known phenomenon of a poorer HRQoL with advanced age. One possible explanation is that our multidimensional approach cancelled the positive and negative influences, rendering age an insignificant contributing factor to the MQoL-HIV index score in the present study.

Japan is considered to be a relatively homogenous and closed society, and hence the prevalence of prejudice towards AIDS patients can easily become widespread. We speculate that such prejudice influences the psychosocial status of the AIDS patients, leading to a poor HRQoL. The scores from the domains of physical health, financial status and medical care were better for those patients who kept their HIV infection a secret from others. However, the interpretation of this observation is complicated by the fact that more support from others is generally needed for those patients who require intensive medical treatments. It is also confounded by the receipt of special governmental medical aid as a result of handicapped status. Another possible reason is that in circumstances where only professional medical staffs are aware of the patient's HIV infection status, the patient generally receives consistent and favourable mental care. In contrast, the HRQoL tends to be better in the domains of social support and partner intimacy in those patients who disclosed their HIV infection to others. This suggests the possibility of the patient maintaining a good relationship with those to whom they have chose to disclose, thus receiving aid and support. There were no statistically significant differences in the MQoL-HIV index score between those patients who have disclosed and those who have not. Therefore, the present study could not demonstrate a correlation between HRQoL and the disclosure of HIV infection to others.

There were no significant differences in the MQoL-HIV index score attributable to route of infection (sexual relations or blood product transfusion). HIV/AIDS patients with haemophilia have problems with physical functioning due to hepatitis C and joint defects caused by repeated haemorrhage. We believe that this is the reason why those patients who were infected through sexual relations showed generally better scores in the domains of physical functioning, social functioning, and medical care. With respect to the presence or absence of ongoing HIV treatment, those patients who were not currently under treatment showed a better score in the physical health domain, whereas those who were currently under treatment showed a better score in the social functioning domain.

There are some limitations in the present study. First, the Cronbach's α coefficient, which indicates internal consistency was found to be low in both the physical functioning and sexual functioning domains. A low value for this coefficient in the sexual functioning domain has also been observed in studies using the original version of the MQoL-HIV in the USA. These results may indicate difficulty in obtaining accurate answers regarding sexual behaviour from the examinees. This is probably not a problem specific to the design of the questionnaire, but rather an issue that should be recognized as a general phenomenon. Second, the responsiveness, which is important to measure outcome of intervention, was not examined in the present study. Therefore, further examination along this line is a subject for future studies.

In conclusion, the MQoL-HIV is reliable, and may possess discriminative properties. However, there were particularly low values in the physical functioning and sexual functioning domains, leaving these domains with uncertain reliability. Among the factors that determined the score on the MQoL-HIV, psychological factors clearly contributed to the largest degree, and the contribution of factors related to functional capacity and social interaction was relatively minor. With 10 domains, this instrument interacts with various contributing factors in a complicated manner. These factors could not predict the whole spectrum of the HRQoL, and the contribution of these particular factors to the HRQoL was limited. Therefore, the HRQoL should be measured directly and results should be based on the score of the instrument rather than by estimating it from individual factors.

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Appendix

The QoL Research Group of the AIDS Clinical Centre and eight regional AIDS treatment hospitals in Japan

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Involvement of IRAK-M in Peptidoglycan-induced Tolerance in Macrophages*

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The molecular mechanisms by which pathogen-associated molecular patterns recognized by TLR2, such as peptidoglycan (PGN), induce homotolerance are largely unknown. It was recently reported that IRAK-M negatively regulates TLR signaling. In this study, we elucidate the molecular mechanisms of tolerance induced by PGN, with a focus on the role of IRAK-M. We demonstrate that pretreatment of macrophage RAW264.7 cells with a high concentration (30 μg/ml) of PGN for 16 h effectively induces tolerance against following stimulation with 30 μg/ml of PGN; while pretreatment with a low concentration (1 μg/ml) of PGN does not. IRAK-M is induced in cells treated with the high concentration of PGN 4–24 h after PGN stimulation, but not in cells treated with the low concentration of PGN up to 24 h after stimulation. Phosphorylation of MAPKs and IκBα is inhibited after the second PGN stimulation in tolerant cells. Kinase activity of IRAK-1 and association between IRAK-1 and MyD88 are also suppressed in PGN-induced tolerant cells. Furthermore, down-regulation of IRAK-M expression by small interfering RNAs specific for IRAK-M reinstates the production of TNF-α after PGN restimulation. These results suggest that induction of IRAK-M and inhibition of kinase activity of IRAK-1 are crucial to PGN-induced tolerance in macrophages.

Essential components of the innate immune system are TLRs,[1] which recognize microbial products termed pathogen-associated molecular patterns (PAMPs). PAMP recognition leads to activation of the innate immune system, which in turn activates adaptive immunity (1). The cytoplasmic portion of TLRs is very similar to that of the IL-1 receptor family and is currently referred to as the Toll/IL-1 receptor (TIR) domain. The signaling pathway via the TLR family is highly homologous to that via the IL-1 receptor family. TLR interacts with adaptor protein MyD88, which recruits IRAK (2, 3). IRAK becomes activated and associates with TRAF6, leading to the activation of several distinct signaling pathways, including MAPKs and NF-κB (4).

Many reports have been published on the reduced capacity of circulating leukocytes from septic patients to produce cytokines as compared with those from healthy controls, a phenomenon referred to as "endotoxin tolerance." It is an adaptive host response that may represent an essential regulatory mechanism during Gram-negative bacterial infection, but it may also promote subsequent infection in survivors of septic shock (5–11). Monocytes from septic patients have a reduced capacity to release TNF-α, IL-1β, IL-6, IL-10, and IL-12 (12–14). A defect in the activation of transcription factor NF-κB also has been reported in endotoxin tolerance (15). An understanding of the molecular mechanisms of the septic shock syndrome is critical, yet despite numerous studies little is known about these mechanisms.

One of the main characteristics of endotoxin tolerance *in vitro* is a change in the pattern of inflammatory gene expression in cells of myeloid lineage (16). Whereas inhibition of cell surface expression of the TLR4/MD-2 complex has been suggested to underlie LPS tolerance in mouse macrophages (17), LPS induces tolerance in CHO cells without affecting cell surface expression of transfected TLR4 and MD-2 (18). In addition, LPS-tolerant cells exhibit significantly suppressed LPS-induced activation and degradation of IRAK and diminished IRAK-MyD88 association (19). These data imply that tolerance induction may affect the expression and/or functions of intracellular intermediates downstream of TLRs (20).

Induction of an endotoxin-tolerant phenotype is not specific to the initiating action of LPS, because engagement of TLR/IL-1 receptor (IL-1R) family members other than TLR4, also results in macrophage resistance to subsequent challenge with respective ligands. However, the molecular mechanisms that induce homotolerance by PAPMs recognized by TLR2, such as PGN and LTA, are largely unknown. THP-1 cells with prolonged LTA treatment develop LTA homotolerance. Stimulation of TLR2 by LTA, although activating IRAK, does not cause IRAK degradation, indicating that molecular mechanism underlying LTA-induced tolerance is clearly distinct from that of LPS-induced tolerance (21). Furthermore, it is speculated that disruption of unique TLR2 signaling components may occur upstream of MyD88/IRAK (21).

IRAK was originally described as a signal transducer for the proinflammatory cytokine, IL-1, and was later implicated in

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[1] The abbreviations used are: TLR, Toll-like receptor; PAMP, pathogen-associated molecular patterns; LPS, lipopolysaccharide; PGN, peptidogycan; LTA, lipoteichoic acid; MALP2, macrophage-activating lipopeptides; IL, interleukin; IRAK, IL-1R-associated kinase; MyD88, myeloid differentiation factor 88; TNF, tumor necrosis factor; TRAF6, TNF receptor-associated factors 6; MAPKs, mitogen-activated protein kinases; ERK, extracellular signal-regulated kinases; JNK, c-Jun N-terminal protein kinase; ELISA, enzyme-linked immunosorbent assay.

signal transduction of other members of the TLR/IL-1R family. To date, four different IRAK-like molecules have been identified: two active kinases, IRAK-1 and IRAK-4, and two inactive kinases, IRAK-2 and IRAK-M. All IRAKs mediate activation of NF-κB and MAPK pathways (22). Furthermore, all IRAKs have been shown to act downstream of MyD88 and be capable of binding to TRAF6 (23–26). Among four IRAKs, IRAK-M is highly restricted to monocytes/macrophages, and initial reports indicated that IRAK-M activates NF-κB when overexpressed in 293T cells and partially restores IL-1 signaling in IRAK−/− cells (23, 27). However, recent reports state that innate immunity was strongly enhanced in IRAK-M−/− mice, and IRAK-M−/− cells have strikingly impaired endotoxin tolerance, showing that IRAK-M negatively regulates TLR signaling (28). Despite potential importance of IRAK-M in signaling via TLRs, however, the relationship between IRAK-M and TLR-mediated tolerance has been fully obscure. In this study we elucidate the molecular mechanisms of homotolerance induced by PGN, with a focus on the role of IRAK-M.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Anti-extracellular-regulated kinase 1 and 2 (anti-ERK), anti-phospho-ERK1/2, anti-IRAK-1, and anti-MyD88 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific antibodies for p38, JNK, and IκBα were purchased from Cell Signaling Technology (Beverly, MA). Anti-murine TLR2 antibody was purchased from eBioscience (San Diego, CA). IRAK-M antibody was purchased from Chemicon International (Temecula, CA) (28). Antibody specific for β-actin was obtained from Abcam. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG were obtained from Dako (Denmark). Peptidoglycan from *Staphylococcus aureus* was purchased from Fluka, and the endotoxin level in the peptidoglycan was less than 0.5 pg/ml in culture medium as determined by a Limulus amehocyte lysate (LAL) assay.

Cell Culture—RAW264.7, a murine macrophage-like cell line, was obtained from the ATCC, and was maintained in Eagle's modified minimal essential medium (EMEM) supplemented with 2 mM glutamine (Sigma), 100 units/ml penicillin, 100 mg/ml streptomycin (ICN, Aurora, OH), and 10% fetal bovine serum (Sigma). RAW264.7 cells were stimulated with 1 or 30 μg/ml of peptidoglycan (PGN) at 37 °C. PGN stimulation was stopped by addition of ice-cold phosphate-buffered saline. The cells were then lysed for immunoprecipitation and immunoblotting.

Immunoprecipitation and Immunoblotting—Immunoprecipitation and immunoblotting were performed as previously reported (29). Briefly, cells were lysed in ice-cold Nonidet P-40 lysis buffer containing 1% Nonidet P-40, 25 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM EDTA, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM leupeptin, and 1 mM phenylmethylsulfonyl fluoride, and cell membrane fractions were prepared as reported previously (30). For immunoprecipitation studies, cell lysates were mixed with the indicated antibodies for 1 h. Cell lysates were then mixed with protein G and coupled Sepharose beads and rotated for 1 h at 4 °C. After the beads were washed 3 times with ice-cold Nonidet P-40 lysis buffer, the precipitated proteins were boiled for 5 min and eluted with sodium dodecyl sulfate, polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For the precipitation of total-cell lysates, cells were lysed directly by addition of SDS-PAGE sample buffer containing 2-mercaptoethanol. Immunoprecipitated proteins and total cell lysates were separated by SDS-PAGE under reducing conditions and were electrically transferred to a polyvinylidene difluoride membrane. The membrane was blocked for 1 h at room temperature with 1% bovine serum albumin in TBS (Tris-buffered saline) buffer. The membrane was then incubated with the indicated antibody, and the reactive bands were visualized with a horseradish peroxidase-coupled secondary antibody via an enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) according to the manufacturer's instructions.

Analysis of TNF-α Production—The production of TNF-α was analyzed using a commercial enzyme-linked immunosorbent assay (ELISA) kit. RAW264.7 cells were seeded at a density of 10^5/ml in 24-well plates, and the supernatants collected 24 h after LPS stimulation. The concentration of TNF-α was measured by ELISA according to the manufacturer's instructions (BIOSOURCE International, Camarillo, CA).

In Vitro IRAK-1 Kinase Assay—The IRAK-1 kinase assay was conducted as described previously (19, 20). Briefly, the immunoprecipitated IRAK-1 complexes were washed four times with lysis buffer and twice with kinase buffer (20 mM HEPES at pH 7.6, 20 mM $MgCl_2$, 20 mM glycerophosphate, 20 mM paranitrophenylphosphate, 1 mM EDTA, 1 mM sodium orthovanadate, and 1 mM benzamidine). Fifty microliters of kinase buffer were then added to each sample, supplemented with 5 μM ATP, 1 μg of myelin basic protein (MBP; Sigma), and 1 μl of [^{32}P]ATP, and incubated at 37 °C for 30 min. Ten microliters of SDS sample buffer was added, and the samples incubated at 50 °C for 10 min and subjected to SDS-PAGE analysis. The gel was dried and exposed to x-ray film.

Constructions and Transfection of Small Interfering RNAs (siRNAs)—The constructions of siRNA molecules were analyzed by B-Bridge International Inc. The oligonucleotide sequences used in the experiments were as follows: 5′-TGGACATTCGAAACCAAGCATAT-3′ (584–606); 5′-TTCCAGTGCAAACATACTCTTGG-3′ (921–943); 5′-TAATGTCCCAAGTATTCCAGTAG-3′ (1455–1477); 5′-AAGTCCTACTGTGATCAGTTT-3′ (1701–1721).

They were synthesized by and purchased from Qiagen. The control RNAs were obtained from Dharmacon. Cocktails of *in vitro* synthesized siRNAs or control RNAs were transfected into RAW264.7 cells using RNAifect transfection reagent (Qiagen). Cells were incubated in 10% fetal bovine serum for 24-h post-transfection and then stimulated with PGN as described above.

RESULTS

Tolerance Induced by Pretreatment with High Concentrations of PGN—Fig. 1*A* shows the dose response curve of TNF-α production by PGN. Levels of TNF-α in the supernatants increased in a dose-dependent manner: a small amount of TNF-α production was observed in cells stimulated with less than 3 μg/ml PGN, while maximal TNF-α production was obtained by stimulation with more than 30 μg/ml PGN. To determine the threshold of tolerance induction by PGN, RAW264.7 cells were pretreated with graded concentrations of PGN for 16 h and then restimulated with 30 μg/ml PGN for additional 16 h. TNF-α production was augmented in cells pretreated with less than 3 μg/ml PGN. In contrast, prior exposure to more than 30 μg/ml PGN markedly suppressed the ability of cells to produce TNF-α in respond to PGN (Fig. 1*B*). These data showed the existence of threshold of PGN concentration in the tolerance induction; pretreatment of cells with higher concentrations of PGN (≥30 μg/ml) for 16 h effectively induced PGN tolerance, while pretreatment with lower concentrations of PGN (<3 μg/ml) did not. In the subsequent experiments, we chose 1 μg/ml and 30 μg/ml of PGN as the low and high concentration of stimulation, respectively.

Phosphorylation of MAPKs and IκB-α Was Inhibited in PGN-induced Tolerant Cells—In the next series of experiments, we compared the time kinetics of phosphorylation of MAPKs (ERK, p38, and JNK) and IκBα between cells stimulated with 1 and 30 μg/ml PGN. As shown in Fig. 2*A*, phosphorylation of these molecules was observed in cells treated with 30 μg/ml PGN 15–30 min after addition. PGN-induced phosphorylation of the MAPKs and IκBα was also observed in cells treated with 1 μg/ml PGN, although the intensity of the phosphorylation was much weaker in these cells. Fig. 2*B* shows time kinetics of PGN-induced phosphorylation of MAPKs and IκBα in cells pretreated for 16 h with 1 μg/ml PGN (PGN-non-tolerant cells) and 30 μg/ml PGN (PGN-tolerant cells). Phosphorylation of MAPKs and IκBα was observed in non-tolerant cells after the second stimulation with 30 μg/ml PGN, but hardly detected in tolerant cells. These results demonstrate that activation of intracellular signaling molecules such as MAPKs and IκBα is exclusively inhibited after the second PGN stimulation in tolerant cells.

Surface Expression Levels of TLR2 Were Not Affected by PGN Stimulation—We then compared the expression levels of TLR2 between cells treated with a low and high concentrations of PGN. The expression levels of TLR2 in the membrane fraction in cells stimulated with a high concentration of PGN were similar to those in cells stimulated with a low concentration up

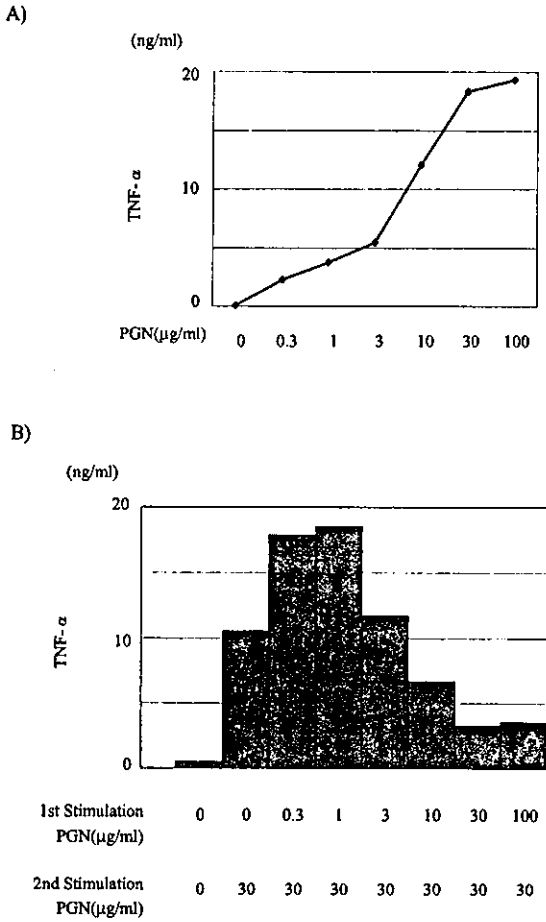

FIG. 1. **Stimulation by PGN induces TNF-α production in a dose-dependent manner and pretreatment with high concentrations of PGN induces homotolerance.** *A*, RAW264.7 cells were stimulated with graded concentrations of PGN, and supernatants were collected in serum-free medium 24 h after stimulation. Samples were analyzed for mouse TNF-α using a commercial ELISA kit (BIOSOURCE) according to the manufacturer's protocol. *B*, RAW264.7 cells were stimulated with graded concentration of PGN, or medium (control) for 16 h. Then the cells were restimulated with 30 μg/ml of PGN. The amount of TNF-α was measured as described above. The data shown are representatives of three independent experiments.

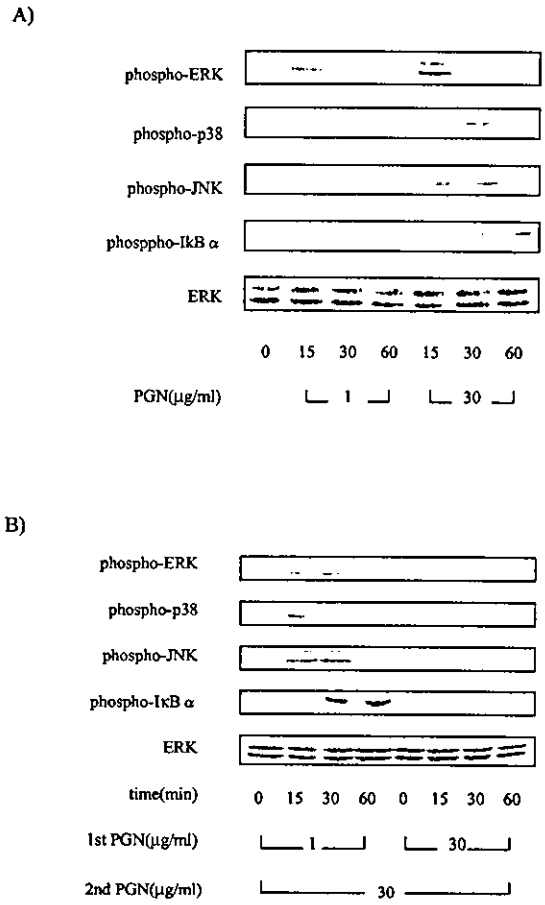

FIG. 2. **Activation of intracellular signaling molecules is exclusively inhibited in PGN-induced tolerant cells.** *A*, RAW264.7 cells were stimulated with 1 or 30 μg/ml of PGN for the indicated times, and total cell lysates were blotted with a phosphospecific antibody (*Ab*) for ERK, p38, JNK, or IκBα. To verify the amount of loaded protein, they were also probed with anti-ERK1/2. *B*, RAW264.7 cells pretreated with 1 or 30 μg/ml PGN for 16 h were stimulated with 30 μg/ml PGN for the indicated times. Total cell lysates were blotted with a phosphospecific antibody (*Ab*) for ERK, p38, JNK, or IκBα. To verify the amount of loaded protein, they were also probed with anti-ERK1/2.

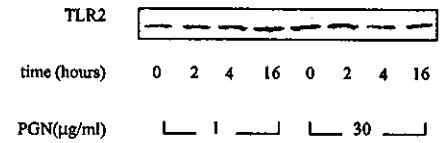

FIG. 3. **Cell surface expression of TLR2 is not affected by PGN treatment.** RAW264.7 cells were stimulated with 1 or 30 μg/ml of PGN for the indicated times. Total cell lysates in the membrane fraction were blotted with an antibody specific for TLR2.

to 16 h after PGN stimulation (Fig. 3). These results indicated that levels of TLR2 expression in the tolerant cells were not different from those in the non-tolerant cells.

IRAK-1 Kinase Activity Was Inhibited in PGN-induced Tolerant Cells—By using the kinase assay, we examined IRAK-1 activation after PGN stimulation. As shown in Fig. 4*A*, in resting cells stimulation with both 1 and 30 μg/ml PGN increased IRAK-1 kinase activity, which was markedly up-regulated at 15 min and decreased at 60 min after stimulation. However, kinase activity of IRAK-1 was much stronger in cells treated with 30 μg/ml PGN than that in cells treated with 1 μg/ml PGN. We also examined whether IRAK-1 is phosphorylated by stimulation with each concentration of PGN. However, IRAK-1 phosphorylation was not observed even in cells treated with 30 μg/ml PGN (date not shown). Fig. 4*B* exhibited the induction of kinase activity of IRAK-1 in PGN-induced non-tolerant and tolerant cells. We observed that kinase activity of IRAK-1 was augmented in non-tolerant cells 15 min after second PGN stimulation, but suppressed in PGN tolerant cells (Fig. 4*B*). Phosphorylation of IRAK-1 was also examined, however, it was not observed for up to 60 min in both cells (date not shown). These results demonstrate that activation of IRAK-1 is exclusively inhibited after the second PGN stimulation in tolerant cells.

Induction of IRAK-M and Inhibition of the Association between MyD88 and IRAK-1 in PGN Tolerant Cells—Previous studies have reported that IRAK-M negatively regulates TLR signaling (28); however, the role of IRAK-M in TLR-mediated tolerance remains fully obscure. Therefore, we compared the time kinetics of expression of IRAK-M in cells treated with 1 and 30 μg/ml PGN. As shown in Fig. 5*A*, the bands corresponding to IRAK-M were not observed in unstimulated cells, indicating little constitutive expression of IRAK-M in resting cells. IRAK-M expression was also not observed in cells stimulated with a low concentration of PGN up to 24 h after stimulation. However, weak but significant expression of IRAK-M was ob-

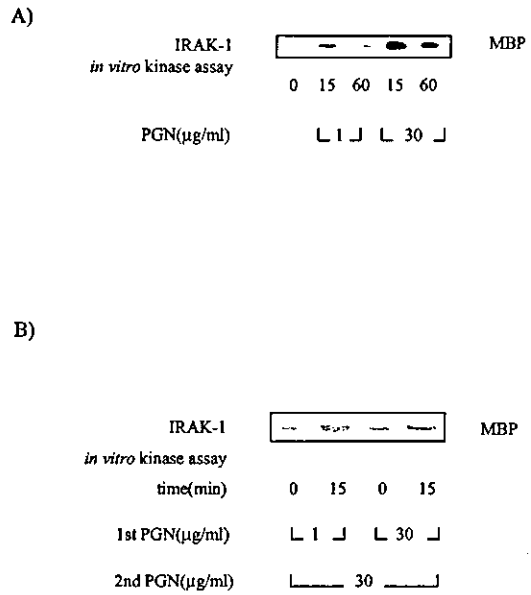

FIG. 4. **IRAK-1 kinase activity is inhibited in PGN tolerant cells.** *A*, cells stimulated with PGN for the indicated times. Cell lysates were immunoprecipitated with an antibody specific for IRAK-1. The immunoprecipitated IRAK-1 complexes in kinase buffer were added to each sample, supplemented with 5 μM ATP, 1 μg myelin basic protein, and 1 μl of [^{32}P]ATP, and incubated at 37 °C for 30 min. Then the samples were eluted in SDS-PAGE sample buffer and subjected to SDS-PAGE analysis. The gel was dried and exposed to x-ray film. *B*, cells pretreated with 1 or 30 μg/ml of PGN for 16 h were stimulated with 30 μg/ml of PGN for the indicated times, and the cell lysates were immunoprecipitated with an antibody specific for IRAK-1. Kinase activity of IRAK-1 was evaluated as described above.

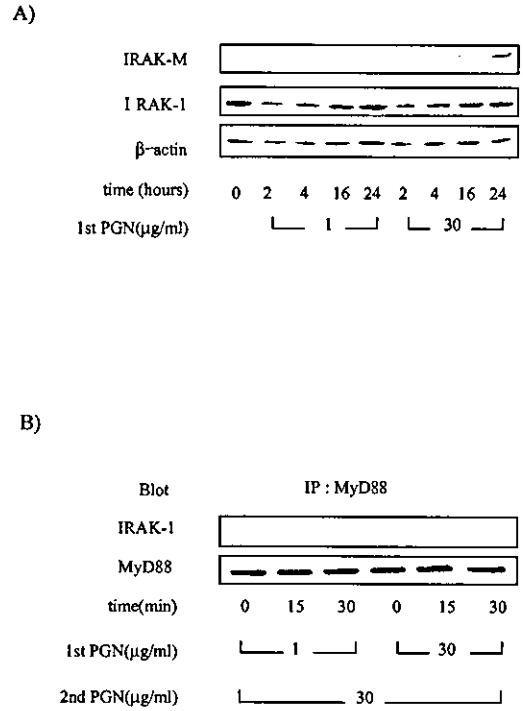

FIG. 5. **IRAK-M is induced by stimulation with a high concentration of PGN and the association between MyD88 and IRAK-1 is inhibited in PGN tolerant cells.** *A*, RAW264.7 cells were stimulated with 1 or 30 μg/ml of PGN for the indicated times. Total cell lysates were blotted with an antibody specific for IRAK-M or IRAK-1. To verify the amount of loaded protein, they were also probed with anti-β-actin. *B*, cells pretreated with 1 or 30 μg/ml of PGN for 16 h were stimulated with 30 μg/ml of PGN for the indicated times. Total cell lysates were immunoprecipitated with an antibody specific for MyD88 and probed with anti-IRAK-1 (*upper lane*) or -MyD88 (*lower lane*).

served in cells treated with a high concentration of PGN 4 h after stimulation. We speculate that the upper bands correspond to phosphorylated IRAK-M. In cells treated with a high concentration of PGN, apparent expression of phosphorylated IRAK-M was observed after 16 h of stimulation, which were slightly augmented 24 h after PGN stimulation (Fig. 5*A*). These results suggested that IRAK-M expression was only slightly induced, if any, in non-tolerant cells, while was apparently induced in tolerant cells. The time course of IRAK-1 protein expression was also studied by Western blotting in these cells. However, we found no significant difference in the protein levels of IRAK-1 between cells treated with 1 or 30 μg/ml PGN during 24 h stimulation, although expression levels appeared to decrease slightly in 2–4 h in both cells (Fig. 5*A*). These results collectively indicated that IRAK-1 was not degraded after PGN stimulation. We finally examined the association between IRAK-1 and MyD88 after restimulation with 30 μg/ml PGN in tolerant and non-tolerant cells. Association between IRAK-1 and MyD88 was clearly enhanced in PGN non-tolerant cells after the second PGN stimulation, but was barely detected in PGN tolerant cells (Fig. 5*B*), indicating that IRAK-1 is not recruited to the receptor/MyD88 complexes in PGN-induced tolerant cells.

Specific Down-regulation of IRAK-M Reinstated the Production of TNF-α after PGN Restimulation—Finally, we examined whether experimental modulation of IRAK-M expression would alter PGN-induced tolerance. As shown in Fig. 6*A*, IRAK-M was induced 16–24 h after PGN stimulation in cells transfected with control RNAs. However, its expression was downregulated in cells transfected with a mixture of four specific siRNAs for IRAK-M (Fig. 6*A*). The intensity of the bands of IRAK-M in siRNA-transfected cells was about 30% of the controls cells (24 h) as judged by a densitometric analysis. In addition, TNF-α production was inhibited in control cells pre-treated with and restimulated with 30 μg/ml of PGN, whereas it was reinstated in cells transfected with IRAK-M siRNAs, in a dose-dependent manner (Fig. 6*B*). These results collectively indicated that down-regulation of IRAK-M expression altered PGN-induced tolerance.

DISCUSSION

Multiple microbial stimulants can induce a wide array of gene expressions through the TLRs. Activation of TLRs subsequently lead to common downstream signaling events, including MyD88/IRAK kinase, MAPKs and NF-κB activation, which contribute to the production of TNF-α. With regard to the common signaling pathway that leads to TNF-α production, prolonged treatment with microbial stimulants has been shown to induce a state of tolerance characterized by decreased TNF-α production upon subsequent challenge (31, 32). Despite numerous studies, however, the molecular mechanism of endotoxin tolerance still remains to be solved, which probably involves down-regulation of multiple TLR signaling components, including down-regulation of the TLR4 receptor, disruption and degradation of IRAK, and reduced activation of MAPKs (17, 33).

Endotoxin tolerance is not limited to the potent TLR4 agonist, but also targets signaling initialized by agonists recognized by other members of the Toll/IL-1R family (18, 31, 34, 35). Among PAMP-induced tolerance, LPS-mediated homo-tolerance has been most extensively studied. Although molecular mechanisms of TLR4-mediated tolerance have still remained to be fully clarified, numerous studies have revealed the involvement of down-regulation of multiple TLR signaling components, including down-regulation of TLR4 receptor expression, disruption and degradation of IRAK, and reduced activation of

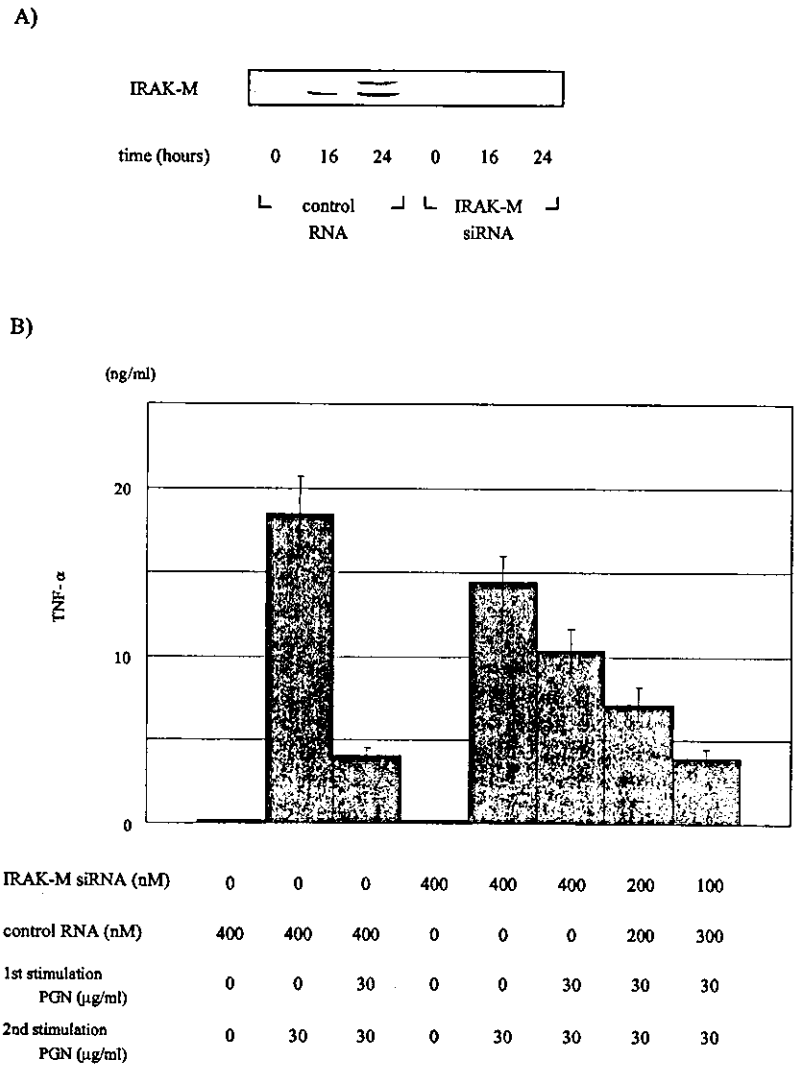

FIG. 6. **Transfection of siRNA for IRAK-M down-regulated the expression of IRAK-M after PGN stimulation and reinstated the production of TNF-α after PGN restimulation.** *A*, RAW264.7 cells were transfected with 400 nM siRNAs for IRAK-M or control RNAs and incubated for 24 h. They were then stimulated with 30 μg/ml of PGN for the indicated times, and total cell lysates were blotted with a specific antibody for IRAK-M. *B*, RAW264.7 cells were transfected with graded concentrations of siRNAs for IRAK-M or control RNAs and incubated for 24 h. Cells pretreated with 30 μg/ml PGN for 16 h were stimulated with 30 μg/ml PGN for 24 h. The amount of TNF-α was measured as described in Fig. 1. Data represent the mean ± S.D., and the data shown are representatives of three independent experiments.

MAPKs, in LPS-mediated homotolerance. On the other hand, little information was available for TLR2-mediated homotolerance. Previous reports state that prolonged LTA treatment of murine monocytes/macrophages and human THP-1 cells can develop similar hyporesponsiveness in TNF-α production to subsequent LTA stimulation (21, 31). In this study, we elucidated the molecular mechanisms of PGN homotolerance. We found that pretreatment with low concentrations of PGN (≤3 μg/ml) for 16 h did not induce PGN homotolerance against following addition of 30 μg/ml PGN as assessed by TNF-α production (Fig. 1*B*). In fact, TNF-α production was rather promoted in these cases. On the other hand, pretreatment with high concentrations of PGN (≥10 μg/ml) for 16 h did clearly induce PGN tolerance (Fig. 1*B*). Furthermore, cells treated with high concentrations of PGN (≥10 μg/ml) for 4 h also became tolerant against subsequent PGN stimulation (date not shown). When phosphorylation of intracellular signaling molecules was compared between non-tolerant and tolerant cells, phosphorylation of MAPKs and IκBα was exclusively suppressed in PGN-induced tolerant cells (Fig. 2*B*). These results indicate that the intensity of phosphorylation of MAPKs and IκBα is well correlated with TNF-α production, implying that phosphorylation of these molecules is also a good indicator for estimating the tolerant state.

LTA-tolerant THP-1 cells are not totally unresponsive to subsequent LTA challenge. Instead, LTA-tolerant cells are capable of responding to further LTA treatment, indicating that the TLR2 receptor is still functional in LTA-tolerant cells (21). It is speculated that the decreased expression of inflammatory proteins such as TNF-α is likely due to disruption of intracellular signaling components downstream of the TLR2 receptor (21). We show that the expression level of TLR2 protein localized in the membrane fraction in tolerant cells is similar to that in non-tolerant cells (Fig. 3), indicating that the expression levels of receptors do not account for the tolerant state.

A previous report showed that IRAK kinase activation and TNF-α protein production were inhibited in LTA-tolerant cells (31). LPS treatment induces rapid degradation as well as inactivation of IRAK (19, 36). Reduced IRAK-1 kinase activity in LPS-tolerant human and murine macrophages has been described previously (17, 19). However, the IRAK protein level does not decrease following prolonged LTA treatment (21). Our presented results demonstrated that stimulation with a high concentration of PGN induced strong IRAK-1 kinase activation in resting cells (Fig. 4*A*). However, the same stimulation causes the suppression of kinase activity of IRAK-1 in PGN-induced tolerant cells, while it was retained in non-tolerant cells (Fig. 4*B*). We examined protein levels of IRAK-1 after PGN stimulation, and found that no significant differences occur in cells treated with either a low or high concentration of PGN (Fig. 5*A*), suggesting that IRAK-1 is activated without degradation, results that agree with previous reports on LTA-induced tolerance (21).

IRAK-M is preferentially expressed in monocytes/macrophages (23), and recent studies have reported that IRAK-M

plays a critical negative regulatory role in signaling via TLRs in these cells. IRAK-M−/− macrophages stimulated with known TLR agonists such as LPS or CpG DNA displayed increased NF-κB and MAPK activation. IRAK-M−/− mice showed increased inflammatory responses to bacterial infection (28). In the present study, we for the first time examined IRAK-M in PGN tolerance. We showed that IRAK-M protein is not detected in cells treated with a low concentration of PGN before and up to 24 h after stimulation (Fig. 5*A*), suggesting that IRAK-M expression is very low. However, IRAK-M was induced in cells treated with a high concentration of PGN 4–24 h after stimulation, implying that induction of IRAK-M is correlated with PGN-induced tolerance. Furthermore, down-regulation of IRAK-M expression by siRNAs specific for IRAK-M reinstated the production of TNF-α (Fig. 6). These results suggest that induction of IRAK-M is crucial to PGN-induced tolerance in macrophages.

MyD88 is essential for TNF-α secretion in response to TLR agonists (37), and association of MyD88 with TLR has been acknowledged as an essential step in signaling via various TLRs. Although the role of MyD88 is fully unknown in TLR-2-mediated tolerance, a recent report demonstrated that LPS-induced tolerance is mediated a failure of MyD88 to associate with TLR4 (20). In the present study, we found that the complex between MyD88 and IRAK-1 is barely detectable after a second PGN stimulation in tolerant cells, while the complex between MyD88 and IRAK-1 is augmented in non-tolerant cells (Fig. 5*B*). These results collectively indicate that interception of recruitment of IRAK-1 to the TLR2/MyD88 complex is an important molecular mechanism of PGN-induced tolerance. One possible scenario is that in tolerant cells, IRAK-M induced by a high concentration of PGN inhibits the activation of IRAK-1 and interferes with the association between IRAK-1 and MyD88. LPS/TLR4 signaling can recruit the specific adaptor protein(s) that leads to IRAK phosphorylation and subsequent degradation, whereas TLR2 signaling recruits adaptor(s) that lead only to IRAK activation, but not phosphorylation/degradation. LTA cannot induce IRAK phosphorylation because IRAK phosphorylation is a prerequisite for subsequent degradation (21). Our results that IRAK-1 is activated but not degraded in a PGN-induced tolerance model agree with the previous report of LTA tolerance. IRAK activation and phosphorylation may be two independent events controlled by distinct regulatory steps, as speculated by Jacinto *et al.* (21).

We have not elucidated the molecular mechanisms that regulate protein expression of IRAK-M and by which IRAK-M regulates IRAK-4, because IRAK-M is reported to inhibit dissociation of IRAK-1 and IRAK-4 from the TLR signaling complex by either preventing phosphorylation of IRAK and IRAK-4 or stabilizing the TLR/MyD88/IRAK complex (28). Nevertheless, our novel finding that IRAK-M is specifically induced in PGN-mediated tolerant state may be important for understanding of the molecular mechanisms of tolerance. Prevention of IRAK-1 activation is an important molecular mechanism of PGN-induced tolerance. An understanding of the molecular mechanisms of tolerance may aid development of improved treatments for inflammatory responses that lead to sepsis.

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PREVALENCE OF MEASLES, RUBELLA, MUMPS, AND VARICELLA ANTIBODIES AMONG HEALTHCARE WORKERS IN JAPAN

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ABSTRACT

OBJECTIVES: To evaluate the immune status of healthcare workers (HCWs) against measles, rubella, mumps, and varicella in Japan, and to promote an adequate vaccination program among HCWs.

SETTING: University of Tokyo Hospital.

PARTICIPANTS: Eight hundred seventy-seven HCWs.

DESIGN: Serologic screening for measles, rubella, mumps, and varicella was performed on HCWs. Antibodies against measles, rubella, and mumps were detected using hemagglutination inhibition (HI) assay ($4.20 per test). If serum was negative by HI assay, enzyme-linked immunosorbent assay (EIA) was performed ($12.60 per test). Anti-varicella antibodies were detected by EIA only.

RESULTS: Among tested HCWs, 98.5%, 90.4%, 85.8%, and 97.2% had immunity to measles, rubella, mumps, and varicella, respectively. All those born before 1970 were seropositive for measles. However, individuals susceptible to rubella, mumps, and varicella were present in all age groups. The sensitivities and negative predictive values of HI assay compared with EIA were 86.6% and 11.3% for measles, 99.1% and 92.2% for rubella, and 47.8% and 24.1% for mumps, respectively. For measles and mumps, prevaccination screening by HI assay in combination with EIA led to significant savings compared with EIA only. In contrast, it was estimated that prevaccination screening using only HI assay would be more economical for rubella.

CONCLUSIONS: Aggressive screening and vaccination of susceptible HCWs was essential regardless of age. Prevaccination serologic screening using a combination of HI assay and EIA was more economical for measles and mumps (*Infect Control Hosp Epidemiol* 2004;25:591-594).

Immunity to measles, rubella, mumps, and varicella is an important part of infection control among healthcare workers (HCWs), especially as epidemics of measles and rubella continue to occur in Japan. The Immunization Law in Japan currently recommends single-dose vaccination of children between 12 and 90 months of age against measles and rubella (preferably 12 to 24 months for measles and 12 to 36 months for rubella).[1] The rate of vaccination against measles in Japan was estimated to be only 81% in 2000,[2] and approximately 60% of children had been vaccinated against rubella in 2001.[3] Although few data are available, vaccine coverage for mumps and varicella, which are non-mandatory vaccinations, is thought to be low. In 1988, live attenuated measles–mumps–rubella vaccine was approved in Japan. However, use of this vaccine was stopped in 1993 due to adverse reactions, mainly aseptic meningitis due to the mumps vaccine component.[1,4-6] Under these circumstances, morbidity from measles and rubella remains high in Japan, with an estimated 100,000 to 200,000 cases of measles each year.[2,7]

Susceptible HCWs are at high risk for acquiring and transmitting measles, mumps, rubella, and varicella.[8-10] Therefore, their immunity to these vaccine-preventable diseases is important. In general, however, HCWs in Japan give little thought to vaccine-preventable diseases. The purpose of this study was to evaluate the immune status of HCWs against measles, rubella, mumps, and varicella in Japan, and to promote an adequate vaccination program among HCWs.

Because HCWs in Japan seem to have little knowledge about their history of these diseases or whether they have been vaccinated, prevaccination serologic screening is essential to detect and vaccinate susceptible individuals efficiently. This study also investigated the most economical approach to prevaccination screening for immunity to measles, rubella, mumps, and varicella.

METHODS

The University of Tokyo Hospital is a 1,193-bed, tertiary-care hospital with 2,100 employees. Between September and October 2002, serologic testing for measles, rubella, mumps, and varicella was recommended to HCWs who did not have any documentation of vac-

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TABLE 1
CHARACTERISTICS OF THE HEALTHCARE WORKERS TESTED

Characteristic	No.
No. of HCWs	877
No. tested for	
Measles	860
Rubella	867
Mumps	867
Varicella	854
Gender	
Male	256 (29.2%)
Female	621 (70.8%)
Age, y	
≤ 25	161 (18.4%)
26 to 35	421 (48.0%)
36 to 45	145 (16.5%)
46 to 55	100 (11.4%)
≥ 56	50 (5.7%)

HCWs = healthcare workers.

TABLE 2
SEROLOGIC RESULTS OF THE HEALTHCARE WORKERS

Disease	No. of HCWs Negative	No. of HCWs Tested	Percentage of HCWs Negative
Measles	13	860	1.5
Rubella	83	867	9.6
Mumps	123	867	14.2
Varicella	24	854	2.8

HCWs = healthcare workers.

cination, history of these diseases, or serologic evidence of immunity, regardless of age.

Antibodies against measles, rubella, and mumps were detected using hemagglutination inhibition assay. If serum samples from HCWs were negative for antibody by hemagglutination inhibition assay (titer less than 8), enzyme-linked immunosorbent assay (EIA), which has a greater sensitivity than hemagglutination inhibition assay, was performed on the same samples.[11-13] Anti-varicella antibodies were detected by EIA only.

Antibody titers on hemagglutination inhibition assay were measured by standard microtiter methods using green monkey erythrocytes for measles, goose erythrocytes for rubella, and guinea pig erythrocytes for mumps. We considered HCWs with a positive result on either hemagglutination inhibition assay or EIA as having evidence of immunity against these diseases. An antibody titer of less than 8 on hemagglutination inhibition assay was considered to be seronegative and a titer of 8 or greater was considered to be seropositive. EIAs for measles-, rubella-, mumps-, and varicella-specific IgG were performed using commercial virus-specific IgG EIA kits [Measles IgG (II)-EIA "SEIKEN," Rubella IgG (II)-EIA "SEIKEN," Mumps IgG (II)-EIA "SEIKEN," and Varicella-zoster IgG (II)-EIA "SEIKEN," Denka Seiken Co., Ltd., Tokyo, Japan]. Optical density values were indexed according to the manufacturer's instructions. EIA values of less than 4.0 were considered negative and values of 4.0 or greater were considered positive. Equivocal values (values of 2.0 to 3.9) were considered negative. Vaccination of susceptible HCWs was recommended for each vaccine-preventable disease. To save costs, not all samples underwent both hemagglutination inhibition assay and EIA, so we calculated the sensitivity of hemagglutination inhibition assay compared with EIA assuming that there were no false-positive reactions on hemagglutination inhibition assay (ie, all sera positive on hemagglutination inhibition assay were positive on EIA).[11]

RESULTS

Eight hundred seventy-seven HCWs (212 physicians, 426 nurses, 45 pharmacists, 130 laboratory technicians, and 64 clerical staff) were tested. Their ages ranged from 20 to 65 years (mean ± standard deviation, 34.4 ± 10.3 years); 256 were men and 621 were women (Table 1). Of 860 HCWs tested for antibodies to measles, 115 (13.4%) were negative on hemagglutination inhibition assay. These 115 HCWs were then tested for measles-specific IgG by EIA. Because 102 of these HCWs were positive for IgG on EIA, only 13 (1.5%) of the 860 HCWs were considered to be truly seronegative for measles (Table 2). Of 867 HCWs tested for antibodies to rubella, 90 (10.4%) were negative on hemagglutination inhibition assay. Because 7 of these HCWs were positive for IgG on EIA, 83 (9.6%) of the 867 HCWs were considered to be seronegative for rubella (Table 2). Of 867 HCWs tested for antibodies to mumps, 511 (58.9%) were negative on hemagglutination inhibition assay. Because 388 of these HCWs were positive for IgG on EIA, 123 (14.2%) of the 867 HCWs were considered to be seronegative for mumps (Table 2). Of the 854 HCWs tested for varicella antibodies by EIA, 24 (2.8%) were susceptible to varicella (Table 2).

On the basis of these results, the sensitivity of hemagglutination inhibition assay compared with EIA for measles, rubella, and mumps antibodies was 86.6%, 99.1%, and 47.8%, respectively, assuming that there were no false-positive reactions on hemagglutination inhibition assay[11] (Table 3). The negative predictive value of hemagglutination inhibition assay compared with EIA for measles, rubella, and mumps antibodies was 11.3%, 92.2%, and 24.1%, respectively. In this study, all HCWs susceptible to measles were younger than 32 years; in other words, all of the HCWs born before 1970 had immunity to measles. In contrast, not all of the HCWs born before 1957 were immune to rubella, mumps, or varicella (Table 4).

A single hemagglutination inhibition assay and EIA cost approximately $4.20 and $12.60, respectively. A sin-

TABLE 3
AGE DISTRIBUTION OF THE HEALTHCARE WORKERS SUSCEPTIBLE TO MEASLES, RUBELLA, MUMPS, AND VARICELLA

Age (y)	No. Susceptible/Total No.			
	Measles	Rubella	Mumps	Varicella
≤ 25	7/153 (4.6%)	13/154 (8.4%)	12/157 (7.6%)	7/150 (4.7%)
26 to 35	6/417 (1.4%)	34/419 (8.1%)	71/417 (17.0%)	12/412 (2.9%)
36 to 45	0/144 (0%)	24/145 (16.6%)	19/144 (13.2%)	1/144 (0.7%)
46 to 55	0/97 (0%)	10/99 (10.1%)	17/100 (17.0%)	2/99 (2.0%)
≥ 56	0/49 (0%)	2/50 (4.0%)	4/49 (8.2%)	2/49 (4.1%)
Total	13/860	83/867	123/867	24/854

gle vaccination for measles, mumps, or rubella costs $25.10, whereas the cost for varicella is $39.90. The overall cost for prevaccination screening of the 877 HCWs was approximately $30,828 and the cost for vaccination (assuming all of the susceptible HCWs would receive vaccination) was approximately $5,498, for a total of $36,326. Prevaccination screening by hemagglutination inhibition assay in combination with EIA led to savings of approximately $12,838 compared with using EIA only. The use of EIA alone for prevaccination screening is associated with high sensitivity, but also high cost. Although hemagglutination inhibition assay is less expensive than EIA, screening by hemagglutination inhibition assay alone is expensive because of the unnecessary vaccination due to excessive false-negative results.

For measles and mumps, the most economical approach to prevaccination screening was to use the less sensitive, less expensive hemagglutination inhibition assay first and then subsequently use the more sensitive EIA for samples with a titer of less than 8 on hemagglutination inhibition assay, as there was a measurable difference in sensitivity between the two tests. In contrast, it was determined that prevaccination screening using only hemagglutination inhibition assay would be more economical for rubella, as the two tests had approximately the same sensitivity.

TABLE 4
RESULTS OF ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE HEALTHCARE WORKERS WHO WERE NEGATIVE ON HEMAGGLUTINATION INHIBITION ASSAY

Disease	HI Assay Results (No.)	EIA Results		Sensitivity*,†	NPV‡
		Positive	Negative		
Measles	Positive (745)	-	-		
				86.6%	11.3%
	Negative (115)	102	13		
Rubella	Positive (777)	-	-		
				99.1%	92.2%
	Negative (90)	7	83		
Mumps	Positive (356)	-	-		
				47.8%	24.1%
	Negative (511)	388	123		

HI = hemagglutination inhibition; EIA = enzyme-linked immunosorbent assay; NPV = negative predictive value.
*Estimate, assuming that there were no false-positive reactions with HI assay compared with EIA.
†Estimated sensitivity of HI assay compared with EIA.
‡NPV of HI assay compared with EIA.

DISCUSSION

Although measles and rubella have been almost eliminated from most industrialized countries due to the development of an aggressive vaccination program,[14,15] epidemics of these diseases still occur regularly in Japan. It is estimated that there are 100,000 to 200,000 cases of measles each year in Japan.[2,7] Vaccination coverage is significantly lower in Japan than in the United States, which has 91.4% coverage for measles, mumps, and rubella and 76% coverage for varicella.[16]

Of the HCWs examined, 98.5% were immune to measles, 90.4% to rubella, 85.8% to mumps, and 97.2% to varicella (Table 2). Immunization rates among HCWs in our hospital for measles, mumps, and varicella were similar to those reported from other countries. In Italy, the rate of seropositivity among HCWs for measles, rubella, mumps, and varicella was reported to be 98.2%, 97.6%, 85.9%, and 97.9%, respectively.[17] In Australia, the rate of seropositivity among HCWs for measles, rubella, and mumps was reported to be 98.3%, 96.6%, and 83.0%, respectively.[18] As in other countries, there is concern that a considerable proportion of HCWs in Japan lack immunity to mumps. The prevalence of antibodies to rubella is considerably lower among HCWs in Japan compared with those in other countries.

Although the Centers for Disease Control and Prevention guidelines have considered birth before 1957 as acceptable evidence of immunity to measles, rubella (except for women of childbearing age), and mumps,[8,9] this may vary from country to country. In our study, the prevalence of measles antibodies in HCWs born before 1970 was 100%. According to the National Epidemiological Surveillance of Vaccine-Preventable Diseases in 2000, more than 99.5% of Japanese adults born before 1970 had measles antibodies and 100% of those born before 1951 did.[19] On piecing these studies together, we find that

almost all Japanese HCWs born before 1970 are considered to have immunity to measles. However, our data showed that HCWs who were susceptible to rubella, mumps, and varicella were present in all age groups (Table 3). Therefore, in Japan, birth before 1957 should not be regarded as evidence of immunity to rubella, mumps, and varicella.

There is a general view that serologic screening need not be done before vaccination of HCWs unless the healthcare facility considers it cost-effective.[8,18,20,21] In Japan, however, prevaccination screening seems to be essential to minimize unnecessary vaccination because most Japanese HCWs seem to have little knowledge about their history of these diseases or whether they have been vaccinated. In such instances, special consideration has been given to the differences in sensitivity and cost between hemagglutination inhibition assay and EIA for serologic screening. So that the results are not misinterpreted, it must be kept in mind that there is a difference in sensitivity between hemagglutination inhibition assay and EIA for some diseases.

The results of hemagglutination inhibition assay were compared with those of EIA. Of the HCWs who were negative for antibodies to measles, rubella, and mumps on hemagglutination inhibition assay, 88.7%, 7.8%, and 75.9% were positive on EIA, respectively. Hemagglutination inhibition assay was less sensitive than EIA for measles and mumps, but the two tests had similar sensitivity for rubella (Table 3). The negative predictive value of hemagglutination inhibition assay compared with EIA for measles, rubella, and mumps antibodies was 11.3%, 92.2%, and 24.1%, respectively (Table 3). Regarding measles and mumps, vaccinating all subjects who are seronegative on hemagglutination inhibition assay leads to a great deal of unnecessary vaccination and a huge cost because of the high false-negative rate of this test. There was a large difference in price between the two tests: EIA in a commercial laboratory costs $12.60 per test and hemagglutination inhibition assay cost $4.20 per test.

In this study, the most sensitive and least expensive way to perform prevaccination serology for measles and mumps was to use hemagglutination inhibition assay first and then subsequently use EIA for samples with a titer of less than 8 on hemagglutination inhibition assay. In contrast, it was determined that prevaccination screening using hemagglutination inhibition assay alone rather than hemagglutination inhibition assay in combination with EIA would be more economical for rubella, as the two tests had approximately the same sensitivity.

In this study, the cost of screening 887 HCWs for antibodies and then vaccinating those susceptible against measles, rubella, mumps, and varicella was $36,326 (ie, $30,828 for serologic testing and $5,498 for each single vaccination). Serologic screening by hemagglutination inhibition assay in combination with EIA led to savings of approximately $12,838 compared with using EIA alone.

The most cost-effective approach to prevaccination serologic screening will depend on the cost per hemagglutination inhibition assay or EIA and the total number of individuals being tested at each institution. It may also be necessary to consider minor geographic differences in seroprevalence rates. Once these factors have been considered, the costs of serologic screening and vaccination can be estimated and the most cost-effective combination of tests can be determined.

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Homozygous *CYP2B6* *6 (Q172H and K262R) correlates with high plasma efavirenz concentrations in HIV-1 patients treated with standard efavirenz-containing regimens

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Abstract

Efavirenz (EFV) is metabolized by cytochrome P450 2B6 (*CYP2B6*) in the liver. We analyzed the genotypes of *CYP2B6* and their contribution to plasma EFV concentrations in 35 EFV-treated patients in International Medical Center of Japan. The mean plasma EFV concentration of patients with *CYP2B6* *6/*6 (Q172H and K262R) ($25.4 \pm 7.5\,\mu M$, $\pm$ SD, $n = 2$) was significantly higher than that of patients with genotypes *6 heterozygote ($9.9 \pm 3.3\,\mu M$, $n = 10$) or without alleles *6 ($8.0 \pm 2.6\,\mu M$, $n = 23$) ($p < 0.0001$). To confirm our result, we further analyzed nine patients (three with high EFV concentrations and arbitrarily selected six with normal EFV concentrations) treated in Osaka National Hospital, and it resulted that the only three patients with the high concentrations were the *6/*6 holder. EFV dose could be decreased in those patients harboring the genotype to reduce toxicity with compromising potency, representing the first step of the Tailor-Made therapy of HIV-1 infection.

Keywords: Cytochrome P450; Genetic polymorphism; HIV-1; Efavirenz; Plasma concentration

Efavirenz (EFV) is a non-nucleoside reverse transcriptase inhibitor that shows potent inhibitory activity against HIV-1 and is stated as a key drug of the first line regimens in the HIV-1 treatment Guideline [1]. However, a number of patients treated with EFV develop central nervous system symptoms including headache, dizziness, insomnia, and fatigue. These side effects are more frequent in patients with high plasma concentration of EFV [2,3] as well as worsen with long-term therapy, and are the main reason for poor adherence or interruption of therapy. EFV is reported to be metabolized by cytochrome P450 (CYP) 3A4 (*CYP3A4*) and 2B6 (*CYP2B6*) to hydroxylated metabolites in the liver [4]. The recent HIV-1 treatment Guideline stated that EFV is metabolized by *CYP3A* [1], whereas an in vitro study indicated that EFV is mainly metabolized by *CYP2B6* [5]. Furthermore, a pharmacogenetic study demonstrated the association of the homozygous variant of multidrug-resistance transporter (*MDR1*; gene product P-glycoprotein) C3435T and good immune recovery in patients treated with EFV-containing regimens [6]. In order to clarify the contribution of polymorphisms to plasma EFV concentration in vivo, we analyzed genotypes of *CYP2B6*, *CYP3A4*, and *MDR1*, and their correlation with plasma EFV concentrations.

Materials and methods

Patients. A total of 60 HIV-1 patients who were treated with EFV-containing regimens at the AIDS Clinical Center, International

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Medical Center of Japan (IMCJ), were examined for their allelic variants of *CYP2B6*, *CYP3A4*, and *MDR1*. Among them, 35 patients were on standard therapy of EFV-containing regimens (600 mg EFV once daily dosing with two nucleotide reverse transcriptase inhibitors) and fully adhered to the regimens based on self-reports. Their plasma EFV concentrations were measured and the correlation between variants and EFV concentrations was analyzed. We excluded those patients who were taking other agents that could potentially interact with plasma EFV concentration such as protease inhibitors and those taking EFV twice daily from the analysis of the correlation. The mean age and body weight of these 35 patients (34 males and 1 female) were 41.6 ± 11.5 years and 63.4 ± 10.9 kg, respectively. The median latency between commencement of treatment and analysis of EFV concentration was 76.9 weeks (range, 4–200). The means ± SD alanine aminotransferase level was 33.1 ± 18.4 U/L. Blood samples were taken between 10 and 14 h (mean, 12.0 h) after dosing. To confirm the results of patients treated at the IMCJ, we further analyzed the allelic variants of nine patients who were treated at the Osaka National Hospital (ONH) [three patients with high plasma EFV concentrations (one patient was taking only 200 mg EFV once daily due to severe side effects) and six patients with normal EFV concentrations]. The Ethics Committee for the Study of Human Genome in each hospital approved this study (IMCJ-H14-36, ONH-23) and all patients gave a written informed consent.

Genotyping. Genomic DNA was isolated from peripheral blood using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). Genotyping of allelic variants of *CYP2B6* [7] [**1* (wild type), **2* (C64T), **3* (C777A), **4* (A785G), **5* (C1459T), **6* (G516T and A785G), **7* (G516T, A785G, and C1459T), and **8* (A415G)], CYP3A4 [**11* (C1088T; unstable form [8]), **12* (C1117T; has an altered testosterone hydroxylase activity [8]), **13* (C1247T; lack of expression [8]), **17* (T566C; exhibits lower turnover numbers for testosterone and chlorpyrifos [9]), and **18* (T878C; exhibits higher turnover numbers for testosterone and chlorpyrifos) [9] and *MDR1* C3435T was carried out using the allelic-specific fluorogenic 5′ nuclease chain reaction assay by the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA). Each 25 μl PCR mixture contained 20 ng genomic DNA, 900 nM primers, 200 nM TaqMan minor groove binder (MGB) probes, and 12.5 μl TaqMan universal PCR master mix (Applied Biosystems). The primers and TaqMan MGB probes used in this study are summarized in Table 1. The thermal cycler program was set up at 50 °C for 2 min and 95 °C for 10 min, and then repeated 40 cycles with 95 °C for 15 s and 60 °C for 1 min.

Plasma efavirenz concentration. Plasma was isolated by centrifugation (10 min at 1800*g*) on the same day as blood sampling and stored at −80 °C until analysis. EFV concentration was measured by reverse-phase high performance liquid chromatography (HPLC) method [10] at BioMedical Laboratory (Saitama, Japan). HPLC was performed on an Inertsil ODS-3 column (5 μm, 250 × 4.6 mm; GL Sciences, Tokyo, Japan) at a flow rate of 1.2 ml/min with ultraviolet-detection at 247 nm. The mobile phase consisted of acetonitrile and water (65:35, v/v).

Statistical analysis. StatView version 5.0 software (SAS Institute, Cary, NC) was used for the comparison of different genotype groups. If one-way analysis of variance (ANOVA) was significant ($p < 0.05$), post hoc Scheffe's F test was applied.

Table 1
Primers and TaqMan MGB probes used in this study

	Forward primer	Reverse primer	VIC probe (wild-type)	6-FAM probe (mutant)
CYP2B6				
C64T	CCTCACAGGACTCTTGCTACTC	AGGCGGTCATGGGTGTTAG	TGGTTCAGCGCCACC	CTGGTTCAGTGCCACC
A415G	CTGTGACCACTATGAGGGACTTC	CTGAGCCTCCTCCTGAATCC	ACACTCCGCTTTCCCAT	CACTCCGCTCTCCCAT
G516T	TCATGGACCCCACCTTCCT	GACGATGGAGCAGATGATGTTG	TTCCAGTCCATTACC	CTTCCATTCCATTACC
C777A	TGGAGAAGCACCGTGAAACC	GAGCAGGTAGGTGTCGATGAG	CCCAGCGCCCCCA	CCCCAGAGCCCCCA
A785G	TGGAGAAGCACCGTGAAACC	TGGAGCAGGTAGGTGTCGAT	CCCCCAAGGACCTC	CCCCAGGGACCTC
C1459T	CCCAGAAGACATCGATCTGACA	GAATGACCCTGGAATCCTTTGAC	AGATCCGCTTCCTG	AGATCTGCTTCCTGC
CYP3A4				
T566C	GGCCTACAGCATGGATGTGAT	TGGATTGTTGAGAGAGTCGATGTT	AGCACATCATTTGGA	AGCACATCATCTGGA
T878C	TCTTTCCTCTCCTTTCAGCTCTGT	GGTTTCATAGCCAGCAAAAATAAAG	CGATCTGGAGCTCG	CGATCCGGAGCTC
C1088T	TGTGCTACAGATGGAGTATCTTGACA	CATCCCATTGATCTCAACATCTTTT	TCTGAGCGTTTCATT	CTGAGCATTTCATTCA
C1117T	TCTTGACATGGTGGTGAATGAAA	CATCCCATTGATCTCAACATCTTTT	CCCTCTCAAGTCTC	CCTCTCAAATCTC
C1247T	AAAGTACTGGACAGAGCCTGAGAAG	GGAGGGCTCCCCTTCCCA	TTCCTCCCTGAAAGG	CCTCCTTGAAAGGTA
MDR1				
C3435T	AACAGCCGGGTGGTGTCA	ATGTATGTTGGCCTCCTTTGCT	CTCACGATCTCTTC	CCTCACAATCTCTT

MGB, minor groove binder; VIC, vasoactive intestinal contractor; 6-FAM: 6-carboxyfluorescein.
Bold indicates the site of substitution.

Results and discussion

Frequency of genotypic variants of CYP2B6, CYP3A4, and MDR1

We first analyzed the frequency of genotypic variants of the 60 patients seen at IMCJ. The *CYP2B6* genotypes were **1/*1* in 28 patients, **1/*2* in 4, **1/*4* in 5, **1/*6* in

Table 2
Frequency of CYP2B6 alleles and genotypes in 60 HIV-1 patients at IMCJ[a]

	Frequency (%)	95% CI
CYP2B6 allele		
**1*	78 (65)	56.5–73.5
**2*	9 (7.5)	3.8–14.2
**4*	10 (8.3)	4.4–15.3
**5*	2 (1.7)	0.3–6.0
**6*	21 (17.5)	10.7–24.3
CYP2B6 genotype		
**1/*1*	28 (46.7)	33.7–60.0
**1/*2*	4 (6.7)	1.8–16.2
**1/*4*	5 (8.3)	2.7–18.4
**1/*6*	13 (21.7)	12.1–34.2
**2/*4*	2 (3.3)	0.4–11.5
**2/*6*	3 (5.0)	1.0–13.9
**4/*4*	1 (1.7)	0.02–8.9
**4/*6*	1 (1.7)	0.02–8.9
**5/*5*	1 (1.7)	0.02–8.9
**6/*6*	2 (3.3)	0.4–11.5

95% CI, 95% confidence intervals.
[a] IMCJ, International Medical Center of Japan.

13, **2/*4* in 2, **2/*6* in 3, **4/*4* in 1, **4/*6* in 1, **5/*5* in 1, and **6/*6* in 2 (Table 2). The *CYP3A4* polymorphisms were only shown in T878C T/C heterozygote in three patients and other alleles were not found. *MDR1* C3435T polymorphisms were C/C in 19 patients, C/T in 31, and T/T in 10.

Correlation between the genotypic variants and EFV concentrations

Among the 35 patients who were on standard therapy of EFV-containing regimens, two had significantly higher plasma EFV concentrations (30.7 and 20.0 μM) than the other patients. *CYP2B6* genotype of the two patients was **6/*6* homozygote. The mean plasma EFV concentrations of patients with *CYP2B6* **6/*6* genotype (25.4 ± 7.5 μM, $n = 2$) were significantly higher than those of patients with **6* heterozygous genotypes (9.9 ± 3.3 μM, $n = 10$) and non-**6* alleles (8.0 ± 2.6 μM, $n = 23$) [one-way ANOVA ($p < 0.0001$) and post hoc Scheffe's F test showed statistically significant difference in plasma EFV concentration between **6/*6* genotype and **6* heterozygous genotypes ($p < 0.0001$), and non-**6* alleles ($p < 0.0001$)]. As shown in Table 3, the differences of patients' characteristics in each *CYP2B6* genotype were not significant, indicating that these characteristics did not influence the difference of EFV concentrations among the three genotypes. Then, we analyzed the additional nine samples (three with high EFV concentrations) obtained from the ONH and found that *CYP2B6* genotypes of the three patients with high EFV concentration were also **6/*6* genotype. Consequently, only five patients whose EFV concentrations were >20 μM had *CYP2B6* **6/*6* genotype (Fig. 1A). There was a significant correlation between *CYP2B6* **6/*6* genotype and high plasma EFV concentrations. In contrast, there was no correlation between *CYP2B6* **5*, *CYP3A4*, *MDR1* genotypes, and plasma EFV concentrations (Figs. 1B–D) in our small number of patients examined in this study.

Homozygous variant of *MDR1* C3435T has been shown to associate with responsiveness to EFV therapy [6]. However, no correlation was found between the C3435T polymorphisms and plasma EFV concentration in our study. Then, the plasma EFV concentration could not explain the favorable clinical result. EFV is a non-nucleoside reverse transcriptase inhibitor and, therefore, plays an anti-HIV-1 activity within HIV-1 infected cells but not in plasma. It remains to be elucidated whether or not the C3435T polymorphisms correlate with high intracellular EFV concentration.

Genetic polymorphism is known to be associated with variable level of *CYP2B6* expression in the liver. Especially, the expression levels of *CYP2B6* **6/*6* genotype are significantly lower than those of wild and other genotypes [7,11]. The high plasma EFV concentration may be explained by the low expression level of this genotype. Based on our new finding, extremely high plasma EFV concentration can be predicted by determining the genotype before commencement of EFV-containing therapy. In such patients, the EFV dose could be decreased to reduce the cost and more importantly the associated toxicity, without compromising its potency. In fact, one patient was treated with 200 mg EFV once daily due to severe side effects but had higher EFV concentrations than other patients with other genotypes. The frequency of the *CYP2B6* **6/*6* genotype in IMCJ patients was 3.3% (2 in 60 patients), whereas

Table 3
Patients' characteristics in each CYP2B6 genotype in 35 patients who were treated with standard EFV-containing therapy at IMCJ[a]

	Non-**6* genotypes	**6* heterozygote genotypes	**6/*6* genotype	*p*
n	23	10	2	
Male:female	23:0	9:1	2:0	n.s.
Age (years) (mean ± SD)	38.8 ± 8.2	45.3 ± 14.8	55.5 ± 19.1	n.s.
Weight (kg) (mean ± SD)	64.3 ± 11.5	58.6 ± 7.5	77.0 ± 5.1	n.s.
Alanine aminotransferase level (U/L) (mean ± SD)	31.0 ± 20.4	35.3 ± 14.0	46.5 ± 3.5	n.s.

n.s., not significant.
[a] IMCJ, International Medical Center of Japan.

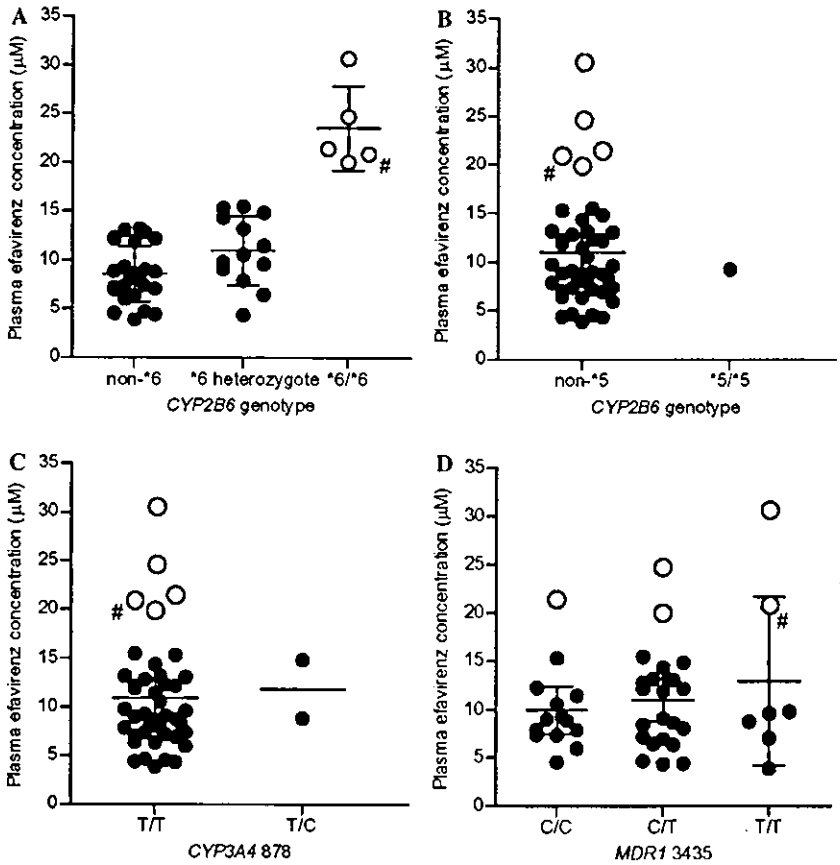

Fig. 1. Correlation between *CYP2B6* *6 genotypes (A), *CYP2B6* *5/*5 genotype (B), *CYP3A4* T878C genotype (C), *MDR1* 3435 genotypes (D), and plasma efavirenz concentrations. A total of 44 HIV-1 patients treated with standard EFV-containing regimens (35 from IMCJ and 9 from ONH) are depicted. Only homozygous genotypes of *CYP2B6* are represented in this figure [A (*6 genotypes) and B (*5/*5 genotype)]. Non-*6 genotypes ($n = 26$) include *1/*1 ($n = 18$), *1/*2 ($n = 2$), *1/*4 ($n = 3$), *2/*4 ($n = 2$), and *5/*5 ($n = 1$). *6 heterozygote genotypes ($n = 13$) include *1/*6 ($n = 9$), *2/*6 ($n = 3$), and *4/*6 ($n = 1$). Numbers of patients of *MDR1* 3435 C/C, C/T, and T/T genotypes are 14, 23, and 7 patients, respectively. Open circles: *CYP2B6* *6/*6 genotype holders, closed circles: other *CYP2B6* genotypes holders. Middle bar indicates mean, and upper and lower bars SD. (#) Patient on 200 mg EFV once daily.

the frequency was 6% in Caucasian population [7]. If these patients could be treated with low dose EFV based on genetic data of *CYP2B6* *6/*6 genotype, it could represent the first step of the Tailor-Made therapy of HIV-1 infection.

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原　著

ANTIBIOTICS & CHEMOTHERAPY
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Nevirapine (BIRG 587) 国内における臨床試験

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Summary

HIV-1感染患者における非核酸系逆転写酵素阻害薬Nevirapine(BIRG587)の有効性，安全性および定常状態における最低血漿中薬物濃度を検討するため，多施設共同オープン臨床試験を実施した。Nevirapineは24週間（200mg/日×2週，400mg/日×22週）投与し，血漿HIV RNAコピー数およびCD4陽性細胞数の変化について評価した。投与完了例は18例であり，中止例13例の内訳は，効果不十分1例，有害事象に起因する患者自己判断に

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よる中止1例，有害事象による中止11例であった。血漿HIV RNAコピー数はNevirapine投与後有意に減少し，投与前4.282logから2週後には2.846logとなり，それ以後検出限界(400copies/mL ≒ 2.6log)付近を24週後まで推移した。また，CD4陽性細胞数では有意な増加を示し，その平均値は24週後に358.1cells/mm³となり，Nevirapineは他の抗HIV薬(核酸系逆転写酵素阻害薬，HIVプロテアーゼ阻害薬)2剤以上との併用により優れた抗HIV-1作用を有することが認められた。副作用は31例中21例(67.7%)にみられ，その主な症状は発熱，発疹，肝機能障害であり，海外で報告されているものと同様であった。定常状態における最低血漿中薬物濃度(400mg/日)は5,078.0 ± 2,298.2ng/mL (n = 93)であり，海外で報告されているものとほぼ同程度の数値であった。

Key Words：Nevirapine／BIRG587／HIV感染／非核酸系逆転写酵素阻害薬

Ⅰ はじめに

ネビラピン（Nevirapine；NVP）は，米国Boehringer Ingelheim Pharmaceuticals Inc.，で合成された非核酸系のHIV-1特異的逆転写酵素阻害薬（NNRTI）である。核酸系逆転写酵素阻害薬(NRTI)が逆転写酵素の基質である核酸と競合して阻害作用を示すのに対して，NVPは核酸とは競合せず，逆転写酵素の疎水ポケット部分に可逆的に結合し，逆転写酵素の触媒活性を阻害する。米国においては1991年から臨床試験が開始され，多くのHIV-1感染症患者に対して優れた臨床効果が得られ，1996年6月21日にFDA(食品医薬品局)で承認後，同年8月から販売された。EUでも1998年2月5日に承認された。

我が国では，1996年12月20日に希少疾病用医薬品に指定され，臨床第Ⅰ相試験，第Ⅱ相試験実施後日本人患者の用法・用量として導入期200mg/日を14日間，その後維持量として400mg/日を経口投与することで1998年11月27日に承認された。

しかしながら，この用法・用量を用いて，日本人患者に対する有効性と安全性を検討し，かつ国内での定常状態における最低血漿中薬物濃度を測定し，その結果を米国の成績と比較することが求められた。そこで，今回市販後臨床試験としてこれらを検討した。

Ⅱ 対象ならびに試験方法

1．対象

対象は，入院・外来の別は問わず，以下の選択基準を満たすHIV-1感染症患者とした。

選択基準

1）血漿HIV RNAコピー数が1×10^3copies/mL以上の患者。
2）CD4陽性細胞数が500cells/mm³以下の患者。
3）Karnofsky Performance Status（KPS，表1）が60%以上の患者。
4）6カ月以上生存可能な患者。
5）年齢16歳以上の患者。
6）試験開始前に文書により本人の自由意思による同意が得られた患者。
7）試験薬投与前の臨床検査で次の基準を満たす患者。
（1）血液学的検査：好中球数：700/mm³以上，ヘモグロビン値：男性8.0g/dL，女性7.5g/dL以上，血小板数：5.0×10^4/mm³以上
（2）血液生化学検査：ALT，AST，γ-GTP値が正常範囲上限の5倍以下，アルカリフォスファターゼ値が正常範囲上限の5倍以下，総ビリルビン値が正常範囲上限の3倍以下，血清クレアチニン値が正常範囲上限の3倍以下

なお，①治験薬が投与されている患者，②既承認薬(抗レトロウイルス薬)による治療で，明らかに血漿HIV RNAコピー数が減少している，あるいはHIV RNAコピー数の減少が維持されている患者，③併用する抗レトロウイルス薬の投与が禁忌とされる患者および併用禁止薬剤の使用が避けられない患者，④重篤な心疾患を有する患者，

表1 Karnofsky Performance Status Scale

Karnofsky の一般全身状態 (Karnofsky Performance Status Scale)	
100% 正常，臨床症状なし 90% 軽い臨床症状があるが正常の活動可能 80% かなりの臨床症状があるが努力して正常の活動可能	正常な活動可能，特に介護する必要なし
70% 自分自身の世話は出来るが正常の活動・労働は不可能 60% 自分に必要なことは出来るが，ときどき介助が必要 50% 病状を考慮した看護および定期的な医療行為が必要	労働不可能，家庭で療養可能，日常の行動の大部分に病状に応じて介助が必要
40% 動けず適切な医療および看護が必要 30% 全く動けず入院が必要だが死は差し迫っていない 20% 非常に重症，入院が必要で精力的な治療が必要 10% 死期が切迫している	自分自身のことをすることが不可能，入院治療が必要 疾患が速やかに進行していく時期
0% 死	

⑤妊婦あるいは妊娠している可能性のある患者，授乳中の患者，⑥その他，試験責任医師あるいは試験分担医師が不適当と判断した患者，は対象としないこととした。

2．患者の同意とGCPの遵守

試験の実施にあたっては，あらかじめ患者本人に，試験について十分な説明を行い，参加に対し自由意思による同意を文書により得ることとした。

なお，本試験は「医薬品の臨床試験の実施に関する基準（GCP）」を遵守して実施した。

3．試験方法

本試験は多施設共同オープン試験として実施した。

投与方法としては，導入期としてネビラピン錠（200mg）1錠を1日1回朝食後に2週間経口投与し（200mg/日），3週目以後は朝食後および夕食後にそれぞれ1錠を経口投与した（400mg/日）。なお，試験薬の投与期間は24週間とした。

4．評価項目

1）血漿HIV RNAコピー数

投与前，2，4，8，12，16，20，24週後に検査を実施し，その推移を観察した。（ロシュ社製Amplicor HIVモニターキットにより測定）

2）CD4陽性細胞数

投与前，2，4，8，12，16，20，24週後に検査を実施し，その推移を観察した。

3）体重，KPS，CD8陽性細胞数，CD4/CD8比

投与前，2，4，8，12，16，20，24週後に検査を実施し，その推移を観察した。

4）血漿中薬物濃度

投与前，2，4，8，12，16，20，24週後に採血し，測定を行った。採血に際しては定常状態における最低値を測定するため非服薬を確認後実施するものとした。

5）有害事象

試験期間中に認められた随伴症状，日和見感染等について，程度，発現時期等を調査し，試験薬との関連性を判定した。

6）臨床評価

（1）HIV-RNAコピー数改善度，CD4陽性細胞数改善度

試験薬投与終了時（または中止・脱落時）に試験薬投与開始日（0週）と比較して，HIV-RNAコピー数とCD4陽性細胞数の改善度を表2に示す基準を参考にして評価した。

（2）総合臨床評価

試験薬投与終了時（または中止・脱落時）にHIV-RNAコピー数改善度とCD4陽性細胞数改善度および自覚症状・他覚所見，体重，KPS等の推移から下記の5段階で判定した。

①著明改善 ②改善 ③不変 ④悪化 ⑤著明悪化

Ⅲ 成績

1．評価対象症例

患者内訳については図1に示した。エントリー