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Relationship between Cytotoxic Necrotizing Factor Production and Serotype in Hemolytic *Escherichia coli*

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We examined the relationship between serotype and cytotoxic necrotizing factor (CNF) production in 123 hemolytic strains of *Escherichia coli* isolated from both stools and extraintestinal infections. Of 76 strains producing both hemolysin (Hly) and CNF, 66 (87%) belonged to one of six serogroups (O2, O4, O6, O22, O75, and O83). In contrast, 47 *E. coli* strains producing Hly only belonged to 21 different O serogroups, and only 2 of these (O6 and O18ac) were widely represented. Generally, CNF-positive and CNF-negative hemolytic isolates were assigned to different O serogroups, with the exception of O6, often present in both categories of isolates. Serogroups O4 and O18ac were significantly more prevalent among strains from extraintestinal infections than among those from stools. In contrast, the Hly-positive, CNF-negative isolates, belonging to numerous less common serogroups, were hardly ever isolated from extraintestinal infections. Serological typing further confirmed that hemolytic isolates of *E. coli* may grossly be divided into two main populations on the basis of the ability to produce CNF. Examination of hemolytic *E. coli* for this property may also be useful in achieving a more detailed characterization of pathogenic clones.

Several studies have shown that hemolysin (Hly) production is a property largely associated with *Escherichia coli* strains which cause extraintestinal infections in humans (6–9), whereas it is rarely found in fecal isolates from healthy persons (2, 6, 7, 10). These epidemiological investigations, as well as genetic studies (19, 20) and in vivo virulence tests (17, 18), have supported the assumption that Hly may act as a virulence factor in *E. coli* infections.

In recent papers (2, 5, 6), we observed that a large percentage (ca. 70%) of hemolytic *E. coli* strains isolated from either stools or extraintestinal infections were able to produce a cytotoxic necrotizing factor (CNF). This toxin, a protein of 110,000 daltons (3), caused necrosis in rabbit skin and induced the formation of large multinucleated cells in tissue cultures (5). Hemolytic strains positive for CNF showed mannose-resistant hemagglutination (MRHA) less frequently than did Hly-positive (Hly^+), CNF-negative (CNF^-) strains, and the patterns of MRHA themselves differed between CNF-positive (CNF^+) and CNF^- isolates (6). All of this evidence led us to hypothesize the existence of two distinct classes of hemolytic strains of *E. coli*. To further support this hypothesis, we examined hemolytic isolates of *E. coli* obtained from different sources between 1980 and 1985 and evaluated the occurrence of specific associations of particular serotypes with either CNF^+ or CNF^- strains. CNF production was also used to characterize particular bacterial clones among hemolytic *E. coli* strains causing extraintestinal infections in addition to the electrophoretic migration of outer membrane proteins (OMPs).

MATERIALS AND METHODS

Bacteria. Fecal strains of *E. coli* were isolated from children with and without diarrhea during investigations carried out in Naples (4), Brescia (2), Orvieto, and Palermo, Italy. Strains from extraintestinal infections were isolated from patients attending three hospitals in Rome, Orvieto, and Perugia, Italy (6). The strains were kept in the form of agar stab cultures stored in the dark at room temperature. The sources of *E. coli* strains serotyped in this study are summarized in Table 1.

Hly and CNF production. *E. coli* strains were tested for Hly and CNF production as previously described (6).

Serotyping. Serotyping of O, K, and H antigens was carried out by standard procedures (12).

OMP patterns. To study the OMP patterns of *E. coli* strains belonging to the same serotype or serogroup, we analyzed cell envelope extracts by sodium dodecyl sulfate-polyacrylamide gel electrophoresis by the methods of Achtman and co-workers (1).

Statistical methods. The Fisher exact test and the χ^2 test were used to analyze the frequency of O serogroups in *E. coli* strains from the different sources.

RESULTS

A total of 76 *E. coli* strains producing Hly and CNF, 47 strains producing Hly only, and 2 strains producing CNF only were serotyped. The O serogroups of Hly^+, CNF^+ strains are shown in Table 2. These strains belonged to 13 different O serogroups, and 66 of them (87%) belonged to 1 of 6 serogroups (O2, O4, O6, O22, O75, and O83). Serogroup O4 was significantly more frequent among strains from extraintestinal infections (24.3%) than among those from stools (5.1%; $P < 0.05$), whereas the other serogroups were found with similar frequencies among strains isolated from both sources.

The 47 *E. coli* strains producing Hly only belonged to 21 different O serogroups (Table 3), among which only O6 was frequent in both categories of hemolytic strains. Serogroups O6 and O18ac accounted for 12 of the 14 strains (85.7%) isolated from extraintestinal infections. Whereas serogroup O6 was found among either fecal or extraintestinal strains, serogroup O18ac was significantly more frequent among the latter (57.1 versus 6.0%; $P < 0.05$). Serogroups O2, O4, O22,

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TABLE 1. Sources of *E. coli* strains

Source of *E. coli*	No. of isolates producing:		
	Hly and CNF	Hly only	CNF only
Stools	39	33	1
Urine	28	12	1
Blood	4	1	
Other extraintestinal infections	5	1	

and O83, altogether representing 42% of Hly^+, CNF^+ strains, were never encountered among Hly^+, CNF^- isolates.

Complete O:K:H serotypes were obtained for 57 strains producing Hly and CNF, for 37 strains producing Hly only, and for the 2 strains producing CNF only (Table 4). A total of 40% (23 of 57) of Hly^+, CNF^+ *E. coli* strains belonged to six serotypes: O4:K12:H5, O4:K3:H5, O6:K2:H31, O6:K13:H1, and O22:K13:H1, mainly isolated from extraintestinal infections, and O75:K95:H5, isolated from stools. Of the 47 Hly^+, CNF^- strains, 15 (40%) belonged to serotype O6:K2:H1, isolated from either stools or extraintestinal infections, and 18ac:K5:H1, isolated from only extraintestinal infections. Both of the Hly^-, CNF^+ *E. coli* strains examined belonged to serotype O2:K7:H−, although they had been isolated in different cities 1 year apart from each other. Only three serotypes (O6:K2:H1, O6:K−:H1, and O75:K5:H−) were found in both CNF^+ and CNF^- hemolytic *E. coli* strains.

To further characterize isolates belonging to the same serotype or the same serogroup, we studied the OMP patterns of 49 strains. Two different membrane patterns were found among 27 *E. coli* strains of serogroup O6 belonging to eight different serotypes (Fig. 1a). One was characteristic of *E. coli* O6:H1 strains, and 20 strains of this O:H type all exhibited this pattern regardless of the K antigen, the production of CNF, or the source, place, and year of isolation. The second OMP pattern was demonstrated in five O6:H31 strains belonging to two different capsular types and in two O6:K14:H− strains. Three different membrane patterns were found among the eight strains belonging to serogroup O2 (Fig. 1b). As described for serogroup O6, strains sharing the same H antigen (H1, H6, and H−) showed the same OMP pattern independent of the other characteristics. In this way, the two O2:K7:H−, Hly^-, CNF^+ strains exhibited the same membrane pattern as did the Hly^+, CNF^+ strain belonging to this serotype.

A different situation was observed when *E. coli* strains belonging to serogroup O4 were analyzed; in fact, all eight isolates examined showed the same OMP pattern (Fig. 1c), although some of them possessed the antigen H1 and others possessed the antigen H5. Similarly, the membrane pattern did not allow us to distinguish among isolates belonging to serogroup O75, even if they differed from one another in H and K antigens and CNF production (Fig. 1d). Only one strain possessing two different capsular antigens (K95 and K5) showed an additional band as compared with the standard pattern of this serogroup. A single common pattern was also found among eight O18ac:K5:H1 strains and one O18ac:K5:H5 strain of Hly^+, CNF^- *E. coli* examined (Fig. 1e).

TABLE 2. O serogroups of 76 *E. coli* strains producing Hly and CNF by source of isolation

Serogroup	No. (%) of isolates from:	
	Stools (n = 39)	Extraintestinal infections (n = 37)
O2	4 (10.2)	2 (5.4)
O4	2 (5.1)	9 (24.3)[a]
O6	12 (30.8)	16 (43.2)
O18ac	2 (5.1)	
O22	5 (12.8)	5 (13.5)
O75	4 (10.2)	2 (5.4)
O83	3 (7.6)	2 (5.4)
O85	2 (5.1)	1 (2.7)
Other (represented by one strain)	5 (12.8)	

[a] Significantly different ($P < 0.05$) from fecal isolates.

TABLE 3. O serogroups of 47 *E. coli* strains producing Hly only by source of isolation

Serogroup	No. (%) of isolates from:	
	Stools (n = 33)	Extraintestinal infections (n = 14)
O6	6 (18.2)	4 (28.5)
O18ac	2 (6.0)	8 (57.1)[a]
O18abc	3 (9.1)	
O15	2 (6.0)	
O17	2 (6.0)	
O21	3 (9.1)	
O143	2 (6.0)	
O166	2 (6.0)	
Other (represented by one strain)	11 (33.3)	2 (14.3)

[a] Significantly different ($P < 0.01$) from fecal isolates.

DISCUSSION

Hemolytic strains of *E. coli* involved in human pathology are known to belong to a restricted number of serogroups (8,

TABLE 4. Serotypes of 96 *E. coli* strains producing Hly or CNF

Serotype	No. of isolates producing:		
	Hly and CNF (n = 57)	Hly only (n = 37)	CNF only (n = 2)
O2:K1:H6	2		
O2:K7:H−	1		2
O4:K12:H5	4		
O4:K12:H1	2		
O4:K3:H5	3		
O6:K2:H1	1	7	
O6:K−:H1	1	2	
O6:K2:H31	3		
O6:K13:H1	7		
O6:K14:H−	2		
O6:K14:H31	2		
O17:K5:H−		2	
O18ac:K5:H1		8	
O18abc:K−:H−		2	
O21:K2:H2		2	
O22:K13:H1	3		
O75:K5:H−	1	1	
O75:K95:H5	3		
O83:K14:H31	2		
O166:K1:H4		2	
Other (n = 31) (represented by one strain)	20	11	

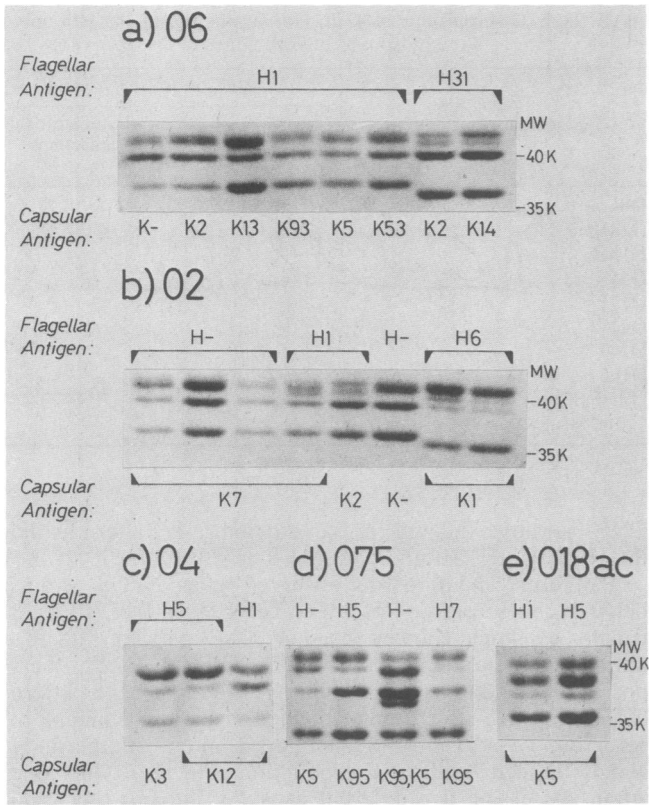

FIG. 1. Membrane patterns found among serogroup O2, O4, O6, O18ac, and O75 isolates. MW, Molecular weight.

9, 13, 14, 16). This study confirms this fact, as most of our strains, and particularly those from extraintestinal infections, belonged to these serogroups. The results of O serotyping also showed that hemolytic *E. coli* strains in the present material could be divided into two distinct classes, a hypothesis which arose from the observation that over one-half of hemolytic strains produce CNF (2, 6). In fact, CNF^+ and CNF^- hemolytic isolates of *E. coli* were shown to belong to different serogroups, with the exception of O6 and O18ac, present in both categories of isolates. While serogroup O18ac was strongly associated with Hly^+, CNF^- isolates (10 of 47 versus 2 of 76; $P = 0.001$), serogroup O6 was frequently associated with both categories of hemolytic *E. coli* isolates. The analysis of the O:K:H serotypes of these strains showed that all of the Hly^+, CNF^- isolates were O6:H1 and generally possessed the K2 antigen, whereas the CNF-producing strains belonged to serotype O6:H31 or O6:K13:H1. Similarly, the complete serotype of the two CNF-producing O18ac strains was O18ac:K10:H16, whereas that of the Hly^+, CNF^- strains was O18ac:K5:H1.

About 90% of CNF- and Hly-producing *E. coli* strains belonged to six O serogroups only (O2, O4, O6, O22, O75, and O83). These groups were encountered either in isolates from stools, which represent the natural reservoir of these organisms, or in isolates from extraintestinal infections. *E. coli* strains producing Hly only belonged to a larger number of O serogroups, and only two of these (O6 and O18ac) were widely represented; the latter two serogroups were virtually the only serogroups encountered among Hly^+, CNF^- strains from extraintestinal infections. The observation that Hly^+, CNF^- *E. coli* strains belonging to numerous less common serogroups were hardly ever isolated from extraintestinal infections suggests that these strains may be weakly virulent. Conversely, it is reasonable to assume that strains belonging to the serogroups found at similar or even higher (O4 and O18ac) rates in extraintestinal infections than in stools may be particularly aggressive. These results indicate that Hly production per se does not make a strain of *E. coli* virulent and confirm that several traits are necessary to make a strain pathogenic. They are also in good agreement with the view that only a limited number of aggressive strains, likely representing the descent of a few bacterial clones, are well adapted to cause extraintestinal infections (1, 9, 11, 13, 15, 16). The examination of CNF production may also be useful in characterizing these hemolytic clones of *E. coli*. Väisänen-Rhen and co-workers (16) identified two uropathogenic hemolytic clones sharing the same OMP pattern among *E. coli* O6:H1 strains isolated in Finland. One was characterized by possession of the K2 antigen, production of P fimbriae, and association with pyelonephritis; the other was characterized by possession of the K13 antigen, lack of P fimbriae, and association with cystitis. It would be interesting to determine if the two Finnish O6:H1 clones could also be differentiated by the ability to produce CNF, since all seven O6:H13:H1 strains identified in our material lacked MRHA and produced CNF and Hly, whereas seven of eight O6:K2:H1, Hly^+, MRHA-positive strains were CNF^-.

Even though hemolytic CNF^+ and CNF^- clones are usually characterized by different O antigens or O:K:H combinations, a few serotypes included both CNF^+ and CNF^- strains, and the membrane pattern did not differentiate between these strains. The CNF^-, Hly^+, O75:K5:H− strain was the sole isolate within serogroup O75 which proved unable to produce CNF. On the contrary, seven of eight *E. coli* O6:K2:H1 isolates were Hly^+ and CNF^-. This result suggests that, in some cases, CNF^+ and CNF^- variants of original bacteria may arise by acquisition or loss of the CNF production character, respectively.

In conclusion, the serological typing of our strains further confirmed that hemolytic isolates of *E. coli* may grossly be divided into two main populations on the basis of the ability to produce CNF. Examination of hemolytic *E. coli* isolates for this property may thus be useful in achieving a more detailed characterization of pathogenic clones.

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