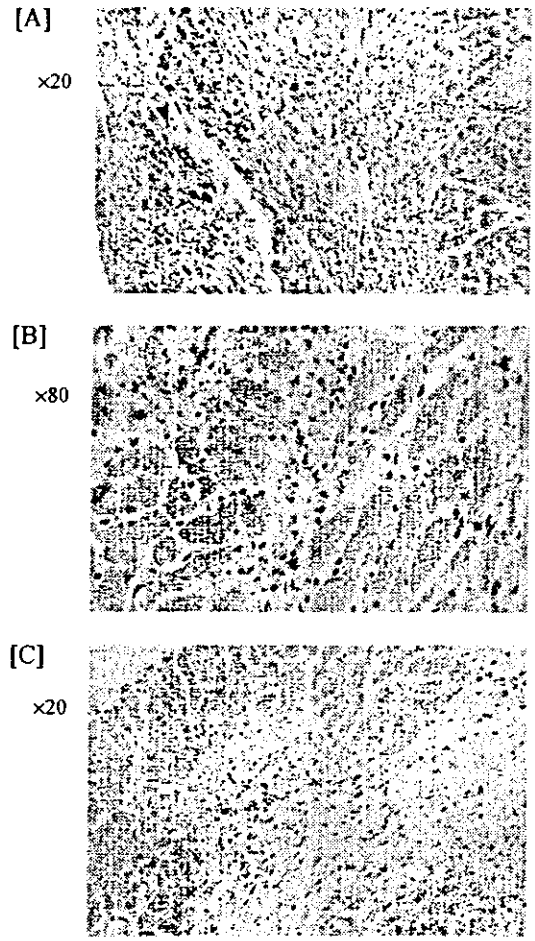

Figure 3. Histological analysis of dead DBA/2 mice administered with CAWS. [A, B, C] CAWS (4mg/mouse) was administered i.p. to DBA/2 mice for five consecutive days in the 1st and 5th week. Thereafter, the hearts of dead mice were stained with hematoxylin-eosin.

Cytokine Production by Spleen Cells of CAWS-Administered Mice on Stimulation with CAWS

As splenocyte counts in DBA/2 mice were high, we examined the production of cytokines in response to CAWS. Spleen cells of mice administered CAWS to induce coronary arteritis were prepared at a concentration of 1×10^7 cells/ml, and cultured for 48 hours in a 5% CO_2 incubator at 37° for observing cytokine production. Following the culture, IL-1β, IL-4, IL-6, IL-10, IL-12, IFN-α, and TNF-α levels in the culture supernatant were measured by ELISA. The strains with severe coronary arteritis, C3H/HeN, DBA/2 and C57BL/6 mice, showed IL-1β and IL-6 production by spleen cells in the CAWS -administered group (Fig. 5). In contrast, IL-10 was significantly produced

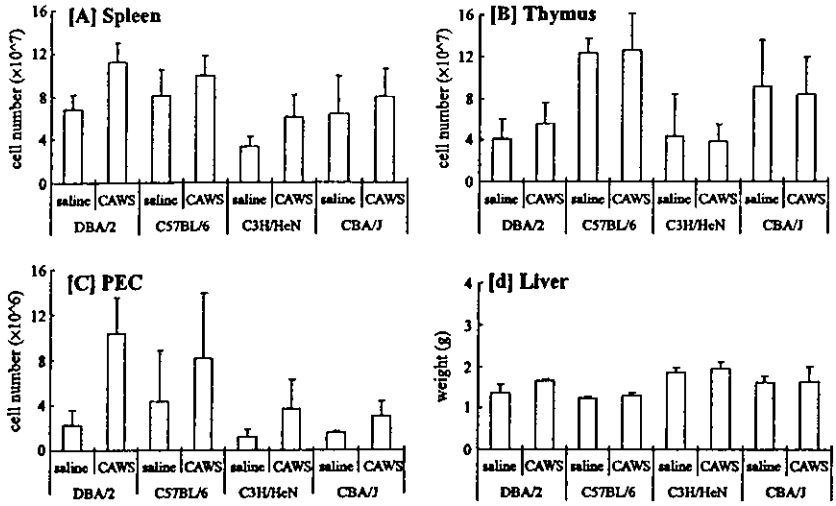

Figure 4. Cell number in peripheral blood and organ weight from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, the internal organs were collected from each mouse. Total cell number was counted with a hemocytometer and organ weight was measured with a analytical balance. The results show the mean ± standard deviation (S.D.). *; $P<0.05$ compared with the control using Student's *t-test*. [A]: Spleen, [B]: Thymus, [C]: PEC, [D]: Liver.

by spleen cells of the CBA/J mouse, which is the strain resistant to the coronary arteritis induced by CAWS.

In order to observe CAWS-specific reactions, the spleen cells of mice administered CAWS were stimulated with CAWS (0, 2.5, 5 or 10 μg/ml) and cultured for 48 hours in a 5% CO_2 incubator at 37°C. IFN-γ, IL-6 and IL-10 levels correlated well with the degree of coronary arteritis induced by CAWS (Fig. 6). IFN-γ and IL-6 production in DBA/2 and C57BL/6 mice tended to increase during the CAWS-specific response in the CAWS groups as compared with the saline groups, but no response was observed in C3H/HeN and CBA/J mice. IL-10 production was particularly enhanced in CBA/J mice treated with CAWS and slightly increased in the saline group, but no significant increase in production was observed even with the administration of CAWS in DBA/2 and C57BL/6 mice, and only a slight increase in C3H/HeN mice. Levels of other cytokines were not correlated with the coronary arteritis caused by CAWS. The amount of IL-4 production was small in all strains, but increased somewhat in CBA/J and C3H/HeN mice. IL-12 was hardly detected in any of the strains.

Measurement of Anti-CAWS Antibody Titer

Serum was obtained from mice administered CAWS in accordance with the coronary arteritis induction protocol, and anti-CAWS antibody in serum was detected with anti-mouse IgG + IgM Ab. Anti-CAWS antibodies were detected in all of the

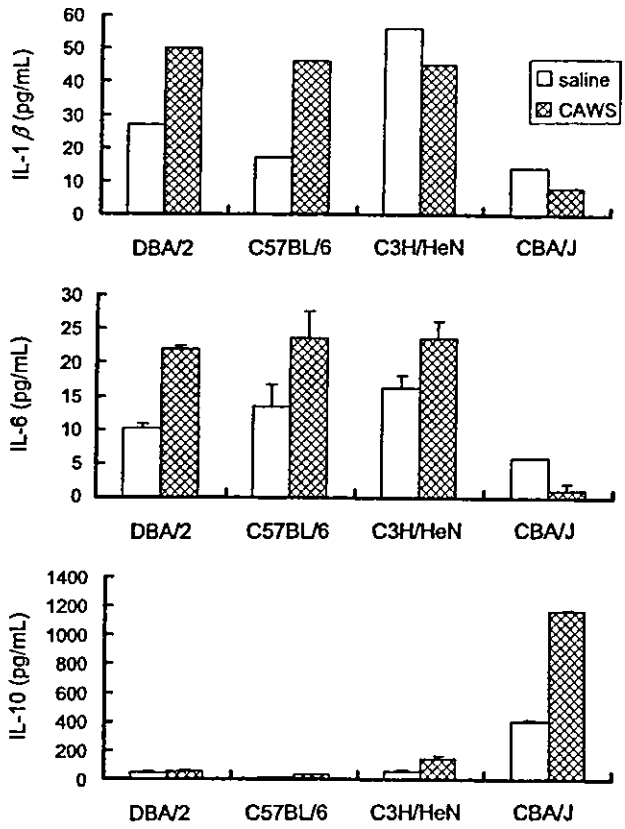

Figure 5. Cytokine production in culture supernatants of splenocytes in vivo from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, splenocytes were collected from each mouse. The splenocytes were cultured for 48 hour at a density of 1×10^7 cells/ml. The culture supernatants were collected and the level of each cytokine was measured by ELISA. The data shows one of four (C3H/HeN and CBA/J), three (DBA/2) or two (C57BL/6) experiments performed with similar results. The results show the mean ± standard deviation (S.D.).

mice, and the titers were extremely high (Fig. 7). Although the IgM titer varied depending on the mouse strain, there was no relationship between the IgM and the incidence of coronary arteritis (Fig. 8). Little IgE was detected in any of the strains. Conversely, IgG1 was detected at high levels in all the strains. CBA/J mice exhibited the lowest IgG2a titers.

On the basis of the above results, the antibodies produced in the form of anti-CAWS antibodies consisted mainly of IgG, followed by IgM. With respect to IgG2a production, although the titers were low in CBA/J and C57BL/6 mice, as the levels in CBA/J mice were roughly half those in C57BL/6 mice, and when considering the results of cytokine production in the spleen, it is possible that CBA/J mice exhibit Th2 bias.

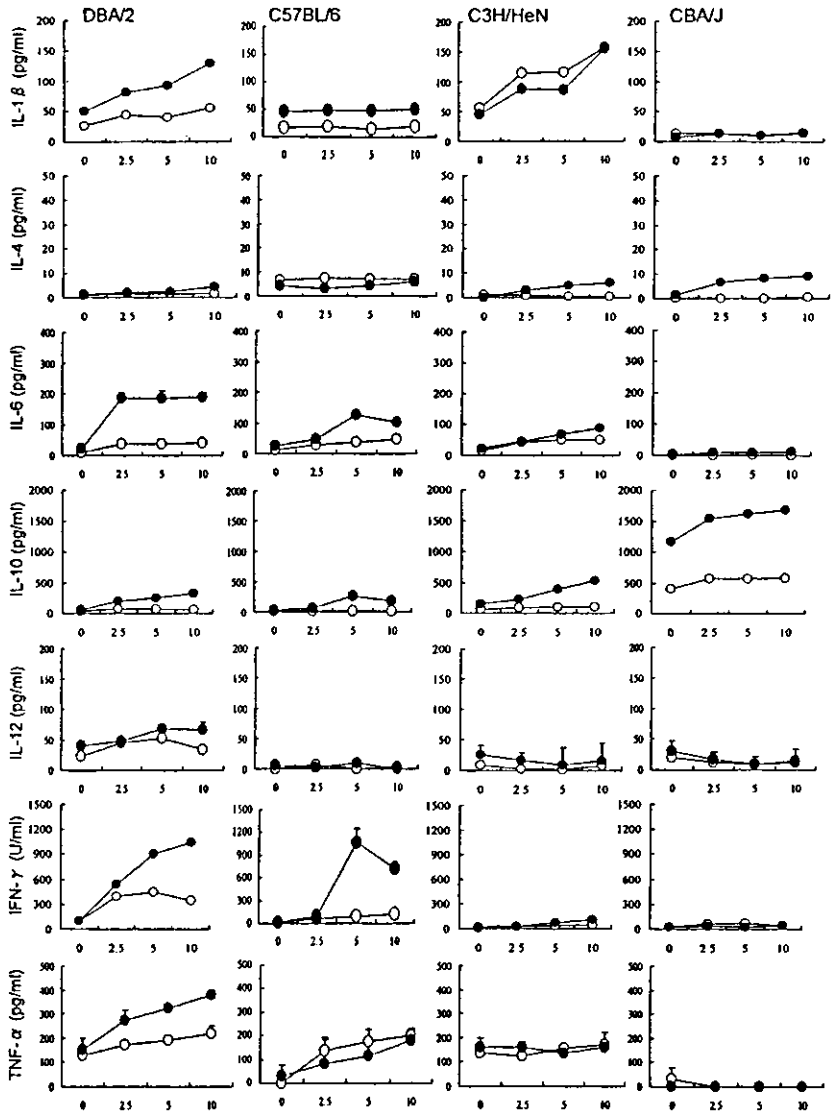

Figure 6. Cytokine production in culture supernatants of splenocytes stimulated with CAWS in vivo from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, splenocytes were collected from each mouse. The splenocytes were cultured with CAWS (0, 2.5, 5 or 10 μg/ml) for 48 hour at a density of 1×10^7 cells/ml. The culture supernatants were collected and the level of each cytokine was measured by ELISA. The data shows one of four (C3H/HeN and CBA/J), three (DBA/2) or two (C57BL/6) experiments performed with similar results. The results show the mean ± standard deviation (S.D.). *; $P < 0.05$ compared with the control using Student's *t-test*.

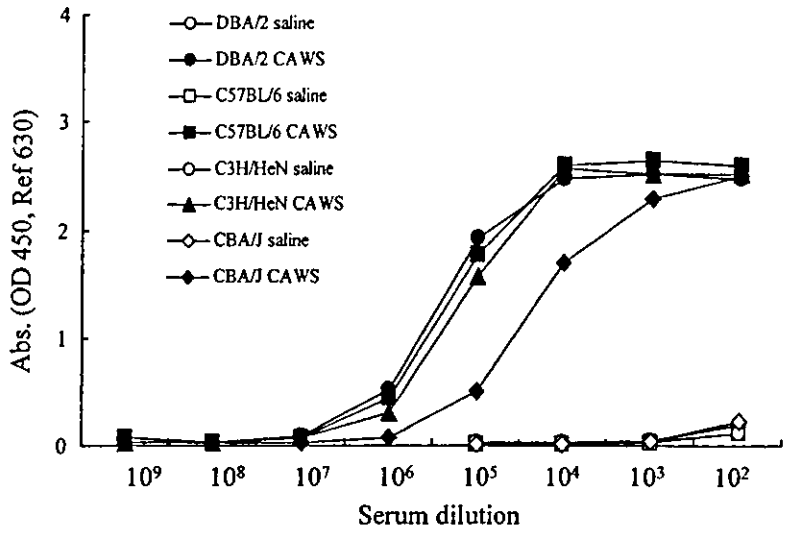

Figure 7. Anti-CAWS antibody in sera from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, sera were collected from each mouse. Anti-CAWS antibody was measured by ELISA. Color development was stopped after 10 min.

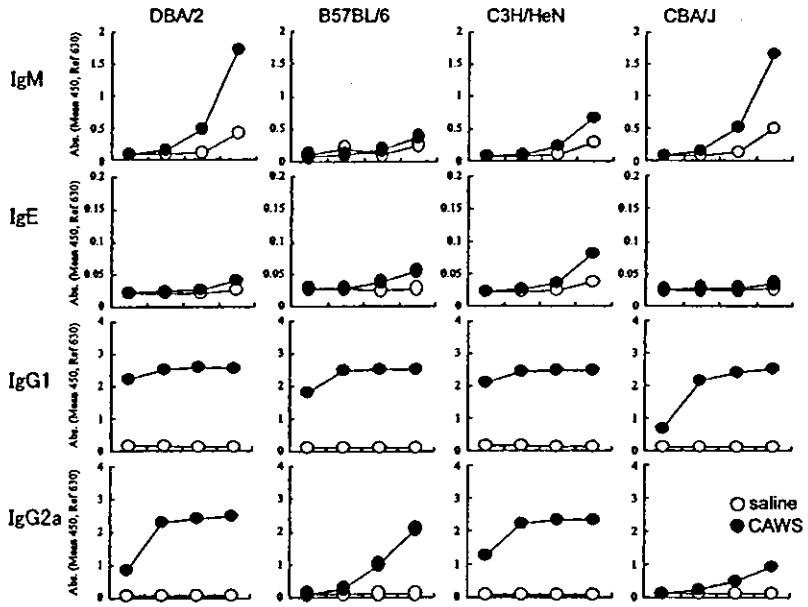

Figure 8. Immunoglobulin subclasses of anti-CAWS antibody in sera from CAWS-administered mice. CAWS (0 or 4mg/mouse) was administered i.p. to DBA/2, C57BL/6, C3H/HeN and CBA/J mice for five consecutive days in the 1st and 5th week. In the 9th week, sera were collected from each mouse. Anti-CAWS immunoglobulin subclasses were measured by ELISA. Color development was stopped after 10 minute.

DISCUSSION

Kawasaki disease is a febrile inflammatory disease that presents with systemic arteritis, and can be fatal particularly in the case of an exacerbation of coronaritis. When CAWS is administered to mice in accordance with the protocol of Murata et al.,[7,8] a Kawasaki-disease-like angiitis is induced at the origin of the coronary arteries. In the present study, strain differences were found to exist with respect to the incidence of the disease induced by CAWS with DBA/2, C3H/HeN and C57BL/6 mice exhibiting sensitivity and CBA/J mice exhibiting resistance. Moreover, histological observation of the sites of coronaritis in DBA/2 mice revealed hypertrophy of the tunica intima and cellular invasion, and the disease occurred with extremely high levels of severity and frequency. DBA/2 mice developed a much more severe coronary arteritis than the other strains. In addition, DBA/2 mice exhibited high mortality during the course of the disease's induction, and based on findings obtained from histological examination of the hearts, the cause of death was suspected to be myocardial infarction attributable to coronary occlusion. Because the difference in sensitivity to CAWS among the mouse strains examined in this study correlates strongly with the diversity of prognoses for Kawasaki disease patients,[19] this model is considered to be effective for elucidating the cause of coronary arteritis associated with Kawasaki disease, analyzing the characteristic condition, and developing more effective treatment methods.

Among patients with Kawasaki disease in the acute stage, cytokines, including IL-1, IL-2, IL-2 receptor, IL-6 and TNF-α, are detected in the serum.[20–23] When the production of cytokines from spleen cells was measured in mice stimulated with CAWS in vitro, a similar trend was demonstrated by the three strains that were sensitive to coronary arteritis induction, namely, DBA/2, C3H/HeN and C57BL/6, and in the case of DBA/2 mice in particular, a prominent CAWS-specific response involving inflammatory cytokines such as IL-6, IFN-γ and TNF-α, was observed, indicating the occurrence of an inflammatory immune response. The levels of inflammatory cytokines produced were high in DBA/2 mice in particular. On the other hand, an increased production of IL-10, which exhibits an immunosuppressive action, was observed in CBA/J mice that exhibited resistance to the occurrence of coronary arteritis. It is interesting to note that these cytokine production patterns resemble those observed in Kawasaki disease patients.

However, as there is one report indicating increased production of IL-4 and IL-10 in patients with Kawasaki disease,[24] dynamics that do not necessarily coincide with the findings of this study are observed. The discrepancies may be related to the stage of the disease (acute, subacute or recovery stage), thus indicating the need for further study of the relationship between cytokine production and the condition of Kawasaki disease.

Analyses have been conducted on the background genes of various diseases, and in the case of Kawasaki disease as well, there is a possibility of some form of involvement by genetic factors.[25,26] During the course of research on genes associated with coronaritis in rodents, numerous analyses have been conducted on the relationship between arteriosclerosis and hyperlipemia, and the IL-10 gene has been reported to have a close relationship with the lesions of coronary arteritis.[27,28] This finding also supports the findings obtained in the present study. In addition, although CBA is a strain derived from DBA, it is quite interesting that there are large differences in the

incidences of coronary arteritis and myocarditis induced by CAWS between the two strains. A survey of the genes involved in mouse coronaritis also suggested the existence of multiple inductive genes and repressor genes. This is a subject that requires detailed analysis.

On the basis of the above results, the activation of lymphocytes, vascular endothelial cells and so forth was prominently induced by means of hypercytokinemia in DBA/2 mice, and the resulting coronaritis promoted the occurrence of chronic myocardial ischemia, which, as a result, was thought to ultimately lead to death caused by fibrosis, infarction and cardiac insufficiency. It is hoped that this model will contribute not only to elucidation of the stage of Kawasaki disease and the associated coronary arteritis, but also to the improvement and development of treatment methods.

ACKNOWLEDGMENTS

This work was partly supported by a grant for private universities provided by Grant-in-Aid for Scientific Research, Ministry of Education, Culture, Sports, Science, and Technology and The Promotion and Mutual Aid Corporation for Private Schools, Japan.

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Jpn. J. Infect. Dis., 57, 2004

Contribution of Peroxidases in Host-Defense, Diseases and Cellular Functions

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SUMMARY: Peroxidases figure prominently in biology and contribute significantly to cell biology, host defense against infection, and pathogenesis of several inflammatory diseases. These varied and diverse aspects of peroxidase biochemistry and its clinical implications will be the subjects of in-depth analysis at the 4th International peroxidase meeting held in Kyoto. Specific topics range from the molecular basis of peroxidase structure and function to the clinical consequences of autoantibodies generated against myeloperoxidase (MPO), the peroxidase present in circulating neutrophils. Consideration of novel aspects of peroxidase biology, both unanticipated biochemical properties of MPO and the potential role of MPO in the pathogenesis of inflammatory diseases such as atherosclerosis, will also be included. In addition to peroxidases, the newly expanded family of NADPH oxidases will be discussed. We hope that this collection of scientists who share a common interest in peroxidase biology but each possess expertise in distinctly different aspects of the subject will provide a setting for spirited discussion and a lively exchange of views to yield advances in understanding and to create new applications of those insights to benefit clinical medicine, agriculture and industry.

1. Peroxidase and related oxidase studies

In 2000, a book entitled "The peroxidase multigene family of enzymes" (1) updated the peroxidase field and summarized the proceedings of the 2nd International Peroxidases Meeting held in Chiemsee and organized by Drs. Petro Petrides and William Nauseef. However since that time, several advances in the peroxidase field have occurred. Recent work has uncovered novel biochemistry, new gene families, and knock-out animals have been used to address important and unanswered questions. We convene now in Kyoto peroxidase scientists from around the world to discuss ongoing studies and share new insights into the biology of this important protein family.

Myeloperoxidase (MPO): The organizing principle of the first peroxidase meeting was an interest in myeloperoxidase, the family member expressed in exclusively in cells of a neutrophilic or monocytic lineage. As neutrophils are the dominant cellular component of the human innate immune system and the oxygen-dependent antimicrobial system of neutrophils is the most efficient defense against microbes, MPO has a central place in neutrophil microbicidal action. Unique among the animal peroxidase family, MPO catalyzes the two electron oxidation of chloride ion in the presence of hydrogen peroxide to generate hypochlorous acid, a potent antimicrobial agent. The MPO-hydrogen peroxide-chloride system is responsible for microbicidal activity against a wide range of organisms and has served as the paradigm for neutrophil oxidative killing of bacteria.

Dysfunction of host-defense due to MPO-deficiency in human: Despite its central role in normal host defense, the phenotype of inherited deficiency of MPO has not been clearly demonstrated as increased risk for infectious complications. Four allelic mutations resulting in inherited MPO deficiency have been previously reported (2-5) R569W, Y173C and M251T and G501S. The defect mechanisms and manner of inheritance has been described in detail (3). The prevalence of complete MPO deficiency in Japan is estimated to be 1.75/100,000, a value 14- to 28-fold lower than that of the United States and Europe, respectively (1,6). The molecular basis of deficiencies in Japan and their relation to the genotypes seen elsewhere are the subject of ongoing study.

MPO-deficient mice: Whereas population studies on the prevalence of complications among human with inherited MPO deficiency have been of limited use, the application of molecular techniques to generate mice deficient in MPO has proven a useful experimental tool. The earlier reports of the clinical consequences of MPO deficiency described the increased risk in affected individuals for disseminated and often fatal candidiasis. This association between MPO activity and host defense against Candida was recapitulated in the mouse model by Aratani et al. (7). The availability of the MPO knock-out mouse also makes possible testing novel hypotheses regarding the role of MPO in pathogenesis of diseases unrelated to infection. Unexpectedly MPO-deficient mice show an increase in experimentally induced atherosclerosis (8), perhaps highlighting important species differences between mouse and man. Nonetheless, the mouse model will provide important and novel insights into MPO biology.

Peroxidase related to Disease Activity: MPO-ANCA related diseases: In addition to host-defense function, MPO is also a target molecule of MPO-specific anti-neutrophil cytoplasmic autoantibody (MPO-ANCA). Antibodies directed against cytoplasmic constituents of the neutrophil, specifically MPO and proteinase 3, have been extensively described as markers for systemic vasculitis and crescentic glomerulonephritis. Evidence further indicates that MPO and the MPO-ANCA are risk factors for the development of immune-mediated renal disease, as the sera of patients with microscopic polyangiitis and crescentic glomerulonephritis (CrGN), high titers of MPO-ANCA are frequently detected. Studies in the mouse model promise to provide important insights into the pathogenesis of this vasculitic disease.

Immunomodulatory therapy for MPO-ANCA related diseases: Many therapeutic trials for MPO-ANCA related diseases have been performed, especially those for rapidly progressive glomerulonephritis (RPGN) due to CrGN. Among the various interventions tested, intravenous immunoglobulin (IVIg) has improved the outcome of this highly life-threatening disease in Europe. Although there are many potential mechanisms underlying the beneficial effect of IVIg, one may be the suppression of the presentation of MPO to stimulated neutrophils. In addition other immunomodulatory effects including the correction of abnormally deviated Th1/Th2 balance and suppression of the highly elevated cytokine activity may play a role (submitted). The favorable outcome of the IVIg for MPO-ANCA related RPGN in Japan and the partial elucidation of the mechanism of action will be presented.

NOX family: MPO action requires hydrogen peroxide and in stimulated neutrophils, the NADPH oxidase generates reactive oxygen species, including hydrogen peroxide, from molecular oxygen. The phagocyte NADPH oxidase is a multicomponent enzyme containing membrane and cytosolic components that assemble at the membrane when neutrophils are stimulated by an appropriate agonist. The membrane component of the NADPH oxidase is a heterodimeric protein composed of gp91*phox* and p22*phox*. Recently homologues of gp91*phox* have been described, giving birth to the NOX (NADPH oxidase) protein family. Previously work from the laboratories of Krause and of Sumimoto were presented at 6th MPO meeting at Atami in 2000 (Abstract Book). As the family grows and new data emerge, it seems that the NOX enzymes have two physiological functions: 1) Host defense, typified by the phagocyte NADPH oxidase and indirectly suggested for NOX1, DUOX1, and DUOX2.

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and 2) Biosynthetic processes, as seen with for DUOX enzymes, implicated in biosynthesis of thyroid hormone in mammals and in the crosslinking of extracellular matrix in *C. elegans*. In addition, NOX enzymes are involved in signaling function of ROS and new information will be presented at the meeting.

2. Role of the International Peroxidase Meeting

Drs. Suzuki and Nauseef have organized this international meeting to extend insights into role of MPO and other peroxidases, as originally intended at the first peroxidase meeting. The first meeting on myeloperoxidase was inspired and organized by Dr. Dolphe Kutter and held in Luxemburg in 1996. It was a small meeting but served to confirm the need for an international meeting where investigators, clinical and basic, who shared an interest in the biology of MPO could come together to discuss important aspects of its biology and role in health and disease. The second meeting was convened to meet this charge and was held in a Benedictine Abbey on Fraueninsel in Lake Chiemsee, in Bavaria, Germany. Organized by Petro Petrides from Munich, Germany and William Nauseef from the University of Iowa, USA, the meeting in 1998 was a great success, generating the publication of a book "The peroxidase multigene family of enzymes: Biochemical basis and clinical applications" and setting the stage for future meetings. The 3rd conference was held in Vienna, Austria in 2002 and was organized by Christian Obinger. Christian expanded the chemistry component of the meeting and expanded the format to include not only other animal peroxidases but also peroxidases from the plant world.

The 4th International Peroxidase Meeting: The 4th International Peroxidase Meeting is held on October 27-30, 2004, Kyoto Palulu Plaza (www.nih.go.jp/MPO/). Based on the background of International Peroxidase Meeting, we will organize the 4th International Peroxidase Meeting joined with the 10th MPO meeting organized by Muso, Kitano Hospital. The MPO meeting has been held in Japan since 1995, making this the 10th anniversary MPO Meeting. Thus it seems appropriate celebrate this special milestone by joining with the 4th International Peroxidase Meeting. The program for the meeting has been organized around the following format: Opening Lecture: Contribution of MPO in vasculitis development by K. Suzuki, and Plenary Lecture: Lessons from MPO deficiency about functionally important structural features by W. Nauseef will be announced. Special lectures-1. Clinical treatment for patients with MPO-ANCA by D. Jayne, E. Muso, and Y. Aratani, and -2. New aspects of peroxidases and oxidases: Nox/Duox family NADPH oxidases: expression patterns and possible physiological functions by K-H Krause will be presented. In addition, five sessions: MPO-ANCA-related diseases, action and molecular aspects of peroxidases, inflammation and peroxidaserelated diseases, peroxidases and NADPH oxidases, and reaction of MPO will be joined with poster presentations. Conferees registered are from Austria, France, Germany, Italy, New Zealand, Russia, Spain, Sweden, Switzerland, UK, and USA in addition to Japan.

Thus, we will have presentations of various peroxidases and other oxidases in this meeting. We intend to provide a venue at these sessions for discussion of all aspects of peroxidase biology. Finally, we hope that the insights and information provided at the meeting will reveal new roles for the peroxidases and other oxidases in health and disease.

The next meeting: The 5th meeting will be held in Christchurch in New Zealand and organized by Dr. Tony Kettle in The Christchurch Medical School.

ACKNOWLEDGMENTS

We thank to all local and international organizers and advisory organizers, speakers, conferees in poster presentation, staffs and the editorial office of Japanese Journal of Infectious Diseases. Also, we represent appreciation to grant supports by 78 companies, which belong to The Pharmaceutical Manufacturers' Associations of Japan, Inoue Foundation for Science, Exoxemis Co., in USA, and all cooperative companies.

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Contribution of Myeloperoxidase in Vasculitis Development

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SUMMARY: Infiltrated neutrophils is believed to contribute to the progression of vasculitis. In particular, myeloperoxidase (MPO)-specific antibodies against neutrophils, anti-neutrophil cytoplasmic antibodies (MPO-ANCA) is involved in the development of vasculitis microscopic polyangiitis etc. In Japan a higher percentage of MPO-ANCA than that in Europe has been reported In addition, we showed a correlation of MPO-ANCA epitopes of Kawasaki disease patients by 47% with that of mothers'. On the other hand, mice having CADS/CAWS-induced vasculitis is a good model for the analysis of the production of MPO-ANCA. We have clarified that MPO is a major antigen for MPO-ANCA production using MPO KO mice. We also investigated the role of activated neutrophils in nephritis renal lesions using SCG/Kj mice. In the phase of nephritis with low grade of proteinuria, the spontaneous release of MPO from peripheral neutrophils increased, indicating that neutrophils are activated and contribute to the development of active crescentic lesion in SCG/Kj mice.

Activated neutrophils in patients with vasculitis suggest that they contribute to the progression of vasculitis has been investigated (1). Target molecules of the antibodies against neutrophils, anti-neutrophil cytoplasmic antibodies (ANCA) related to the develop-

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ment of vasculitis are myeloperoxidase (MPO) and proteinase-3 (PR3) contained in the granules of neutrophils. In particular, MPO-ANCA is associated with certain subtypes of primary vasculitis. Thus, MPO-ANCA has been demonstrated to be involved in the development of vasculitis microscopic polyangiitis etc. (2). Patients with MPO-ANCA related glomerulonepheritis (GN) also show an increase in the activated neutrophils in peripheral blood (1) in addition to Kawasaki disease. In Japan a higher percentage of MPO-ANCA than that in Europe has been reported (3). Recently, role of ANCA by the European Vasculitis Study Group trials have also been studied (4).

Furthermore, in addition to these diseases, elevation in the levels of MPO-ANCA in sera of patients with Kawasaki disease and systemic lupus erythematosus (SLE) has also been observed. Then, we analyzed a correlation of MPO-ANCA epitopes of Kawasaki disease patients with their mother to know the etiology related to MPO-ANCA. Most of healthy mothers showed MPO-ANCA positive in their sera with lower titer. Epitopes in sera of patients were coincident by 47% with that of mothers', but less father's (Table 1), suggesting that source of auto-antibody MPO-ANCA may be same to that of patient's mother (5).

Table 1. Correlation of epitopes of MPO-ANCA of KD patients with their parents

MPO-ANCA Positive 85.7 %		
Correlation	Epitopes	% Prevalence
with Father	Ha	5.9
	Hg	5.9
	No-relation	0
with Mother	Ha	17.6
	Hg	29.4
	No-relation	11.8
with parents		5.9

Eighteen families were examined in 42 patients in Hiroshima City Hospital from Mar. 1998 to Dec. 2000.
Ha: N-terminus of heavy chain, Hg: C-terminus.

On the other hand, ANCA may be important in the pathophysiology of necrotizing vasculitis due to neutrophils activated with inflammatory cytokines such as tumor necrosis factor- α (TNF-α), IL-6 and IL-8 in blood circulation. Interestingly, it has been demonstrated that ANCA activates neutrophils primed with TNF-α in vitro, resulting in the translocation of ANCA antigens to the cell surface.

As the basis for clinical studies, animal models are often used to understand the mechanisms of the development of vasculitis, and to establish therapeutic strategies. Both MRL lpr/lpr, and SCG/Kj strains are known to show high levels of MPO-ANCA in association with renal lesions including GN and vasculitis. On the other hand, CADS or CAWS-induced vasculitis have been used for the analysis of the development and progression of vasculitis (6). CADS/CAWS-induced vasculitis with coronary arteritis is a good model for the analysis of the production of MPO-ANCA. We have clarified that MPO is a major antigen for MPO-ANCA production using MPO KO mice (7). Moreover, the study using NZB/W F1 mice with the Fcγ receptor-deficiency has shown that Fcγ receptor on neutrophils and/or macrophages has been demonstrated to be necessary in the occurrence of GN. However, the more precise pathogenic roles of MPO-ANCA and neutrophils in the development of GN and vasculitis in these murine models are undetermined. We investigated the role of activated neutrophils in nephritis renal lesions using SCG/Kj mice. The mice having spontaneous CrGN and vasculitis showed higher levels of MPO-ANCA and TNF-α than those of normal mice C57BL/6. In the phase of nephritis with low grade of proteinuria, the spontaneous release of MPO from peripheral neutrophils increased, while superoxide generation increased before spontaneous MPO release occurred. In addition, the renal lesion in histological observations aggravated with aging and the glomerular neutrophil infiltration was positively correlated with MPO-ANCA levels as well as with histological indices of nephritis, active renal injury score, especially crescent formation was correlated with spontaneous MPO release. These findings indicate that neutrophils are activated and contribute to the development of active crescentic lesion in SCG/Kj mice (8).

The certain neutrophil infiltration into tissue showing vasculitis suggests that neutrophils may cause the development of vasculitis. MPO released from activated neutrophils occasionally causes self-damage to tissues due to the toxicity of its product OCl^- or other radicals such as O_2^-, H_2O_2, OCl^-, NO as well killing fungi improved with MPO-KO mice.

ACKNOWLEDGMENTS

This study is supported in part by a grant of Ministry of Health, Labour and Welfare, Japan.

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Jpn. J. Infect. Dis., 57, 2004

Genomic Variations in Myeloperoxidase Gene in the Japanese Population

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SUMMARY: Myeloperoxidase (MPO; EC 1.11.1.7) is a lysosomal hemeprotein that plays an important role in the host defense mechanism against microbial diseases. This neutrophil disorder, characterized by the lack of MPO, may result in a weakened defense activity. Complete MPO deficiency has been postulated to be to originate from genomic mutation. Recently, two Japanese patients were reported with MPO deficiency. Both had base substitutions in the exon 9 region of the MPO gene; a region in close proximity functionally important residue, His502. Genomic DNA from 387 Japanese individuals was examined to determine the prevalence of these recently discovered base substitutions. None of these DNA samples possessed the mutations found in the MPO deficient cases, though two synonymous and one non-synonymous mutation were found. The frequency of mutation in the exon 9 coding region was estimated to be one heterozygote in 129, thus the homozygote of such mutations would be revealed one in 16,000 in the Japanese population.

Myeloperoxidase (MPO) is a lysosomal hemeprotein located in azurophilic granules of neutrophils and monocytes. MPO is part of the host defense system and is responsible for microbicidal activity against a wide range of organisms. A deficiency in MPO is speculated to be associated with a decreased level of immunity (4). Aratani et al. (1) has described the association with this deficiency and continuous infection of *Candida albicans* in MPO knock-out mice.

In the human population, the prevalence of complete MPO deficiency in Japan is estimated to be 1.75/100,000, a value 14- to 28-fold lower than that of the United States and Europe, respectively (8). Three allelic mutations related to MPO deficiency have been previously reported: R569W (5), Y173C (3), and M251T (7). MPO research is now making headway with the genetic analysis of patients with complete and partial MPO deficiency.

Research conducted over the past year entailed identifying mutations found in cases afflicted with MPO deficiency and estimating the prevalence of these mutations in a control cohort. Two novel non-synonymous mutations were researched during this time period: a glycine to serine substitution (G501S) and an arginine to cysteine substitution (R499C), both found on the exon 9 region of the MPO gene. The G501S mutation, first reported in the Japanese population, (6) was found originally in a patient with complete MPO deficiency. Neutrophil function analysis revealed that MPO activity was significantly diminished with slightly elevated superoxide production.

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Another patient was later identified with complete MPO deficiency. As with the first case, MPO activity was diminished with increased superoxide production. In this case, a new mutation was also found in the region responsible for coding MPO: a point mutation in exon 9 region that resulted in an arginine to cysteine substitution (R499C) (Persad, in preparation). Primer sets used in the recognition of mutations found in both patients are described in (6).

A total of 387 DNA samples served as a comparison cohort in the investigation of a possible link between these identified mutations and the presence of MPO deficiency. Due to difficulties in obtaining samples from a large number of healthy individuals, the control group used consisted of DNA from rheumatoid arthritis samples (21%), hepatitis C samples (41%) and healthy blood donors (38%), none of which had information on levels of MPO activity or superoxide production. Among these samples, three isolated point mutations were found in exon 9, all of which were heterozygous, with two of the mutations being synonymous in nature (1434 G/A, 1478 C/A; the numbers indicate the base position from Adenine of first ATG in mRNA). The third isolated mutation (1464 T/C) would result in an amino acid substitution from isoleucine to threonine. This mutation has not been confirm nor is MPO activity available for this DNA sample. All 387 samples did not possess the non-synonymous mutations found in the MPO deficient cases, thus drawing a more defined postulation that G501S and R499C may be associated with complete MPO deficiency.

Complete MPO deficiency is hereditary and requires the possession of two recessive alleles. This knowledge, coupled with population dynamics in Japan, produced a scenario in which cases with this type of deficiency can serve as sentinels in the detection of clusters of individuals that are heterozygous for these novel mutations.

Both novel mutations, G501S and R499C, have thus far been only found Japanese individuals. An interesting phenomenon, unlike previously identified mutations, is the proximity of these mutations to each other as well as to the histidine at codon 502 that is pivitol to heme binding (2). Based on this research, it is speculated that mechanism of action of these mutations to induce MPO deficiency is via the interruption of heme binding due to the amino acid substitution caused.

ACKNOWLEDGMENTS

This study is supported in part by a grant of Ministry of Health, Labour and Welfare, Japan.

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In Vivo Role of Myeloperoxidase for the Host Defense

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SUMMARY: Myeloperoxidase (MPO) is located within neutrophils capable of producing HOCl. To define the in vivo role of MPO, we have generated MPO-knockout (MPO-KO) mice. The mice without MPO developed normally. However, MPO-KO mice showed severely reduced cytotoxicity to various microorganisms such as *Candida albicans*, *Aspergillus fumigatus*, and *Klebsiella pneumoniae*, demonstrating that MPO-dependent oxidative system is important for host defense against fungi and bacteria, although the effect varies from species to species of pathogens. To compare the importance of MPO and NADPH-oxidase for host defense, MPO-KO and chronic granulomatous disease (CGD) mice were infected with different doses of *C. albicans*, and their infection severity was analyzed. CGD mice exhibited increased mortality and tissue fungal burden in a dose-dependent manner, whereas normal mice showed no symptoms. Interestingly, at the highest dose, the mortality of MPO-KO mice was comparable to CGD mice, but was the same as normal mice at the lowest dose. These results suggest that MPO and NADPH-oxidase are equally important for early host defense against a large inocula of *Candida*.

Neutrophils are believed to be the first line of defense against invading microorganisms, but in vivo roles of reactive oxygens produced by neutrophils are not well known. Myeloperoxidase (MPO) catalyzes reaction of hydrogen peroxide with chloride ion to produce hypochlorous acid that is used for microbial killing by phagocytic cells. To define the in vivo role of MPO, we have generated mice having no peroxidase activity in their neutrophils and monocytes (1). MPO-deficient (MPO-KO) mice showed severely reduced cytotoxicity to *Candida albicans*, *Aspergillus fumigatus*, *Trichosporon asahii*, and *Pseudomonas aeruginosa*, and others (Table 1) (1-3), demonstrating that MPO-dependent oxidative system is important for host defense against fungi and bacteria.

However, the significance of MPO compared to the NADPH-oxidase is still unclear because individuals with MPO deficiency are usually healthy in contrast to the patients with chronic granulomatous disease (CGD) who present clinical symptoms early in life and die with recurrent infections during childhood. To better understand the contributions of MPO and NADPH-oxidase to antifungal defense mechanisms, we compared the susceptibility of MPO-KO mice and CGD mice to the infections with *C. albicans*. Interestingly, at the highest dose, the mortality of MPO-KO mice was comparable to CGD mice, but was the same as normal mice at the lowest dose (Fig. 1). At the middle dose, the number of fungi disseminated into various organs of the MPO-KO mice was comparable to the CGD mice in one week after infection, but it was significantly lower in 2 weeks (4). These results suggest that MPO and NADPH-oxidase are equally important for early host defense against a large inocula of *Candida*.

Table 1. Recovery of fungi and bacteria from the lungs of wild-type and MPO-KO mice after intranasal inoculation

Organism	log CFU/lung			B/A
	0.5 h	48 h		
		(A) Wild type	(B) Mutant	
Candida albicans	6.7	4.8 ± 0.2	6.3 ± 0.2	30.1
	5.7	3.6 ± 0.2	5.4 ± 0.1	66.1
Cadida tropicalis	6.0	4.1 ± 0.2	5.6 ± 0.1	33.1
	5.1	3.0 ± 0.2	3.5 ± 0.1	3.0
Trichosporon asahii	6.0	4.7 ± 0.1	6.1 ± 0.1	26.3
	5.1	3.6 ± 0.2	4.2 ± 0.1	3.9
Aspergillus fumigatus	5.7	2.2 ± 0.2	3.6 ± 0.2	22.9
	5.2	1.8 ± 0.5	2.9 ± 0.2	12.9
Pseudomonas aeruginosa	5.8	3.5 ± 0.3	6.3 ± 0.3	550.0
	5.0	2.8 ± 0.1	2.9 ± 0.2	1.3
Klebsiella pneumoniae	6.8	3.3 ± 0.3	4.3 ± 0.3	9.3
	5.2	<1.0	1.9 ± 0.9	>8.1

CFU of inoculated fungi and bacteria in mouse lungs was assessed at indicated times.

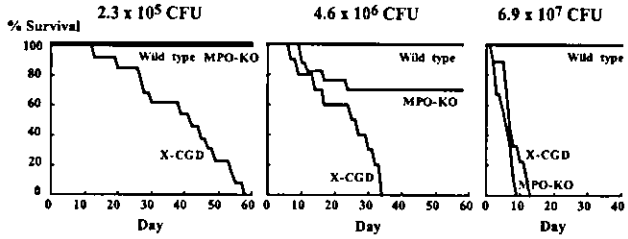

Fig. 1. Survival of mice after *C. albicans* infection. Wild-type, MPO-KO, and X-CGD mice were intraperitoneally infected with the indicated doses of *Candida*.

Hereditary MPO deficiency is a common neutrophil defect with estimated incidence of 1 in 2,000 in the United States, and of 1 in 50,000 in Japan. Our present results suggest that MPO-deficient individuals could exhibit similar problems as CGD patients if exposed to a large amount of microorganisms.

ACKNOWLEDGMENTS

This study is supported in part by a grant of Ministry of Health, Labour and Welfare, Japan.

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Intravenous Immunoglobulin (IVIg) Therapy in MPO-ANCA Related Polyangiitis with Rapidly Progressive Glomerulonephritis in Japan

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SUMMARY: For 30 myeloperoxidase (MPO) antineutrophil cytoplasmic antibody (ANCA) related rapidly progressive glomerulonephritis patients (male 17, female 13 , average age of 68 ± 11.8 years old), intravenous immunoglobulin (IVIg) (400 mg/kg/day) was administered for 5 consecutive days before or along with conventional immunosuppressive therapy in Japan. Twenty patients were treated with IVIg before the start or newly increase of conventional therapy and evaluated the independent effect of this therapy. In these patients, just after IVIg, significant decrease of CRP from 8.61 ± 5.77 to 5.47 ± 4.50 mg/dl ($P < 0.001$) was noted with improvement of elevated serum creatinine in 12 out of 19 patients (63%). In the analysis of the overall outcome of 30 patients, at 3 months after IVIg and following conventional therapy, no patients showed renal death except 4 for whom hemodialysis had been started before IVIg. At 6 months, renal survival rate were 92% (newly renal death 2 out of 26) and 2 patients died due to cerebral bleeding (survival rate was 93%). No fatal infection was noted. IVIg might be the potent inducible therapy which can be promoted before the beginning of conventional immunosuppressant treatment for relatively aged and lower immunopotent MPO-ANCA patients in Japan.

Rapidly progressive glomerulonephritis (RPGN) is often associated with systemic vasculitis presenting antineutrophil cytoplasmic antibody (ANCA) (1). These ANCA-related RPGN often necessitated aggressive immunosuppressive treatment using high dose corticosteroid and cyclophosphamide (CYC) which sometimes brought about severe side effects especially sometime fatal infections, since these diseases often occur in relatively aged populations. To avoid these fatal side effects, intravenous immunoglobulin (IVIg) therapy has been utilized in Europe for these ACNA-related vasculitis and has been proved to be clinically safe, suppress disease activity for at least 1 year, and reduce the total dose of immunosuppressive agents (2-4). Although these reports of this therapy are useful, it is necessary to be prudent for the direct application of these results for those in Japan because in Europe, the distribution of the type of disease tends to orient to Wegener's granulomatosis (WG) which are not so frequently experienced as the causative disease of RPGN in Japan. Recent survey for the incidence of RPGN in Japan revealed that 62% of 593 RPGN patients from 1996 to 2000 were MPO-ANCA positive, in contrast to only 4% of PR3-ANCA positive patients (5). Therefore, the independent survey is necessary to prove the efficacy of this therapy as the safe and potent induction therapy for MPO-ANCA related RPGN.

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Patients and methods: **Patients:** Thirty MPO-ANCA related RPGN patients (male 17, female 13) in Japan were treated with IVIg before or during the conventional immunosuppressant therapy using corticosteroid and CYC. These patients were treated in 5 hospitals (Kitano Hospital: 12 cases, Ehime Prefecture Hospital: 8, Osaka University Hospital: 4, Tsukuba University Hospital: 4 Kyoto University Hospital: 4) in Japan separately from 2001 to 2003. Average age of the patients were 68 ± 11.8 from 36 to 83 years old. All patients showed elevated serum MPO-ANCA as well as characteristic pathology observed in the renal biopsy specimen. All patients were provided written informed consent for renal biopsy and the present treatment protocol.

Treatment protocol: For all patients except one, IVIg was administered intravenously once for 5 consecutive days (400 mg/kg/day) (Kenketus Venilon-I, Teijin Co., Ltd., Tokyo, or Kenketsu Glovenin-I, Nihon Pharmaceutical Co., Ltd., Tokyo, Japan). One patient was treated with IVIg twice during her hospital course. Twenty patients (male 12, female 8, average age: 71.3 ± 8.82) were treated with IVIg before the start or newly increase of conventional immunosuppressive therapy and the effect of the IVIg could be evaluated independently.

Clinical features before IVIg treatment: All patients showed elevation of creatinine (Cre) before IVIg with mean value of 4.04 ± 2.94 mg. Twenty-one of them were diagnosed RPGN with rapid increase of Cre more than double within 3 months before entry. For four patients, hemodialysis had to be started before IVIg therapy. The activity of the inflammation were severe with the mean CRP of 7.2 ± 5.5 mg/dl. All patients were MPO-ANCA positive with 243.7 ± 355.2 EU.

These patients were under various complicated diseases prior to the burst of MPO-ANCA disease. Ten cases showed one or more pulmonary diseases such as pulmonary fibrosis: 7 cases, latent tuberculosis: 2, aspergilosis: 1, other bacterial infections: 3. Other complicasions were as follows: Idiopathic thrombocytic purpura 3, hepatitis B virus carrier 2, diabetes mellitus 2, aortic aneurysm 1, mononeuropathy 1, malignancy 1 (laryngeal cancer). In addition, 2 patients were MRSA carrier.

RESULTS

Response to the IVIg therapy: For 20 patients independently treated with IVIg before the start or the increase of the immunosuppressants, the evaluation of the response to this therapy was performed separately within 14 days for 19 out of 20 patients whose data just after the IVIg were available. The significant decrease of the CRP was noted just after the IVIg from 8.61 ± 5.77 to 5.47 ± 4.50 mg/dl ($P < 0.001$). Although the average level of Cre did not show a significant decrease within such short period of observation (from 3.46 ± 2.34 to 3.39 ± 2.16 mg/dl P:n.s.), it was noteworthy that the elevation of Cre before IVIg stopped in one and rather decreased in 12 patients.

The evaluation of the effect of IVIg on MPO-ANCA titers was available in 13 of 19 patients. There was no significant decrease of these titers just after the IVIg (from 253.30 ± 275.40 to 410.07 ± 621.56 EU).

Outcome of the patients: Following or along with IVIg, patients were treated with conventional immunosuppressants including corticosteroid. Two out of 30 patients did not need to add additional therapy after IVIg. For other 28 patients, average initial dose of 33.4 ± 11.2 mg of Prednisolone (0.6 ± 0.1 mg/kg/day) were administered. For those without complicated infectious diseases, pulse therapy of methylprednisolone (0.5-1 g/day for 3 days) were performed in 8 and oral CYC 50 mg/day for 9 patients for their severely active state of the disease. Plasma exchange was also performed for 2 patients. After 3 months of these treatments, activity of the disease was completely suppressed with average CRP value of 0.80 ± 2.44 mg/dl ($P < 0.001$ v.s. before IVIg). The elevated Cre was also significantly suppressed to 2.20 ± 1.20 mg/dl ($P < 0.01$) and 25 out of 30 patients showed the improvement of renal function. Significant decrease of the MPO-ANCA titers were also noted with the mean value of 41.44 ± 81.42 EU ($P < 0.001$). In 12 patients, ANCA were completely negative at this point. As for the overall outcome of renal function, except four patients who were started hemodialysis before treatment, no renal death was noted at 3 months, in 1 at 6 months (overall renal survival rate: 77%, and 92% except for those who were hemodialyzed before IVIg) and in another one patient after 6 months until the end of year 2003. As for the life survival, before 6 months 2 patients (survival rate 93%) and more 3 patients died after 6 months. The causes of death were cerebral bleedings for two and malignancies such as malignant lymphoma for 2 and one gastric cancer after 6 months following IVIg. It should be noted that there was no fatal infection in all IVIg treated patients.

DISCUSSION

Recently we have experienced the favorable results of IVIg monotherapy for 15 RPGN patients showing rapid decrease of CRP, WBC and ANCA titers in association with down regulation of the serum inflammatory cytokines especially of TNF α (Ito-Ihara, in submission). In the current survey, a significant anti-inflammatory effect of IVIg therapy was also proved even though the increase of the samples. In Japan survey of RPGN, 6 months renal survival rate was 70% and survival rate was 74% in MPO-ANCA positive RPGN with conventional immunosuppressant therapy (5). Comparing with these results, the 92% of renal survival rate and especially 93% life survival were remarkably high. In particular, the absence of the death due to fatal infection even following usage of the conventional immunosuppressive agents should be highly evaluated. Although more qualified evidence of beneficial effect of this therapy remains to be established in randomized controlled study, considering not only the high survival rate but the low cost for treatment of the complicated infections, the IVIg should be the potent inducible therapy which can be promoted before the beginning of conventional immunosuppressant treatment for relatively aged and lower immuno-potent MPO-ANCA patients in Japan.

ACKNOWLEDGMENTS

This study is supported in part by a grant of Ministry of Health, Labour and Welfare, Japan.

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Microbiol. Immunol., 48(9), 669–675, 2004

Editor-Communicated Paper

On the Cyto-Toxicity Caused by Quantum Dots

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Communicated by Dr. Hidechika Okada: Received June 21, 2004. Accepted June 29, 2004

Abstract: **Quantum dots (QDs) such as CdSe QDs have been introduced as new fluorophores. The QDs conjugated with antibody are starting to be widely used for immunostaining. However there is still not sufficient analysis of the toxicity of QDs in the literature. Therefore we evaluated the cell damage caused by the quantum dots for biological applications. We performed cell viability assay to determine the difference in cell damage depending on the sizes and colors of mercapto-undecanoic acid (MUA) QDs and the cell types. The results showed that the cell viability decreased with increasing concentration of MUA-QDs. But in the case of Vero cell (African green monkey's kidney cell) with red fluorescence QD (QD640), the cell damage was less than for the others. Furthermore through the flow cytometry assay we found that this cell damage caused by MUA-QD turned out to be cell death after 4–6-hr incubation. From the two assays described above, we found that there is a range of concentration of MUA-QDs where the cell viability decreased without cell death occurring and thus we conclude that attention should be given when MUA-QDs are applied to living organisms even in low concentrations.**

Key words: **Cell damage, MUA-QD, Cell death**

Quantum dots (QDs) such as CdSe QDs are nano-sized metal clusters. QDs have specific characteristics such as the quantum effect, which is a special photo quality caused by the widening of the band gap when the spatial dimension is reduced. Kubo et al. predicted the specific character of the quantum dot theoretically in 1962 (14–16). Since then, research concerning the applications of QDs has gained a great amount of interest. For example, in the field of Information Technology and optical-engineering (3, 10, 21, 29, 30), QDs have been proposed for use as a new material for memory, and as miniature laser-beam emitting devices. Furthermore, the biological applications of QDs conjugated with antibody have started to attract much attention, especially in immunostaining, separating cells, and diagnostics, because of their advantages such as longer lifetime and higher fluorescence over conventional organic fluorophores (1, 2, 8, 27). The first synthesized QDs are insoluble in biological solvents because non-polar groups of organic molecules are exposed on the surface of QDs. However the water-soluble QDs covered with mercapto-undecanoic acid (MUA) have been reported (2). In addition, the MUA-QD covered with sheep serum albumin (SSA) is well dispersed in water (2, 9). The advantages of MUA-QDs described above make it possible to consider the application of MUA-QDs to drug delivery systems (6, 20, 25, 28) as a drug-carrier and cell delivery system. Quantum dots have a longer lifetime compared to conventional organic fluorophores and thus make it easier to trace the drug delivered in living organisms. To make sure the application is feasible, an in-depth evaluation using MUA-QD in living organisms is needed. In fact cadmium (13) and selenium (24) are known to be toxic. Though the use of MUA-QDs for organisms has been known and some other studies about the actual injections into organisms have been conducted, the toxicity of MUA-QDs has not been reported in detail yet. Published works regarding

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Abbreviations: DMEM, Dulbecco's Modified Eagle's Medium; FCS, fetal calf serum; HC, human primary hepatocyte; MUA-QD, mercapto-undecanoic acid quantum dot; PBS, phosphate-buffered saline; PI, propidium iodide; SSA, sheep serum albumin; TOPO, tri-*n*-octylphosphine oxide.

QDs have so far only effectively assumed that QDs are safe. In this paper, we proceeded one step further by investigating the cell damage caused by MUA-QDs through an extensive and comprehensive experiment. We chose CdSe QDs because they are one of the QDs that have the strongest emission and they are used the most in many fields. In order to analyze the cell damage caused by MUA-QDs, a cell viability assay, which assesses the mechanism of glycolytic pathways, was conducted (12, 17, 26). Then in order to figure out whether the cell damage was cell death or not, we examined cell death using the flow cytometry assay.

Materials and Methods

Preparation of MUA-QDs. CdSe/ZnS QDs were synthesized in tri-*n*-octylphosphine oxide (TOPO) in accordance with the standard method (5, 11, 18, 19). For these experiments, three MUA-QDs were prepared; QD520, QD570 and QD640 which emitted green, yellow, and red, respectively.

Preparation of MUA-QDs solution with sheep serum albumin. The same volumes of 10 mg/ml MUA-QD and 10 mg/ml sheep serum albumin (SSA) were mixed as described in Hanaki et al. (9). Then we centrifuged this solution with a 0.45 μm filter at 5,000×*g* for 5 min at room temperature. The MUA-QD/SSA solution for all the cells was diluted with DMEM into several concentrations.

Cell viability assay. The cell viability was measured after the exposure of cells with MUA-QD to 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt to generate hormazan. The number of the living cells is known to be proportional to the concentration of the generated hormazan (12, 26). Cell viability was measured for the following two cell lines and a primary cell culture; Vero cells (African green monkey kidney cells), HeLa cells, and human primary hepatocyte (HC). The above-mentioned cell types were cultured at 37 C, in 5% CO_2 in DMEM, supplemented with 5% heat-inactivated fetal calf serum (FCS). All the cells were suspended in DMEM, supplemented with 5% FCS and 50 μg/ml gentamicin after they had been treated with trypsin and centrifuged at 1,800 rpm for 5 min at room temperature. The cell count was performed for the three types of cells respectively. Each cell was plated into a 96-well plate (Iwaki Co., Tokyo) at 3×10^4 cells/well (100 μl/well). After a 24-hr incubation, the DMEM was removed and the prepared MUA-QD/SSA solution diluted to several different concentrations was poured into the wells. After another 24-hr incubation period, a Cell Counting Kit8 (Dojindo Laboratories Co., Kumamoto, Japan) was added into the 110 μl/well. The Cell Counting Kit8 was diluted with DMEM (Cell Counting Kit8:DMEM=1:10). Then the absorbances were measured at 450 nm by an absorptiometer (Molecular Devices Co.).

Flow cytometry assay. For the flow cytometry assay (23), in all the experiments, each cell was plated into a 12-well plate (Iwaki Co., Tokyo) at 10^6 cells/well (1,000 μl/well).

The cells were incubated for 24 hr. The culture medium was removed, and then the prepared MUA-QD/SSA solution diluted to several different concentrations was poured into the wells. After incubation, the cells were washed with PBS and the dead cells were stained with propidium iodide (PI) (4, 7) (0.1 mg/ml) for 5 min at room temperature, followed by treatment with Puck's EDTA solution (4 mM, $NaHCO_3$; 136 mM, NaCl; 4 mM, KCl; 1 mM, EDTA; 1 mg/ml, glucose), which will do less damage to cells than trypsin. The cells were suspended in PBS after they were fixed with 3% formaldehyde. Then, the fluorescence intensity of PI and QD520 was measured using the flow cytometry (Cyto Ace 300 JASCO, Tokyo) assay.

Results and Discussion

Cell Viability Assay

We conducted the cell viability assay to confirm whether the MUA-QDs do damage to the cells or not (12, 26). We used three cell types; Vero cell, HeLa cell, and primary human hepatocyte for three MUA-QDs (QD520, QD570, and QD640). Their spectrums are shown in Fig. 1. The result showed that MUA-QDs affect the cell viability even in rather low concentrations (Fig. 2). The tendencies of the cell viability with QD570 and QD520 were almost the same. However only in the case of QD640 with Vero cells does the result show a difference in cell viability of less than 0.4 mg/ml. The cell damage was less than for the others only in this experiment though the tendency was the same.

Flow Cytometry Assay

Cell viability assay is easy to handle and quantitatively good as well. However, if the intracellular activity is affected; for example, that of NADH-Dehydrogenase, the results will not reflect the true number of cells. Therefore fluorescence intensity of PI was measured using flow cytometry; another method of counting living cells based on a different principle. Figure 3 shows the result of the experiment incubated for 24 hr with QD520 (23).

Collins et al. reported that living cells do not take in

propidium iodide (PI), which has 610 nm fluorescent (4). Only QD520 was used for the flow cytometry assay because the emission peaks of QD570 and QD640 could not be distinguished from that of PI. The top two Figs. (without MUA-QD) show that the emission intensity obtained with a PI filter and that obtained with a QD filter were both quite low. The emission intensity obtained from the PI filter, however, increased gradually, according to the concentration of the MUA-QDs. At more than 0.15 mg/ml concentration of MUA-QDs, the emission intensity of PI was split into two peaks; the left peak shows the living cells, and the right peak shows the dead cells. On the other hand, in the right lane (with the QD filter), in the cases where the concentration of MUA-QD was more than 0.15 mg/ml, the emission intensity of MUA-QD increased, depending on the concentration of MUA-QDs. The higher emission peak contains both the damaged cells and the undamaged cells in the left panel. At the same time, however, the intensity of PI also increased steadily, which means that the population of dead cells increased from 0.15 mg/ml upward. The results showed the cell damage caused by MUA-QD is cell death.

To analyze the dependence on the incubation time, we measured the ratio of the damaged cells (PI stained cells) against the total number of the cells chronologically (Fig. 4). The ratio of damaged cells increased sharply from 4-hr incubation in 0.2 mg/ml concentration of MUA-QD, and slowly in 0.1 mg/ml. On the other hand, we cannot observe any difference between the result obtained from the concentration of 0.05 mg/ml and that from the control. The result from the flow cytometry assay is compatible with that from the cell viability assay in the view of the concentration of MUA-QD causing cell damage. Cell damage caused by MUA-QD probably occurs because the connection of SSA that covers MUA-QD is not a chemical bond; it just attaches to the surface of the MUA-QD (9). Therefore SSA is easy to remove from the surface of MUA-QD and MUA comes out to the surface. To solve this problem, the surface-processing should be reexamined. Safer materials should be used to coat the surface of QDs or new safer QDs, such as silicon-QD, etc., can be considered for use for the DDS. As for its application for the DDS, the coating with peptide is effective because the tagging of target-molecules will be necessary: Peptide is more easily applicable for pharmaceutical biology and it is much safer. What is more, we have seen the difference in the extent of the cell damage only in the case of the combination of Vero cells and

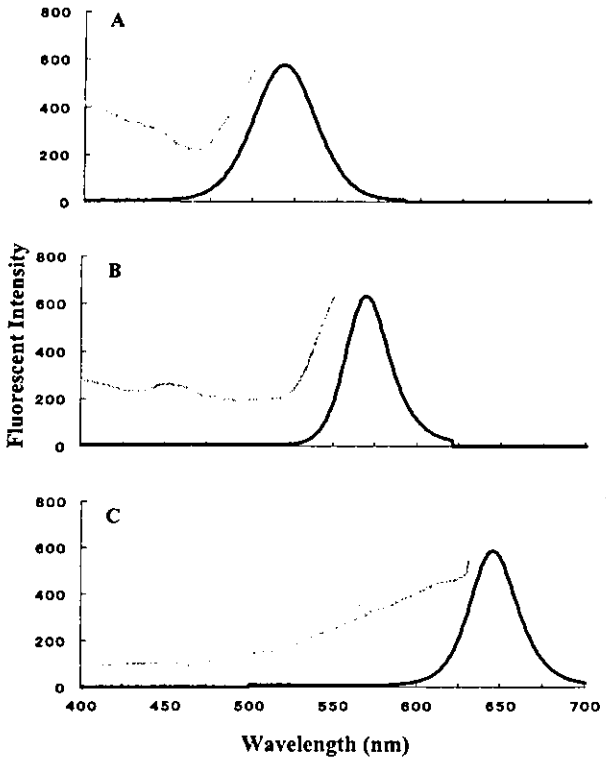

Fig. 1. Photoluminescent properties of three different MUA-QDs. (A) QD520, (B) QD570, and (C) QD640. Three different MUA-QDs were dissolved in DW, and their photoluminescent properties were measured with FP-6500. Emission spectra of QD520 excited at 300 nm, QD570 excited at 350 nm, and QD640 excited at 360 nm, represented as black lines. Excitation spectra represented as gray lines collected with detection at the respective peak spectra.

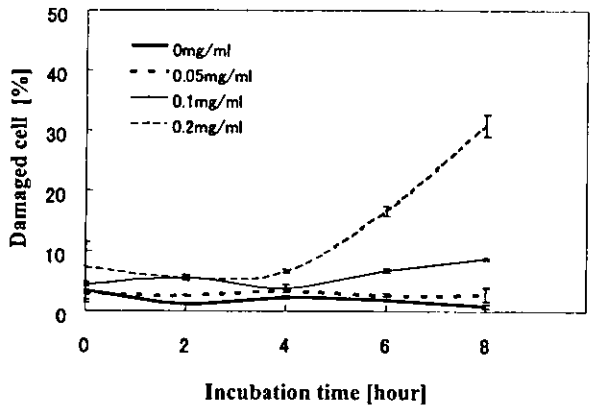

Fig. 4. Flow cytometry assay for the effect of the incubation time difference and concentration of QD. Vero cell and QD520 were used for this experiment. The vertical axis stands for the damaged cell % (the ratio of the number of the PI stained cell against the total number of cells). The intensity of PI is measured between 565 and 605 nm, and the intensity of QD520 is measured between 515 and 545 nm. The horizontal axis stands for the incubation time of the cell. The bold line stands for a concentration of QD520 at 0 mg/ml, the broken line stands for a concentration of 0.05 mg/ml, the solid line, 0.1 mg/ml, and the dotted line, 0.2 mg/ml. The vertical lines are the error bars.