and indirect Coombs' reactions were also encountered.

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Response of Germ-Free Animals to Experimental Virus Monocontamination. I. Observation on Coxsackie B Virus.* (28105)

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The demonstration of the pathogenicity of Coxsackie viruses for newborn suckling mice, by Dalldorf and Sickles(1), suggested that an animal with an immature defense system and poorly defined intestinal flora might be advantageous for the study of virus-host relationships. The germ-free animal provides the advantages of a relatively immature defensive mechanism, a sterile environment, and the absence of microbial flora in the intestinal tract.

Our purpose here is to summarize the results of experiments with germ-free, suckling mice challenged with Coxsackie B_1 virus, and the modification of the host's response to virus in relation to concomitant monocontamination with a species of bacteria. Comparative histopathologic changes in the animals are described elsewhere(2).

Materials and methods. Lobund (N.D. #1) Webster Swiss mice maintained in sterile isolators for 10 to 16 generations were available for the studies. Animals in plastic isolators, as designed by Trexler(3), were maintained on germicide-free San-i-cel bedding (Paxton Processing Company, Inc., Paxton, Ill.) and 50-10-C ration obtained from the Ralston Purina Co., St. Louis, Mo. Environmental temperature was stabilized at 25.5°C, and a light cycle of 12 hours "on" and 12 hours "off" was followed. The microbiologic procedures used in detection of bacteria, fungi, and protozoa were performed in accordance with the plan described by Wagner(4). Twenty-four-hour suckling animals were inoculated intraperitoneally with a 0.05-ml dose of a $10^{-8.8}$, $10^{-7.8}$, or $10^{-6.8}$ dilution of Coxsackie B_1 virus. Dilutions of virus correspond, respectively, to 0.1, 1, and 10 LD_{50}

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doses, as determined by means of titration in conventional Webster mice, on basis of the method described by Reed and Muench(5). The interval of time until death of each of the animals was recorded, and other animals were sacrificed in groups of 4, at intervals of 3, 7, 10, and 14 days after inoculation. Specimens of mice were ground in a mortar with alundum, using 1.25 ml of Eagle's BSS per brain and 2.5 ml of BSS per carcass (with skin, forefeet, and skull removed). All of the specimens were centrifuged at 12,000 rpm for 30 minutes at 4°C, then assayed for virus in tissue culture, using 1 ml of supernatant fluid from the suspensions of tissue and organs. Tissue cultures used were of human (Henle's intestine) and simian origin (primary monkey kidney). The titer of virus was regarded as acceptable only after the cytopathic effects in tissue culture were negated by the use of type-specific antisera. Histopathologic examinations were performed on formalin-preserved animals.

The initial monocontamination of the germ-free animals with *Staphylococcus aureus* (*albus* variant) was inadvertent. Subsequently, monocontamination with the bacteria was perpetuated in order to acquire data for confirmation of the earlier observations. This strain of *Staphylococcus* was penicillin-sensitive, coagulase-negative and mannite-negative, not typable with bacteriophage.

The virus used was Coxsackie B_1 (Connecticut 5-Prototype), maintained in the laboratory by means of serial passage in mice and tissue culture. The $TICD_{50}$ titer in PMK was $10^{6.5}$/ml. Death of the suckling mice was interpreted as being caused by Coxsackie virus on the basis of 3 criteria: (1) survival for 72 hours after inoculation; (2) isolation of the same virus in monkey kidney tissue culture cells, inoculated with suspensions of minced tissues from the infected suckling mice; (3) development of histopathologic changes that are consistent with those known to occur in lesions caused by Coxsackie virus.

Results. Table I represents a summary of 3 experiments based on inoculation of a minimum of 16 suckling mice for each dilution of

TABLE I. Mortality of Mice Experimentally Inoculated with Virus of the Coxsackie B Group.*

Dose of virus (dilution)	Germ-free				Conventional				Germ-free monocontaminated with *Staphylococcus*			
	Group 1	Group 2	Group 3	Total	Group 1	Group 2	Group 3	Total	Group 1	Group 2	Group 3	Total
$10^{8.8}$	4/16	3/16	3/16	10/48	0/16	0/16	0/16	0/48	0/16	0/16	0/16	0/48
$10^{7.8}$	3/16	4/16	3/16	10/48	0/16	2/16	1/16	3/48	0/16	0/16	0/16	0/48
$10^{6.8}$	4/16	3/11	†	7/27	5/16	6/16	4/16	15/48	0/16	0/16	0/16	0/48
Total				27/123				18/144				0/144

* In the various columns, numerator indicates No. of deaths during experimental period of 14 days, and denominator indicates total No. of mice in the respective groups.

† No experimental animals were available for valid results due to maternal cannibalism.

TABLE II. Presence of Virus in Brain and in Total Remaining Portions of Bodies of Suckling Mice Inoculated with 0.05 ml of $10^{-6.8}$ Dilution of Coxsackie B Virus.*

	Length of time after inoculation with virus							
	3 days		7 days		10 days		14 days	
Type of animal	Brain	Body	Brain	Body	Brain	Body	Brain	Body
Germ-free	++++	+++	+	+++	++	+++	++	++
Conventional	0	++	+++	+++	0	0	0	0
Germ-free monocontaminated with *Staphylococcus*	0	0	0	0	0	0	0	0

* In the various columns, zero indicates that virus was not recovered in tissue cultures. Plus signs represent the greatest dilution of suspensions of the respective tissues that yielded proliferating virus in tissue cultures, as follows: + = virus isolated only from undiluted suspension; ++ = positive results with 1:10 dilution and with undiluted; +++ = positive results with 1:100 dilution and with greater concentrations; ++++ = positive results with suspension diluted as much as 1:1000.

Experiments indicated for the 3 groups of mice (as listed in left column) were repeated with inoculums of 0.05 ml of $10^{-7.8}$ and with 0.05 ml of $10^{-8.8}$ dilution of virus; results were similar to those summarized above, and were comparable and consistent with the varied conditions.

virus under variable environmental conditions. The obvious exception in the germ-free mice with a virus dilution of $10^{-6.8}$ was the result of maternal cannibalism. The mortality in the germ-free, suckling mice after inoculation with Coxsackie B virus was uniform and independent of the dose of virus in the range of 0.1 to 10 LD_{50}. The mortality in the conventional, suckling mice was related to the dose of virus, no deaths with 0.1 LD_{50} and 15 of 48 with 10 LD_{50}. A conspicuous lack of mortality was observed in the germ-free, suckling mice in the presence of bacterial monocontamination with *Staphylococcus* organisms.

Deaths of suckling mice in the germ-free and also the conventional groups occurred within a post-inoculation interval of 7 days. No deaths or illness, *e.g.*, spasticity, were observed after 7 days in either the germ-free or conventional groups of mice, although there was unquestionable persistence of demonstrable Coxsackie virus in the suspensions of brain and of the other tissues (remainder of the body, except for skin, forefeet, and skull) from animals sacrificed at 10 and 14 days after inoculation with virus (Table II). No illness or virus was detected in the mice associated with the staphylococci (Table II) during the experimental observation period of 14 days. Unfortunately, the role of antibody in the various groups of mice was not evaluated, inasmuch as attempts to collect specimens of serum were not successful.

A test of significance in the proportions between the germ-free group and the conventional group of mice indicated that a deviation of this magnitude would have occurred less than 2 times, and slightly more than once, in a hundred on a chance basis ($p = 0.02$). Thus, the probability of chance being a valid explanation for the difference is slight, and the difference in proportions is interpreted as a function of the varying experimental conditions in the 2 groups. The results in germ-free mice associated with *Staphylococcus* bacteria deviated most from those in the other groups. Statistical analysis indicated that the proportion could have increased from 0/144 to 5/144, with difference from the conventional group of mice still having a probability of chance occurrence amounting to less than 0.01. The differences in proportions observed in any of the 3 possible pairs of groups of mice, however, are statistically significant.

Discussion. Protection of a host against infection is the result of many factors. Resistance may be enhanced by mechanisms that are independent of specific acquired immunity. Recent experiments with germ-free animals(6,7) demonstrated that the previous experience of a host with a pathogen can greatly affect the response to the experimental introduction of a variety of organisms. Florman *et al.*(8,9) demonstrated the significant role of an extract (devoid of endotoxin), from the supernatant fluid of a culture of an epidemic strain of *Staphylococcus,* in the rapid enhancement of resistance in mice and rab-

bits to 2 strains of bacteria (*Escherichia coli* and *Staphylococcus*) and a virus (*Herpes simplex*). Protection was demonstrated in the mice at 3 days of age, after a pretreatment interval of 20 hours. The most reasonable explanation of the induced resistance is a stimulation of an intrinsic, general mechanism of natural resistance. Stimulation of the reticuloendothelial system seems to be an unlikely explanation of the observations in our study, owing to the fact that cecal reduction and typical, relatively clear-centered, germinal foci in the lymphoid tissue were not observed during histopathologic studies of mice associated with staphylococci. Furthermore, no gross or microscopic evidence of staphylococcal infection was observed in any of the mice.

Significant observations and evaluation of various features pertaining to the "host-parasite" association of a single strain of specific microorganisms (*i.e.*, a strain of *Staphylococcus*) in conventional mice was not feasible; that is, such a study was not practicable in relation to the experimental design and ultimate objectives in the total research project. The specific effect of a single strain of microorganisms in the relatively complex flora of the intestines of conventional mice, in relation to modifying their susceptibility to a certain virus, would probably be unrecognizable and virtually impossible to evaluate. On the other hand, preliminary observations on animals treated with a cell-free extract of a culture of the *Staphylococcus* strain isolated in this study reveal *an increased degree of resistance in conventional suckling mice* on inoculation with Coxsackie B virus.

The role of antibody and the development of hypersensitivity in the recovery of germ-free mice that are experimentally infected with Coxsackie virus deserve continued study.

Summary. An inadvertent monocontamination of germ-free mice with a so-called "non-pathogenic" strain of an *albus* variant of *Staphylococcus aureus* was observed to enhance the systemic resistance of suckling mice to lethal infections with Coxsackie B virus in doses ranging from 0.1 to 10 LD_{50}. The response of germ-free suckling mice to intraperitoneal injections of virus was uniform, with mortality seeming to be independent of the dose of virus. Mortality was not observed after a post-inoculation interval of 7 days, although there was significant persistence of virus in the brain and other organs of the germ-free mice.

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