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Identification of a Clone of *Escherichia coli* O103:H2 as a Potential Agent of Hemolytic-Uremic Syndrome in France

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In a French multicenter study, six verocytotoxin-producing *Escherichia coli* strains were isolated from the stools of 6 of 69 children suffering from hemolytic-uremic syndrome. All strains belonged to serotype O103:H2, a serotype commonly associated with diarrhea in weaned rabbits in France. To determine whether the strains from humans and rabbits were genetically related, they were compared by analyzing their esterase electropherotypes and the restriction fragment length polymorphisms of the ribosomal DNA regions. A common clonal origin of these pathogenic strains was suggested by their identical esterase electropherotypes and their identical ribotypes, in addition to their identical serotypes. However, strains from humans, which are cytotoxic for HeLa cells through the production of verocytotoxin type 1, do not show adhesion in vitro to HeLa 229 cells and cannot infect rabbits. On the other hand, strains from rabbits do not carry the verocytotoxin type 1 gene, are not cytotoxic for Hela cells, and adhere to ileal villi and HeLa 229 cells because of the expression of their 32-kDa adhesin. Our results therefore identify a clone of verocytotoxin-producing *E. coli* O103:H2 as a potential agent of hemolytic uremic syndrome in France. They further suggest that clones from humans and rabbits probably have a common origin but that adaptation to the two species occurred by different mechanisms. Thus, they eliminate the hypothesis that the species is horizontally transmitted between rabbits and humans.

Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) are generally thought to be involved in the pathogenesis of the hemolytic-uremic syndrome (HUS) (19), the most common cause of acute renal failure in children (7). VTEC organisms are widely distributed in the intestinal tracts of domestic animals, particularly bovines, and the major source of human infection is ingestion of uncooked meat (18). VTEC strains produce one or two VTs (VT type 1 [VT1] and/or VT2) which are closely related to the Shiga toxin and which are encoded by bacteriophages (31). There is increasing evidence that HUS results from the systemic action of VT on vascular endothelial cells (17). *E. coli* O157:H7 is the most common serotype of VTEC isolated from the stools of patients with HUS (13, 18, 33). However, at least 50 other serotypes of *E. coli* also produce these cytotoxins (6, 18, 20).

In a French multicenter study, six VTEC isolates, all belonging to serotype O103:H2, were identified in the stools of 6 of 69 children suffering from HUS. No other VTEC strains belonging to a different serotype were isolated during the study. *E. coli* O103:H2 is infrequently isolated from humans, but it has often been identified as a cause of diarrhea in weaned rabbits (23).

The aim of the present study was (i) to determine whether the pathogenic *E. coli* O103:H2 strains, isolated from six different patients with HUS, were genetically related to each other and the O103:H2 strains from rabbits and (ii) to compare the virulence mechanisms of these strains.

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MATERIALS AND METHODS

Bacterial strains. Six *E. coli* O103:H2 strains of human origin (PMK1 to PMK6) were isolated between 1987 and 1989 from 6 of 69 patients with HUS; patient ages ranged between 4 months and 4.6 years. These strains were selected on the basis of their cytotoxicities among 292 isolates obtained from primary fecal cultures of 69 patients with HUS originating from different areas of France (Table 1). Seven *E. coli* O103:H2 strains of rabbit origin, which were isolated from the feces or the cecal contents of diarrheic weaned rabbits in France from 1984 to 1987, were also studied. These strains originated from an epidemiological survey involving 119 farms in different areas of France. Among them, five strains (B10, E1, E31, E37, C148) were highly pathogenic and two strains (C124, C127) were nonpathogenic in an experimental model in which the strains were tested for their ability to adhere in vitro to rabbit intestinal villi and HeLa 229 cells (23). Two control strains were used in the study: a VT1- and VT2-producing *E. coli* O157:H7 strain and a *Shigella dysenteriae* type 1 strain obtained from A. D. O'Brien; a VT-negative *E. coli* strain (CNCM 8185) and the *E. coli* type strain of the species (ATCC 11775) were also included. Serotyping was kindly performed by F. Orskov and F. Orskov from the International *E. coli* and *Klebsiella* Reference Center in Copenhagen, Denmark.

Cytotoxicity assay for VTs. Bacteria were grown for 48 h in 200 ml of iron-depleted medium M9. Filter-sterilized culture supernatants were assayed for cytotoxicity on HeLa cells as described previously (8, 9). HeLa cells were grown in minimal essential medium with Earle's salts and *N*-glu-

TABLE 1. Characterization of the O103:H2 *E. coli* strains studied

Strain no.	Geographical area in France	Date of HUS (yr/mo)	Clinical data	
			Diarrhea	Bloody stools
PMK1	Rhône-Alpes	1987/10	+	−
PMK2	Ile de France	1988/03	+	−
PMK3	Rhône-Alpes	1988/11	+	+
PMK4	Aquitaine	1989/01	+	−
PMK5	Touraine	1989/01	+	−
PMK6	Nord-Pas de Calais	1989/07	+	+
B10	Midi-Pyrenées	1984/02	+	
E1	Charentes	1987/01	+	
E31	Provence	1987/02	+	
E37	Languedoc	1987/03	+	
C148	Rhône-Alpes	1985/05	+	
C124	Rhône-Alpes	1985/04	+	
C127	Nord-Pas de Calais	1985/04	+	

tamine (GIBCO, Paisley, Scotland) supplemented with 10% fetal calf serum (GIBCO). Serial dilutions of toxin in cell culture medium (100 μl) were made in a microdilution plate. Each well was inoculated with 2×10^4 cells in 100 μl of medium. Plates were incubated at 37°C in 5% CO_2 for 24 h. Neutralization assays were performed with either VT1 or VT2 monoclonal antibodies, which were a kind gift from A. D. O'Brien. Plates were stained with Giemsa and observed with an inverted microscope. Cytotoxicity was assessed on detached cells and was calculated as the 50% cytotoxic dose per milligram of proteins in the extract.

PCR analysis of the VT genes. DNA was obtained by boiling a colony resuspended in 500 μl of distilled water for 10 min. Two pairs of oligonucleotide primers ($VT1_1$, 5′-TAATAGTTCTGCGCACCCAGAGTGGATGTA-3′ and $VT1_u$, 5′-TTCATCCACTCTGGGTGCGCAGAACTATTA-3′; $VT2_1$, 5′-AACTGCTCTGGATGCATCTCTG-3′; and $VT2_u$, 5′-CAACGGTTTCCATGACAACG-3′) were chosen to amplify 149- and 164-bp fragments from the VT1 and VT2 genes, respectively (26). The DNA preparation (25 μl) was subjected to polymerase chain reaction (PCR) as described by Denamur et al. (5), except that 3.5% (vol/vol) formamide was added. To improve the specificity, DNA was added last, after heating the amplification mixture at 80°C (24). Samples were subjected to 35 cycles of amplification in a DNA thermal cycler (Perkin-Elmer Cetus). Cycling conditions were as follows: denaturation, 1 min at 94°C; primer annealing, 30 s at 55°C; and primer extension, 30 s at 72°C. Five microliters of the PCR was analyzed by electrophoresis on 8% polyacrylamide gels as described previously (5).

Esterase electrophoresis. Protein extracts from bacterial cells were prepared as described previously (10). Horizontal slab gel electrophoresis was performed in composite polyacrylamide-agarose gels, and esterases were stained on the gel by the method of Uriel (34). The relative mobility value was calculated as the ratio between the progression of the esterase band and the progression of a dye front. These relative mobilities were used for comparison only (12).

Analysis of DNA restriction fragment length polymorphism (RFLP). Bacterial DNA was digested independently with three restriction endonucleases (*Eco*RI, *Hin*dIII, *Bam*HI) and was Southern blotted (30) as described previously (1). Then, two probes were used in two separate experiments. The first one was an *E. coli* 16S plus 23S ribosomal DNA (rDNA)-derived probe (14). The second probe was the 149-bp fragment of the VT1 gene obtained by PCR. The probe labeling, hybridization, washing, and procedures for autoradiography of the membranes were described previously (1).

Plasmid DNA profiles. Plasmid DNA was prepared by the alkaline extraction procedure (2), analyzed by electrophoresis in a 0.6% agarose gel in TBE buffer (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA), and visualized under transillumination after ethidium bromide staining. Molecular weights were measured relative to three molecular size standards (54, 90, 103 kb).

Adhesion to HeLa cells and identification of the 32-kDa adhesin. Adhesion to the HeLa 229 cell line was tested by the technique of Scaletsky et al. (28) modified by Milon et al. (23). We searched for the 32-kDa adhesin in hot urea bacterial surface extracts prepared by the technique of de Graaf (4). Extracts were prepared from 18-h bacterial cultures in Penassay broth (Difco Laboratories, Detroit, Mich.), a medium which favors the expression of the adhesin by O103 strains of rabbit origin (23). Approximately 10 μg of protein extracts was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (21), and Western blots (immunoblots) were performed by using rabbit antisera against the 32-kDa protein (23).

Infection of rabbits with human O103:H2 strains. Each strain from humans (PMK1 to PMK6) was inoculated orally to a group of six weaned 34-day-old New Zealand White rabbits (INRA strain 1077) that were free of coccidia. Cultures of bacteria grown for 18 h in Trypticase soy broth at 37°C were used to infect the animals. Each rabbit was given approximately 2×10^9 CFU through an oral cannula. The animals were checked daily for clinical symptoms of diarrhea and dehydration and for mortality. They were weighted twice a week. *E. coli* counts on fecal samples were performed by dilution on MacConkey agar twice a week during the first 2 weeks after the experimental infection and then once a week. For each sample, six colonies of *E. coli* were reisolated on Trypticase soy agar and were serogrouped by slide agglutination by using anti-O103 rabbit serum. Coccidia controls remained negative at all times.

RESULTS

The results of the present study are summarized in Table 2. The six strains from humans (PMK1 to PMK6) were positive for VT1 both by the cytotoxicity assay and by PCR analysis, whereas the strains from rabbits (B10, E1, E31, E37, C148, C124, C127) did not harbor VTs by either technique.

Four distinct zymotypes were detected among the 14 strains studied. The six strains isolated from humans and the five pathogenic strains isolated from young rabbits exhibited zymotype 1, whereas strains C124 and C127 were distinguished by the electrophoretic mobility of esterase I (relative mobility, 68). The esterase electrophoretic profile of the *E. coli* O157:H7 strain was very different.

For analysis of the rDNA RFLP, *Hin*dIII was the most discriminant enzyme, and thus, the patterns it generated (Fig. 1) were used to define ribotypes (Table 2). The pathogenic strains isolated both from the patients with HUS and from rabbits produced indistinguishable patterns (pattern A in Fig. 1 is ribotype A in Table 2). The nonpathogenic strains from rabbits (C124, C127), however, were distinguished from the pathogenic ones by a different pattern (pattern B) (Fig. 1). The VT1- and VT2-producing *E. coli* strain exhib-

TABLE 2. Properties of the *E. coli* O103:H2 strains studied

Strains	VT1	VT2	Zymotype	Ribotype pattern	Plasmid profile pattern	Adhesion to HeLa cells	32-kDa adhesin	Infection of rabbits
PMK1 to PMK6	+	−	1	A	a	−	−	−
B10, E1, E31, E37, C148	−	−	1	A	b, b, c, d, e	+	+	+
C124, C127	−	−	2	B	f	−	−	−

ited a third pattern (pattern D) that was distinct from those of the other strains, including the type strain of the species (pattern C) (Fig. 1). When we studied the RFLP in the VT1 gene region, we observed a single fragment with each of the three restriction enzymes in VT1-producing *E. coli* strains only. For example, the results obtained after *Eco*RI digestion are shown in Fig. 2. All of the O103:H2 VTEC strains from children with HUS produced a VT1 fragment of the same size. The VT1- and VT2-producing O157:H7 *E. coli* strain produced a fragment of a larger size, and an *S. dysenteriae* type 1 strain studied gave a fragment of a smaller size. No DNA fragments that hybridized to the VT probe were detected in the strains from rabbits.

All of the strains from humans displayed strictly identical plasmid profiles (Fig. 3) with a plasmid of circa 54 kb in addition to two small cryptic plasmids (pattern a). The enteropathogenic strains from rabbits carried plasmids of similar sizes, but they also carried a larger molecule of circa 110 kb. In addition, their plasmid profiles appeared to be heterogeneous (patterns b, c, d, e) and were different from those observed for the nonenteropathogenic rabbit strains (pattern f) and for *E. coli* O157:H7 (pattern h).

The O103:H2 *E. coli* strains which are pathogenic in rabbits have been shown to adhere in vitro to the ileal villi of 8-day- or 6-week-old rabbits as well as to HeLa 229 cells. Adhesion of these strains is correlated with the expression of a 32-kDa surface protein (23). When tested on HeLa 229 cells, the O103:H2 strains of human origin (PMK1 to PMK6) did not adhere to the cells, while a concurrent set of O103:H2 pathogenic strains from rabbits (B10, E1, E31, E37, C148) showed a diffuse adhesion pattern. The 32-kDa adhesin was not found in surface protein extracts of the human strains, as shown by SDS-PAGE and Western blot analysis (Fig. 4).

In 35 of the 36 rabbits inoculated with the strains from humans, only transient weight loss was registered, and the weight curves remained normal with daily weight gains compatible with those of healthy animals (33.4 to 36.2 g/day between days 0 and 28). The kinetics of the human O103:H2 *E. coli* strain in the feces of the animals confirmed the in vitro adhesion results. Indeed, inoculation of pathogenic

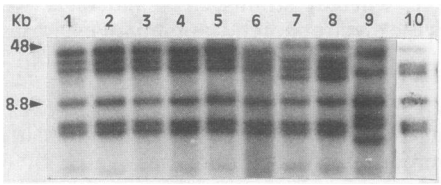

FIG. 1. RFLP patterns of the rDNA regions of strains from humans and rabbits after *Hin*dIII digestion. Lane 1, PMK1 (pattern A); lane 2, B10 (pattern A); lane 3, E1 (pattern A); lane 4, E31 (pattern A); lane 5, E37 (pattern A); lane 6, C148 (pattern A); lane 7, C124 (pattern B); lane 8, C127 (pattern B); lane 9, ATCC 11775 (pattern C); lane 10, *E. coli* O157:H7 (pattern D).

O103:H2 strains of rabbit origin under the same conditions and doses induced diarrhea in 90% of the animals and mortality in more than 70% of them (3). Bacteria reach a level of 10^9 CFU/g of feces or cecal content within 3 to 4 days and remain at that level for up to 20 days (22a). In the present study, the strains from humans (PMK1 to PMK6) showed only slight colonization properties and remained as a subdominant component of the flora, with a peak at day 7 that did not exceed 10^7 CFU/g of feces. However, one rabbit, which was inoculated with strain PMK1, was found dead 2 days after inoculation with signs of hemorragic diarrhea and dehydration. At the time of necropsy, the cecum showed hemorrhagic lesions of the serosa and mucosa and contained blood. It also contained 4×10^8 O103:H2 *E. coli* per ml, no coccidia, and no *Clostridium spiroforme*. Still, it is difficult to conclude that death was due to the O103:H2 *E. coli* strain. One cannot exclude the possibility that this rabbit may have represented a genetic variant with an enhanced susceptibility to the toxin. No lesions, however, were detected outside of the digestive tract in this animal, and other rabbits of the same litter were not sensitive to inoculation with PMK strains.

DISCUSSION

E. coli of the O103:H2 serotype is not frequently isolated from humans. They cause gastroenteritis and hemorrhagic colitis (15, 16) and are now considered enterohemorrhagic *E. coli* because they produce a Shiga-like toxin. Conversely, the O103:H2 *E. coli* strains first described in 1983 as pathogenic for rabbits are now considered enteropathogenic *E. coli* because (i) they induce in vivo attaching-effacing lesions on the brush border of enterocytes, (ii) they do not produce heat-stable or heat-labile enterotoxins, and (iii) they are not invasive (22).

The most striking feature of the present study is that strains of human and rabbit origin appeared to be genetically closely related. Indeed, a clonal origin of these strains can be suggested on the basis of their identical serotypes (O103:H2), their identical esterase electropherotypes, and their identical RFLP patterns in the rDNA regions. The nonpathogenic strains (C124, C127) from rabbits were slightly different regarding their esterase electropherotypes and the RFLP patterns of their rDNA regions. They may represent a recent divergence from the pathogenic strains. Although the strains were isolated from different parts of France, the strains from children with HUS were highly homogeneous because their rDNA regions and VT1 genes displayed identical RFLP patterns. These patterns were different from the RFLP patterns observed with *E. coli* O157:H7. A clonal origin of the human *E. coli* O157:H7 strains has already been reported in the United States by Whittam and colleagues (29, 35) by multilocus enzyme polymorphism. Esterase electropherotyping (12) and ribotyping (14) have been shown independently to represent discriminant epidemiologic tools in *E. coli* (11, 25). Actually, esterase electropherotyping of *E. coli*

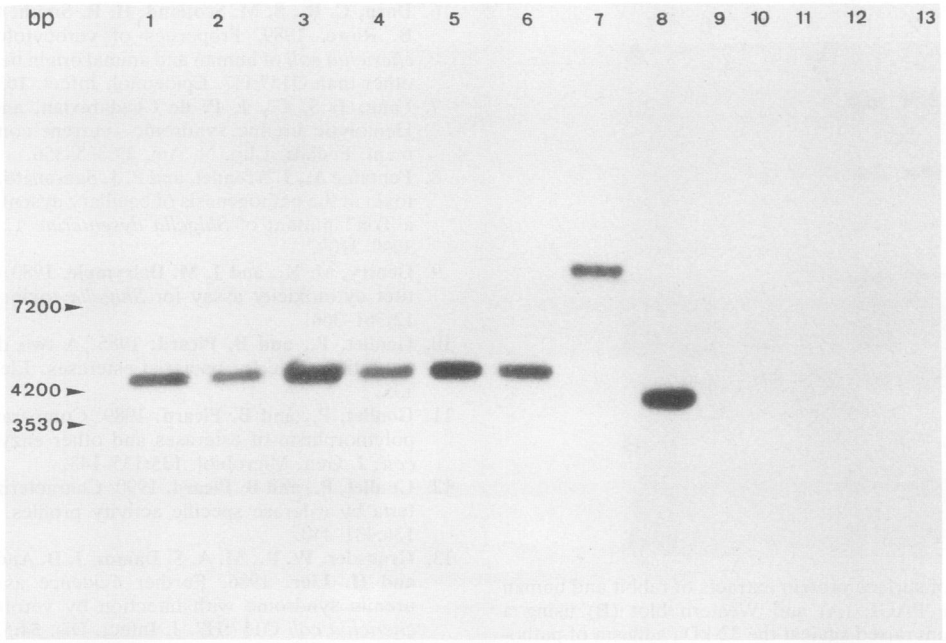

FIG. 2. *Eco*RI pattern of the VT1 gene. Lanes 1 to 6, PMK1 to PMK6, respectively; lane 7, *E. coli* O157:H7; lane 8, *S. dysenteriae* type 1; lane 9, B10; lane 10, E1, lane 11, E31; lane 12, E37; lane 13, C148.

is, by itself, as discriminant as multilocus enzyme analysis (11). In the present study, these two typing approaches were used concomitantly and their results did converge. RFLP of the Shiga toxin gene has also been evaluated for epidemiological studies (32). In the present study, RFLP of the VT1 gene clearly distinguished our human clinical O103:H2 isolates from the O157:H7 *E. coli* strain and from the *S. dysenteriae* type 1 strain that was studied for comparison. However, analysis of the RFLPs of virulence genes has the disadvantage of being limited to bacteria that harbor those genes. Of the 69 patients with HUS, the 6 O103:H2 strains were the only VT-producing isolates that we found. No common exposure that could relate the patients was found. The finding that this *E. coli* clone is associated with HUS can be interpreted in two ways. First, there could be an unforeseen selection bias in our detection techniques. Second, the epidemiology of HUS in France has not yet been documented and it might, indeed, be truly different from the epidemiology described in other countries.

The pathogenicities of the strains from humans and rabbits do not involve the same mechanisms. Strains from humans are cytotoxic for HeLa cells through the production of VT1, but they do not show adhesion in vitro to HeLa 229 cells because they do not carry the gene for the 32-kDa adhesin. On the other hand, strains from rabbits do not carry the VT1 gene and they are not cytotoxic to HeLa cells, but they adhere to the ileal villi because they express the 32-kDa adhesin. Thus, the low sensitivity of rabbits to the human strains probably results from the absence of specific adhesins at the bacterial surface that are necessary for colonization of the digestive tract and infection.

The O103:H2 strains from humans and rabbits differed in their plasmid contents. Recently, a self-transferable 117-kb plasmid (pREC-1) has been described in a pathogenic O103:H2 strain of rabbit origin (27). This plasmid is involved in the in vivo colonization of the rabbit intestinal tract, as judged by the differential colonization and pathogenic properties of the wild-type strain, its cured derivative, and a K-12 conjugate. All pathogenic O103:HH2 strains from rabbits tested bore a conjugative plasmid with a size within the range of 110 to 135 kb; this plasmid does not carry the gene for the 32-kDa adhesin (22a). The O103:H2 strains of human origin did not show any plasmid within this size range but shared a 54-kb plasmid with some strains of rabbit origin.

Taken as a whole, our results show that even though they share an identical serotype, an identical RFLP of rDNA regions, and an identical esterase electrophoretic type, the strains from humans and rabbits exhibited different adhesive properties and plasmid contents, which are involved in

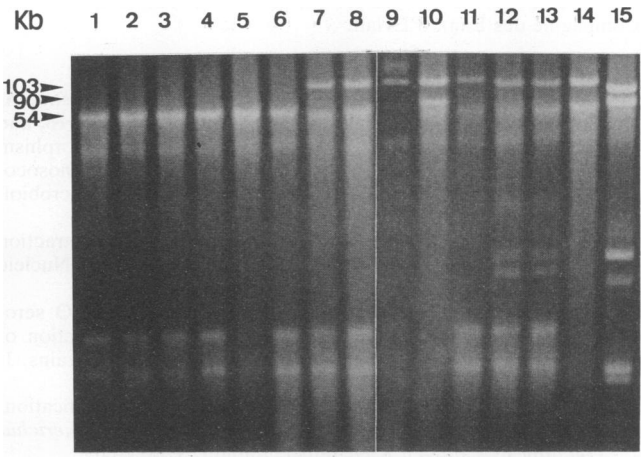

FIG. 3. Plasmid profiles. Lanes 1 to 6, PMK1 to PMK6 (pattern a), respectively; lane 7, B10 (pattern b); lane 8, E1 (pattern b); lane 9, E31 (pattern c); lane 10, E37 (pattern d); lane 11, C148 (pattern e); lane 12, C124 (pattern f); lane 13, C127 (pattern f); lane 14, CNCM 8185 (pattern g); lane 15, *E. coli* O157:H7 (pattern h).

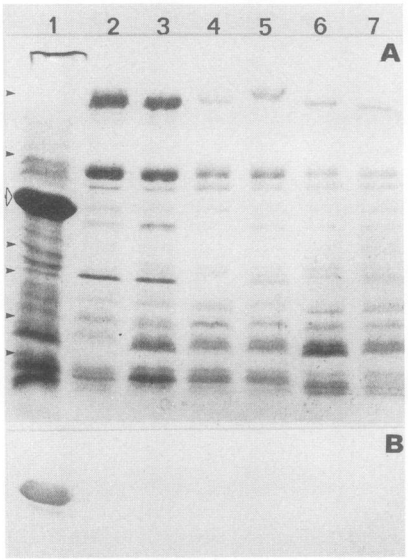

FIG. 4. Analysis of surface protein extracts of rabbit and human strains by SDS–15% PAGE (A) and Western blot (B) using a polyclonal rabbit serum raised against the 32-kDa adhesin of pathogenic *E. coli* O103:H2 from a rabbit. Lane 1, B10; lanes 2 to 7, PMK1 to PMK6, respectively. Black arrowheads show the positions of molecular mass markers (68, 50, 28.5, 25, 18.8, and 14.3 kDa from top to bottom, respectively). The white arrow shows the position of the 32-kDa adhesin of strain B10.

colonization of the digestive tract. Our observation suggests that these isolates were originally derived from the same clone but that adaptation to the rabbit species has probably necessitated the presence of the observed larger plasmid. These data eliminate the hypothesis of horizontal transmission between rabbits and humans in the pathogenesis of HUS.

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