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Further Observations on the Association of the Colicine V Plasmid of *Escherichia coli* with Pathogenicity and with Survival in the Alimentary Tract

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SUMMARY

A high proportion of invasive strains of *Escherichia coli* produced colicine V. This property was easily eliminated from 20 of 21 of these strains by 'curing' agents, especially sodium lauryl sulphate, indicating that the genes determining it were located on a plasmid (ColV) which was transmitted from ten of the strains by conjugation. Inoculated intramuscularly, the ColV$^-$ forms of all 17 strains tested were less pathogenic for chickens than the corresponding ColV$^+$ forms. The pathogenicity of the ColV$^-$ forms of four strains was increased, usually to that of the ColV$^+$ form from which they were derived, by implanting other ColV determinants in them. Much higher concentrations of organisms were found in the blood and liver of chickens infected with ColV$^+$ forms than in chickens infected with ColV$^-$ forms. Inoculated intraperitoneally, the ColV$^+$ form of one of the strains, BI88, was more pathogenic for mice than the ColV$^-$ form; much higher concentrations of organisms were found in the peritoneal fluid and blood of ColV$^+$-inoculated mice than of ColV$^-$-inoculated mice. Inoculated orally and intravenously, ColV$^+$ forms were more pathogenic for colostrum-deprived calves than the corresponding ColV$^-$ forms. After mixtures of ColV$^+$ and ColV$^-$ organisms of the same strain in ratios of 1:10, 1:100 or 1:1000 were given orally, they were found in a similar ratio in the contents of the alimentary tract 1 to 2 days later, when the calves were near to death. Many more ColV$^+$ than ColV$^-$ organisms were found in the mesenteric lymph nodes, the deeper tissues and the blood; in the urinary and gall bladders, locations remote from the defence mechanisms of the body, the numbers of ColV$^-$ organisms sometimes exceeded those of ColV$^+$ organisms. Colicine V, although demonstrated in the blood at death, did not appear adversely to influence the concentration of ColV$^-$ organisms in these calves.

Several days after mixtures of ColV$^+$ and ColV$^-$ organisms of strain BI88 were taken orally by two human beings, the ColV$^+$ organisms became much more numerous in their faeces than the ColV$^-$ ones. Similar results were obtained when *col*V^r organisms of BI88 were included in the inoculum or when a strain whose ColV$^-$ form was completely colicine V-resistant was studied.

A ColE$^+$ form of BI88 ColV$^-$ was no more pathogenic for chickens, mice or colostrum-deprived calves than was a ColE$^-$ form of this strain, and it did not persist in the faeces of the two human beings for longer than the ColE$^-$ form.

INTRODUCTION

In a recent study (Smith, 1974), plasmids determining colicine V production were transferred from a variety of wild-type strains of *Escherichia coli* to laboratory strains of this organism. As a result, the laboratory strains became more pathogenic for experimental animals, the increased pathogenicity being associated with a greater ability of the ColV$^+$

forms of these strains to resist the defence mechanisms of the host. Principally because the increased pathogenicity of the strains could not be separated by laboratory procedures from their colicine V production, it was tentatively concluded that either the genes determining each of these properties were located on the same plasmid or colicine V itself was responsible for the increased pathogenicity.

The subsequent finding that 'curing' agents eliminated ColV from organisms of invasive strains of *E. coli* (Smith and Huggins, unpublished work) presented an opportunity to obtain more information on the relationship of colicine V to pathogenicity. It seemed important to do this, because the fact that ColV bestows increased pathogenicity on the laboratory strains of *E. coli* does not necessarily imply that it does so in invasive strains of *E. coli* or that it plays any part in the pathogenesis of disease caused by these strains. The laboratory strains (including *E. coli* K12) used in previous experiments were not regarded as natural pathogens, and large doses had to be injected, usually intravenously, into experimental animals to cause death. By contrast, invasive strains usually produce death in much lower dosage and by more natural routes of infection, including the oral route in colostrum-deprived calves, and with lesions resembling those present in the natural disease. The results of these observations are reported, as are others on the ability of $ColV^+$ and $ColV^-$ organisms of the same strain to survive in the alimentary tract.

METHODS

Only the methods not described by Smith (1974) are dealt with here.

Experimental animals. Male calves from three Jersey herds were brought to the laboratory 3 to 12 h after birth and immediately used in experiments; the immune-globulin-negative status of the serum of the colostrum-deprived ones was confirmed by the zinc sulphate flocculation test (Aschaffenburg, 1949). They were given 1 l heat-sterilized cows' milk twice daily. Chickens used to study the survival of *E. coli* in the alimentary tract were six weeks old; those used in all other experiments were four weeks old.

E. coli strains. Apart from K12, all the strains had been isolated from the blood or internal organs of human beings (prefix H), calves (prefix B), lambs (prefix S) or chickens (prefix F) suffering from bacteraemia.

Culture media and cultural conditions. Except where stated, all media were incubated at 37 °C for 24 h. Broth cultures consisted of organisms grown in 10 ml nutrient broth, Oxoid No. 2 (CM67), in a shaking water bath, and contained approximately 10^9 viable organisms/ml. Only in mating experiments were broth cultures not shaken. The broth was not suitable for colicine V production. The nutrient agar employed was Difco tryptose agar (B64) and the MacConkey's agar was Oxoid (CM7). Synthetic medium consisted of (g/l): K_2HPO_4, 7; KH_2PO_4, 3; $(NH_4)_2SO_4$, 1; $MgSO_4.7H_2O$, 0·1; NaCl, 5; glucose, 5; agar, 15. Colicine V-containing plates were plates of nutrient agar whose entire surface had been inoculated with a broth culture of a $ColV^+$ *E. coli* strain, incubated and then maintained at 60 °C for 1 h to kill the *E. coli*; a thin layer of nutrient agar was then poured over their surface.

Demonstration of ColV transmissibility in E. coli strains. Strains that produced colicine V were grown in broth with a nalidixic acid-resistant mutant (*nal-r*) of a *lac*$^-$ *E. coli* K12 strain. The mixed cultures were inoculated on to colicine V-containing plates that had been overlaid with nutrient agar containing 30 μg sodium nalidixate/ml. Colonies that grew after incubation were then checked to confirm that they were of $ColV^+$ *E. coli* K12 organisms. Using antibiotic resistance determinants as markers, transfer factors F (Lederberg, Cavalli

& Lederberg, 1952; Hayes, 1953) and I [I = I*drd*16(I); Hardy *et al.* 1973] were implanted in those strains whose colicine V production was not shown to be transmissible, and the strains were then re-examined.

Preparation of colicine V-resistant mutants (colV^r) of E. coli strains. Colicine V-containing plates were inoculated heavily with broth cultures of strains from which it was desired to isolate *col*V^r mutants and incubated. Colonies that grew were purified and checked for resistance to colicine V.

Elimination of ColV from E. coli strains. The sodium lauryl sulphate method of Tomoeda *et al.* (1968), the acridine orange method of Hirota (1960) and the ethidium bromide method of Bouanchaud, Scavizzi & Chabbert (1968) were employed. Strains were transferred several times in broth containing these agents if success did not follow the first transfer. Unless stated, any culture of an invasive strain referred to below as ColV$^-$ was obtained by sodium lauryl sulphate treatment; a culture of the same strain referred to as ColV$^+$ was a ColV$^+$ isolate from the same transfer in sodium lauryl sulphate as that in which the ColV$^-$ culture was obtained, i.e. it was not the original ColV$^+$ strain before sodium lauryl sulphate treatment.

The re-introduction of ColV into ColV$^-$ forms of E. coli. The ColV$^-$ forms were grown in broth with an auxotrophic ColV$^+$Tet$^+$I$^+$ strain of *E. coli* K12 (Tet = tetracycline resistance determinant) and the mixed culture then inoculated on to plates of synthetic medium containing 20 μg tetracycline/ml. Colonies that grew on this medium were tested to find ones that were ColV$^+$. In one case, the ColV$^+$ K12 donor strain employed possessed the transfer factor of an invasive strain instead of I.

Differentiation of ColV$^+$, ColV$^-$ and colV^r colonies from each other. Colonies from plates of MacConkey's agar that had been inoculated with material containing ColV$^+$, ColV$^-$ and *col*V^r organisms of the same *E. coli* strain were spot-inoculated on to nutrient agar plates spread with a broth culture of *E. coli* K12 to differentiate the ColV$^+$ colonies from the ColV$^-$ and *col*V^r ones. Those found not to produce colicine were then inoculated over small areas of a colicine V-containing plate to differentiate the ColV$^-$ ones from the *col*V^r ones.

Determination of colicine V in cultures, body fluids and tissues. These were centrifuged to remove solid matter and most of the bacteria they contained and then heated to 60 °C for 1 h, a procedure that had no effect on their colicine V content. Tissues such as muscle were first ground in a pestle and mortar with the smallest amount of broth necessary to form a thick suspension. Successive twofold dilutions were prepared from the heated fluids and 0·03 ml of each spotted on to the surface of a plate of nutrient agar over which a lawn of a diluted broth culture of *E. coli* K12 had been spread. The plates were incubated and the highest dilution that prevented bacterial growth was recorded as the titre.

The survival of ColV$^+$, ColE$^+$ and ColV$^-$ organisms in the alimentary tract of chickens or human beings. Groups of chickens were given orally 5×10^9 viable ColV$^+$ or ColV$^-$ organisms of the same *nal-r spc-r* strain of *E. coli* (spc = spectinomycin). Six days later they were killed; the total *E. coli* in their caecal contents was estimated by counting on MacConkey's medium, and the number of inoculated *E. coli* estimated by counting on this medium containing 20 μg each of spectinomycin and sodium nalidixate/ml. Mixtures of ColV$^+$, ColV$^-$ and *col*V^r; of ColV$^+$ and ColV$^-$; or of ColE$^+$ and ColE$^-$ organisms of the same *nal-r str-r* or *nal-r spc-r* strain of *E. coli* were taken orally by two human beings (str = streptomycin). Their faeces were cultured at intervals on MacConkey's agar containing, per ml, 20 μg of each of the antibiotics to which the inoculated organisms were resistant. Colonies that grew were then classified according to their colicine status. On some occasions

the total number of viable *E. coli* in the faeces was estimated by counting on MacConkey's agar and the number of inoculated *E. coli* by counting on antibiotic-containing MacConkey's medium.

Preparation of ColE$^+$ and ColE$^-$ forms of E. coli B188 *ColV$^-$*. A ColE$_1$$^+Tet^+F^+$ strain of *E. coli* K12, in which Tet and F but not ColE were linked, was mated with a *nal-r str-r* mutant of B188 ColV$^-$, and a ColE$^+$Tet$^+$F$^+$ and a ColE$^-$Tet$^+$F$^+$ segregant of B188 selected. The segregants were then treated with sodium lauryl sulphate to remove Tet and F.

RESULTS

Prevalence of the ColV$^+$ form in invasive strains of E. coli

Epidemiologically-unrelated strains, most of which had been maintained in the laboratory for several years, were tested for colicine V production. Examination of serotype O78:K80 cultures showed that 25 of 31 cattle strains, 3 of 5 sheep strains and 36 of 44 chicken strains were positive. Of 54 chicken strains belonging to serotype O2:K1, 40 were positive. Examination of miscellaneous untyped cultures showed that 50 of 68 chicken strains and 10 of 45 human strains were positive.

The nature of the ColV determinant in invasive strains of E. coli

Twenty-one invasive strains of *E. coli*, of human, bovine, ovine and avian origin and belonging to several different antigenic types, were tested for their ability to mobilize a streptomycin/sulphonamide resistance determinant in a Tra$^-$ *E. coli* K12 strain. The examination revealed that ten possessed transfer factors (Tra$^+$) and transmitted their ColV determinants, four were Tra$^+$ but did not transmit their ColV determinants, and seven were Tra$^-$; the determinants in the strains belonging to the last category could not be mobilized by implanting transfer factors F or I in them. Strains of serotype O78:K80 were found in all three categories. *Escherichia coli* K12 that had acquired ColV from five of the ten strains in which it was transmissible became fully susceptible to the MS2 phage, indicating that the transfer factors in these five strains were F-like. In general, ColV$^-$ forms were obtained more easily by sodium lauryl sulphate treatment from these ten than from the other eleven; from only one strain were they not obtained by this method, even after four transfers. Forms that had lost ColV were also obtained from three of eight strains by acridine orange treatment and from one of five by ethidium bromide treatment. An occasional ColV$^-$ form was obtained from six of the 21 strains during four transfers in plain nutrient broth. The ColV$^-$ forms of six of the 21 strains were resistant to colicine V, but the remainder were susceptible, though less sensitive than *E. coli* K12.

The effect of growing ColV$^+$ and ColV$^-$ forms of the same strain together in broth

Nutrient broth was inoculated with approximately equal amounts of ColV$^+$ and ColV$^-$ organisms of one or other of eleven invasive *E. coli* strains and incubated. The ColV status of 50 or 100 colonies obtained from each of the eleven mixed cultures at 0 and 24 h was determined. The total number examined at each occasion was 800. Of those examined at 0 and 24 h, 365 and 294, respectively, were ColV$^+$. In only two mixed cultures had the proportion of ColV$^+$ organisms increased during the 24 h incubation period and in neither of these was the increase statistically significant. Colicine V was not detected in any of the mixed cultures.

Two additional mixed cultures of *E. coli* B188 were studied. One contained ColV$^+$, ColV$^-$ and colicine V-resistant mutant (*col*V^r) organisms, and the other contained ColV$^-$

Table 1. *The lethality for chickens of different forms of E. coli strain* F65 *given intramuscularly*

10^{-6} × no. of viable organisms inoculated	No. of chickens that died when given*: ColV$^+$	ColV$^-$	ColV$^+$(V$^-$)	ColV$^-$(V$^-$)
300	10	9	8	10
100	10	0	8	0
30	8	0	8	0
10	8	1	6	0
3	3	0	5	0

* The ColV$^+$(V$^-$) form was the ColV$^-$ form in which ColV, Tet and transfer factor I were implanted; the ColV$^-$(V$^-$) form was the ColV$^-$ form in which only Tet and I were implanted. Each form was given to ten chickens.

and *col*V^r organisms. In both cultures, the ColV$^-$ and the *col*V^r organisms multiplied at the same rate.

Seven of the mixed culture experiments in which the ColV determinant of the ColV$^+$ form was transmissible were repeated employing a *nal-r* mutant of the ColV$^-$ form. All of 100 *nal-r* colonies isolated from each of the mixed cultures after incubation were ColV$^-$. Thus it appeared that ColV$^-$ forms of invasive strains multiplied at approximately the same rate as the corresponding ColV$^+$ forms in broth, and that while doing so they had only a low probability of acquiring ColV.

The pathogenicity of ColV$^+$ and ColV$^-$ forms of the same invasive E. coli strain for chickens

Groups of ten chickens were injected intramuscularly (i.m.) with successive doses (each at one-third the concentration of the one before) of the following forms of *E. coli* F65: the ColV$^+$ form, the ColV$^-$ form, the ColV$^-$ form into which a ColV determinant had been introduced with transfer factor I, and the ColV$^-$ form into which only transfer factor I had been introduced (Table 1). The two ColV$^+$ forms were clearly more lethal than the two ColV$^-$ forms; they also produced severe lesions in many survivors, whereas the two ColV$^-$ forms did so in only a few. Comparable results were obtained in substantially similar experiments with strains B188, F185 and H247.

The macroscopic lesions found in chickens that died from infection with ColV$^+$ and ColV$^-$ forms of F65 and of other invasive strains of *E. coli* resembled those found in the natural disease. They consisted principally of a proliferative pericarditis, peritonitis and air-sacculitis. Most deaths occurred two to four days after infection. They were uncommon after five days, although at seven days, when experiments were concluded, severe lesions were still present in some of the chickens.

Intramuscular pathogenicity tests with 13 further strains invariably gave clear evidence of the greater lethality of ColV$^+$ forms than ColV$^-$ forms. These experiments included two in which ColV had been removed by acridine orange treatment and one in which it had been lost during passage in broth.

Two groups of 47 chickens were also inoculated i.m. with organisms of either a ColE$^-$ or a ColE$^+$ form of a *nal-r str-r* strain of B188 ColV$^-$. Nine of the chickens inoculated with the ColE$^+$ form and ten of the chickens inoculated with the ColE$^-$ form died.

The concentration of ColV+ and ColV− organisms in the tissues of infected chickens

Ten chickens were inoculated i.m. with 5×10^8 viable ColV+ organisms of the Tra− strain, B188, and ten further birds were similarly treated with ColV− organisms. Slaughter and examination 24 h later revealed that the ColV+ form was much more invasive than the ColV−. The median values of ColV+ organisms in blood and ground tissue were ($10^{-6} \times$ viable counts/ml, with ranges in parentheses): blood, 80 (9 to 600); liver, 30 (2 to 800); inoculated muscle, 5000 (3000 to 8000). The corresponding figures for ColV− organisms were: blood, 0·04 (0·01 to 0·9); liver, 0·5 (0·15 to 2); inoculated muscle, 2500 (1000 to 5000).

Four chickens died 1 to 3 days after inoculation into the breast muscle with the non-colicine V-transmitting strain B188 in a total dose of 5×10^8 viable organisms, consisting of ColV+, ColV− and *col*V^r forms in a ratio of 1:4·5:4·5. Practically all organisms isolated from liver and blood were ColV+. Examination of 50 organisms from the inoculated muscle of each of the four birds showed that 0·5, 26·5 and 73% of the total of 200 organisms consisted of ColV−, *col*V^r and ColV+ forms respectively. No colicine V was detected in blood, liver or muscle.

An exactly similar inoculum was given to three further chickens, but in this instance the ColV+ organisms were injected into the left leg muscle and the ColV− and *col*V^r forms into the right breast muscle. Death occurred in 2 to 3 days and liver, blood and leg muscle contained organisms that were almost exclusively ColV+. Breast muscle contained 53·5 and 31% of ColV+ and *col*V^r forms, respectively, and a higher proportion of ColV− than in the previous experiment, namely 15·5%.

Three more chickens that died after receiving a mixture of ColV− and *col*V^r organisms i.m. yielded the two forms from inoculated muscle, liver and blood in approximately the same proportions as in the inoculum.

Mixtures of ColV+ and ColV− organisms of three invasive strains, B192, F185 and H261, whose ColV− forms were completely resistant to colicine V, were each inoculated i.m. into a chicken; the ColV+:ColV− ratio in the inocula was 1:10. All of 50 isolates obtained from the liver and from the blood of each of these three chickens after death were ColV+, and so were most from the inoculated muscle.

The pathogenicity for mice of ColV+, ColV− and ColV−ColE+ organisms of E. coli B188; *the concentration of ColV+ and ColV− organisms in the peritoneal fluid and blood of mice*

When the ColV+ form of strain B188 was injected intraperitoneally (i.p.) into four groups of 25 mice at doses of 10^8, 10^7, 10^6 and 10^5 viable organisms, the deaths produced were 25, 23, 13 and 2, respectively. The corresponding results for a parallel virulence titration of the ColV− form were 18, 0, 0 and 0.

No difference in mouse lethality could be shown between the ColV− and the ColV−ColE+ forms of the *nal-r str-r* mutant of strain B188; each form produced a single death in a group of 25 mice when the dose was 10^7 viable organisms i.p., and >20 deaths when the dose was 10^8.

The ColV+ and ColV− forms of strain B188 were each injected i.p. into ten mice in doses of 10^7 viable organisms. After 24 h, five mice given ColV+ had just died and the other five were moribund; mice that received ColV− were apparently healthy. The survivors were killed and all mice were examined. The median values of the ColV+ organisms in blood and

Table 2. *The concentration of E. coli organisms in the intestinal contents and tissues of colostrum-deprived calves given ColV$^+$ or ColV$^-$ and colV^r forms of E. coli* B188 *orally*

Calf A was given ColV$^+$ organisms and calves B and C were given a mixture of ColV$^-$ and *col*V^r organisms; the infecting dose for all three calves was $1{\cdot}5 \times 10^{10}$ viable organisms. Calves A and B were killed 24 h after infection and calf C 48 h after infection. The small intestine was sampled from three regions, No. 1 being nearest the stomach and No. 3 nearest the caecum; the lymph nodes associated with these regions were similarly identified.

	$10^{-3} \times$ no. of organisms/ml, in calf:		
Materials examined	A	B	C
Small intestinal contents 1	4000	1000	4000
Small intestinal contents 2	400	1000	40000
Small intestinal contents 3	100000	150000	300000
Caecal contents	200000	300000	3000000
Faeces	5000000	1000000	5000000
Retro-pharyngeal lymph node	10000	80	70
Small intestinal lymph node 1	7000	800	1000
Small intestinal lymph node 2	10000	1500	500
Small intestinal lymph node 3	7000	1500	600
Popliteal lymph node	20000	60	50
Blood	50000	100	30
Muscle	300	8	1
Lung	5000	500	200
Spleen	1500	200	700
Liver	1500	80	200
Bile	200	0	25000
Kidney	100000	9000	1000
Urine	1	0	100000
Brain	5000	100	20
Cerebro-spinal fluid	700	25	10

peritoneal washings were ($10^{-6} \times$ viable counts/ml, with ranges in parentheses): blood, 250 (3 to 500); peritoneal washings, 50 (6 to 150). The corresponding results for ColV$^-$ organisms were: blood, 0·0001 (0·00001 to 0·01); peritoneal washings, 0·004 (0·0005 to 0·015).

The pathogenicity for calves of ColV$^+$ and ColV$^-$ forms of invasive strains of E. coli; their concentrations in the tissues of infected calves

Two colostrum-deprived calves inoculated orally with mixtures of B188 ColV$^-$ and *col*V^r organisms appeared normal 24 h later when one of them was killed. The other was killed at 48 h, by which time it had become moderately unwell. By contrast, one of two calves infected with the same dose of B188 ColV$^+$ organisms was found dead 24 h later and the other was collapsed and near to death. Higher concentrations of organisms were present in the tissues of the latter calf than in the corresponding tissues of the two calves given ColV$^-$ and *col*V^r organisms (Table 2), this difference being most apparent in the blood, and least apparent in organs such as the liver and spleen which by virtue of their high content of reticulo-endothelial cells constitute the main defence mechanism of the body against invading bacteria. The main difference between the two calves given the ColV$^-$ and *col*V^r forms was the high concentrations of organisms in the bile and urine of the calf killed at 48 h; the ratio of ColV$^-$ to *col*V^r organisms in all the materials examined from these two calves was approximately the same as their ratio, 3 to 1, in the inoculum.

Isolates were examined from 12 colostrum-deprived calves infected orally with mixtures of ColV$^+$ and ColV$^-$ organisms of B188 or of three other invasive strains (Table 3 and Fig. 1)

Table 3. *The ratio of ColV$^+$ and ColV$^-$ organisms of the same E. coli strain in the tissues of colostrum-deprived calves that had been inoculated orally with these organisms*

ColV$^+$(V$^-$) is the ColV$^-$ form after ColV has been implanted in it with transfer factor I; ColV$^-$(V$^+$) is the ColV$^+$(V$^-$) form after ColV and I have been removed from it. The inoculum for all calves contained $1{\cdot}5 \times 10^{10}$ viable organisms; half the inoculum for calf K comprised *col*V^r organisms. Fifty isolates were tested in each case.

Calf	Inoculum	ColV$^+$:V$^-$ ratio in inoculum	No. of isolates that were ColV$^+$							
			Intestinal contents*	Intestinal lymph node*	Blood	Liver	Bile	Muscle	Kidney	Urine
D	B188 ColV$^+$ and ColV$^-$	1:10	3	50	50	50	50	50	50	50
E	B188 ColV$^+$(V$^-$) and ColV$^-$	1:10	4	25	46	21	47	45	50	0
F	B188 ColV$^+$(V$^-$) and ColV$^-$	1:10	3	49	50	50	50	50	50	28
G	B188 ColV$^+$(V$^-$) and ColV$^-$(V$^+$)	1:10	9	50	50	50	50	50	50	17
J	B188 ColV$^+$ and ColV$^-$	1:100	2	50	50	44	50	50	50	0
K	B188 ColV$^+$ and ColV$^-$	1:100	1	48	50	50	42	—	50	0
L	B188 ColV$^+$ and ColV$^-$	1:1000	0	43	49	38	46	47	49	0
M	B188 ColV$^+$ and ColV$^-$	1:1000	0	37	50	47	0	50	50	0
N	H247 ColV$^+$ and ColV$^-$	1:100	—	—	50	50	—	50	50	—
P	B192 ColV$^+$ and ColV$^-$	1:10	3	43	50	50	50	50	50	19
R	B4 ColV$^+$ and ColV$^-$	1:10	6	50	50	46	50	48	50	40
S	B4 ColV$^+$ and ColV$^-$	1:10	—	—	50	50	50	50	50	50

* Average for three regions of the small intestine and for three lymph nodes associated with these regions.

The results of examining 50 isolates from the stomach, caecum and faeces resembled those quoted for intestinal contents; those of examining retro-pharyngeal and popliteal lymph nodes, spleen, lung, brain and cerebrospinal fluid resembled those quoted for liver.

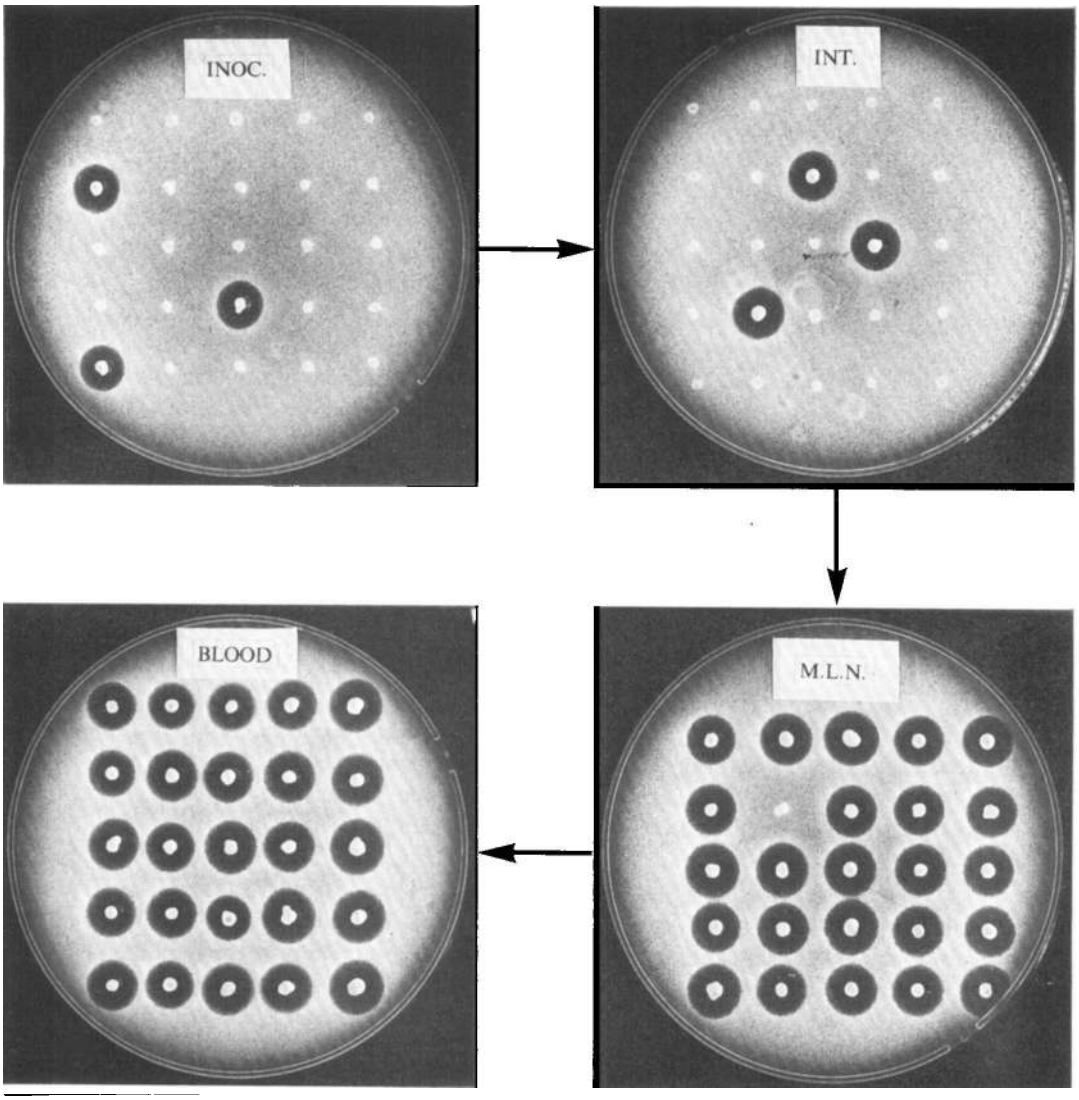

Fig. 1. Colicine tests on *E. coli* isolates from a colostrum-deprived calf that had been inoculated orally with ColV$^+$ and ColV$^-$ organisms of *E. coli* B188 in a ratio of 1:10. Small portions of *E. coli* colonies cultured from the inoculum (INOC.), the intestinal contents (INT.), a mesenteric lymph node (M.L.N.) and the blood were placed on the surfaces of the plates of nutrient agar after they had been spread with a lawn of *E. coli* K12. Magnification × 0.8.

and killed when near to death. The ratios of ColV$^+$ to ColV$^-$ organisms in the contents of different regions of the alimentary tract were similar to that in the inoculum; in the lymph nodes, however, blood, liver, spleen, lung, muscle, kidney, brain and cerebro-spinal fluid ColV$^+$ organisms greatly outnumbered the ColV$^-$ ones. The concentrations of organisms in the different materials examined from these calves resembled those for calf A quoted in Table 2 except that high concentrations were found in the urine and bile of some of these calves. ColV$^+$ organisms dominated the isolates from the tissues of the calves infected with

B188 even when their numbers in the inoculum were 1000 times fewer than those of the ColV$^-$ ones and when the inoculum contained *col*V^r organisms in addition to ColV$^+$ and ColV$^-$ ones; the few non-colicine-producing isolates from the calf given these three forms of B188 (calf K) consisted of approximately the same number of ColV$^-$ and *col*V^r forms. The dominance of ColV$^+$ isolates also applied in the case of the calves given B192 and B4 (calves P, R and S), strains whose ColV$^-$ forms were completely insusceptible to colicine V; those of B188 and H247 were moderately susceptible. In contrast to the results for the tissues, most of the isolates from the urine of six calves and the bile of one calf – fluids remote from the defence mechanisms of the body – were ColV$^-$. Similar results to those quoted in Table 3 were obtained by infecting a colostrum-deprived calf intravenously instead of orally with 10^5 viable organisms of a mixture of ColV$^+$, ColV$^-$ and *col*V^r forms of B188 in the ratio 1:4·5:4·5. Apart from two isolates, one of which was ColV$^-$ and the other *col*V^r, all of the 350 isolates from the blood, kidney, spleen, muscle, mesenteric lymph node, bile and urine were ColV$^+$. In contrast with the results of all the ColV experiments, the ratio of ColE$^+$ to ColE$^-$ organisms amongst isolates made from the alimentary contents and tissues of a colostrum-deprived calf 26 h after it had been infected orally with B188 ColV$^-$ColE$^+$ and ColV$^-$ organisms was the same as in the inoculum, 1 to 10.

Blood samples were taken from some of the calves referred to in Table 3 on several occasions before they were killed. ColV$^+$ organisms became dominant amongst the isolates from these samples at a time when they contained only low concentrations of organisms. For example, of 50 isolates obtained from calf M at 20, 24, 30 and 40 h, the numbers that were ColV$^+$ were 1, 3, 30 and 50. At these times, the numbers of viable organisms/ml of blood ($\times 10^{-4}$) were 8, 7, 2 and 20, respectively.

The numbers of ColV$^+$, ColV$^-$ and *col*V^r organisms in the blood of a colostrum-deprived calf were estimated on several occasions during the 9 h period between the time it was inoculated intravenously with these organisms and the time it was killed when near to death (Table 4). Similar estimates were made on a sample of blood removed 3 min after inoculation, on two pre-inoculation serum samples (one raw and one heated to destroy complement), and on broth; the three forms of B188 were added to the serum samples and the broth in the same proportions as they were given to the calf. All the samples and the broth were held at 37 °C. Neither the concentrations of organisms in the circulating blood of the calf or the ratio of ColV$^+$, ColV$^-$ and *col*V^r organisms amongst the isolates changed during the first 5 h. The concentrations of organisms then increased, as did the proportion of isolates that were ColV$^+$; the proportion of ColV$^-$ to *col*V^r isolates remained approximately the same. The inoculated bacteria multiplied rapidly in the blood and serum maintained at 37 °C. The proportion of ColV$^-$ organisms amongst the isolates from the blood and serum samples after 4 to 5 h was very low; the proportion of *col*V^r organisms amongst the isolates from both serum samples showed little alteration during the whole observation period but after 6 h the proportion of these organisms in the blood sample decreased slightly. The proportion of ColV$^+$, ColV$^-$ and *col*V^r organisms in the broth, which was known to be unsuitable for colicine V production, remained constant throughout. The colicine V titre of the serum of the blood taken from the calf at the end of the experiment was 1 in 1; that of the incubated blood and serum samples was in the region of 1 in 8; no colicine V was detected in the broth culture. A specimen of serum obtained from the calf at the end of the experiment was heated to 60 °C for 1 h to destroy the bacteria it contained, held at 37 °C and inoculated, at 10^5 viable organisms/ml, with a mixture of equal numbers of ColV$^-$ and *col*V^r organisms of B188 growing exponentially in the serum of another colostrum-deprived calf. The ratio of ColV$^-$ to *col*V^r organisms was the same at 0·5 and

Table 4. *The numbers of ColV$^+$, ColV$^-$ and colV^r organisms of E. coli* B188, *at different times after inoculation, in blood, in vivo and in vitro, and in serum of a colostrum-deprived calf and in broth*

The calf was given an intravenous inoculation of 10^9 viable organisms of a mixture of ColV$^+$, ColV$^-$ and *col*V^r organisms of B188 in a ratio of 1:4·5:4·5. Blood removed 3 min after inoculation, heparinized and held at 37 °C constituted the *in vitro* specimen. An immediate count performed on it was recorded as the 0 h count for 'Blood *in vivo*' and 'Blood *in vitro*'. The experiments with the calf's raw and heated (62 °C, 1 h) serum and with broth were performed on another occasion, and employed a similar-sized inoculum to that used for the blood.

	Material examined																			
	Blood *in vivo*				Blood *in vitro*				Raw serum				Heated serum				Broth			
		No. of isolates				No. of isolates				No. of isolates				No. of isolates				No. of isolates		
Time (h)	Count*	V$^+$	V$^-$	V^r	Count*	V$^+$	V$^-$	V^r	Count*	V$^+$	V$^-$	V^r	Count*	V$^+$	V$^-$	V^r	Count*	V$^+$	V$^-$	V^r
0	2	9	18	23	2	9	18	23	4	6	16	28	2	5	29	16	3	4	20	26
2	2	7	27	16	60	6	19	25	40	7	20	23	30	4	26	20	27	9	21	20
3	—	—	—	—	65	3	13	34	160	9	13	28	200	16	8	26	220	6	28	16
4	3	12	25	13	170	16	1	33	700	10	7	33	1500	10	9	31	2700	6	18	26
5	3	8	29	13	580	30	0	20	3500	14	0	26	5200	13	1	36	8000	3	30	17
6	6	33	11	6	3000	36	0	14	14000	20	2	28	14000	17	1	32	10000	0	29	21
7	25	46	4	0	5200	45	0	8	10000	18	0	32	22000	12	0	38	18000	5	19	26
8	120	49	1	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
9	220	49	1	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

* 10^{-5} × No. of organisms/ml.

Table 5. *The ratio of ColV$^+$, ColV$^-$ and colV^r organisms of E. coli* B188 *in the faeces of two human beings after they had consumed mixtures of these organisms*

In expt 1 the human beings consumed 10^{10} viable ColV$^+$ and ColV$^-$ organisms and in expt 2 a similar dose of ColV$^+$, ColV$^-$ and *col*V^r organisms; 1 in 10 of the organisms consumed in both experiments were ColV$^+$. The organisms consumed by human being No. 1 were *nal-r spc-r* mutants and those consumed by human being No. 2 were *nal-r str-r* mutants. They were recovered from the faeces of the human beings by culturing on MacConkey's medium containing the two antibiotics to which they were resistant. Fifty isolates were tested in each case.

		No. of isolates from:					
		Human being No. 1, that were:			Human being No. 2, that were:		
	Time after consumption (days)	ColV$^+$	ColV$^-$	*col*V^r	ColV$^+$	ColV$^-$	*col*V^r
Expt 1	1	10	40	—	4	46	—
	2	17	33	—	10	40	—
	3	24	26	—	12	38	—
	4	25	25	—	42	8	—
	5	35	15	—	39	11	—
	6	50	0	—	40	10	—
	7	49	1	—	50	0	—
	9	49	1	—	50	0	—
	11	50	0	—	50	0	—
	13	50	0	—	50	0	—
	16	50	0	—	50	0	—
Expt 2	1	5	9	36	4	21	25
	2	3	14	33	1	25	24
	3	2	10	38	3	24	23
	4	1	9	40	8	21	21
	5	3	11	36	44	3	3
	6	41	0	9	45	4	1
	7	50	0	0	48	1	1
	8	50	0	0	50	0	0
	10	—	—	—	50	0	0
	17	—	—	—	50	0	0

1 h as at 0 h, but at 1·5 h the proportion of *col*V^r organisms had increased slightly. When mixtures of ColV$^+$ and ColV$^-$ organisms of F185, an invasive strain whose ColV$^-$ form was completely insusceptible to colicine V, were incubated in the serum of a colostrum-deprived calf, the ColV$^+$: ColV$^-$ ratio was the same at 24 h as at 0 h.

The comparative survivability of ColV$^+$ and ColV$^-$ organisms of the same strain in the alimentary tract

ColV$^+$ forms gradually became more dominant amongst the B188 isolates from the faeces of two human beings who had consumed mixtures of ColV$^+$ and ColV$^-$ or of ColV$^+$, ColV$^-$ and *col*V^r organisms of this strain, until eventually they were the only form isolated, a situation that persisted until B188 organisms disappeared from the faeces (Table 5). After each occasion that they were taken by human being No. 1, B188 organisms formed the major component of his *E. coli* faecal flora for several weeks. In human being No. 2 they formed the major component for only a few days although they persisted in his faeces for several weeks. At the end of one experiment, none of the 50 ColV$^+$ isolates obtained from one faecal specimen of human being No. 2 were found to be able to transmit ColV, an indication that none of them were really ColV$^-$ forms of B188 that had acquired ColV plasmids from other

E. coli that were inhabiting the alimentary tract (the ColV of B188 ColV$^+$ is non-transmissible). In another experiment human being No. 1 took a mixture of equal quantities of B188 ColV$^-$ and *col*V^r organisms. In all subsequent faecal examinations they were found in approximately this proportion. When both human beings took, in a ratio of 1:10, ColV$^+$ and ColV$^-$ organisms of B192 (a strain whose ColV is non-transmissible and whose ColV$^-$ form is completely resistant to colicine V), the results were substantially the same as those obtained following the consumption of the ColV$^+$ and ColV$^-$ forms of B188. This state of affairs continued until the end of the observation period.

In a further experiment, the two human beings consumed 10^{10} viable organisms consisting of ColE$^+$ and ColE$^-$ forms of a *nal-r str-r* mutant of strain B188 ColV$^-$ in a ratio of 1:10. ColE$^+$ organisms did not become dominant amongst the B188 isolates obtained subsequently from the faeces by culturing on MacConkey's agar containing sodium nalidixate and streptomycin. In one individual, repeated sampling showed that the percentages of faecal B188 organisms that proved to be ColE$^+$ ranged from 4 to 42 (mean 18) over the first 6 days, and had fallen to nil by the 9th day. In the second individual, the corresponding figures were 6 to 72 (mean 21) over the first 12 days and had fallen to nil by the 13th day.

The ratios of the numbers of *E. coli* F65 organisms to the numbers of the general *E. coli* organisms, in the caecal contents of ten chickens 6 days after they had been inoculated orally with 5×10^9 viable organisms of a ColV$^+$ form of a *nal-r spc-r* mutant of F65, were 1:1, 1:5, 1:5, 1:10, 1:10, 1:20, 1:50, 1:50, 1:100 and 1:500; in ten given the ColV$^-$ form instead of the ColV$^+$ form the ratios were 1:300, 1:1000, 1:1000, 1:1000, 1:3000, 1:3000, 1:30000, 1:50000, 1:200000 and 1:>1000000. The concentration of the general *E. coli* in the caeca of the chickens in both groups was similar, the median concentration for each group being 50×10^6 viable organisms/ml.

DISCUSSION

The elimination of ColV in various ways from human, bovine, ovine, and avian *E. coli* strains belonging to a number of serological types was always accompanied by a distinct decrease in pathogenicity. Furthermore, the pathogenicity of ColV$^-$ forms could be increased, usually to that of the ColV$^+$ forms from which they were derived, by implanting other ColV plasmids in them. These observations are in accord with earlier ones made on essentially non-pathogenic strains of *E. coli*, such as K12, in which different ColV plasmids had been implanted. However, as the present ones were made on invasive strains employing inoculation methods that gave rise to a disease in chickens and colostrum-deprived calves indistinguishable ftom that which these strains cause under natural conditions, they suggest even more strongly that ColV is an important pathogenic character of *E. coli*. It is particularly significant in this respect that a high proportion of the invasive strains examined produced colicine V. Of those that did not produce it, some may have possessed a ColV determinant when originally isolated from a disease process but may have lost it during the several years they were maintained in the laboratory before their ColV status was determined; ColV was shown to be unstable in some of our stock cultures. Although the genes determining colicine V production could not be transmitted from some of the invasive strains, even after transfer factors F and I were established in the strains, they were easily eliminated during sodium lauryl sulphate treatment, which strongly suggested that they were located in plasmids. For this reason it still cannot be concluded that colicine V or some related substance determined by the ColV gene is responsible for the increased pathogenicity, since it is always possible that other genes present in these plasmids are really responsible for this increased pathogenicity. Whatever substance is implicated it must

function by remaining intimately associated with the bacteria that form it, to provide increased protection against the defence mechanisms of the body. It does not function by reducing the effectiveness of the defence mechanisms in the manner of an exotoxin. If it did so, then in the chickens and calves used in the mixed infection experiments the ColV$^-$ organisms would have proliferated to the same extent as the ColV$^+$ ones, whereas they did not appear to proliferate to any greater extent than when they were used alone.

The mixed infections in the colostrum-deprived calves provided the best illustration of the differing *in vivo* behaviour of ColV$^+$ and ColV$^-$ forms. During the 1 to 2 days that the calves survived, the ratio of the two forms in the alimentary tract remained the same as in the inoculum. In the early stages of invasion, the ColV$^-$ forms predominated in the blood but to a lesser extent than they did in the inoculum. Later, while the blood concentrations were still low, the ratio altered in favour of the ColV$^+$ organisms. These then proliferated and when the calves were killed they dominated the isolates from the blood and tissues. The frequent predominance of ColV$^-$ organisms in the urine samples and, occasionally, in the bile samples examined at this time was probably the consequence of these fluids becoming infected in the early stages of infection when the ColV$^-$ organisms were still more numerous in the blood than the ColV$^+$ ones. Their continued dominance can be explained by the fact that, being away from the defence mechanisms of the body, they were no longer competing unequally with the ColV$^+$ ones.

Braude & Siemienski (1965) found that after subcutaneous injection of colicine V the sera of mice became strongly bactericidal for colicine V-sensitive *E. coli*. Concentrations of colicine V bactericidal for *E. coli* K12 were found in the present studies in blood samples taken from infected calves when they were near to death. However, the concentrations were not sufficiently high to influence significantly the multiplication of the less colicine V-susceptible B188 ColV$^-$ organisms. It therefore seemed unlikely that colicine V production *in vivo* had influenced the B188 ColV$^-$:ColV$^+$ ratio in the calves inoculated with mixtures of these organisms, particularly since the ColV$^+$ organisms assumed their dominant role in the blood of these calves at a time when their concentrations were low, approximately one-hundredth of that present at death. Furthermore, *col*V^r organisms survived no better than ColV$^-$ ones in calves infected with both these forms of B188 in addition to ColV$^+$ forms. The degree of ColV$^+$ dominance found in calves infected with mixtures of ColV$^+$ and ColV$^-$ forms of strains whose ColV$^-$ forms were completely colicine V-resistant, too, resembled that observed with B188. The *col*V^r form of B188 appeared little more susceptible to complement than did the ColV$^+$ form, in that its rate of multiplication in raw and heated samples of colostrum-deprived calf serum resembled that of the ColV$^+$ form. The ColV$^-$ form with which these samples were also inoculated disappeared fairly rapidly; this clearly was due to the colicine V produced by the ColV$^+$ organisms. Chicken blood also proved a good medium for the *in vitro* production of colicine V, though on occasion the colicine content of chicken and of calf blood cultures was low, possibly due to the antagonizing effect of endotoxin (Braude & Siemienski, 1965). This may explain our failure to demonstrate colicine V in the inoculation site of the dead chickens that had had mixtures of ColV$^+$, ColV$^-$ and *col*V^r B188 organisms injected into the same muscle; our failure to isolate ColV$^-$ organisms, but not *col*V^r organisms, from the site strongly suggested that the very high concentrations of ColV$^+$ organisms present – some 1000 times higher than that found in the blood or tissues of dying calves – must at some time have produced sufficient colicine V adversely to influence the survival of the B188 ColV$^-$ organisms.

The experiments in chickens and human beings revealed that ColV confers on organisms an increased ability to survive in the alimentary tract in addition to the tissues. Support for

this conclusion with regard to the experiments with B188 and B192 in human beings derives from the following observations: (i) As far as can be determined the ColV$^+$ and ColV$^-$ forms of B188 and of B192 differ only in the possession of a ColV plasmid. (ii) The ColV of both strains is non-transmissible, which eliminates the possibility of ColV transfer from the ColV$^+$ to the ColV$^-$ organisms occurring in the alimentary tract. (iii) None of 50 B188 ColV$^+$ isolates from human being No. 2 at the end of one experiment could transmit their ability to produce colicine, indicating they were not B188 ColV$^-$ organisms that had acquired ColV from any ColV$^+$ *E. coli* other than B188 that might have been present in his alimentary tract. (iv) In the experiments in which mixtures of B188 ColV$^+$, ColV$^-$ and *col*V^r organisms were ingested, the ColV$^-$ organisms survived as long as the *col*V^r ones, indicating that colicine V was not providing any selection pressure in the alimentary tract as far as B188 organisms were concerned; ColV$^-$ and *col*V^r organisms survived equally well in the absence of ColV$^+$ ones. Furthermore, colicine V could not influence the B192 experiments because the ColV$^-$ form of this strain was resistant to colicine V, and its bactericidal effect also did not appear to play a part in the chicken experiments, in that the general *E. coli* population of the caeca of the chickens given the ColV$^+$ form of F65 was no lower than that of the caeca of the chickens given the ColV$^-$ form.

After taking mixtures of different forms of the same *E. coli* strain, several days always elapsed before the ColV$^+$ form dominated the other forms in the alimentary tract of the human beings, indicating that the survival value conferred by possession of ColV, although definite, was not intense. Its expression, therefore, may sometimes be masked in experiments in which wild-type strains that do or do not produce colicine V are compared, because they can differ in many respects other than in ability to produce colicine V; this may explain why such experiments have not yielded unequivocal results.

The fact that the ColE$^+$ form of *E. coli* B188 ColV$^-$ did not survive better than the ColE$^-$ form in the alimentary tract of the two human beings indicates that all colicine determinants do not confer increased survival on *E. coli* possessing them. The ColE$^+$ form also was no more pathogenic for chickens, mice and calves than the ColE$^-$ form, and those colicines (other than V) tested by Smith (1974) did not confer increased pathogenicity on *E. coli* K12. These findings, together with those made on ColV, may explain, at least in part, why colicine V is the most common colicine produced by *E. coli* (Wilson & Miles, 1964).

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