Chapter Sixteen

Development of Gnotobiotics and Contamination Control in Laboratory Animal Science

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&
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Introduction: Nomenclature

While gnotobiotics and "germfree" rodents were being developed at the University of Notre Dame, a series of staff meetings was held to devise a proper nomenclature (1) since the term germ-free was occasionally confused with pathogen-free, and the only scientific term then available, axenic (2), did not lend itself to broader applications, i.e., germ-free animals associated with one or more organisms. One of us [Trexler] proposed the term gnotobiota from the Greek, *gnotos*, well known, combined with biota, the combined fauna and flora of a region. Thus the meaning of the word can be gleaned from its roots "well known biota." Gnotobiotics was proposed for the science itself, using the Greek suffix –"ics" meaning "pertaining to" as used in the terms mathematics and genetics. The use of the suffix -ology, a branch of learning, to form gnotobiotology was considered a bit long, while gnotobiology was rejected since the roots did not provide a clue as to its meaning. However, T. D. Luckey, who had participated in the meetings, used it for his monograph (3) and this term has also been used widely thereafter.

As noted by a National Academy of Sciences' Committee within the Institute for Laboratory Animal Resources [ILAR], many terms were subsequently used to describe gnotobiotic animals, some of which were quite misleading (4). The committee agreed that a gnotobiotic animal, or gnotobiote, is one in which all life forms are fully defined. If there are no other life forms, the gnotobiote should be referred to as a "germ-free" or "axenic" (no strangers) animal. An axenic animal intentionally colonized or "associated" with other life form(s) should be referred to as a "defined flora and/or fauna" animal. However, even though germ-free animals are also gnotobiotes, the term gnotobiote has been used colloquially when referring to germ-free animals which have been intentionally associated with one or more organisms. The use of such terms as "monocontaminated" or "polycontaminated" when referring to defined flora and/or fauna gnotobiotes was considered inappropriate, since the introduction of any and all additional organisms into these animals must be intentional.

It was also recognized that the presence of murine "leukemogenic" viruses in all "germ-free" mice meant that these mice were not really germ-free at all. The committee stated, "These animals may be considered gnotobiotes, but they can no longer be called 'germ-free.'" This particular recommendation has not been adopted by the biomedical research community. The state of the art today is such that we can only produce "adventitious" virus free mice. In all probability, it will eventually be shown that the axenic state is only hypothetical in all species. Nevertheless, it can be argued that once an organism has incorporated it's genome into the genome of every cell of its host, both somatic and germ, it has then become part of its host's genome and therefore part of the host itself, and no longer a "stranger".

Initial Development of Isolators and Germ-free Animals

by P. C. Trexler

The gnotobiotic principles used in the production of infection-free laboratory animals evolved from the efforts to rear and study animals in the absence of microbes or in association with one or more pure cultures of microbes (5). These efforts were initiated by Louis Pasteur, who speculated as to whether or not higher animals could live in their absence since they had evolved in their presence. In 1895, Nuttall and Thierfelder, (6) published a report on the successful maintenance of caesarian-derived guinea pigs in a bacteria-free environment for a fortnight using an isolator built around a glass bell-jar. Because vitamins had not yet been discovered, it was not possible to prepare an adequate sterile diet so the animals merely survived rather than grew. Kuster

Editor's Note

The authors of this chapter were directly and intimately involved in the events they describe, as were many of the other chapter authors in this publication. They have appropriately chosen to relate this history in the first person, hence the editors would like to briefly describe the background of each author of this chapter.

Philip C. Trexler served as associate director of the Lobund Institute of the University of Notre Dame where the first germ-free rats and mice were developed. He developed the flexible film isolator. In 1960 he conducted a workshop in which he taught the major suppliers of lab animals how to derive their animal stocks and stains into the "germ-free" or axenic state, thereby freeing them of the numerous infectious disease agents which were plaguing biomedical research. This workshop was a seminal event in enabling suppliers to produce animals free of interfering infectious disease agents.

Roger P. Orcutt received his doctorate under Russell W. Schaedler, MD. In the early days of gnotobiotics, Dr. Schaedler provided numerous micofloras of bacteria to multiple laboratory animal suppliers for "normalizing" germ-free mice. Dr. Orcutt developed an "Altered Schaedler Flora" which has become the standard adopted by all major suppliers for their nucleus, isolator-maintained animals. Dr. Orcutt introduced chlorine dioxide as a replacement sterilant for peracetic acid for maintaining isolators and filter topped cages. He has shown the effectiveness of filter-topped cage level contamination control systems for laboratory animals.

(7) avoided this problem by feeding caesarian-derived kids milk obtained from the mothers and carefully heated by steam in the entry port of a sterile glove box. These animals remained free of bacterial contamination for about a month and grew as well as those born normally and suckled.

In 1933, James Arthur Reyniers, then instructor in bacteriology at the University of Notre Dame, reported his efforts to rear guinea pigs in a sterile environment (8). Both apparatus and results were similar to that obtained by Nuttall and Thierfelder, though he was not aware of the earlier work. Reyniers was interested in studying bacterial variation since at that time there was considerable interest in differentiating between bacterial contamination and possible life-cycles or variation. However, the problems involved with rearing the animals in a sterile environment soon took most of our attention. Since we were interested in avoiding all forms of contamination, as well as the toxic effects of germicides, steam under pressure was used for sterilization wherever possible. The isolators were cylindrical, stainless steel pressure vessels with sturdy glass sight ports and arm-length rubber gloves sterilized in place. The neonates were entered directly into the isolator to avoid potential contamination from passage through an open room. A caesarian section was performed by means of a thermal cautery through a plastic membrane, which formed a portion of the isolator wall and adhered to the prepped skin of a gravid dam. In addition, a germicidal dunk-bath was used for passing in gravid uteri or embryonated eggs.

In 1939 a symposium was held at the University of Notre Dame on Micrurgical and Germ-free Methods. The proceedings were published as a hardcover book in 1943, edited by James A. Reyniers (9). Micrurgy (microsurgery), the manipulation and dissection of cells and subcellular particles under the microscope, formed an important part of the proceedings because of the need to make certain bacterial cultures were derived from single cells to avoid contamination and because of our interest in intracellular symbionts. The rest of the meeting really formed the First Symposium on Gnotobiotic Technology. The 11 papers presented included the rearing of germ-free protozoa, nematodes and insects. There was also a paper on growing germ-free plants and plant tissues as well as the microbiological use of rodent fetuses *in utero* for the propagation of viruses. The steam sterilized isolator system, manufactured by Reyniers and Son of Chicago, was described in detail, as well as its use in rearing germfree guinea pigs, chickens, rats, rabbits, and a monkey. It is shown in Figure 1.

During WW II, part of our apparatus and facilities was devoted to war-related research. We had demonstrated a capability of maintaining a sterile workspace for long periods without contamination, even though just a single viable bacterial cell or mold spore was sufficient to contaminate the animal and the environment within the isolator. Our system was also effective for containment. For this reason, I didn't hesitate to spray-dry hazardous toxins and pathogens-which was considered to be too dangerous by the safety officer at Fort Detrick to be undertaken there. The process of spray-drying permits considerable control over the shape and size of the dry particles. A technician and myself carried on this work with little risk since containment isolation was easier to achieve in our apparatus than exclusion. For the latter operation the apparatus had to be leak-tested then subjected to internal steam pressure followed by a vacuum for drying and cooling. When used for containment, the apparatus was subjected to steam

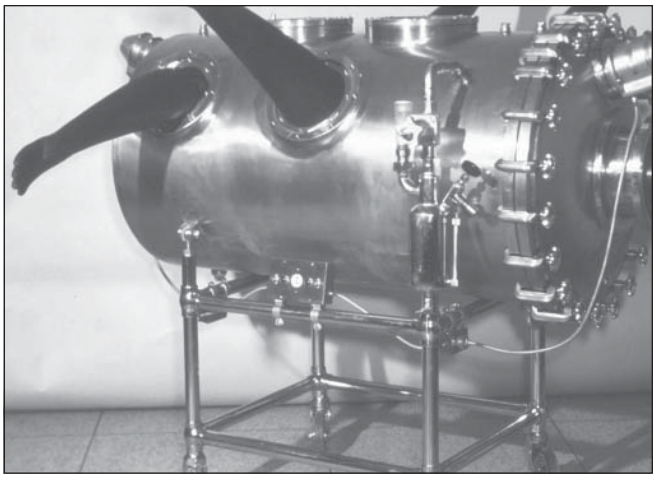

FIG. 1. The steam sterilized isolator system.

or a chemical sterilant only after the operation. A vacuum was not required other than to maintain a pressure slightly less than ambient during operations. The operation proceeded without incident; thereby demonstrating the suitability of our isolators for containment isolation.

In order to expand upon these studies and the technology, the University established an Institute, Lobund (Laboratories of Bacteriology University of Notre Dame) with Reyniers as its director. There were many advantages rearing isolator animals in colonies rather than continual derivation from contaminated stocks. The feasibility of this was shown when, at last, we succeeded in weaning a few rats in a sterile environment and they reproduced. The animals were used initially in a study on the etiology of dental caries (10). Females from this germ-free colony were also used as foster mothers to derive other lines of rodents, thereby circumventing the tedious and time-consuming process of hand feeding around the clock.

The cost of routinely producing animals in the Reyniers Isolator System was far too high. An attempt was made to lower production costs by using a steam sterilized room, actually a steel tank (11,12), 2.4 m in diameter by 4.6 m in length, serviced by attendants in sealed, ventilated protective garments made of polyvinylchloride (PVC). Entry to the room was gained by donning the garment and passing through a germicidal shower and dunk bath. During five years of operation (1951-6) only 286 rats were produced (13). Ten contaminations occurred during this period, only one of which was due to a fault in the protective garment.

The search for a suitable germicide to use for sterilizing PVC lead to the discovery of the sporicidal properties of peracetic acid vapor (PAA) (14). Since PVC is available in a great variety of forms including sheets and films, which can be readily fabricated, the discovery of a practical way for cold sterilization made possible the design of a far less expensive and easier-to-use isolator system. However, since PAA is effective against bacteria, molds and viruses, but not many of the eggs and cysts of parasites, it is best to keep floors wet mopped and free of large dust particles. Reyniers, the director of Lobund, refused to permit the development of such a system for laboratory animals since he had submitted a plan to the University for an expanded Institute using his expensive steam sterilized equipment . As the principle investigator for a classified project supported by Fort Detrick (1953-4), I used the PAA/plastic technology and volunteered to work with the agent that presented the most difficult containment risk, *Pasteurella*

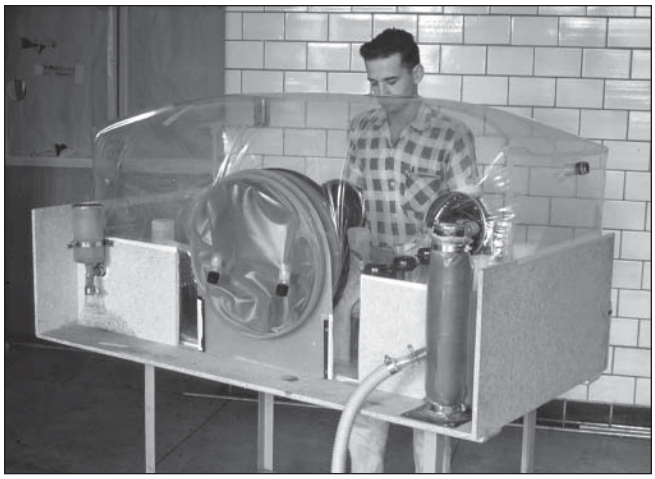

FIG. 2. A flexible film islolator system.

tularensis [subsequently renamed, *Francella tularensis*]. The use of this organism at Fort Detrick was permitted only in buildings in which all personnel were vaccinated. We used it in a building with a traffic of 750 students and staff, none of whom were vaccinated. The Ft. Detrick safety officer stated that he had never seen an installation such as ours, but did not see why it would not work. None of the 750 students and staff became infected. Later on, the group then published the portion of the technology involving isolators (15). At the completion of the project, I was able to continue to develop the flexible film isolator system (13). An early model is depicted in Figure 2.

Reyniers desired that gnotobiotic work only be performed at a few select sites within the country, analogous to that of the atomic research program of the U.S. In 1955, at the insistence of the president of the University, the Rev. Theodore Hesburgh, we assisted in the design and installation of another germ-free animal laboratory at the Walter Reed Army Institute of Research (WRAIR) using the Reyniers Stainless Steel Isolator System. The following year, equipment was installed at the National Institutes of Health. Gustafsson (16) in Sweden developed a different type of steam sterilized isolator, which was lightweight but had to be sterilized inside a large steam autoclave. He also established and maintained a colony of gnotobiotic rats and was engaged in dental caries research.

Reyniers provided John B. Nelson (17) with some gnotobiotic rats that had become accidentally contaminated with a single nonpathogenic bacterium. This made it possible, for the first time, to produce a colony of rats free from chronic respiratory disease, thereby demonstrating another important way in which gnotobiotics might benefit laboratory animal science. We joined the Animal Care Panel in order to become better acquainted with this science. At the 1956 National Meeting of the Animal Care Panel, Reyniers described the expensive steam sterilized isolator system and proposed a way to use it with so called specific-pathogen-free, or "SPF," animal colonies (18). At this meeting, two commercial and an institutional breeder presented their methods of producing "SPF" colonies. In subsequent meetings, I reported progress with the development of the flexible film isolators (19,20). The barrier room concept and "SPF" animals will be dealt with further in this chapter.

In 1959 Reyniers left the University of Notre Dame, whereupon I provided WRAIR and the NIH with breeding stock from our gnotobiotic rat and mouse colonies and organized the Second Symposium on Gnotobiotic Technology (21). The ILAR, NIH, and the Office of Naval Research [ONR] cosponsored the symposium. Nineteen papers were presented-including papers from the four laboratories having gnotobiotic animals, two producers of SFP laboratory animals, and the use of the technique for livestock production. Dale W. Jenkins, chairman of ILAR, discussed laboratory animal standards and announced the establishment of a Committee on Gnotobiotic and SPF Laboratory Animals. Arnold Wedum, M.D, Head of the Safety Division at Fort Detrick, discussed safety while working with hazardous materials including pathogens. The isolators and methods used were then described.

Table 1. Participants in the June 1960 ILAR Workshop, held at the LOBUND Institute at the University of Notre Dame by P.C.Trexler in order to teach lab animal suppliers how to free their stocks of infectious diseases agents by utilizing gnotobiotic techniques.

Blue Spruce Farms
Carworth Farms
Charles River Breeding Laboratories
National Laboratory Animal Company
Cumberland View Farms
Fort Detrick
Manor Farms
Millerton Research
Schmidt Animal Industries
Simonsen Laboratories
Taconic Farms

The following year, as chairman of the ILAR Committee and with the help of the new ILAR director, Bert Hill, a workshop (22) was organized in order to acquaint animal breeders with gnotobiotic technology. The workshop was attended by 10 commercial and one institutional breeder, as illustrated in Table 1. To complete the course, attendees were required to maintain a gnotobiotic colony on their own premises. The following year a meeting was held at which time certificates were awarded to those completing the course (10 of the original 11 attendees) and the Association for Gnotobiotics was organized. The first meeting of the Executive Committee was held that year in the office of Walter Newton at the NIH.

Since the development of the gnotobiotic apparatus and method, as well as its operation, was supported by federal grants, patent applications were made where appropriate. Under the direction of Reyniers, all grant applications for the development of apparatus or methods were submitted to the Department of Defense because they permitted ownership of patents to be retained by the grantee. However, when Reyniers resigned, an acting director was appointed from the University of Notre Dame's administrative staff who was a lawyer. Since we were exploring applications of the technology in human medicine, as well as laboratory animal science, the acting director insisted that I submit an application to the NIH for isolator development. Shortly thereafter we received a letter from the NIH stating that they had put all of the isolator patents obtained with federal funds into the public domain. Six months later the head of the study section reviewing our applications informed me that they considered the research aspects of isolator development to be completed and I now had to depend upon industry for further development. However, much further development was required to provide an efficient and economical operation. Without patent protection, it was impossible to interest a company with sufficient resources to make a substantial contribution to development and marketing. As a result of giving away

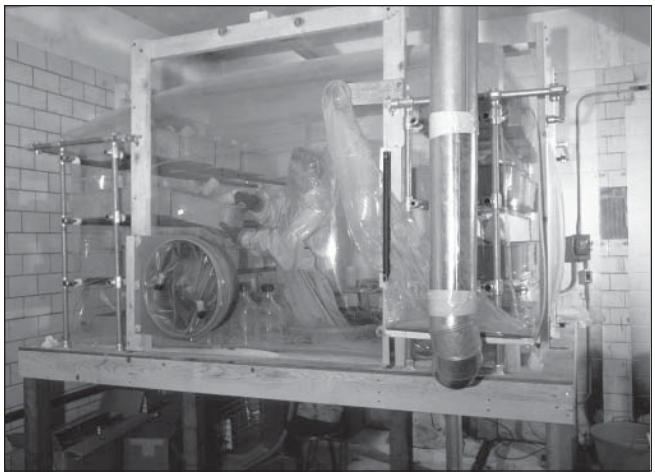

FIG. 3. A half-suit isolator.

the patents, at one time seven companies offered isolators for a market that was so small that none of them could invest much in either development or marketing.

Upon appointment of a new director of the Lobund Institute, Dr. Morris Pollard, the earlier criticism by a review committee of the National Science Foundation (NSF) was satisfied. The NSF had considered the technology to be sound, but indicated it should have been developed at a large center for biological research where it could have been used more effectively. I continued to develop the flexible film technology with the goal of greater economy and a microenvironment in an isolator comparable to that of an animal room. When a dental caries research project was started, control animals having a full microflora did not survive for more than a week in the steel isolators. These isolators were suitable for axenic animals; but lacked adequate ventilation for animals with a normal flora. The airflow was increased in the stainless steel isolators so that these animals could at least survive. The amount of airflow, and its direction, could be readily controlled in the flexible film isolators.

Our development group had, in addition to numerous glove isolators, five half-suit isolators in operation-each capable of containing 80 breeding cages, see Figure 3. One isolator was used for an SPF mouse colony. To improve the quality of the gnotobiotic mice, we undertook a study of the enlarged cecum, which is the most adverse anomaly of the germ-free state, and discovered that either *Clostridium difficile* or two bacteroides species in combination would produce gnotobiotic mice with a cecum within the normal size range (23). At the time *C. difficile* was considered nonpathogenic, so we discarded the bacteroides cultures. A pathologist at WRAIR examined the *C. difficile* monoassociated mice and found them to be similar to axenic mice except for the small cecum and an inflamed pharyngeal lymph gland. This feature attracted little attention, though a colony of these mice was kept for three years at the Ohio State University.

In 1962 the Third Symposium on Gnotobiotic Technology was held at Lobund and cosponsored by ILAR. There were 110 participants at the two-day meeting, the proceedings of which were published in *Laboratory Animal Care* (24). Over 40 laboratories were using flexible film isolators and there were four commercial sources of gnotobiotic laboratory rodents. The forum provided by the Animal Care Panel accounted for the rapid spread of the technology. In addition, there were several papers on gnotobiotic swine and a round-table discussion.

Since it appeared that the developments in gnotobiotic technology had out-stripped the current need for gnotobiotic animals, I left for the Albert Einstein College of Medicine in 1962 in order to continue the development of isolators for use in the human hospital. In 1966, I joined the staff at the Royal Veterinary College in London in order to develop effective isolators for managing the common farm animals (25). The National Research and Development Corporation, an organization of the British government, supported the research required for applications in veterinary and human medicine. This support made it possible to obtain the necessary equipment and train technicians to fabricate, modify, and repair isolators, much as was done at Lobund. There were two main gnotobiotic animal projects: rearing piglets for the production of monospecific antisera against all of the common viral diseases of the pig and the production of gnotobiotic calves and foals for the study of a variety of viral diseases.

After the crew had been trained and the sources of all contaminations were determined and eliminated, 900 large white piglets were used over a three-year period without a single contamination (see Figure 4). Many of the animals were kept for two months. The antisera were used for diagnostic purposes and distributed commercially. Half-suit isolators were required for the calves and foals since the animals frequently weighed 500 lbs. at postmortem. These isolators were inexpensive since they were made on-site, using 12-gauge polyethylene layflat tubing. The half-suits were developed in-house and then made commercially. The internal isolator pressure was controlled to within less than a millimeter of water pressure either above or below ambient by connecting the top of the inflated chamber to a control valve in the exhaust duct. The top of the isolator chamber acts as a control diaphragm providing a considerable force. The design principles, which had been developed in the animal laboratory, were then used for isolator applications in human medicine.

The isolators I was able to design and build while in both the US and England demonstrated that isolators could be inexpensive and secure for either exclusion or containment isolation. Indeed, in a properly managed facility, an accidental contamination is a rare event.

There is little or no competition in the market place for isolators since the quantities made are very small for the plastics fabricating industry. For this reason, manufacturers favor complicated and expensive designs to increase profits. As an example, the simplest and most comfortable device for attaching gloves to sleeves is a grooved support ring with a rubber O ring and adhesive tape as described in '57 (13). It even provided good service when managing 500 lb. animals and these support rings have been used for human surgery with surgical gloves having a rolled cuff. In my view, there is still a void in the marketplace for a simple, inexpensive flexible film isolator with the minimum of equipment necessary for gnotobiotics. The source of a contamination is easier to locate in such equipment, which is essential for its elimination and a reliable operation. With an increase in the numbers and cost of infection-prone animals used, it is likely that the need for isolators and other microbe constraining devices will increase. The principles of microbial contamination control are simple to master so that a large proportion of the equipment required can be designed, fabricated and repaired in house economically, provided there is a convenient source of supplies available.

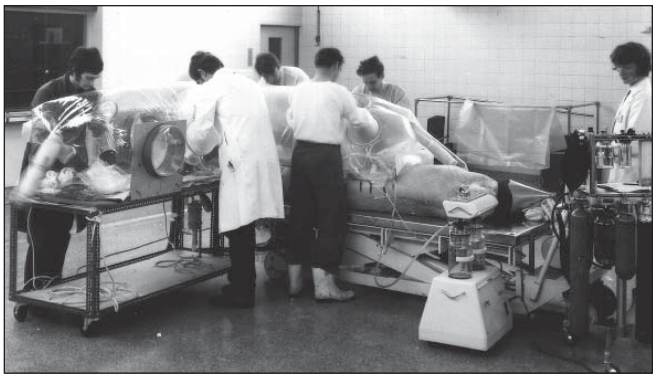

FIG. 4. Isolators built for the Royal Veterinary College in London in the late 1960s resulted in 900 large white piglets being used over a three-year period without a single contamination.

Application of Gnotobiotic Techniques for Contamination Control

by Roger P. Orcutt

After the introduction of gnotobiotics into lab animal science, it was originally thought that suppliers and investigators alike would soon be raising and experimenting with gnotobiotic animals in isolators. However, the harsh reality of the relative costs of maintaining gnotobiotes versus the classic approach of an open room soon made it readily apparent that this was not practical-to say nothing of the added inconvenience of having to manipulate animals and cages inside a plastic bubble. Therefore, the barrier room concept was conceived in which the room is made to be as much like an isolator as possible. Feed, bedding, and cages are steam autoclaved into the room, air is sterilized with HEPA filters, water is decontaminated by acidification, hyperchlorination and/or "filter sterilization," and items that could not be steam autoclaved are sterilized with ethylene oxide. Personnel are required to take a shower while entering and then don sterilized garments including hair nets, face masks, and jump suits. Obviously, however, none of these procedures are sufficient to prevent personnel from introducing bacteria into the "barrier room." The human body is estimated to be comprised of 10 trillion cells (10^{13}), but it is colonized with 10 times as many bacterial cells (10^{14}) representing some 400 different species, some of which are able to colonize, and sometimes infect, rodents (26).

It was soon observed that germ-free rodents were predisposed to opportunistic infections when removed from their isolators and introduced into barrier rooms, whereas gnotobiotes colonized with just a small moiety of their "normal" flora did not undergo these lethal infections. During the mid-to-late 1960s, Dr. Russell W. Schaedler was isolating the bacteria comprising the autochthonous, or "normal," flora of the mouse while conducting host/parasite studies at the Rockefeller University in the laboratory of Dr. Rene Dubois. In order to assist animal suppliers in preventing the loss of gnotobiotes after transfer into barrier facilities, Dr. Schaedler provided various bacteria to multiple suppliers over several years as he isolated them. This resulted in many different microfloras, or so-called "Schaedler cocktails." After receiving my PhD under Dr. Schaedler in 1972, while studying the autochthonous microflora of the mouse, I entered the animal supply industry. In 1978, Dr. Joseph Mayo of the National Cancer Institute (NCI) within the NIH called for the standardization of the associating microflora or "cocktail" among all his contract suppliers, and asked me to undertake this task.

The industry was very receptive to this concept as the contractors felt that the microfloras in use were needlessly difficult to monitor (27). The various Schaedler cocktails had aerobic organisms, such as an atypical *Escherichia coli* (var. mutabilis) which fermented lactose very slowly, thereby mimicking a *Salmonella* species on primary isolation and it even was agglutinated by many lots of *Salmonella* Group B antisera. In addition, there was a strain of *Streptococcus fecalis* which also overgrew on aerobic plates making it more difficult to detect aerobic isolator contaminants. Numerous selective media were therefore required to inhibit the aerobic members of the microflora in order to detect many contaminants. Since it had been learned that the anaerobic moiety of the microflora of the mouse could be cultured using strict anaerobic techniques, and this moiety comprised 99.999 percent of the total bacteria in the "normal" microflora of the laboratory and wild mouse, deletion of the aerobic members of the original Schaedler cocktails was deemed appropriate.

It was requested that the total number of bacteria in the microflora not exceed eight, as used previously, to keep the monitoring at a reasonable cost. Out of the purported 130 members of the normal mouse microflora, I chose four of the original Schaedler bacteria and replaced the two previously mentioned aerobes, as well as the anaerobic *Streptococcus* and the anaerobic *Clostridium* species contained in the original "Schaedler Cocktail," used by NCI's largest supplier. By eliminating these species, the microflora was devoid of any cocci or spore-forming, blunt-ended rods, which together represent the great majority of isolator contaminants. Therefore, direct phase microscopic examination of isolator samples, especially feces, allowed for the rapid detection of the preponderance of isolator contaminations. I then added four additional anaerobes, each with a different cellular morphology distinguishable under phase microscopy, from the myriad of anaerobes comprising the autochthonous microflora of the mouse. I named the new microflora "The Altered Schaedler Flora" (28) and it contained the two Schaedler lactobacilli, which were only moderately anaerobic or "microaerophilic," so some slow, low level growth was observed on aerobic plates, but not to the extent of hindering the rapid detection of common aerobic contaminants. Also retained was the Schaedler 19X *Bacteroides* (*B. distasonis*) and the Schaedler fusiform-shaped anaerobe. This microflora was quickly adopted by all major suppliers in the US and abroad, for both NCI contract and commercial nucleus stock mice and rats, making it the one common denominator for the great majority of laboratory animals used in biomedical research throughout the world. It should be noted, however, that these animals are also colonized with many other bacteria when they are removed from their isolators to stock barrier room facilities, where they come into contact with literally hundreds of additional bacteria and other microorganisms. Sometimes even primary murine pathogens gain entry into these barrier rooms, which house so-called specific-pathogen-free or "SPF" animals. It should be noted that this term is absolutely meaningless unless one specifies exactly which pathogens the animals are free of, and what kinds of tests were employed to prove that they are free of the specified pathogens (29).

While looking for an alternative to the barrier room concept, without the inconveniences of manipulating animals in isolators, I met Robert "Bob" Sedlacek of the Massachusetts General Hospital at Harvard University. He took me on a tour of his facility

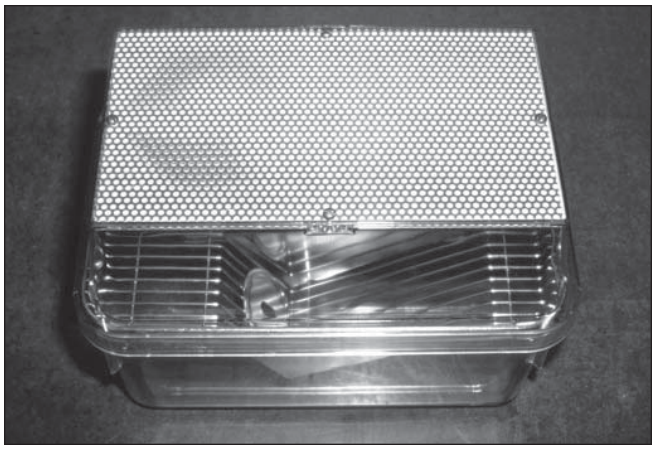

FIG. 5. A custom designed filter top cage consisting of a plastic cage bottom fitted with a heavy plastic top containing a filter medium protected with a perforated aluminum plate.

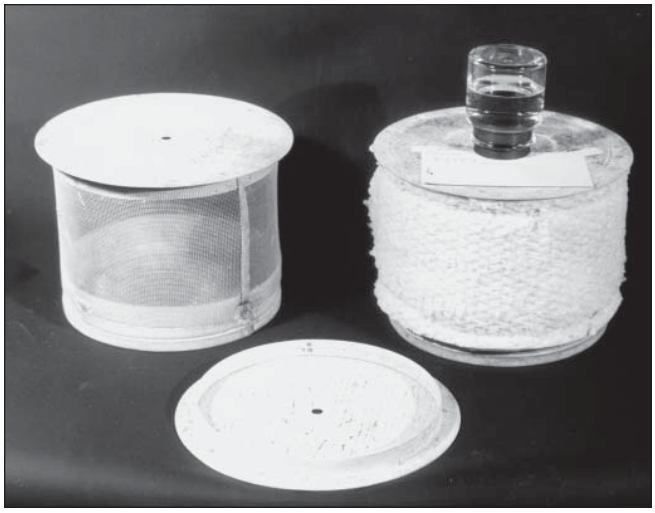

FIG. 6. The first filtered cage.

where he showed me how he was raising mice with a limited microflora in his custom designed filter top cage. This consisted of a plastic cage bottom fitted with a heavy plastic top containing a filter medium protected with a perforated aluminum plate, as shown in figure 5. It was manipulated aseptically under a flow of sterile air in a laminar airflow workbench, very similar to the method described in 1958 by the inventor of barrier-at-cage level, and Griffin Award-winning veterinarian for that work, Dr. Liz Kraft (30). The very first filtered cage can be seen in Figure 6 and the hood it was manipulated within is shown in Figure 7. Unfortunately, following Dr. Kraft's papers, many individuals decided not to bother with the hood, witnessed contaminations and then concluded that barrier-at-cage level didn't work! Indeed, Sedlacek also tried to circumvent the cost of using a hood, but quickly found that it was essential (31). Then, in order to use makeup cages without tops, and save on labor and material costs, Sedlacek also supplied sterile air in his animal room with the crucial design of supply at ceiling level and exhaust at floor level. Personnel had to gown with full surgical head wraps, face masks, etc., but the savings obtained by fewer autoclave loads and fewer filter tops more than justified these minor inconveniences (32). Personnel did not have to shower. Also, because his mice had a flora free of urease-positive bacteria, there was no ammonia in his animal room. This allowed him to recycle the air and keep

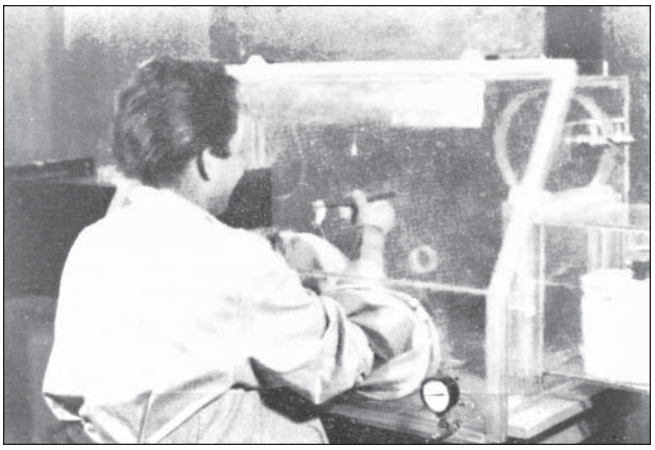

FIG. 7. A hood for manipulating the early filtered cages.

excellent control of the relative humidity, because there was no ammonia to erode the copper coils in his cooling system. I tested "The Sedlacek Cage" for maintaining mice in the gnotobiotic state; however, not in a room with mass air flow.

In 1981, I presented a paper at the 32nd National AALAS Meeting in Salt Lake City demonstrating the ability to keep mice germ-free in "The Sedlacek Cage" (renamed the Micro-Isolator™ Cage the following year by the manufacturer) for a period of one month, while it sat on the top of a filing cabinet in my office (33). This work was confirmed 13 years later using many more mice over a much longer period of time and was conducted in a conventional animal facility. Under these field conditions, two layers of Reemay 2024 filter medium were found to be needed (34). Therefore, this cage was also shown to be effective in preventing contamination, even when the ambient environment was conventional and not treated with a supply of sterile air-although it did require more labor and autoclave usage since makeup cages were not stacked when sterilized as in Sedlacek's procedure. Nevertheless, the ability to conduct research in a conventional setting without contamination and without facility renovation to supply sterile air from ceiling to floor, more than offset the costs of repeating experiments previously lost to contamination. Although gnotobiotes can be maintained in filter top cages, personnel must practice scrupulous aspectic technique at all times. In an isolator, personnel do not have to decontaminate their gloves in between cages, nor pay attention to what they have just touched inside the isolator, but they certainly do have to take such notice when working with filter top cages inside a "hood" supplying sterile air either vertically, as in the case of a Class II or III biological safety cabinet, or horizontally, as in a laminar air flow workbench. The former provides both personnel and product protection (33,34,35), while the latter provides product protection only (36), but both will suffice for maintaining mice contamination free.

The NIH used the generic term "microisolation" to address all filter top cages in its 1985 edition of the *Guide for the Care and Use of Laboratory Animals*. In this edition it also dropped it's previous requirement that multiple species could not be housed in the same room. This was appropriate since the room was no longer the barrier when filter top cages were used (barrier-at-cage level).

In 1989, Gary Novak of Johns Hopkins University and I called for the formation of a Microisolation User's Group, or MUG, via an open letter to the AALAS membership (37). Approximately 300 veterinarians, facility managers and other lab animal care personnel responded. Our first meeting was held at a very suc-

cessful Seminar during the 1989 AALAS National Meeting and the first speaker was the famous veterinarian who started it all, Dr. Liz Kraft. Dr. Kraft came out of retirement to deliver her lecture, "Overview of Barrier at Cage Level" and was a guest of AALAS. The first *MUG Newsletter* was published the following year. The executive committee of the Microisolation User's Group consisted of one member each from academia (Gary Novak), government (Rosemary Riggs) and industry (Dr. David K. Johnson), all of whom still serve in this capacity today; while I continue to serve as the secretary/treasurer and newsletter editor.

Although mice can be maintained gnotobiotic in filter top cages, it should be noted that the inventor of the Micro-Isolator™ cage, Bob Sedlacek, maintained his nucleus stocks in flexible-film isolators. An isolator may require more labor and be more inconvenient, but it provides an intact barrier to contamination without any open areas for insects, to enter, and therefore provides a safer barrier to contamination. One can also drop a cage inside an isolator and not alter its microbiological status. The precious, nucleus stock animals at major rodent suppliers are also maintained in isolators to this day. Some major suppliers also find it necessary to raise all of their immunodeficient rodents in isolators due to their susceptibility to opportunistic pathogenic microorganisms, which eventually appear in barrier rooms.

Lastly, attempting to maintain animals free of cross-contamination by using mass air flow (MAF) rooms containing open cages of animals was finally put to rest by the controlled studies of Dr. Julius Thigpen and Paul Ross at the NIEHS (38). Not only does cross-contamination occur from open cage to open cage within a mass air flow room supplied with sterile air from ceiling to floor, but personnel wearing face masks and hair nets are obvious sources of contagion as well. The air in these rooms may be sterile when it comes out of the ceiling, but it is then free to blow contagion off the foreheads of personnel and down into open animal cages below. However, rooms supplied with sterile air from ceiling to floor have been used successfully in conjunction with filter top cages to raise gnotobiotes in order to dramatically reduce costs as described by Sedlacek. In addition, rooms can be sealed and used as giant isolators with personnel totally and contiguously separated from the interior wearing either full or half suits make of plastic.

In summary, filter top caging systems utilized in a conventional setting within multipurpose animal facilities have been the method of choice for housing barrier reared animals on study, while gnotobiotes have continued to be maintained almost exclusively in flexible film isolators. As stated earlier by Dr. Trexler, less costly isolators can be made, and more efficient procedures adopted, so that isolators could become more common for also housing barrier-reared animals free of contamination. The historical limiting factor for the adoption of isolators for this purpose has been the unwillingness of investigators to work with animals within an enclosure like an isolator, which requires the operator to wear heavy gloves, prevents animals from being taken to research laboratories, and other such inconveniences. However, there is a clear trend in lab animal medicine to have animal care personnel perform all animal manipulations for investigators and therefore the use of isolastors may become more common for also housing barrier reared animals on study. However, the most probable scenario will be the continued increase in popularity of filter top cages, especially now that automatic air and water have been introduced to dramatically reduce labor costs. Therefore, the availability of resources, coupled with the level of inconvenience that animal care and research personnel will agree upon, will decide which animal housing systems are appropriate in any given circumstance.

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