J. Hyg., Camb. (1962), **60**, 175
Printed in Great Britain

Experimental intestinal coliform infections in mice

By FRANCES M. ASHBURNER and ROSE MUSHIN
The School of Bacteriology, University of Melbourne, Parkville, Victoria, Australia

(*Received* 9 *October* 1961)

INTRODUCTION

The evidence of the aetiological significance of *Escherichia coli* serotypes found in association with infantile diarrhoea rests mainly on clinical and epidemiological data. Studies on the pathogenesis of these strains in laboratory animals have failed to reproduce disease simulating the essential features of the human infection and to distinguish the enteropathogenic from normal coliforms (Neter, 1959; Braun, Resemann & Stöckle, 1953). Olitzki (1956) reported that 3 weeks' old mice on a milk diet were susceptible to oral coliform infections. Our attempts to apply this method by challenging mice with an *E. coli* 055:B5 strain, recently isolated from a fatal case of gastro-enteritis, gave inconclusive results.

Freter (1955, 1956) used streptomycin, erythromycin and mycostatin to suppress the normal intestinal flora of mice, thereby facilitating the colonization of the gut with orally administered *Vibrio cholerae* or *Shigella flexneri*. The present communication describes the application of a similar technique, modified to some extent by Cooper (1959), to study intestinal coliform infections in mice. In this paper the term 'infection' is used as defined by Jawetz, Melnick & Adelberg (1960), as 'the process whereby the parasite enters into a relationship with the host'. The term refers to the successful colonization of the gut by both the enteropathogenic and normal coliforms.

The experiments were set up with five main aims in view: (1) to establish intestinal infections in mice with enteropathogenic coliforms, (2) to show the extent of colonization of the gut, (3) to record any macroscopic or histological evidence of disease, (4) to observe any immune response, and (5) to compare differences, if any, between infections with enteropathogenic *E. coli* serotypes and normal coliform strains, cultured from faecal specimens from healthy babies.

MATERIALS AND METHODS

White mice of both sexes of average weight 25 to 30 gm. were used. Chlorhexidine ('Hibitane', 1% in alcohol, an I.C.I. product) was sprayed on the cages before placing the mice in them and at various intervals thereafter, as this helped to minimize the introduction of organisms other than those under study, and subsequent cross-infection.

The protocol of the experiments was as shown below.

Day: Before administration of infecting dose	Day: After administration of infecting dose	Procedure
3	—	Mice were put into individual cages. Faeces were sampled
2	—	Mice were given S and E in 0·2 ml. water by stomach tube. S–E water was supplied *ad lib.*
0	0	Faeces were sampled. Mice were given S + $CaCO_3$ in 0·2 ml. water by stomach tube, followed by 0·2 ml. *E. coli* (SR) suspension
—	1, 2, 3*	Faeces were sampled.
	4, 5, 6	*Post mortem* examinations were made.
	7, 14, 21, 28	Serum and copro-antibody titrations were carried out

S = streptomycin; E = erythromycin; SR = streptomycin-resistant strain.
* Days of examination were as indicated in the text.

Bacteriological sampling prior to infection

Before the first administration of antibiotics a sample of faeces was collected and screened for the presence of *Salmonella*, enteropathogenic coliforms and, on occasions, for the normal coliforms under test. The faecal suspension was plated out on deoxycholate agar with a low sodium deoxycholate and citrate content (Leifson, 1935), on SS agar (Difco), and cultivated for 24 hr. in selenite enrichment medium, from which subcultures were made to SS agar. The non-lactose fermenting colonies were classified on biochemical reactions. The coliform organisms were tested with fourteen diagnostic sera as previously described (Mushin, Lowson & Bishop, 1960). Agglutination tests were also done with antisera specific for the normal coliforms under study.

Infecting dose

The enteropathogenic *Escherichia coli* strain used was serotype 0111:B4 Stoke W. Normal coliform strains, labelled *E. coli* N 1 and N 2, were isolated by us from the faeces of normal infants. The streptomycin resistant (SR) strains were maintained on Dorset's egg medium and gave the same biochemical and serological reactions as parent organisms. Control mice were given the antibiotics only.

The infecting doses in the earlier experiments were prepared from an overnight growth on nutrient agar slope. Suspensions were made in 0·85% saline and diluted to correspond with the required opacity on the Brown scale using Burroughs Wellcome opacity tubes. When it became apparent that infections were readily set up, serial tenfold dilutions of overnight broth cultures were used. Viable bacterial counts were made on pour-plates with nutrient agar which contained 100 μg./ml. streptomycin.

Bacterial sampling after infection

The faecal suspensions were plated out on deoxycholate agar using a standard technique. The degree of growth was assessed as abundant or moderate, and the colonies were counted when sparse. Six lactose-fermenting colonies were tested with the diagnostic anti-serum specific for the administered organism. Mice were killed 1, 2, 3, 7, 14, 21 and 28 days after the administration of coliforms. When the chronic character of the infection became evident, the examinations were made 1, 3 and 7 days after the oral challenge.

On *post mortem* the peritoneal cavity was examined for macroscopic changes. Incisions were made with sterile scissors in the stomach, duodenum, caecum and colon and a loopful of the exudate was plated on deoxycholate agar. The degree of growth was assessed as previously, and six lactose-fermenting colonies were serologically tested.

Histological examination

Sections from duodenum, caecum and colon from infected and antibiotics-treated mice were stained with haematoxylin-eosin stain, and with a fluorescent stain specific for mucin (Hicks & Matthaei, 1958).

Serum and copro-antibody tests

Blood was taken from the axillary veins of anaesthetized mice and the serum was titrated against sheep red blood corpuscles sensitized with the 'O' antigen of a steamed suspension of the infecting organism (Neter, Bertram & Arbesman, 1952). This technique was chosen as the simplest, after finding no significant difference in employing antigens prepared by other procedures, such as alcohol-acetone extract by Roschka (Kauffmann, 1954), filtrates from steamed 5 day broth cultures (Neter *et al.* 1952), and alkaline extracts followed by acetone precipitation (Cooper & Pillow, 1959). The initial serum dilution was 1 in 10, or occasionally less, and the days of testing were as for bacterial sampling. A known positive serum was included as control.

Copro-antibody tests were carried out in a similar manner using supernatant fluid from the faecal suspensions at the initial dilution 1 in 2. The tests were done 1, 2, 3 and 7 days after the oral challenge, or on each day in the first week and after 2 weeks.

Serum and copro-antibody titrations were made in plastic trays containing small cups. The reading was recorded after 2 hr. incubation at 37° C.

RESULTS

Infections with Escherichia coli *strains*

The normal intestinal flora of the experimental mice did not include *Salmonella*, the coliform serotypes associated with infantile diarrhoea or *E. coli* N1 and N2 strains. In all cases the faeces of mice given streptomycin were cleared of aerobic Gram-negative intestinal bacteria after 2 days, that is before *E. coli* SR strains

were introduced. In the infected animals abundant faecal excretion of the administered organisms was evident after 24 hr.

As shown in Table 1 it has been found possible to infect mice with as few as ten, or less, enteropathogenic or normal coliform cells. However, to obtain more regular infections, larger doses should be given, for example, 300 cells or more. In the absence of other intestinal flora the coliform infection persisted in the gut for at least 28 days, which was the longest period in which mice were kept under observation.

Table 1. *Infection and implantation of* Escherichia coli *in various parts of the intestine in experimental mice*

Organism	No of organisms in infecting dose	No. of animals		No. of mice with *E. coli* in			
		Challenged	Infected	Stomach	Duodenum	Caecum	Colon
E. coli 0111:B4	10^9	26	26	25	25	26	26
	9	9	2	1	1	2	2
	85	9	9	5	6	9	9
	850	9	9	8	7	9	9
	8500	9	9	6	6	9	9
E. coli N1	3	9	5	5	3	5	5
	30	9	6	5	4	6	5
	280	9	7	7	7	7	7
	2800	9	9	9	8	9	9
E. coli N2	3	9	0	0	0	0	0
	33	9	7	6	6	7	7
	340	9	9	9	7	9	9
	3400	9	9	7	7	9	9
		No. of normal mice examined					
		20		9*	6*	20	20

* 15 or less colonies on deoxycholate agar in all except three instances.

Considerable trouble was experienced owing to cross-infection with other streptomycin-resistant organisms, mainly coliforms and paracolons. The results of several series of oral challenges with enteropathogenic and normal *E. coli* strains were not included in the Table as the findings were obscured by mixed infections with accidentally introduced organisms. In two mice at least, the accidentally introduced coliforms colonized the gut to the exclusion of the experimentally administered strain. It seemed that growth advantages for both the enteropathogenic and normal coliforms, which were purposely implanted in the bowel, were not significantly in competition with the accidentally acquired organisms. However, an initial infection with large numbers, e.g. of the order 10^9 cells was followed, with one exception, by colonization of the gut through its full length. The use of chlorhexidine spray proved to be very valuable in almost eliminating cross-infection.

Site of implantation of coliforms

Successful infection of mice with an introduced coliform strain was indicated by the colonization of caecum and colon.

As shown in Table 1, the stomach and duodenum were more consistently colonized when the larger infecting doses were given. It was of interest to note that when the organisms became established in the upper region of the gut, their growth, as a rule, was abundant or moderate. In contrast, *post mortem* examinations on twenty normal mice revealed a less frequent occurrence of bacterial flora in the upper bowel. It was significant that on deoxycholate agar these organisms appeared in small numbers only, usually a count of less than fifteen colonies of coliforms, paracolons or *Providencia* was recorded, and one gained the impression that these organisms belonged to a transient, rather than a resident population.

Macroscopic examination after infection

On autopsy of infected mice, some degree of distension was noticed, probably due to the administration of antibiotics. No peritoneal exudate was observed and the mesenteric lymph nodes were not obviously enlarged. In animals given antibiotics only, a comparison with the untreated animals showed that the caecum and appendix increased markedly in size and some thickening of the duodenum occurred. Blood vessels in the mesentery became more obvious. However, no difference in the colour of the intestinal tract, or in the size and appearance of other organs could be observed between the infected animals and the streptomycin-treated controls. Similarly, no difference was noted between mice infected with *E. coli* 0111:B4 and those with normal coliforms.

The infected mice were clinically well with no symptoms of diarrhoea. Faecal pellets from mice given antibiotics were much more bulky and moister than normal. However, no significant difference could be observed in the consistency or total volume of faeces from infected and uninfected antibiotics-treated mice.

Histological examination after infection

Sections from various organs of mice which were given doses of 10^9 enteropathogenic coliforms were examined. No definite pathological changes were observed by comparison with sections from the antibiotics-treated uninfected animals.

Immune response to infection

Apart from one exceptional case in which serum antibody to *E. coli* 0111:B4 in a titre 64 was observed 28 days after infection with 10^9 bacteria, serum or faecal antibodies were not found in mice of any series at the dilutions tested.

DISCUSSION

An oral dose of ten or less cells of *Escherichia coli* was occasionally sufficient to initiate an intestinal infection in antibiotics-treated mice, while larger doses of over 300 bacteria produced a more regular colonization of the gut. No obvious difference was shown between enteropathogenic and normal coliforms in that both

types were able to colonize the bowel. The significant feature was the frequently recorded multiplication of the bacteria in the stomach and duodenum in the experimental mice, while the normal animals had a scanty flora in the upper gut.

It is of interest to note that Koya, Kosakai, Kono, Mori & Fukasawa (1954) observed the proliferation of *E. coli* 0111:B4 in the small intestine of adult human volunteers who were given this organism orally. Thomson (1955) noted that the small intestine of babies with coliform gastro-enteritis harboured large numbers of these organisms. In contrast, the small intestine of humans with no symptoms of gastro-enteritis had a transient and scanty flora (Cregan & Hayward, 1953; Thomson, 1955). These observations in humans showed some similarity with the findings on autopsy in the experimental mice.

It is of interest to compare the results of experimental coliform infections in mice with those by intestinal organisms of accepted pathogenicity. Both *Salmonella* and *Shigella* infections have been set up in mice by suppression of the normal intestinal flora with antibiotics. Bohnhoff, Drake & Miller (1954) established *Salmonella enteritidis* infection in mice and demonstrated positive faecal cultures. No indication was given of the sites of implantation in the intestinal tract but streptomycin-resistant *Salmonella* were frequently cultured from the heart, blood and spleen. Freter (1956) established experimental shigellosis in mice but could find no gross symptoms on autopsy. Cooper (1959) produced a chronic infection in mice with *Shigella dysenteriae* 2 and obtained macroscopic and histological evidence of pathological damage. The organisms were isolated *post mortem* from the appendix, caecum and colon, less frequently from the upper intestine and rarely from gastric contents.

In the present communication it has been shown that no serum or copro-antibodies against the introduced coliforms were detected (with one exception) in mice. Owing to the benign nature of the infection the lack of immunological response should not be surprising. Cooper & Pillow (1959) demonstrated serum and copro-antibodies in experimental shigellosis in mice.

The problem of the potential virulence of intestinal coliforms has attracted much attention. Taylor, Wilkins & Payne (1961) using ligated loops of rabbit small intestine showed histological changes on injection with cultures of enteropathogenic coliforms. It was suggested that a positive result expressed the ability of an organism to produce toxin, which may be lost under laboratory conditions. This may have accounted for the lack of inflammatory response in our experiments and possibly, a more recently isolated virulent strain may have produced toxic symptoms. However, several subcultures of the organism are necessary for the production of streptomycin-resistant mutant and this may be a factor difficult to overcome.

The readiness of colonization of the gut by coliforms, both enteropathogenic and normal, was demonstrated in our experiments by either purposely or accidentally introduced infections. Both Freter (1956) and Cooper (1959) observed that the presence in the gut of streptomycin-resistant *E. coli* caused the elimination of *Shigella* from faeces and Freter (1956) could not attribute this overgrowth to antibiotic action. The nature of the successful coliform colonization is not yet elucidated.

SUMMARY

A technique involving the suppression of normal intestinal flora with antibiotics, and the introduction of streptomycin-resistant coliforms was used for the study of infections in mice.

It was shown that *Escherichia coli*, both the enteropathogenic and normal faecal strains, tend to colonize the upper intestinal tract as well as the lower region. An oral dose of ten or less cells could produce a chronic infection. Infections were asymptomatic even with an initial dose of 10^9 bacteria. There was no indication of growth advantages for enteropathogenic coliforms in competition with normal.

Thanks are expressed to Prof. S. D. Rubbo for his interest and support of this work. Thanks are also due to G. G. Blake for technical assistance.

REFERENCES

Bohnhoff, M., Drake, B. L. & Miller, C. P. (1954). Effect of streptomycin on susceptibility of intestinal tract to experimental Salmonella infection. *Proc. Soc. exp. Biol. Med., N.Y.*, **86**, 132.

Braun, O. H., Resemann, G. & Stöckle, Ch. (1953). Untersuchungen über die Tierpathogenität der Dyspepsiecolibakterien. *Z. Hyg. InfektKr.* **137**, 581.

Cooper, G. N. (1959). Experimental shigellosis in mice. I. *Aust. J. exp. Biol. med. Sci.* **37**, 193.

Cooper, G. N. & Pillow, J. A. (1959). Experimental shigellosis in mice. II. *Aust. J. exp. Biol. med. Sci.* **37**, 201.

Cregan, J. & Hayward, N. J. (1953). The bacterial content of the healthy human small intestine. *Brit. med. J.* i, 1356.

Freter, R. (1955). The fatal enteric cholera infection in the guinea-pig achieved by inhibition of normal enteric flora. *J. inf. Dis.* **97**, 57.

Freter, R. (1956). Experimental enteric Shigella and Vibrio infections in mice and guinea pigs. *J. exp. Med.*, **104**, 411.

Hicks, J. D. & Matthaei, E. (1958). A selective fluorescence stain for mucin. *J. Path. Bact.*, **75**, 473.

Jawetz, E., Melnick, J. L. & Adelberg, E. A. (1960). *Review of Medical Microbiology*. 4th edn. Los Altos, California: Lange Medical Publications.

Kauffmann, F. (1954). *Enterobacteriaceae*. Copenhagen: Einar-Munksgaard.

Koya, G., Kosakai, N., Kono, M., Mori, M. & Fukasawa, Y. (1954). Observations on the multiplication of *Escherichia coli* 0111 : B4 in the intestinal tract of adult volunteers in feeding experiments. *Jap. J. med. Sci. Biol.* **2**, 197.

Leifson, E. (1953). New culture media based on sodium desoxycholate for the isolation of intestinal pathogens and for the enumeration of colon bacilli in milk. *J. Path. Bact.* **40**, 581.

Mushin, R., Lowson, F. M. & Bishop, R. F. (1960). Bacteriological and epidemiological studies on coliform diarrhoea in children. *Med. J. Aust.* ii, 365.

Neter, E. (1959). Enteritis due to enteropathogenic *Escherichia coli*. *J. Pediat.* **55**, 223.

Neter, E., Bertram, L. F. & Arbesman, C. E. (1952). Demonstration of *Escherichia coli* 055 and 0111 antigens by means of hemagglutination test. *Proc. Soc. exp. Biol. Med., N.Y.*, **79**, 255.

Olitzki, A. L. (1956). Investigations on infantile gastro-enteritis in Jerusalem 1953–1956. *Acta med. or., Jerusalem*, **15**, 137.

Taylor, J., Wilkins, M. P. & Payne, J. M. (1961). Relation of rabbit gut reaction to enteropathogenic *Escherichia coli*. *Brit. J. exp. Path.* **42**, 43.

Thomson, S. (1955). The role of certain varieties of *Bacterium coli* in gastro-enteritis of babies. *J. Hyg., Camb.*, **53**, 357.