with anit-CD3 Ab induced tyrosine phosphorylation of TLR5. We demonstrated that stimulation with anti-CD3 did not induce tyrosine phosphorylation of TLR5 (Fig. 1C). These results indicate that TLR5 was tyrosine-phosphorylated by flagellin.

Flagellin activated NF-κB and mitogen-activated protein kinases, but not NFAT

As activation of NF-κB and mitogen-activated protein kinases (MAPKs) is the hallmark of TLR-mediated signalling cascades (Muzio *et al.*, 1997; 1998; Yu *et al.*, 2003; Khan *et al.*, 2004), we first examined whether flagellin induced NF-κB activation using a luciferase reporter assay. We demonstrated that the activity of NF-κB was increased in a dose-dependent manner, while *Salmonella* LPS did not activate NF-κB in Jurkat T cells (Fig. 2A). We then examined phosphorylation of MAPKs. Maximal phosphorylation of ERK, c-Jun N-terminal protein kinase (JNK) 1/2 and p38 was observed 15 min after flagellin stimulation (Fig. 2B). These results collectively indicate that flagellin stimulation induced activation of both NF-κB and MAPKs.

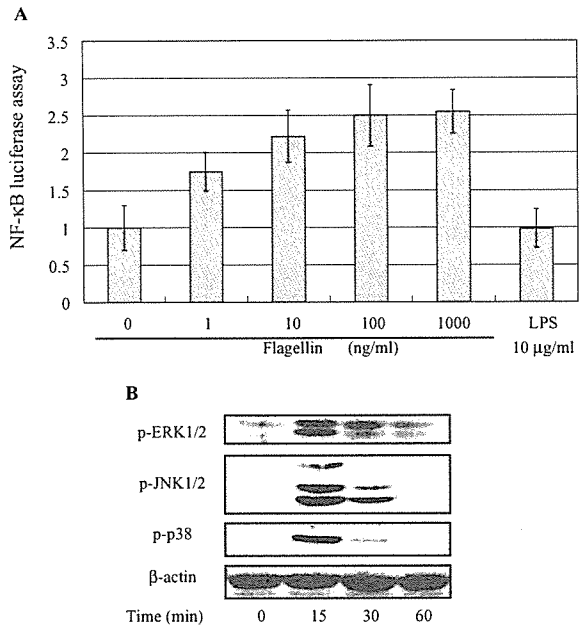

Fig. 2. Stimulation by TLR5 induced activation of Jurkat T cells.
A. A total of 1×10^7 Jurkat T cells were transiently transfected with 30 μg of NF-κB luciferase reporter construct along with 0.03 μg of pRL-TK by electroporation. Twenty-four hours after transfection, the cells were stimulated with the graded concentrations of flagellin or 10 μg ml^{-1} LPS. Six hours after stimulation, the cells were harvested and lysed, and the activities of control *Renilla* luciferase and *Firefly* luciferase (experimental) were measured in triplicate. After normalization according to the *Renilla* luciferase activity, the promoter activity was calculated as fold increase of the control. The data represent the mean ± SD of three independent experiments.
B. Jurkat T cells were stimulated with 10 ng ml^{-1} flagellin for the indicated times. Cell lysates were probed with a phosphospecific Ab for ERK1/2, JNK1/2 and p38, as indicated. Cell lysates were also blotted with β-actin Ab as a loading control.

Pre-treatment of flagellin suppressed TCR-mediated activation of NFAT

We attempted to examine whether pre-treatment of flagellin modulated the TCR-mediated activation of T cells. As NFAT is activated by TCR stimulation (Kane *et al.*, 2000), we analysed TCR-mediated NFAT activities using a luciferase assay in cells pre-treated with flagellin. NFAT activities were significantly suppressed in cells pre-treated with 100 ng ml^{-1} flagellin 6 or 18 h before stimulation with anti-CD3 (Fig. 3A). NFAT activities were also suppressed in a dose-dependent manner in cells pre-treated with graded concentrations of flagellin for 6 h and re-stimulated with anti-CD3 (Fig. 3B). However, TCR-mediated NFAT activities were reinstated 36 h after stimulation with 100 ng ml^{-1} flagellin (Fig. 3C). Furthermore, to exclude the possibility that stimulation with flagellin itself induces NFAT activation in Jurkat T cells, we examined flagellin-induced NFAT activation using a luciferase reporter assay. As shown in Fig. 3D, stimulation with flagellin alone did not activate NFAT, while treatment of PMA plus ionomycin or anti-CD3 Abs activated NFAT. These results suggest that pre-treatment of flagellin inhibited the TCR-mediated activation of T cells, as judged by NFAT activation. However, the inhibitory effect of flagellin on the TCR-mediated activation of T cells was reversible.

Flagellin stimulation regulated TCR signalling, but not TCR surface expression

We then analysed the molecular mechanisms by which pre-treatment of flagellin inhibited the TCR-mediated activation of T cells. First, we examined whether surface expression of TCR was downregulated by pre-treatment with flagellin using a FACS analysis. The levels of TCR expression in cells stimulated with flagellin for 1 or 6 h were almost the same as those in unstimulated cells (Fig. 4A), indicating that expression levels of TCR at the cell surface were unaffected by treatment with flagellin. Next, we compared TCR-mediated intracellular signalling cascade with NFAT activation in cells pre-treated with or without flagellin. Zeta-associated protein 70 (Zap-70) is a tyrosine kinase which is positioned just downstream of TCR in TCR-mediated signalling cascade and is pivotal for TCR-mediated T cell activation, including NFAT activation. Tyrosine phosphorylation of Zap-70 was observed clearly after TCR stimulation in control cells (Fig. 4B). However, pre-treatment with flagellin significantly reduced tyrosine phosphorylation of Zap-70 (Fig. 4B), indicating

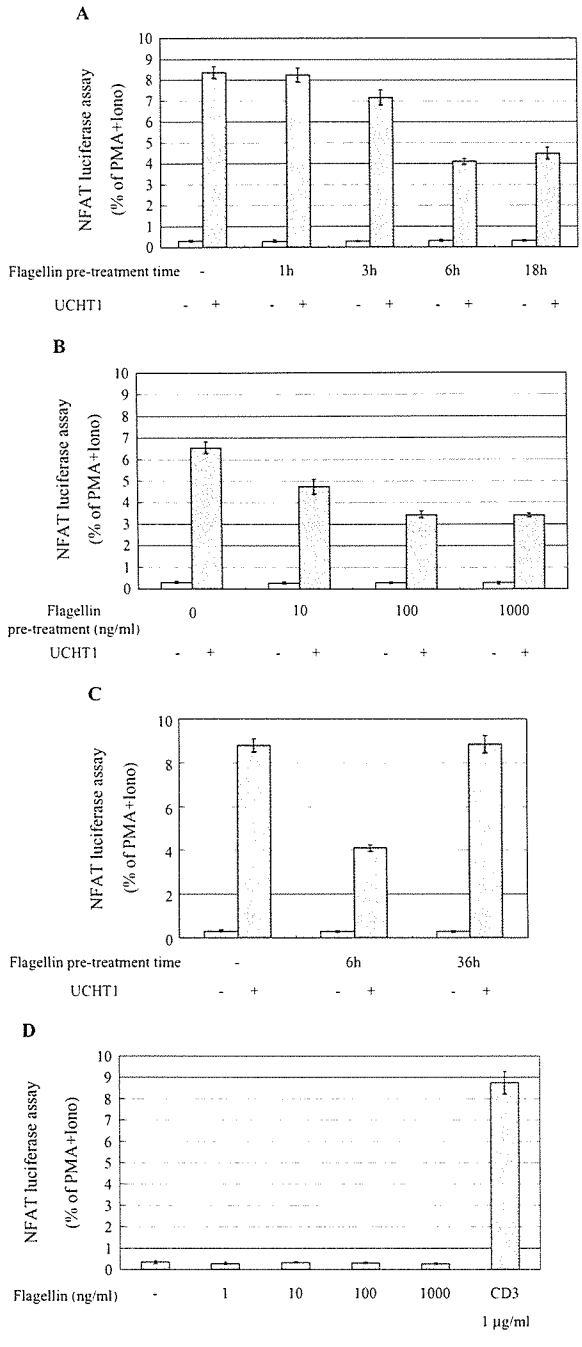

Fig. 3. Flagellin pre-treatment inhibited NFAT activation via T cell receptors.
A. Jurkat T cells transfected with NFAT reporter construct and pRL-TK were treated with 100 ng ml^{-1} flagellin for the indicated times prior to anti-CD3 Ab (UCHT1) stimulation. Six hours after stimulation with anti-CD3 Ab, *Renilla* luciferase and *Firefly* luciferase (experimental) were measured in triplicate. After normalization according to the *Renilla* luciferase activity, the promoter activity was expressed as the percentage of response obtained with PMA plus ionomycin. The data represent the mean ± SD of three independent experiments.
B. Jurkat T cells transfected with the NFAT reporter construct and pRL-TK were treated with graded concentrations of flagellin for 6 h. Six hours after stimulation with anti-CD3 Ab, luciferase activities were measured as described in Fig. 3A.
C. Jurkat T cells transfected with NFAT reporter construct and pRL-TK were treated with 100 ng ml^{-1} flagellin for 6 or 36 h prior to anti-CD3 Ab (UCHT1) stimulation. Six hours after stimulation with anti-CD3 Ab, NF-AT luciferase activities were measured as described in (A).
D. Jurkat T cells were transfected with the NFAT reporter construct and pRL-TK as an internal control and were stimulated with graded concentrations of flagellin, 1 μg ml^{-1} anti-CD3 Ab (UCHT1) or PMA plus ionomycin as positive controls. Luciferase activities were examined as described in Fig. 2A.

that flagellin inhibited TCR-mediated signalling events just downstream of TCR.

SOCS-1 induced by flagellin negatively regulated TCR-mediated T cell activation

SOCS proteins are known to be negative regulators in a variety of signalling cascades including LPS or TCR-mediated signalling (Marine *et al.*, 1999; Matsuda *et al.*, 2000; Banerjee *et al.*, 2002; Kinjyo *et al.*, 2002; Nakagawa *et al.*, 2002; Egan *et al.*, 2003). We speculated that SOCS-1 was one of the candidates that negatively regulate TCR-mediated responses in cells pre-treated with flagellin, and examined the level of SOCS-1 expression

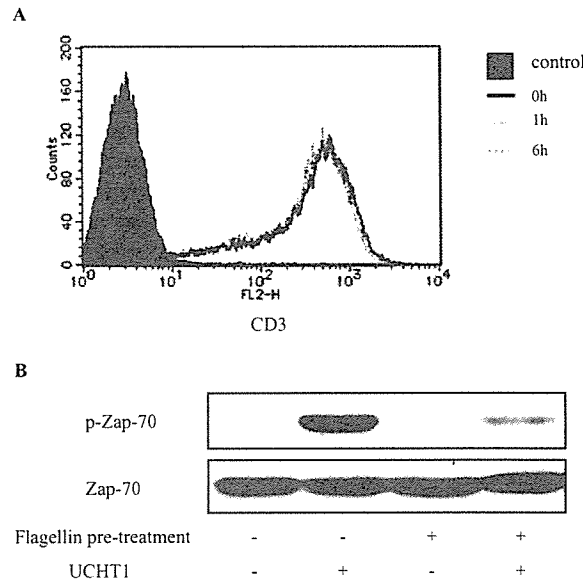

Fig. 4. Flagellin pre-treatment had no effect on the surface expression of TCRs, but inhibited TCR-induced tyrosine phosphorylation of Zap-70.
A. Jurkat T cells were stimulated with or without 100 ng ml^{-1} flagellin for the indicated times, and stained with PE-conjugated anti-CD3 Ab. The surface expression of flagellin was analysed by FACS.
B. Jurkat T cells were pre-treated with or without 100 ng ml^{-1} flagellin for 6 h prior to anti-CD3 Ab (UCHT1) stimulation. Then cells were stimulated with 1 μg ml^{-1} anti-CD3 Ab for 1 min. Cell lysates were blotted with phospho-Zap-70 Ab (top) and Zap-70 Ab as a loading control (bottom).

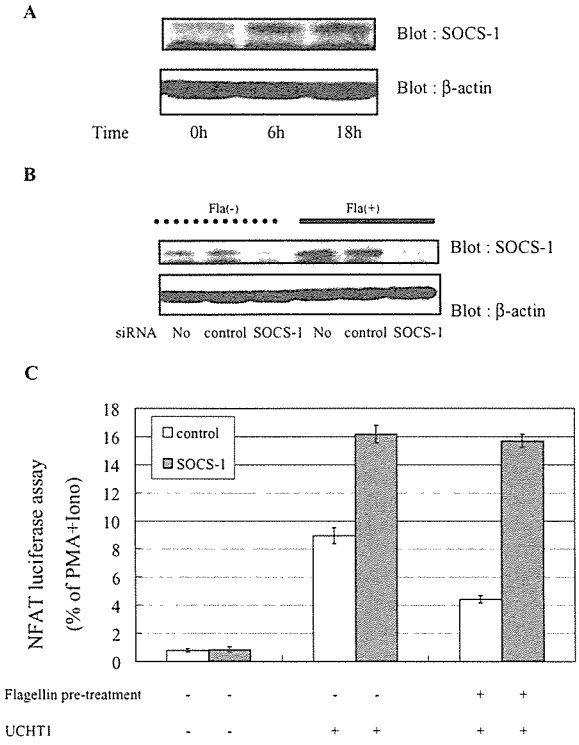

Fig. 5. Flagellin stimulation induced SOCS-1, and siRNA specific for SOCS-1 inhibited flagellin-induced SOCS-1 production and reinstated TCR-induced NFAT activation in cells pre-treated with flagellin.
A. Jurkat T cells were stimulated with 100 ng ml^{-1} flagellin for the indicated times. Cell lysates were probed with anti-SOCS-1 Ab. Then the Ab was stripped, and re-probed with a β-actin as a loading control. The data shown are the representative of three experiments.
B. Jurkat T cells were transfected with 10 μg of siRNA specific for SOCS-1, control siRNA or vehicle by electroporation. After incubation for 24 h, cells were stimulated with or without 100 ng ml^{-1} flagellin for 6 h. Cells were lysed and subsequently subjected to SDS-PAGE for Western blotting. The blots were probed with anti-SOCS-1 Ab. Then the Ab was stripped, and the blots were re-probed with a β-actin as a loading control. The data shown are the representative of three experiments.
C. SOCS-1 siRNAs or non-specific control siRNAs were co-transfected with the NFAT luciferase reporter construct and the pRL-TK internal control into Jurkat T cells by electroporation. Then the cells were treated with or without 100 ng ml^{-1} flagellin for 6 h, and stimulated in the presence or absence of anti-CD3 Ab (UCHT1) for 6 h. The luciferase activities were calculated as described in Fig. 3A.

after flagellin stimulation in Jurkat T cells. As shown in Fig. 5A, expression levels of SOCS-1 were very low in unstimulated Jurkat cells, while they were augmented in cells treated with flagellin for 6 or 18 h, indicating that SOCS-1 was induced by flagellin stimulation in Jurkat T cells.

To confirm that flagellin-induced SOCS-1 protein inhibited TCR-mediated NFAT activity, we inhibited expression of SOCS-1 protein by transfection of small interfering RNA (siRNA) specific for SOCS-1. Cells transfected with non-specific siRNA induced the same levels of basal and flagellin-induced SOCS-1 protein as those in control cells (Fig. 5B). However, transfection of siRNA specific for SOCS-1 inhibited expression levels of SOCS-1 protein in both cells pre-treated with or without flagellin (Fig. 5B), indicating that the siRNA inhibited both basal and flagellin-induced SOCS-1 protein expression. We then examined whether inhibition of SOCS-1 protein by siRNA would alter TCR-mediated NFAT activity in cells treated with flagellin. We compared TCR-mediated NFAT luciferase activity between cells transfected with siRNAs specific or non-specific for SOCS-1. As shown in Fig. 5C, NFAT activities were inhibited by flagellin pre-treatment in cells transfected with non-specific SOCS-1 siRNA. However, pre-treatment of siRNA specific for SOCS-1 augmented TCR-mediated activation of NFAT, and flagellin pre-treatment did not inhibit TCR-mediated activation of NFAT (Fig. 5C). These results indicate that flagellin-induced SOCS-1 inhibited TCR-mediated activation of Jurkat T cells.

SOCS-1 forms a complex with Zap-70 after TCR stimulation

To explore the molecular mechanism by which SOCS-1 protein suppressed TCR-mediated NFAT activity, we established a stable cell line that expressed SOCS-1. As shown in Fig. 6A, SOCS-1 was expressed constitutively in these cells. We next compared NFAT luciferase activity and tyrosine phosphorylation of Zap-70 between cells stably transfected with or without SOCS-1. Both TCR-mediated NFAT activity and tyrosine phosphorylation of Zap-70 were inhibited in cells that expressed SOCS-1 constitutively compared with control cells (Fig. 6B and C). In addition, we examined whether SOCS-1 forms a complex with Zap-70 after TCR stimulation. As shown in Fig. 6D, Zap-70 interacted with SOCS-1, and the interaction between Zap-70 and SOCS-1 was dependent on TCR stimulation. These results collectively indicate that SOCS-1 was a negative regulator of TCR-mediated activation of T cells, inhibiting activation of Zap-70 and NFAT.

Effects of flagellin stimulation on human primary T cells

We also determined whether pre-treatment of flagellin inhibited TCR-mediated activation of primary T cells. First, we examined whether flagellin stimulation induced activation of human primary T cells, and analysed flagellin-induced phosphorylation of MAPKs as a representative. As shown in Fig. 7A, three MAPKs, ERK, JNK and p38, were all phosphorylated by flagellin stimulation. Next, we examined whether pre-treatment of flagellin suppressed TCR-mediated phosphorylation of Zap-70. As observed in Jurkat T cells, it was also inhibited in cells pre-treated with flagellin for 6 h before stimulation with anti-CD3 Ab (Fig. 7B). Finally, we showed that SOCS-1 was induced

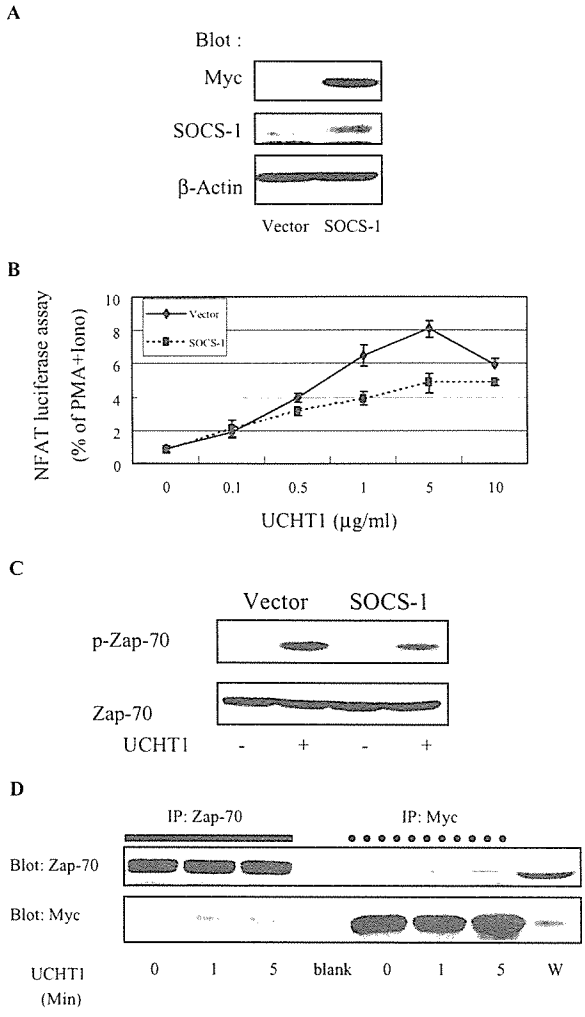

Fig. 6. TCR-induced activation of NFAT and tyrosine phosphorylation of Zap-70 was suppressed in cells stably expressed with SOCS-1.
A. To establish a cell line that stably expresses SOCS-1, Jurkat T cells were transfected with pcDNA3-SOCS1-Myc or pcDNA3 (control), and selected in the medium with 600 μg ml^{-1} G418. Protein expression of SOCS-1 in cells stably expressing SOCS-1 or control cells was examined by Western blotting with anti-Myc Ab.
B. Cells stably expressing SOCS-1 or control cells were transfected with the NFAT luciferase reporter construct and pRL-TK by electroporation. Twenty-four hours after transfection cells were stimulated with anti-CD3 Ab (UCHT1). Six hours after stimulation luciferase activities were measured as described in Fig. 3A.
C. Cells stably expressing SOCS-1 or control cells were stimulated with anti-CD3 Ab for 1 min. Then the cell lysates were blotted with a phosphospecific Ab for Zap-70 or Zap-70 Ab as a loading control.
D. Jurkat cells stably expressing SOCS-1 or control cells (E6.1) were stimulated with anti-CD3 Ab for indicated times. Then the cell lysates were immunoprecipitated with anti-Myc or anti-Zap-70 Ab and blotted with anti-Zap-70 or anti-Myc Ab. Whole-cell lysates of Jurkat T cells stably expressing SOCS-1 (W) were used as a control.

by flagellin stimulation in primary T cells (Fig. 7C). These results collectively indicated that pre-treatment of flagellin inhibited the TCR-mediated activation of primary T cells and that flagellin stimulation induced SOCS-1.

Discussion

We demonstrated for the first time that stimulation by flagellin itself induced tyrosine phosphorylation of TLR5 (Fig. 1B). TLR2 has a YXXM motif whose phosphorylation is important for phosphatidylinositol 3-kinase (PI3-K)

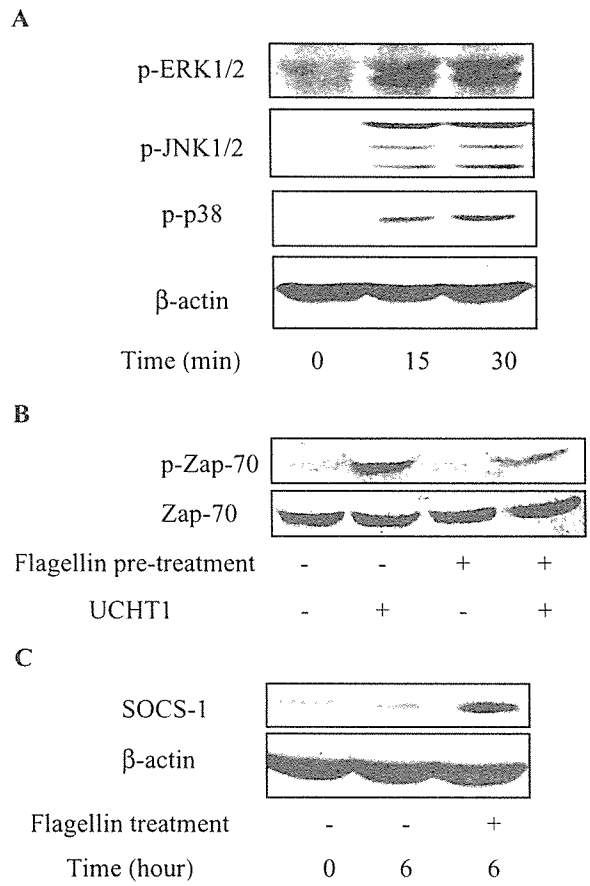

Fig. 7. Flagellin stimulation led to phosphorylation of MAPKs, inhibited TCR-mediated activation and induced SOCS-1 in human primary T cells.
A. Human primary T cells were stimulated with 100 ng ml^{-1} flagellin for the indicated times. Cell lysates were probed with a phosphospecific Ab for ERK1/2, JNK1/2 and p38, as indicated. Cell lysates were also blotted with β-actin Ab as a loading control.
B. Primary T cells were pre-treated with or without 100 ng ml^{-1} flagellin for 6 h prior to anti-CD3 Ab (UCHT1) stimulation. Then cells were stimulated with 1 μg ml^{-1} anti-CD3 Ab for 1 min. Cell lysates were blotted with phospho-Zap-70 (top) or Zap-70 Ab as a loading control (bottom).
C. Primary T cells were stimulated with 100 ng ml^{-1} flagellin for the indicated times. Cell lysates were probed with anti-SOCS-1 Ab. Then the Ab was stripped, and re-probed with a β-actin as a loading control. The data shown are the representative of three experiments.

association and activation of the TLR2-mediated signal transduction cascade (Arbibe *et al.*, 2000), while phosphorylation of TLR4 is important for activation of NF-κB and IL-8 production (Chen *et al.*, 2003). We speculated that phosphorylation of TLR5 is also important for the activation of the TLR5-mediated signal transduction cascade, although which tyrosine is phosphorylated in TLR5 remains to be determined. In our studies, tyrosine phosphorylation of TLR5 indicates that flagellin activated the cells through TLR5. As Jurkat T cells do not express TLR4 and were not activated by LPS in our study (data not shown), activation was carried out by flagellin via TLR5, not by impure ingredients such as LPS. Flagellin stimulation induced activation of both MAPKs (Figs 2A and 7A) and NF-κB (Fig. 2B), which are important for the TLR-mediated signal transduction cascade in T cells. Although stimulation by flagellin is involved in cytokine production in macrophages or dendritic cells, we did not detect flagellin-induced production of cytokines, such as TNF-α, IL-1β, IL-6 and IL-2, in Jurkat T cells (data not shown), indicating that the role of flagellin-induced activation of T cells may be different from that of macrophages or dendritic cells.

Stimulation by flagellin itself did not induce NFAT activation in T cells, while stimulation via TCR lead to the activation of NFAT (Fig. 3C). However, TCR-mediated NFAT activation was inhibited in cells pre-stimulated by flagellin (Fig. 3A). We speculated that flagellin stimulation might downregulate cell surface expression of TCR or inhibit molecules in the signal transduction cascade downstream of TCR. However, pre-treatment of flagellin had no effect on the expression of TCR on the cell surface (Fig. 4A). Interestingly, pre-treatment of flagellin inhibited tyrosine phosphorylation of Zap-70 which is a crucial tyrosine kinase in TCR-mediated activation of T cells (Figs 4B and 7B), thus indicating that pre-treatment of flagellin interfered with activation of the molecules just downstream of TCR in TCR-mediated signal transduction cascade. The inhibitory effect of flagellin on Zap-70 phosphorylation appears to be greater than that on NFAT-mediated gene expression. One possibility is that NFAT gene expression might be regulated in part by the molecules that is not located downstream of Zap-70 in the TCR-mediated signal cascade and that is not affected by flagellin pre-stimulation. Another possibility is that the difference might be due to using different assay systems, Western blotting and luciferase assay.

Proteins in SOCS family inhibit signal transduction cascades mainly through cytokine receptors (Elliott and Johnston, 2004), and negatively regulate TCR-mediated activation of T cells (Marine *et al.*, 1999; Matsuda *et al.*, 2000; Egan *et al.*, 2003). For example, SOCS-3 inhibits TCR-mediated NFAT activation by binding to calcineurin (Banerjee *et al.*, 2002). In addition, SOCS-1 is induced by LPS stimulation (Kinjyo *et al.*, 2002; Nakagawa *et al.*, 2002). As T cells over-respond to stimulation through TCR in SOCS-1-deficient mice (Egan *et al.*, 2003), and activation of NFAT is suppressed by binding Splenic Tyrosine Kinase (Syk) to SOCS-1 in HEK293 cells that express Syk and SOCS-1 (Matsuda *et al.*, 2000), we speculated that SOCS-1 might be involved in suppression of TCR-mediated activation of NFAT in cells pre-stimulated with flagellin. We showed that SOCS-1 is induced by flagellin stimulation (Figs 5A and 7C). Furthermore, inhibiting SOCS-1 expression by SOCS-1-specific siRNA reinstates TCR-mediated activation of NFAT in cells pre-stimulated with flagellin (Fig. 5C). These results collectively indicated that SOCS-1 inhibited TCR-mediated NFAT activation directly or indirectly in cells pre-treated with flagellin. In addition, TCR-mediated activation of NFAT was also inhibited in cells stably expressing SOCS-1 and pre-stimulated with flagellin (Fig. 6B). However, the level of inhibition of NFAT-mediated gene expression was lower in cells stably expressing SOCS-1 than that in control cells stimulated with flagellin. We speculated that NFAT-mediated gene expression might be inhibited in part by molecules other than SOCS-1. SOCS-1 is associated with activated Jak family kinases through its SH2 domain in interferon or cytokine receptor signalling cascades, and negatively regulates their signal transduction cascades (Elliott and Johnston, 2004). As co-stimulation by interferons or cytokines also augments TCR-mediated T cell activation (Romano *et al.*, 1996; Zella *et al.*, 2000), we cannot deny the possibility that SOCS-1 might inhibit action of interferons or cytokines, and thus negatively regulate T cell activation via TCR. However, we demonstrated that activation via TCR leads to a complex forming between SOCS-1 and Zap-70 upon induction of the TCR-mediated signalling cascade in Jurkat T cells (Fig. 6D). We also observed that the degrees of TCR-mediated NFAT activation in Jurkat T cells stably expressing SOCS-1 were significantly lower than those in control Jurkat T cells when they were cultured in the absence of serum overnight (data not shown). Thus, we speculated that SOCS-1 induced by flagellin stimulation directly inhibited TCR-mediated NFAT activation, probably by interfering with activation of Zap-70.

In this article, we report that pre-stimulation by flagellin inhibited TCR-mediated activation in T cells. Our study is important for understanding the molecular mechanisms of host defences mediated by T cells. Why are T cells that are stimulated with flagellin made refractory to TCR-mediated activation? We speculate that inhibition of TCR-mediated activation by flagellin might represent a host mechanism aimed at limiting inflammatory damage on activation of the immune system by flagellated bacteria because excessive inflammatory responses is potentially harmful to the host and may lead to microcirculatory dysfunction, causing tissue damage and septic shock. Thus,

our study may lead to the development of improved treatments for inflammatory responses to flagellated bacteria that may cause sepsis.

Experimental procedures

Purification of human primary T cells

Peripheral blood mononuclear cells (PBMC) were isolated from human healthy volunteers by a standard density gradient centrifugation on Ficoll-Paque (MP Biomedicals, OH), and T cells were subsequently purified by negative selection with magnetic beads according to the manufacturer's instructions (StemCell Technologies, Canada). Purity of T cells were about 95–97% measured by flow cytometry using Phycoerythrin (PE)-conjugated anti-CD3 mouse monoclonal Ab.

Cell line, reagents and antibodies

The human acute leukaemia T cell line, Jurkat E6.1 (American Type Culture Collection, Manassas, VA) and purified T cells were maintained in RPMI 1640 supplemented with 2 mM glutamine (Sigma, St Louis, MO), 100 units ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (ICN, Aurora, OH) and 10% fetal bovine serum (Sanko, Japan). *Salmonella muenchen* flagellin was purchased from Calbiochem (San Diego, CA). *Salmonella typhosa* LPS, phorbol myristate acetate (PMA), ionomycin calcium salt were purchased from Sigma. The following Abs were used in the experiments: anti-TLR5 goat Ab, c-Myc mouse monoclonal Ab (9E10), fluorescein isothiocyanate (FITC)-conjugated anti-goat donkey Ab, control goat IgG Ab and PE-conjugated mouse IgG Ab (Santa Cruz Biotechnology), anti-phosphotyrosine mouse monoclonal Ab (4G10; Upstate, Lake Placid, NY), anti-β-actin mouse monoclonal Ab (Abcam, Cambridge, UK), anti-phosphorylated JNK1/2 Ab and anti-phosphorylated p38 Ab (Cell Signalling Berverly, MA), anti-CD3 mouse monoclonal Ab and PE-conjugated anti-CD3 mouse monoclonal Ab (Pharmingen, San Diego, CA), horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Dako, Denmark), anti-SOCS-1 Ab (Zymed Laboratories, South San Francisco, CA).

Flow cytometric analysis

Flow cytometric analysis was performed essentially as described previously (Nagase *et al.*, 2000). Briefly, cells were incubated with anti-TLR5, followed by staining with FITC-conjugated anti-goat donkey Ab, or cells were stained with PE-conjugated anti-CD3 mouse monoclonal Ab. Expression of TLR5 or CD3 was analysed by using a BD LSR (BD biosciences, Franklin Lakes, NJ).

Establishing a stable cell line that expresses SOCS-1

To establish a cell line that stably expresses SOCS-1, 1×10^7 Jurkat T cells were transfected by electroporation with 50 μg of pcDNA3MycSOCS-1 construct (kindly provided by Dr A. Yoshimura), using a Gene Pulser II (Bio-Rad) set at 290 V, 970 μF. Cells that expressed SOCS-1 were selected by incubation in media containing 600 μg ml^{-1} G418 (Life Technologies, Gaithersburg, MD) and cloned by a standard limiting dilution method. Protein expression of SOCS-1 in cells was examined by Western blotting.

Transfection and dual luciferase reporter assay

Transfection and luciferase reporter assays were performed essentially as previously reported (Nakayama *et al.*, 2003). Briefly, 1×10^7 Jurkat T cells were co-transfected by electroporation with 30 μg of NFAT luciferase reporter plasmid (kindly provided by Drs B. Barbara and R.L. Wange) or 30 μg of pNF-κB luciferase reporter plasmid (Stratagene, La Jolla, CA), and 0.03 μg of *Renilla* luciferase reporter vector pRL-TK (Promega, Madison, WI). Twenty-four hours after transfection, cells were either left untreated or were incubated with anti-CD3 monoclonal Ab UCHT1, flagellin, LPS or PMA (30 ng ml^{-1}) plus ionomycin (1.5 μM). Six hours after stimulation, cells were lysed, and luciferase activities were measured by a dual luciferase reporter assay system (Promega, Madison, WI). Data were obtained by calculating the ratio of *Firefly* luciferase activity (experimental) and *Renilla* luciferase activity (control) and were expressed as the relative luciferase activities representing the mean ± SD of triplicate experiments. NFAT luciferase activities were reported as the percentage of those produced by stimulation with PMA and ionomycin.

Immunoprecipitation and immunoblotting

Immunoprecipitation and Western blotting were performed as previously described (Okugawa *et al.*, 2003). Briefly, cells were lysed in ice-cold NP-40 lysis buffer containing 1% NP-40, 25 mM Tris-HCl (pH 7.5), 150 mM sodium chloride, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM leupeptin and 1 mM phenylmethylsulphonyl fluoride. For immunoprecipitation studies, cell lysates were mixed with the indicated Abs for 1 h. Cell lysates were then mixed with protein G-coupled Sepharose beads and rotated for 1 h at 4°C. After the beads were washed three times with ice-cold NP-40 lysis buffer, the precipitated proteins were boiled for 5 min and eluted with sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer. For the precipitation of total-cell lysates, cells were lysed directly by the addition of SDS-PAGE sample buffer containing 2-mercaptoethanol.

Immunoprecipitated proteins and 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 Ab and the reactive bands were visualized with an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia Biotech, UK).

Constructions and transfection of siRNA

The constructions of siRNA molecules specific for SOCS-1 were evaluated by B-Bridge International (Sunnyvale, CA). The oligonucleotide sequences used in the experiments were as follows: 5′-CggAACTgCTTTTTCgCCCTT-3′. SOCS-1-specific and negative control RNAs were obtained from Dharmacon (CO). siRNAs specific for SOCS-1 or control RNAs were transfected into Jurkat T cells by electroporation.

Acknowledgements

We are grateful to M. Kataoka for preparation of the manuscripts. This work was supported in part by a grant-in-aid for scientific research to S.O. and Y.O. from the Japanese Ministry of Education, Culture, Sports, Science and Technology.

References

Aderem, A., and Ulevitch, R.J. (2000) Toll-like receptors in the induction of the innate immune response. *Nature* **406:** 782–787.

Alexander, W.S., Starr, R., Fenner, J.E., Scott, C.L., Handman, E., Sprigg, N.S., *et al.* (1999) SOCS1 is a critical inhibitor of interferon gamma signaling and prevents the potentially fatal neonatal actions of this cytokine. *Cell* **98:** 597–608.

Arbibe, L., Mira, J.P., Teusch, N., Kline, L., Guha, M., Mackman, N., *et al.* (2000) Toll-like receptor 2-mediated NF-kappa B activation requires a Rac1-dependent pathway. *Nat Immunol* **1:** 533–540.

Baetz, A., Frey, M., Heeg, K., and Dalpke, A.H. (2004) Suppressor of cytokine signaling (SOCS) proteins indirectly regulate toll-like receptor signaling in innate immune cells. *J Biol Chem* **279:** 54708–54715.

Banerjee, A., Banks, A.S., Nawijn, M.C., Chen, X.P., and Rothman, P.B. (2002) Cutting edge: suppressor of cytokine signaling 3 inhibits activation of NFATp. *J Immunol* **168:** 4277–4281.

Brightbill, H.D., and Modlin, R.L. (2000) Toll-like receptors: molecular mechanisms of the mammalian immune response. *Immunology* **101:** 1–10.

Caramalho, I., Lopes-Carvalho, T., Ostler, D., Zelenay, S., Haury, M., and Demengeot, J. (2003) Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. *J Exp Med* **197:** 403–411.

Caron, G., Duluc, D., Fremaux, I., Jeannin, P., David, C., Gascan, H., and Delneste, Y. (2005) Direct stimulation of human T cells via TLR5 and TLR7/8: flagellin and R-848 up-regulate proliferation and IFN-gamma production by memory CD4+ T cells. *J Immunol* **175:** 1551–1557.

Chen, L.Y., Zuraw, B.L., Zhao, M., Liu, F.T., Huang, S., and Pan, Z.K. (2003) Involvement of protein tyrosine kinase in Toll-like receptor 4-mediated NF-kappa B activation in human peripheral blood monocytes. *Am J Physiol Lung Cell Mol Physiol* **284:** L607–L613.

Ciacci-Woolwine, F., McDermott, P.F., and Mizel, S.B. (1999) Induction of cytokine synthesis by flagella from gram-negative bacteria may be dependent on the activation or differentiation state of human monocytes. *Infect Immun* **67:** 5176–5185.

Cookson, B.T., and Bevan, M.J. (1997) Identification of a natural T cell epitope presented by *Salmonella*-infected macrophages and recognized by T cells from orally immunized mice. *J Immunol* **158:** 4310–4319.

Crespo, A., Filla, M.B., Russell, S.W., and Murphy, W.J. (2000) Indirect induction of suppressor of cytokine signalling-1 in macrophages stimulated with bacterial lipopolysaccharide: partial role of autocrine/paracrine interferon-alpha/beta. *Biochem J* **349:** 99–104.

Crespo, A., Filla, M.B., and Murphy, W.J. (2002) Low responsiveness to IFN-gamma, after pretreatment of mouse macrophages with lipopolysaccharides, develops via diverse regulatory pathways. *Eur J Immunol* **32:** 710–719.

Didierlaurent, A., Ferrero, I., Otten, L.A., Dubois, B., Reinhardt, M., Carlsen, H., *et al.* (2004) Flagellin promotes myeloid differentiation factor 88-dependent development of Th2-type response. *J Immunol* **172:** 6922–6930.

Eaves-Pyles, T., Murthy, K., Liaudet, L., Virag, L., Ross, G., Soriano, F.G., *et al.* (2001a) Flagellin, a novel mediator of *Salmonella*-induced epithelial activation and systemic inflammation: I kappa B alpha degradation, induction of nitric oxide synthase, induction of proinflammatory mediators, and cardiovascular dysfunction. *J Immunol* **166:** 1248–1260.

Eaves-Pyles, T.D., Wong, H.R., Odoms, K., and Pyles, R.B. (2001b) *Salmonella* flagellin-dependent proinflammatory responses are localized to the conserved amino and carboxyl regions of the protein. *J Immunol* **167:** 7009–7016.

Egan, P.J., Lawlor, K.E., Alexander, W.S., and Wicks, I.P. (2003) Suppressor of cytokine signaling-1 regulates acute inflammatory arthritis and T cell activation. *J Clin Invest* **111:** 915–924.

Elliott, J., and Johnston, J.A. (2004) SOCS: role in inflammation, allergy and homeostasis. *Trends Immunol* **25:** 434–440.

Gewirtz, A.T., Navas, T.A., Lyons, S., Godowski, P.J., and Madara, J.L. (2001a) Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol* **167:** 1882–1885.

Gewirtz, A.T., Simon, P.O., Jr, Schmitt, C.K., Taylor, L.J., Hagedorn, C.H., O'Brien, A.D., *et al.* (2001b) *Salmonella typhimurium* translocates flagellin across intestinal epithelia, inducing a proinflammatory response. *J Clin Invest* **107:** 99–109.

Gingras, S., Parganas, E., de Pauw, A., Ihle, J.N., and Murray, P.J. (2004) Re-examination of the role of suppressor of cytokine signaling 1 (SOCS1) in the regulation of toll-like receptor signaling. *J Biol Chem* **279:** 54702–54707.

Gomez-Gomez, L., and Boller, T. (2000) FLS2: an LRR receptor-like kinase involved in the perception of the bacterial elicitor flagellin in *Arabidopsis*. *Mol Cell* **5:** 1003–1011.

Hayashi, F., Smith, K.D., Ozinsky, A., Hawn, T.R., Yi, E.C., Goodlett, D.R., *et al.* (2001) The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature* **410:** 1099–1103.

Kane, L.P., Lin, J., and Weiss, A. (2000) Signal transduction by the TCR for antigen. *Curr Opin Immunol* **12:** 242–249.

Khan, M.A., Kang, J., and Steiner, T.S. (2004) Enteroaggregative *Escherichia coli* flagellin-induced interleukin-8 secretion requires Toll-like receptor 5-dependent p38 MAP kinase activation. *Immunology* **112:** 651–660.

Kinjyo, I., Hanada, T., Inagaki-Ohara, K., Mori, H., Aki, D., Ohishi, M., *et al.* (2002) SOCS1/JAB is a negative regulator of LPS-induced macrophage activation. *Immunity* **17:** 583–591.

Kubo, M., Hanada, T., and Yoshimura, A. (2003) Suppressors of cytokine signaling and immunity. *Nat Immunol* **4:** 1169–1176.

Lodes, M.J., Cong, Y., Elson, C.O., Mohamath, R., Landers, C.J., Targan, S.R., *et al.* (2004) Bacterial flagellin is a dominant antigen in Crohn's disease. *J Clin Invest* **113:** 1296–1306.

McDermott, P.F., Ciacci-Woolwine, F., Snipes, J.A., and Mizel, S.B. (2000) High-affinity interaction between gram-

negative flagellin and a cell surface polypeptide results in human monocyte activation. *Infect Immun* **68:** 5525–5529.

McSorley, S.J., Cookson, B.T., and Jenkins, M.K. (2000) Characterization of CD4+ T cell responses during natural infection with *Salmonella typhimurium*. *J Immunol* **164:** 986–993.

McSorley, S.J., Ehst, B.D., Yu, Y., and Gewirtz, A.T. (2002a) Bacterial flagellin is an effective adjuvant for CD4+ T cells *in vivo*. *J Immunol* **169:** 3914–3919.

McSorley, S.J., Asch, S., Costalonga, M., Reinhardt, R.L., and Jenkins, M.K. (2002b) Tracking salmonella-specific CD4 T cells *in vivo* reveals a local mucosal response to a disseminated infection. *Immunity* **16:** 365–377.

Marine, J.C., Topham, D.J., McKay, C., Wang, D., Parganas, E., Stravopodis, D., *et al.* (1999) SOCS1 deficiency causes a lymphocyte-dependent perinatal lethality. *Cell* **98:** 609–616.

Matsuda, T., Yamamoto, T., Kishi, H., Yoshimura, A., and Muraguchi, A. (2000) SOCS-1 can suppress CD3zeta- and Syk-mediated NF-AT activation in a non-lymphoid cell line. *FEBS Lett* **472:** 235–240.

Matsuguchi, T., Takagi, K., Musikacharoen, T., and Yoshikai, Y. (2000) Gene expressions of lipopolysaccharide receptors, toll-like receptors 2 and 4, are differently regulated in mouse T lymphocytes. *Blood* **95:** 1378–1385.

Means, T.K., Golenbock, D.T., and Fenton, M.J. (2000) The biology of Toll-like receptors. *Cytokine Growth Factor Rev* **11:** 219–232.

Medzhitov, R., and Janeway, C., Jr (2000) Innate immune recognition: mechanisms and pathways. *Immunol Rev* **173:** 89–97.

Mizel, S.B., and Snipes, J.A. (2002) Gram-negative flagellin-induced self-tolerance is associated with a block in interleukin-1 receptor-associated kinase release from toll-like receptor 5. *J Biol Chem* **277:** 22414–22420.

Mokuno, Y., Matsuguchi, T., Takano, M., Nishimura, H., Washizu, J., Ogawa, T., *et al.* (2000) Expression of toll-like receptor 2 on gamma delta T cells bearing invariant V gamma 6/V delta 1 induced by *Escherichia coli* infection in mice. *J Immunol* **165:** 931–940.

Moors, M.A., Li, L., and Mizel, S.B. (2001) Activation of interleukin-1 receptor-associated kinase by gram-negative flagellin. *Infect Immun* **69:** 4424–4429.

Muzio, M., Ni, J., Feng, P., and Dixit, V.M. (1997) IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* **278:** 1612–1615.

Muzio, M., Natoli, G., Saccani, S., Levrero, M., and Mantovani, A. (1998) The human toll signaling pathway: divergence of nuclear factor kappaB and JNK/SAPK activation upstream of tumor necrosis factor receptor-associated factor 6 (TRAF6). *J Exp Med* **187:** 2097–2101.

Nagase, H., Miyamasu, M., Yamaguchi, M., Fujisawa, T., Ohta, K., Yamamoto, K., *et al.* (2000) Expression of CXCR4 in eosinophils: functional analyses and cytokine-mediated regulation. *J Immunol* **164:** 5935–5943.

Nakagawa, R., Naka, T., Tsutsui, H., Fujimoto, M., Kimura, A., Abe, T., *et al.* (2002) SOCS-1 participates in negative regulation of LPS responses. *Immunity* **17:** 677–687.

Nakayama, K., Ota, Y., Okugawa, S., Ise, N., Kitazawa, T., Tsukada, K., *et al.* (2003) Raf1 plays a pivotal role in lipopolysaccharide-induced activation of dendritic cells. *Biochem Biophys Res Commun* **308:** 353–360.

Ogushi, K., Wada, A., Niidome, T., Mori, N., Oishi, K., Nagatake, T., *et al.* (2001) *Salmonella enteritidis* FliC (flagella filament protein) induces human beta-defensin-2 mRNA production by Caco-2 cells. *J Biol Chem* **276:** 30521–30526.

Okugawa, S., Ota, Y., Kitazawa, T., Nakayama, K., Yanagimoto, S., Tsukada, K., *et al.* (2003) Janus kinase 2 is involved in lipopolysaccharide-induced activation of macrophages. *Am J Physiol Cell Physiol* **285:** C399–C408.

Romano, M.F., Lamberti, A., Petrella, A., Bisogni, R., Tassone, P.F., Formisano, S., *et al.* (1996) IL-10 inhibits nuclear factor-kappa B/Rel nuclear activity in CD3-stimulated human peripheral T lymphocytes. *J Immunol* **156:** 2119–2123.

Samakovlis, C., Asling, B., Boman, H.G., Gateff, E., and Hultmark, D. (1992) *In vitro* induction of cecropin genes – an immune response in a *Drosophila* blood cell line. *Biochem Biophys Res Commun* **188:** 1169–1175.

Sierro, F., Dubois, B., Coste, A., Kaiserlian, D., Kraehenbuhl, J.P., and Sirard, J.C. (2001) Flagellin stimulation of intestinal epithelial cells triggers CCL20-mediated migration of dendritic cells. *Proc Natl Acad Sci USA* **98:** 13722–13727.

Steiner, T.S., Nataro, J.P., Poteet-Smith, C.E., Smith, J.A., and Guerrant, R.L. (2000) Enteroaggregative *Escherichia coli* expresses a novel flagellin that causes IL-8 release from intestinal epithelial cells. *J Clin Invest* **105:** 1769–1777.

Winstanley, C., and Morgan, J.A. (1997) The bacterial flagellin gene as a biomarker for detection, population genetics and epidemiological analysis. *Microbiology* **143** (Pt 10)**:** 3071–3084.

Wyant, T.L., Tanner, M.K., and Sztein, M.B. (1999a) Potent immunoregulatory effects of *Salmonella typhi* flagella on antigenic stimulation of human peripheral blood mononuclear cells. *Infect Immun* **67:** 1338–1346.

Wyant, T.L., Tanner, M.K., and Sztein, M.B. (1999b) *Salmonella typhi* flagella are potent inducers of proinflammatory cytokine secretion by human monocytes. *Infect Immun* **67:** 3619–3624.

Yasukawa, H., Sasaki, A., and Yoshimura, A. (2000) Negative regulation of cytokine signaling pathways. *Annu Rev Immunol* **18:** 143–164.

Yu, Y., Zeng, H., Lyons, S., Carlson, A., Merlin, D., Neish, A.S., and Gewirtz, A.T. (2003) TLR5-mediated activation of p38 MAPK regulates epithelial IL-8 expression via post-transcriptional mechanism. *Am J Physiol Gastrointest Liver Physiol* **285:** G282–G290.

Zella, D., Romerio, F., Curreli, S., Secchiero, P., Cicala, C., Zagury, D., and Gallo, R.C. (2000) IFN-alpha 2b reduces IL-2 production and IL-2 receptor function in primary CD4+ T cells. *J Immunol* **164:** 2296–2302.

J Infect Chemother (2006) 12:227–232
DOI 10.1007/s10156-006-0460-0

REVIEW ARTICLE

Kazuhiko Koike

Antiviral treatment of hepatitis C: present status and future prospects

Received: June 7, 2006

Abstract Hepatitis C virus (HCV) infection is a major cause of chronic hepatitis. A substantial proportion of patients with chronic hepatitis C eventually develop hepatocellular carcinoma (HCC), which is one of the leading causes of death worldwide. Therefore, efficient antiviral treatments for HCV have long been needed. A recently developed combination therapy of pegylated interferon and ribavirin has dramatically improved the outcome of antiviral therapy for HCV infection. In genotype 1b HCV infection, 48 weeks of the combination therapy achieved eradication of the virus in 50% of patients, and in genotype 2 HCV infection, 24 weeks of the therapy resulted in viral eradication in 80%–90% of patients. By this eradication, an improvement in the hepatic fibrosis, an inhibition of HCC development, and an improvement in life expectancy were attained. Patients who did not respond to the combination therapy may be treated with long-term interferon monotherapy, which is not intended to eradicate HCV, but will lower the serum alanine aminotransferase (ALT) level. Thus, the treatment for HCV infection has progressed significantly, but therapies with new modalities, such as inhibitors of viral protease or RNA polymerase, are still being awaited.

Key words Hepatitis C · Interferon · Treatment

Introduction

Hepatitis viruses mainly infect the liver, causing hepatic diseases in humans. To date, five types of hepatitis virus, B, A, D, E, and C, have been found, in this order, and subjected to medical treatment. Hepatitis C virus (HCV) and hepatitis B virus (HBV) infections can develop into persistence, while hepatitis A virus and hepatitis E virus cause only transient infection. In Japan, chronic hepatitis caused by HCV infection currently poses the greatest problem because of the large number of patients affected and the high rate of patient mortality from complications, particularly hepatocellular carcinoma (HCC).[1]

K. Koike (✉)
Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan
Tel. +81-3-5800-8800; Fax +81-3-5800-8799
e-mail: kkoike-tky@umin.ac.jp

Chronic hepatitis C

It is estimated that there are approximately 170 million HCV carriers or patients with persistent HCV worldwide, and approximately 1.8 million patients in Japan. HCV infection occurs when blood contaminated with HCV enters the body directly. The infection routes include blood transfusion with HCV-contaminated blood products obtained a long time ago, sharing of needles among drug abusers, and the use of inappropriately disinfected acupuncture needles and tattoo needles, among others.[2] People undergoing folk remedies and hair-removal treatments should also be regarded as susceptible to HCV infection if these are invasive practices and nondisposable devices are used.

The problem with HCV infection resides in the very high rate of general HCV infections which are becoming chronic (approximately 70%). However, in the case of HCV infection via blood transfusion, the rate of reaching chronicity has been reported to reach 80%, probably because of a high virus load.

Virus markers of HCV infection required for the treatment of hepatitis

Some virus markers of HCV infection are available, as described below. Figure 1 shows a progress observation flowchart for anti-HCV antibody-positive patients obtained using these virus markers.

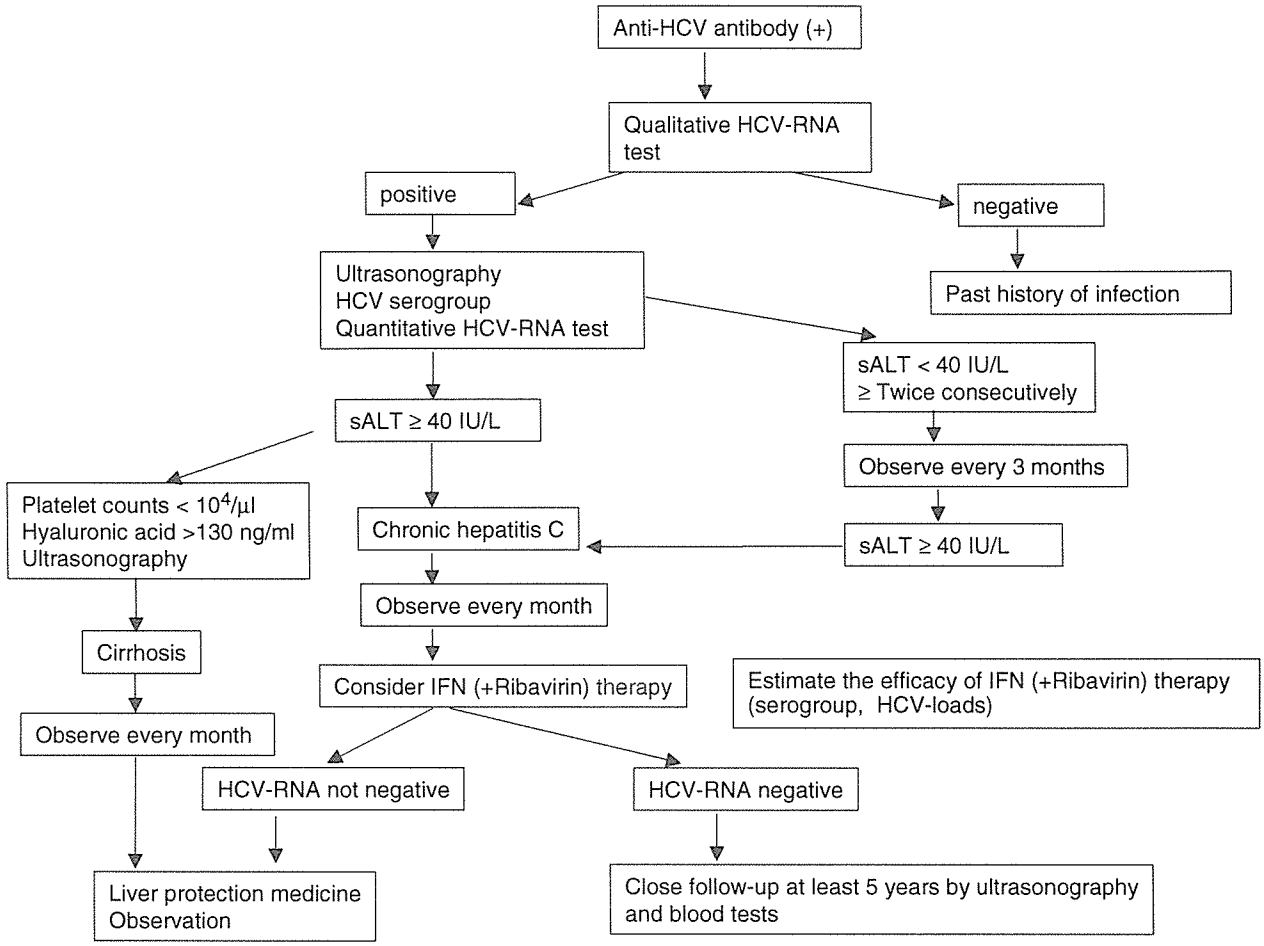

Fig. 1. Progress observation flow chart for anti-HCV antibody-positive patients. *HCV*, hepatitis C virus; *ALT*, alanine aminotransferase; *IFN*, interferon

Anti-HCV antibody

Anti-HCV antibody of low titer is frequently detected using sensitive HCV kits currently available in Japan. Patients with low anti-HCV antibody titers mostly have a history of remote HCV infection, while those with high titers generally have an ongoing infection. Hence, patients who test positive for anti-HCV antibodies are not necessarily infected with HCV at present. When the antibody titer is found to be low, a history of infection (i.e., currently cured) should be suspected. To verify this, a sensitive qualitative HCV-RNA measurement is required (reverse transcriptase–polymerase chain reaction (RT-PCR) method).

Meanwhile, it should be noted that during the early stage of HCV infection (2–3 months from the initial HCV exposure), patients do not test positive for anti-HCV antibody (window period).

HCV-RNA

To confirm the presence of HCV, we use an HCV-RNA assay by RT-PCR. There are two types of RT-PCR assay, a qualitative one and a quantitative one. However, the latter has a relatively low sensitivity. Therefore, the qualitative RT-PCR assay PCR is used to monitor the presence or absence of HCV, and hence the efficiency of an antiviral drug. For an estimation of the efficacy of antiviral treatment with interferon (IFN), a quantitative RT-PCR assay must be used.

Genotypes and serogroups of HCV

Many genotypes of HCV have been identified (i.e., there are HCV groups whose gene or genomic sequences differ to some extent). HCV genotypes are clinically important because the efficacy of IFN therapy varies depending on the HCV genotype. In Japan, the majority of HCV patients have HCV genotypes 1 or 2. Because the HCV genotype is determined on the basis of restriction fragment length polymorphism (RFLP) by PCR assay, the determination procedure is somewhat complicated. In order to determine the responsiveness of patients with chronic hepatitis C to IFN therapy easily (rapidly and accurately), serogroup (SG) identification by enzyme immunoassay is useful.[3] Patients

are classified as SG-1 (corresponding to HCV genotype 1) or SG-2 (corresponding to HCV genotype 2). Many patients classified as SG-1 are resistant to IFN, whereas many patients classified as SG-2 are generally responsive to IFN therapy.

Natural course of HCV infection

HCV patients commonly develop "acute hepatitis" 2 or 3 months after the initial exposure. However, many patients are unaware of this development because they have minor subjective symptoms and hardly exhibit jaundice. About 20% to 30% of patients exhibiting acute hepatitis recover spontaneously from the disease, but acute hepatitis develops into chronic hepatitis in the remaining 70% to 80% of patients (hepatitis persisting for more than 6 months is defined as chronic hepatitis). In general, these patients enter an "inactive phase" of hepatitis C, which persists for more than 10–15 years. The serum alanine aminotransferase (ALT) level, which indicates the extent of hepatocytic damage, is within the normal range during the inactive phase, but viral replication continues even during this period (Fig. 2).

Chronic hepatitis C frequently enters the "active phase" after an inactive phase of 10–15 years; however, this period varies greatly depending on the individual. In the active phase, the serum ALT level becomes approximately 2–3 times higher than the normal level. The problem with chronic hepatitis C is that it does not resolve spontaneously once it enters the active phase. If chronic hepatitis is left untreated, the risk of progression to cirrhosis increases without the patient realizing it. Thus, hepatitis C is characterized by its gradual but steady progression.[4]

With the progression to cirrhosis, there is an increasing risk of developing HCC. This risk has been reported to have an annual rate of 5% to 7%.[5] Ideally, HCV-infected patients should have the disease diagnosed during the inactive phase of chronic hepatitis so that, upon transition to the active phase, the patients can start receiving antiviral therapy for HCV.

Treatment of HCV infection

HCV infection is treated using mainly IFN preparations. These IFN preparations are outlined below in their order of development.

IFN monotherapy

IFN monotherapy was first introduced for the treatment of chronic hepatitis C. In Japan, the treatment of chronic hepatitis C generally starts with the daily administration of 6–10 million units of IFN for 2–4 weeks, followed by administration three times weekly for 6 months. In Europe and the USA, 3 million units of IFN are administered three times weekly from the start, and this is continued for a year. The efficacy of the therapy is evaluated after 6 months of IFN treatment. If an HCV-RNA test is negative by a qualitative RT-PCR assay at this time, it indicates that the patient obtained a sustained virological response (SVR) and is considered to be practically free of HCV.

IFN monotherapy had conventionally been used for non-A/non-B hepatitis from around 1985, prior to the discovery of HCV. A nationwide survey carried out by a research group supported by the former Ministry of Health and Welfare in 1995 showed that the overall SVR rate following IFN monotherapy for chronic hepatitis C (the administration of 6–10 million units per day) for 6 months was approximately 30%. SVR rates at facilities across Japan were nearly equal to this value. However, among patients with HCV genotype 1, who accounted for approximately 70% of all Japanese patients infected with HCV, and particularly those with a high viral load (defined as HCV-RNA >100 KIU/ml in Japan), a SVR was obtained in only 2% to 7% of cases; i.e., the efficacy of treatment by IFN

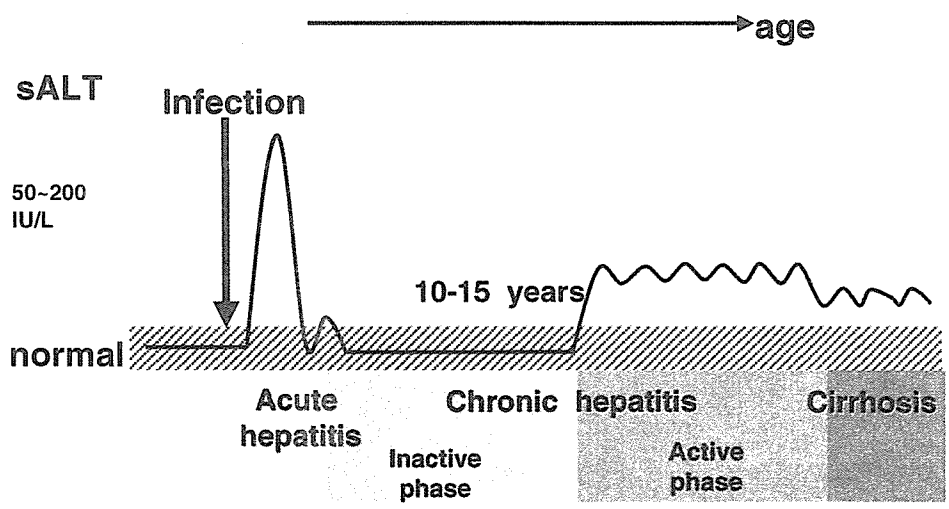

Fig. 2. Natural course of HCV-infected patients. Approximately 70% of acutely HCV-infected people develop persistent infection. After 10–15 years of the inactive phase, most chronic hepatitis C patients move into the active phase. One-third of chronic hepatitis C patients are assumed to develop cirrhosis. *sALT*, serum alanine aminotransferase

monotherapy was low. These patients with HCV genotype 1 at a high viral load have what is called "intractable hepatitis C."

IFN therapy in combination with ribavirin

IFN is also administered in combination with ribavirin, an antiviral drug. In Japan, the use of ribavirin was approved in December 2001. Ribavirin (600–800 mg daily, divided into two doses) is taken orally throughout the period of IFN injections. Ribavirin is a synthesized nucleic acid derivative and, when administered in combination with IFN, shows an increased antiviral activity.

In clinical studies of IFN therapy in combination with ribavirin conducted in Japan, a SVR rate of approximately 20% was obtained even in patients with HCV genotype 1 at a high viral load, i.e., "intractable hepatitis C," and who were less responsive to IFN monotherapy. Because patients on IFN monotherapy used as the control showed a SVR rate of only 2.3%, the concomitant use of ribavirin contributed to an approximately 10-fold increase in antiviral activity.[6]

The efficacy of IFN therapy in combination with ribavirin after its inclusion in the health insurance program is very similar to that found in a clinical study in Japan. However, the adverse effects of this combinational therapy have generally been more severe than those observed during the clinical study period. The drop-out rates of patients who could not complete the combinational therapy were as high as 15%–20%, and this led to a decrease in SVR rate calculated on intention-to-treat (ITT). In other words, the number of patients who dropped out of the treatment is added to the denominator. Adverse drug reactions that reduce the quality of life (QOL), such as hemolytic anemia, severe malaise, anorexia, and taste disorders, are frequently observed, particularly in many elderly patients. Indications for IFN therapy in combination with ribavirin should be considered carefully for patients aged 65 years or older.

Long-term interferon therapy

In cases of long-term IFN therapy, IFN is administered two or three times a week for a period of 2 years or more. The purpose of this therapy is not the eradication of HCV, but the normalization of serum ALT levels and eventually the suppression of HCC development. This is a promising therapy for patients who cannot be treated with ribavirin because of its adverse effects, or for those who were not able to continue with the combination therapy of IFN and ribavirin.

PEG–IFN therapy in combination with ribavirin

PEG–IFN is an interferon molecule covalently bonded to polyethylene glycol (PEG), which shows a sustained release. PEG–IFN characteristically requires subcutaneous administration only once weekly, as compared with the conventional type of IFN which requires administration three times a week. PEG–IFN therapy alone has a higher efficacy than the conventional IFN monotherapy, but it has been demonstrated that PEG–IFN therapy used in combination with ribavirin shows an even higher efficacy[7,8] (Fig. 3).

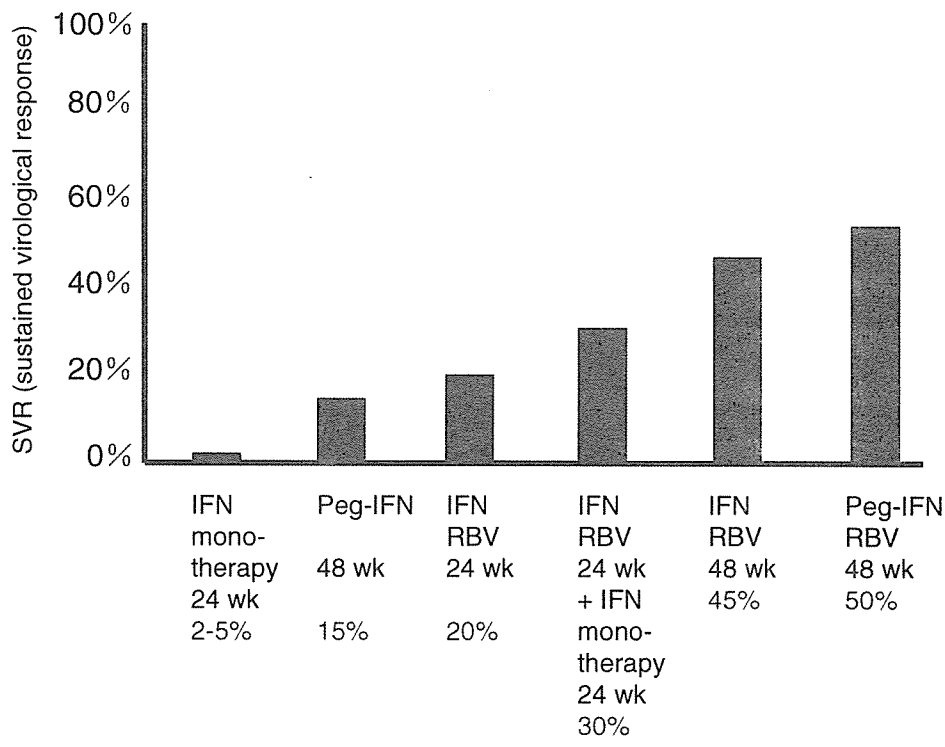

Fig. 3. Changes in anti-HCV therapy, including interferon for intractable (genotype 1b, high viral loads) chronic hepatitis C patients. After the introduction of IFN monotherapy for chronic hepatitis C, the efficacy of IFN therapy has gradually increased with the addition of ribavirin, the introduction of pegylated IFN, and an extension of the duration of therapy. *IFN*, interferon; *RBV*, ribavirin

PEG–IFN therapy in combination with ribavirin is expected to increase the SVR rate to approximately 50% even in patients infected with HCV genotype 1 at a high viral load, and to approximately 60% in all patients infected with HCV. The efficacy in those infected with genotype 2 HCV reaches 80%–90%. In Japan, treatment with PEG–IFN α-2a (Pegasys) alone was approved in December 2003. The combined use of PEG–IFN α-2b (PegIntron) and ribavirin (Rebetol) was also approved in December 2004. These treatments with PEG–IFN are generally administered for 48 consecutive weeks. Continuation of the treatments for 48 consecutive weeks is important, although it may be necessary to decrease the dose owing to adverse drug effects.

The adverse effects of PEG–IFN therapy in combination with ribavirin are almost the same as those of conventional IFN therapy. However, such adverse effects are generally minor (for example, fever), and the therapy requires administration only once per week, thereby improving the patient's QOL. Because there is the possibility of drug accumulation in the body and an associated exacerbation of adverse effects owing to the sustained-release formulation, very careful observation of patients is required. There have been reports of other problematic adverse effects of this combinational therapy compared with those of the conventional IFN preparation, e.g., decreased counts of leukocytes, and particularly of neutrophils. Some patients exhibit severe thrombocytopenia. It is mandatory to confirm neutrophil count immediately before every administration.

It is currently specified that PEG–IFN therapy used in combination with ribavirin is the best choice in the treatment of intractable hepatitis C of genotype 1 at a high viral load. This combinational therapy is thus administered first. It has recently been suggested that an extended administration period of 72 weeks for PEG–IFN therapy in combination with ribavirin proves effective in patients who are slow in showing a SVR.

Efficacy of antiviral therapy and its effect on patient prognosis

The following points have been reported in the literature: in patients in whom HCV was eradicated mainly by IFN monotherapy, hepatic fibrosis is improved,[9] the development of HCC is inhibited,[10] and life expectancy is also improved.[11] It has thus been indicated that if the eradication of HCV can be achieved, chronic hepatitis C prognosis is improved. It has also been reported that in patients in whom serum ALT level was normalized (even if this was transient), despite the failure to eradicate HCV (cases referred to as a biochemical response (BR)), the development of HCC was delayed in the short term. However, because no improvement in fibrosis was observed, it will probably be impossible in the long term to block the development of HCC. It has also been demonstrated that when curative treatment is carried out even after the development of HCC, subsequent IFN-based treatment could inhibit the recurrence of HCC.

Treatment of hepatitis C patients who do not respond sufficiently to IFN

Liver-protection therapy

Liver-protection therapy aims to delay the progression of chronic hepatitis by controlling inflammation in patients in whom HCV could not be eradicated. An ursodeoxycholic acid preparation (Urso) and a glycyrrhizin preparation (Stronger Neo Minophagen C) are used in combination as a liver-protection therapy. These drugs inhibit hepatic inflammation and decrease serum ALT level, but they do not decrease HCV load. It was reported that Stronger Neo Minophagen C delays the progression of chronic hepatitis and the onset of HCC.[12] The ursodeoxycholic acid preparation decreases serum ALT level, but its action of delaying the progression of chronic hepatitis has not yet been verified.

Hepatitis C generally progresses slowly and is less likely to aggravate rapidly, unlike hepatitis B, which may aggravate very rapidly, and progresses steadily. Liver-protection therapy, which retards the progression of the disease by controlling inflammation, can therefore be considered significant in hepatitis C. This therapy is applied mainly when it is impossible to use IFN due to its adverse effects, or when patients do not respond sufficiently to IFN therapy, including in combination with ribavirin. Liver-protection therapy is also administered as a temporary measure until a therapy in combination with IFN is started.

Phlebotomy

Iron deficiency leads to a decrease in serum ALT level, and its use as a therapy for chronic hepatitis C has begun to be appreciated. This is based on the observation that reactive oxygen species (ROS) production increases in hepatitis C patients, which leads to the development of liver disease and eventually HCC. Because intrahepatic iron plays an important role in ROS production (Fenton reaction), phlebotomy is designed to suppress ROS production by inducing intrahepatic iron deficiency. In fact, decreasing the serum ferritin level (an indicator of iron store) to 10 ng/ml or lower leads to a significant decrease in serum ALT level.[13] This is a promising therapy for patients who do not respond sufficiently to IFN therapy, or who are unable to receive it and do not respond to the above-mentioned liver-protection therapy either.

Conclusions

An overview of the current status of research on the progression of chronic hepatitis C and the treatment methods available has been presented and discussed in terms of the effects and limits of these methods. The early detection of HCV infection makes it possible to apply antiviral therapy at the appropriate time. It is particularly worth noting that

it has become possible for antiviral therapies to eradicate viruses in a majority of HCV patients, and to suppress and control the progression of HCV infection (or acute hepatitis C) to chronic hepatitis and subsequently to HCC. However, the limits of the current IFN-based therapies have also become evident. Specific antiviral drugs targeting HCV enzymes (including viral proteases, helicase, and RNA polymerase) have recently been developed. The development of one antiviral drug has advanced to phase II clinical trials as of 2006.

References

1. Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. Proc Natl Acad Sci USA 1990;87:6547–9.
2. Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. Hepatology 1990;12:671–5.
3. Tanaka T, Tsukiyama-Kohara K, Yamaguchi K, Yagi S, Tanaka S, Hasegawa A, et al. Significance of specific antibody assay for genotyping of hepatitis C virus. Hepatology 1994;19:1347–53.
4. Koike K. Hepatitis viruses update. Intern Med 2001;40:173–5.
5. Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. J Hepatol 1998;28:930–8.
6. Tsubota A, Arase Y, Suzuki F, Suzuki Y, Akuta N, Hosaka T, et al. High-dose interferon alpha-2b induction therapy in combination with ribavirin for Japanese patients infected with hepatitis C virus genotype 1b with a high baseline viral load. J Gastroenterol 2004;39:155–61.
7. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–82.
8. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. Lancet 2001;358:958–65.
9. Sobesky R, Mathurin P, Charlotte F, Moussalli J, Olivi M, Vidaud M, et al. Modeling the impact of interferon alfa treatment on liver fibrosis progression in chronic hepatitis C: a dynamic view. The Multivirc Group. Gastroenterology 1999;116:3783–6.
10. Nishiguchi S, Kuroki T, Nakatani S, Morimoto H, Takeda T, Nakajima S, et al. Randomized trial of effects of interferon-alpha on incidence of hepatocellular carcinoma in chronic active hepatitis C with cirrhosis. Lancet 1995;346:1051–5.
11. Okanoue T, Itoh Y, Minami M, Sakamoto S, Yasui K, Sakamoto M, et al. Interferon therapy lowers the rate of progression to hepatocellular carcinoma in chronic hepatitis C, but not significantly in an advanced stage: a retrospective study in 1148 patients. Viral Hepatitis Therapy Study Group. J Hepatol 1999;30:653–9.
12. Arase Y, Ikeda K, Murashima N, Chayama K, Tsubota A, Koida I, et al. The long-term efficacy of glycyrrhizin in chronic hepatitis C patients. Cancer 1997;79:1494–500.
13. Kato J, Kobune M, Nakamura T, Kuroiwa G, Takada K, Takimoto R, et al. Normalization of elevated hepatic 8-hydroxy-2′-deoxyguanosine levels in chronic hepatitis C patients by phlebotomy and low iron diet. Cancer Res 2001;61:8697–702.

J Gastroenterol 2006; 41:981–986
DOI 10.1007/s00535-006-1888-9

Journal of Gastroenterology

Molecular epidemiology of hepatitis A virus in metropolitan areas in Japan

Hideaki Takahashi[1], Hiroshi Yotsuyanagi[1,3], Kiyomi Yasuda[4], Tomohiko Koibuchi[5], Michihiro Suzuki[1], Tomohiro Kato[2], Tetsuya Nakamura[5], Aikichi Iwamoto[5], Kusuki Nishioka[2], Shiro Iino[1,4], Kazuhiko Koike[5], and Fumio Itoh[1]

[1]Department of Internal Medicine, Division of Gastroenterology and Hepatology, Institute of Medical Science, St. Marianna University, Kawasaki, Japan
[2]Division of Molecular Immunology, Institute of Medical Science, St. Marianna University, Kawasaki, Japan
[3]Department of Infectious Diseases, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan
[4]Center for Liver Diseases, Seizankai Kiyokawa Hospital, Tokyo, Japan
[5]Department of Internal Medicine, Division of Infectious Diseases and Immunology, Institute of Medical Science, University of Tokyo, Tokyo, Japan

Background. Transmission routes of hepatitis A virus (HAV) in Japan have changed. The present study investigated changes of transmission routes in relation to genetic drift. ***Methods.*** All 60 patients who were admitted between 1993 and 2003 with a diagnosis of hepatitis A were retrospectively analyzed. Nucleotide sequences of the VP1/2A region of the HAV recovered from their sera were determined. ***Results.*** The suspected transmission routes were household contact, 19 (31%); food or waterborne, 16 (27%); homosexual activity, 11 (18%); international travel, 4 (7%): and unknown 10. (17%). The 11 patients presumably infected through homosexual activity were found exclusively in 1998 and 1999. The proportion of patients exposed through homosexual behavior and household contact was higher in those 2 years than in other years. Nucleotide sequences could be determined for 58 patients. Fifty-seven of the 58 sequences belonged to genotype IA HAV, with less than 10% nucleotide diversity. Of the 27 sequences isolated during 1998 and 1999, 25 had an identical nucleotide sequence regardless of the suspected transmission route. In contrast, sequences obtained in the other years differed from one another. A phylogenetic tree constructed from sequences recovered from patients without a history of travel abroad showed several clusters. ***Conclusions.*** Our results suggest that (1) HAV acquired through homosexual activity may be transmitted to nonhomosexual individuals; (2) hepatitis A in metropolitan areas in Japan is caused mainly by sporadic infection with genotype IA HAV; and (3) several subtypes of genotype IA HAV are endemic in Japan.

Key words: hepatitis A virus, epidemiology, transmission route, sexuality, Japan

Received: July 10, 2006 / August 3, 2006
Reprint requests to: H. Yotsuyanagi
None of the authors has a potential conflict of interest.

Introduction

Hepatitis A virus (HAV) is one of the major causes of viral hepatitis and a worldwide problem. The annual incidence of hepatitis A is 1.5 million cases of clinical disease, and the true incidence, including subclinical disease, may be much higher.[1] Fulminant hepatic failure is a complication for some patients with clinical disease.[2] Therefore, preventing the spread of HAV is an important issue.

Good sanitation and a sterilized water supply are essential for the prevention of hepatitis A. In developing countries with a high incidence of hepatitis A, the main transmission route of hepatitis A is the fecal–oral route caused by poor sanitation, which increases the chance of ingesting contaminated food or water.[2,3] In contrast, in developed countries with good sanitation and a sterilized water supply, such as North America, Western Europe, Australia, and New Zealand, the incidence of hepatitis A is low and transmission is caused by personal contact with an infected person, homosexual activity, or transfusion of contaminated blood products, in addition to ingestion of contaminated food or water.[2–5]

The incidence of hepatitis A in Japan has markedly decreased recently. National surveillance of HAV in Japan has shown that more than 90% of people over 65 but fewer than 10% of people under 34 are positive for anti-HAV.[6] The difference can probably be attributed to changes in sanitation. If this hypothesis is true, then the transmission route of HAV in Japan may have changed with time. Studying changes in HAV transmission routes in Japan may therefore elucidate the influence of sanitation on transmission routes.

Molecular epidemiological approaches may also be useful for studying transmission routes. Studies from European countries have shown that several clusters of viral strains from various genotypes prevail in those countries.[7,8] The heterogeneity of isolated strains

suggests multiple transmission routes. Information on transmission routes is, however, not available in these reports. Sequential molecular epidemiological studies linked to transmission routes may elucidate native strains in Japan and provide new information for the control of this disease.

The aim of this study was to understand both clinical and molecular epidemiology of HAV infection in Japan.

Methods

Patients

Sixty patients admitted to our institutions between 1993 and 2003 who were diagnosed with hepatitis A were analyzed retrospectively. The patients comprised 39 men (65%) and 21 women (35%), and their median age was 34.0 years (range, 22–55 years). The diagnosis of hepatitis A was based on a high titer serum IgM anti-HA level with acute liver injury. Coinfection with hepatitis B virus, hepatitis C virus, or other hepatotropic viruses was excluded by serological testing. Serum samples were available from all patients on admission. Fifty-eight of the 60 samples were positive for HAV RNA by reverse transcription (RT)-nested polymerase chain reaction (PCR) with the protocol outlined below. None of the 58 patients had fulminant hepatic failure. Intrahepatic cholestasis was a complication in one patient. The other 57 patients underwent a noncomplicated and self-limited clinical course.

Informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

Detection of hepatitis A viral RNA in serum

RNA was extracted from sera using the acid guanidinium-phenol-chloroform method.[9] In brief, 100 µl of sample was mixed with 300 µl of solution D (guanidinium solution), 60 µl of chloroform, and 40 µl of NaOAc (pH 5.2) and precipitated with 1 ml of ethanol. The RNA pellet was washed twice with 70% ethanol and dissolved in 25 µl of RNase-free distilled water.

For reverse transcription, 1 µl of RNA solution, extracted from 100 µl of sera using the acid guanidinium-phenol-chloroform method and dissolved in 25 µl of RNase-free distilled water, was heat-denatured at 68°C for 10 min. It was chilled rapidly on ice and mixed with 4 µl of 1.5 mM $MgCl_2$ solution, 2 µl of 10× RNA PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 8.5 µl of RNase-free distilled H_2O, 2 µl of dNTP mixture (10 mM dATP, dCTP, dGTP, dTTP), 1 µl of random 9-mers (5′-NNNNNNNNN-3′), 0.5 µl of RNase inhibitor (Takara-Shuzo, Kyoto, Japan), and 1 µl of reverse transcriptase (Takara-Shuzo). After incubation at 30°C for 10 min, reverse transcription reaction was carried out at 42°C for 30 min, followed by inactivation at 95°C for 5 min.

In the first PCR, 5 µl of the 20 µl cDNA solution was used. The first PCR was performed in 50 µl of reaction mixture containing 1.0 µM each of outer sense primer (5′-GGTTTCTATTCAGATTGCAAATTA-3′ nt. 2891–2914) and antisense primer (5′-AGTAAAAACT CCAGCATCCATTTC-3′ nt. 3398–3375), 200 µM of each dNTP, 5 µl of 10× PCR buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl; 15 mM $MgCl_2$; 0.01% (w/v) gelatin), and 2.5 U of *Ex Taq* polymerase (Takara) with proofreading activity. The amplification conditions were 94°C for 16 min followed by 40 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min.

One microliter of the first PCR product was used for the second PCR. The reaction mixture contained 1.0 µM each of inner sense primer (5′-TTGCAAATTACAAT CATTCTG-3′ nt. 2905–2925) and inner antisense primer (5′-TTCAAGAGTCCACACACTTCT-3′ nt. 3377–3367), 5 µl of 10× PCR buffer, 35 µl of RNase-free dH_2O, 5 µl of dNTP mixture (2 mM dATP, dCTP, dGTP, dTTP), and 0.5 µl of amplitaq gold (Roche Diagnostics, Branchburg, NJ, USA). The amplification conditions for the second PCR were the same as those of the first PCR. The second PCR products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized by UV transillumination. Standard precautions to avoid contamination were taken during PCR, with a negative control serum included in each run.

Sequencing of PCR products

Amplification products were purified on Wizard PCR Preps DNA purification resin (Promega, Madison, WI, USA), and sequenced bidirectionally with the Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, CA, USA) using the above PCR primers. Sequencing was performed on an automated DNA sequencer ABI 377 (PE Applied Biosystems).

The nucleotide sequences of HAV isolates from the patients were compared with those of seven reference HAV strains retrieved from the DDBJ/EMBL/GenBank databases, representing each of the seven major genotypes (I–VII). Phylogenetic trees were constructed with the Mega program version 2.1 using the Kimura two-parameter matrix and the neighbor-joining method.[10] To confirm the reliability of the phylogenetic tree analysis, bootstrap resampling and reconstruction were carried out 500 times.

Statistical analysis

Data were analyzed by a χ-squared test. P values less than 0.05 were regarded as statistically significant.

Results

Transmission routes

Table 1 shows the numbers of patients and transmission routes. An epidemic of hepatitis A among homosexuals was reported in metropolitan areas in Japan between 1998 and 1999.[11,12] Our results showed that more patients were admitted during that period. The increase was caused not only by patients involved in homosexual activity but also by individuals without that risk factor.

The suspected transmission routes for the patients were as follows: household contact, 19 (31%); food or water, 16 (27%); homosexual activity, 11 (18%); international travel, 4 (7%); and unknown, 10 (17%). In 1998 and 1999, the suspected transmission routes were as follows: household contact, 13 (45%); homosexual activity, 11 (38%); food or water, 1 (3%); international travel, 1 (3%); and unknown, 3 (11%). The proportion of cases associated with homosexuality and household contact was higher in those 2 years than in the other years (homosexuality-associated, $P = 0.0006$; household contact-associated, $P = 0.034$). Figure 1 shows the time of onset for all patients over the 2 years. The times of onset for those 2 years were from July 1998 to July 1999 for homosexual patients and from February 1998 to September 1999 for nonhomosexual patients. After excluding two patients with different sequences, the onset of nonhomosexual patients varied from August 1998 to September 1999. The periods of transmission were similar between homosexual and nonhomosexual groups.

Sequence analysis of HAV RNA

The sequence between nucleotides 3024 and 3191 of the VP1/2A region was determined for the 58 patients. Many nucleotide sequences were closely related to HAS-15, a representative HAV genotype IA strain. Twenty-five of the 27 viral sequences recovered during 1998–1999 were identical. This sequence is identical to IMSTU, which is prevalent among homosexuals in metropolitan areas.[12] As shown in Table 2, all sequences except one showed more than 90% identity with the reference sequences.

We then performed a phylogenetic analysis of the region between nucleotides 3024 and 3191 and classified the virus strains (Fig. 2). Bootstrap analysis to evaluate the statistical reliability of the phylogenetic tree revealed 500/500 (100%) reliability. All strains belonged to genotype IA, except for one that belonged to genotype IIIA. The patient harboring the genotype IIIA virus had a history of travel to Africa 1 month before admission.

Table 1. Numbers of patients and routes of transmission

	1993–1997	1998–1999	2000–2004	Total
Household contact	4 (29%)	13 (45%)*	2 (12%)	19 (31%)
Food or waterborne	4 (29%)	1 (3%)	11 (64%)	16 (27%)
Homosexual activity	0	11 (38%)**	0	11 (18%)
Foreign travel	1 (7%)	1 (3%)	2 (12%)	4 (7%)
Unknown	5 (35%)	3 (11%)	2 (12%)	10 (17%)
Total	14	29	17	60

* $P = 0.034$, ** $P = 0.0006$; χ-squared test

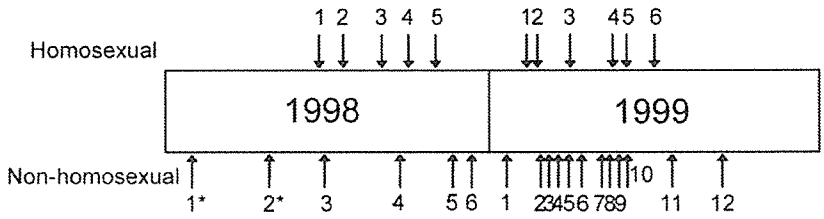

Fig. 1. Time of first visit of patients with hepatitis A in 1998 and 1999. *Arrows* in the upper part show the times of visit of homosexual patients. *Arrows* in the lower part show those of nonhomosexual patients. All except two (cases 1 and 2) had the same sequences. The period of transmission was similar for homosexual and nonhomosexual groups. * shows the sequences that were different

Table 2. Homology with recovered sequences and representative strains (HAS-15 and IMSTU)

Homology with IMSTU			Homology with HAS-15		
	Nucleic acid	Amino acid		Nucleic acid	Amino acid
HAS-15	95.7	98.2	IMSTU	95.7	98.2
1993	97.6	100	1993	94.4	98.2
1994	77.1	89.1	1994	73.8	87.3
1995	95.7–98.8	98.2–100	1995	93.8–94.4	96.4–98.2
1996	97.6	100	1996	94.4	98.0
1997	95.7–98.8	96.4–100	1997	92.4–94.4	94.5–98.2
1998	95.7–100	98.2–100	1998	93.7–95.7	96.4–98.2
1999	100	100	1999	95.7	98.2
2000	90.4–95.7	92.7–98.2	2000	86.8–92.4	90.9–96.4
2001	93.7–97.6	96.4	2001	92.4–94.4	94.5–98.2
2002	93.1–96.3	100	2002	92.4–96.3	94.5–98.2
2003	96.7	100	2003	95.0	98.2

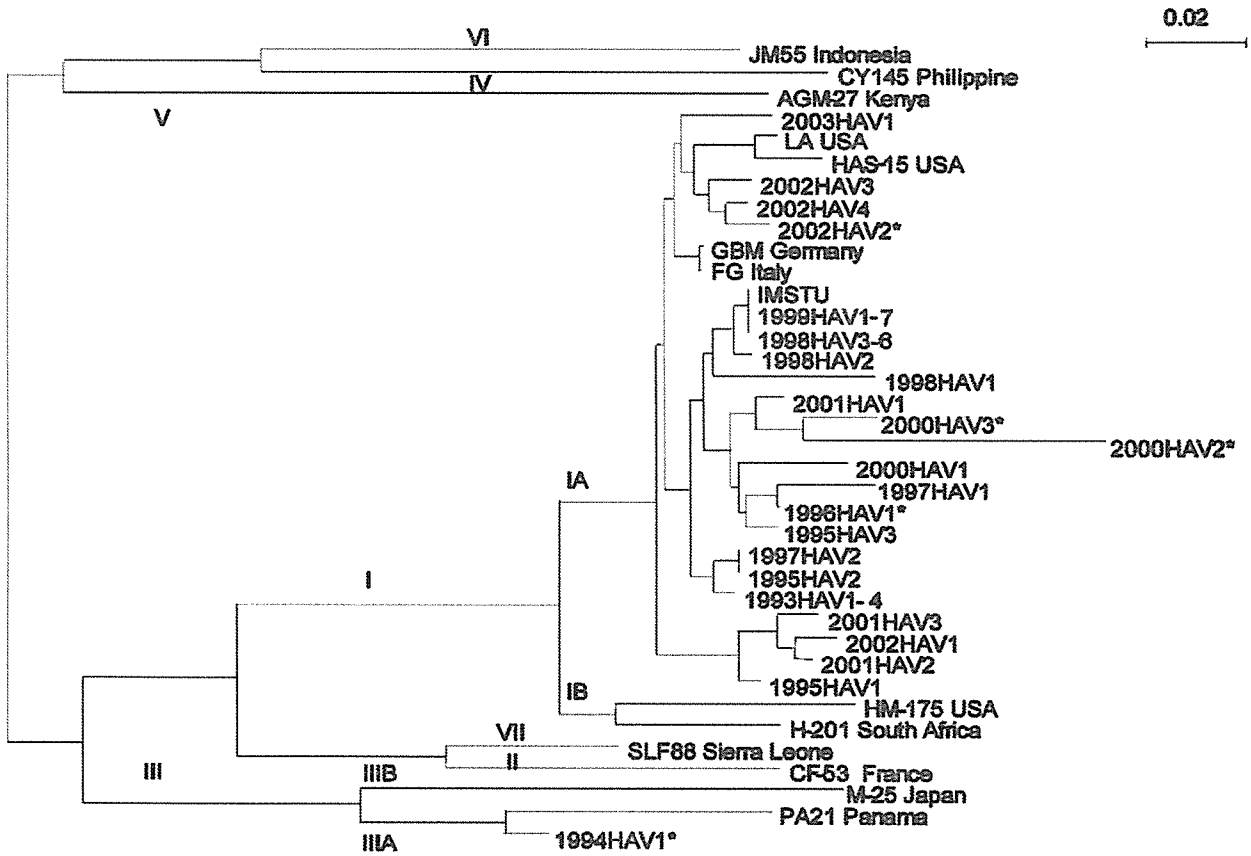

Fig. 2. A phylogenetic tree constructed for RNA sequences located in the VP1/2A region of hepatitis A virus (HAV) genomes reported previously. Accession numbers are shown for the isolates that have been deposited in the DDBJ/EMBL/GenBank databases. Many nucleotide sequences were close to that of HAS-15, a representative HAV genotype IA strain. Twenty-five of 27 viral sequences recovered during 1998–1999 were identical to IMSTU. *shows the sequences that were acquired abroad

Discussion

In this study, the most frequent transmission route was personal contact, which is also the case in the United States (www.cdc.gov/ncidod/diseases/hepatitis/resource/PDFs/hep_surveillance_60.pdf). The next most frequent cause was contaminated food, which is different from the United States. Additionally, the

proportion of patients whose hepatitis was caused by contaminated food was highest in recent years. As mentioned above, anti-HAV prevalence, which may reflect poor sanitation, has decreased in Japan. Therefore, the high incidence of hepatitis A with food/water as a transmission route may not result from poor sanitation. The reason for the high percentage is presumably related to diet. Japanese people often eat raw fish or shellfish, which increases the chances of transmission. Indeed an outbreak caused by eating raw oysters has been reported.[13] In other words, the transmission routes of HAV in Japan are different from those in other developed countries irrespective of improved sanitation.

The molecular epidemiological study showed interesting results. The sequences recovered in the years other than 1998 and 1999 were heterogeneous (Fig. 1). Furthermore, the phylogenetic analysis showed several clusters of genotype IA HAV strains, after excluding cases presumably acquired abroad. This suggests that several subgroups of genotype IA HAV strains are endemic in Japan and cause sporadic hepatitis. A large-scale epidemiological study may be useful for testing this hypothesis.

In contrast, an epidemic caused by homosexual activity was observed in 1998 and 1999.[11,12] The same nucleotide sequences were detected among patients for more than 1 year (Fig. 1), which suggests that the same strain was transmitted secondarily. Among 24 patients whose sera harbored the same HAV sequences in this period, 11 were presumably infected through homosexual activity. Among the other patients, four were infected through close person-to-person contact (heterosexual activity, familial transmission, or transmission in day-care facilities), while the other four had no relevant history. These findings suggest that homosexual persons can transmit HAV to nonhomosexual persons through close contact or heterosexual activity.

It is interesting that 24 separate sequences in 1998 and 1999 were identical to the IMSTU recovered from ten patients in another institution. A serum sample recovered 2 months before the epidemic had a similar sequence, with 99.4% homology. Because a homosexual patient may have sexual contact with multiple partners within that community, the 34 patients may have been infected by the same strain. Therefore, our results suggest that the rate of mutation in this region of 168 base pairs is probably very low. A previous study has shown that the mutation rate of HAV within a person is very low;[14] our results are consistent with that observation.

A previous report has shown that sequences recovered in Japan in the early 1990s are variable and belong to multiple genotypes.[14] Our results are contrary to those of that study. This suggests that improvements in sanitation decrease both the number of patients and viral heterogeneity. The endemicity in low-HAV-prevalence countries may be caused by highly related viral strains. A report from the United States showing that most patients infected in a community-wide outbreak were infected by the same strain supports this hypothesis.[15]

In developed countries, a substantial number of patients with hepatitis A acquired HAV through homosexual activity (www.cdc.gov/nciod/diseases/hepatitis/h96surve.htm). Recent studies using PCR analysis have shown that the fecal excretion of HAV continues even after recovery,[16] which suggests that hepatitis A patients may transmit the virus even after recovery. This is in accordance with the fact that positivity for anti-HAV among homosexual people is very high.[17–19] Therefore, people who engage in homosexual activity should be considered for HAV vaccination regardless of human immunodeficiency virus coinfection.

To conclude, recent hepatitis A in metropolitan areas is caused predominantly by sporadic infection by genotype IA HAV. Homosexual activity may cause an HAV epidemic among not only homosexuals but also heterosexuals, and involve homogeneous viral strains.

Acknowledgments. We thank Ms. Mie Kanke for her excellent technical assistance. This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labour and Welfare, Japan.

References

1. Hepatitis A vaccines. Wkly Epidemiol Rec 2000;75:38–44.
2. Cuthbert JA. Hepatitis A: old and new. Clin Microbiol Rev 2001;14:38–58.
3. Gust ID. Epidemiological patterns of hepatitis A in different parts of the world. Vaccine 1992;10 Suppl 1:S56–8.
4. Dentinger CM, Bower WA, Nainan OV, et al. An outbreak of hepatitis A associated with green onions. J Infect Dis 2001;183:1273–6.
5. Hepatitis A among persons with hemophilia who received clotting factor concentrate—United States, September–December 1995. MMWR Morb Mortal Wkly Rep 1996;45:29–32.
6. Kiyohara T, Satoh T, Yamamoto H, Totsuka A, Moritsugu Y. The latest seroepidemiological pattern of hepatitis A in Japan. Jpn J Med Sci Biol 1997;50:123–31.
7. Bruisten SM, van Steenbergen JE, Pijl AS, Niesters HG, van Doornum GJ, Coutinho RA. Molecular epidemiology of hepatitis A virus in Amsterdam, the Netherlands. J Med Virol 2001;63:88–95.
8. Tallo T, Norder H, Tefanova V, Ott K, Ustina V, Prukk T, et al. Sequential changes in hepatitis A virus genotype distribution in Estonia during 1994 to 2001. J Med Virol 2003;70:187–93.
9. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987;162:156–9.
10. Sugita S, Yoshioka Y, Itamura S, Kanegae Y, Oguchi K, Gojobori T, et al. Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. J Mol Evol 1991;32:16–23.
11. Baba M, Oka H, Asayama M, Yoshinaga S, Yamashita H, Tachikawa N, et al. Acute hepatitis outbreak in Tokyo caused by

hepatitis A virus of common origin transmitted through oral and sexual routes. Jpn J Infect Dis 2000;53:127–9.
12. Ishida T, Nakamura T, Ajisawa A, Negishi M, Kashiyama T, Takechi A, et al. Outbreak of hepatitis A virus infection among HIV-1 seropositive men who had sex with men. Jpn J Infect Dis 1999;52:131–2.
13. Ohara H, Naruto H, Watanabe W, Ebisawa I. An outbreak of hepatitis A caused by consumption of raw oysters. J Hyg (Lond) 1983;91:163–5.
14. Robertson BH, Jansen RW, Khanna B, Totsuka A, Nainan OV, Siegl G, et al. Genetic relatedness of hepatitis A virus strains recovered from different geographical regions. J Gen Virol 1992; 73(Pt 6):1365–77.
15. Robertson BH, Averhoff F, Cromeans TL, Han X, Khoprasert B, Nainan OV, et al. Genetic relatedness of hepatitis A virus isolates during a community-wide outbreak. J Med Virol 2000;62:144–50.
16. Yotsuyanagi H, Koike K, Yasuda K, Moriya K, Shintani Y, Fujie H, et al. Prolonged fecal excretion of hepatitis A virus in adult patients with hepatitis A as determined by polymerase chain reaction. Hepatology 1996;24:10–3.
17. Villano SA, Nelson KE, Vlahov D, Purcell RH, Saah AJ, Thomas DL. Hepatitis A among homosexual men and injection drug users: more evidence for vaccination. Clin Infect Dis 1997;25:726–8.
18. Diamond C, Thiede H, Perdue T, Secura GM, Valleroy L, Mackellar D, et al. Viral hepatitis among young men who have sex with men: prevalence of infection, risk behaviors, and vaccination. Sex Transm Dis 2003;30:425–32.
19. Koff RS. Seroepidemiology of hepatitis A in the United States. J Infect Dis 1995;171 Suppl 1:S19–23.