INFECTION AND IMMUNITY, Sept. 1977, p. 497-503

Vol. 17, No. 3
Printed in U.S.A.

Production of Colicin V In Vitro and In Vivo and Observations on Its Effects in Experimental Animals

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Received for publication 3 March 1977

In recent years, a possible relationship between pathogenicity and colicinogeny in some *Escherichia coli* strains responsible for gastrointestinal infection and bacteremia in man and animals has been inferred. Using enterotoxigen-negative, colicin V-producing *E. coli* strains, we have (i) elaborated a simple in vitro method for producing greater yields of colicin V free of bacterial cells and large, non-dialyzable molecules; (ii) detected the presence of the bacteriocin in peritoneal fluids of moribund mice injected intraperitoneally 18 h previously with colicin V-producing strains (in these mice, Col V^+ exconjugants survived and multiplied more extensively than the Col V^- recipient strains from which they were derived; and (iii) observed an increased vascular permeability and inflammatory response in rabbits and guinea pigs when a culture supernatant demonstrating colicin activity was injected intradermally. The vascular response obtained after the injection of either a colicin V-containing dialysate alone or that of a trypsinized colicin-containing supernatant was always smaller than when the colicin V-active supernatant was injected. An enterotoxin-free dialysate containing colicin V also increased markedly in rabbits and guinea pigs the mild inflammatory reaction that occurred in the skin when purified endotoxin was injected subcutaneously in microgram doses. Our results are consistent with the hypothesis that colicin V may act as a virulence factor in some *E. coli* strains.

Some colicins, like colicin V, are plasmid-determined, narrow-spectrum antibiotic substances produced by *Escherichia coli* or closely related bacterial strains. Although the lethal effect of colicins on bacterial cells is well documented (4, 11, 12), we know very little about the possible action of colicins upon eucaryotic cells; recent work with *Euglena gracilis* (15), with *Candida albicans* (C. D. Jeffries and R. E. Schileru, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, p. 60, D52), and with experimental animals (16, 17) strongly suggests that some colicins, in addition to their action on procaryotes, may act upon eucaryotic cells, including mammalian cells; the fact that mammalian cells harbor organelles (mitochondria) reminiscent of procaryotic microorganisms may not contradict this (15). For workers on infection and immunity, a renewal of interest in the ill-known colicin V (4) arises from the facts that (i) it has been shown by Smith (16) to be closely associated or identical with a plasmid-controlled lethal character found in some virulent *E. coli* strains, and (ii) the Col V plasmid of certain strains is transmissible by bacterial conjugation (4, 16). Our recent experiments on the possible relationships between colicin V and the virulence of *E. coli* strains support previous observations by other workers (16, 17) that colicin V can be related to virulence, and they indicate that it may potentiate at least one of the effects of endotoxins in rabbits and guinea pigs.

MATERIALS AND METHODS

Organisms. The *E. coli* strains used in our experiments as a source of colicin V were: strain 44B from the American Type Culture Collection (ATCC), which produces colicin V_1, and strain RC414 (ATCC), which synthesizes colicin V_2. The colicin V-sensitive indicator strains used were *E. coli* RC511 (ATCC) and the *E. coli* K-12 Row strain from our collection. A colicin V-resistant mutant of the Row strain (Row R/V) was included in all experiments as a control that any inhibitory effect exerted on the indicator Row strain was due to colicin V and not to other unknown factors. Transfer of the Col V_2 plasmid by bacterial conjugation was performed according to techniques we have already described (6). The colicin V_2-producing donor strain was *E. coli* RC414, and the recipient strains used were *E. coli* RC511, *E. coli* K-12 Row, and *E. coli* K-12 Row R/V; the respective exconjugants were identified as *E. coli* RC511-V_2, *E. coli* K-12 Row-V_2, and *E. coli* K-12 Row^1-V_2. All the strains used were tested for enterotoxinogenic activity in S. Larivière's laboratory (Department of Microbiology and Pathology, Faculty of Veterinary Medicine, Ste-

Hyacinthe, Quebec, Canada) and were reported negative on the basis of (i) alteration of the Chinese hamster ovary cell morphology test, (ii) the ligated swine intestinal loop assay, and (iii) the suckling mouse test.

Media. The yields of colicin V, a bacteriocin considered to be noninducible by mitomycin C or ultraviolet rays (12), are low in most usual culture media. After a series of trials involving several culture media (brain heart, nutrient broth, Mueller-Hinton broth, nutrient broth supplemented with 4 μg of methionine per ml [Sigma Chemical Co.], and others), it became apparent that, for unknown reasons, in heated (60°C, 10 h) tryptic soy broth (TSB) we repeatedly obtained higher yields of the bacteriocin. Consequently, commercial (Difco) TSB powder was first heated at 60°C in wet heat for 10 h and dried at 37°C in dry heat for 72 h. This "modified TSB" (TSBM) was used according to the specifications for ordinary TSB.

Production of colicin V in vitro. Since previous investigations (1, 5) had shown that colicin V may cross dialysis membranes and since it is believed that the heat-labile toxin of *E. coli* has a large molecular weight (19), a cellophane dialysis bag (Fisher, no. 8-667-B) containing 150 ml of sterile distilled water was tied securely and suspended in a closed 1-liter graduated cylinder containing 500 ml of TSBM culture medium. The medium was inoculated with 0.1 ml (10^8 cells) of washed colicinogenic cells, and the cylinder was incubated for 72 h at 37°C. The dialysis bag acts as an exchange recipient which lets some of the colicin produced enter and at the same time excludes the bacterial cells and the large macromolecules like the lipopolysaccharide (endotoxin). The dialysate was heat sterilized (60°C for 90 min); then sterility tests were run on samples of its content, and its colicinogenic activity was tested on both colicin V-sensitive and -resistant strains. Colicin V being relatively heat stable, this last treatment of the culture dialysate was also intended to decrease the possibility that a biological activity observed in subsequent tests with the dialysate would be due to heat-labile enterotoxin that may be involved in vascular permeability changes (3).

Detection of colicin V and titration of activity. We used a simple detection test in which we spotted drops of heat-sterilized dialysate on lawns of a colicin V-sensitive strain to detect bactericidal colicinogenic activities and on lawns of a colicin V-resistant strain as controls. For sterility tests, we dropped 1-ml samples of dialysate on brain heart agar plates and incubated them for 48 h. For titration of bactericidal activity of our colicinogenic dialysates, we followed the technique described by Mayr-Harting et al. (10), which assesses the number of lethal units of colicin per milliliter.

Survival of colicinogenic strains and production of colicin in vivo. To measure the survival of Col V_2^+ exconjugant and Col V_2^- recipient strains in vivo, we injected groups of 72 mice weighing approximately 20 g with each bacterial strain in three different experiments. Equal numbers of viable units (VU) of Col V_2^- recipient and Col V_2^+ exconjugant strains were injected intraperitoneally (i.p.) into the mice of each group. At time zero, immediately after all mice had been injected, we took three mice in each group and washed their peritoneal cavity with 5 ml of isotonic saline (9). We counted the VU in 1 ml of peritoneal fluids, and this was recorded as the initial number of VU. Every 2 h during a period of 24 h, we washed the peritoneal cavity of three randomly selected mice in each group and counted the VU. By this method, we followed the fate of each strain in the mouse peritoneal cavity. Tests for colicinogenic activity of peritoneal fluids were also performed on each sample obtained from the mice. We also made smears with 10-μl portions of peritoneal fluids from mice injected i.p. with the Col V_2^+ or Col V_2^- strains and from mice injected with isotonic saline as a control. Standardized smears were stained with acridine orange as described previously (8, 9), and we counted the number of phagocytic cells in 50 differents fields examined under the fluorescence microscope (magnification, ×730).

Vascular permeability test. We followed the Evans blue technique (3) used for the assay of production of vascular permeability factor in enteropathogenic *E. coli* strains; the substances to be tested were injected intradermally (0.1 ml) to 500-g guinea pigs (or to 1.5-kg New Zealand rabbits) in cleanly shaved areas on the backs of the animals. Twenty-four hours later, a solution (2% in 0.15 M NaCl) of Evans blue dye (Matheson, Coleman and Bell) at a dose of 40 mg per kg of body weight was administered intravenously. Two hours were allowed for permeation of the dye, after which we measured the diameter of the blue zones in the skin of the experimental animals.

In vivo tests with sources of colicin V and Col V^- cells. (i) With live Col V^- cells. To verify whether a colicin V-containing dialysate could increase the virulence of Col V_2^- strains for mice when both were administered together, we injected i.p. (i) groups of 10 mice weighing approximately 20 g with 2.5×10^8 VU of *E. coli* RC511 suspended in 1 ml of colicin V-containing dialysate, and (ii) groups of 10 mice with 6×10^8 VU of the same strain under the same experimental conditions. Controls were mice injected i.p. with the bacterial strain alone. Deaths were recorded during 24 h.

(ii) With heat-killed cells as a source of endotoxin. From previous experiments, we knew that colicin V is produced in vivo. Therefore, groups of 10 mice were injected i.p. with 10^8 VU of *E. coli* RC511-V_2 Col V_2^+ mixed with 10^8 heat-killed (60°C for 90 min) cells of *E. coli* RC511 Col V_2^- in 1 ml of saline to see whether the lethality of the Col V_2^+ dose would be increased by the addition of bacterial endotoxin. Groups of mice injected i.p. with 10^8 VU of *E. coli* RC511 mixed with 10^8 heat-killed cells of *E. coli* RC511-V_2, with 10^8 VU of *E. coli* RC511-V_2 alone, or with *E. coli* RC511 alone served as controls. Deaths were recorded after 24 h.

RESULTS

Colicin V production. The colicin V concentration in TSBM was 5 to 10 times higher than in the other media tested. Concentrations around 4×10^7 lethal units of colicin V per ml could be obtained in the dialysis bag, whereas final numbers of viable microorganisms were

near 10^9/ml in the surrounding medium. We also observed that incubation in an ambient temperature exceeding 40°C or under anaerobic conditions inhibited colicin V production; shaking (aeration) did not seem to increase the final numbers of lethal units of colicin V per milliliter. Figure 1A shows the inhibition obtained when drops of sterile, colicin V-containing dialysate were spotted on a lawn of a sensitive strain.

Survival of Col V_2^+ exconjugant and Col V_2^- recipient strains in the mouse peritoneal cavity. We compared the survival in the peritoneal cavity of mice of the *E. coli* RC511-Col V_2^- recipient and the *E. coli* RC511-V_2^+ exconjugant strains (Fig. 2). When 2×10^8 viable cells were injected, the number of VU in peritoneal fluids was similar for both strains during the first 6 h. Then the number of VU began to increase for the colicin-producing strain and continued to decrease for the nonproducing strain. After 12 h, there was a difference of 3 log units between the numbers of viable cells of the two strains in the peritoneal cavity of the mice, and those that had received the RC511-V_2 exconjugant began to die. No deaths were recorded in the groups injected with the Col V_2^- strain. We repeated the same experiment with the *E. coli* Row and Row-V_2 strains, and the results after 24 h, at the end of the experiment, were similar. The Col V_2^+ exconjugant strain displayed a greater ability to survive in the mouse peritoneal cavity and was more lethal than the Col V_2^- recipient strain (Fig. 3). Also, we observed that the number of phagocytic cells (counted in 50 different fields examined under the microscope) in 10 μl of peritoneal fluids was lower in mice injected i.p. 16 to 20 h previously with the *E. coli* RC511-V_2 strain than in mice injected with the *E. coli* RC511 strain. We recorded an average of four macrophages per field when the mice had been injected with the Col V_2^+ strain and a mean of nine macrophages per field for mice challenged with the Col V_2^- strain. On the average, four macrophages per

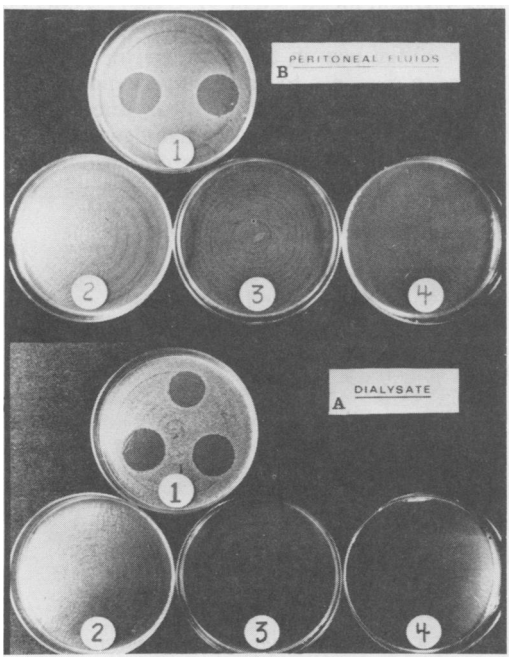

FIG. 1. *Presence of colicin V in a culture dialysate of E. coli strain 44B (colicin V producing) and in the peritoneal fluids of mice injected with the same strain. (A) In culture dialysate: 3 drops of heat-sterilized culture dialysate were spotted on a lawn of colicin V-sensitive RC511, resulting in three large zones of inhibition (dish 1); when the same dialysate was spotted on a lawn of a mutant E. coli K-12 Row resistant to colicin V (dish 2) or on a lawn of the colicin V-producing strain itself (dish 3), no inhibition zones were observed. Continuous concentric rings of growth can be seen. In dish 4, the dialysate was spread on agar to check for its sterility; no growth resulted. (B) The same tests were performed with a sample of sterile peritoneal fluids from mice injected i.p. with the same strain, except that 2 drops were spotted instead of 3.*

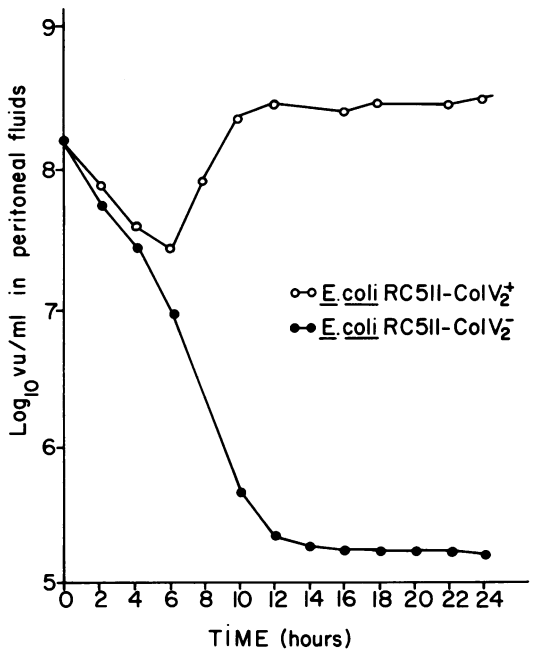

FIG. 2. *Comparison between E. coli RC511 (Col V_2^-) recipient and E. coli RC511-V_2 (Col V_2^+) exconjugant strains for survival in the mouse peritoneal cavity. There was a difference of 3 log units between the numbers of viable cells harvested from the peritoneal cavity of the mice 24 h after the injections.*

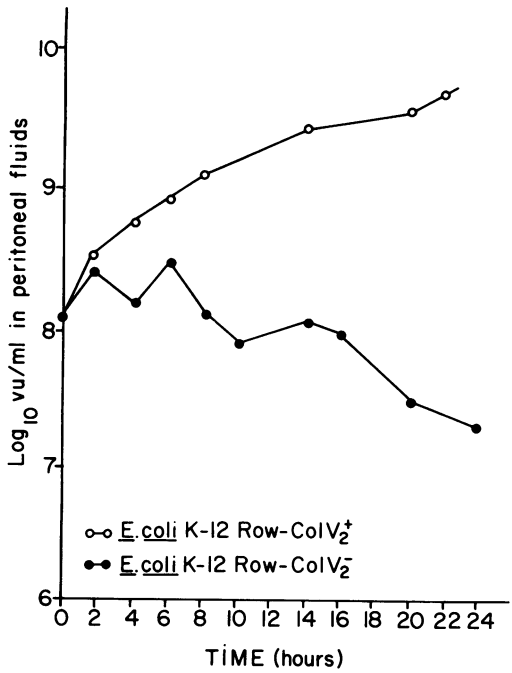

FIG. 3. *Fate of E. coli K-12 Row (Col V_2^-) recipient and E. coli K-12 Row-V_2 (Col V_2^+) exconjugant strains in the mouse peritoneal cavity. The difference between the two strains after 24 h was 2 log units.*

field were present in the smears of peritoneal fluids from mice injected with isotonic saline (control). One hundred percent death was recorded in the group of mice injected with the Col V_2^+ strain, and no mice died in the group injected with the Col V_2^- strain.

Production of colicin V_2 in vivo. Colicin V_2 was present in detectable quantities in the peritoneal fluids of mice about 10 to 12 h after the injection of colicin-producing strains, which corresponded with the active exponential phase of multiplication of the bacteria. We also detected colicinogenic activity in peritoneal fluids from agonizing mice injected with any of the Col V_2-producing strains (see Fig. 1B).

Vascular permeability. We had observed that agonizing mice injected with Col V_2-producing strains showed symptoms like those observed in endotoxic shock (14). We hypothesized that the Col V_2^+ *E. coli* strains could leave the peritoneal cavity and invade the circulatory system more rapidly than the Col V_2^- *E. coli* strains used in our experiments. We carried out tests to see whether the colicin V preparation alone (dialysate) or the colicin preparation and endotoxin (broth culture) could influence vascular permeability. It is known that endotoxin injected subcutaneously in guinea pigs or rabbits causes a mild inflammatory reaction and vascular changes (2). Under our experimental conditions, we found that colicinogenic dialysate alone had no effect on vascular permeability (Fig. 4A); negative results were also obtained with trypsinized dialysate (Fig. 4B) and sterile TSBM dialysate (Fig. 4G). However, the centrifuged (5,000 rpm for 20 min), heat-sterilized broth culture of Col V^+ *E. coli* 44B increased vascular permeability (Fig. 4C) as measured by the Evans blue technique. The diameter of the zone colored by the dye was 15 mm, whereas trypsinized centrifuged broth culture (Fig. 4D) and sterile broth culture (Fig. 4E) did not give colored zones. We knew that there was no detectable colicin V production at 41°C, so we also verified that broth culture inoculated with *E. coli* 44B and incubated at 41°C had no effect on vascular permeability (Fig. 4F). There was a great difference in vascular permeability increase in response to broth culture incubated at 41°C (Fig. 5B) and broth culture incubated at 37°C and shown to have colicinogenic activity (Fig. 5A). These results, reproduced several times in both rabbits and guinea pigs, indicated

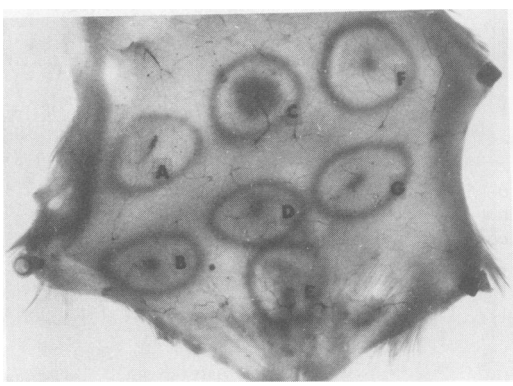

FIG. 4. *Detection of increased vascular permeability by the technique of Evans et al. (3) in the skin of guinea pigs injected intradermally with colicin V-containing and colicin V-free preparations. (A) Colicin V-containing dialysate of E. coli 44B (Col V^+) culture in TSBM; (B) trypsinized dialysate of E. coli 44B culture in TSBM, without detectable colicinogenic activity; (C) centrifuged (5,000 rpm for 20 min) broth culture of E. coli 44B (Col V^+), with detectable bactericidal (colicinogenic) activity; (D) trypsinized broth culture used in (C), without detectable colicinogenic activity; (E) sterile culture broth (TSBM); (F) centrifuged broth culture of E. coli 44B (Col V^+) incubated at 41°C, without detectable bactericidal activity; (G) dialysate of sterile culture broth. A large zone of extravasation of the dye was seen only in (C), the only preparation expected to contain a mixture of untreated (not trypsinized) colicin and endotoxin.*

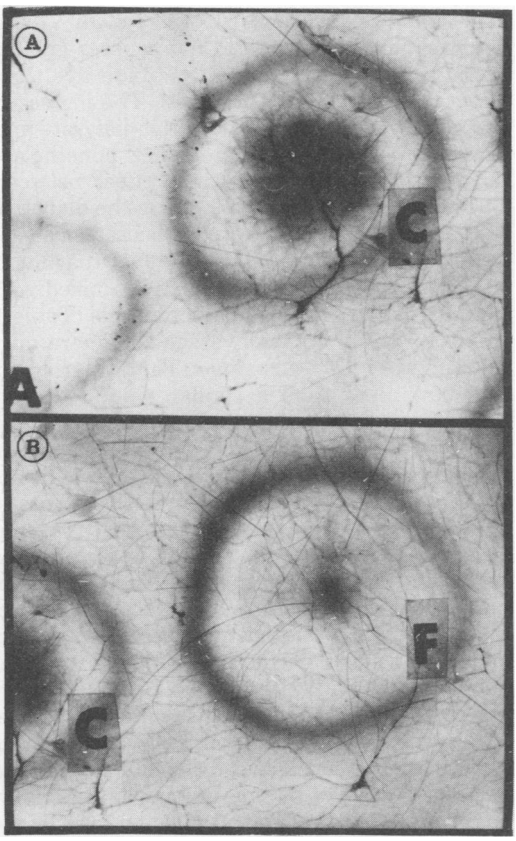

FIG. 5. *Close-ups from Fig. 4. (A) Extravasation zone observed in Fig. 4C (centrifuged broth culture of Col V⁺ E. coli 44B strain); (B) Extravasation zone observed in Fig. 4F (same as in [C], except that the culture was incubated at 41°C). One can observe the great differences in diameter and intensity of zone C (15 mm) in comparison with zone F, which is smaller (2 mm).*

that apparently colicin V affected the vascular response to an *E. coli* culture supernatant presumably containing endotoxin. In other experiments with rabbits, the colicin V-containing dialysate was injected alone and with purified *Serratia marcescens* endotoxin (lipopolysaccharide) (Sigma catalog, 1976, no. L-3380). There was a greater permeation of the dye in the area where the two substances had been injected.

Interaction between colicin V and bacterial cells of the Col V_2^- strain in vivo. There was no significant difference in the mortality rates when Col V^- cells were injected into mice with or without the colicin V-containing dialysate. The lower cell concentration (2.5×10^8 cells) caused no death, whereas for the higher one (6×10^8 cells) the mortality rate (45%) was not increased by the presence of colicin-containing dialysate. It was not possible, under our experimental conditions, to find a bacterial concentration with lethality that could be significantly increased by the concomitant injection of our colicin V-active preparation. That is why, in another series of experiments, we injected live cells of the Col V_2^+ strain to have production of colicin V in situ. When live Col V_2^+ cells were mixed with dead Col V_2^- cells, 90% of the mice died; when live Col V_2^- cells were mixed with dead Col V_2^+ cells and injected i.p., no deaths were recorded (Table 1). Control live Col V_2^+ cells injected i.p. alone killed 50% of the mice, and Col V_2^- cells caused no death.

DISCUSSION

In the past, *E. coli* strains associated with infections in man (and animals) have been identified as belonging to a small number of enteropathogenic serotypes or to enterotoxigenic strains (13), but little consideration (4) has been given to colicins as factors of invasiveness or pathogenicity. The discovery by Smith (16) of a plasmid coding for colicin V production and associated with lethality stemmed from an observation that several different passaged mating cultures grown with a Col V *E. coli* strain previously associated with an outbreak of bacteremia in chickens were lethal for chickens upon intravenous injection. When the Col V plasmids of another six wild strains of *E. coli* of varied origin were transferred to organisms of *E. coli* K-12, a lethality increase was also observed by the same author. Our study was triggered by Smith's report, and we felt it desirable to determine conditions under which colicin V can be readily obtained in vitro to be used in studies of pathogenicity with and without bacterial cells.

Our past experience with several colicins led us to expect that the choice of the culture me-

TABLE 1. *Mortality rates in mice injected i.p. with a colicin V-producing E. coli exconjugant strain and heat-killed cells as a dose of endotoxin*

Mouse group[a]	Mortality[b] after 24 h (%)
A	90
B	0
C	50
D	0

[a] Ten mice per group. Group A received 10^8 VU of *E. coli* RC511-V_2 mixed with 10^8 heat-killed cells of *E. coli* RC511; group B was injected with 10^8 VU of *E. coli* RC511 mixed with 10^8 heat-killed cells of *E. coli* RC511-V_2; group C was injected with 10^8 VU of *E. coli* RC511-V_2; and group D was injected with 10^8 VU of *E. coli* RC511.

[b] Average of three experiments.

dium (7) and the growing conditions, as we have shown, could be critical. Other workers (18) had demonstrated that the incubation temperature and rate of oxygenation can influence bacteriocin production. Our own results with colicin V suggest that the colicin is not produced at 41°C or when the oxygenation rate is decreased by anaerobiosis. The use of a dialysis bag (suggested also by the results of other workers [5]) represents an easy method to separate the colicin from the cells and the endotoxin considered as a large macromolecule unable to pass through the pores of cellophane.

Smith (16) reported an increased lethality for mice of a Col V^+ strain (B188) over its Col V^- form. In our laboratory, we have found similar results with a Col V_2^+ exconjugant (*E. coli* RC511-V_2) and the wild-type recipient strain (RC511 Col V_2^-). In these experiments, repeated several times and also performed with another exconjugant–wild-type system (Row-V_2 and Row), we showed that the increased lethality for mice was accompanied by a significant multiplication of the colicin V-producing exconjugants (Fig. 2 and 3) in the peritoneal cavity of mice, whereas the Col V_2^- wild types were rapidly being eliminated by the defense systems of the mice. In relation with this, we observed that the number of phagocytic cells in peritoneal fluids from mice injected i.p. with the Col V_2^+ strain was twice as low as in peritoneal fluids from mice injected i.p. with the Col V_2^- strain. Since the only difference between the two strains is the presence of the Col V_2 plasmid and since we found colicin V_2 in the peritoneal fluids from mice injected with the colicin V_2-producing strain, the possibility that colicin is active against mouse peritoneal phagocytic cells is suggested.

We hypothesized that the colicin could play a role in the invasion of the blood vessels, and we checked the possible influence of the colicin on vascular permeability, which could be done easily with Evans technique. We recognize here the possibility that the biological effects observed in our experiments may have been due to some form of *E. coli* enterotoxin. It is important to recall, however, that (i) all the strains used in our experiments had been previously tested and found negative for enterotoxin and permeability factor by another laboratory where such tests are routinely done (see Materials and Methods); (ii) the better-known permeability factor of *E. coli* seems to be a function of the heat-labile enterotoxin (3) (our colicin V dialysate did not lose its activity after heating at 60°C for 90 min [3], and the cellophane bag used in our experiments would normally not allow the entry of the heat-labile toxin, which presumably has a large molecular weight); and (iv) the colicin V-containing dialysate alone did not cause a greater vascular permeability to Evans blue dye than did any of the control solutions. The increased response obtained when both the dialysate and endotoxin were injected together nonetheless suggests a possible synergistic effect between one or possibly more substances in the dialysate and endotoxin when injected subcutaneously.

This is supported also by the fact that living cells of a colicin V-producing strain mixed with a nonlethal dose of dead bacterial cells (Table 1, group A) killed on the average 40% more mice than the Col V_2^+ cells alone (Table 1, group C) and 90% more than a similar number of live cells of a nonproducing strain mixed with killed bacterial cells of the Col V-producing strain (Table 1, group B). Increased mortality was not caused by additional endotoxin itself because in group B (Table 1) we observed no deaths even if the total amount of endotoxin was compared with that of group A, the numbers of cells injected being the same. These results suggest again a possible synergistic effect between colicin V (or a substance closely associated with it) and bacterial endotoxin in vivo. The fact that it was not possible to increase significantly the lethality of non-colicinogenic cells by injecting them simultaneously with a colicin V-containing dialysate may be due in part to the following: (i) the traces of colicin injected may be absorbed or destroyed rapidly; and (ii) it is difficult to find the right number of live cells to be injected, the margin between a lethal and a nonlethal dose being small. Indeed, when the dose administered is low (e.g., 10^8), the bacteria are cleared within a few hours by the phagocytic cells; if more bacteria are used (e.g., 6×10^8), the mortality rate may be high enough to make it difficult to measure any additive effect of the colicin.

The observation that a centrifuged broth culture of *E. coli* 44B (Col V^+) with detectable bactericidal (colicinogenic) activity increased vascular permeability (Fig. 4C) in experimental animals, whereas the same preparation treated with trypsin and no longer showing bactericidal activity failed to affect permeation of Evans dye (Fig. 4D), suggests a possible involvement of colicin V as a factor of invasion (16). This is further supported by the fact that a colicin V dialysate alone had no significant action on vascular permeability, but in association with *S. marcescens* endotoxin it increased vascular permeability as evidenced by a greater permeation of intravenously injected Evans blue dye. Colicin V alone, as reported by Smith (16) and observed by us, is not lethal, but the facts that it is produced in vivo, that it seems to affect the

phagocytic ability of macrophages, and that it may potentiate at least one of the effects of endotoxin support a possible implication of colicin V_2 in the pathogenicity of some *E. coli* colicin V_2-producing strains.

ACKNOWLEDGMENTS

This investigation was supported by grant MA-4586 from the Canadian Medical Research Council.

We wish to thank sincerely Réal Lallier, who performed the different tests for enterotoxigenicity of the strains in S. Larivière's laboratory.

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