INTRODUCTION

Haemolytic uraemic syndrome (HUS) is a serious affliction that involves acute renal failure, coagulation abnormality and blood-cell destruction. Approximately 90% of HUS cases are preceded by diarrhoea (Declrudt et al., 2000; Gianviti et al., 1994), most of which can be attributed to Shiga toxin (Stx)-producing bacteria, such as enterohaemorrhagic Escherichia coli (Karmali et al., 1985; Banatvala et al., 2001) or Shigella dysenteriae (Bhimma et al., 1997). Stxs are believed to damage endothelial cells of the microvascular system especially in the kidney, lung and colon epithelium (Laurence & Mitra, 1997). The endothelial cells express the specific receptor for Stxs, globotriaosyl ceramide (Gb3), and its content is enhanced by various pro-inflammatory cytokines, such as TNF-α (Molostvov et al., 2001; Eisenhauer et al., 2001). After endocytosis, Stxs travel through the Golgi apparatus (Arab & Lingwood, 1998), are processed by furin (Garred et al., 1995) and then, finally, reach their cellular target, the 60S ribosome. The A-subunit N-glycosidase activity inactivates the ribosome, resulting in protein-synthesis inhibition (Endo et al., 1988).

Previous studies have shown that human umbilical vein endothelial cells (HUVECs) or adult human saphenous vein endothelial cells (HSVECs) are resistant to nanomolar Stx concentrations in vitro (van de Kar et al., 1992; Keusch et al., 1996). In contrast, a 50 ng Stx1 kg⁻¹ dose, which would lead to picomolar concentrations in tissues, was lethal for primates in vivo (Taylor et al., 1999). Our previous data indicated that both HUVECs and HSVECs were moderately sensitive to Stxs at picomolar concentrations in the absence of cytokine stimulation at the beginning of the primary culture, whereas they obtained over a 1000-fold resistance to Stxs after only 7–14 days of in vitro passage (Yoshida et al., 1999). The HUVEC and HSVEC resistant phenotype after passage was consistent with previous studies and the native response of these endothelial cells in vivo might be different from in vitro results. According to our preliminary trials, the conversion rate toward the toxin-resistant phenotype in vitro was accelerated by IFN addition (T. Yoshida et al., unpublished data), suggesting a possible application to induce such a toxin-resistant phenotype in other cells. The endothelial cells from a microvascular origin stayed highly sensitive to Stxs at femtomolar concentrations during in vitro passage (Ohmi et al., 1998; Pijpers et al., 2001). Such sensitivity agreed with the observation of microvascular damage in the in vivo primate model (Siegler et al., 2001). In this study, IFN-γ pre-treatment was shown to induce strong resistance against Stxs in human lung microvascular endothelial cells (HLMECs). This was a peculiar but intriguing phenomenon in that a pro-inflammatory cytokine could reduce, instead of enhance, the lethal response of human cells to Stxs.

Abbreviations: CD50, 50% cytotoxic dose; HLMEC, human lung microvascular endothelial cell; IFN-γ, γ interferon; Stx, Shiga toxin; TNF-α, tumour necrosis factor α.
METHODS

Reagents. Human plasma fibronectin, 0-25% trypsin/1 mM EDTA solution and recombinant Insulin-like growth factor-1 were purchased from Gibco-BRL. Recombinant human TNF-α was obtained from PeproTech EC (20 U ng⁻¹). Recombinant vascular endothelial cell growth factor and IFN-γ were from Wako Pure Chemical Industries and Shionogi & Co., respectively. Purified preparations of Stx1 and Stx2, which gave single peaks on HPLC (Kondo et al., 1997) and contained endotoxin at 3.10⁻⁶ g ml⁻¹, were kindly supplied by the Aichi Prefectural Institute of Public Health, Japan.

Cell culture conditions. Four independent lots of HLMCECs (4th passage) were purchased from BioWhittaker and were cultured in MCDNB131 medium supplemented with 10% fetal calf serum, VEGF, IGF-1 and Serum extender (Collaborative Biomedical Products) (complete medium). All plastic culture plates were coated with MCDB131 medium supplemented with 10% fetal calf serum, VEGF, passage) were purchased from BioWhittaker and were cultured in complete medium. All plastic culture plates were coated with human fibronectin at 5 μg cm⁻² before use. All experiments were done within three generations of passage. The IFN-γ treatment was performed in sub-confluent conditions in a 24-well plate.

Cell viability analysis. Cells were harvested from the 24-well plates by using 0-25% trypsin/1 mM EDTA and plated onto a Terasaki plate (Greiner Labotechnik) at 10⁵ cells per well or in a 96-well plate at 5 x 10⁵ cells per well and subsequently exposed to Stxs for 24-72 h in sub-confluent conditions. Then, the viabilities of the cells was assessed by the uptake and digestion of calcine/AM (Molecular Probes) as described previously (Yoshida et al., 1999) or by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] reduction. The data obtained on calcine/AM uptake were comparable to the MTT reduction readout. The CD₅₀ values were calculated using the ALLFIT program (Guardabasso et al., 1988) from the means of triplicate data. The cell growth rate was measured by [³H]thymidine uptake for 24 h using 37 kBq per 5 x 10⁵ cells.

Protein synthesis analysis. The degree of protein synthesis was assessed by the incorporation of [³H]-labelled methionine (Tran³H-label; ICN Biomedicals) into intracellular proteins. HLMECs were plated into a 96-well plate (5 x 10³ cells per well) and treated with 1000 U IFN-γ ml⁻¹ for 3 days. Cells were exposed to Stx2 for 24 h and pulsed with 37 kBq of [³H]-labelled methionine per well for 1 h at 37°C. The cells were dislodged with 0-25% trypsin/1 mM EDTA and harvested on a glass fibre filter using 0-5% EDTA/PBS. After proteins were denatured with 10% trichloroacetic acid, the filter was rinsed four times and subjected to liquid scintillation counting.

Toxin binding and uptake analysis. Stxs (10 pmol) were labelled with ¹²⁵I by incubation with 18-5 MBq Na¹²⁵I (ICN Biomedicals) for 2 min at 26°C in the presence of Iodo-beads (Bio-Rad). The labelled protein was purified on a PD-10 column (Pharmacia-Biotech). The specific radioactivities were 14-0 and 24-6 Bq fmol⁻¹ for Stx1 and Stx2, respectively. To analyse toxin binding, the ¹²⁵I-labelled Stx1 or Stx2 was added to 10⁵ cells at 1 nM in complete medium, followed by incubation at 4°C for 3 h. After washing three times, the cells were subjected to γ-counting. The value obtained in the presence of 50 nM cold Stxs was assumed as the non-specific binding value. For toxin uptake analysis, 3 x 10⁶ cells were incubated with 109 pM ¹²⁵I-labelled Stx2 for 20 h at 37°C in the complete medium, harvested in 0-25% trypsin/1 mM EDTA, washed four times and then counted.

Subcellular localization of Stx2. The cell suspension (100 μl) from the toxin uptake analysis was homogenized in the presence of protease inhibitor cocktail (Sigma) and centrifuged at 500 g for 5 min. The supernatant was overlaid on 0-9 ml of 22% Percoll (Pharmacia-Biotech) in 0-25 M sucrose and 15 mM HEPES/NaOH (pH 7-4), and spun at 28000 g for 30 min in a TLA100.2 rotor in a TL-100 centrifuge (Beckman Instruments). The resultant density gradient was fractionated into 50 μl fractions and counted for radioactivity. The locations of the cytoplasmic and endosomal fractions were determined by the enzymic activities of lactate dehydrogenase and β-hexosaminidase, respectively (Wanders et al., 1989).

RESULTS

HLMECs became resistant to Stxs by IFN-γ

All four of the employed HLMCEC lots were extremely sensitive to Stxs (Table 1, Fig. 1) during three passages without any pre-treatments, in which the CD₅₀ values were comparable with previous reports (Ohmi et al., 1998; Pijpers et al., 2001). The Stx2 toxicity exceeded the toxicity of Stx1, reproducing results from other studies. In our hands, cell death did not apparently involve apoptosis, as suggested by negative TUNEL (terminal deoxynucleotidyl transferase mediated dUTP nick end-labelling) staining (data not shown). However, HLMEC responses to Stxs were surprisingly modified by prior IFN-γ treatment. Outstanding resistance to Stx2 was induced by 3–4 days treatment with IFN-γ (1000 U ml⁻¹) by 1000- to 100 000-fold in all tested

<table>
<thead>
<tr>
<th>Cell lot no.*</th>
<th>Treatment</th>
<th>CD₅₀ (pM)†</th>
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<tbody>
<tr>
<td></td>
<td>Stx1</td>
<td>Stx2</td>
</tr>
<tr>
<td>1</td>
<td>None</td>
<td>0-063</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
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</tr>
<tr>
<td>2</td>
<td>None</td>
<td>0-023</td>
</tr>
<tr>
<td></td>
<td>1000 U IFN-γ ml⁻¹, 1-5 days</td>
<td>0-036</td>
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<td>1-32</td>
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<tr>
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<td>100 U IFN-γ ml⁻¹, 3 days</td>
<td>0-028</td>
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<tr>
<td></td>
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</tr>
<tr>
<td>3</td>
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<td>0-098</td>
</tr>
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<td>21-7</td>
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<td>0-359</td>
</tr>
<tr>
<td></td>
<td>1000 U IFN-γ ml⁻¹+100 U TNF-α ml⁻¹, 4 days</td>
<td>1-35</td>
</tr>
</tbody>
</table>

*Each lot was obtained from an independent subject according to the manufacturer (BioWhittaker).
†After treatment, cells were exposed to Stxs for 24 h (lot nos 1 and 2) or 48 h (lot no. 3), and CD₅₀ values were calculated from the mean calcine/AM uptake of triplicate data using the ALLFIT program (Guardabasso et al., 1988).
lots (Table 1, Fig. 1). A lower but significant effect was observed at 100 U IFN-γ ml⁻¹ (Table 1). Interestingly, the degree of CD₅₀ increase was smaller in the case of Stx₁ after 3 days treatment, but it surged to a level analogous to that of Stx₂ in 6 days. The minimal required pre-treatment period until the appearance of the effect was reproducibly longer than 2 days in multiple experiments (data not shown), as represented by the data in Table 1. Notably, IFN-γ was not required to be present continuously, which was shown by a significant effect of an 8-h pulse and a 3-day incubation without IFN-γ (Table 1). As suggested by the comparable CD₅₀ between 24 h (lot nos 1 and 2) and 48 h (lot no. 3) exposure to Stxs, the apparent toxin resistance was not an artefact due to a slower rate of cell death. The idea was further supported by an increase in viability (30–38 %) from 48 to 72 h exposure to Stx₂ at 1 nM, which was still possible that the intracellular toxin uptake was highly affected by minute differences in the specific binding. However, the intracellular ¹²⁵I-labelled Stx₂ amounted to 2-64 ± 0-63 fmol per 10⁵ cells by 20 h incubation with IFN-γ at the CD₅₀ concentration of 10⁹ pM (lot no. 3). Furthermore, the intracellular ¹²⁵I-labelled Stx₂ was distributed in the cytoplasmic fraction (37 %), as determined by subcellular fractionation analysis, indicating that the major part of Stx₂ was not associating with the plasma membrane. Also, it exhibited an intact molecular size on gel permeation chromatography (data not shown). In contrast, the intracellular Stx₂ required to damage 50 % of the native HLMECs should be less than 0-011 fmol per 10⁵ cells, which was the entire Stx₂ input at the CD₅₀ concentration, and was far below the detection limit (0-1 fmol). Thus, the amount of intracellular Stx₂ required to cause equivalent damage in the IFN-γ pre-treated HLMECs was apparently over 100-fold greater than in the native cells.

Protein synthesis was inhibited by CD₅₀ or a lower dose of Stx₂ after IFN-γ treatment

Since a large amount of Stx₂ was incorporated into the cytoplasm even after IFN-γ treatment, the degree of protein synthesis was measured to confirm the toxin activity inside the cell. When 100 pM of Stx₂, an approximate CD₅₀ concentration (lot no. 3), was applied to HLMECs after IFN-γ treatment, the rate of protein synthesis was highly suppressed to 11-8 % of the control levels after 24 h exposure (Fig. 3). Moreover, a 10-fold lower concentration, 10 pM, which allowed for 70 % cell viability, also decreased protein synthesis to 24-8 % (Fig. 3), indicating that the Stx₂ N-glycosidase activity is indeed functioning inside the cells. In contrast, the degree of protein synthesis inhibition in the
native HLMECs was consistent with the viability throughout the doses examined (Fig. 3).

**TNF-α neutralized the protective effect of IFN-γ against Stxs**

A significant but weak sensitization was induced in native HLMECs by a 4-day treatment with a high dose of TNF-α (1000 U ml⁻¹, 50 ng ml⁻¹) (Table 1, Fig. 1), but not by a 1-day treatment (data not shown). In contrast, TNF-α addition to IFN-γ treatment reverted the protective effect of IFN-γ by nearly 100- (Stx1) or 1000-fold (Stx2) (Table 1, Fig. 1). It is noteworthy that the resultant CD₅₀ values were still higher than the native ones.

**DISCUSSION**

In this study, a representative of adult microvascular endothelial cells, HLMECs, which are quite sensitive to femtomolar doses of Stxs, could be rendered highly resistant to Stxs by IFN-γ treatment. The extent of the sensitivity difference exceeded three to four orders of magnitude in terms of the CD₅₀. The required pre-treatment time of 3 days until the appearance of the effect might suggest that certain sequential signalling steps are required after IFN-γ stimulation. Moreover, a weaker but significant effect induced by an 8-h pulse treatment and incubation without IFN-γ indicated that the initial triggering was the crucial event to induce the phenotype conversion. Although the IFN-γ concentration employed here was relatively high compared with physiological conditions, it is not impractical under pharmacological settings (Wills, 1990).

Whether the decreases in receptor expression and toxin uptake were responsible for the Stx resistance was examined by comparing the amounts of bound or intracellular Stx before and after IFN-γ treatment. Stx binding at 4°C decreased by no greater than twofold after IFN-γ treatment, which was measured at the same 1 nM Stx concentration to reflect the receptor content at equilibrium. Next, the amounts of Stx2 uptake were compared at the CD₅₀ toxin concentrations, which damage equivalent proportions of the native and Stx-resistant cells. However, the amount of Stx2 inside the native HLMECs was too small to be detected, even with the radioisotopic labelling. On the other hand, the intracellular toxin in the Stx-resistant phenotype was present at 2·6 fmol per 10⁵ cells and was more than 100-fold greater than in the native ones, even if the native cells incorporated the total toxin input (0·011 fmol). There still remained a possibility that Stxs were transferred to lysosomes and degraded, instead of travelling through the Golgi apparatus to the cytoplasm in an active form (Arab & Lingwood, 1998). However, a significant proportion of the intracellular Stx2 was recovered from the cytoplasmic fraction without any apparent degradation, where the intracellular toxin inactivated ribosomes by N-glycosidase activity (Endo et al., 1988). Correspondingly, a remarkable inhibition of protein synthesis was observed, which indicated that the intracellular Stx2 inactivated ribosomes, but did not terminate cell viability. In contrast, the inhibition of protein synthesis coincided well with the cell viability in the case of the native HLMECs, possibly because the viability decrease might have resulted in the overall reduction in protein synthesis. These data indicated that the mode of toxin action might be different in the native and resistant phenotypes and also showed a discrepancy between the ribosome inactivation and the cytotoxicity of Stxs.

To date, many *in vitro* studies have indicated that pro-inflammatory cytokines are intimately involved in the endothelial damage caused by Stxs, through enhancing receptor expression and sensitizing the target cells (Molostrov et al., 2001; Eisenhauer et al., 2001). In the particular case of native HLMECs, which exhibit high sensitivity to Stxs in the basal state like other microvascular endothelial cells (Ohmi et al., 1998; Pijpers et al., 2001), the CD₅₀ decreased only moderately even after 4 days treatment with a high TNF-α dose (Table 1) and not at all after 1 day of treatment (data not shown). In contrast, TNF-α addition almost reverted the Stx-resistant phenotype induced by IFN-γ to basal sensitivity. The residual resistance of HLMECs would exclude a synergy of these cytokines here. This phenomenon corresponded with other results on the effects of pro-inflammatory cytokines and supports the hypothesis that various endothelial cells *in vitro* have already obtained a Stx-resistant phenotype, as observed in human umbilical vein endothelial cells, and respond to pro-inflammatory cytokines to a higher degree.
It is reported that IFN-γ, but not TNF-α, induces apoptosis in human endothelial cells and the degree is enhanced by both IFN-γ and TNF-α (Wang et al., 1999). Correspondingly, the combination of these cytokines reduced HLMEC viability to about 40% of the control levels (data not shown), although only the growth rate was moderately suppressed by IFN-γ alone. The surviving HLMECs from the pre-treatment with these cytokines still showed the weak but significant Stx-resistant phenotype described above. This cytotoxic effect would endanger a simple IFN-γ application as a novel therapy. However, the concept of converting the host-cell response rather than inhibiting toxin binding would be feasible, especially given that the factors responsible for the phenotypic difference have been elucidated.

Collectively, a highly resistant phenotype could be induced in human microvascular endothelial cells by IFN-γ treatment for a few days and neutralized by TNF-α addition. This is the first case of a cytokine reducing the lethal response of human cells to Stxs. It was intriguing that protein synthesis inhibition by Stxs was observed, despite the maintenance of cell viability, suggesting an unknown mechanism of Stx toxicity. In addition, these phenomena raise the possibility of a novel therapeutic approach to focus on the host-cell response to Stxs.

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