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Abstract

The pathogenesis of diffusely adherent *Escherichia coli* (DAEC) remains to be elucidated. Previously, we found that afimbrial adhesin gene (*afa*)-positive motile DAEC strains isolated from patients with diarrhoea induce high levels of IL-8 secretion in Caco-2 cells via toll-like receptor 5 (TLR-5), while non-motile strains did not. The aim of this study was to compare virulence properties, including the phylogenetic groups, *afa* subtypes, IL-8 secretion levels, and the effects on tight junctions, of DAEC strains isolated from healthy persons with those isolated from patients with diarrhoea. Induction of IL-8 secretion in Caco-2 cells was examined for a total of 36 *afa*-positive strains: 19 from diarrhoeal patients and 17 from healthy carriers. Irrespective of the source, all strains were classified into the phylogenetic group B2 or D, with the exception of two strains. All 7 motile strains isolated from diarrhoeal patients induced high levels of IL-8 secretion, while only 6 of 15 motile strains from healthy carriers induced IL-8 secretion to the same levels as the diarrhoeal strains. We speculated that additional virulence factors other than *afa* and motility cause the loosening of tight junctions that allows flagellin to reach TLR-5 located on the basolateral side of the epithelium. However, no differences in the TER and dextran permeability were observed between cells infected with diarrhoeal strains and those from healthy persons. Thus, diarrhoeagenic DAEC seems to possess additional factors, in addition to adhesin and flagellin, which can induce high IL-8 secretion.

Keywords: DAEC; Virulence; Flagella; IL-8; Tight junction

1. Introduction

Escherichia coli is the predominant facultative anaerobe of the normal colon flora. However, particular strains can cause sepsis, urinary tract infections, and diarrhoeal disease. On the basis of pathogenic features, diarrhoeagenic *E. coli* (DEC) are classified into at least six categories, including enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), Shiga toxin-producing *E. coli* (STEC), enteroaggregative *E. coli* (EAEC) and diffusely adherent *E. coli* (DAEC) (Croxen and Finlay, 2010). Colonization of the human intestine is an essential part of the infection process. Diffusely adherent *E. coli* (DAEC), the sixth DEC class, shows diffuse adhesion to HEp-2 cells (Scaletsky et al., 1984). Its role in diarrhoeal disease, however, has been controversial. DAEC isolated from children with and without diarrhoea showed no diarrhoeagenicity in adult volunteers (Giron et al., 1991; Echeverria et al., 1992; Jallat et al., 1993). Several groups have suggested that an age-dependent susceptibility may explain the epidemiological discrepancy, because a positive association with diarrhoea was found when study populations were age-stratified (Scaletsky et al., 1984; Giron et al., 1991; Baqui et al., 1992; Gunzburg et al., 1993; Levine et al., 1993; Scaletsky et al., 2002). However, clinical microbiologists continue to hesitate to classify DAEC isolates as causative agents. DAEC likely comprises a heterogeneous group of organisms with variable enteropathogenicity (Poitrineau et al., 1995). Measuring diffuse adhesion alone is insufficient to evaluate the diarrhoeagenicity of strains, and therefore, other distinguishing characteristics are needed.

DAEC possesses no typical virulence markers associated with diarrhoea other than a characteristic adherence to cultured epithelial HEp-2 or HeLa cells. Various operons

with a genetic organization similar to that of afimbrial adhesive sheath (Afa), which was identified as an adhesin of uropathogenic *E. coli* (Labigne Roussel et al., 1984), have been recognised as genes of colonization antigens (Le Bouguénec et al., 2001), although F1845 was initially thought to be a fimbrium that was unique to DAEC (Bilge et al., 1989). Germani et al. (1996) reported that DAEC isolation rates differ significantly between patients with diarrhoea and healthy controls only when the presence of the Afa sequence was considered.

The Afa/Dr family currently includes at least 13 adhesins, comprising both fimbrial and afimbrial adhesins (Servin, 2005). Servin proposed two classes of Afa/Dr: Afa/Dr_{DAF}, which recognise Dr antigen with an exposed decay-accelerating factor (DAF) domain (Telen et al., 1988) of the Cromer blood-group system; and Afa/Dr^{-}, which do not bind to human DAF. Afa/Dr_{DAF} are further subdivided into two subtypes: Afa/Dr_{CEA} (AfaE-III, Dr, and F1845), which bind to human carcinoembryonic antigen (CEA; CD66e) and CEA-related cell adhesion molecules 1 (CEACAM1; CD66a) and 6 (CEACAM6; CD66c); and Afa/Dr_{CEA}^{-} (AfaE-I and Dr-II), which do not exhibit such binding. The other Afa/Dr_{DAF} (AfaE-II, AfaE-V, and NFA-I) remained to be subtyped yet. DAEC possessing Afa/Dr^{-} adhesins (AfaE-VII and AfaE-VIII) is likely present in animals as well as in humans.

Bétis et al. (Bétis et al., 2003a; Bétis et al., 2003b) found that two DAEC strains induce interleukin-8 (IL-8) in the human colonic epithelial cell line T84. Compared to low virulent strains, diarrhoeagenic enteroaggregative *E. coli* (EAEC) reportedly causes a larger amount of the proinflammatory chemokine IL-8 to be released from human intestinal epithelial cells. Faecal IL-8 elevation may correlate with the severity of clinical symptoms (Steiner et al., 1998; Steiner et al., 2000; Jiang et al., 2002).

Subsequently, we found that some DAEC strains that cause epithelial cells to secrete high levels of IL-8 are likely to be diarrhoeagenic in paediatric patients (Arikawa et al., 2005; Meraz et al., 2006; Meraz et al., 2007).

A previous study clearly showed that motile Afa/Dr DAEC strains isolated from diarrhoeal patients induce IL-8 secretion via signal induction by the interaction between TLR-5 and bacterial flagella (Arikawa and Nishikawa, 2010); however, it was suggested that motile strains isolated from asymptomatic people failed to induce IL-8 secretion even in the presence of flagella (Fujihara et al., 2009). Unless epithelial polarity is changed, the flagella cannot, theoretically, interact with the innate receptor toll-like receptor 5 (TLR-5) since it is normally located on the basolateral side of epithelia (Gewirtz et al., 2001). In this study, we investigated whether motile Afa/Dr DAEC strains always induce high amounts of IL-8 secretion. Furthermore, the effects of Afa/Dr DAEC infection on tight junctions (TJs) of tissue culture epithelial cells were compared between strains isolated from patients with diarrhoea and those from healthy carriers.

2. Materials and methods

2.1. Bacterial strains

A total of 36 *afa*-positive DAEC strains were used in this study (Table 1). Nineteen strains were isolated as causal agents from stool specimens of 924 patients with diarrhoea in a surveillance study (Nishikawa et al., 2003); they were not included as etiologic agents in that report because the enteropathogenicity of DAEC is still

controversial. The remaining 17 strains were isolated from stool specimens of 2,230 healthy persons in a surveillance study (Fujihara et al., 2009). EAEC strain V546, and *E. coli* laboratory strains DH5α and HB101 were used as positive and negative controls, respectively, in the IL-8 induction assays. Bacterial inocula were prepared by culturing the strains in Luria Broth (Becton Dickinson and Co., Sparks, MD) at 37°C for 16 h.

2.2 Phylogenetic classification

Phylogenetic grouping of the DAEC strains was performed by a simple, rapid PCR-based technique that uses a combination of three DNA markers (*chuA*, *yjaA* and *tspE4.C2*), generating 279, 211, and 152-bp fragments, respectively (Clermont et al., 2000). A triplex PCR was performed using the six primers in a single reaction. The results of these three amplifications allowed classification of the strains into one of four major phylogenetic groups: A, B1, B2 or D. AfaE subtypes of the strains were based on our previous reports (Arikawa et al., 2005; Fujihara et al., 2009).

2.3. Epithelial cell line

Caco-2 cells were grown in 25-cm^2 polystyrene tissue culture flasks with EMEM containing 2 mM L-glutamine, 0.15% $NaHCO_3$, and 10% FBS at 37°C in a 5% CO_2 incubator.

2.4. IL-8 induction test

Infection of the epithelia was performed as previously described (Arikawa and Nishikawa, 2010). Briefly, Caco-2 cells were seeded at high density (approximately $5 \times 10^4 – 10^5$ cells/well) in 24-well tissue culture plates. For each experiment, Caco-2 cells were cultured for at least 2 weeks, with the medium changed twice a week. When confluent, the monolayers were washed three times with PBS in the 24-well plates. Then, 1 ml of EMEM containing D-mannose (1%, w/v) and FBS (0.5%, v/v) without antibiotics was added to each well. The cells were infected with the bacteria suspended in EMEM at a bacteria-to-cell ratio of 10:1, and the mixtures were incubated at 37°C for 22 h. A 100-µl aliquot of culture medium was used for enzyme-linked immunosorbent assay (ELISA) of IL-8 using the Quantikine Human IL-8 Immunoassay (R&D System, Minneapolis, MN).

2.5. Test for epithelial barrier integrity

Caco-2 cells were seeded on 6.5-mm Transwell inserts with a 0.4-µm pore size membrane insert (Corning Inc., Corning, NY) in 24-well plates and grown for 14 days before the experiment, and the medium was changed twice a week until the transepithelial electrical resistance (TER) reached the range of 230 – 330 $\Omega \cdot cm^2$. The TER was measured using a Millicell Electrical Resistance System (Millipore Corporation, Bedford, MA) and was calculated as $\Omega \cdot cm^2$ using the measured electrical resistance and the surface area of the filter (0.33 cm^2). Bacterial inoculation was performed as described above. The reading from a cell-free control filter was subtracted from the obtained values to remove background resistance.

The permeability of Caco-2 cell monolayers grown on the Transwell inserts with a

0.4-μm pore size membrane insert (Corning) was determined by measuring the paracellular passage of FITC-dextran (molecular weight, 4 kDa) (Sigma, St. Louis, MO) from the apical to the basolateral compartment. The bacterial inoculum was prepared in EMEM containing FITC-dextran, and the 200-μl mixture was loaded onto the apical side of the monolayer. The FITC-dextran concentration in the basolateral compartment was determined longitudinally on the basis of fluorescence intensity analysed using a spectrofluorophotometer (Wallac 1420 ARVOSX; PerkinElmer Life Sciences, Boston, MA) at an excitation wavelength of 485 nm and an emission wavelength of 535 nm.

2.6. Statistical analysis

All post hoc multiple comparisons between the groups were performed using Tukey's test with the statistics add-in software Statcel 2 (OMS, Tokorozawa, Japan) for Microsoft Excel, unless otherwise stated.

3. Results

All 7 motile Afa/Dr DAEC strains isolated from diarrhoeal patients induced higher amounts of IL-8 secretion compared with the non-motile strains and negative controls, whereas only 6 out of 15 motile strains isolated from healthy carriers triggered IL-8 secretion to similar levels (Fig. 1). To clarify whether this difference in IL-8 secretion directly reflects the capacity of DAEC strains to loosen/open TJs, we longitudinally measured the TER and paracellular passage of FITC-dextran as indices of barrier integrity of cultured epithelial cells. After inoculation of Afa/Dr DAEC strains into the

apical medium, the TER increased over the first 6 h. However, it started to decrease after 9 to 12 h (Fig. 2A). Subsequently, FITC-dextran passed through the epithelial monolayers to the basolateral compartment, but this was independent of the level of IL-8 induced by the inoculated strains (Fig. 2B). In contrast, the non-pathogenic *E. coli* strains, DH5α and HB101, did not affect the barrier integrity of the epithelium.

The phylogenetic grouping did not reveal any significant differences between the 19 strains isolated from diarrhoeal patients and the 17 strains isolated from healthy carriers; the former comprised of one strain of A, 9 strains of B2 and 7 strains of D, while the latter comprised one strain of A, 8 strains of B2 and 10 strains of D (Table 1). No strain was identified as belonging to the phylogenetic group B1. Subtyping of the AfaE gene showed that 13 of 19 clinical isolates had AfaE1 or AfaE3 and only 6 strains had untypable genes. In contrast, with the exception of 2 strains, all 15 other strains from healthy carriers had untypable AfaE. Typable AfaE was significantly more prevalent among patient strains ($p < 0.001$, Fisher's exact probability test).

4. Discussion

Although DAEC is often isolated from faecal specimens (Jallat et al., 1993), it is unknown whether these strains are all enteropathogenic. If only certain strains of DAEC are diarrhoeagenic, methods for the discrimination between diarrhoeagenic and non-diarrhoeagenic strains are required. Previously, we found that some Afa/Dr DAEC strains induce IL-8 secretion from cultured human intestinal cells, whereas others cause only a weak reaction despite their diffuse adhesion (Arikawa et al., 2005); if chemokine

induction is an essential step for full virulence, only a subset of Afa/Dr DAEC strains would be expected to be enteropathogenic. Thus, diffuse adhesion itself does not necessarily indicate that the strain can cause inflammation in the intestine. This may explain why epidemiological studies have so far failed to yield conclusive answers regarding the role of DAEC in diarrhoeal disease. In fact, we found that high IL-8-inducing strains were significantly more prevalent among 1- to 4-year-old diarrhoeal patients, who are reported to be prone to DAEC infection (Meraz et al., 2007).

The present study showed that motile Afa/Dr DAEC strains from patients with diarrhoea induce high levels of IL-8 secretion, but that strains from healthy carriers often induce much lower levels of the cytokine. Arikawa et al. (2010) suggested that flagella play important roles in inducing IL-8, and that levels of the inflammatory chemokine induced after DAEC infection is higher in Caco-2 cells with an initial TER below 150 $\Omega \cdot cm^2$; TLR-5 is reported to be located on the basolateral side of intestinal epithelia (Gewirtz et al., 2001), and few flagella can reach from the apical side to the basolateral side when the barrier function of TJs is normal. Although partially purified flagella alone induced IL-8 secretion (data not shown), larger amounts of flagella were required compared to the amount of flagella estimated to be present on inoculated DAEC organisms. We hypothesised that motile strains isolated from healthy carriers would not reduce barrier integrity and that induction of IL-8 secretion by these motile strains would be weaker than that induced by motile strains isolated from diarrhoeal patients. However, some motile strains that induced little IL-8 secretion were able to affect TJs to the same degree as high IL-8-inducing strains. Pathogens must co-ordinately regulate motility and adhesion (Pesavento et al., 2008; Simms and Mobley,

2008). Upon adhesion to surfaces, *Pseudomonas aeruginosa* alters its outer membrane composition, thereby shedding flagellin (Gerstel et al., 2009). Likewise, high IL-8-inducing strains of Afa/Dr DAEC may also have additional unknown mechanisms for shedding flagella, allowing for stable colonization and efficient delivery of flagella or flagellin to TLR-5.

Since the role of inflammatory cells is to remove harmful pathogens, it is likely that the low IL-8 inducers isolated from healthy carriers lost the virulence to avoid the activation of epithelial cells or have unknown mechanisms that prompt epithelial cells to secrete tolerance signals to maintain the epithelial cells in a quiescent state (Neish, 2009); if this is the case, the benefits of IL-8 induction in highly IL-8-inducing strains is of great interest. A theory suggesting that inflammation is unlikely to be detrimental for all pathogens has been proposed (Stecher et al., 2007). Commensal microflora inhabit and shield the intestine from infection; this is referred to as colonization resistance. Inflammatory host responses induced by salmonella can change the surrounding microflora to help the pathogen overcome colonization resistance. It is possible that high IL-8-inducing DAEC may also utilise the host response to overcome colonization resistance in a similar manner.

It is generally accepted that human faecal isolates belong to groups A and B1, and extraintestinal pathogenic *E. coli* belongs to groups B2 and D (Duriez et al., 2001). Although a recent review indicates that strains of group A are predominant, followed by B2 strains, and that B1 and D strains are less common (Tenaillon et al., 2010), in this study, phylogenetic grouping clearly showed that Afa/Dr DAEC strains were present at different degrees in the B2 and D groups, and few were present in the A and B1 groups. Since Afa/Dr was first identified as a colonization factor for urinary tract infection, the

accumulation of Afa/Dr DAEC in the phylogenetic groups of B2 and D is concordant with previous reports (Zhang et al., 2002; Anastasi et al., 2010). Among animal strains, group B1 is predominant, followed by groups A, B2, and D. The present results indicate that Afa/Dr DAEC is unlike Shiga toxin-producing *E. coli*, of which the main reservoir is domestic animals. Since DAEC belonged to groups B2 and D, this supports our recent findings that Afa/Dr DAEC cycles from person to person rather than among animals (Wang et al., 2011).

5. Conclusion

Diffuse adhesion and the presence of flagella are two possible criteria for predicting the ability of DAEC isolates to induce high amounts of IL-8 secretion in clinical laboratories. However, strains isolated from healthy individuals often do not trigger IL-8 secretion. No difference was found between DAEC strains isolated from patients with diarrhoea and healthy carriers in their ability to loosen epithelial TJs. It is possible that high IL-8 inducers have a third factor that contributes to the enhancement of IL-8 secretion, or that low IL-8 inducers have other factors that can maintain the epithelial cells in a quiescent state.

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Conflict of interest

We declare that we have no conflicts of interest in connection with this paper.

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Table 1. Properties of Afa/Dr+ DAEC strains used in this study.

Strain	Origin	Motility[a]	Adhesion[b]	*afaE* subtype	Phylogenetic Group
V1	Patient	+	DA	AfaEX	D
V19	Patient	+	DA	AfaEX	D
V36	Patient	+	DA	AfaE1	D
V64	Patient	+	DA	AfaEX	D
V554	Patient	+	DA	AfaEX	D
V561	Patient	+	DA	AfaE1	B2
V827	Patient	+	DA	AfaEX	D
V205	Patient	-	DA	AfaEX	D
V242	Patient	-	DA	AfaE3	B2
V289	Patient	-	DA	AfaE1	A
V547	Patient	-	DA	AfaE3	B2
V550	Patient	-	DA	AfaE3	B2
V582	Patient	-	DA	AfaE1	D
V599	Patient	-	DA	AfaE1	D
V679	Patient	-	DA	AfaE3	B2
V720	Patient	-	DA	AfaE1	D
V725	Patient	-	DA	AfaE3	B2
V880	Patient	-	DA	AfaE3	B2
V922	Patient	-	DA	AfaE3	B2
SK164	Carrier	+	DA	AfaEX	D

SK189	Carrier	+	DA	AfaEX	B2
SK237	Carrier	+	DA	AfaEX	D
SK279	Carrier	+	DA	AfaEX	D
SK324	Carrier	+	DA	AfaEX	D
SK407	Carrier	+	DA	AfaEX	B2
SK476	Carrier	+	DA	AfaEX	B2
SK493	Carrier	+	DA	AfaEX	D
SK577	Carrier	+	DA	AfaEX	B2
SK832	Carrier	+	DA	AfaEX	B2
SK1144	Carrier	+	DA	AfaEX	B2
SK1157	Carrier	+	DA	AfaEX	B2
SK1402	Carrier	+	DA	AfaEX	B2
SK1431	Carrier	+	DA	AfaE2	D
SK1472	Carrier	+	DA	AfaE3	D
SK1247	Carrier	-	DA	AfaEX	B2
SK1256	Carrier	-	DA	AfaEX	A
V546	Patient	+	AA	EAEC	A
DH5α		-	-	-	A
HB101		-	-	-	A

[a]Motility was determined in lysine-indole motility medium at 37°C.

[b]DA: diffused adherence; AA: aggregative adherence

Figure captions

Fig. 1. IL-8 secretion from Caco-2 cells induced by Afa/Dr DAEC strains. (A) IL-8 induction by DAEC strains isolated from diarrhoeal patients. Black and white columns indicate IL-8 secretion induced by motile and non-motile strains, respectively. The average secretion of IL-8 induced by each non-motile strain was significantly lower than that induced by the reference strain V546 of enteroaggregative *Escherichia coli*, which was used as a positive control ($p < 0.01$). DH5α and HB101 were used as negative controls. EMEM containing TNF-α (100 ng/ml) was used as an additional positive control for IL-8 induction to validate the appropriateness of V546 as the reference strain. IL-8 secretion induced by motile strains showed no significant difference from that induced by strain V546. (B) Except for 6 strains (SK279, 324, 476, 577, 1157, 1402), IL-8 induction by DAEC strains isolated from healthy carriers was significantly lower than that induced by the positive control V546. Values are the mean ± standard deviation of three independent experiments.

Fig. 2. Effect of DAEC on barrier integrity of Caco-2 monolayers. (A) Comparison of longitudinal changes in transepithelial electrical resistance of Caco-2 monolayers infected with DAEC strains. The circlets and triangles indicate high and low IL-8 inducers, respectively. The closed symbols represent motile strains and the open symbols represent non-motile strains. Plain lines represent strains isolated from patients and broken lines represent strains from healthy persons. Compared to the negative control DH5α, all DAEC strains caused significant decreases in electrical resistance 12 h after inoculation ($p < 0.01$), irrespective of their source, motility, and ability to induce IL-8 secretion. Values are expressed as the percentage of the electrical resistance immediately before bacterial inoculation. Values are the mean ($n = 3$) ± standard deviation. (B) Compared with cells inoculated with

non-pathogenic *E. coli* DH5α, paracellular passage of FITC-dextran (molecular weight, 4 kDa) in Caco-2 cell monolayers significantly increased when cells were infected with DAEC for 12 h or more ($p < 0.01$), irrespective of their source and ability to induce IL-8 secretion. FITC-dextran quickly diffused through the 0.4-μm pores of the polycarbonate membrane insert from the upper to the lower chambers (cell-free membrane). Values are the mean ($n = 3$) ± standard deviation.

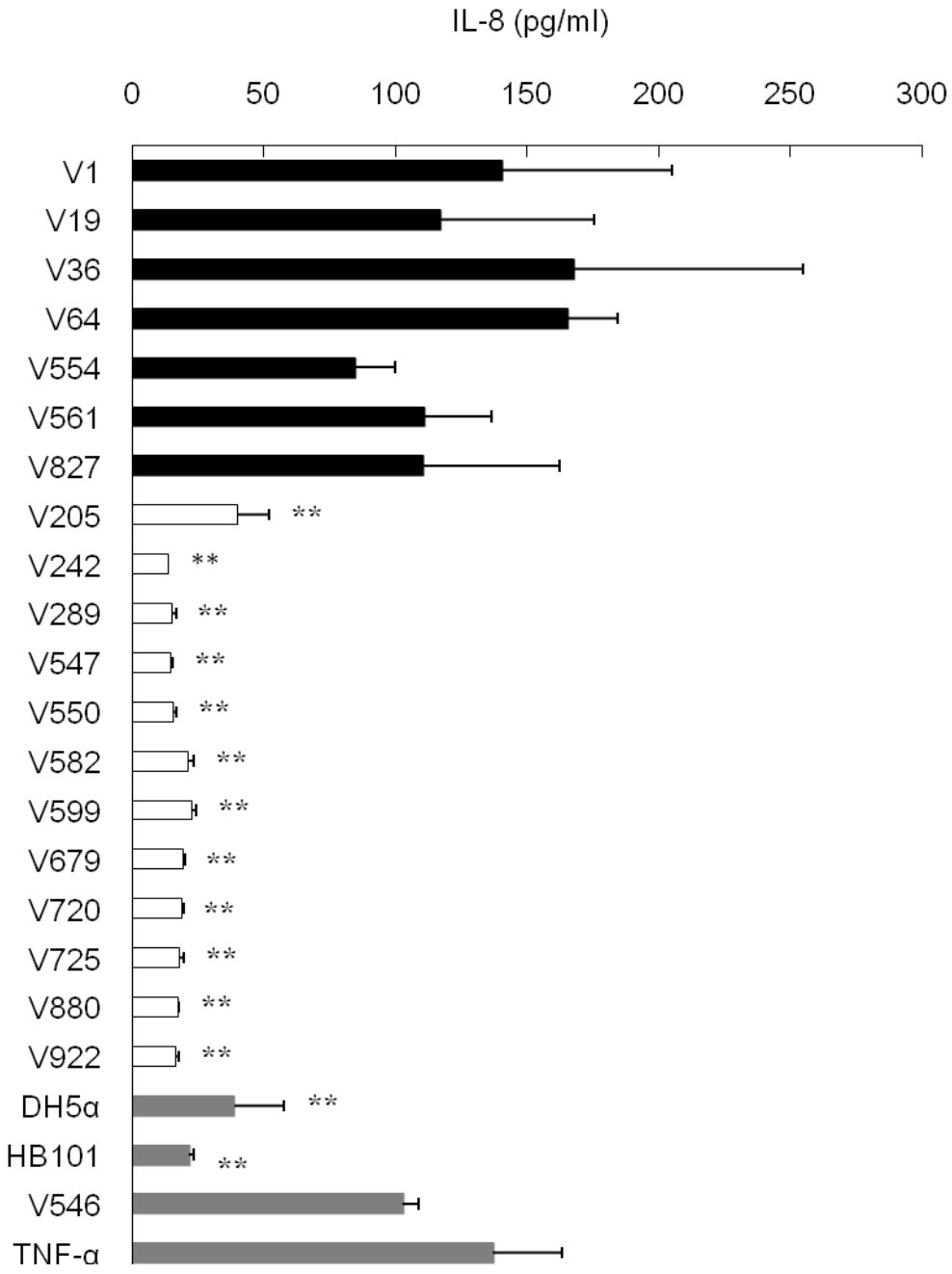
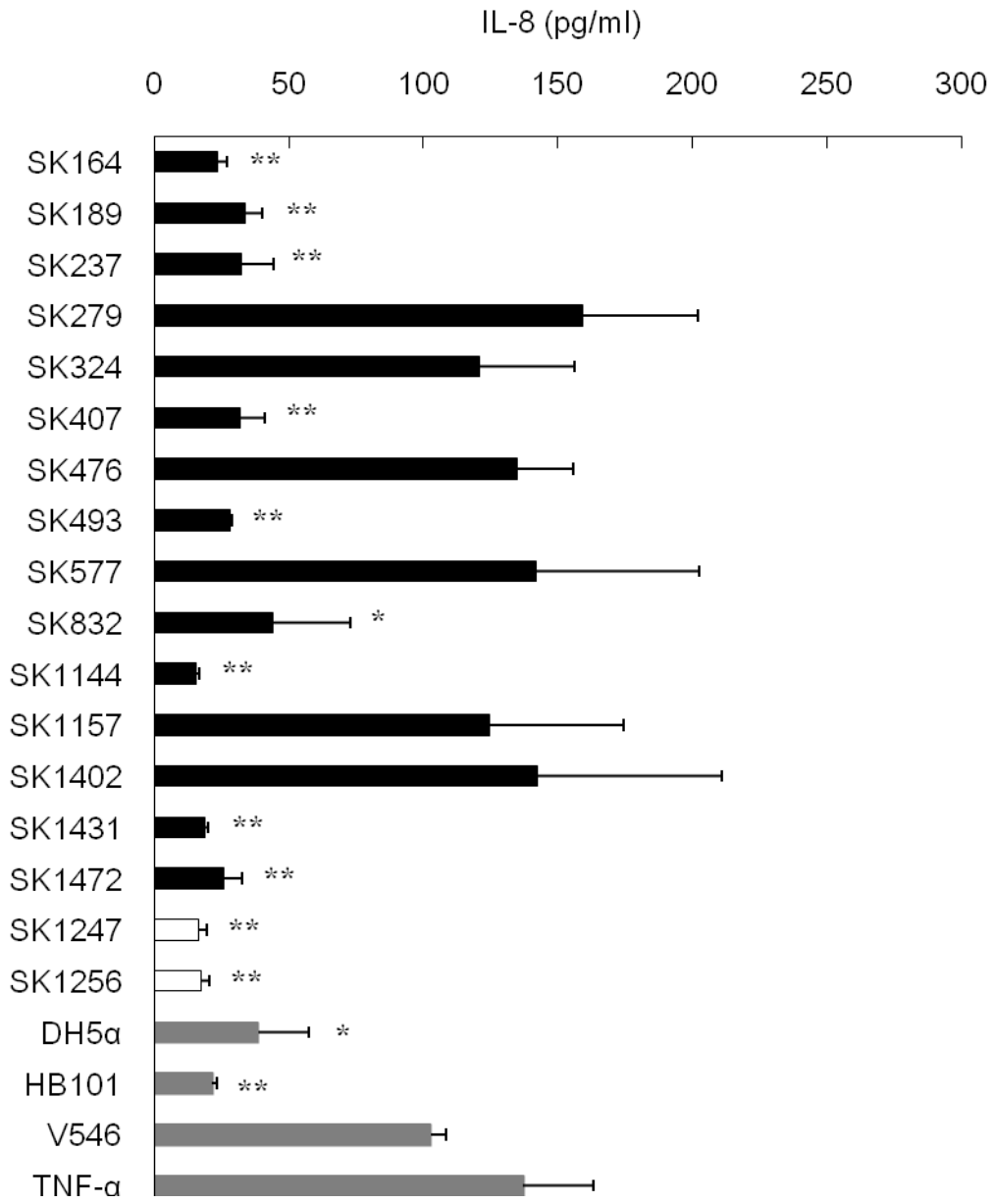

IL-8 (pg/ml)
0
50
100
150
200
250
300
V1
V19
V36
V64
V554
V561
V827
V205
V242
V289
V547
V550
V582
V599
V679
V720
V725
V880
V922
DH5α
HB101
V546
TNF-α
**

Fig. 1A


IL-8 (pg/ml)
0
50
100
150
200
250
300
SK164
SK189
SK237
SK279
SK324
SK407
SK476
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SK577
SK832
SK1144
SK1157
SK1402
SK1431
SK1472
SK1247
SK1256
DH5α
HB101
V546
TNF-α
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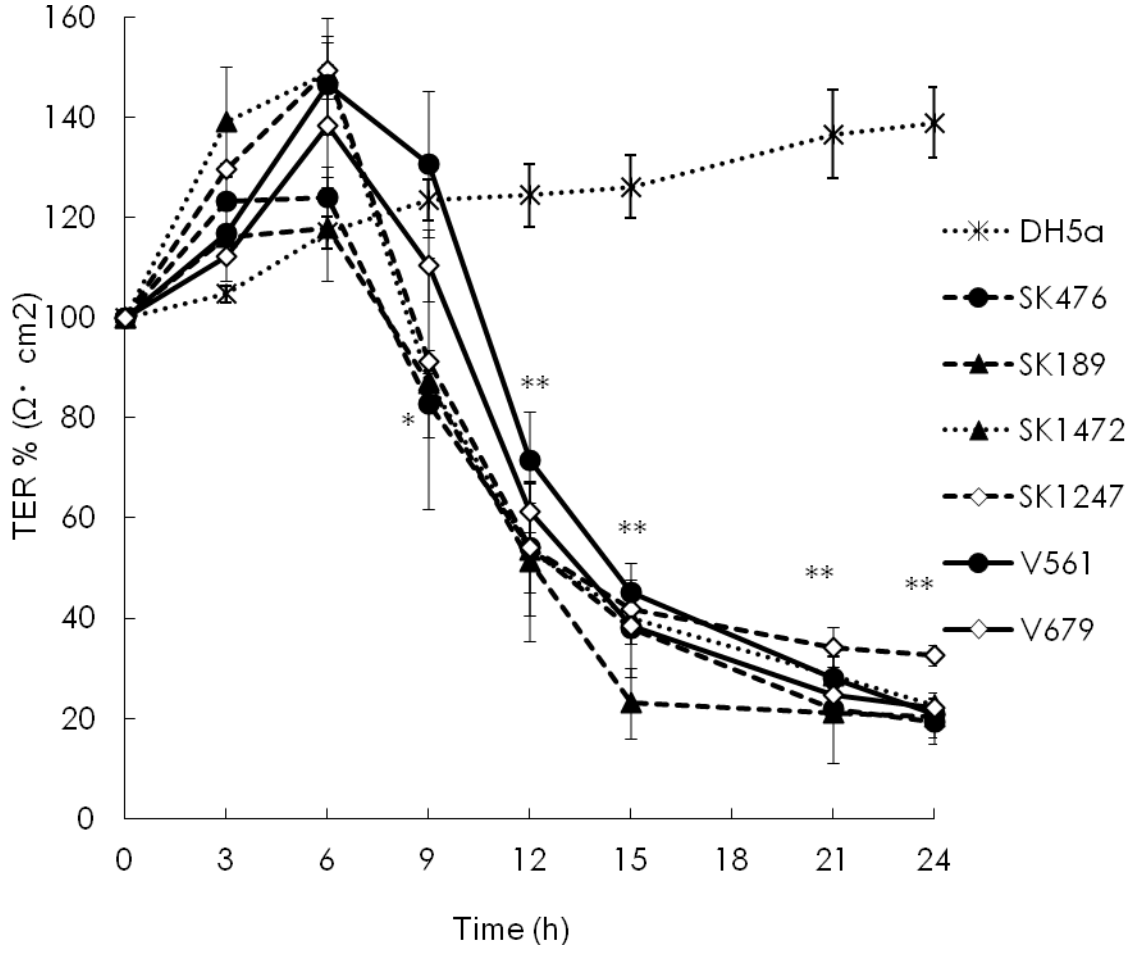
Fig. 1B


160
140
120
100
80
60
40
20
0
TER % (Ω· cm2)
0
3
6
9
12
15
18
21
24
Time (h)
DH5a
SK476
SK189
SK1472
SK1247
V561
V679
*
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**
**

Fig. 2A

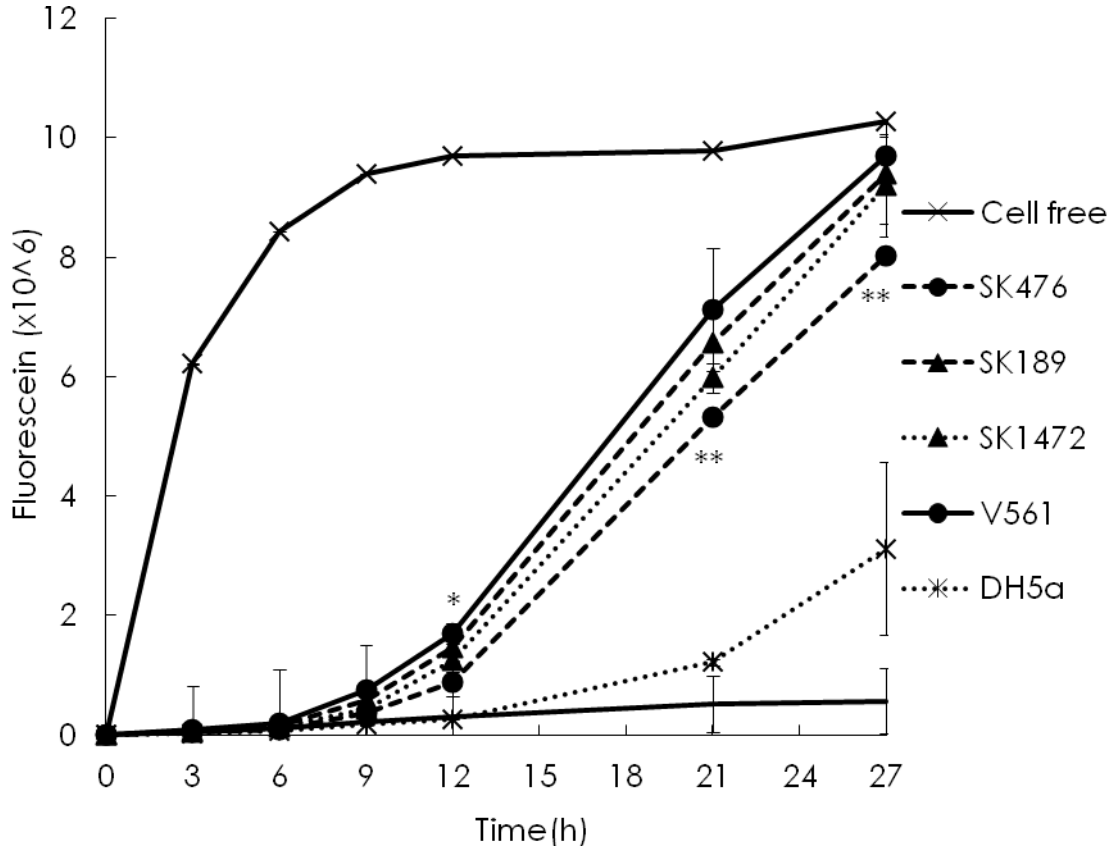
12
10
8
6
4
2
0
Fluorescein (x10^6)
0
3
6
9
12
15
18
21
24
27
Time(h)
*
**
**
Cell free
SK476
SK189
SK1472
V561
DH5a

Fig. 2B