COLONIZATION OF THE MOUSE INTESTINE WITH ESCHERICHIA COLI*

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(Received for publication, June 10, 1965)

Although enteropathogenic strains of *Escherichia coli* are a frequent cause of disease in human infants, it has proven difficult to establish experimental infections in animals with these organisms. The most successful results so far have been obtained by feeding cultures to mice treated with antibacterial drugs prior to the test in order to suppress their indigenous intestinal flora (1). Useful as it is, this method is obviously artificial and fails to reproduce the conditions under which the disease is commonly observed in man. The availability at The Rockefeller University of a colony of mice (NCS) maintained under so called "specific pathogen-free" conditions, and especially free of *E. coli*, prompted us to investigate whether these animals would prove more receptive to human strains of *E. coli* than mice raised under usual conditions.

The experiments reported in the present paper were carried out with a culture of *E. coli*, serotype 026:K60 (026:B6). This type has been found to be associated with infantile diarrhea and is also occasionally recovered from animals (2); the strain used here was isolated from an infant. While the ultimate purpose of our study was to determine whether NCS mice can be used to differentiate virulent enteropathogenic strains of *E. coli* from ordinary strains, it seems worthwhile reporting at this time a finding of more general importance. It was found namely that the age of the animal host has a profound effect on the ability of the bacteria to colonize the intestine and on their persistence in this organ. Colonization uniformly took place when very young mice received *E. coli per os*, whereas adult animals usually failed to become infected under the same conditions. Indeed the population of *E. coli* in the gastrointestinal tract abruptly fell to very low levels around the time of weaning.

Materials and Methods

The NCS Mouse Colony.—As described in earlier publications (3), the NCS colony was derived from the colony of albino "Swiss" mice maintained at The Rockefeller University. When first obtained, and for 4 years thereafter, the NCS colony was free of *E. coli* and, in

* These studies have been supported by the Health Research Council of the City of New York, Research Project No. U-1049.

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fact, was characterized by an intestinal flora far simpler than that of ordinary mice. Following an accident in the breeding room, reported in an earlier publication, many of the NCS animals became contaminated and the colony thereby lost some of its unusual characteristics (4). Fortunately, it was possible to save a number of litters which had not been involved in the accident, and which had not acquired *E. coli*. A subcolony developed from these litters and maintained in our own laboratory has remained free of *E. coli* ever since. It will be designated as NCS-D.

In all the experiments to be described here, the mice were given acidified water and commercial pellets essentially free of bacteria, as described in reference 5.

The experiments were conducted with animals of precisely known age. To this end the breeding units were maintained in the laboratory and each litter of young mice was kept in a separate cage; woodshaving was used for bedding.

Indigenous Gastrointestinal Flora.—Studies of the indigenous flora of NCS and NCS-D animals were carried out prior to attempts at colonization with *E. coli* 026:K60.

Postmortem samplings prior to and after experimental infection included the stomach, the duodenum, the lower colon, and often the lungs, heart, and spleen. The individual organs were homogenized in 5 ml charcoal water in a teflon grinder. Bacteriological studies were also carried out on fecal material obtained from mice 18 days old or older; in this case the fecal samples were collected directly in tubes containing 4 ml charcoal water, and were emulsified in a mechanical shaker (5).

Bacteriological Techniques.—The selective media used for the recovery and enumeration of lactobacilli, enterococci, and coliforms have been described elsewhere (5). In addition, nonselective nutrient agar was also used. All cultures were incubated under aerobic conditions overnight at 37°C; an atmosphere enriched with CO_2 was provided for the cultivation of lactobacilli.

The sizes of bacterial populations in the various organs were not determined quantitatively, but only evaluated by approximate methods. To this end, a standardized loopful of the sample to be tested was spread on the surface of the appropriate agar medium. The abundance of the growth was recorded by symbols as indicated for each table; this estimate was of comparative value only.

Experimental Infection with E. coli 026:K60.—The organisms used for the infection tests were obtained by incubating for 16 hours 10 ml of meat infusion broth inoculated with a loopful of a 16 hour broth culture. The average viable count of such cultures, estimated from surface platings of tenfold dilutions, was approximately 9×10^8 per ml.

The infective doses were administered *per os* in the form of one drop of bacterial suspension containing either 23×10^2, or 23×10^6, or 230×10^6 viable bacterial cells. Because of difficulties in handling very young mice, the dose taken by animals younger than 10 days could not be precisely assessed.

In general, the drop of bacterial suspension was deposited into the mouth of the animal with a syringe, the tip of the needle being covered by a polyethylene tube. In other cases, the drop was introduced directly into the stomach through a fine polyethylene tube attached to a syringe. In one experiment a culture of *E. coli* 026:K60 was added on 3 consecutive days to the drinking water given to 24-day-old mice.

Needless to say, colonial examination would not have been sufficient to identify the cultures of *E. coli* 026:K60 recovered from mice infected *per os* with this strain. Identification, and differentiation from the strains present in the indigenous flora, were carried out by the use of biochemical tests and serotyping, as will be described in a subsequent publication.

RESULTS

The Indigenous Flora of NCS and NCS-D Mice at Various Ages.—Fig. 1 illustrates the sizes of the population of lactobacilli, enterococci, and coliforms[1] recovered from the digestive tract of mice at various times after birth. Seven age groups were investigated, the numbers of animals in each group ranging

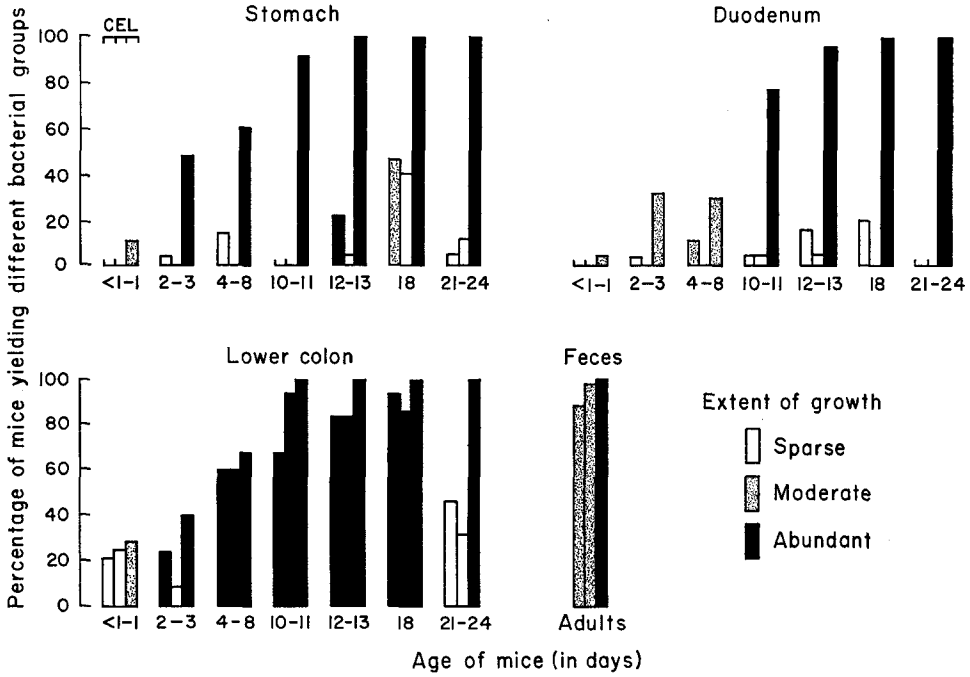

FIG. 1. Coliforms (*C*), enterococci (*E*), and lactobacilli (*L*) in normal NCS and NCS-D mice at various ages. The figure summarizes the data on the extent of growth of the above organisms in the various sites of the gastrointestinal tract.

from 19 to 63; the results shown in Fig. 1 correspond to the averages for each group.

Lactobacilli could be occasionally recovered from the stomachs of mice less than 1 day old and were abundant in a large percentage of mice 2 to 3 days old. These organisms were the only ones to achieve large populations in the duodenum; they were most abundant in the lower colon. The coliforms occurred,

[1] Throughout this paper, the expression coliforms will be used to denote both the true lactose-fermenting (LF) and the slow lactose-fermenting bacteria (SLF). As mentioned earlier, many animals of the NCS colony are now contaminated with *E. coli*, whereas the NCS-D colony is still free of this organism but harbors slow lactose-fermenting coliforms (SLF).

but only in very small numbers, in the stomachs of mice less than 12 days old; then their numbers increased somewhat but fell again at the age of 21 days. The populations of coliforms were very small at all times in the duodenum, but much larger in the lower colon. In this segment of the intestine they increased up to 18 days of age, then fell sharply. Although coliforms could be consistently recovered from the feces of adult mice, their numbers were much smaller than in the case of lactobacilli. The enterococci exhibited population trends very similar to those of coliforms, except that they were far less abundant in the stomach and duodenum.

Lactobacilli, enterococci, and coliforms were not found in the heart, spleen, or lungs. The only limitation to this statement is that small numbers of coliforms were recovered from the heart and lungs of 2 animals in which the coliform flora was extremely abundant in the stomach and lower colon, and moderate in the duodenum.

Other ill defined gram-negative organisms were occasionally recovered from the intestine of young mice; they did not belong to the family Enterobacteriaceae, were facultative anaerobes, and gave either yellow or colorless colonies on nutrient agar. *Pseudomonas aeruginosa* was recovered on a few occasions from the stomach and lower colon of young mice and from the feces of adults. Organisms of the *Proteus* species were not encountered.

Special attention was naturally directed to the coliform flora of mice at various ages, prior to their infection with *E. coli* 026:K60. The observations extended to 235 young and 199 adult animals of the NCS colony, and to 136 young and 69 adults of the NCS-D colony. In agreement with earlier findings, lactose fermenters were detected in the NCS, but not in the NCS-D colony (Fig. 2).

Experimental Infection per Os with E. coli.—

Mice were infected with various doses of *E. coli* by introducing one drop of the bacterial suspension into their mouths as described under Materials and Methods. Postmortem bacteriological examinations were carried out 24 and 48 hours after infection. As the results were essentially the same whether NCS or NCS-D mice were used, it seems justified to group them in the statement of findings for the different age groups (Table I, A).

Irrespective of the infective dose used, *E. coli* 026:K60 became established in all animals 13 days old or younger. In contrast, the percentage of colonization in mice 18 days old was related to the dose; it occurred in all animals only when very large numbers of organisms (230×10^6) were administered. Infection rates were even lower in 24-day-old animals; in this case, furthermore, the numbers of *E. coli* tended to decrease rapidly in the gastrointestinal tract within 48 hours after infection.

As seen in Table I, A, many young mice died following infection (usually within 2 to 3 days). In mice 1 to 8 days old, mortality occurred usually when

the infective dose was large. The highest mortality rate was observed among mice infected at 10 to 13 days of age, and it was clearly related to the size of the infective dose. The mortality rate decreased markedly as older animals were used. In fact, the few deaths recorded for animals 24 days old were probably the result of an accident since they occurred in only one litter of animals infected with a small dose.

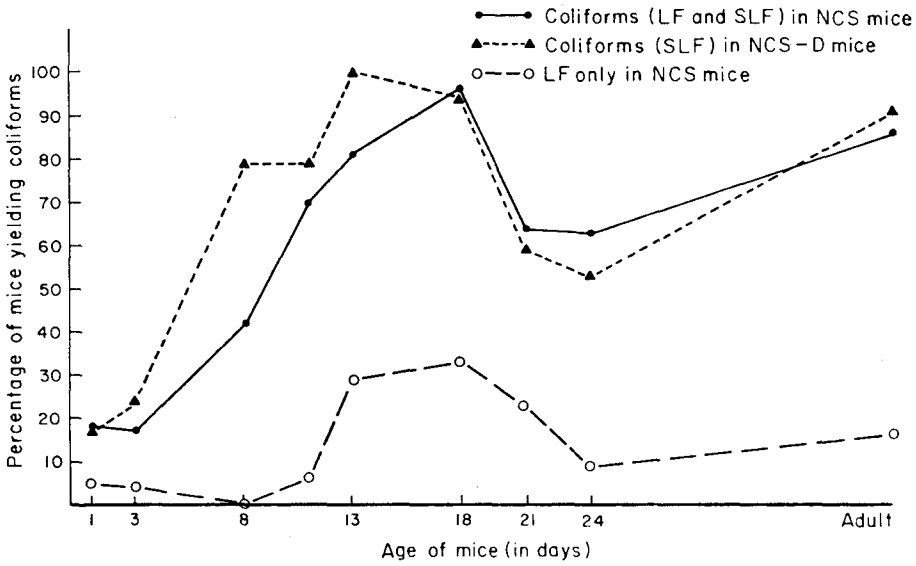

FIG. 2. Incidence of coliforms in normal mice at various ages. The incidence of coliforms was highest (100 per cent) in NCS-D mice at 13 days of age, and in NCS mice (96 per cent) at 18 days. A sharp decrease in incidence, and also in numbers (see Fig. 1), occurred at 21 days of age.

Table II presents typical protocols of the extent of colonization of the various organs, in mice given *E. coli* when 10 to 12 or 18 days of age. It is apparent that infection became generalized in the animals receiving the largest infective dose; it affected the whole length of the gastrointestinal tract, and usually the lungs and heart as well. With the lower doses, *E. coli* could not be recovered from the lungs or the heart, and it achieved only a low degree of colonization of the stomach and the duodenum. In all cases, colonization was most intense and persistent in the lower colon, but as noted earlier, it often failed even in this organ when the smaller infective doses were given to mice 18 days of age or older.

Duration of the Colonization with E. coli 026:K60.—Administration *per os* of 23×10^6 bacteria proved a convenient method for studying the effect of the age of the host on the intensity and duration of the colonization (Fig. 3).

TABLE I

Effect of Age and of Infective Dose on Infection and Mortality Rates in Mice Given E. coli

Age at time of infection	No. of bacteria in infective dose	Mice carrying *E. coli* 24 hours after infection		Occurrence of deaths following infection		
				Litters	Individual mice	
days		*No.*	*per cent*		*No.*	*per cent*
		A. Oral infection				
1–3	230×10^6	15/15	100	10/10	43/94	46
	23×10^6	32/32	100	2/21	3/177	2
	23×10^2	10/10	100	1/5	1/47	2
6–8	230×10^6	10/10	100	10/10	43/73	59
	23×10^6	3/3	100	0/3	0/20	0
10–13	230×10^6	8/8	100	8/8	62/72	86
	23×10^6	34/34	100	30/36	109/300	36
	23×10^2	6/6	100	0/6	0/46	0
18	230×10^6	6/6	100	0/3	8/24	33
	23×10^6	5/6	83	0/3	0/25	0
	23×10^2	1/6	17	0/3	0/25	0
24	230×10^6	15/18	83	0/3	0/27	0
	230×10^6	7/27*	26			
	23×10^6	9/21	43	1/8	3/71	4
	23×10^6	2/25*	8			
	23×10^2	1/14	7	0/3	0/21	0
	23×10^2	0/21*	0			
		B. Infection by stomach tube				
12	230×10^6	2/2		2/2	2/13	15
	23×10^6	4/4		0/4	0/28	0
	23×10^2	8/10		0/10	0/72	0
24	230×10^6	17/17	100	1/3	1/20	5
	230×10^6	4/14‡	25			
	23×10^6	21/23	91	0/3	0/23	0
	23×10^6	1/20‡	5			
	23×10^2	4/22	18	0/3	0/22	0
	23×10^2	0/19‡	0			

* Infection rate at 48 hours after challenge.

‡ Infection rate at 3 days after challenge.

TABLE II

Effect of Age and Infective Dose on the Extent of Colonization of Various Organs by E. coli*

No. of bacteria in infective dose	Bacterial populations in the various organs at indicated age					
	Age	Stomach	Duodenum	Colon	Lungs	Heart
	days					
	A. Mice challenged at 10 to 12 days of age					
230×10^6	11–13	∞+	∞+	∞	∞++	∞+
	18–21	++<10	+	∞	++<10	<100
23×10^6	11–13	∞+	++++0	∞+	∞+	∞0
	18–21	+++0	+0	∞0	++0	0
	28	0	0	++0	0	0
23×10^2	11–13	+0	+0	∞++++	0	0
	18–21	0	0	+0	0	0
	28	0	0	0	0	0
	B. Mice challenged at 18 days of age					
230×10^6	19	++++	+	∞++++	++++	+0
	28	0	0	+0	+0	0
23×10^6	19	+0	0	∞0	+++0	+0
	28	+0	0	+0	+0	0
23×10^2	19	+0	0	++++0	0	0
	28	0	0	0	0	0

∞ indicates confluent growth.
++++ indicates crowded colonies (reduced size).
+++ indicates crowded colonies (normal size).
++ indicates 51 to <100 colonies.
+ indicates 1 to <50 colonies.
0 indicates no growth.
* Highest and lowest extent of colonization are recorded.

Colonization took place in all mice which were 1 to 3 days old, or 10 to 13 days old, at the time of infection. In these two groups, the carrier rate had fallen respectively from 100 per cent to 86 and 70 per cent when the animals were 21 days old, and to 8 and 4 per cent at 28 days of age. Colonization was less general and its duration shorter in animals infected when 18 days old, and the same trend became even more evident when the experimental infection was delayed until the 24th day after birth. In fact, most animals had overcome the infection

when 4 to 5 weeks of age, whatever the age at which colonization had taken place.

Other experiments have shown that, as was to be expected, the period of colonization is somewhat more prolonged when the size of the infective dose is increased. It seems certain, however, that the age of the animal at the time of infection is the most important factor in determining the duration of the carrier state.

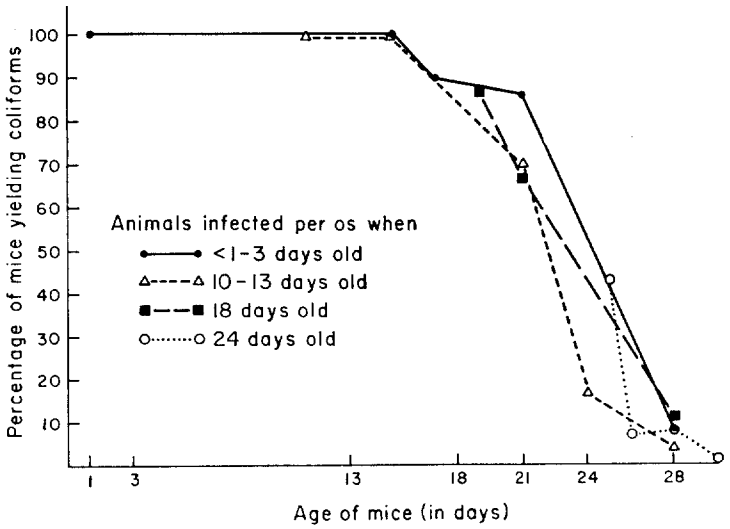

FIG. 3. Duration of infection with *E. coli* 026:K60 in mice. Animals of 4 age groups were given orally a dose of 23 × 10^6 bacteria. *Results:* Duration of infection depended on the age at which the mice were initially infected, the bacteria being essentially eliminated at 27 days of age.

Administration of E. coli 026:K60 to Young Mice by Stomach Tube.—In all experiments described so far, infection was carried out by introducing one drop of bacterial suspension into the mouths of the test animals. Table I, B presents the results of two other experiments in which the infective dose was introduced by stomach tube in mice 12 or 24 days old.

As can be seen, infection was readily established by this method in the younger group of animals. It is true that colonization failed to take place in 2 of the 10 mice receiving the small infective dose, but this may be related to the fact that these animals harbored a large number of lactose fermenters prior to experimental infection. In general, the duration of colonization was the same as when the bacteria were placed in the mouth.

In mice infected by the stomach tube method when 24 days old, *E. coli*

026:K60 colonized a higher percentage of mice than by the oral route, but disappeared just as promptly from the gastrointestinal tract.

A few of the animals died as a result of administration of the large doses of bacteria by stomach tube. Postmortem examinations revealed the presence of *E. coli* in the lungs and the heart.

Contamination of Adult Mice with E. coli 026:K60.—It has been our experience that adult mice of the NCS colony rarely acquire the bacterial flora of other adult mice with which they come into contact. This fact is obviously related to the difficulty reported in the preceding pages of establishing infection with *E. coli* in mice past the weaning age. A few other observations made in the course of the present study provide further evidence of the ability of adult mice to resist colonization with *E. coli*.

Mice 24 days old were given drinking water heavily contaminated with *E. coli* 026:K60 on 3 consecutive days. Under these conditions, the bacteria could be recovered from the stomach and the whole length of the intestine, but not from the heart, spleen, and lungs. Furthermore, after clean drinking water was substituted for contaminated water *E. coli* disappeared promptly from the duodenum, then from the stomach, and finally from the rest of the intestine. At the most, small numbers of the introduced bacteria persisted for a week in the lower colon in a few of the animals.

A record was kept of the carrier state due to cross-infection, among 231 parents in contact with their offspring which had been infected at various ages with *E. coli* 026:K60. The occurrence of cross-infection, which was extremely irregular, can be typified by the following findings. The rate of isolation of *E. coli* 026:K60 from parents was occasionally as high as 88 per cent when they had been in contact for 4 days with their young challenged at 1 to 3 days of age. It was 61 per cent at 2 days, then rapidly fell to 20 per cent, when the young had been challenged at 10 to 12 days of age. It was essentially nil when the young had been challenged at 24 days. Clearly then, the carrier state in the parents was merely the reflection of the intensity and persistence of infection in the young.

Effect of Coliform Colonization.—The initial presence in the gastrointestinal tract of lactobacilli and enterococci did not seem to interfere with the implantation of *E. coli* (see reference 6). However, the initial presence of coliforms apparently exerted a competitive effect, as colonization with *E. coli* 026:K60 gradually evolved into colonization with a mixed coliform flora, containing the originally resident strains. On some occasions, the failure to colonize the intestine, especially with a small dose, may have been due to the fact that the coliform flora was very abundant. Some coliform strains, which were cultivated from the intestine of NCS and NCS-D mice, proved to be endemic in these colonies. The serological identification of those resident strains will be considered in a subsequent communication.

DISCUSSION

The findings reported in the present paper show that very young mice of the NCS and NCS-D colonies are extremely susceptible to infection *per os* with an enteropathogenic strain of *E. coli* (serotype 026:K60), originally isolated from a human infant. Let it be noted outright that the word infection has been used throughout this paper to denote implantation of *E. coli* in the gastrointestinal tract, without regard to the production of disease. In fact, diarrhea was not observed in any age group, even when *E. coli* had extensively colonized the stomach and intestine.

Colonization of the whole gastrointestinal tract could be established almost without fail by introducing a small infective dose either directly into the mouth of the young animals, or by stomach tube. In contrast, adult animals of the NCS and NCS-D colonies proved resistant to colonization, even when large amounts of bacterial culture were added to their drinking water for 3 consecutive days. The period of greatest susceptibility was of short duration. It lasted from the day of birth to a few days before weaning. Beyond the 14th day of life, extensive infection of the intestinal tract could be established only with large infective doses. These findings account in part at least for the difficulties experienced heretofore in establishing experimental infections with enteropathogenic strains since the common practice is to use laboratory animals past the weaning age.

In young NCS and NCS-D mice, *E. coli* 026:K60 colonized the stomach, the duodenum and the lower colon; many animals died of the infection. However, if they survived, and irrespective of the infective dose, *E. coli* 026:K60 eventually disappeared completely or almost completely from their gastrointestinal tract. The characteristics of this clearing process are so striking as to deserve some emphasis.

Whatever the age at which the mice were initially infected with *E. coli* 026, these organisms disappeared from their organs and from the feces at the same age. For example, with an infective dose of 23×10^6 (administered *per os*) the elimination of the infective bacteria was essentially eompleted in 90 to 96 per cent of the challenged animals by the 28th day of age. In other words, the colonization lasted about 28, 16, or 4 days depending upon whether the infective dose had been given respectively at 1, 12, or 24 days of age. Increasing the size of the infective dose prolonged only little the duration of the carrier state. In all cases studied, the fall in the *E. coli* population became precipitous toward the end of the 3rd week of life, in other words around weaning time.

The *E. coli* ingested by weaned or adult mice, whether inoculated *per os* or taken in the drinking water, or acquired by contact with infected animals, could be recovered in large numbers after 24 hours from the stools or from the lower colon. It is therefore unlikely that the acidity of the stomach, or other bactericidal agencies of the gastrointestinal tract, played a significant role in the power of adult mice to overcome *E. coli* infection. The clearing mechanism did

not seem to consist either in an immunological reaction since clearance took place almost immediately when the animals were infected during the 4th week of life, in other words before humoral or cellular immunity could develop. It appears that some change which occurred in the animal around weaning time brought about a sudden increase in its resistance to *E. coli*. Two mechanisms come to mind in this regard. One would involve the histological structure and physiological attributes of the gastrointestinal tract, the other the composition of its indigenous flora. Several observations suggest indeed that the indigenous flora plays a role in resistance to *E. coli* infection.

First is the fact, already mentioned, that the only known way to render adult mice receptive to *E. coli* administered *per os* is to treat them with antimicrobial drugs that eliminate many components of their indigenous microbiota (1, 7). This artificially induced susceptibility presents similarities with findings in germfree mice. It has been found, for example, that when germfree mice are contaminated with coliforms (SLF) isolated from ordinary mice, these organisms invade the whole gastrointestinal tract and persist in it at very high population levels. However, the populations of coliforms fall to very low levels within a few days after the animals have been further contaminated with the intestinal contents of ordinary mice (6).

Granted the likelihood that the indigenous microbiota can be antagonistic to *E. coli* and other coliforms, there is no precise information concerning the identity of the microbial species responsible for the antagonistic effect. In the course of the present investigation, it was observed that young NCS mice retained a high degree of susceptibility to *E. coli* implantation for longer than a week after lactobacilli and enterococci had extensively colonized the gastrointestinal tract. Similarly, the establishment and extensive multiplication of lactobacilli, anaerobic streptococci, enterococci, and bacteroides in germfree mice failed to affect the population size and organ distribution of coliforms (SLF) subsequently introduced (6). On the basis of these two independent groups of observation, it seems necessary to postulate that the indigenous microbiota exerts its antagonistic effect through microbial species which are susceptible to streptomycin but have not yet been identified.

In several cases, the presence of many coliforms in the indigenous flora seemed to interfere with the establishment of *E. coli* 026:K60 especially when the infective dose was small. Furthermore, colonization with this strain was frequently followed by the progressive multiplication of the indigenous coliforms which eventually replaced it. Thus, it is possible that the dynamics of the infectious process involved a competition between the introduced and the indigenous strains. Although the existence of competition between various strains of coliforms must remain subjudice for lack of convincing evidence, the possibility of such competitive processes is worth mentioning here because of their potential relevance to the great susceptibility of young NCS and NCS-D mice to *E. coli*

infections. Ordinary mice harbor very much larger numbers of coliforms (including their indigenous strains of *E. coli*) than do NCS and NCS-D mice, and might therefore exhibit a higher degree of so called "natural" resistance to enteropathogenic strains of *E. coli*.

Several different mechanisms have been invoked to account for the antagonistic effect exerted by certain strains of coliforms on others *in vivo*. No evidence for the role of colicin-like products has been obtained so far. On the other hand, it appears that competition for carbon or energy sources is a factor in determining the ability of an invader strain of *E. coli* to establish itself in the mouse intestine (7). Studies focused on the ability of *Salmonella* to multiply in the mouse large intestine have pointed furthermore to the inhibitory effect exerted by certain short chain fatty acids and by low oxidation reduction potentials (8, 9).

Whatever the explanation of the findings recorded in the present paper, they give substance to the hope that young NCS mice (preweaning) can be used for testing the comparative ability of strains of *E. coli* to colonize the gastrointestinal tract. They also make it worth while to use these animals for the analysis of the factors which account for the high susceptibility of the young age group to this bacterial species, and for the occasional breakdown of resistance to it among the adults. If it is true that resistance to *E. coli* enteritis is dependent upon the antagonistic activity of the indigenous microbiota, then it becomes of practical as well as theoretical importance to identify the microbial species responsible for the antagonistic effect, and to determine the conditions most favorable for their persistence and proliferation in the gastrointestinal tract.

SUMMARY

Young albino Swiss mice, of the NCS and NCS-D colonies, proved highly susceptible to the establishment of intestinal infection with an enteropathogenic strain of *E. coli* administered *per os* or by stomach tube.

The period of highest susceptibility was rather short, extending from the day of birth to approximately 2 weeks of age. Adult NCS and NCS-D mice failed to become experimentally colonized with *E. coli*, even when large doses were administered *per os* on 3 consecutive days.

The extent of colonization of the various parts of the gastrointestinal tract was related to the size of the infective dose. Many of the young mice died within 2 to 3 days following *per os* infection with large doses of enteropathogenic *E. coli*. However, practically all the animals which survived cleared their intestinal infection at approximately the same age. For example, in mice infected with 23×10^6 bacteria, colonization of the intestinal tract usually came to an abrupt end when the animals were 24 to 28 days old, irrespective of the age at which they had been infected.

There is suggestive evidence that the acquisition of resistance with age, and

the ability of adult animals to control the intestinal infection, are related to the development in the gastrointestinal tract of a microbiota which is antagonistic to *E. coli*.

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Note Added in Proof.—A recent report of a study, carried out under conditions very different from those described in the present paper, confirms that newborn mice are highly susceptible to infection with *E. coli*. Kétyi, I., Modellversuche an Sauglingsmausen mit einem enteropathogenen Coli-Stamm, *Zact. Bakt.*, 1964, **194,** 332.