MATERIALS AND METHODS

The patient

A 3-year-old boy suffering from abdominal pain and bloody diarrhea was admitted to a hospital in Kagoshima, Japan in 1999. On the second hospital day, he had a few convulsions and a platelet count of 70,000 per cubic millimeter, and development of HUS was diagnosed. The patient fell into a deep state of unconsciousness and barely responded to painful stimulation. On the third hospital day, hemodialysis for HUS was begun. However, his pupils were mydriatic and the electroencephalographic examination showed null waves on the fourth hospital day. The patient died of HUS on the sixth hospital day.

Bacterial strains

A stool specimen of the patient (boy) obtained on the first hospital day, described above, yielded colonies of EHEC belonging to serotype O86:NM. This strain was designated 1076. EHEC strains employed in this study (including the serotype O86:NM strain 1076) are summarized in Table 1. They were all clinical isolates in Japan. Serotype O157:H7 strains S1 and OB1 were derived from outbreaks at Sakai City and Obihiro City, respectively, in 1996. All other EHEC strains were from sporadic cases. Serotype O157:H7 strain U8 was isolated from a 1-year-old female with HUS (deceased) at Chiba City in 1996[8].

Media and bacterial growth

For bacterial growth, we used L broth (Difco Laboratories, Detroit. Mich., USA), colonization factor antigen (CFA) broth[14], and Eagle MEM (Nissui Pharmaceutical, Tokyo) supplemented with 6% fetal bovine serum as liquid meium. This was followed by incubation at 37℃ for 18 to 20 h with agitation. L (2%) agar, CFA (2%) agar, and MacConker agar (Eiken Chemical, Tokyo) were used as solid media.

Stx assay

Bacteria were grown for 18 h at 37℃ in CA-YE broth. The bacterial concentration was adjusted to Klett 300 units (measured in a Klett-Summerson photoelectric colorimeter with a red filter; Klett Manufacturing, Long Island City, NY). This concentration of strain 1076 corresponded to 1.2×10^9 CFU/ml. The amount of Stx in the culture supernatants was determined by passive latex agglutination using a VT detection kit (Denka Seiken Co., Tokyo). The Stx titers (the levels of the Stx production) represents the highest dilution [fold] to yield positive results.

The Stx gene (*stx*) was examined by PCR as described previously[15]. PCR primers used were a set of V1 (5'-AGTTAATGTGGTGGCGAA) and V5 (5'-GACTCTTCCATCTGCCG) generating a 811-bp product for Stx1, and a set of V3 (5'-TTCGGTATCCTATTCCCG) and V4 (5'-TCTCTGGTCATTGTATTA) generating a 471-bp product for Stx2[16].

Intimin gene (*eae*) assay

The *eae* gene was examined by PCR as described previously[15]. PCR primers used were IntF (5'-GACTGTCGATGCATCAGGCAAAG) and IntR (5'-TTGGAGTATTAACATTAACCCCAGG), generating a 368-bp product[17].

Table 1. Bacterial strains used in this study

Strain	Serotype	Shiga toxin type	Intimin gene (*eae*)
1076	O86:NM	Stx2	−
S1	O157:H7	Stx1, Stx2	+
OB1	O157:H7	Stx1, Stx2	+
U8	O157:H7	Stx2	+
T1	O26:H11	Stx1	+
T2	O26:H11	Stx1	+
E11	O111:HUT	Stx1, Stx2	+
F59	O111:NM	Stx1, Stx2	+
F60	O128:H12	Stx1, Stx2	−
E10	O145:NM	Stx1, Stx2	+

HA assay

HA activities were examined by a 24-well plate method[18,19]. Briefly, bacterial cells were grown in liquid cultures or on agar plates for 18 h at 37℃ and suspended in phosphate-buffered saline (PBS; pH 7.4) to a concentration of 600 Klett units. Twofold serial dilutions were then made with PBS, and 100 μl samples were mixed with 100 μl of 3% erythrocytes in a 24-well multidish plate (diameter of each well, 15 mm; A/S Nunc, Roskilde, Denmark). D-mannose-resistant HA (MRHA) activities were examined with human (group A), bovine, horse, guinea pig, sheep, rabbit, and goat erythrocytes in the presence of D-Mannose (0.5%, wt/vol). The MRHA titers were determined with a light microscope after 20 min of incubation at room temperature (ca. 22℃); the MRHA titers represent the highest bacterial dilution [fold] to yield positive results. The concentration of undiluted bacterial samples corresponding to a MRHA titer of 1 was 600 Klett units.

RESULTS

Stx production and lack of the *eae* gene

Strain 1076 was positive for the Stx2 gene but negative for the Stx1 gene in the PCR assay. The Stx2 production of strain 1076 was confirmed by the latex agglutination test. The level of Stx2 (Stx2 titers) in the culture supernatants was 512. The corresponding Stx2 titers for EHEC O157 strains S1, OB1, and U8 were 2,048, 1,024, and 128, respectively, indicating that the Stx2 production level of strain 1076 was comparable to or only slightly lower than the levels of the serotype O157:H7 strains.

Strain 1076 was negative for the *eae* gene in the PCR assay, in contrast to the EHEC strains belonging to serotypes O157, O26, O111, and O145.

HA activity

Strain 1076 exhibited strong MRHA activities detected with human and bovine erythrocytes but not with guinea pig, horse, sheep, rabbit, or goat erythrocytes (Fig. 1 and Table 2). The MRHA activities increased greatly when the bacteria were grown in liquid media rather than on solid media (Table 2).

Under these conditions, the EHEC strains belonging to serotypes O157, O26, O111, O128, or O145 exhibited no detectable MRHA activities.

DISCUSSION

EHEC strains belonging to serotype O157:H7 adhere to the tissue culture cells in a clustering pattern[8] (or a diffuse pattern[20]). The adherence factor (s) involved in this stage has not been established. At the next stage of adherence, EHEC O157:H7 tightly binds to the membrane, and causes the characteristic membranous lesions (called attaching and effacing) with the bacterial outer membrane protein, intimin[9,10]. During this stage, bacterial secretion proteins (including Tir, a receptor protein to intimin) are inserted into the human epithelial cells through the bacterial type III secretion system and modify the host signaling pathways[10–12,21], resulting in the enwrapping of the adherent bacteria by the elongated cell membrane[8]. This enwrapping may facilitate the translocation of Stx across the intestinal epithelial cells.

In this study, we demonstrated that EHEC strain 1076 belonging to serotype O86:NM (which was iso-

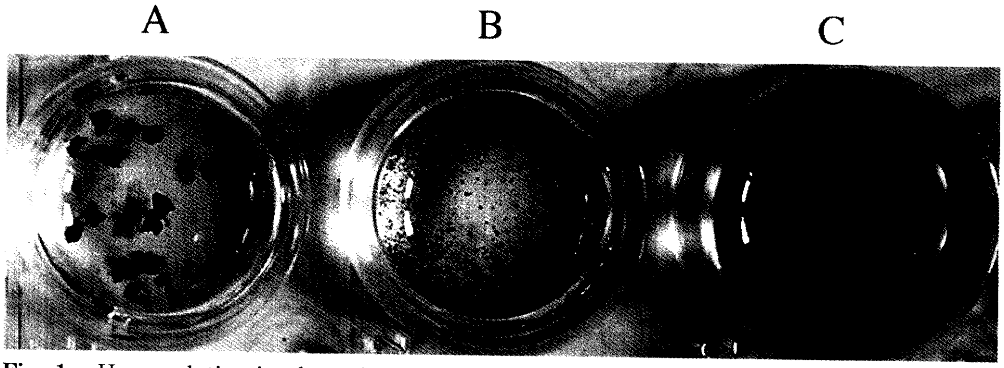

Fig. 1. Hemagglutination by a 24-well plate method. EHEC 1076 suspensions at Klett 150 were mixed with human (**A**) and bovine (**B**) erythrocytes in the presence of D-Mannose. C, negative control without EHEC 1076 cells.

Table 2. MRHA levels of EHEC strains belonging to various serotypes, which were grown in liquid media or on solid media

Strain (Serotype)	Medium	MRHA activity[a] against the following erythrocytes:						
		Human	Bovine	Guinea pig	Horse	Sheep	Rabbit	Goat
1076 (O86:NM)	MEM	16	64	<1	<1	<1	<1	<1
	L broth	8	32	<1	<1	<1	<1	<1
	CFA broth	8	32	<1	<1	<1	<1	<1
	L agar	2	2	<1	<1	<1	<1	<1
	CFA agar	4	8	<1	<1	<1	<1	<1
S1 (O157:H7)	L broth	<1	<1	<1	<1	<1	<1	<1
OB1 (O157:H7)	L broth	<1	<1	<1	<1	<1	<1	<1
U8 (O157:H7)	L broth	<1	<1	<1	<1	<1	<1	<1
T1 (O26:H11)	L broth	<1	<1	<1	<1	<1	<1	<1
T2 (O26:H11)	L broth	<1	<1	<1	<1	<1	<1	<1
E11 (O111:HUT)	L broth	<1	<1	<1	<1	<1	<1	<1
F59 (O111:NM)	L broth	<1	<1	<1	<1	<1	<1	<1
F60 (O128:H12)	L broth	<1	<1	<1	<1	<1	<1	<1
E10 (O145:NM)	L broth	<1	<1	<1	<1	<1	<1	<1

a) Data (MRHA titer) indicate the highest dilution which yielded positive results by the 24-well plate method (Fig. 1) and are representative of at least three trials. D-mannose was added to 0.5% (wt/vol).

lated from a HUS patient) lacked the intimin-encoding *eae* gene. Instead, this strain was found to possess a novel and strong hemagglutinin as a putative adherence factor. The possibility exists that EHEC O86:NM strain 1076 can adhere better to the intestinal epithelial cells than do EHEC O157:H7 strains.

Several *eae*-negative EHEC strains have been shown to be associated with HUS. Morabito et al. demonstrated that *eae*-negative EHEC of serotype O111:H2 produced Stx2, and displayed the characteristic aggregative adherence of enteroaggregative *E. coli* (EAggEC)[22]. Paton et al. reported that *eae*-negative EHEC of serotype O113:H21 produced a Stx2-related toxin (Stx20113) and possessed a high adherence ability[23]. In those *eae*-negative strains, however, no HA activities were reported.

This study demonstrates the first case of HUS due to an *eae*-negative, MRHA-positive EHEC. In the case of *eae*-negative EHEC, a combination of a highly adhesive property and an Stx2 (or related toxin) production may be important factors for the development of HUS. The tight attachment of EHEC (e. g., O86:NM strain 1076 exhibiting a great MRHA activity) to the intestinal epithelial cells must facilitate the translocation of Stx2 across the intestinal epithelial cells.

EHEC O128:H12 strain F60 was also *eae*-negative, but the association of this serotype with HUS remains uncertain.

The most common source of EHEC O157:H7 infection is undercooked ground beef, with other causes being milk, vegetables, or fruits (including apple juice)[3,24]. Person-to-person infection has also been reported[3,25-27]. Stx-producing, intimin-positive *E. coli* (STEC) of serotype O157:H7 can also be isolated from cattle, which therefore are considered to be a reservoir of EHEC O157:H7[28-30].

EHEC O86:NM strain 1076 exhibited a strong MRHA activity which was detected only toward human and bovine erythrocytes. This result suggests that the receptor for MRHA (a putative adherence factor) of strain 1076 is present on human and bovine erythrocytes, but not on guinea pig, horse, sheep, rabbit, or goat erythrocytes. EHEC O86:NM strain 1076 may be able to colonize the intestines of cattle (in addition to the human intestines).

EHEC O86:NM strain 1076 induced larger aggregates for human erythrocytes than for bovine erythrocytes at high bacterial concentrations (as shown in Fig. 1), but HA titers were greater for bovine erythrocytes (Table 2). There is a possibility that human erythrocytes possess a large number of receptors on the surface, and bovine erythrocytes possess a limited number of receptors but with a higher binding efficiency.

Finally, EHEC O86:NM strain 1076 exhibited MRHA activities to a much greater extent when the bacteria were grown in liquid media rather than on solid media. Some bacterial adherence factors are tightly regulated by environmental and host factors. In the case of type 1 pili, which are an important adherence factor of uropathogenic *E. coli* (UPEC), a piliated phase of *E. coli* is obtained by culturing in liquid media and not on solid media[31,32]. Moreover, the expression of an adherence factor of enteropathogenic *E. coli* (EPEC) (bundle-forming pili, BFP; an adherence factor at the first stage of adherence)[33] and *Vibrio cholerae* O1 (toxin-coregulated pilus, Tcp)[34] is not constitutive, but is induced by specific conditions of growth. Further studies investigating the regulation of MRHA expression as well as the molecular nature of MRHA are necessary for EHEC O86:NM strain 1076.

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