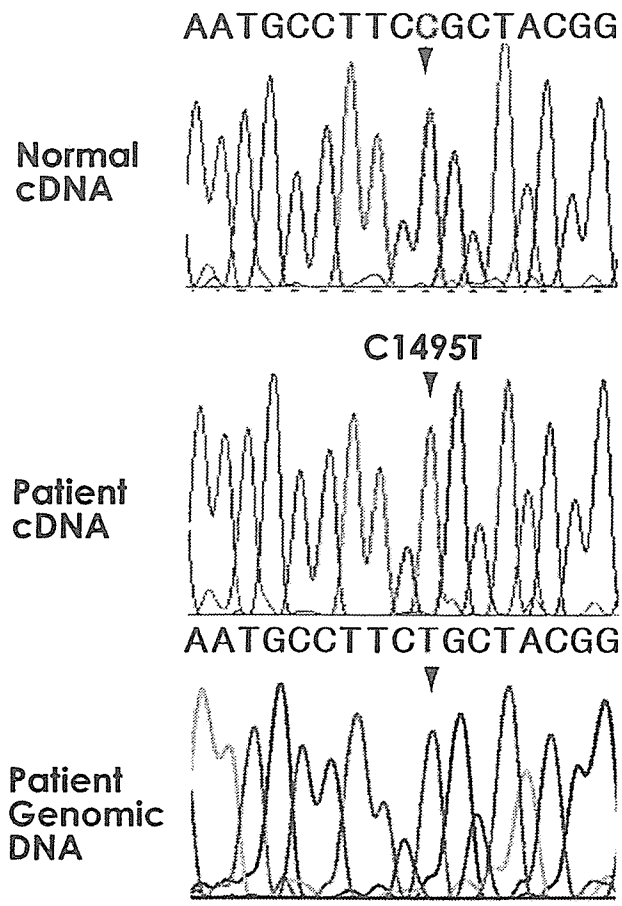

Figure 2. Sequence histogram around the mutation point of cDNA and genomic DNA of the patient. Top panel shows a histogram of the same region from normal control. In the middle and bottom are histograms of cDNA and genomic DNA from the patient. Arrowheads indicate the mutation position 1495.

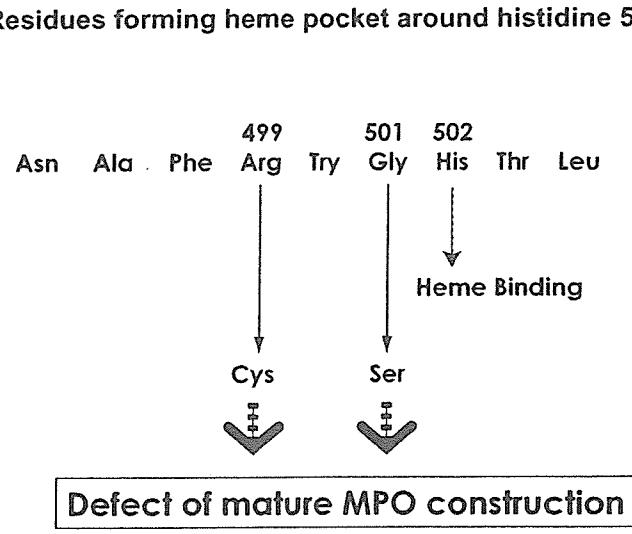

Figure 3. Schematic diagram of the amino acid residues around histidine 502. R499C substitution causes a similar effect on mature MPO construction with G501S.

Mutational Analysis

No alternative splice variations were found in the complementary DNA (cDNA) of the MPO mRNA, obtained from the patient's monocytes. Notably, this observation was also recorded by Ohashi et al. (13). The sequenced cDNA fragment lengths were found to be the appropriate length for each region. This finding shows that the MPO gene in the patient was normally transcribed without any alternative transcription. Both the genomic and cDNA revealed a point mutation in the patient sample (Fig. 2): a nonsynonymous homozygous mutation in the coding region of exon 9 with thymine (T) being substituted for cytosine (C) at the 1495th position from the adenine of the first AUG codon. This C to T substitution resulted in an arginine to cysteine substitution (R499C). None of the 387 DNA samples were found to possess the novel R499C mutation identified in the patient.

DISCUSSION

Complete deficiency of MPO, originating from a novel mutation, is a unique phenomenon, requiring two recessive alleles due to the bi-allelic manner of MPO gene expression. With rare migration rates throughout different areas of Japan, these homozygous patients may serve as a sentinel in identifying clusters with the same mutation. The biosynthesis of MPO includes N-linked glycosylation, heme insertion, proteolytic processing, and dimerization (5). Our findings reveal another mutation in the MPO gene that may be responsible for MPO deficiency. Both the R499C mutation and the previously published G501S mutation found in MPO-deficient Japanese individuals are proximal to the histidine in position 502 (H502) in the myeloperoxidase structure (3,19). This amino acid is strongly involved with heme binding and thus a mutation near this area might interfere with subsequent development of mature MPO (Fig. 3). Our findings also support the hypothesis that immature peptides are disrupted, as the Western blot analysis did not reveal any peptides with the same antigenic determinants of MPO. Lastly, among the almost 400 Japanese individuals tested no nonsynonymous mutations in exon 9 genetic coding region were identified.

ACKNOWLEDGMENTS

This study was supported partly by a grant from the Ministry of Health, Labour and Welfare and Human Science Foundation Japan.

REFERENCES

1. Aratani, Y.; Kura, F.; Watanabe, H.; Akagawa, H.; Takano, Y.; Suzuki, K.; Maeda, N.; Koyama, H. Differential host susceptibility to pulmonary infections with bacteria and fungi in mice deficient in myeloperoxidase. J. Infect. Dis. 182:1276–1279; 2000.
2. Aratani, Y.; Koyama, H.; Nyui, S.; Suzuki, K.; Kura, F.; Maeda, N. Severe impairment in early host defense against *Candida albicans* in mice deficient in myeloperoxidase. Infect. Immun. 67:1828–1836; 1999.
3. Davey, C. A.; Fenna, R. E. 2.3 A resolution X-ray

crystal structure of the bisubstrate analogue inhibitor salicylhydroxamic acid bound to human myeloperoxidase: A model for a prereaction complex with hydrogen peroxide. Biochemistry 35:10967–10973; 1996.
4. DeLeo, F. R.; Goedken, M.; McCormick, S. J.; Nauseef, W. M. A novel form of hereditary myeloperoxidase deficiency linked to endoplasmic reticulum/proteasome degradation. J. Clin. Invest. 101:2900–2909; 1998.
5. Gullberg, U.; Andersson, E.; Garwicz, D.; Lindmark, A.; Olsson, I. Biosynthesis, processing and sorting of neutrophil proteins: Insight into neutrophil granule development. Eur. J. Haematol. 58:137–153; 1997.
6. Hashinaka, K.; Nishio, C.; Hur, S. J.; Sakiyama, F.; Tsunasawa, S.; Yamada, M. Multiple species of myeloperoxidase messenger RNAs produced by alternative splicing and differential polyadenylation. Biochemistry 27:5906–5914; 1988.
7. Johnson, K. R.; Nauseef, W. M.; Care, A.; Wheelock, M. J.; Shane, S.; Hudson, S.; Koeffler, H. P.; Selsted, M.; Miller, C.; Rovera, G. Characterization of cDNA clones for human myeloperoxidase: predicted amino acid sequence and evidence for multiple mRNA species. Nucleic Acids Res. 15:2013–2028; 1987.
8. Kawai, Y.; Okawarab, A. I.; Okuyama, H.; Kura, F.; Suzuki, K. Modulation of chemotaxis, O_{2-} production and myeloperoxidase release from human polymorphonuclear leukocytes by the ornithine-containing lipid and serineglycine-containing lipid of Flavobacterium. FEMS Immunol. Med. Microbiol. 28:205–209; 2000.
9. Morishita, K.; Kubota, N.; Asano, S.; Kaziro, Y.; Nagata, S. Molecular cloning and characterization of cDNA for human myeloperoxidase. J. Biol. Chem. 262:3844–3851; 1987.
10. Nauseef, W. M.; Cogley, M.; Bock, S.; Petrides, P. E. Pattern of inheritance in hereditary myeloperoxidase deficiency associated with the R569W missense mutation. J. Leukoc. Biol. 63:264–269; 1998.
11. Nauseef, W. M.; Cogley, M.; McCormick, S. Effect of the R569W missense mutation on the biosynthesis of myeloperoxidase. J. Biol. Chem. 271:9546–9549; 1996.
12. Nauseef, W. M.; Brigham, S.; Cogley, M. Hereditary myeloperoxidase deficiency due to a missense mutation of arginine 569 to tryptophan. J. Biol. Chem. 269: 1212–1216; 1994.
13. Ohashi, Y. Y.; Kameoka, Y.; Persad, A. S.; Koi, F.; Yamagoe, S.; Hashimoto, K.; Suzuki, K. Novel missense mutation found in a Japanese patient with myeloperoxidase deficiency. Gene 327:195–200; 2004.
14. Romano, M.; Dri, P.; Dadalt, L.; Patriarca, P.; Baralle, F. E. Biochemical and molecular characterization of hereditary myeloproliferative deficiency. Blood 90: 4126–4134; 1997.
15. Suzuki, K.; Nunoi, H.; Miyazaki, M.; Koi, F. Prevalence of inherited myeloperoxidase deficiency in Japan. In: Petrides, P. E.; Nauseef, W. M., eds. The peroxidase multigene family of enzymes. Berlin: Springer-Verlag; 2000:45–149.
16. Suzuki, K.; Yamada, M.; Akashi, K.; Fujikura, T. Similarity of kinetics of three types of myeloperoxidase from human leukocytes and four types from HL-60 cells. Arch. Biochem. Biophys. 245:167–173; 1986.
17. Suzuki, K.; Ota, H.; Sasagawa, S.; Sakatani, T.; Fujikura, T. Assay method for myeloperoxidase in human polymorphonuclear leukocytes. Anal. Biochem. 132: 345–352; 1983.
18. Yamada, M.; Hur, S. J.; Hashinaka, K.; Tsuneoka, K.; Saeki, T.; Nishio, C.; Sakiyama, F.; Tsunasawa, S. Isolation and characterization of a cDNA coding for human myeloperoxidase. Arch. Biochem. Biophys. 255: 147–155; 1987.
19. Zeng, J.; Fenna, R. E. X-ray crystal structure of canine myeloperoxidase at 3 A resolution. J. Mol. Biol. 226: 185–207; 1992.

ELSEVIER

Atherosclerosis 186 (2006) 310–320

ATHEROSCLEROSIS

www.elsevier.com/locate/atherosclerosis

Lethal and severe coronary arteritis in DBA/2 mice induced by fungal pathogen, CAWS, *Candida albicans* water-soluble fraction

Noriko Nagi-Miura[a], Toshie Harada[a], Hiroyasu Shinohara[a], Kiyoshi Kurihara[a], Yoshiyuki Adachi[a], Akiko Ishida-Okawara[b], Toshiaki Oharaseki[c], Kei Takahashi[c], Shiro Naoe[c], Kazuo Suzuki[b], Naohito Ohno[a,*]

[a] *Laboratory for Immunopharmacology of Microbial Products, School of Pharmacy, Tokyo University of Pharmacy and Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*

[b] *Department of Bioactive Molecules, National Institute of Infectious Diseases, Tokyo, Japan*

[c] *Department of Pathology, Ohashi Hospital, Toho University School of Medicine, Tokyo, Japan*

Received 4 May 2005; received in revised form 23 July 2005; accepted 8 August 2005
Available online 12 September 2005

Abstract

CAWS is a microbial pathogen-associated molecular patterns (PAMPs) produced by *Candida albicans*. CAWS is a mannoprotein–beta-glucan complex and secreted into the culture supernatant. CAWS has various biological effects, causing acute shock and disrupting vascular permeability. Intraperitoneal administration of CAWS induces coronary arteritis in various strains of inbred mice. The CAWS-induced coronary arteritis is strain-dependent and most severe in DBA/2 mice with a significant number of these animals expiring with cardiomegaly during the observation period. In vivo and in vitro, splenocytes of DBA/2 mice produced various cytokines, such as IL-6, TNF-α, and IFN-γ in response to CAWS. GM-CSF was also produced in response to CAWS. The production of cytokines was significantly enhanced in the presence of recombinant GM-CSF. In contrast, anti-GM-CSF significantly reduced the production of TNF-α and IFN-γ. Augmented production of cytokines in response to CAWS would be a key to the severity of coronary arteritis.

Keywords: *Candida albicans*; Induction of arteritis; Polysaccharide; DBA/2 mice

1. Introduction

Cardiovascular disease is now one of the most life-threatening diseases, and various new compounds and also new methods of pharmaceutical care, such as gene therapy and drug delivery systems, have been developed to treat it [1–7]. A central concept with regard to its pathogenesis is that of endothelial cell dysfunction, which is associated with the release of numerous mediators secreted by leukocytes that are present in large numbers at the sites where atheroma form [8–11]. There are several lines of evidence that inflammatory cells, particularly macrophages, regulate endothelial cell function and dysfunction in atherosclerosis, via the release of mediators that display pro-inflammatory activity. In addition, studies have recently shown that a correlation exists between serum neutrophil levels and atherosclerosis [12]. Most importantly, the neutrophil concentration is particularly high within atherosclerotic plaques. Neutrophil activation produces diverse biochemical changes [13]. TNF-α and IL-6 are the main pro-inflammatory cytokines associated with atherosclerosis and other inflammatory diseases, and these cytokines are abundantly expressed in atherosclerotic lesions [14–16]. TNF-α plays a major role in the recruitment and activation of inflammatory cells and stimulation of matrix metalloproteinase production, which promotes plaque rupture. Circulating IL-6 is the central mediator of the acute-phase response and the primary inducer of C-reactive protein production in liver, which, along with TNF-α and IL-6, has been associated with coronary artery disease risk

* Corresponding author. Tel.: +81 426 76 5561; fax: +81 426 76 5570.
E-mail address: ohnonao@ps.toyaku.ac.jp (N. Ohno).

0021-9150/$ – see front matter © 2005 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.atherosclerosis.2005.08.014

in a number of studies. Thus, TNF-α and IL-6 have been speculated to be pro-atherogenic based on their inflammatory activities.

Kawasaki disease, also referred to as acute febrile mucocutaneous lymph node syndrome or MCLS, was first reported by Kawasaki in 1967 [17,18]. A disease of unknown cause, it affects mainly children aged 4 and under. Kawasaki disease is characterized by fever, bilateral non-exudative conjunctivitis, erythema of the lips and oral mucosa, changes in the extremities, rash, and cervical lymphadenopathy. Coronary artery aneurysms or ectasia develop in 15–25% of untreated children with the disease and may lead to myocardial infarction (MI), sudden death, or ischemic heart disease [19,20]. Although the occurrence of such coronary artery disorders has decreased with γ-globulin therapy, the mechanism of occurrence along with the pharmacological mechanism of treatment is unknown [21,22].

Murata et al. [23,24] reported that Kawasaki-disease-like coronary arteritis was induced in mice by administration of an alkaline extract of *C. albicans* (CADS) isolated from patients with Kawasaki disease. This model has been extensively examined from various points of view, i.e. anti-myeloperoxidase antibody production, susceptibility loci, and histopathological features [25–27]. We have prepared a water-soluble polysaccharide fraction of *C. albicans* released into the culture supernatant (*Candida albicans* water-soluble fraction or CAWS), and performed various analyses. Even its wide variety of biological activities, CAWS is considered as microbial pathogen-associated molecular patterns (PAMPs) [28–32]. Previously, we have found that the intraperitoneal administration of CAWS to mice induces a coronary arteritis similar to that induced by CADS [33]. It is of note that the CAWS-induced arteritis showed significant strain dependency. 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 coronary arteritis induced by CAWS was accompanied by hypertrophy of the tunica intima, the rupture of elastic fibers and a diffuse invasion by lymphocytes, histiocytes, fibroblasts, smooth muscle cells and eosinophils of vascular endothelial cells and the regions surrounding blood vessels. On the basis of such characteristics, the coronary arteritis induced by CAWS was presumed to be the so-called proliferative granulomatous coronary arteritis, and is clearly different from fibrinoid arteritis [27]. In DBA/2 mice, it was observed to cover nearly the entire periphery of the vessels, and those mice were considered to demonstrate the most virulent form of coronary arteritis. CAWS/CADS-induced vasculitis may provide additional information in this field. Because symptoms of Kawasaki disease include lymphadenopathy which may be a principal mechanism of the disease, in the present study, we have examined CAWS-induced vasculitis, especially in DBA/2 mice, from the view point of cytokine synthesis in lymphoid tissue.

2. Materials and methods

2.1. Mice

Male DBA/2 and C3H/HeN mice were purchased from Japan SLC. The mice were housed in a specific pathogen-free (SPF) environment and were used at 5–14 weeks of age.

2.2. Preparation of CAWS

Candida albicans strain IFO1385 was purchased from the Institute for Fermentation, Osaka (IFO), stored at 25 °C on Sabauroud's agar (Difco, USA) and passaged once every 3 months. CAWS was prepared from *C. albicans* strain IFO1385 in accordance with conventional methods [28]. The procedure used was as follows: 5 L of medium (C-limiting medium) was added to a glass incubator and cultured for 2 days at 27 °C with air supplied at a rate of 5 L/min and rotation at 400 rpm. Following the culture, an equal volume of ethanol was added and after the mixture was allowed to stand overnight, the precipitate was collected. The precipitate was dissolved in 250 mL of distilled water, ethanol was added and the mixture was left to stand overnight. The precipitate was collected and dried with acetone to obtain CAWS.

2.3. Administration schedule for induction of coronary arteritis [33]

CAWS (4 mg/mouse) was administered intraperitoneally for five consecutive days to each mouse in week 1. The hearts of the animals were fixed with 10% neutral formalin and prepared in paraffin blocks. Tissue sections were stained with HE stain. Cells were prepared from the spleen and cultured.

2.4. Measurement of cytokines

Concentrations of cytokines were measured by ELISA. All of the monoclonal antibodies and corresponding recombinant cytokines used were purchased from Pharmingen.

2.5. Measurement of myeloperoxidase

Myeloperoxidase activity was measured by using 3,3′,5,5′-tetramethyl benzidine (TMB) and hydrogen peroxide as substrate by the method of Suzuki et al. [34].

2.6. In vitro spleen 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 $KHCO_2$, and 37.2 mg/L EDTA/2Na) to lyse red blood cells. After centrifugation, splenocytes were maintained in RPMI 1640 medium supplemented with 50 μg/mL of gentamycin sulfate and 10%

heat-inactivated fetal calf serum (FCS). Cells were cultured in 24-well flat-bottomed plates at 5×10^6 per well in 1 mL of culture medium, and stimulated with saline or CAWS (up to 10 μg/mL). Splenocytes were cultured at 37 °C for 48 h in a humidified 5% CO_2, 95% air atmosphere.

2.7. Preparation of dendritic cells (DCs)

Bone marrow was removed from mice. Bone marrow cells obtained by flushing femoral shafts were suspended in HBSS containing 50 μg/mL of gentamycin sulfate. After centrifugation, the single cell suspension was treated with an ACK-lysing buffer (8.29 g/L NH_4Cl, 1 g/L $KHCO_2$ and 37.2 mg/L EDTA/2Na) to lyse red blood cells. After centrifugation, the cells were maintained in RPMI 1640 medium supplemented with 50 μg/mL of gentamycin sulfate containing 5% heat-inactivated FCS, 10 ng/mL of recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF; Pharmingen, San Diego, CA) and 5 ng/mL of recombinant mouse interleukin-4 (IL-4; Pharmingen) and cultured in 24-well flat-bottomed plates at 1×10^6 per well in 1 mL of culture medium at 37 °C in a humidified 5% CO_2: 95% air atmosphere. Non-adherent and loosely adherent cells were removed by pipetting on day 2 and replated with fresh cytokine-containing medium in the plate. CAWS was added on day 5. On day 7, non-adherent and loosely adherent cells were collected.

2.8. FACS analysis

Spleen cells were stained with FITC- and PE-conjugated mAbs. To block FcR-mediated binding of the mAb, anti-mouse CD16/CD32 FcBlockTM (Pharmingen Co. USA) was added. All incubation steps were performed at 4 °C for 30 min. The cells were analyzed on a FACS. Calibur and data were processed by using the CELLQuest program.

2.9. Test for significant differences

Tests for significant differences in this study were performed using Student's t-test and values of $P < 0.05$ were judged significant.

3. Results

3.1. Response of spleen cells to CAWS in DBA/2 mice

The administration of CAWS induced coronary arteritis in various strains of mice, including C3H/HeN, C57Bl/6, and DBA/2 [33]. In DBA/2 mice, the arteritis was observed to affect nearly the entire periphery of the vessels, and those mice were considered to demonstrate the most virulent form of coronary arteritis (Fig. 1). In addition, only DBA/2 mice exhibited high mortality during the observation period (Fig. 1). Fig. 2 shows histological features of the heart of a CAWS-administered DBA/2 mouse on days 3, 10, 17 and 24 after the final administration. Hyperplasy and remodeling of the artery were significantly induced around day 10 and the ventricles had a denatured histology. As stated in the introduction, Kawasaki disease is a systemic disease associated with lymphadenopathy, so we compared the response to CAWS of spleen cells of DBA/2 mice. We used C3H/HeN mice as a control, in which CAWS induced coronary arteritis but was not lethal [33].

To demonstrate the reactivity of spleen cells to CAWS, 2 or 4 mg of CAWS was administered for five consecutive days and then the responses of the cells were examined. Fig. 3 shows the number of spleen cells in CAWS-administered mice from immediately after to 5 weeks after the administration. Cell numbers were significantly increased just after the administration and remained high for at least 5 weeks. The total cell number was increased in both strains of mice, but much more so in DBA/2 mice. From the splenic cell numbers (Fig. 3) and the histological changes of arteries (Fig. 2), repeated administration of CAWS induced not only acute but also chronic immune/inflammatory cascades.

Fig. 4 shows cytokine levels in spleen cells cultured without further stimulation. It is of note that cytokines were spontaneously produced just after the administration and disappeared 1 week later. We tested IFN-γ, IL-6, and IL-10, and found IL-6 and IL-10 levels to be similar between DBA/2 and C3H/HeN. However, IFN-γ levels were significantly elevated in DBA/2 mice, especially just after the administration (Fig. 4(A)). The accumulation of neutrophils and macrophages in spleen cells was detected by flow cytometry using Gr-1 and Mac-1 antibodies. As shown in Fig. 5, PMN as well as macrophage numbers were almost doubled in both strains. Neutrophil activity was further examined using a specific enzyme, myeloperoxidase. Fig. 6 shows myeloperoxidase (MPO) activity in the supernatant of a spleen cell culture. MPO was spontaneously released immediately after the administration of CAWS, but disappeared later. However, MPO activity was maintained in splenocytes of both strains of mice for more than 5 weeks (Fig. 6). Considering these results, strain-dependent changes were only observed upon the release of IFN-γ. IFN-γ would be a key to the severity of coronary arteritis.

3.2. Production of IFN-γ by DBA/2 spleen cells in response to CAWS

The data shown in the above section suggested that the synthesis of IFN-γ is a key reaction of the CAWS-induced lethal toxicity in DBA/2 mice. We have recently demonstrated that spleen cells of DBA/2 mice responded to fungal branched 1,3-β-glucan (BG) to produce IFN-γ in vitro and the process was mediated by GM-CSF [35,36]. Addition of recombinant GM-CSF to the spleen cell culture augmented the production of IFN-γ in response to BG. Thus we tested GM-CSF levels in DBA/2 mice exposed to CAWS in vitro. As shown in Fig. 7(left), DBA/2 splenocytes produced GM-

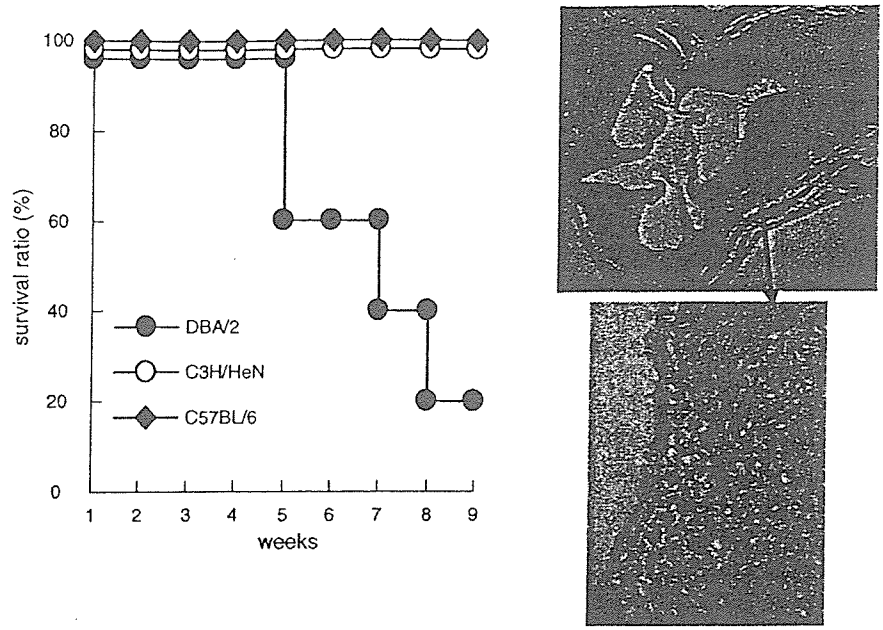

Fig. 1. Survival and histological examination of CAWS-induced coronary arteritis in mice. CAWS (0 or 4 mg/mouse) was administered i.p. to DBA/2, C3H/HeN and C57BL/6 mice for five consecutive days in the 1st and 5th week. Survival had been observed for 9 weeks (left). CAWS (4 mg/mouse) was administered i.p. to DBA/2 mice for five consecutive days. Five weeks later, the hearts of DBA/2 mice were fixed in buffered formalin solution, embedded in paraffin, thin-sectioned, and stained with hematoxylin–eosin (right).

CSF in response to CAWS dose-dependently. It is of note that production of GM-CSF was not induced in C3H/HeN mice. We also tested the reaction of DBA/2 mice to CAWS in the presence of anti-GM-CSF. As shown in Fig. 7(right), production of IFN-γ in response to CAWS was significantly inhibited in the presence of anti-GM-CSF. In contrast, addition of recombinant GM-CSF increased production of IFN-γ in spleen cell cultures in the presence of CAWS (Fig. 8(left)). Levels of other cytokines such as TNF-α and IL-6 were also elevated. From the results of in vitro experiments, production of IFN-γ induced by CAWS would be closely related to levels of GM-CSF.

3.3. Response of adherent cells to CAWS

Dendritic cells (DCs) are distributed throughout organs to collect information on antigens including microbial pathogens, toxins, cancers, and aged and altered cells. Thus,

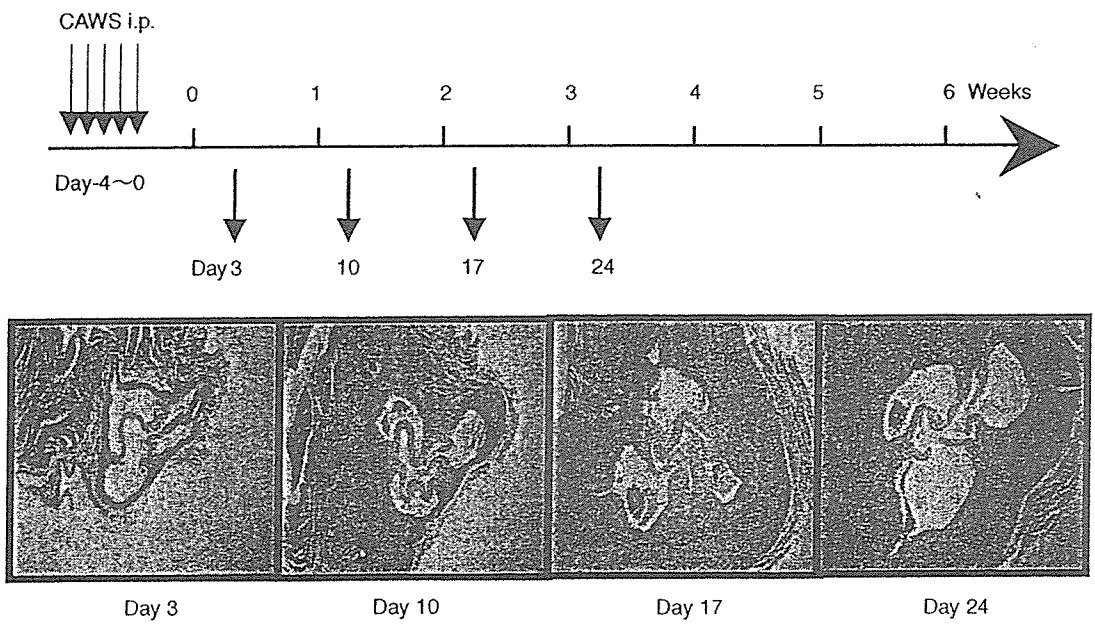

Fig. 2. Kinetic histological observation of arteries of DBA/2 mice administered CAWS. CAWS (4 mg/mouse) was administered i.p. to DBA/2 mice for five consecutive days. On days 3, 10, 17 and 24, the hearts of these mice were fixed and stained with hematoxylin–eosin.

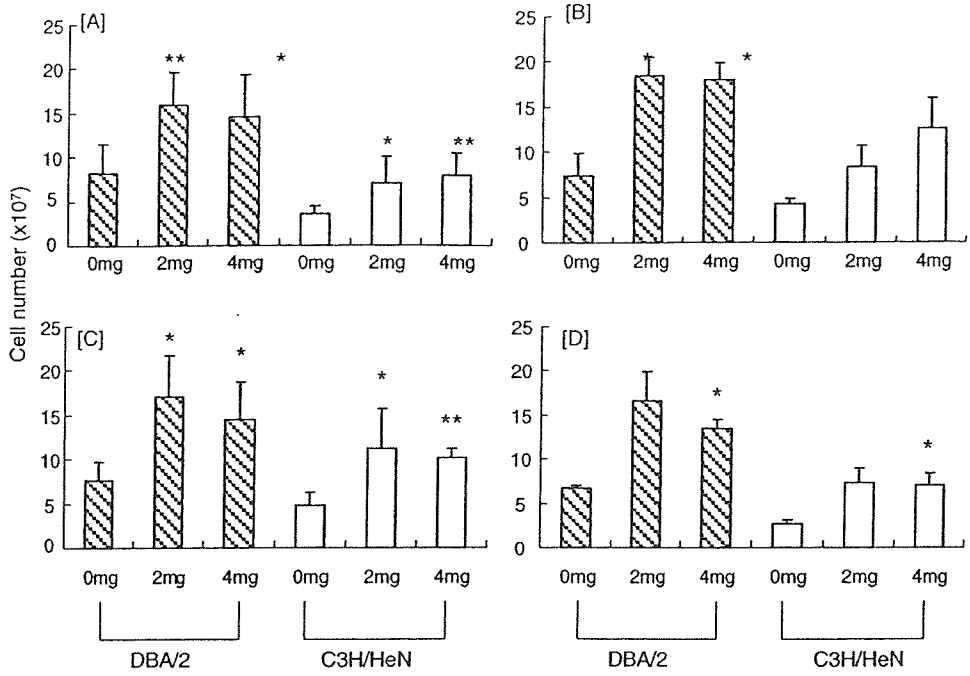

Fig. 3. Number of splenocytes from CAWS-administered mice. CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90 min, 1 week, 3 weeks or 5 weeks after the final administration. Total cell number was counted with a hemocytometer. Oblique and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean ± S.D. $^{*}P<0.05$ and $^{**}P<0.005$ compared with control (0 mg) using Student's *t*-test. (A) 90 min after final administration. (B) 1 week after final administration. (C) 3 weeks after final administration. (D) 5 weeks after final administration.

the DC is a key leukocyte, which initiates and maintains immune responses as well as inflammatory responses. Thus we also tested for the activation of DCs by CAWS. A DC fraction was prepared using standard methods, by stimulating bone marrow cells with GM-CSF and IL-4. The supernatant of the DC culture contained higher concentrations of IL-6 and TNF-α in response to CAWS, however, IFN-γ could not be detected in this culture (Fig. 8(right)). The activation of DC by CAWS was also demonstrated by enhanced expression of CD80, MHC class I antigens, as well as MHC class II antigens (data not shown). These results suggested that even though CAWS activated the DC fraction to some extent, splenic mature leukocytes would be necessary for the production of IFN-γ.

4. Discussion

CAWS-induced coronary arteritis in mice and the severity was found to differ significantly with strain: CBA/j mice were most resistant and showed almost no signs of arteritis, C3H/HeN mice were mildly sensitive and developed a moderately severe arteritis, and DBA/2 mice were most sensitive and developed an arteritis that was lethal. Severe lesions gradually formed over several weeks after which the mice began to die (Figs. 1 and 2). Heart weights of the dead mice were increased, suggesting that the lethality is related to the dilated cardiomyopathy (unpublished results). We have shown that during the period of vascular dysfunction, splenomegay was also induced chronically (Fig. 3). These results strongly suggested that the biological responses triggered during coronary arteritis not only occurred at the site of local inflammation but also were systemically induced. These observations have some similarity to the acute and systemic symptoms of Kawasaki disease. Understanding the molecular events during coronary arteritis and developing new therapeutic strategies for vascular biology will require research in various areas. We have already shown that CAWS induces several responses [30]; i.e. the production of thrombomodulin by HUVEC, the activation of complement, the aggregation of platelets, the synthesis of cytokines and so on. In the present study, the response of spleen cells to CAWS was investigated focusing on two strains of mice, DBA/2 and C3H/HeN.

On comparing the two strains, we found several interesting differences. The degree of splenomegary differed and leukocyte infiltration was more significant in DBA/2 mice. The cells that had infiltrated the spleen were found to be mainly neutrophils and macrophages, and numbers of leukocytes remained high for 5 weeks, suggesting the induction of a systemic/chronic inflammatory response. It is of note that infiltrated leukocytes were activated as assessed by the synthesis of IFN-γ and release of MPO especially just after the administration of CAWS, and that these changes were specific to DBA/2 mice. MPO is a key molecule for the oxygen dependent organ failure induced by neutrophils to produce hypochlorous acid. Oxidative damage of the vascular wall might be a very early event of CAWS-induced vasculitis.

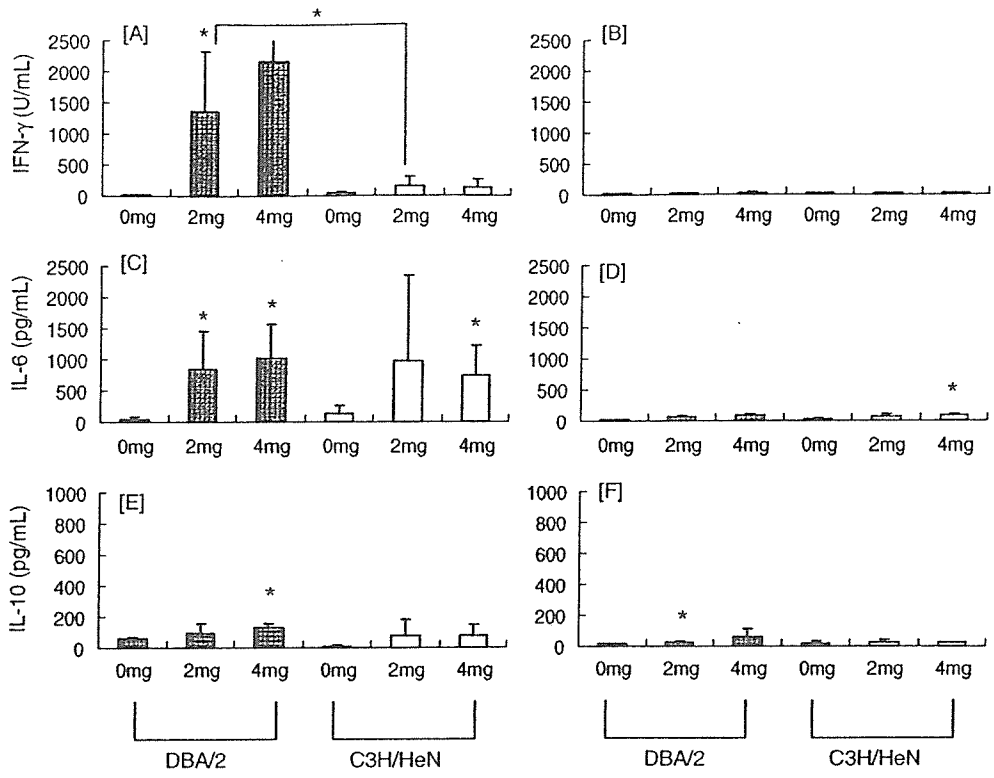

Fig. 4. Cytokine production in culture supernatants of splenocytes from CAWS-administered mice. CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90 min or 1 week after the final administration. IFN-γ, IL-6 and -10 concentrations were measured by ELISA. Oblique and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results represent the mean ± S.D. $^{*}P<0.05$ and $^{**}P<0.005$ compared with control (0 mg) using Student's *t*-test. (A) IFN-γ, 90 min after final administration. (B) IFN-γ, 1 week after final administration. (C) IL-6, 90 min after final administration. (D) IL-6, 1 week after final administration. (E) IL-10, 90 min after final administration. (F) IL-10, 1 week after final administration.

The in vitro spleen cell culture revealed that the production of cytokines, especially GM-CSF, in response to CAWS depended significantly on strain. Harada et al recently found that fungal BG induced the production of IFN-γ by splenocytes of DBA/2 mice in vitro [35,36]. In splenocytes derived from almost all inbred strains of mice except for DBA/1 and DBA/2, IFN-γ was not produced in response to BG. The level of IFN-γ was most significantly increased by contact between adherent and non-adherent splenocytes. In addition to IFN-γ, TNF-α, GM-CSF and IL-12p70 were also increased. The addition of recombinant murine GM-CSF (rMuGM-CSF) to spleen cell cultures from various strains of mice syner-

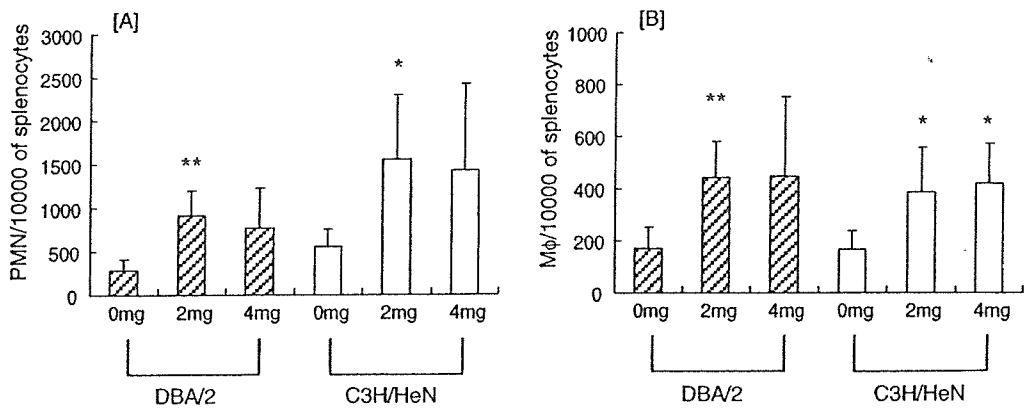

Fig. 5. Ratio of granulocytes and macrophages in splenocytes from CAWS-administered mice. CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of sacrificed mice at 90 min after the final administration. Cell counts of granulocytes and macrophages were made by flow cytometry as described in Section 2. Oblique and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean ± S.D. $^{*}P<0.05$ and $^{**}P<0.005$ compared with control (0 mg) using Student's *t*-test. (A) PMN. (B) *Mφ*.

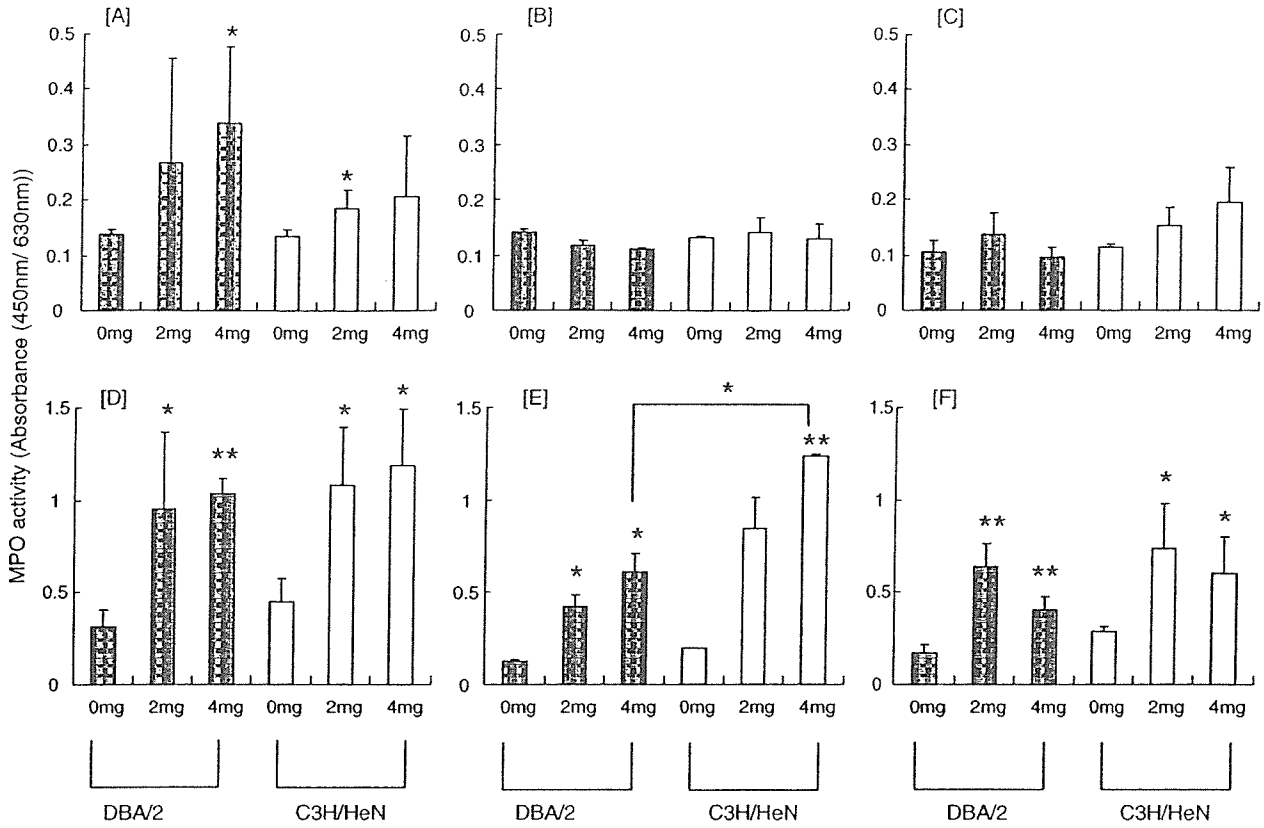

Fig. 6. Myeloperoxidase activity in the cell suspension and the culture supernatants of splenocytes from CAWS-administered mice. CAWS (0, 2 or 4 mg/mouse) was intraperitoneally administered to DBA/2 and C3H/HeN mice for five consecutive days. The splenocytes were collected from each group of mice at 90 min, 1 week, or 5 weeks after the final administration. Myeloperoxidase activity in the culture supernatant and the cell suspension was measured as described in Section 2. Oblique and white bars show the results from DBA/2 mice and C3H/HeN mice, respectively. The results shown represent the mean ± S.D. $^{*}P<0.05$ and $^{**}P<0.005$ compared with control (0 mg) using Student's *t*-test. (A)–(C) culture supernatant, (D)–(F) cell suspension, (A) and (D) 90 min after the final administration. (B) and (E) 1 week after the final administration. (C) and (F) 5 weeks after the final administration.

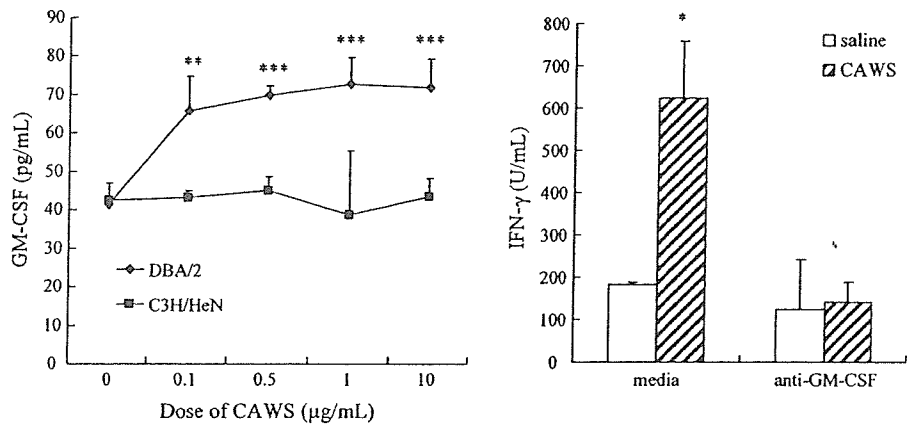

Fig. 7. Contribution of GM-CSF to responses of spleen cells stimulated with CAWS: (left) splenocytes were removed from DBA/2 and C3H/HeN mice. The spleen was teased apart in RPMI 1640 medium, and treated with ACK-lysing buffer. After centrifugation, splenocytes were suspended in RPMI 1640 medium supplemented with 50 μg/mL of gentamycin sulfate and 10% heat-inactivated fetal calf serum (FCS) and cultured in 24-well flat-bottomed plates at 5×10^6 cells per well in 1 mL of culture medium in the presence or absence of CAWS (up to 10 μg/mL). Splenocytes were cultured at 37 °C for 48 h in a humidified 5% CO_2, 95% air atmosphere. GM-CSF concentrations in the resulting culture supernatants were measured by ELISA as described in Section 2. (right) Splenocytes were removed from DBA/2 mice. The spleen was teased apart in RPMI 1640 medium, and treated with ACK-lysing buffer. After centrifugation, splenocytes were suspended in RPMI 1640 medium supplemented with 50 μg/mL of gentamycin sulfate and 10% heat-inactivated fetal calf serum (FCS). The resulting suspensions were cultured with CAWS (10 μg/mL) in 24-well flat-bottomed plates at 5×10^6 cells per well in 1 mL of culture medium in the presence or absence of anti-GM-CSF (1 ng/mL, Pharmingen). Splenocytes were cultured at 37 °C for 48 h in a humidified 5% CO_2, 95% air atmosphere. IFN-γ concentrations in the resulting culture supernatants were measured by ELISA as described in Section 2.

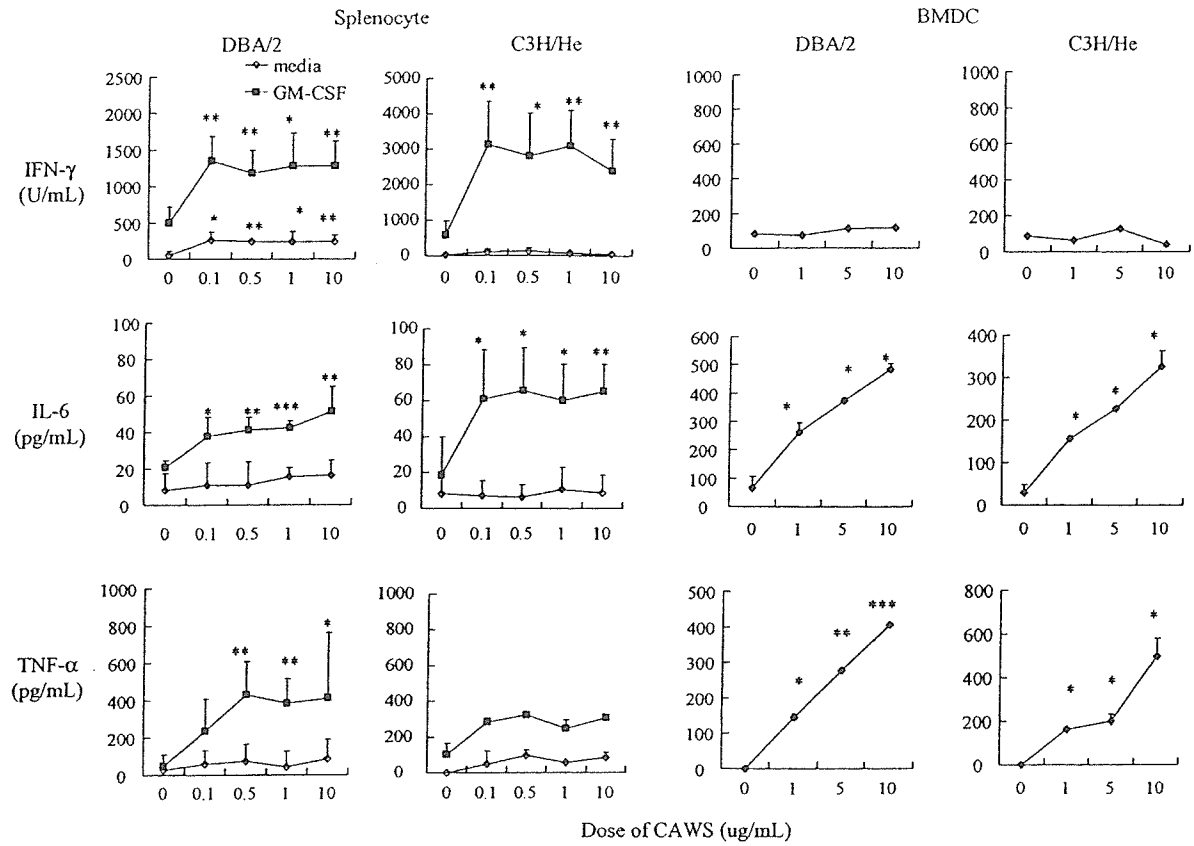

Fig. 8. Production of IFN-γ, IL-6, and TNF-α by spleen cells and bone marrow cells stimulated with CAWS in the presence or absence of GM-CSF. (left) Splenocytes were removed from DBA/2 and C3H/HeN mice. The spleen was teased apart in RPMI 1640 medium, and treated with ACK-lysing buffer. After centrifugation, splenocytes were suspended in RPMI 1640 medium supplemented with 50 μg/mL of gentamycin sulfate and 10% heat-inactivated fetal calf serum (FCS). The resulting suspensions were cultured with CAWS (up to 10 μg/mL) in 24-well flat-bottomed plates at 5×10^6 cells per well in 1 mL of culture medium in the presence or absence of GM-CSF (1 ng/mL, Pharmingen). Splenocytes were cultured at 37 °C for 48 h in a humidified 5% CO_2, 95% air atmosphere. Cytokine concentrations in the resulting culture supernatants were measured by ELISA as described in Section 2. (right) Bone marrow cells of DBA/2 and C3H/HeN mice obtained by flushing femoral shafts were suspended in RPMI 1640 medium supplemented with 50 μg/mL of gentamycin sulfate containing 5% heat-inactivated FCS, 10 ng/mL of recombinant murine granulocyte-macrophage colony stimulating factor (GM-CSF; Pharmingen, San Diego, CA) and 5 ng/mL of recombinant mouse interleukin-4 (IL-4; Pharmingen) and cultured in 24-well flat-bottomed plates at 1×10^6 cells per well in 1 mL of culture medium at 37 °C in a humidified 5% CO_2: 95% air atmosphere. Non-adherent and loosely adherent cells were removed by pipetting on day 2 and replated with fresh cytokine-containing medium in the plate. CAWS was added on day 5. On day 7, the resulting culture supernatants were collected and concentrations of cytokines were measured by ELISA as described in Section 2.

gistically enhanced the production of IFN-γ, TNF-α and IL-12p70 in the presence of BG. In contrast, neutralizing GM-CSF using anti-GM-CSF monoclonal antibody (mAb) significantly inhibited production of IFN-γ, TNF-α and IL-12p70 elicited by BG. These findings strongly suggested that GM-CSF is a key molecule in the production of cytokines induced by BG, and that synthesis of GM-CSF is a specific step in DBA/2 mice in vitro. In the present study, CAWS induced production of IFN-γ and GM-CSF by spleen cells of not C3H/He but DBA/2 mice, and the activity was inhibited by adding anti-GM-CSF antibody. These results suggested that GM-CSF is a key to the severity of the coronary arteritis induced by CAWS.

GM-CSF is known to play crucial roles in various diseases, such as pulmonary alveolar proteinosis, acute pancreatitis-associated lung injury, collagen-induced arthritis, atherosclerosis, acute coronary syndromes, multiple sclerosis, leukocyte-mediated immune glomerular injury, LPS-induced lung injury and zymocel-induced hepatic granuloma formation [37–44]. These roles have been established by using either knockout mice or a monoclonal antibody. GM-CSF is an aggravating factor in all of these diseases, and thus is a target for therapeutic intervention.

GM-CSF is a growth factor for granulocytes and macrophages. Biochemical and molecular biological analyses have revealed additional functions of GM-CSF in the differentiation, maturation and activation of leukocytes, i.e. GM-CSF activates neutrophils and inhibits programmed cell death [45,46]. Increased numbers of activated neutrophils in lesions thus induced more significant damage. A representative toxic molecule, MPO, was released from activated neutrophils in response to FMLP [47]. The release was enhanced by treatment with GM-CSF. The presence of hydrogen peroxide induced production of hypochlorous acid, thus leading to more severe lesions. In addition, mature macrophages usually contain little MPO, but in some instances, macrophages