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Effect of a thiol proteinase inhibitor, E-64-d, on susceptibility to infection with *Staphylococcus aureus* in Chediak–Higashi syndrome (beige) mice

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Abstract

We previously reported that abnormally down-regulated protein kinase C (PKC) activity is responsible for the impaired cellular function of natural killer cells and polymorphonuclear cells (PMNs), and the giant granule formation in fibroblasts in the beige mouse, an animal model of Chediak–Higashi syndrome. Here, we examine the effect of oral or intraperitoneal administration of E-64-d, which protects PKC from calpain-mediated proteolysis, on the impaired cellular function in PMNs from beige mice. We found that oral administration of E-64-d (12.5 mg/kg body weight per day) for three consecutive days, significantly improved the abnormally increased concanavalin A (Con A) cap formation and the decreased lysosomal enzyme activity in beige PMNs. In addition, E-64-d significantly improved the delayed bactericidal activity against *Staphylococcus aureus*. In contrast, E-64-d at the same dose did not affect these cellular functions in PMNs from C57BL/6J mice. We confirmed that the abnormal down-regulation of PKC after Con A stimulation was eliminated in PMNs from E-64-d-treated beige PMNs. We then examined whether the administration of E-64-d to beige mice improved the susceptibility to experimental infection with *S. aureus* (2×10^8/mouse). Both intraperitoneal and oral administration of E-64-d to beige mice resulted in a significant increase in survival, whereas E-64-d at the same dose did not alter the survival rate in normal mice. These results suggest that the administration of E-64-d may be effective against severe bacterial infection in Chediak–Higashi syndrome.
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Keywords: Chediak–Higashi syndrome; beige mice; protein kinase C; calpain; bacterial infection

1. Introduction

Chediak–Higashi syndrome (CHS) is a rare autosomal recessive disorder characterized by partial oculocutaneous albinism, recurrent pyogenic infections, defective natural killer (NK) activity and abnormal giant lysosomes [1–4]. There is no specific treatment, and most patients succumb to frequent bacterial infections.

Susceptibility to infection in CHS patients is associated with granulocytopenia, abnormal chemotaxis and a delay in the killing of phagocytized bacteria in polymorphonuclear cells (PMNs) [5–8]. It has been

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reported that ascorbic acid, a known stimulant of cyclic GMP levels, improves the chemotaxis and bactericidal activity against *Staphylococcus aureus* in PMNs from beige mice [9]. In that report, however, the leukocyte dysfunction is not improved by ascorbic acid in human CHS patients.

The beige mouse has been used as an animal model of CHS. Human CHS patients and beige mice have homologous disorders associated with *CHS 1* mutation [10–12]. However, the precise function of the CHS 1 protein has not been elucidated, although it was suggested that the CHS 1 protein regulates lysosomal fission [13].

Protein Kinase C (PKC) is a Ca^{2+}, phospholipid-dependent serine/threonine protein kinase that plays an essential role in intracellular signal transduction [14]. We previously reported that PKC activity is abnormally down-regulated after stimulation with phorbol ester or concanavalin A (Con A) in PMNs, NK cells and fibroblasts from beige mice [15–17]. This abnormal down-regulation of PKC in beige cells is eliminated by treatment of cells with potent inhibitors of calpain, which is a thiol proteinase and proteolyses PKC to an inactive form [16–18]. In addition, we showed that the formation of giant granules, increased Con A cap formation, defective NK activity and decreased elastase and cathepsin G activity in beige mice are recovered by treatment of cells *in vitro* with thiol proteinase inhibitors.

In the present study, we examined whether susceptibility to infection with *S. aureus* and the associated cellular functions of PMNs are improved when E-64-d, a cell-permeable thiol proteinase inhibitor, is given orally or intraperitoneally to beige mice.

2. Materials and methods

2.1. Mice

C57BL/6J(+/+) and C57BL/6J-beige(bg/bg) mice were originally obtained from CLEA Japan Inc. (Tokyo, Japan). Eight- to twelve-week-old mice of the same age and sex (weighing 20–25 g) were used in all experiments. All animal experiments were approved by the Animal Experiment Committee at the University of Yamanashi.

2.2. Reagents

E-64-d [ethyl (+)-(2S,3S)-3-[(*S*)-3-methyl-1-(3-methylbutylcarbamoyl) butyl-carbamoyl]-2-oxiranecarboxylate] was kindly provided by Taisho Pharmaceutical Co. (Saitama, Japan). Hanks' balanced salt solution (HBSS) and phosphate-buffered saline (PBS) were from Invitrogen Co. (Carlsbad, CA, USA). [γ-^{32}p] ATP and the PKC enzyme assay system were purchased from GE Healthcare Bio-Science Co. (Piscataway, NJ, USA). The substrate of elastase was from Peptide Institute Inc. (Osaka, Japan). The substrates of cathepsin G and β-galactosidase, fluorescein isothiocyanate (FITC)-Con A, lysostaphin and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA). SCD agar and heart infusion broth were obtained from Eiken Chemical Co. (Tokyo, Japan).

2.3. Bacteria

The coagulase-positive and methicillin-sensitive strains of *S. aureus* used in this study were clinical isolates from the University of Yamanashi Hospital. Bacteria were grown for 12 h in heart infusion broth at 37 °C. Subsequently, the bacterial culture was kept at 4 °C until further use. Serial 10-fold dilutions were made from this bacterial culture and plated on SCD agar plates. After overnight culture, the colonies were counted and the bacterial culture was diluted to the desired concentrations.

2.4. Administration of E-64-d

E-64-d was suspended in carboxymethylcellulose solution containing 0.1% Tween 80 according to the method described by Tamai et al. [19]. E-64-d was given to mice orally by gastric intubation (0.1 ml) using a 22-gauge intubation tube (CLEA Japan Inc., Tokyo, Japan) for three consecutive days. Alternatively, E-64-d was dissolved in dimethylsulfoxide and diluted with PBS and given to mice by intraperitoneal injection (0.5 ml).

2.5. Isolation of murine PMNs

PMNs were obtained by lavage with 5 ml of HBSS 16–20 h after intraperitoneal injection of 1 ml of sterile thioglycolate broth as previously described [15]. The cells obtained ($6–10 \times 10^6$/mouse) were 80–85% PMNs as determined by May–Giemsa staining: the remainder were macrophages and lymphocytes.

2.6. Con A cap formation

FITC-conjugated Con A-induced cap formation was determined according to a method described previously [18,20]. Cells (1×10^6/ml) were incubated with FITC-Con A at a concentration of 20 μg/ml at 37 °C. The cells were fixed with 2% paraformaldehyde for 10 min at 37 °C Wet mounts were prepared by using 10 μl of each cell mixture on a glass side with cover slips. The cells were observed with an epifluorescence microscope fitted with an FITC interference filter and a 20× or 40× objective. Scoring was carried out in two categories with respect to distribution of the label as random clusters or capped. Two hundred cells were counted, and the percentage of capped cells was calculated.

2.7. Assay for lysosomal enzyme activity

Cells (5×10^6) were resuspended in cold 0.2% Triton X-100, 0.2 M sucrose, 5 mM EDTA in 20 mM imidazole–HCl, pH 7.3 [21]. The cells were then disrupted by sonication for 20 s three times at 4 °C and were centrifuged at 15000 rpm for 15 min at 4 °C. The supernatant was used for the following enzyme assay. Elastase activity was measured with the fluorogenic substrate methoxysuccinyl-Ala-Ala-Pro-Val-4 methylcoumarinyl-7-amide by the method of Barrett [22]. The regular reaction mixture (100 μl) contained 40 mM Tris–HCl (pH 7.5), 8% (v/v) dimethylsulfoxide, 0.4 M NaCl and 0.8 mM substrate. The mixture was incubated at 37 °C for 15 min after which the reaction was terminated by the addition of 1 ml of 5 M formic acid. The fluorescence product, AMC, was measured by its fluorescence intensity at 460 nm with excitation at 370 nm on a fluorescence spectrophotometer F-4500 (Hitachi, Tokyo, Japan). Cathepsin G activity was assayed by using Suc-Ala-Ala-Pro-Phe-pNA as the substrate according to the method developed by Barrett [23]. The mixture was incubated at 50 °C for 15 min after which the reaction was terminated by adding 200 μl of 0.3 mg/ml of trypsin inhibitor. The reaction product was measured at 410 nm on an Ultrospec 2100 pro UV/visible spectrophotometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). One unit of enzyme activity is expressed as 1 nmol product/h. The activity of β-galactosidase was assayed according to the method of Brandt et al. [24].

2.8. Bactericidal assays

Bactericidal assays were performed according to the method described by Gallin et al. [9]. For these studies, 5×10^6 PMNs in HBSS with 10% fresh normal mouse serum were preincubated at 37 °C for 10 min and then 5×10^7 bacteria were added. The PMNs suspension was then tumbled for 20 min to allow phagocytic uptake to occur. Then, lysostaphin (final concentration 10 U/ml), which kills extracellular bacteria, was added and the tumbling continued. Portions (0.1 ml) were removed at intervals (at 30, 45 and 120 min) and washed three times with ice-cold HBSS and then placed into 1 ml of distilled water to rupture the cells for total viable bacterial counts. Serial 10-fold dilutions were made and plated onto SCD agar plates. After overnight culture at 37 °C, the number of colonies was counted.

Table 1
Effect of intraperitoneal administration of E-64-d on Con A cap formation in PMNs

Administration	Con A-capped cells (%)	
E-64-d (mg/kg per day)	C57BL/6J	Beige
0	12.6±0.8	27.9±1.6[a]
3.125	12.8±1.0	23.4±2.2[b]
6.25	13.0±0.8	19.8±2.4[b]
12.5	12.5±0.6	15.9±1.8[b]
25	13.1±1.2	15.2±1.2[b]

Various doses of E-64-d were administered intraperitoneally to normal and beige mice for three consecutive days. Data represent means±SE of at least three experiments.

[a] $p<0.01$, significant when compared with C57BL/6J mice without E-64-d.

[b] $p<0.01$, significant when compared with beige mice without E-64-d.

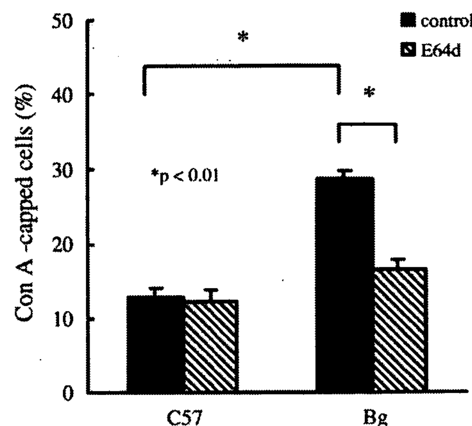

Fig. 1. Effect of oral administration of E-64-d on Con A cap formation in PMNs E-64-d (12.5 mg/kg per day) or control solution was administered orally to normal and beige mice for three consecutive days. Con A cap formation in PMNs was then examined as described in Materials and methods. The data represent the mean±SE of at least three experiments.

2.9. Assay for PKC activity

Cells (5×10^6) were disrupted by sonication for 10 s three times at 4 °C in 20 mM Tris–HCl (pH7.5), 0.25 M sucrose, 2 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsulfonylfluoride, 0.01% leupeptin and 50 mM 2-mercaptoethanol. The cytosolic and the membrane fractions were prepared as previously described [15]. PKC activity was assayed by using a PKC enzyme assay system according to the manufacturer's protocol.

2.10. Susceptibility to infection with S. aureus

Beige and normal mice were divided into two groups. One group was administered E-64-d (12.5 mg/kg per day) orally or intraperitoneally for three consecutive days. The other group received control solution given in the same manner. Five hours after the final administration, all mice were inoculated intraperitoneally with viable *S. aureus* (2×10^8) at the same time. The mice were observed at 12-h intervals, and the number of surviving mice was tabulated. Deaths within the first 4 h were recorded as stemming from technical causes, these mice were excluded from this study.

Table 2
Effect of oral administration of E-64-d on lysosomal enzyme activity in PMNs

Mice	Administration	Elastase (U/10^6 cells)	Cathepsin G (U/10^6 cells)	β-Galactosidase (%)
C57BL/6J	Control	3.66±0.2	5.55±0.1	100
	E-64-d	3.73±0.3	5.50±0.3	99.5
Beige	Control	0.12±0.2[a]	1.43±0.3[a]	111.5
	E-64-d	3.20±0.4[b]	5.02±0.4[b]	108.5

E-64-d (12.5 mg/kg per day) or control solution was administered orally to normal and beige mice for three consecutive days. One unit of elastase and cathepsin G activity is expressed as 1 nmol products/h. Data are expressed as means±SE of at least three experiments. The activity of β-galactosidase is expressed as means of the percent of enzyme activity of C57BL/6J control mice.
[a] $p<0.01$, significant when compared with C57BL/6J control mice.
[b] $p<0.01$, significant when compared with beige control mice.

At least fifteen mice were examined in each group. Mortality rates were compared by using the Wilcoxon test [25].

2.11. Statistics

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

3. Results

3.1. Effect of administration of E-64-d on Con A cap formation

We examined whether intraperitoneal or oral administration of E-64-d affects Con A cap formation in beige mice. As shown in Table 1, Con A-capped cells were significantly increased in beige mice compared with C57BL/6J mice. We also examined Con A-capped cells after administering various doses of E-64-d intraperitoneally to both groups of mice for three consecutive days. Table 1 also shows that Con A cap formation in E-64-d-treated beige mice significantly decreased in a dose-dependent manner, compared with that in untreated beige mice. E-64-d did not significantly affect Con A capping in PMNs from control mice. The results were similar when E-64-d (12.5 mg/kg body weight per day) was administered orally for 3 days (Fig. 1). The number of peritoneal PMNs was not significantly altered by the administration of E-64-d at these doses (data not shown).

3.2. Effect of administration of E-64-d on lysosomal enzyme activity

As shown in Table 2, elastase and cathepsin G activity in beige PMNs were significantly lower than in normal PMNs, whereas β-galactosidase activity was not significantly different. We found that oral administration of E-64-d (12.5 mg/kg per day) for 3 days significantly increased elastase and cathepsin G activity in beige PMNs, whereas E-64-d at this dose did not affect enzyme activity in normal PMNs. The results were similar when E-64-d was administered intraperitoneally (Table 3).

3.3. Effect of administration of E-64-d on bactericidal activity against S. aureus

As shown in Table 4, the killing rate of *S. aureus* in PMNs from beige mice was significantly lower than that in PMNs from normal mice ($p<0.05$). The bactericidal activity of PMNs from beige mice significantly increased after oral administration of E-64-d (12.5 mg/kg per day) for 3 days, at 30 min, 45 min and 120 min ($p<0.05$). In contrast, the oral administration of E-64-d at the same dose had no significant

Table 3
Effect of intraperitoneal administration of E-64-d on lysosomal enzyme activity in PMNs

Mice	Administration	Elastase (U/10^6 cells)	Cathepsin G (U/10^6 cells)	β-Galactosidase (%)
C57BL/6J	Control	3.70±0.2	5.56±0.1	100
	E-64-d	3.72±0.2	5.50±0.2	98.5
Beige	Control	0.11±0.2[a]	1.38±0.2[a]	108.5
	E-64-d	3.58±0.3[b]	5.28±0.4[b]	110.5

E-64-d (12.5 mg/kg per day) or control solution was administered intraperitoneally to normal and beige mice for three consecutive days. One unit of elastase and cathepsin G activity is expressed as 1 nmol products/h. Data are expressed as means±SE of at least three experiments. The activity of β-galactosidase is expressed as means of the percent of enzyme activity of C57BL/6J control mice.
[a] $p<0.01$, significant when compared with C57BL/6J control mice.
[b] $p<0.01$, significant when compared with beige control mice.

Table 4
Effect of oral administration of E-64-d on the bactericidal activity of PMNs

Mice	Administration	Bactericidal Activity (% *S. aureus* killed)		
		30 min	45 min	120 min
C57BL/6J	Control	27.1±5.1	28.1±3.7	51.6±2.4
	E-64-d	27.5±5.1	29.4±2.2	51.7±2.3
Beige	Control	10.3±3.2[a]	10.6±1.7[a]	43.5±2.6[a]
	E-64-d	20.1±6.3	29.3±7.2	50.4±3.0

E-64-d (12.5 mg/kg per day) or control solution was administered orally to normal and beige mice for three consecutive days. Data represent means±SE of at least three experiments.
[a] $p<0.05$, significant when compared with C57BL/6J control mice and E-64-d-treated beige mice.

effect on bactericidal activity of PMNs from normal mice. The results were similar when E-64-d was administered intraperitoneally (data not shown).

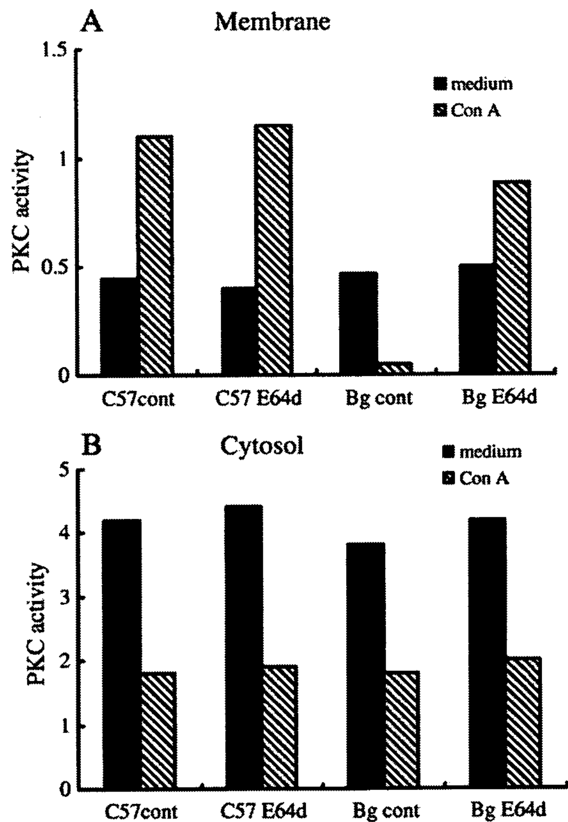

Fig. 2. Effect of oral administration of E-64-d on PKC activity in Con A-stimulated PMNs E-64-d (12.5 mg/kg per day) or control solution was administered orally to normal and beige mice for three consecutive days. After PMNs were stimulated with Con A (20 μg/ml) for 10 min, PKC activity in the membrane (A) and the cytosolic (B) fractions were assayed as described in Materials and methods. The data represent the mean (pmol/min/10^7 cells) of at least three experiments. The SE of each column was less than 10%.

3.4. *Effect of administration of E-64-d on PKC activity*

Fig. 2 shows the PKC activity in PMNs from beige mice and normal mice. In normal mice, membrane-bound PKC activity increased after 10 min of Con A stimulation (Fig. 2A), whereas cytosolic enzyme activity decreased (Fig. 2B). In contrast, membrane-bound PKC activity in beige PMNs drastically decreased after Con A stimulation compared with that in normal mice. The oral administration of E-64-d (12.5 mg/kg per day) for 3 days markedly increased the down-regulated membrane-bound PKC activity in beige PMNs. PKC activity in normal mice was not significantly altered by the same dose of E-64-d. The results were similar when E-64-d was administered intraperitoneally (Fig. 3).

3.5. *Effect of E-64-d on susceptibility to infection with S. aureus*

Because the administration of E-64-d to beige mice improved the bactericidal activity and lysosome enzyme

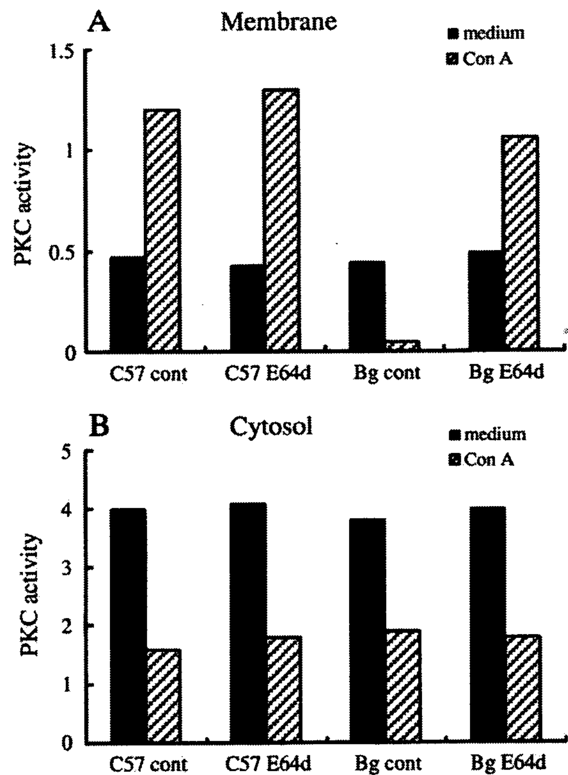

Fig. 3. Effect of intraperitoneal administration of E-64-d on PKC activity in Con A-stimulated PMNs E-64-d (12.5 mg/kg per day) or control solution was administered intraperitoneally to normal and beige mice for three consecutive days. After PMNs were stimulated with Con A (20 μg/ml) for 10 min, PKC activity in the membrane (A) and the cytosolic (B) fractions were assayed as described in Materials and methods. The data represent the mean (pmol/min/10^7 cells) of at least three experiments. The SE of each column was less than 10%.

activity that are strongly linked to the defense mechanism for bacterial infection, we examined the effect of E-64-d on the susceptibility of beige mice to infection with *S. aureus* (2×10^8/mouse). As shown in Fig. 4, 75% of the beige mice died within 5 days after intraperitoneal inoculation, whereas only 33.3 % of the normal mice died. In contrast, survival increased significantly ($p<0.01$) in beige mice treated with intraperitoneal administration of E-64-d (12.5 mg/kg per day) for 3 days (Fig. 4A). However, E-64-d at this dose did not significantly affect survival in the normal mice. As shown in Fig. 4B, survival increased significantly ($p<0.01$) in beige mice treated with oral administration of E-64-d (12.5 mg/kg per day) for 3 days. The beige mice administered E-64-d orally had a percentage cumulative mortality approximately midway between that of control beige mice and that of normal control mice. The survival in normal mice was not altered by oral administration of E-64-d at this dose.

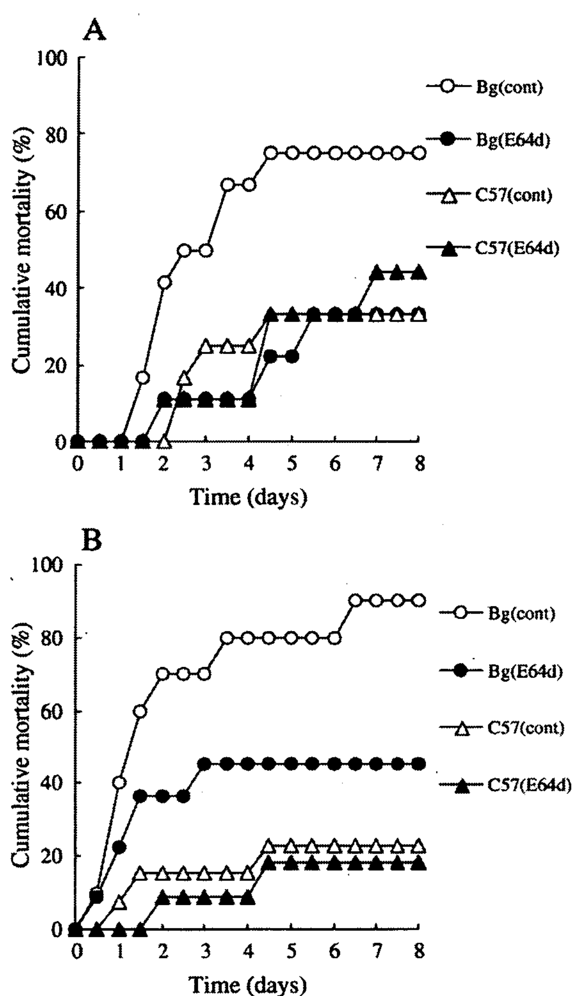

Fig. 4. Effect of administration of E-64-d on susceptibility to infection with *S. aureus* E-64-d (12.5 mg/kg per day) or control solution was administered intraperitoneally (A) or orally (B) to normal and beige mice for three consecutive days. Five hours after the final administration, all mice were intraperitoneally inoculated with viable *S. aureus* (2×10^8/mouse) and then observed as described in Materials and methods. Number of mice dead at 12-h intervals was recorded and the percentage cumulative mortality was calculated. At least fifteen mice were examined in each group.

4. Discussion

We show here for the first time that oral or intraperitoneal administration of E-64-d to beige mice improves the defective leukocyte function in these mice, such as Con A cap formation, lysosomal enzyme activity and bactericidal activity against *S. aureus*. In addition, we show that the administration of E-64-d decreases the percentage cumulative mortality of beige mice from systemic infection with *S. aureus*. E-64-d, which is isolated from the culture of *Aspergillus japonicus*, is a specific inhibitor of cysteine proteinases, including calpain [26]. E-64-d is the ethyl ester of E-64-c, a synthetic analogue of E-64, and is readily absorbed through the intestinal membrane because it is more lipophilic than E-64-c [19].

We previously reported that E-64-d prevents giant granule formation in beige fibroblasts [17]. E-64-d eliminates the calpain-mediated proteolysis of PKC, not only in beige fibroblasts but also in beige PMNs and NK cells, and in human CHS cell lines [15,18,27]. Other proteinase inhibitors, leupeptin and Z-Leu-Leu-H, which inhibit calpain, significantly decreased the increased capping in CHS cell lines [27]. This abnormal down-regulation of PKC is suggested to cause the cellular defects in CHS.

Con A cap formation is known to be associated with membrane-cytoskeleton interaction [28–30]. We previously showed that PKC inhibitors enhance Con A cap formation in normal murine PMNs [31]. In beige PMNs, abnormal down-regulation of PKC occurs after Con A stimulation, and E-64-d which eliminates PKC breakdown corrects the increased cap formation [18]. Because colchicine which disrupts microtubules also enhances cap formation [18], PKC may play an important role in microtubule function.

It has been reported that lysosomal elastase activity and cathepsin G activity are selectively reduced in beige PMNs [21]. This reduced lysosomal enzyme activity may contribute to the high susceptibility of beige mice to infection. We have shown that E-64-d almost corrects the enzyme activity in CHS cell lines and beige fibroblasts [17,27]. These two enzymes are believed to undergo similar processing in the Golgi apparatus [32]. It was also reported that elastase is present in granules as

a 46-kDa proenzyme in beige neutrophils [33]. Thus, the processing and the intracellular transport of these enzymes are considered to be deficient in CHS. PKC may be involved in generating the active form of these enzymes by some unknown mechanism.

The intracellular destruction of *S. aureus*, Group D *streptococci* and Type II *pneumococci has* been reported to be impaired in leukocytes from CHS patients [8]. Gallin et al. [9] reported that ascorbic acid improved PMN chemotaxis and bactericidal activity against *S. aureus*. Intraperitoneal administration of ascorbic acid (20 mg/mouse) for 5 days prolonged the survival of beige mice from lethal infection with *Candida albicans*, although the survival of ascorbic acid-treated beige mice was not as good as that of saline-treated control mice. However, leukocyte dysfunction was not improved by ascorbic acid (6 g/day for 8 months) in human CHS patients [9]. In addition, this therapy had no effect on the clinical course of these patients and resulted in no change in abnormal giant lysosome.

We have shown that ceramide which is produced by sphingomyelinase (SMase) activation promotes calpain-mediated PKC proteolysis [34]. C_2-ceramide, a cell-permeable ceramide analogue, was also shown to increase Con A cap formation in human cell lines and to reduce elastase and cathepsin G activity in beige fibroblasts and human cell lines [17,27]. C_2-ceramide also promotes giant granule formation in normal murine fibroblasts [17]. In the previous study, we found that both acidic SMase and neutral SMase are significantly enhanced after Con A stimulation in CHS cell lines [27]. Although acidic SMase is known to be associated with lysosomes, both SMases may be linked to ceramide production in CHS cells.

The genetic defect of CHS and beige mice (*CHS 1*) has been identified [10–12]. The CHS 1 protein is a 400-kDa cytosolic protein; however, the precise role of this protein has not been clarified. Perou et al. [13] reported that cultured fibroblasts in which the CHS 1 protein is overexpressed have smaller than normal lysosomes that are more peripherally distributed than in control cells. It was suggested that the CHS 1 protein regulates lysosomal fission. The BEACH domain in the CHS 1 protein is considered to be the functional domain and to be crucial for vesicle trafficking, membrane dynamics and receptor signaling [35,36]. However, the relationship between abnormal PKC breakdown and CHS 1 protein remains unclear, and further work is needed.

In summary, we have shown that E-64-d not only corrects the cellular defects seen in CHS mice *in vitro* but also improves the susceptibility to infection with *S. aureus* in CHS mice *in vivo*. These findings suggest that E-64-d may be effective for preventing severe bacterial infection in patients with CHS.

Acknowledgments

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CORRESPONDENCE

External Validation of FIB-4: Diagnostic Accuracy Is Limited in Elderly Populations

To the Editor:

We read with interest the articles by Stering et al.[1] and Vallet-Pichard et al.[2] The former authors developed the FIB-4 index, a non-invasive method for assessing liver fibrosis in patients with HIV/HCV coinfection. The variables used are age, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and platelet (PLT) count, and the formula is as follows: (age [yr] $\times$ AST [U/L])/ ((PLT[10^9/L]) $\times$ (ALT[U/L])$^{1/2}$). They showed that over 70% of patients could be classified into either absence or presence of advanced fibrosis by cutoff of <1.45 or >3.25 respectively, with diagnostic accuracy of 87%. The latter authors expanded the applicability of the FIB-4 index to HCV-monoinfected patients and showed that 73% of patients were classified with diagnostic accuracy of 93%, an excellent performance in both classification and accuracy of diagnosis.

Because the mean age of patients was young in these studies (40 years[1] and 44 years[2]), we wondered whether this index could also fit to Japanese patients who are rather older than the Western patients. We validated the FIB-4 index in a retrospective cohort of 1,405 patients who underwent liver biopsy at our hospital. The mean age was 55 ± 12 years. The distribution of METAVIR fibrosis scores was as follows: 1.6% showed no fibrosis (F0), 44.8% showed mild fibrosis (F1), 29.5% showed moderate fibrosis (F2), 20.2% showed severe fibrosis (F3), and 3.9% showed cirrhosis (F4). The proportion of advanced fibrosis (F3 or F4) was slightly higher in our population compared to the former studies (24.1% vs. 20.7% [1] and 17.2% [2]). As shown in Table 1, only 53% of patients were classified to either <1.45 or >3.25, a much lower rate than previous reports. The diagnostic accuracy was excellent in patients with a FIB-4 index <1.45 (94%), however, it was relatively poor in patients with a FIB-4 index >3.25 (50%) making the overall accuracy as low as 67%.

We supposed this discordance with previous reports may be derived from the older age of our populations and thus we categorized patients into three groups according to age and analyzed separately. In patients with age ≤50 years, 64% of patients were classified, and the diagnostic accuracy was 94% for a FIB-4 index <1.45 and 68% for a FIB-4 index >3.25 making the overall accuracy of 90%, a result comparable to previous reports. In older patients, however, diagnostic accuracy was significantly low compared to those with age ≤50 years (56% for age 51-60 years, $P < 0.0001$ and 51% for age ≥60 years, $P < 0.0001$). Because patients with a FIB-4 index >3.25 increased according to age (6%, 34%, and 53% for ages ≤50, 51-60 and >60 years), and the diagnostic accuracy was low in these patients (48% to 50%), these results suggest that, in elderly patients, a variable "age" generates excessively high FIB-4 index leading to misclassification of no-moderate fibrosis (F0-F2) into a FIB-4 index >3.25.

In conclusion, the FIB-4 index could accurately differentiate advanced fibrosis in young Japanese patients with chronic hepatitis C but the diagnostic accuracy is limited in the elderly. Thus, in elderly patients, some sort of adjustment for the effect of age on FIB-4 index may be necessary for more precise classification.

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Table 1. Comparison of FIB-4 Index and Liver Biopsy Results in Terms of Age

		METAVIR Fibrosis Score			
	FIB-4	F0-2	F3-4	Total	Diagnostic Accuracy
All patients					
	<1.45	283 (20%)	18 (1%)	301 (21%)	94%
	>3.25	228 (16%)	226 (16%)	454 (32%)	50%
	1.45-3.25	556 (40%)	94 (7%)	650 (47%)	
	Total	1067 (76%)	338 (24%)	1405 (100%)	67%
Age ≤50	<1.45	240 (54%)	16 (4%)	256 (58%)	94%
(Mean 40 yrs)	>3.25	9 (2%)	19 (4%)	28 (6%)	68%
	1.45-3.25	126 (28%)	38 (8%)	164 (36%)	
	Total	375 (84%)	73 (16%)	448 (100%)	90%
Age 51-60	<1.45	30 (7%)	2 (1%)	32 (8%)	94%
(Mean 56 yrs)	>3.25	76 (18%)	69 (16%)	145 (34%)	48%
	1.45-3.25	215 (50%)	36 (8%)	251 (58%)	
	Total	321 (75%)	107 (25%)	428 (100%)	56%
Age >60	<1.45	13 (2%)	0 (0%)	13 (2%)	100%
(Mean 66 yrs)	>3.25	143 (27%)	138 (26%)	281 (53%)	49%
	1.45-3.25	215 (41%)	20 (4%)	235 (45%)	
	Total	371 (70%)	158 (30%)	529 (100%)	51%

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The presence of steatosis and elevation of alanine aminotransferase level are associated with fibrosis progression in chronic hepatitis C with non-response to interferon therapy.

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Title: Potential relevance of cytoplasmic viral sensors and related regulators involving innate immunity in antiviral response

Short title: Innate immunity and therapeutic response

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Technology; and the Japanese Ministry of Welfare, Health and Labor.

Abbreviations: HCV, hepatitis C virus; PEG-IFN, pegylated interferon; NVR, non-virological responders; RIG-I, retinoic acid-inducible gene I; MDA5, melanoma differentiation associated gene 5; CARD, Caspase-recruiting domain; Cardif, caspase-recruiting domain adaptor inducing IFN-beta; IPS-1, IFN-beta promoter stimulator I; MAVS, mitochondrial antiviral signaling protein; VISA, virus-induced signaling adaptor; RNF125, ring-finger protein 125; ISG15, IFN-stimulated gene 15; USP18, ubiquitin-specific protease 18; UBP43, ubiquitin-specific protease 43; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; HMBS, hydroxymethylbilane synthase; PBMC, peripheral blood mononuclear cell; SVR, sustained viral responder; TR, transient responder; ROC, receiver operator characteristic; JAK, Janus kinase.

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Jan 31, 2008

Gastroenterology Ms. No. GASTRO-D-07-01373R1
Potential relevance of cytoplasmic viral sensors and related regulators involving innate immunity in antiviral response

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Comments from the Editors and Reviewers:

AE: Congratulations! Kind regards, Kyong-Mi Chang and Anil Rustgi (for the Board)

Reviewer #1: None

Reviewer #2: No further comments.

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Pharmacokinetics and enhanced PKR response in patients with chronic hepatitis C treated with pegylated interferon alpha-2b and ribavirin

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SUMMARY. This study investigated the molecular and pharmacokinetic mechanisms of the enhanced antiviral efficacy associated with pegylated interferon (PEG-IFN) alpha-2b and ribavirin. The study involved comparing the expression of serial double-stranded RNA-activated protein kinase (PKR) before and during treatment in 26 PEG-IFN alpha-2b and 26 conventional IFN alpha-2b recipients matched for age, body weight and dose of ribavirin. The pharmacokinetics of PEG-IFN alpha-2b and ribavirin was analysed in 15 of the 26 PEG-IFN recipients. There was a rapid increase in PKR expression in both treatment groups, although expression from day 2 onwards was maintained at a significantly higher level in the PEG-IFN recipients ($P < 0.05$). C_{max} of PEG-IFN occurred 12–48 h after the initial administration, with $t_{1/2}$ and C_{min} being 49 h and 190 pg/mL, respectively. In contrast to ribavirin, accumulation of PEG-IFN was minimal. There was no association between serum PEG-IFN and ribavirin levels and virological response. Although baseline expression of PKR before treatment was marginally higher in nonresponders (NRs), from day 2 onwards, sequential PKR expression in response to PEG-IFN was higher in sustained viral responders compared with the NRs ($P < 0.05$). Significant correlations were found between kinetics of PKR expression and viral decline rates in each phase of hepatitis C virus dynamics (first phase, $r = 0.67$, $P = 0.0006$; second phase, $r = 0.67$, $P = 0.001$). In conclusion, improvement in pharmacokinetics following pegylation led to higher intracellular PKR expression, which was associated with enhanced virological efficacy of PEG-IFN-based combination therapy. The concentrations of both ribavirin and PEG-IFN alpha-2b were not associated with viral response and PKR expression.

Keywords: hepatitis C virus, hepatitis C virus dynamics, interferon-stimulated gene, treatment.

INTRODUCTION

Combination therapy with pegylated interferon (PEG-IFN) alpha and ribavirin results in a higher sustained virological response (SVR) rate than conventional IFN alpha and ribavirin therapy [1,2] and is now established as the standard treatment for chronic hepatitis C virus (HCV) infection. However, the mechanism responsible for this improved response rate remains to be elucidated.

Interferon induces transcription of IFN-stimulated genes (ISG), including double-stranded RNA-activated protein kinase (PKR) [3]. PKR has many cellular roles, including inhibition of translational responses to viral infection, growth control, differentiation activity and proapoptotic functionality [4,5]. However, the clinical significance of PKR expression during PEG-IFN therapy is not fully understood. Moreover, the pharmacokinetic effects of PEG-IFN on PKR expression and the relationship between the expression of PKR and viral response remain unknown.

In addition, although the serum concentration of ribavirin has been reported to affect the outcome of conventional IFN alpha and ribavirin combination therapy [6], the relationship between serum ribavirin, PEG-IFN concentrations and viral response has not been studied.

Abbreviations: PEG-IFN, pegylated interferon; SVR, sustained virological response; HCV, hepatitis C virus; ISG, IFN-stimulated genes; PKR, double-stranded RNA-activated protein kinase; ALT, alanine aminotransferase; NR, nonresponder; PBMC, peripheral blood mononuclear cells; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction.

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In the present study, we sought to elucidate the underlying mechanism of the enhanced antiviral efficacy seen with PEG-IFN alpha-2b and ribavirin combination therapy by analysing PKR gene expression and pharmacokinetics of PEG-IFN and ribavirin in patients with chronic HCV genotype 1b infections. The relationships between the viral response and PKR expression and pharmacokinetics of PEG-IFN and ribavirin were also studied.

MATERIALS AND METHODS

Patients

Fifty-two patients infected with chronic hepatitis C of genotype 1b and high viral load, admitted between November 2001 and June 2002, were included in the study. Twenty-six patients were treated with PEG-IFN alpha-2b and ribavirin combination therapy, with the remaining 26 patients matched for age, body weight and dose of ribavirin being treated with conventional IFN alpha-2b and ribavirin. The inclusion criteria for the study were as follows: Persistent elevation of serum alanine aminotransferase (ALT) levels above the upper limit of the normal for ≥6 months prior to therapy; the presence of HCV genotype 1b in the serum; the presence of serum HCV-RNA of >100 000 IU/mL detected by the Amplicor-HCV monitor assay (Roche Molecular Diagnostic Co., Tokyo, Japan); no evidence of hepatocellular carcinoma in an ultrasound examination; a haemoglobin level ≥14 g/dL, neutrophil count ≥1500/mm^3, platelet count ≥100 × 10^3/mm^3, creatinine clearance ≥51 mL/min and fasting blood sugar <110 mg/dL. Exclusion criteria included the presence of hepatitis B surface antigen or human immunodeficiency viral antibodies and a history of excess alcohol consumption. Eleven of the 26 PEG-IFN alpha-2b recipients and all 26 conventional IFN alpha-2b recipients had been enrolled previously in a viral dynamics study [7].

Written informed consent was obtained from all the patients and the study protocol was approved by the institutional ethical committee in accordance with the revised version of the Helsinki Declaration of 1983.

Treatment

Twenty-six patients were treated for 48 weeks with subcutaneous injections of PEG-IFN alpha-2b (PegIntron®; Schering-Plough Corporation, Kenilworth, NJ, USA) at a dose of 1.5 μg/kg/week. Ribavirin (Rebetol®, Schering-Plough Corporation) was administered concomitantly over the 48-week period, provided orally twice daily at a total daily dose of 800 mg. At the start of the study, 400 mg of ribavirin was administered, with serum concentrations being measured after 48 h. As the body weight of the patients in the study ranged between 60 and 80 kg, the dose of ribavirin for the remainder of the study period was fixed at 800 mg/day. The dose of PEG-IFN alpha-2b was reduced to 0.75 μg/kg/week when either the neutrophil count was <750/mm^3 or the platelet count was <80 × 10^3/mm^3. The dose of ribavirin was reduced to 600 mg/day when the haemoglobin concentration decreased to <10 g/dL.

The remaining 26 patients were treated for 48 weeks with intramuscular IFN alpha-2b (Intron-A®; Schering-Plough Corporation) in combination with daily oral ribavirin at a dose of 800 mg. For the first 2 weeks of therapy, 6 MU of IFN alpha-2b was administered daily, followed for the next 46 weeks by 6 MU given three times a week.

Measurement of PKR mRNA before and during therapy

Serial measurements of PKR expression before and during treatment were determined in both treatment groups. Peripheral blood mononuclear cells (PBMCs) were obtained from whole blood samples collected before, and at 4, 8, 24 h and 2, 4, 7, 14, 21, 28, 56, 84, 112, 140, 168 and 336 days after the initiation of either PEG- or conventional IFN alpha-2b and ribavirin combination therapy. After extraction of total RNA from the PBMCs, the expression of PKR mRNA was quantified at each specified time point using real-time quantitative polymerase chain reaction (PCR) as described previously [8]. The assays were performed in triplicate, and as an internal control, the expression levels of PKR transcript were normalized to glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression quantified by real-time quantitative PCR. The level of PKR gene expression at each time point during IFN treatment was calculated relative to baseline expression levels measured prior to IFN treatment.

Pharmacokinetics of pegylated interferon alpha-2b and ribavirin

The pharmacokinetics of PEG-IFN and ribavirin was analysed in 15 PEG-IFN alpha-2b recipients who consented to be enrolled in the additional pharmacokinetic study. Of these 15 patients, two were naïve , nine had relapsed and four had not responded to previous conventional IFN monotherapy. Blood samples were collected immediately before, and at 2, 4, 6, 8, 10, 12, 14, 16, 24, 36, 48, 72, 96, 120, 144 and 168 h after the first dose of PEG-IFN alpha-2b and ribavirin. Blood samples were also collected immediately before each administration at weeks 5, 9, 13, 25 and 37 and the trough values measured. At week 48 (final dose), blood was drawn immediately before, and at 2, 4, 6, 8, 10, 12, 14, 16, 36, 48, 72, 96, 120, 144, 168, 366, 504 and 672 h after administration. The sera were harvested immediately after blood collection and stored frozen at −20°C.

Serum PEG-IFN alpha-2b levels were determined using an electrochemiluminescent immunoassay (IGEN International, Inc., Gaithersburg, MD, USA), with the lower limit of detection for this assay being 27 pg/mL. Serum ribavirin levels were measured by high-performance liquid chromatography

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in conjunction with tandem mass spectroscopy (MDS Pharma Services Inc., Montreal, QC, Canada) according to a method reported previously [9]. The maximum serum concentration (C_{max}), time to maximum serum concentration (t_{max}) and C_{168h} (trough value of ribavirin) were then determined. Confirmation of the steady state using circadian changes of the trough value, estimation of the time to reach the steady state, the cumulative coefficient (Rods) based on the area under the curve (AUC), the clearance half-life in the terminal excretion phase ($t_{1/2\lambda}$) and comparison of AUC_{0-168h} (PEG-IFN alpha-2b) or AUC_{0-12h} for the first and final administrations were also determined. One patient whose IFN concentration exceeded the upper limit of the therapeutic range was excluded from this analysis.

Final virological response and hepatitis C virus dynamics in serum

Patients who were HCV-RNA negative at week 24 following completion of treatment were defined as having achieved an SVR. Patients who did not achieve an SVR were classified as nonresponders (NRs).

To analyse the effect of treatment on HCV dynamics, the amount of HCV-RNA was quantified at the following time points: immediately before initiation of the therapy and 4, 8, 24 h and 2, 4, 7, 14, 21, 28, 56, 84, 112, 140, 168 and 336 days after initiating therapy. The total RNA was extracted from the serum, and the amount of HCV-RNA at each time point was quantified by real-time detection PCR as reported previously [7,10]. The detection sensitivity of this assay was approximately 10 copies/mL, and the dynamic range for the method was from 10 to 1×10^8 copies/mL [11]. The viral decline curve was plotted on a semilogarithmic graph, and the slope of the exponential viral decline was calculated individually by a straight-line fit to the data for each viral decline phase.

Statistical analysis

Categorical data were compared by the chi-square test or Fisher's exact test. Distributions of continuous variables in the two treatment groups were analysed by Student's *t*-test. All tests of the confidence interval were two tailed, with the level of confidence level being set at 95%. *P*-values of <0.05 were considered statistically significant.

In order to analyse the pharmacokinetics of PEG-IFN alpha-2b and ribavirin, descriptive statistics were calculated at each blood collection, and the relationship between the time point of blood collection and the measured levels of the two drugs displayed graphically for each subject. These graphs included the mean value, standard error and the measured concentrations of the drugs at the first and after the final administration. In addition, these analyses were used to confirm the circadian trough values and to estimate the time to reach the steady state, based on AUC (Rods) and clearance half-life ($t_{1/2\lambda}$).

RESULTS

The demographics of the patients are shown in Table 1. No significant differences were found in mean age, gender proportionality, activity and stage of liver histology, serum ALT level and initial viral load between the PEG-IFN alpha-2b and non-PEG-IFN alpha-2b treatment groups. SVR rates in the PEG-IFN alpha-2b and non-PEG-IFN alpha-2b treatment groups were 69% (18/26) and 31% (8/26), respectively.

Differences in PKR mRNA expression in response to the different interferon treatment regimens

Sequential transcript analysis demonstrated an approximately 15-fold increase in PKR mRNA expression within 4 h following administration of conventional IFN alpha-2b. At

Table 1 Clinical characteristics of the patients in the two treatment groups of the study

	PEG-IFN alpha-2b plus ribavirin	IFN alpha-2b plus ribavirin	*P*-value (95% CI)
No. of patients	26	26	
Age (years), median (range)	53 (29–67)	53 (29–70)	0.66 (−4.18–6.57)*
Gender (male/female)	14/12	13/13	0.78†
Histology of the liver			
A1/A2/A3	12/11/3	14/11/1	0.56†
F1/F2/F3	14/10/2	13/7/6	0.28†
ALT (IU/L)	93 (72–113)	84 (63–105)	0.54 (−38.2–20.2)*
Haemoglobin (g/dL)	14.6 (14.2–15.0)	14.2 (13.6–14.9)	0.26 (−1.11–0.31)*
Platelet count ($\times 10^3$/mL)	179 (164–195)	171 (151–190)	0.47 (−3.32–1.56)*
Viral load ($\times 10^6$ copies/mL)	14.6 (9.00–20.2)	8.35 (3.77–12.9)	0.11 (−14.1–1.55)*
Ribavirin concentration at 4 W (ng/mL)	2413 (1451–3376)	2266 (1568–2963)	0.79 (−1281–985)*

Values are expressed as mean (95% CI).
*Unpaired *t*-test. †Chi-square test.

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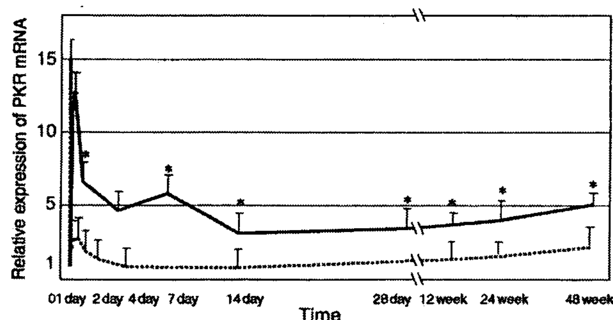

Fig. 1 Sequential expression of PKR mRNA in PBMCs during PEG- (solid line) and conventional (dotted line) IFN alpha-2b and ribavirin combination therapy. Expression of mRNA is shown as the expression level relative to baseline expression. The error bars indicate the standard error. An asterisk indicates a statistically significant difference in relative expression values between the two different IFN regimens ($P < 0.05$).

8 h, the level of PKR mRNA had fallen to a level that was twofold greater than the pre-treatment level (Fig. 1). With PEG-IFN alpha-2b administration, PKR mRNA expression reached a peak at 8 h at a level 12-fold greater than the pre-treatment level. At 24 h post-administration, the level of PKR mRNA had fallen but was still sixfold greater than the pre-treatment level (Fig. 1). This level was maintained until the next dose. No significant difference was observed in peak PKR mRNA expression between conventional IFN alpha-2b and PEG-IFN alpha-2b. However, from the second day of administration onwards, the expression was maintained at a significantly higher level in the PEG-IFN alpha-2b group compared with the conventional IFN alpha-2b group ($P < 0.05$) (Fig. 1).

Pharmacokinetics of serum pegylated interferon alpha-2b

The pharmacokinetic parameters for PEG-IFN alpha-2b at weeks 1 (first administration) and 48 (final administration) are shown in Table 2. Although the trough value of serum PEG-IFN alpha-2b varied between individuals, it almost reached a plateau at week 8. Accumulation of IFN was minimal in the PEG-IFN alpha-2b treatment regimen.

The level of serum PEG-IFN alpha-2b at week 1 increased gradually up to 12–24 h with a $t_{1/2\lambda_z}$ of 40.2 h. These levels were measurable up to 168 h after administration or immediately before the next administration. The trough value following administration showed no significant increase during the 48-week treatment phase (Fig. 2). The blood level after the final administration increased gradually for 12–24 h, remained high for approximately 48 h, and then decreased slowly with a $t_{1/2\lambda_z}$ of 55.3 h. The drug remained measurable up to 2 weeks post-administration. The cumulative coefficients (Rods) of repeated administrations calculated on the basis of C_{max}, C_{168h} and AUC_{0-168h} were 0.917, 2.11 and 1.12, respectively. When a comparison was made between the first and final administrations (weeks 1 and 48), $t_{1/2}$ of serum PEG-IFN alpha-2b levels was slightly prolonged after the final administration, although no changes were observed in C_{max}, AUC and plasma clearance (CL/F) (Table 2; Fig. 3).

Pharmacokinetics of serum ribavirin

The pharmacokinetic parameters for ribavirin at weeks 1 (first administration) and 48 (final administration) are summarized in Table 3. The trough value of serum ribavirin almost reached a plateau 8 weeks after the initial administration. In contrast to PEG-IFN alpha-2b, ribavirin was accumulated significantly during the first 4–8 weeks.

Serum ribavirin levels after the first administration (first day) reached t_{max} by 3.33 h and then decreased rapidly with a $t_{1/2\lambda_z}$ of 27.1 h. In contrast, serum ribavirin levels reached t_{max} by 2.73 h after the final administration and then decreased slowly with a $t_{1/2\lambda_z}$ of 296 h. A comparison of the cumulative coefficient (Rods) in the steady state was made between the first and final administrations and was calculated on the basis of C_{max}, C_{12h} and AUC_{0-12h}. This showed that by the final administration, there was a marked increase in C_{max} and AUC in serum ribavirin levels, an approximately 10-fold prolongation of $t_{1/2\lambda_z}$, a decrease in CL/F of about 1/3, and an approximately threefold increase in Vz/F. There was no change evident in t_{max} (Table 3; Fig. 4).

Clinical and virological response and serum pegylated interferon alpha-2b and ribavirin levels

The dose of PEG-IFN alpha-2b was reduced in two patients after 4 and 25 weeks of treatment because of neutropoenia. Similarly, the dose of ribavirin was reduced in three patients

Table 2 Pharmacokinetic parameters of the patients who received PEG-IFN alpha-2b at weeks 1 (first administration) and 48 (final administration)

	t_{max} (h)	C_{max} (pg/mL)	C_{168h} (pg/mL)	$t_{1/2\lambda_z}$ (h)	AUC (pg h/mL) 0–168 h	CL/F (mL/h/kg)	Vz/F (L/kg)
First	23.1	874	99	40.2	68 926	21.4	1.18
Final	22.2	774	185	55.3	77 039	–	–
Rods	–	0.917	2.11	–	1.12	–	–

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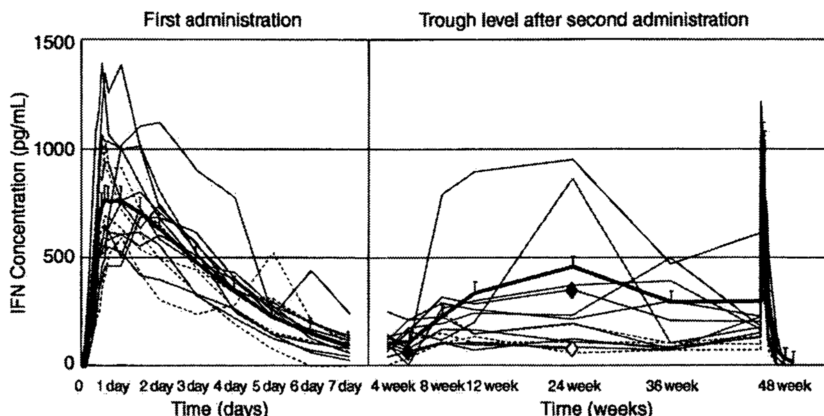

Fig. 2 Changes in serum IFN levels during PEG-IFN alpha-2b and ribavirin combination therapy. No significant increase in the trough value of serum IFN level was found during the 48-week treatment period. The bold lines indicate mean values, while the error bars indicate the standard error. Fine solid lines indicate a sustained virological responder and broken lines a nonresponder. The diamond-shaped symbol indicates a time point and IFN concentration at which either dose reduction (closed diamonds) or discontinuation (open diamonds) occurred.

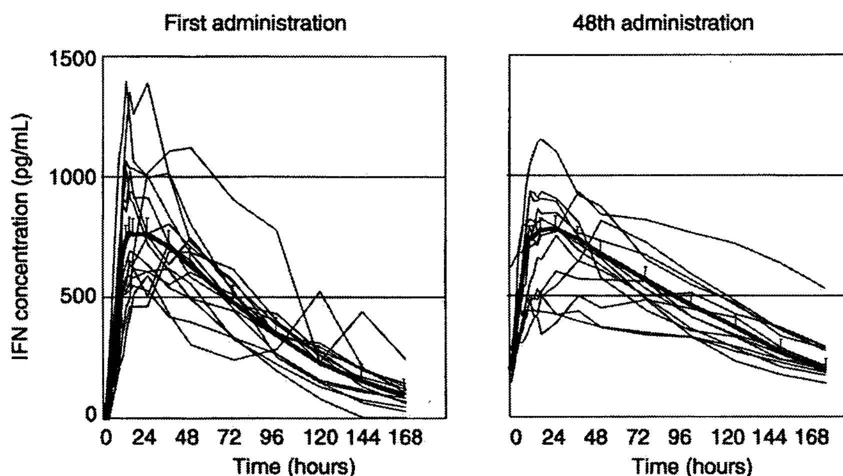

Fig. 3 A comparison of serum IFN levels between the first and 48th doses. Both show very similar values and no accumulation of IFN. It should be noted that PEG-IFN alpha-2b was detectable in all but one patient at 168 h after initial administration. Bold lines indicate mean values and the error bars indicate the standard error.

Table 3 Pharmacokinetic parameters of the patients who received ribavirin at weeks 1 (first administration) and 48 (final administration)

	t_{max} (h)	C_{max} (pg/mL)	C_{168h} (pg/mL)	$t_{1/2\lambda_z}$ (h)	AUC(pg h/mL) 0–168 h	CL/F (mL/h/kg)	Vz/F (L/kg)
First	3.33	604	221	27.1	4019	37.8	1472
Final	2.73	3449	2422	296	33 060	12.7	5374
Rods	–	6.53	12.2	–	9.42	–	–

after 12 and 16 weeks of treatment because of anaemia. In Figs 2 & 4, the individual time points and drug concentration following dose reduction are indicated by closed diamonds. No association could be found between dose reduction and serum concentration for both agents. Treatment was discontinued in 1 of the 15 patients because of depression, as indicated by open diamonds in Figs 2 & 4. Eleven patients including this patient achieved an SVR, with the remaining four patients being classified as NRs.

In order to demonstrate the association between virological response and pharmacokinetics, the final virological response for each individual is indicated in Figs 2 & 4. Serum IFN levels at 2 weeks post-dose tended to be slightly higher in NRs when compared with patients who achieved an SVR. This difference was not statistically significant. There was also no significant difference in serum ribavirin levels between these two groups from the time of the first administration until the completion of the 48-week treatment period.

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