THE EFFECT OF THE INTESTINAL FLORA ON THE GROWTH RATE OF MICE, AND ON THEIR SUSCEPTIBILITY TO EXPERIMENTAL INFECTIONS

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During recent years, attempts have been made to establish colonies of laboratory animals free of certain types of pathogenic agents. These animals are commonly designated as "specific pathogen-free" (SPF), or less frequently as "disease-free." They must be clearly differentiated from the so called "germ-free" animals which are raised in a completely sterile environment and which must by definition be bacteriologically sterile.

We wish to report here some observations made on albino mice from a new colony recently established at The Rockefeller Institute by Dr. John Nelson and Mr. George Collins. It was derived from the so called Swiss albino mice which have been raised on a large scale at The Rockefeller Institute since 1932. In order to avoid confusion with the "SPF" or "disease free" colonies maintained in other laboratories, we shall designate the new colony as NCS (Nelson-Collins-Swiss). The albino mice raised under ordinary conditions—from which the NCS animals were derived—will be referred to as SS (standard Swiss). The present paper summarizes the results of extensive comparative studies carried out between May and December, 1959, on almost four thousand animals of each colony. All comparisons refer to animals of the same sex and of the same age (within 4 days).

Materials and Methods

Full details concerning the development of the NCS colony will be presented elsewhere by Dr. John Nelson and Mr. George Collins. Suffice it to mention here that the new colony was started from three pregnant SS animals delivered with sterile technique by Caesarian section on October 8, 1958. The twelve surviving young (7 males and 5 females) were nursed by foster mothers from a colony of the Princeton strain maintained by Dr. Nelson and known to be free of PPLO organisms and of the virus of enzootic bronchiectasis. The new colony derived from these twelve animals was freed of intestinal parasites and ectoparasites by adequate therapies. It has been maintained continually for the past year under conditions designed to prevent contact with outside sources of infection. Some of the preliminary work leading to the development of the NCS colony has been described by Dr. Nelson (1).

In most of the experiments to be reported here, the animals were housed in individual cages on wire grids. Extensive bacteriological studies of the stools and of the intestinal tract were carried out to test for contamination from the outside. The details of these tests will be

presented in later publications. Food and water were provided *ad lib.* The bacterial cultures, bacteriological techniques, composition, and preparation of the various diets used, have been described in earlier publications from this laboratory (2, 3).

RESULTS

1. Growth Rates.—

Animals were placed in individual cages within 1 week after weaning, fed various experimental diets, and weighed individually at regular intervals of time. Food and water were given *ad lib.*

TABLE I A

Diet* (*ad lib.*)	Wks. on diet	SS mice							NCS mice						
		Weights of individual mice after indicated times on diet,‡ *gm.*													
Pellets	0	19.4	20.9	19.0	14.0	20.1	18.5	15.8	13.4	15.3	16.7	17.7	12.7	12.9	17.3
	1	22.4	24.0	21.3	18.0	23.8	23.8	19.1	21.0	23.0	20.5	22.3	21.8	20.8	21.2
	2	23.5	24.8	23.1	17.6	25.1	24.4	18.2	22.4	24.6	23.3	23.9	24.1	23.3	23.1
	3	25.5	24.8	25.6	19.0	23.4	27.3	20.2	25.3	26.1	24.1	25.8	25.8	26.6	24.4
15 per cent casein	0	16.0	19.7	17.0	14.5	16.5	15.7	19.5	16.6	19.4	16.6	14.1	12.0	18.3	11.9
	1	20.9	23.3	18.6	16.4	18.9	20.2	19.7	19.0	21.7	21.2	20.7	17.8	22.2	16.0
	2	20.2	22.0	19.2	18.7	20.6	22.5	21.8	21.5	24.2	23.9	23.4	20.5	26.2	17.5
	3	20.0	25.4	21.1	20.5	22.0	24.5	23.2	24.3	26.6	25.3	25.7	20.9	25.1	19.2
15 per cent gluten	0	17.1	20.1	17.0	16.5	18.3	15.8	16.3	14.5	19.2	13.3	13.8	15.4	12.7	14.9
	1	15.6	19.9	17.9	14.8	18.2	15.9	15.1	17.5	21.5	15.2	15.7	17.5	14.9	17.1
	2	16.3	21.7	18.7	14.3	19.6	17.7	15.9	22.0	23.6	17.9	17.3	21.4	18.0	—
	3	17.2	22.7	19.5	14.3	21.6	19.7	16.3	26.1	24.3	23.7	20.3	23.9	22.1	—
15 per cent gluten + lysine	0	18.6	18.5	16.4	16.0	20.4	20.3	18.0	13.1	10.3	10.7	13.3	15.8	17.6	12.9
	1	20.7	19.6	18.6	18.8	22.5	22.3	18.3	17.9	15.1	16.1	17.5	22.2	20.6	18.1
	2	23.3	20.9	20.7	18.9	23.7	24.5	19.3	21.9	19.6	21.4	20.8	25.7	22.1	22.4
	3	24.2	22.7	22.3	18.7	22.1	25.0	19.4	25.5	20.4	24.3	23.5	29.0	23.6	24.1

* The composition and preparation of these diets have been described in references 2 and 3.

‡ These figures correspond to the first 7 animals of groups of 11 mice. The animals were female mice 4 weeks old at the beginning of the experiment. Their initial weights are indicated at time 0.

Tables I A and I B present the results of one particular experiment. Table I A shows the weights of individual mice at different intervals of time after being placed on the experimental diets. Table I B gives the arithmetic averages of weight changes of 10 animals for each group and each diet.

When comparisons were made with regard to initial size, it was found that the NCS animals gained weight much more rapidly than did SS animals of the same age and sex. As shown in Tables I A and I B, this was true whether the animals were fed commercially available pellets, or a semisynthetic complete diet prepared in our laboratory with 15 per cent casein supplemented with cystine (2, 3).

When casein was replaced in the semisynthetic diet by 15 per cent wheat gluten, the SS mice lost weight rapidly—as was to be expected from the low

lysine content of gluten. Contrary to expectation, however, the NCS mice continued to gain weight on the gluten diet, although not as fast as on the casein diet. When the gluten diet was supplemented with lysine, SS mice gained weight satisfactorily, and NCS mice gained even more rapidly.

TABLE I B

Diet* (*ad. lib.*)	Wks. on diet	SS mice		NCS mice	
		Average weight change‡ (*gm. per mouse*) after indicated times on diets			
		Weekly	Cumulative	Weekly	Cumulative
Pellets	0	(18.3)‡		(15.2)‡	
	1	+3.5‡		+6.4‡	
	2	+0.6	+4.1	+2.0	+8.4
	3	+1.3	+5.4	+1.9	+10.3
15 per cent casein	0	(17.4)		(14.5)	
	1	+2.6		+4.5	
	2	+1.0	+3.6	+2.8	+7.3
	3	+1.2	+4.8	+1.7	+9.0
15 per cent gluten	0	(17.4)		(14.7)	
	1	−0.4		+2.2	
	2	+0.8	+0.4	+3.0	+5.2
	3	+1.1	+1.5	+2.7	+7.9
15 per cent gluten + lysine	0	(19.1)		(15.2)	
	1	+1.3		+4.7	
	2	+1.7	+3.0	+3.6	+8.3
	3	+0.9	+3.9	+2.2	+10.5

* See Table I A.

‡ These figures correspond to arithmetic averages for 10 (4 week old) female animals; the sign − indicates loss of weight.

The figures in parenthesis (at 0 time) indicate the average initial weight for each group of 10 animals.

Individual weights for the first 7 animals of each group are given in Table I A.

Table II presents in a summary form the results of another experiment with a similar design. In this case, the weight changes after 1 week on the experimental diets are given as the arithmetic average for 10 mice of each group.

2. Susceptibility to Experimental Infections.—

In a large number of comparative experiments, animals were infected by the intravenous route with cultures of *Klebsiella pneumoniae* type C (0.01–0.002 ml.), *Staphylococcus aureus* (strain Giorgio) (0.05–0.03 ml.), *Mycobacterium fortuitum*, (0.1 ml.), *Mycobacterium tuber-*

culosis var. *bovis* (strain Vallée) (0.05 ml.). In other experiments, staphylococci were introduced by the intraperitoneal route, and tubercle bacilli by inhalation in aerosol. Details concerning the characteristics of the pathogens used and the mode of infection have been presented in other publications from this laboratory (2, 3). The animals varied from 4 to 12 weeks of age at the time of infection, but in all cases the comparisons between SS and NCS mice were carried out on mice of the same age and sex.

TABLE II

Diet* (*ad. lib.*)	SS mice	NCS mice
	Average weight change (*gm. per mouse*)‡ after 1 wk. on experimental diet	
Pellets	+3.3	+6.3
15 per cent casein	+3.7	+6.5
15 per cent gluten	−0.6	+3.2
15 per cent gluten + lysine	+2.0	+7.1
Corn§	−2.0	+0.5

* See Table I A.

§ Corn grain was the only source of food.

‡ Average for 10 mice.

TABLE III

Diets* (*ad lib.*)	Infective dose	Days post-infection	SS mice	NCS mice
			No. of deaths at indicated times postinfection	
Pellets	*Kl. pneumoniae*, 0.01 ml., i.v.	2	2/7	7/7
"	" " 0.002 ml., "	"	1/7	6/7
"	*Staph. aureus*, 0.05 ml., "	"	5/15	10/15
"	" " 0.03 ml., "	"	0/10	7/10
"	*Myco. fortuitum* 0.2 ml., "	8	2/10	8/10
Corn	*Myco. tuberculosis*, 0.05 ml., i.v.	25	5/12	12/12
"	" " aerosol	15	1/12	8/13

* All animals fed pellets throughout the experiment except the animals infected with *Myco. tuberculosis* (either i.v. or by aerosol) which received only corn grain and water during the first 2 weeks after infection.

As illustrated by the representative results in Table III, NCS mice proved more susceptible to experimental bacterial infections than did SS mice of the same age and same sex. This was true irrespective of the route of infection, and whether susceptibility was measured in terms of survival time following administration of a given infective dose, or in terms of minimal lethal dose.

3. Susceptibility to Endotoxin.—

Two endotoxins were used: one prepared in our laboratory from *Klebsiella pneumoniae* type C (4); the other prepared from *Escherichia coli* (distributed by Difco Laboratories, Lot

109,981). The materials were dissolved in physiological saline and the desired amount injected in a final volume of 0.2 ml., either by the intraperitoneal or the intravenous route.

The LD_{50} of both endotoxins was approximately 150 μg. for SS mice—whether the material was administered intravenously or intraperitoneally. In contrast, none of the NCS mice (out of several hundred tested) succumbed following injection of either endotoxin, even when the dose was raised to 600 μg. It is important to point out that, as will be shown later, this statement is valid only for NCS mice not contaminated with the bacterial flora of SS mice (see Table VII).

TABLE IV

Staphylococcus aureus	Endotoxin*	SS mice	NSC mice
		Deaths within 36 hrs. after infection	
ml. i.p.	*μg. i.p.*		
0.1	0	0/6	0/6
0.1	1	2/6	2/6
0.1	3	4/6	3/5
0.1	0.1	Not done	1/5
0.1	0.3	" "	4/4
0.07	0	Not done	0/5
0.07	3	" "	3/5
0.07	10	" "	5/5

* The endotoxin was injected intraperitoneally in admixture with the staphylococci into female mice 4 weeks old.

In the three experiments presented in Table IV, the endotoxin had been prepared from *Kl. pneumoniae* type C (see reference 4). Similar results were obtained with an endotoxin derived from *E. coli*.

Although NCS mice survived following injection of even large doses of endotoxins, they responded nevertheless to these substances as revealed by other kinds of tests. In fact, a degree of responsiveness of the same order as that exhibited by SS mice could be brought out by the following technique.

Animals received by the intraperitoneal route 0.1 ml. or 0.07 ml. of a culture of *Staph. aureus* (strain Giorgio)—either alone or in admixture with various amounts of endotoxin. It was known from previous experience that intraperitoneal injection of these doses of staphylococci did not cause progressive disease in mice. The effect of adding endotoxin to the inoculum is reported in Table IV.

As was to be expected, no deaths occurred among mice of either the SS or NCS groups receiving staphylococcus alone. In contrast, addition of 1 to 3 μg. of endotoxin to the infective inoculum was sufficient to bring about a fulminating staphylococcal infection in both groups. In several experiments, en-

hancement of staphylococcal infection could be achieved with doses of endotoxin as small as 0.3 μg. It appears, therefore, that although NCS mice are resistant to the lethal effect of endotoxin, they are highly susceptible to an unidentified pharmacological effect of these materials which greatly increases their susceptibility to certain bacterial infections.

4. Intestinal flora of NCS Mice.—Bacteriological studies on various culture media have revealed the presence of a large bacterial population in the different parts of the intestine, as well as in the stools, of NCS mice. Although these bacteriological studies have not yet been concluded, it can be said that the most prominent microorganisms detected on ordinary culture media appear to be lactobacilli and enterococci, with smaller numbers of certain Gram-negative bacilli as yet unidentified. It is of special interest that organisms of the coliform group (lactose fermenters) have not been found so far in any of the NCS animals protected from contamination, and that organisms of the para colon group (slow lactose fermenters) have been very few in number or altogether absent. In contrast, typical lactose fermenters, tentatively identified as strains of *Escherichia coli*, have been consistently recovered from the intestines and the stools of SS mice, whatever their age and the composition of the diet.

In several experiments, NCS mice were placed in contact with SS mice for several consecutive days. In other experiments, the drinking water was contaminated for 24 hours with either the intestine of SS mice homogenized in a tissue grinder or with a dilution of *E. coli* culture isolated from the SS animals. These three techniques rapidly brought about contamination of the intestinal contents of the NCS mice. Moreover, the contaminated NCS mice became persistent carriers of *E. coli*[1] even after discontinuance of contact with SS animals or discontinuance of feeding the intestine homogenate or the bacterial culture. In fact, preliminary studies indicate that the numbers of *E. coli* in the intestines of NCS mice remain large during the first 2 weeks after contamination and then fall to a lower level similar to that usually observed in SS mice. The findings suggest that the establishment of *E. coli* in contaminated NCS mice corresponded to an infection of the intestinal tract rather than to mere multiplication of the bacteria in the intestinal contents.

5. Effect of Contamination with E. coli on Some Characteristics of NCS Mice.—When NCS mice were tested at different intervals of time after contamination with the intestinal contents of SS mice, they were found to acquire progressively most of the characteristics exhibited by the latter animals of the same age and sex.

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[1] Recent experiments, to be reported later, have revealed that the various strains of *E. coli* differ in their ability to establish the carrier state in NCS mice. Of four strains of *E. coli* tested so far, the one isolated from SS mice proved the most effective by far in this regard.

Table V presents the results of an experiment in which NCS mice were contaminated by adding to their drinking water for 1 day the intestine of SS mice homogenized in a teflon tissue grinder. These contaminated animals were fed either pellets or corn grain and their weights were compared with those of uncontaminated NCS mice and of SS mice of the same age and sex.

As seen in Table V, NCS mice contaminated with ground intestine from SS mice gained weight less rapidly on pellets than did uncontaminated animals. Moreover, contamination caused them to suffer a great loss of weight when their diet consisted exclusively of corn grain and water.

TABLE V

Diet*	Wks. on diet	SS mice		NCS mice		NCS mice contaminated‡	
		Average weight change,§ *gm. per mouse*					
		Weekly	Cumulative	Weekly	Cumulative	Weekly	Cumulative
Pellets	0	(20.6)		(21.3)		(20.1)	
	1	+3.3		+2.6		+2.5	
	2	+2.7	+6.0	+2.7	+5.3	+2.2	+4.7
	3	+1.8	+7.8	+2.9	+8.2	+2.4	+7.1
Corn	0	(19.9)		(21.2)		(21.6)	
	1	−3.4		−2.0		−2.9	
	2	−0.1	−3.5	+1.4	−0.6	−1.8	−4.7
	3	+1.1	−3.4	+1.6	+1.0	+1.1	−3.6

* See Table 1 A.

‡ Fed suspension of ground intestine from SS mice for 48 hours.

§ Average for 10 mice. The − sign indicates loss of weight. The figures in parenthesis at 0 time give the average initial weight for the group.

Similar results were obtained by contaminating NCS mice with suspensions of a culture of *E. coli* isolated from the intestine of SS mice. Thus, contaminated NCS mice gained weight less rapidly than uncontaminated animals when they were fed either pellets or the 15 per cent casein diets. Even more strikingly, they initially lost weight when fed the 15 per cent gluten diet which allowed weight gain of the uncontaminated animals (Table VI).

Injection of endotoxin into NCS mice shortly after their contamination with *E. coli* (within 1 to 3 days) did not cause any significant number of deaths. However, susceptibility to the lethal effect of endotoxin was usually established 2 weeks after contamination with either homogenized intestine or with the culture of *E. coli* derived from SS mice (Table VII).

Studies of the effect of contamination on susceptibility to various bacterial infections are still underway. The preliminary results summarized in Table

TABLE VI

Diet* (*ad lib.*)	Wks. on diet	NCS mice		NCS mice contaminated‡	
		Average weight change,§ *gm. per mouse*			
		Weekly	Cumulative	Weekly	Cumulative
15 per cent casein	0	(18.2)		(19.7)	
	1	+3.1		+0.6	
	2	+2.6	+5.7	+1.4	+2.0
	3	+2.0	+7.7	+2.9	+4.9
	4	+1.5	+9.2	+2.1	+7.0
15 per cent gluten	0	(19.4)		(19.1)	
	1	+1.1		−1.2	
	2	+1.4	+2.5	+0.4	−1.0
	3	+3.2	+5.7	+4.3	+3.3
	4	+1.8	+7.5	+1.5	+4.8

* See Table I A.

‡ NCS mice contaminated by feeding for 24 hours a suspension of *E. coli* culture derived from SS mice.

§ Average for 8 mice; the − sign indicates loss of weight. Figure in parenthesis (at 0 time) gives average initial weight for each group of mice.

TABLE VII

Endotoxin*	SS mice	NCS mice	NCS mice contaminated
	Deaths‡ within 3 days after injection		
mg. i.p.	*per cent*	*per cent*	*per cent*
0.15	50	0	?
0.60	70	0	75

* Results were essentially the same whether the endotoxin was prepared from *E. coli* or from *Kl. pneumoniae.*

‡ These results correspond to averages for 4 independent experiments, totalling approximately 30 animals for each group. NCS mice were contaminated either by contact with SS mice, or by feeding ground intestine from these animals.

VIII show that the resistance of NCS mice to *Kl. pneumoniae* and to *Staph. aureus* could be appreciably increased by feeding to these animals 2 weeks before infection either a culture of *E. coli* or a suspension of homogenized intestine of SS mice (Table VIII). Worth mentioning also is the fact that uncontaminated NCS mice treated with large doses of endotoxin were found to exhibit 2 weeks later a resistance to infection at least equal to that of untreated SS mice.

TABLE VIII

Diets* (*ad lib.*)	Infective dose	Days postinfection	NCS mice	NCS mice contaminated with		SS mice
				Intestine	*E. coli*	
			Cumulative Nos. of deaths (out of 10 animals) at indicated days postinfection			
	ml. i. v.					
Pellets	*Staph. aureus*, 0.05	3	4	2		3
		5	7	5		4
		6	10	6		4
15 per cent casein	*Staph. aureus*, 0.05	2	4		2	
		3	7		7	
Pellets	*Kl. pneumoniae*, 0.004	1	3	1		
		2	5	3		
		3	6	4		
15 per cent casein	*Kl. pneumoniae*, 0.004	1	5		2	
		2	7		5	

* See Table I A.

DISCUSSION

As already pointed out, the NCS colony was derived from the SS colony by breeding 12 young obtained by Caesarian section from 3 pregnant SS mice. It is possible therefore, and indeed probable, that the new colony does not possess the total genetic endowment of the parent group. In point of fact, however, it is not necessary to appeal to genetic factors to account for the differences observed so far between the two colonies. It has been consistently found that the intestinal flora of the NCS mice differs qualitatively from that of the SS group, lacking in particular the Gram-negative lactose fermenters (*E. coli*) which are always found in the latter animals. The most striking peculiarities of NCS mice: namely their very rapid weight gain on complete diets, their ability to gain weight on deficient diets, their great susceptibility to experimental bacterial infections, and their resistance to the lethal effect of endotoxins—can be rapidly obliterated by the mere artifice of contaminating these animals with a culture of *E. coli* derived from SS mice.

The implications of these findings are many. For example, they compel a reinterpretation of the meaning of nutritional requirements of animals and men; they also raise new problems with regard to factors which condition resistance to microbial disease. It is clear that many characteristics assumed to be inher-

ent in an individual can in reality be determined by the microbial flora of the intestinal tract. For example, the fact that a given dose of endotoxin is or is not lethal for the mice of a given colony, need not depend on the genetic makeup of the animals, but may merely be the expression of a susceptibility state elicited by certain components of the intestinal flora.

While the larger implications of the facts revealed by the present study cannot be discussed here, it seems worth pointing out that the NCS mice have proved very useful in a variety of experimental programs at The Rockefeller Institute. They have been found to develop a marked increase in resistance to tuberculosis following vaccination with living BCG or with killed mycobacterial antigens; they can be rendered solidly and lastingly immune to *Kl. pneumoniae* type C by one single injection of 0.1 μg. of the specific antigen; their resistance to a variety of bacterial infections can be increased in a non-specific manner by prior treatment with unrelated endotoxins. Results obtained by our colleague, Dr. Philip McMaster, reveal that NCS mice lend themselves well to studies of antibody production and that they are more resistant than ordinary mice to repeated injections of large doses of cortisone.

While this report was in the course of preparation, there appeared a short paper describing the production of "disease-free" rats in an English laboratory by techniques very similar to those used for the production of NCS mice at The Rockefeller Institute (5). The English paper does not provide any information concerning the characteristics of the rats raised in the "disease-free" state, but it indicates that their production on a large scale is a practical possibility. This fact, and our own findings, would seem to make it worthwhile to develop breeding programs for the production of experimental animals free of some of the most common pathogens.

SUMMARY

Mice delivered by Caesarian section were used to develop a new mouse colony which has been maintained in an environment protected from contact with common mouse pathogens, but not in the germ-free state. These mice, designated as NCS, were compared with animals of the same sex and age coming from the parent colony maintained under ordinary conditions.

The NCS mice grew more rapidly than ordinary mice on complete diets; moreover, they continued to gain weight—although at a slower rate—when fed deficient diets which caused ordinary mice to stop growing, or to lose weight.

The NCS mice proved much more susceptible than ordinary mice to certain experimental bacterial infections. In contrast, they were much more resistant than ordinary mice to the lethal effect of large doses of endotoxins. However, they responded to injection of minute amounts of these endotoxins by a marked increase in susceptibility to staphylococcal infection.

Bacteriological studies revealed striking qualitative differences between the

intestinal flora of NCS and ordinary mice. When NCS mice were contaminated—either by contact or by feeding—with a strain of *Escherichia coli* recovered from the intestine of ordinary mice, they acquired the characteristics of the latter animals with regard to weight gain on various diets, and to response to bacterial pathogens and endotoxins.

NCS mice have been found well suited to the study of several nutritional, bacteriological, and immunological problems and it appears that their production on a large scale will not present unsurmountable difficulties.

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