Enhanced Sensitivity of Germ-Free Mice to the Botulinal Neurotoxin (36335)

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It has been observed that adult germ-free mice differ from conventional mice in their susceptibility to the toxic effects of a number of dissimilar drugs acting on the nervous system (1). A hint that this phenomenon includes botulinal toxin has been confirmed and is reported in this paper. An advantage for study of botulinal toxin is the fact its primary physiological action is restricted strictly to the nervous system. Experience with this toxin indicates a need to consider the nervous system among those organ systems of the body whose development may be influenced by contact with microbes during the course of normal existence.

Materials and Methods. The study was conducted by ip injection of crystalline type-A botulinal toxin kindly made available by Dr. Edward A. Schantz. The toxin was diluted 20 times with Difco nutrient broth. Sterilization was achieved by filtration of the solution through a millipore filter. One ml aliquots were frozen in separate vials and stored at −20°. When toxin was desired for use, 9 ml of sterile nutrient broth was added to one ml of frozen toxin solution, and further dilution as required was made by conventional procedure in nutrient broth.

Unsexed, 5-week-old germ-free and parent strain conventional mice of 18–21 g weight, raised by Charles River Breeding Laboratories, North Wilmington, MA, were used. In addition, mice from Fort Detrick colony, both conventional and specific pathogen-free were studied. Germ-free and specific pathogen-free and their counterpart conventional mice were all F_3 generation from a common parent.

The comparison of germ-free with conventional mice was conducted at the Charles River Breeding Laboratories employing their germ-free facilities and equipment. The advantage of this procedure is that it eliminates variables attendant on transportation of animals between laboratories.

Data were collected for graded and quantal responses. For the graded analysis, time of death was the response measure. These test animals were under constant observation for death 28 hr after challenge with toxin. For the quantal analysis animals were observed for death each 4 hr for 120 hr postinjection. In the quantal analysis the proportion of animal deaths is the response variable and probit of percentage of fatalities is the value used in the statistical analysis. For both types, a linear model was used; $y = a + bx$, where y was the response variable and x was the logarithm of the dose. The least squares method was used to estimate the parameters for the graded responses and the probit method was used for the quantal responses.

Results and Discussion. Data and statistical comparison of the time it takes for toxin to kill germ-free relative to conventional mice is recorded in Table I. Since by the conventional statistical test the slopes of the time–dose regression lines, 0.2146 and 0.1961, for the nongerm-free and germ-free mice, respectively, were alike the data were also analyzed using a parallel slopes technique. Parallelism implies equal rates of response to the treatment variable, and in this procedure the horizontal distance between lines is a quantitative measure of relative effect such as potency, and the relative effect is constant over the dose level. On this basis germ-free mice are estimated to be 2.3 times more sensitive than the conventional mice. The greater rapidity with which germ-free mice succumb to the toxin includes doses both greater and less

TABLE I. Time to Death in Minutes of Charles River Mice Exposed to Graded Doses of Toxin.[a]

Toxin dilution	Germ-free						Mean time to death	Conventional						Mean time to death
1:200	75	75	80	80	85	90	81	75	77	78	79	86	[b]	87
1:2000	95	97	105	111	130	135	112	73	103	108	138	168	188	128
1:20,000	120	125	170	175	180	[b]	152	123	157	182	197	227	287	196
$1:2 \times 10^5$	270	275	300	305	315	[b]	293	275	280	285	295	310	375	306
$1:2 \times 10^6$	425	495	505	505	565	575	434	443	523	583	650	650	[b]	580
$1:2 \times 10^7$	410	505	525	560	560	590	607	680	705	725	820	850	925	920
	590	590	625	625	730	935		940	940	1000	1050	1160	1250	
$1:2 \times 10^8$	600	730	780	830	860	920		1415	1460	1490	1490	1620	1620	
	950	1070	1070	1130	1130	1150	1160	1750	1750	1805	1805	1805	1950	1992
	1225	1225	1250	1300	1340	1485		2265	2365	2410	2490	2550	2820	
	1585	1705						2890						
Dose–response regression														
Intercept							1.6730							1.6685
Slope							0.1961							0.2146
Dose–response regression by parallel slope analysis														
Intercept							1.6432							1.7168
Slope							0.2021							0.2021

[a] Values of relative susceptibility:
Logarithm of relative susceptibility (M): .3642
As a multiplicative statistic ($\hat{R}$): 2.31; lower 95% confidence limit 1.51, upper confidence limit 3.55

[b] No test animal.

TABLE II. Comparative LD_{50} Assays in Germ-free and Conventional Mice.

Toxin dilution	Germ-free mice: No. of deaths/ no. injected	Germ-free mice: Time to death, hr. av.	Germ-free mice: Time to death, hr. median	Conventional mice: No. of deaths/ no. injected	Conventional mice: Time to death, hr. av.	Conventional mice: Time to death, hr. median
7.7×10^6	23/24	22.2	21	20/24	37.4	38
1×10^7	17/24	20.8	21.5	8/24	36.1	35.5
1.25×10^7	6/24	33.5	33	2/24	51.5	
2.8×10^7	3/24	47		2/24	59.5	
3.1×10^7	0/20[a]			0/20[a]		
5×10^7	0/12[a]			0/12[a]		
Totals	49/128			32/128		
LD_{50} statistics calculated by parallel slopes:						
LD_{50}	1.21×10^7			8.58×10^6		
95% confidence limits	8.59×10^6–1.69×10^7			5.96×10^6–1.23×10^7		
Relative susceptibility (germ-free to conventional):				1.4		
95% confidence limits	1.125–1.75					

[a] The fact that these animals do not die is not only indicative of dilution of toxin to non-lethal levels but also an indication of the fact that the injection procedure and handling of the animals is a nontraumatic experience. In this respect these animals are sham-controls and the germ-free and conventional mice do not differ.

than the minimal lethal dose (Tables I and II).

A comparative study of the quantal response of the Charles River mice is recorded in Table II. Using the probit approach in which the probit response is related to the logarithm of the dose, a linear function was found to be satisfactory for both germ-free and conventional mice. The slopes were essentially identical. When the data were reanalyzed under the restriction of parallel slopes the germ-free mice prove to be 1.4 times more susceptible than the conventional mice (Table II).

A comparison in two trials was run between conventional and specific pathogen-free mice from the Ft. Detrick colony (Table III). Since the difference between trials was not larger than the variation between mice at a given dose, the data of the two trials were combined to calculate a mean-dose response regression. By this analysis, though the slopes for the regressions are statistically alike, the specific pathogen-free mice are significantly but not more than twice as sensitive. Since the Ft. Detrick mice have no immediate genetic relation to the germ-free mice studied, it is not legitimate to compare them. Yet the greater susceptibility of the specific pathogen-free mouse relative to the conventional mouse may rest on a lack of contact with a variety of microbial induced stresses in extension of the germ-free situation which is a lack of experience with any microbial contact at all. It would appear that sensitivity to botulinal toxin is influenced by contact with microbes and that this is itself a function of the opportunity for contact with a variety of microbial species.

It is of interest to record the fact that the Ft. Detrick mouse colony has remarkable constancy as regards susceptibility to botulinum toxin since the slope of response for tests conducted in 1957–58 (2) and in the present work are alike, 0.22 and 0.21, respectively.

The problem for future investigations is to determine if the differences in susceptibility between germ-free and conventional mice rests on an intrinsic difference in the nervous system. An alternative possibility is that detoxification mechanisms are dissimilar in kind

TABLE III. Comparison of Conventional and Specific Pathogen-free Mice from the Fort Detrick Colony.

Toxin dilution		Nov. 6, trial		Nov. 10, trial	
		Conventional	Pathogen-free	Conventional	Pathogen-free
1:20	No. deaths/no. injected	4/4	5/5	7/7	7/7
	Median death time (min)	64	57	52	50
	Mean death time (min)	66	59	56	50
1:200	No. deaths/no. injected	5/5	6/6	8/8	7/7
	Median death time (min)	84	95	71	69
	Mean death time (min)	89	98	66	68
1:2000	No. deaths/no. injected	5/5	6/6	8/8	8/8
	Median death time (min)	148	143	137	106
	Mean death time (min)	151	141	147	107
1:20,000	No. deaths/no. injected	6/6	6/6	8/8	7/7
	Median death time (min)	232	227	255	175
	Mean death time (min)	263	239	227	177

or rate. It should be pointed out that botulinal toxin does not disappear rapidly from the blood stream (3). The death times reported are within the time limits observed for plateauing of toxin concentrations in blood of injected animals. This argues against the difference being due to variation in detoxification rate.

Summary. Germ-free and conventional mice were tested for their response to crystalline botulinal type-A toxin. Data were analyzed on the basis of graded and quantal response. Slopes of responses were linear throughout the entire response range, and that of the germ-free animals was slightly smaller than for conventional animals. This observation was statistically significant when all tests were combined. Based on time-to-death analysis, the germ-free animals were 2.3 times more susceptible than conventional animals. An increased susceptibility of 1.4 times was observed for the quantal response. Specific pathogen-free mice by time of death test appear more susceptible to the toxin than conventional mice.

In conducting the research reported herein, the investigators adhered to "Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources," NAS-NRC.

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