other cells retain their normal 3-dimensional morphology when dried by the critical point technique yet nuclear structure and cytoplasmic components are well visualized in the electron microscope. A delicate intercellular fibrillar network is described as are some aspects of virus uptake by these cells.

1. Anderson, T. F., *Trans. N. Y. Acad. Sci.*, 1951, v13, 130.

2. ———, in *Physical Techniques in Biological Research*, G. Oster and A. W. Pollister, Eds., Academic Press, N. Y., 1956, III, 178.

3. Overman, J. R., PROC. SOC. EXP. BIOL. AND MED., 1961, v107, 895.

4. Overman, J. R., Eiring, A. G., *ibid.*, 1961, v107, 812.

Received April 26, 1963. P.S.E.B.M., 1963, v113.

Susceptibility of Conventional and Germfree Mice to Lethal Effects of Endotoxin. (28469)

S. BORGLUM JENSEN, STEPHAN E. MERGENHAGEN, ROBERT J. FITZGERALD AND HAROLD V. JORDAN

National Institute of Dental Research, National Institutes of Health, U. S. Department of HEW, Bethesda, Md.

Recent studies have indicated that the composition of the intestinal flora greatly affects the reaction of mice to bacterial endotoxins(1,2,3). Utilizing a strain of mice with a predominantly gram-positive intestinal flora, Schaedler and Dubos(2) found that colonization of the intestinal tract by certain gram-negative bacilli rendered the mice susceptible to the lethal effect of endotoxin. This altered reactivity suggested a sensitization by bacterial products absorbed through the intestinal wall.

To obtain further evidence for this hypothesis, it was thought of interest to investigate the response of germfree mice, reared and maintained in an environment without contact with living bacteria, to challenge with endotoxin, and to compare the lethal effect of endotoxin in germfree mice with the effect in conventional mice of the same genetic stock. Additional information on the influence of the intestinal flora on the reaction of mice to endotoxin challenge was provided through experiments with monoinfected and conventionalized ex-germfree mice, and by utilizing a strain of specific pathogen-free mice (SPF mice) with a known and controlled intestinal flora.

Materials and methods. Conventional animals. Conventional mice of the General Purpose (G.P.) and NIH stock (NIH) derived from the albino Swiss-Webster colony were obtained from the NIH Animal Production Section.

Conventional Lobund mice (CVN) derived from the Univ. of Notre Dame germfree stock were obtained originally from Dr. W. L. Newton, Laboratory of Germfree Animal Research, Nat. Inst. of Allergy and Infectious Diseases. These animals were bred in clean, but not germfree, quarters which had not previously housed animals and were provided with sterilized food, water and bedding(4).

Germfree animals. Germfree mice of the NIH and Lobund stocks were supplied by Dr. C. E. Miller of the Germfree Unit, Laboratory Aids Branch, NIH, and housed in plastic isolators of the type described by Trexler(5).

Specific pathogen-free animals. The SPF mice were derived from the NIH germfree stock. They were maintained under the same conditions as their germfree counterparts but harbored an intestinal flora composed of 2 strains of lactobacilli, an enterococcus and a pseudomonad. These bacteria were obtained from Dr. R. W. Schaedler. They were isolated from the NCS mice of Dubos and Schaedler.

Bacterial strains. Earlier experiments indicated that increased susceptibility of mice to endotoxins induced by parenteral injection

of heat-killed gram-negative bacteria exhibits a certain degree of specificity(2). Therefore, it was deemed important in our experiments to use bacteria indigenous to the intestinal tract of the strains of conventional mice utilized in this investigation for preparation of endotoxins.

Coliform bacteria, strain C-BJ-3(04-H5), strain NIH-8(091-H21), strain ND-13(0 unknown-H21), all showing fermentation patterns characteristic of *Escherichia coli,* were isolated from the stools of the 3 strains of conventional mice. All strains of bacteria were transferred biweekly in broth and periodically checked for purity on blood agar plates.

For the purpose of monoinfection of mice kept in the isolators, 18 hour broth cultures of coliform bacteria were dispensed in sterile glass ampoules, sealed, and transferred to the isolator by means of a peracetic acid lock(5). The culture was inoculated in the oral cavity of each mouse by means of a cotton swab and some of the bacterial culture was poured over the food and into the drinking water. The stools of monoinfected mice were checked bacteriologically at intervals during the 3 week period of each experiment.

Stool cultures. Fresh fecal samples were collected in sterile culture tubes and homogenized in broth. Various dilutions were surface streaked or prepared as pour plates using appropriate agar media. Plates were incubated at 37°C aerobically and anaerobically in an atmosphere of 5% CO_2-95% N_2.

Several different types of culture media were used for detection and enumeration of bacteria present in the stools: 5% Sheep Blood Agar was used as a general purpose medium. MacConkey agar and Eosin-methylene blue agar were used for enumeration of coliform bacteria. Mitis-salivarius agar was used for detection of enterococci. Chapman-Stone medium was used for selective cultivation of micrococci. S-L medium(6) prepared as pour plates was used for differentiation of lactobacilli. Tergitol-7 agar supplemented with 4 mg/liter tetrazolium was used for differentiation of coliform bacteria and pseudomonads in stools from SPF mice superinfected with *E. coli.* As the coliform bacteria tended to outgrow the pseudomonads on these plates, 100 μg/ml neomycin sulfate was added to the medium. This was found to inhibit *E. coli* and allow detection and enumeration of the pseudomonads.

Endotoxin preparations. Endotoxins from the 3 strains of *E. coli* described earlier were prepared by phenol-water extraction procedure similar to that used previously(7). Lyophilized endotoxin preparations were solubilized in pyrogen-free saline (Baxter Laboratories), distributed in sealed glass ampoules and sterilized by exposure to 3 Megarads of electron beam radiation at 2.5 mev from a Van der Graff accelerator prior to transfer to the germfree isolators. In each experiment the same batch of endotoxin, whether irradiated or not, was used simultaneously in germfree and conventional animals. All injections were given intraperitoneally and animals were observed at frequent intervals during the first 96 hours after endotoxin administration. Per cent mortality was calculated by the method of Reed and Muench(8), using groups of 6 to 10 mice per dose of endotoxin injected. Rigid bacteriological control was undertaken in all cases to detect any bacterial contamination of the isolators during the experimental period.

Results. Repeated experiments with different preparations of endotoxins from 3 strains of *E. coli,* indigenous to the intestinal tract of the conventional laboratory mouse, have shown that germfree mice are significantly more resistant to the lethal effect of these endotoxins than their conventional counterparts. More than 200 germfree mice and a comparable number of conventional animals were utilized in the type of experiment shown in Fig. 1. The rather high dosages of endotoxin necessary to produce an LD_{50} in conventional mice in these experiments are due to the method of sterilization. The irradiation procedure used to sterilize the endotoxins reduces the toxicity of the preparations considerably. The LD_{50} for conventional NIH mice of irradiated endotoxin was found to be 830 μg while the LD_{50} for the same animal of nonirradiated material was < 250 μg. In later experiments, the isolation of more potent endotoxins enabled us to obtain an LD_{50}

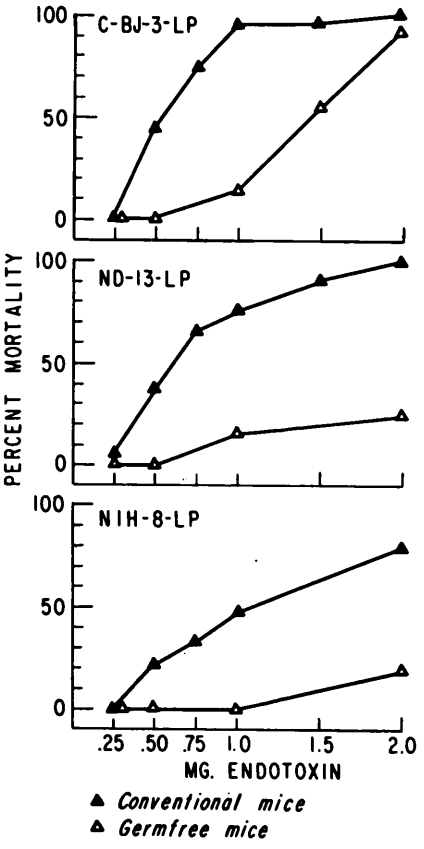

FIG. 1. Susceptibility of conventional and germfree NIH mice to lethal effects of endotoxin (LP) isolated from 3 strains of *E. coli*.

in conventional mice with as little as 125 μg of irradiated material.

The greater resistance of germfree mice to challenge with endotoxin was not due to any alteration of the lipopolysaccharide by irradiation. A few experiments done with nonirradiated material in parallel with irradiated endotoxin gave basically the same results although, as expected, the nonirradiated material had the greater toxicity.

Whereas no difference was observed in the reaction of germfree mice of the Lobund strain and of the NIH strain to challenge with endotoxin, conventional NIH mice were generally more susceptible to the lethal effect of endotoxin than the conventional Lobund mice (Table I).

A survey of the intestinal flora of these 2 strains of mice showed that the NIH mouse harbored 10–100 times more coliform bacteria in their intestinal contents than the Lobund mouse. Streptococci and lactobacilli were also much more numerous in fecal samples from NIH mice, whereas micrococci seemed to be somewhat more prevalent in stools from the Lobund mice. An explanation for the difference in the intestinal flora in the 2 strains of conventional mice might be found in the different environmental conditions to which the 2 groups of mice were subjected.

The observation of differences in the intestinal flora paralleling differences in the reactions of the 2 strains of mice to endotoxin challenge led to the following experiments pertaining to the influence of the intestinal flora on susceptibility and resistance to endotoxin.

Germfree NIH mice were monoinfected with a viable strain of coliform bacteria while kept in the isolator. During a 3 week period fresh fecal samples were cultured periodically and consistently showed very high numbers of coliform organisms in the stools (10^8–10^9 per gram feces). Other germfree mice of the same litters were transferred from the isolators to the conventional mouse room and caged with conventional mice of the same strain, age, and weight. Stool cultures showed that the ex-germfree mice under these conditions acquired a bacterial flora similar to the conventional mouse.

Isolator bred SPF mice known to harbor an intestinal flora consisting only of lactobacilli, enterococci, and pseudomonads were divided in groups of which one was kept in an isolator, another superinfected in a second isolator with a strain of coliform bacilli, and a third caged in the general animal room together with conventional NIH mice. Super-

TABLE I. Lethal Effect of Endotoxin* in NIH and Lobund Mice.

Mice	Mortality data No. dead/No. injected μg 250	500	1000	2000
Lobund germfree		0/12	0/11	5/12
NIH "	0/ 5	0/ 6	0/ 6	2/ 6
Lobund conventional	0/11	1/11	4/16	10/11
NIH "	2/17	12/25	10/16	10/10

* *E. coli* endotoxin (ND-13) irradiated.

TABLE II. Lethal Effect of Endotoxin in Germfree, Monoinfected, Conventionalized and Conventional NIH-Mice.

E. coli endotoxin (NIH-8) irradiated (μg)	NIH-germfree		NIH-monoinfected (*E. coli*, NIH-8)		NIH-conventionalized		NIH-conventional	
	Dead/Total	% mortality	Dead/Total	% mortality	Dead/Total	% mortality	Dead/Total	% mortality
500	5/13	38	4/14	28	14/15	93	12/12	100
250	3/14	21	0/13	0	8/11	73	9/11	82

TABLE III. Lethal Effect of Endotoxin in SPF Mice.

E. coli endotoxin (NIH-8) irradiated (μg)	SPF-isolated		SPF-monoinfected (*E. coli*, NIH-8)		SPF-conventionalized		NIH-conventional	
	Dead/Total	% mortality	Dead/Total	% mortality	Dead/Total	% mortality	Dead/Total	% mortality
500	8/14	57	3/15	20	16/16	100	15/15	100
250	0/14	0	1/15	6	13/16	81	14/15	93

infection with coliform bacilli did not alter the relative numbers of the original SPF intestinal flora although coliforms were present in the stools in numbers of more than 10^8 per gram feces.

After 3 weeks all animals were challenged with *E. coli* endotoxin and the results are shown in Tables II and III. The experiments have been repeated utilizing another strain of *E. coli* for monoinfection and have given the same results.

It is evident from these results that the SPF mouse is just as resistant to challenge with endotoxin as the germfree mouse. Monoinfection with coliform bacteria and the establishment of a coliform flora in the intestinal tract over a period of 3 weeks did not alter the resistance of either germfree mice or SPF mice. If anything, the monoinfected mice were even more resistant to challenge with endotoxin than the noninfected counterpart. On the other hand, transfer of SPF mice or germfree NIH mice to a conventional environment and the caging together with conventional animals was sufficient to alter the reactivity to endotoxin and make the mice just as susceptible to the lethal effect as the conventional mouse.

Discussion. The observation originally made by Dubos and Schaedler(2) that the intestinal bacterial flora influences the response of mice to challenge with bacterial endotoxins seems to have been substantiated by the experiments reported here. Germfree mice, as well as SPF mice, which, although of another genetic strain, harbor the same bacterial flora as the NCS mouse utilized by Schaedler and Dubos, are more resistant to the lethal effect of endotoxin than conventional mice.

On the other hand, Landy *et al.*(4) have found that germfree mice are just as susceptible to endotoxins from *Salmonella enteritidis* and *Shigella flexneri* as are conventional mice. The different results reported by Landy *et al.* as compared to those reported here might be due to the particular bacterial endotoxins utilized. In the experiments reported here only endotoxins derived from *E. coli* strains indigenous to the intestinal tract of conventional mice have been used, whereas Landy *et al.* used endotoxins from organisms not indigenous to the mouse. The different results obtained in the two investigations might reflect a different immunological response in the animals challenged.

The mechanism by which conventionalization of germfree mice and SPF mice induces an altered reactivity of the animals to endotoxin is still unclear. It has been suggested that endotoxins liberated from the intestinal pool of gram-negative bacteria are resorbed through the alimentary tract and contribute materially to the reaction evoked by parenterally administered endotoxin(9). However, monoinfection with coliform bacteria alone does not induce susceptibility in ex-germfree mice to challenge with endotoxin derived from homologous or heterologous strains of *E. coli*, although the period of contamination

is of the same duration as the period used for conventionalization of ex-germfree mice. The number of coliform bacteria in the stools of monoinfected mice is of the same order or even higher than in conventional mice or conventionalized ex-germfree mice. The same has been found to be true when *E. coli* is superimposed on the intestinal flora of SPF mice kept in isolators.

Other factors obviously play a role. One might suggest that other microbial products could affect the resorption of bacterial lipopolysaccharides from the intestinal tract and thus indirectly contribute to the induction of susceptibility primarily inflicted by the lipopolysaccharides.

Summary. Germfree mice of the NIH and Lobund strains, as well as specific pathogen-free mice, are significantly more resistant to the lethal effect of endotoxin, derived from *E. coli* strains indigenous to the mouse, than conventional mice of the same genetic stock. Conventional NIH mice were generally more susceptible to *E. coli* endotoxin than conventional Lobund mice. The NIH conventional mice harbored 10-100 times more coliform bacteria in their intestinal contents than the Lobund conventional mice. Differences in the gram-positive flora were also noted. Whereas conventionalization of germfree mice or SPF mice enhanced their susceptibility to endotoxin, contamination of germfree mice or SPF mice with single strains of *E. coli* failed to increase susceptibility to endotoxin.

The authors would like to express thanks to Mr. Robert W. Swain, Radiation Branch, Nat. Cancer Inst., for irradiating the endotoxin preparation utilized in this investigation and to Dr. W. H. Ewing for typing of the mouse strains of *E. coli*.

1. Dubos, R. J., Schaedler, R. W., *J. Exp. Med.*, 1960, v111, 407.

2. Schaedler, R. W., Dubos, R. J., *ibid.*, 1961, v113, 559.

3. ———, *ibid.*, 1962, v115, 1149.

4. Landy, M., Whitby, J. L., Michael, J. G., Woods, M. W., Newton, W. L., PROC. SOC. EXP. BIOL. AND MED., 1962, v109, 352.

5. Trexler, P. C., *Ann. N. Y. Acad. Sci.*, 1959, v78, 29.

6. Rogosa, M., Mitchell, J. A., Wiseman, R. F., *J. Bact.*, 1951, v62, 132.

7. Mergenhagen, S. E., Jensen, S. B., PROC. SOC. EXP. BIOL. AND MED., 1962, v110, 139.

8. Reed. L. J., Muench, H., *Am. J. Hyg.*, 1938, v27, 493.

9. Ravin, H. A., Fine, J., *Fed. Proc.*, 1962, v21, 65.

Received April 17, 1963. P.S.E.B.M., 1963, v113.

A Micromethod for Determination of Acidic Substances of Biological Significance. (28470)

HARBHAJAN S. SODHI AND PETER D. S. WOOD (Introduced by Laurance W. Kinsell)
Institute for Metabolic Research, Highland-Alameda County Hospital, Oakland, Calif.

Currently available methods for determination of minor acidic components of plasma and other biologic material, including free fatty acids (FFA) and bile acids (conjugated or free) may not be sufficiently specific and sensitive at the micro level. We wish to report preliminary studies utilizing (a) the quantitative methylating properties of diazomethane, (b) the excellent fractionation capabilities of thin-layer chromatography and (c) the extreme sensitivity of radioactivity determinations following introduction of C^{14}-methyl groups of high specific activity.

Diazomethane is known to react with compounds containing a variety of structures, but the reaction with carboxylic acids to form methyl esters occurs more readily and under milder conditions than with most other groups(1). Thin-layer chromatography on silicic acid offers excellent prospects of isolating the methyl esters formed. When such esters are radioactive, they may be eluted from silicic acid and counted. Provided methylation is complete, the amount of radioactivity will be directly proportional to the amount of acid originally present. Absolute