JOURNAL OF CLINICAL MICROBIOLOGY, Jan. 1987, p. 146–149
0095-1137/87/010146-04$02.00/0

Vol. 25, No. 1

Cytotoxic Necrotizing Factor Production by Hemolytic Strains of *Escherichia coli* Causing Extraintestinal Infections

ALFREDO CAPRIOLI,[1]* VINCENZO FALBO,[1] FRANCO M. RUGGERI,[1] LUCILLA BALDASSARRI,[1] ROSARIO BISICCHIA,[2] GIUSEPPE IPPOLITO,[3] EDOARDO ROMOLI,[4] AND GIANFRANCO DONELLI[1]

Laboratorio di Ultrastrutture, Istituto Superiore di Sanità, 00161 Rome,[1] Ospedale dei Bambini Umberto I, Brescia,[2] Ospedale Lazzaro Spallanzani, Rome,[3] and Presidio Ospedaliero, Unità Sanitaria Locale, Orvieto,[4] Italy

Received 4 September 1986/Accepted 22 September 1986

Two hundred and nineteen strains of *Escherichia coli* from extraintestinal infections and feces of healthy subjects were examined for hemolysin (Hly) and cytotoxic necrotizing factor (CNF) production and for mannose-resistant hemagglutination. Of 105 strains from extraintestinal infections, 42 (40.0%) were positive for production of both Hly and CNF, and 21 (20.0%) were positive for Hly alone; on the contrary, only 1 Hly- and CNF-positive strain and 2 Hly-positive strains were found among 114 strains from normal stools. CNF production was not found to occur among the nonhemolytic strains, confirming the close association existing between these toxic factors. Hemolytic strains positive for CNF showed mannose-resistant hemagglutination less frequently than did Hly-positive, CNF-negative strains (25.6 versus 82.6%), suggesting the existence of two distinct classes among hemolytic strains of *E. coli*.

In a recent paper, we described the production of a cytotoxic necrotizing factor (CNF) by some human enteric isolates of *Escherichia coli* (3). This toxin, which is a protein of 110,000 daltons in size (2), causes necrosis in rabbit skin and induces the formation of large multinucleated cells in tissue cultures. All but one CNF-producing strain studied so far have also been found to produce hemolysin (Hly) (1, 3). Hly production is rarely found in fecal isolates of *E. coli* (4, 13), but is very common among strains from extraintestinal infections (4, 6, 9, 12); on this basis, we have hypothesized that CNF production could be an additional feature of these virulent strains. In this paper, we describe the examination of *E. coli* isolates from extraintestinal infections for CNF and Hly production in comparison with strains isolated from feces of healthy subjects. All strains were also tested for hemagglutination (HA), a property which is often associated with Hly production (6, 12).

MATERIALS AND METHODS

Bacterial strains. All *E. coli* strains from extraintestinal infections were isolated from patients at two large hospitals. Isolates from urinary tract infections were obtained by cultures of 91 different urine specimens. Patients were either symptomatic (cystitis or pyelonephritis) or asymptomatic (10^5 or more microrganisms per ml of urine). Ten strains were isolated from bronchial aspirates, two were isolated from infected wounds, one was from blood, and one was from a patient with neonatal conjunctivitis. Moreover, 114 strains of *E. coli* were obtained from the feces of 113 healthy subjects (77 children and 36 adults). Isolation and identification of *E. coli* were performed by current methods.

Hly production. *E. coli* strains were tested on washed blood agar plates. Defibrinated sheep blood was washed three times with 0.01 M Tris chloride buffer (pH 7.5), containing 0.135 M NaCl and added to blood agar base no. 2 (Oxoid Italiana, Garbagnate Milanese, Italy) to a final concentration of 5%. Hemolysis was read after overnight incubation at 37°C. Production of soluble alpha-Hly in fresh alkaline meat extract broth (18) was tested as described by Van Den Bosch et al. (19).

CNF production. The method used to detect CNF production is described in detail elsewhere (3) and summarized here. *E. coli* strains were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.), sonicated, and centrifuged; supernatants were filter sterilized and inoculated in HEp-2 cells (American Type Culture Collection, Rockville, Md.) in 96-well microdilution plates. Monolayers were examined during a 4-day period, and the presence of CNF was revealed by multinucleation in at least 50% of the cells.

HA typing. HA typing was carried out as described by Evans and co-workers (7) with human (type A), guinea pig, bovine, adult chicken, and African green monkey erythrocytes. Tests were performed by slide agglutination with bacterial cells grown for 18 h on CFA agar (5). HA was noted as mannose resistant (MRHA) if the same degree of agglutination occurred with and without 1% mannose and was classified into types V, VI and VII according to the scheme of Evans et al. (7).

Statistical methods. The Fisher exact test and the χ^2 test were used to analyze the incidence of virulence factors in strains of *E. coli* from the different sources (17).

RESULTS

A total of 219 strains of *E. coli* isolated from extraintestinal infections and from feces of healthy subjects were studied. All strains were tested for Hly and CNF production and for the presence of mannose-resistant hemagglutinins (Table 1). All three virulence markers were significantly associated with disease ($P < 0.01$). Interestingly, 42 of 105 strains from extraintestinal infections (40%) were positive for CNF production (CNF^+) as against one strain in the group from feces (0.9%).

On the basis of the three markers considered in the study, our strains were divided into six groups (Table 2). Most of the strains from normal feces (85.1%) exhibited no virulence-associated properties, whereas these negative strains represented only 36.2% of the extraintestinal isolates ($P < 0.001$). All of the 43 CNF-producing strains identified were also

* Corresponding author.

TABLE 1. Detection of virulence factors in *E. coli* strains from different sources

Source	No. of isolates	No. (%) of isolates identified as:		
		Hly$^+$	CNF$^+$	MRHA$^+$
UTI	91	53 (58.2)	34 (37.4)	26 (28.5)
Other[a]	14	10 (71.4)	8 (57.1)	5 (35.7)
Total extraintestinal infections	105	63 (60.0)[b]	42 (40.0)[b]	31 (29.5)[b]
Normal stool	114	3 (2.7)	1 (0.9)	16 (14.0)

[a] See Materials and Methods.
[b] Significant difference ($P < 0.01$) between strains from extraintestinal infections and normal stools.

hemolytic (Hly$^+$). The pattern Hly$^+$ CNF$^+$ was very common among strains from extraintestinal infections (30.5%) and did not occur among the 114 strains from normal intestinal flora. The strains showing the patterns Hly$^+$ MRHA positive (MRHA$^+$) and Hly$^+$ CNF$^+$ MRHA$^+$ were also more frequent in extraintestinal infections ($P < 0.001$), accounting for 16.2 and 9.5% of isolates, respectively. On the contrary, strains showing only mannose-resistant adhesins were more commonly found in the group from feces ($P < 0.05$).

The correlations between production of toxins and MRHA are shown in Table 3, where *E. coli* strains are grouped by their toxin pattern, regardless of the source of isolation. MRHA was found in a high percentage (45.4%) of all strains producing Hly. Striking differences were observed when hemolytic strains were divided according to their ability to produce CNF; in fact, MRHA was revealed in 82.6% of Hly$^+$ CNF$^-$ strains, compared with 25.5% of Hly$^+$ CNF$^+$ strains ($P < 0.001$). Moreover, the two groups of hemolytic strains appeared unevenly distributed among MRHA patterns (Table 4). Of the 19 Hly$^+$ CNF$^-$ MRHA$^+$ strains, 14 belonged to HA type VI, and 5 belonged to HA type V; in contrast, 7 of the 11 Hly$^+$ CNF$^+$ MRHA$^+$ strains belonged to HA type V, and 4 belonged to HA type VI ($P < 0.05$); further significant differences within the HA types V and VI could not be reckoned because of the small number of strains considered. However, strains belonging to the HA type V showed a large variability in their erythrocyte reactivity whereas most of the HA type VI isolates fitted in the VI D pattern, which has been reported to be closely associated with Hly production (7).

After the first screening on blood agar, hemolytic strains were further tested in fresh alkaline meat extract broth for production of soluble alpha-Hly. All the 66 strains examined, regardless of their ability to produce CNF, gave the same result, releasing the alpha-Hly into the liquid medium.

TABLE 2. Prevalence of different phenotypes among *E. coli* isolates from normal stools and extraintestinal infections

Phenotype	No. (%) of fecal isolates	No. (%) of extraintestinal isolates
Hly$^+$ CNF$^+$ MRHA$^+$	1 (0.9)	10 (9.5)[a]
Hly$^+$ CNF$^+$	0 (−)	32 (30.5)[a]
Hly$^+$ MRHA$^+$	2 (1.8)	17 (16.2)[a]
Hly$^+$	0 (−)	4 (3.8)
MRHA$^+$	14 (12.3)	4 (3.8)[b]
Other	97 (85.1)	38 (36.2)[a]

[a] Significant difference ($P < 0.001$) between fecal and extraintestinal isolates.
[b] Significant difference ($P < 0.05$) between fecal and extraintestinal isolates.

DISCUSSION

Many virulence factors have been shown to be associated with *E. coli* strains causing extraintestinal infections, in particular MRHA antigens and hemolysin (6, 11, 12). In the present study, we have examined *E. coli* isolates from patients with extraintestinal infections and from healthy subjects for the presence of these virulence markers, as well as for the production of the cytotoxic necrotizing factor, a toxin formerly described in hemolytic strains of *E. coli* from stools (1, 3).

As previously reported by others (6, 9, 14, 20), the hemolytic property was strongly associated with *E. coli* strains causing extraintestinal infections. Similarly, CNF production appears to be a common property among the pathogenic strains of *E. coli*.

On the basis of the three markers studied, we have identified six different phenotypes, distributed at significantly different rates between the two populations of *E. coli* considered. Most of MRHA$^+$ isolates from extraintestinal infections (27 of 31, 87%) were found to produce Hly; about one-third of these also produced CNF. On the contrary, hemolytic strains accounted for a low percentage (2 of 16, 17.6%) of MRHA$^+$ strains from feces. Moreover *E. coli* strains exhibiting only MRHA were more frequent in the group from stools than in the group from extraintestinal infections. These observations suggest that the higher prevalence of MRHA among our strains from patients with respect to fecal strains (Table 1) would be merely due to the close association existing between MRHA and Hly and CNF production. The analysis of the phenotypes of pathogenic isolates of *E. coli* also showed that only 6% of hemolytic strains (4 of 66) exhibited Hly production as their unique virulence marker; rather, this property appears alternatively associated with CNF production or MRHA and, less frequently, with both of these factors.

The present investigation further confirms the close association of CNF with Hly production (1, 3); CNF was produced by 65.2% of the hemolytic strains, whereas we were not able to find any CNF producer among the 153 nonhemolytic *E. coli* strains examined. At the present time, among over 100 CNF-producing strains identified in our laboratory, only 1 isolate did not produce Hly; this strain was isolated from the stool of a child with diarrhea (1) and showed the phenotype CNF$^+$ Hly$^-$ MRHA$^-$.

The possibility that two different types of Hly were produced by CNF$^+$ and CNF$^-$ strains was also investigated. It is known that *E. coli* can produce two main kinds of hemolysin: one is filterable and is termed alpha-Hly (18, 21); the other is cell bound and nonfilterable and is termed beta-Hly (16, 18). All of the hemolytic strains examined, regardless of their ability to produce CNF, were shown to produce alpha-Hly, even though the possibility cannot be

TABLE 3. MRHA in *E. coli* strains showing different toxin patterns

Toxin pattern	No. of isolates	No. (%) of MRHA$^+$ isolates
Hly$^+$	23	19 (82.6)[a]
Hly$^+$ CNF$^+$	43	11 (25.6)
Hly$^-$ CNF$^-$	153	18 (11.8)

[a] Significant difference ($P < 0.001$) between Hly$^+$ and Hly$^+$ CNF$^+$ strains.

TABLE 4. Relationship between CNF production and HA type in hemolytic MRHA$^+$ strains of *E. coli*

HA type	MRHA or MSHA with[a]:					No. of Hly$^+$ strains (%; n = 19)	No. of Hly$^+$ CNF$^+$ strains (%; n = 11)
	Hu	Bv	Ck	Mk	Gp		
VB	–	R	–	–	–	1	
VF	R	–	S	S	S	3	1
VG	R	–	–	S	S	1	1
VG	R	S	S	S	S		1
VG	S	R	S	S	S		1
VG	S	–	S	R	S		1
VG	S	–	S	S	R		2
V (total)						5 (26.3)[b]	7 (63.6)
VI B	R	–	R	R	S	1	
VI C	R	–	–	R	–	1	
VI D	R	–	S	R	S	12	2
VI E	R	–	R	R	R		2
VI (total)						14 (73.7)[b]	4 (36.4)

[a] Erythrocytes tested: Hu, human; Bv, bovine; Ck, chicken; Mk, monkey; Gp, guinea pig. R, MRHA; S, mannose-sensitive HA (MSHA); –, negative for HA.
[b] Significant difference ($P < 0.05$) between Hly$^+$ and Hly$^+$ CNF$^+$ strains.

excluded that further analyses might reveal slight biochemical or immunological differences between the hemolysins produced by strains CNF$^+$ and CNF$^-$. On the other hand, these two categories of hemolytic isolates of *E. coli* behave differently as concerns MRHA, which was more frequently detected in CNF$^-$ strains; the patterns of HA themselves differed between CNF$^+$ and CNF$^-$ isolates.

Whereas the genetic determinant for Hly production can be located either on the bacterial chromosome (10, 15) or on transmissible plasmids (8), the genes encoding CNF production appear to be chromosomally inherited. In fact, the analysis of the plasmid content carried out on 19 Hly$^+$ CNF$^+$ strains revealed that 9 of them were plasmid free; the remaining strains shared no similar plasmids (unpublished data). Just as the genes encoding alpha-Hly and MRHA are closely linked in the chromosomal DNA of some strains of *E. coli* from urinary tract infections (10, 15), the association between the production of Hly and CNF might be explained by the presence of a gene cluster governing the synthesis of the two toxins. Our results also suggest that *E. coli* strains producing CNF may belong to a large extent to the restricted number of serotypes which are known to be associated with Hly production and MRHA (6, 9, 12). An important subsequent step will be the serotyping of our hemolytic strains to verify whether there is a more specific association of such serotypes with either the CNF$^+$ or CNF$^-$ strains.

In conclusion, our results suggest the following: (i) the production of toxic factors is a main feature of *E. coli* strains causing extraintestinal infections, whereas the ability to agglutinate erythrocytes would play only a secondary role; (ii) hemolytic *E. coli* strains can be divided into two separate populations differing from each other both in their ability to produce CNF and in other characteristics, i.e., the presence and kind of hemagglutinins.

A better understanding of the association between CNF and Hly will help clarify the respective roles of the two toxins in the pathogenesis of *E. coli* infections.

ACKNOWLEDGMENT

This work was partially supported by grant 84.03102.52 of the Consiglio Nazionale delle Ricerche Progetto Finalizzato per il Controllo delle Malattie da Infezione.

LITERATURE CITED

1. **Bisicchia, R., R. Ciammarughi, A. Caprioli, V. Falbo, and F. M. Ruggeri.** 1985. Toxin production and hemagglutination in strains of *Escherichia coli* from diarrhoea in Brescia, Italy. J. Hyg. **95**:353–361.
2. **Caprioli, A., G. Donelli, V. Falbo, R. Possenti, L. G. Roda, G. Roscetti, and F. M. Ruggeri.** 1984. A cell division-active protein from *E. coli*. Biochem. Biophys. Res. Commun. **118**:587–593.
3. **Caprioli, A., V. Falbo, L. G. Roda, F. M. Ruggeri, and C. Zona.** 1983. Partial purification and characterization of an *Escherichia coli* toxic factor that induces morphological cell alterations. Infect. Immun. **39**:1300–1306.
4. **Cooke, E. M., and S. P. Ewins.** 1975. Properties of strains of *Escherichia coli* isolated from a variety of sources. J. Med. Microbiol. **8**:107–111.
5. **Evans, D. G., D. J. Evans, Jr., and W. Tjoa.** 1977. Hemagglutination of human group A erythrocytes by enterotoxigenic *Escherichia coli* isolated from adults with diarrhea: correlation with colonization factor. Infect. Immun. **18**:330–337.
6. **Evans, D. J., Jr., D. G. Evans, C. Höhne, M. A. Noble, E. V. Haldane, H. Lior, and L. S. Young.** 1981. Hemolysin and K antigens in relation to serotype and hemagglutination type of *Escherichia coli* isolated from extraintestinal infections. J. Clin. Microbiol. **13**:171–178.
7. **Evans, D. J., Jr., D. G. Evans, L. S. Young, and J. Pitt.** 1980. Hemagglutination typing of *Escherichia coli*: definition of seven hemagglutination types. J. Clin. Microbiol. **12**:235–242.
8. **Goebel, W., B. Royer-Pokora, W. Lindenmaier, and H. Bujard.** 1973. Plasmids controlling synthesis of hemolysin in *Escherichia coli*. I. Molecular properties. J. Bacteriol. **118**:964–973.
9. **Green, C. P., and V. L. Thomas.** 1981. Hemagglutination of human type O erythrocytes, hemolysin production, and serogrouping of *Escherichia coli* isolates from patients with acute pyelonephritis, cystitis, and asymptomatic bacteriuria. Infect. Immun. **31**:309–315.
10. **Hacker, J., S. Knapp, and W. Goebel.** 1983. Spontaneous deletions and flanking regions of the chromosomally inherited hemolysin determinant of an *Escherichia coli* O6 strain. J. Bacteriol. **154**:1145–1152.
11. **Hagberg, L., U. Jodal, T. K. Korhonen, G. Lidin-Janson, U. Lindberg, and C. Svanborg Eden.** 1981. Adhesions, hemagglutination, and virulence of *Escherichia coli* causing urinary tract infections. Infect. Immun. **31**:564–570.
12. **Hughes, C., J. Hacker, A. Roberts, and W. Goebel.** 1983. Hemolysin production as a virulence marker in symptomatic and asymptomatic urinary tract infections caused by *Escherichia coli*. Infect. Immun. **39**:546–551.
13. **Hughes, C., D. Muller, J. Hacker, and W. Goebel.** 1982. Genetics and pathogenic role of *Escherichia coli* alpha-hemolysin.

Toxicon **20**:247–252.
14. **Lidin-Janson, G., L. A. Hanson, B. Kaijser, K. Lincoln, U. Lindberg, S. Olling, and H. Wedel.** 1977. Comparison of *Escherichia coli* from bacteriuric patients with those from feces of healthy schoolchildren. J. Infect. Dis. **136**:346–353.
15. **Low, D., V. David, D. Lark, G. Schoolnik, and S. Falkow.** 1984. Gene clusters governing the production of hemolysin and mannose-resistant hemagglutination are closely linked in *Escherichia coli* serotype O5 and O6 isolates from urinary tract infections. Infect. Immun. **43**:353–358.
16. **Short, E. C., Jr., and H. J. Kurtz.** 1971. Properties of hemolytic activities of *Escherichia coli*. Infect. Immun. **3**:678–687.
17. **Siegel, S.** 1956. Nonparametric statistics for the behavioural sciences. McGraw-Hill Book Co., New York.
18. **Smith, H. W.** 1963. The hemolysins of *Escherichia coli*. J. Pathol. Bacteriol. **85**:197–211.
19. **Van Den Bosch, J. F., P. Postma, J. De Graaf, and D. M. Mac Laren.** 1980. Determination of the alpha-hemolytic activity of hemolytic urinary *Escherichia coli* strains. FEMS Microbiol. Lett. **8**:75–77.
20. **Van Den Bosch, J. F., P. Postma, P. A. R. Koopman, J. De Graaf, D. M. MacLaren, D. G. Van Brenk, and P. A. M. Guinee.** 1982. Virulence of urinary and fecal *Escherichia coli* in relation to serotype, hemolysis and hemagglutination. J. Hyg. **88**: 567–577.
21. **Williams, P. H.** 1979. Determination of the molecular weight of *Escherichia coli* alpha-hemolysin. FEMS Microbiol. Lett. **5**:21–24.