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Germfree Animals and Biological Research

The gnotobiote is an improved investigative tool for biology.

Morris Pollard

During the past 35 years a methodology has been developed whereby animals can be maintained and propagated free of a demonstrable microbial flora (*1*). They are referred to as germfree, axenic, or gnotobiotic animals, as distinguished from "conventional" animals.

Originally the laboratories involved in germfree research were few. They were located at l'Institut Pasteur, the University of Notre Dame, and the University of Lund. The first of these laboratories is no longer active, the third has been moved to Stockholm, and a number of new laboratories have been added to the list. The era of "gadgeteering" in developing essential equipment and procedures was so successful that definitive experiments with germfree animals are now being conducted in increasing numbers of laboratories the world over.

The Lobund Laboratory of the University of Notre Dame (*2*) has aided in the establishment of germfree laboratories in France, Holland, Japan, and England and in several areas of the United States. Frequent exchanges of information and of personnel among germfree laboratories have helped to clarify the range of application of germfree methodology as a unique tool for biological research. It is to the credit of such contemporary investigators as Reyniers, Glimstedt, Gustafsson, Trexler, Miyakawa, and their associates that germfree research has now attained "respectability" and acceptance in the circles that advise on the support of research programs. Even greater credit is due the institutions with which these workers are associated and the government agencies that supported programs which were at times vague in purpose or generally unpopular. Valuable support for activities of the Lobund Laboratory came from the Office of Naval Research, the National Institutes of Health, the Department of the Army, the Nutrition Foundation, the National Science Foundation, the Atomic Energy Commission, the Tobacco Research committee, county cancer societies, and the University of Notre Dame.

The early developments in germfree methodology received a good deal of publicity, much of it in semitechnical publications or in popular magazines and journals. Undoubtedly the need for support may have been a stimulating factor in the earlier splashes of publicity. As important as the early developments, however, is the more recent technical breakthrough whereby animals in large numbers can be maintained, propagated, and utilized for experimentation under germfree conditions.

The maintenance, production, and utilization of germfree animals requires rigid attention to procedures which only experienced technicians can carry out (*3*). Once mastered, the procedures become routine. Any investigator who is willing to devote time, effort, and money to this problem can eventually develop a team of competent technicians for this work. Standard procedures have been recorded, established laboratories are willing to train visiting neophytes, and training workshops are held annually at which new developments are disclosed.

The Lobund Laboratory has provided nuclei of breeding stock to technically qualified laboratories newly engaged in such work. Germfree animals have been flown from the University of Notre Dame to laboratories in Paris, Amsterdam, Nagoya, and London and to a number of laboratories in the United States. The animals were delivered germfree in portable isolators, and they constituted the breeding nucleus of germfree animals, or of pathogen-free derivatives of such animals. If the investigator has no inclination to master the methodology, or if he has only limited need for germfree animals, he can purchase germfree mice and rats in a completely stocked isolator through commercial channels. Animals provided in a stocked isolator should be used for only a limited time, since the chemical quality of the food deteriorates after a certain period.

It should be emphasized that germfree animals must be maintained under controlled (gnotobiotic) conditions throughout the experiment. If they are not, there is no procedural advantage in using them. I make this somewhat obvious statement in response to a suggestion from a prominent microbiologist that the expense of germfree research could be reduced through removal of the animals from the controlled environment at the beginning of an experiment. Orientation in using the techniques of this discipline is clearly needed.

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Technical Details

The sterile units into which animals are delivered by cesarian section and where they are maintained germfree are called isolators (*3, 4*). The Reyniers stainless steel isolator (Fig. 1) (sterilized by steam) is expensive and relatively inflexible, but it is the instrument with which the basic methodology was developed. Many of the early investigators have great confidence in steel equipment. As new materials have become available the Reyniers isolator has been supplemented, and sometimes replaced, by the flexible plastic isolator developed by Trexler (Fig. 2). An isolator of the Trexler type is sterilized by peracetic acid, it permits great flexibility in the design of experiments, and it is inexpensive.

Germfree animals are expensive to produce, and maintenance is costly, in view of the fact that some experiments requiring germfree conditions may last for several months. In one experiment concerned with aging, colonies of rats and mice have been maintained under germfree conditions for over 2½ years (*5*). The following animals have been propagated under germfree conditions at Lobund Laboratory and elsewhere: mice, rats, guinea pigs, rabbits, Japanese quail, and chickens. Turkeys, sheep, dogs, cats, monkeys, pigs, and goats have been introduced into the germfree environment and maintained in it, but not propagated under germfree conditions. The Lobund Laboratory propagates and maintains seven genetic strains of mice, three strains of rats, and one strain each of guinea pig and rabbit. Production figures for 1963 indicate that 6000 mice and 2000 rats were made available for the research programs. Both the Lobund germfree Wistar rat and the Lobund germfree Swiss-Webster mouse have been propagated through 21 successive germfree generations.

The germfree animal is an invaluable tool for studies in which microbial flora might have a modifying influence, beneficial or harmful, on the physiological status of the host. Obviously the indiscriminate use of germfree animals would be expensive, wasteful, and valueless.

Before an animal can be successfully used as an instrument of research, its parameters of physiological "normalcy" should be known. How does the germfree animal compare with its "conventional" counterpart? The germfree rodents have been examined thoroughly for parasites, fungi, bacteria, and mycoplasma. None have been found (*6*). However, these rodents have a distinct anatomic anomaly which is related to the absence of bacterial species: the cecum is thin-walled, enlarged, and filled with fluid; it sometimes occupies as much as half the abdominal cavity, and it can weigh as much as 20 percent of the total body weight (*7*). The reticuloendothelial system is poorly developed though functional; it can be activated by antigenic stimuli (*8*). Individual lymph nodes are underdeveloped and contain predominantly primitive lymphoid follicles in the cortex; there are occasional secondary "reaction" zones, especially in the mesenteric lymph node. The low levels of immune globulins in serum reflect the absence of viable microorganisms (*9*).

The quality of germfree animals is determined by their appearance, growth rate, reproductive capacity, and longevity. In all such respects the germfree animal compares favorably with, or is superior to, its conventional counterpart. In addition, these animals are not subject to intercurrent infection, which can interrupt or alter critical experiments with conventional animals.

Germfree rats, mice, rabbits, chickens, and turkeys may acquire a microbial flora, by exposure to the normal "contaminated" environment, without ensuing fatalities (the germfree guinea pig seldom survives under such circumstances). They are not exceptionally likely to die from the effects of bacterial contamination, and, surprisingly, they show no pathogenic effects on exposure to some pure cultures of pathogenic agents—for example, *Histomonas meleagridis*, in the intestine of turkeys (*10*). A nutritional factor essential to the parasite may be required for the manifestation of pathogenic effects, and this may be lacking in the germfree animal. Elements of the microbial flora synthesize nutritional factors essential to the host or to such pathogenic contaminants as may be in the gastrointestinal tract. Where the nutrients are readily available, as vitamin K or folic acid are, the conventional animal does not show a requirement for such factors, but its germfree counterpart does (*11*).

In an episode in our laboratory, reproductive sterility resulted from heat sterilization of the diet, to which in-

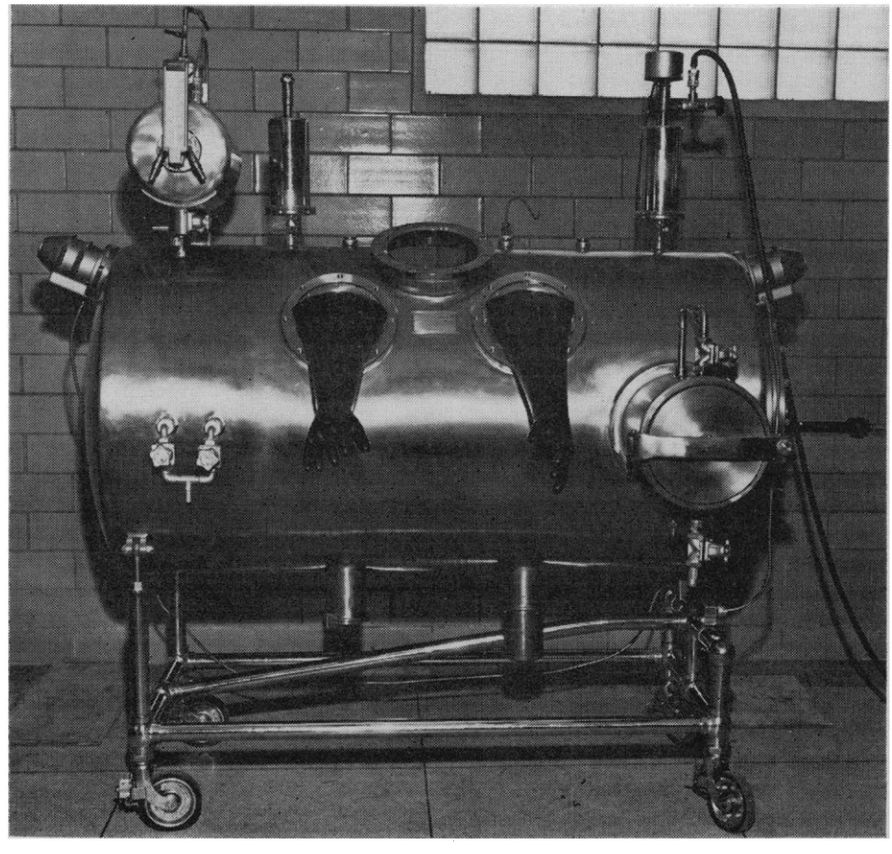

Fig. 1. A Reyniers-type stainless steel isolator.

creasing amounts of thiamine had been added. It was thought, but not conclusively demonstrated, that an antimetabolite, oxythiamine, was being formed as a result of the heat sterilization, and that this interfered with reproduction. This problem was corrected through filter-sterilization, instead of heat-sterilization, of the thiamine added to the diet.

Studies on Germfree Animals

Some of the programs at the Lobund Laboratory in which germfree animals are being used as tools for the study of biological phenomena are as follows.

1) Virology. A question relevant to the germfree status of the animals concerns the existence of a viral flora. The detection of some viruses requires techniques so diversified and subtle that the absence of these viruses is difficult to substantiate. Do germfree animals have a viral flora? Viral agents have been looked for in germfree rats and mice by means of serology, tissue culture, challenge inoculations with standard viruses, histology, and electron microscopy. Thus far no virus has been isolated from germfree animals. While none has as yet been detected by conventional procedures (*12*), we could not disregard the possibility that some viruses are transmitted congenitally (or "vertically") through the ovum or the placentum, as is indeed described in connection with leukemia later in this article.

2) Oncology. Traditional concepts of cancer causation suggest that chemical, physical, and viral agents can act as initiators of the carcinogenic process. Since conventional animals are frequently contaminated with viruses, some of which may be oncogenic, evidence for these concepts has been equivocal. If the ultimate results indicate that germfree rodents are virus-free, then such rodents provide an uncomplicated medium in which to examine the effects of nonviral carcinogenic initiators.

In a search for baseline information, animals in the germfree colonies were thoroughly screened for spontaneous tumors. Tumors were found only in the Wistar rats, not in Fischer and Sprague-Dawley rats, and those that were found involved the mammary gland predominantly (*13*). The rat tumors were localized fibroadenomas and adenocarcinomas; one of the latter showed metastasis to the kidneys. Pulmonary adenomas have been observed, rarely, in aged germfree Swiss-Webster mice. Thus far, no spontaneous tumors have been found in mice of strains ICR, CFW, C3H, and Balb/C, and standard detection methods involving tissue culture and electron microscopy revealed no viruses in the neoplastic tissue cells of these rats and mice.

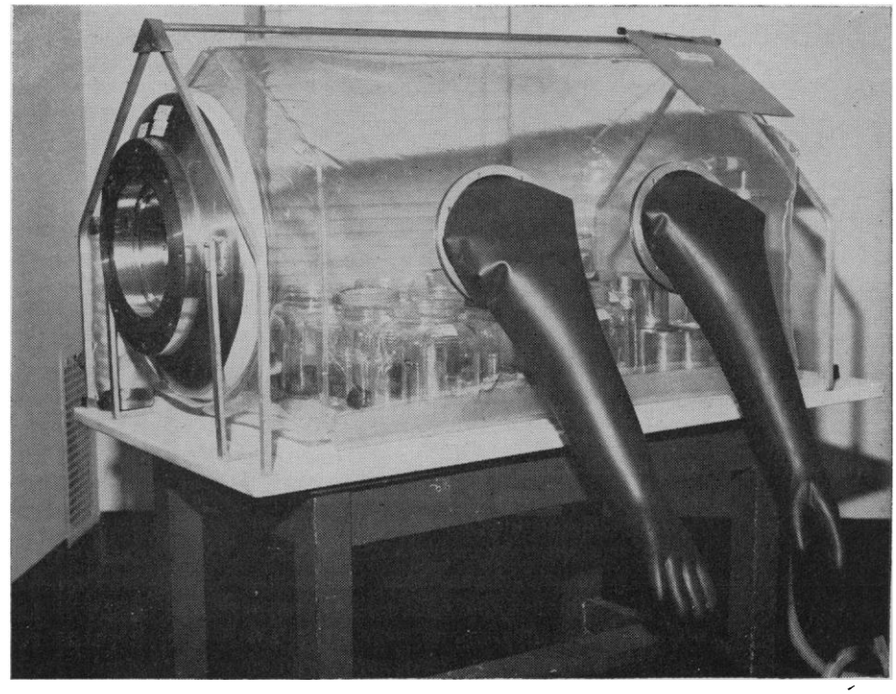

Fig. 2. A Trexler-type plastic isolator.

Germfree rodents developed tumors after inoculation with heat-sterilized carcinogenic chemical agents. Pulmonary adenomas developed only in Swiss-Webster mice which, at birth, had been inoculated subcutaneously with 3-methylcholanthrene in olive oil (*14*); no lesions developed in the lungs of mice of strains C3H and ICR, or in the lungs of germfree rats, inoculated with 3-methylcholanthrene. Inoculation with mouse adenovirus subsequent to inoculation with 3-methylcholanthrene had no effect on the number or nature of the lung tumors that developed. Transplantable fibrosarcomas developed in germfree adult mice of the Swiss-Webster, ICR, CFW, and C3H strains and in rats of the Fischer, Wistar, and Sprague-Dawley strains in the areas inoculated with 3-methylcholanthrene (*15*). Breast tumors were induced in germfree female Sprague-Dawley rats after one feeding of sterile 7, 12-dimethylbenzanthracene (20 mg) in sesame oil (*16*). Tumors seemed to appear earlier in the germfree rats than in their conventional counterparts, but tumors in the two groups were indistinguishable histologically.

Viruses have been detected in tumor cells induced by chemical agents in conventional animals, particularly in tumors transplanted serially through series of animals. The tumor cells acted in a manner analogous to a "vacuum sweeper," picking up agents which occupied the successive contaminated hosts (*17*). Some of the viruses were of unknown oncogenic potential and were thought to be "passengers" in the tumor tissue cells. A fibrosarcoma similarly induced in germfree Swiss-Webster mice through inoculation with 3-methylcholanthrene was transplanted nine times to other germfree mice of the same strain. The tumor cells of each passage were examined by means of various tissue culture procedures; no cytopathology suggestive of viral action was observed. Electron microscopy revealed no virus-like structures, either in the original or in the passaged germfree tumor cells. Thus, findings for germfree and for conventional mice and rats inoculated with sterile chemical carcinogenic agents were similar with respect to genetic susceptibility, tumor type, tumor distribution, and latency periods, and no virus could be detected (*15*).

3) *Can viruses induce tumors in germfree mice?* Newborn germfree C3H mice were inoculated with Gross

leukemia virus, strain A. The mice developed dyspneic symptoms 9 weeks later; however, lesions of leukemia had been detected in asymptomatic mice as early as 3 weeks after inoculation (*18*). At autopsy examination the dyspneic animals had great enlargement of the thymus and lymph glands, and the spleens, livers, and kidneys were swollen and discolored. All of the organs were heavily infiltrated with lymphoid cells, many of them in mitosis.

By examining tissues from infected "germfree" animals at intervals after virus inoculation, evolution of a leukemia lesion could be observed. In the early stages of leukemia, the lymph nodes had reaction (germinal) zones in the cortical areas, structures which were not observed in the uninoculated germfree controls. As the disease evolved, the thymus expanded until it occupied half the chest cavity (and until its weight was 5 percent of body weight). The germinal zones of the lymph nodes enlarged progressively and contained numerous large lymphoid-type cells, many of them in mitosis. As each germinal zone expanded, the peripheral collar of small lymphocytes shrank, and eventually the germinal zones occupied most of the lymph node cortex and extended into the intermediate and medullary regions.

From the results noted, it appears that viral leukemogenesis can occur in the absence of a bacterial flora. The lymph nodes of germfree mice provide a structural baseline in which to study the pathogenesis of leukemia. It appears that the lesion evolves through hyperactivity of the structures associated with immunity: the thymus, then the lymph glands. Does this mean that leukemia actually represents an abnormal immune response of the host?

4) *Radiation leukemia.* The leukemogenic effect of x-irradiation provides a means of determining whether occult leukemia virus is present in certain strains of mice. Groups of 1-month-old germfree and 1-month-old conventional mice of strains Swiss-Webster, C3H, and C57 Bl were subjected to x-irradiation through whole-body exposure; the dosage was 150 roentgens, and each mouse received four doses, separated by 1-week intervals. Four months after the last dose, lymphatic leukemia with enlargement of the thymus developed in the germfree and the conventional mice of the three strains (*18*). The visceral organs were infiltrated with lymphoid cells. Leukemia in mice is generally thought to be a viral disease; and virus-like structures have been detected in the cytoplasm of thymus cells from the leukemic germfree mice. Demonstration of the occurrence of radiogenic leukemia in germfree mice provides the first suggestion that a virus exists in these mice. It gives support to the concept that the occult leukemogenic agent or agents in mice may be disseminated from generation to generation by "vertical" passage. Demonstration of the presence of other viable occult agents in germfree animals may depend on the development of new procedures for "unmasking" them. In this regard, a more appropriate designation for germfree mice which develop radiogenic leukemia would be gnotobiotic mice.

5) *Lymph node reaction.* The lymph nodes of germfree mice and rats are small and contain occasional germinal zones; these zones occur particularly in the mesenteric nodes. The lymph nodes of germfree rats are more uniformly lacking in reaction zones than those of germfree mice. With the low antigen diet developed by Pleasants and Wostmann (*19*), a structural baseline of even greater uniformity becomes available. In response to inoculation with individual viruses such as polyoma virus, mouse adenovirus, "K" virus, and mouse hepatitis virus, reaction zones appear in the primary lymph follicle in the cortex of the lymph node. This is interpreted as an immunological response.

Viruses induce the formation of reaction zones in the lymph nodes of germfree rats and mice. With Gross leukemia virus these changes may play an important role in pathogenesis of the disease. Germfree mice develop fibrosarcomas in the areas previously inoculated with 3-methylcholanthrene in oil; in such tumorous animals the lymph nodes show marked zones of reactivity in the cortical follicles. Germfree Sprague-Dawley female rats with breast tumors induced by feeding the animals 7, 12-dimethylbenzanthracene show no reaction zones in the cortical follicles. The lymph node reaction in the mice may reflect either (i) "unmasking" of a latent viable agent, (ii) a unique antigenic quality in tumors induced by methylcholanthrene, or (iii) a more sensitive system in the mouse than in the rat. The significance of reaction zones in lymph nodes of tumorous animals warrants further investigation.

6) *Current studies.* A number of interesting studies with germfree animals are in progress at the Lobund Laboratory. Germfree mice tolerate whole-body irradiation better than their conventional counterparts, and unique bacterial monocontaminants such as *Clostridium difficile* and agents such as endotoxin induce even greater tolerance (*20*). Surgical procedures have been developed for thymectomy of newborn mice under germfree conditions. The "runting syndrome" of conventional mice thymectomized at birth has not yet been observed in the germfree mice (*21*). Germfree rats developed dental caries when they were fed a high sugar diet and inoculated with, or fed, a single species of bacterium—for example, *Streptococcus faecalis* (*22*). Attempts to immunize the rats against bacteria that cause dental caries are in progress (*23*).

The effects of bacterial species in the intestinal flora on the nutritional status of the host are being determined, with particular relation to vitamin B_{12}, to cholesterol metabolism, and to the enlarged cecum. It has been shown that the action of bacteria in the "normal" intestinal flora accelerates oxidative catabolism of cholesterol; the rate is 50 percent higher in the conventional rat than in the germfree rat (*24*). It is hoped that an organism will be found which will accelerate the breakdown of cholesterol in the intestine of the germfree animal. It was shown that the cholesterol-lowering activity of certain lipid fractions, often ascribed to changes induced in the composition and characteristics of the intestinal flora, occurs in both germfree and conventional rats. With germfree rats it was possible to establish the superiority of vitamin K_1 over vitamin K_3 as a means of preventing or curing the hemorrhagic syndrome resulting from vitamin K deficiency (*25*). It was demonstrated in germfree rats dying of vitamin K deficiency that the oxidative phosphorylation in the mitochondria of the liver was not impaired—a finding which rules out vitamin K as a factor of major importance in this metabolic system. The physiology of antibody response to highly purified antigens is being determined. In seeking a more precise test system, low-molecular, water-soluble, chemically defined diets were developed which could be sterilized by filtration (*19*). Three successive generations of newborn germfree rats derived from germfree mothers

have been maintained on the low-antigen diets. Adult rats on this low-antigen diet have shown a low level of 7*S* gamma globulin. Germfree mice show an age-related immunity to viral infection (*26*), and studies are in progress to determine the nature of this resistance.

In studies on wound healing, germfree animals provide uniform baseline information on enzymological response of tissues to injury (*27*). Possibly, the addition of single species of bacteria to damaged tissue may retard or accelerate the rate of healing. From present trends in germfree research we can anticipate some worthwhile and exciting new applications. Species of laboratory animals (guinea pigs and rabbits) are being propagated in the germfree state, and special strains of germfree rodents (rats with genetical resistance to dental caries, and mice of strain AKR with a high rate of spontaneous leukemia) should provide needed information on important aspects of pathogenesis.

For studying certain problems, utilization of the germfree animal has practical advantages. Since these animals live in a controlled environment, they may be used as monitors of the microflora in an environment of unknown character. They may be used in certain kinds of experimental surgery, especially in tissue transplantation, since germfree animals are not subject to infection when given drugs which reduce antibody production. With this methodology, controlled environments can be provided (i) for surgery under unsanitary conditions (portable operation rooms), (ii) for protection of human burn patients against contamination by the antibiotic-resistant flora in hospitals, and (iii) as portable, inexpensive isolation rooms for patients with contagious diseases.

Prospects

The use of germfree animals may be of help in solving many biological problems. Because of the technical details and the expense involved, such animals will probably not be used extensively in biological research; however, if information is needed on the relation of host to environment, germfree animals should provide some significant answers.

I do not mean to imply that research with germfree animals is a concern only of the Lobund Laboratory. The summary of activities given here covers only a small part of the current research programs with germfree animals. Discussion of many important and exciting programs in other laboratories has been omitted intentionally in order to avoid premature disclosure of confidential information, misinterpretation of results, or inadvertent omission of an important finding.

Further use of germfree animals in research programs will increase the need for such animals. They do provide unique experimental advantages which should be exploited. The results will surely justify the faith and perseverance of the pioneers in germfree research.

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