Germfree Animals and Technics in Surgical Research

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Germfree animals have been reared to a size, weight, and age permitting the performance of major surgical procedures and the pursuit of a variety of surgical research problems. Germfree dogs have been maintained in the isolator system through three generations, indicating that life, reproduction, and growth are all possible in the absence of microbial contamination.

The value of the germfree approach to surgical problems has been utilized in studies of a variety of gastrointestinal problems, shock, cancer, immunology, burns, wound healing, and in direct patient application. Patients have been maintained in isolator environments for prevention of infection, for operative procedures, for treatment of extensive burns, and for management of immune-suppressed individuals.

We conclude that germfree animals and germfree technics provide a valuable addition to the armamentarium of the surgeon in both research and clinical applications.

The Germfree Laboratory at Louisiana State University was begun in 1961 as part of a continuing study of the role of bacteria in the fatal outcome of strangulation intestinal obstruction. The obvious advantages of this approach to any problem with bacterial implications stimulated us to extend the studies to other areas and to become a part of what was then a growing worldwide interest in the use of gnotobiotes (germfree animals and/or specifically contaminated animals with pure microbial strains).

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Germfree animals and technics were well established in other centers when our work began, and the first extensive reports in the surgical literature of the potential uses of this "tool" had appeared in 1959 [*1*] and in 1961 [*2*]. Dramatic changes in the equipment necessary to house these animals made the technics much more widely applicable and led to an enlarging group of researchers with ever-widening interests who became involved in the field. The development of the flexible-film, vinyl plastic isolator to replace the old standard stainless-steel isolator, which was basically a modified autoclave, decreased the cost and the complexity, increased the mobility, and made possible more extensive modifications in the utilization of the technics. Earlier work, based on mice, rats, guinea pigs, and chickens, dealt mainly with life cycles, body and organ weight, and the refinement of the technology required to keep animals alive and allow them to reproduce in the germfree environment, but studies involving surgical experiments were limited. Our work necessitated our learning the technics of delivering and rearing the animals, plus the development of special isolators in which germfree animals could be operated on, observed, and studied in the postoperative period. As the work progressed, it became obvious that larger animals would be more useful for surgical experiments, and this led to our interest in delivering, rearing, and using dogs suitable for the specific interests at hand. Special stainless-steel cages had to be devised, designed in such a way that they would be sturdy enough to hold a fully grown dog but modular enough so that sections could be passed through the relatively small ports of entry of the isolators and then reconstructed inside the isolator. Important lessons in nutrition had to be learned and sources found to provide the proper nutrients to wean pups in a sterile environment, separated from the bitch from which they had been removed by Caesarian section. Eventually, mature dogs were allowed to mate in the germfree environment, leading to the first second-generation germfree dogs anywhere; this was a major advance, though in retrospect it does not sound as difficult as it was at the time. Maintaining sterility of the internal atmosphere and the food and water, providing for sterile exit of waste, arranging for sterile entry of equipment for surgical procedures, and taking and removing specimens for study all presented problems. In addition, since the inflation of the flexible isolators depends on a continuous source of forced filtered sterile air, provisions had to be made for emergency power in case of electrical failure, hurricanes, or other disasters—natural or manmade. This recitation of the problems involved in germfree research is not made to garner credit, particularly since many advances were the result of the work of others, but is intended to make others aware of the complexities of the field, including the cost, which could not have been met without significant support from the National Institutes of Health and others; to give the reader some insight into the problems inherent in any unexplored field that seems to offer research opportunities; and to let interested workers know the technology is available to them. Was it worth the effort? The results of the numerous investigators in the field provide ample evidence for a clearly positive reply.

Direct surgical application of the germfree technics was applied first and most extensively by Levenson and associates [*1*], who demonstrated that it was possible to do a number of standard surgical procedures utilizing the germfree technic to envelop the patient, or at least to exclude the incision from contact with the outside atmosphere and to conduct the operative procedure within the isolator. This involves some cumbersome equipment, the learning of a completely different approach to the handling of the instruments and tissues, and certain difficulties for nursing personnel. But the lessons learned from these studies have aided in the use of the technics in other applications, perhaps most publicized in the use of entire room isolators for patients with specific immune-suppressed problems. Other surgical areas where isolator technics have been used include neurosurgery, cardiac surgery, bone marrow transplantation, post-transplantation immune suppression, burns, and infected cases where reverse isolation is possible. Orthopedists have extended these uses to procedures where they are most concerned about infection—hip replacements—and then modified them to laminar flow and other technics, all of which came partially from previous experience with germfree technology.

INTESTINAL OBSTRUCTION

Our earlier studies had shown that bacteria played a major role in the death of animals subjected to strangulation intestinal obstruction, even when the animals received adequate fluid and electrolyte support. Further studies showed that antibiotics could prevent the fatal outcome, and therefore the dominant role of bacteria appeared to be established. There remained the lingering question as to whether the antibiotics might be acting through some other mechanism rather than just the control of the bacterial flora in the segment of bowel subjected to the obstruction and loss of its blood supply. This same question about the action of antibiotics has persisted through other areas and was a further stimulus to our work in the germfree field. A clear-cut answer should be available in the total absence of bacteria—the germfree animal.

Germfree dogs were subjected to the same strangulation obstruction procedure as conventional animals and treated only with fluid replacement. Six germfree dogs and a total of 13 gnotobiotic dogs contaminated only with a *Bacillus* or a *Bacillus* and *Staphylococcus albus* all survived to 100 hours, in contrast to the conventional animals that all died within 37 hours [*3,4*]: "These results convinced us of the importance of bacteria in the pathogenesis of the lethal results in strangulation obstruction. Gnotobiotic dogs that received only fluid support survived, had a strangulated segment of bowel return to grossly normal appearance [as well as normal histologic appearance], and failed to produce any lethal fluid in the strangulated segment. This was in contrast to all the opposite findings in conventional animals. The vital role of bacteria in the outcome of strangulation obstruction has been demonstrated." [*5*] Subsequent confirmation of these results has been reported by Amundsen and Gustafsson [*6*] and Yale and Altemeier [*7*].

Major differences were noted in the fluid requirements and the output by vomiting and other routes in the germfree versus the conventional animals, but there was no explanation for these differences. Subsequent studies of the fluid flux in, above, and below an obstructed segment of small bowel in germfree animals demonstrated that above the obstructed loop in germfree animals, there is absorption of water and electrolytes, in contrast to the secretory state in conventional animals [*8*]. Thus, the "normal" microbial flora of the intestine are important in the loss of fluids in the presence of intestinal obstruction.

The blind loop syndrome has been blamed on bacterial overgrowth within the loop, and there can be no doubt that such growth does occur. However, whether that growth is responsible for the loss of fecal fat is not so clear. Studies in conventional and germfree rats with a blind loop [*9*] found that germfree rats excreted approximately the same percentage of their fat intake as those without a blind loop, in contrast to conventional rats, which had a doubling of fat in the stool after establishment of a blind loop. Germfree rats did not increase fat excretion even with monocontamination or dicontamination with *Bacteroides* sp. and *Clostridium* sp. When they were removed from the germfree environment, the rate of fecal fat excretion increased five- or seven-fold, in contrast to nonoperated germfree rats, which increased excretion only slightly when removed from their isolator. These studies need to be pursued with other species of gnotobiotic animals to determine which organisms or combinations are responsible for the malabsorption. It is difficult to extrapolate the data from one species of gnotobiotic animal directly to man, but improved knowledge of how various bacteria produce their lethal effects in each gnotobiotic species should aid the understanding of the disease process in man.

BILIARY/HEPATIC PROBLEMS

Bile peritonitis was studied by establishing a fistula into the peritoneal cavity from the gallbladder [*10*]. Conventional animals subjected to this procedure died within 26 hours. Conventional animals or gnotobiotic animals with one or two known contaminants and treated with antibiotics also survived. Germfree animals that received no therapy of any kind all survived to reoperation at 15 days, at which time they showed no ill effects other than the presence of a sterile bile ascites, amounting to 2,500 mL in one animal. Thus, bacteria were shown to have a commanding role in the fatal effects of bile peritonitis.

Ligation of the hepatic artery was thought to be fatal for many years, but then became a popular technic for the treatment of portal hypertension. Conventional dogs subjected to this operation had a 100% mortality, with *C.*

welchii given credit for the lethal effects. Antibiotics prevented the lethal outcome, and it was shown that even small doses of antibiotic administered prior to the hepatic artery ligation would also prevent the fatal result. Still, the possibility that the antibiotics might be acting in some other way prevented the full acceptance of the bacterial etiology as the complete answer to this problem. Repetition of the experiment in germfree dogs without any antibiotic or other therapy resulted in survival, eliminating any question about the importance of bacteria in this condition [*11*].

Bacteria have been implicated in the production of blood ammonia levels, particularly in the elevations found in patients with liver disease. Germfree guinea pigs were shown to have blood ammonia levels about one fourth that of conventional animals and to exhibit an increase when given a high-protein diet, although not to the same extent as conventional guinea pigs [*12*]. Subsequent studies using the Eck fistula as a technic for measuring various parameters of hepatic encephalopathy indicated that germfree dogs develop encephalopathy with histologic, biochemical, and clinical evidences of hyperammonemia and hepatic coma [*13*]. Ingestion of blood led to increases in blood ammonia levels comparable with that in conventional dogs. Thus, bacteria are not essential for production of blood ammonia nor for the development of hepatic encephalopathy, though bacteria may exaggerate such a condition.

PANCREATITIS

The role of bacteria in pancreatitis has not yet been resolved. The Pfeffer technic, which uses a closed loop of duodenum that includes the pancreatic duct but does not include bile since the common duct is ligated, was employed for this study. Germfree animals developed severe pancreatitis in this model, as did the conventional animals, even though cultures of the germfree animals and all their secretions continued to be sterile [*14*]. The closed-loop obstruction was not a cause of death in the germfree animals, although it could be implicated as such in the conventional animals. However, the conclusion drawn from this work was that bacteria are not a cause of death in this form of experimental pancreatitis, and this was the first clear-cut study where other nonbacterial factors could be excluded. Other reports with different animals and different technics confirmed these results in germfree animals. A paradox arises from the observation that antibiotics instilled into the Pfeffer loop prevent the development of pancreatitis, even in germfree animals [*15*].

SHOCK

Fine focused modern interest on the role of the microbial flora and endotoxins in shock. Thus, the germfree animal offered an opportunity to refine our understanding of shock. The early studies of shock in germfree animals failed to give the anticipated increase in survival, even though fluid therapy did increase survival. A partial explanation was provided by studies which showed that the large cecum in germfree rats, which were the experimental animals, contributed to the death of the animals, since prior cecectomy prolonged survival [*16*].

More recent studies have shown that germfree rats had significantly better survival than their conventional controls when both groups were subjected to a "fluid-treated" model of hemorrhagic shock in unanesthetized unrestrained rats [*17*]. The germfree animal should be a fertile source for defining the role of the microbial flora in the shock syndrome.

IMMUNOLOGY

All germfree species studied demonstrated reduced development of the peripheral lymphoid organs. This is most noticeable in the organs close to the gastrointestinal tract, which normally would be influenced by the microflora or microbial products. The degree of retardation of lymphoid system development is diet-dependent and is most striking in the colostrum-deprived germfree piglet and in other species maintained on a low molecular weight, water-soluble, antigen-free diet. All species of germfree animals have reduced or absent immunoglobulins, which is also diet-dependent. When immunized with specific antigens or stimulated with nonspecific mitogens, all species have been capable of an adequate response, except the germfree guinea pig, which fails to develop a typical delayed-type hypersensitivity response. There is usually an increase in the lag phase of approximately 24 to 48 hours in the response curves. Once the germfree animal has responded, the response usually reaches a greater magnitude and is more prolonged than that observed in the conventional counterpart [*18*]. A study of allograft rejection, using transplanted thyroid, showed no difference in any of the parameters studied between germfree and conventional animals [*19*]. Thus, germfree animals provide yet another tool for the evaluation of a number of immunologically related problems in surgical research.

CANCER

Spontaneous prostate adenocarcinomas developed in aging germfree Lobund Wistar strain rats, but not in Sprague-Dawley rats maintained under the same conditions, emphasizing the role of genetics in the development of cancer [*20*]. Tumors have been induced in germfree animals with dimethylbenzanthracene (DMBA), but the incidence was higher in conventional than in germfree rats for colon, small intestinal, breast, salivary gland, and ear duct tumors [*21*].

Early studies with Ehrlich ascites tumor in germfree mice showed that transplanted tumors could grow in the germfree animal, and therefore these animals were suitable for the study of certain effects of immunologic and microbiologic factors on tumor growth [*22*]. Later studies with nude mice seemed to indicate that transplanted human tumor cells would grow but not metastasize in these animals [*23*]. It was later found that if the tumor were debulked from time to time, so that the weight of the tumor did not cause death of the host animal, metastases did occur. Further evaluation of the potential of germfree animals in cancer research is warranted.

BURNS

An early report on the value of the isolator for patients with extensive burns summarized the experience gained from 149 patient-days in isolators and concluded that the isolator is an effective barrier against cross-contamination, that the device was practical, and that the usual care of patients with serious burns was possible without undue difficulty [*24*].

Experimental studies on animals indicated that a 25% full-thickness burn was not lethal to germfree rats and that the eschar did not separate even after prolonged intervals, emphasizing the importance of bacteria in the elimination of the early eschar. If the animals were specifically contaminated with *Pseudomonas* after the burn, the subsequent course was similar to that of conventional rats, except that the gnotobiotic rats had a more rapidly lethal course. High immunoglobulin M titers developed but were not protective. Heptavalent *P. aeruginosa* vaccine increased immunoglobulin G response and prolonged survival time. Some forms of antibacterial therapy increased survival under these conditions, but no benefit was detectable from the use of the isolator system [*25*].

PATIENT ISOLATORS

David, the Houston "bubble-boy," was an infant with severe combined immune deficiency disease (SCID), who lived a full life within germfree isolation chambers for more than 12 years and thus became the longest living untreated survivor of SCID. From him "much was learned about the role of the environment on mental, psychomotor, and psychosocial development. The isolation systems had worked better than anticipated, proving to be adaptable to David's changing needs as he grew from a baby to a young man. He was kept free of infection throughout his entire life" [*26*]. David died of massive β-cell proliferation (probably stimulated by an Epstein-Barr virus) following an attempt to reconstitute his bone marrow with a bone marrow graft.

Germfree isolator technology can be utilized to protect cancer patients from infection during immunosuppressive chemotherapy, for patients with burns, and in patient isolation [*27*].

MISCELLANEOUS

The relatively few studies of wound healing in germfree animals point to the fact that the rate of wound healing is essentially normal in the germfree animal even if there is less inflammatory reaction, granulation tissue, and epithelialization in the germfree than in the conventional animal [*28*].

The formation of a capsule around a prosthetic breast implant, with its attendant contracture and loss of compressibility, was shown not to be bacteria-dependent since there was no difference between conventional and germfree animals in the response to a silicone implant [*29*].

Within the gastrointestinal tract, there have been studies of Crohn's disease, ulcerative colitis, the relation between severe diarrhea in *C. difficile* infection in germfree animals, the role of intestinal bacteria in bilirubin metabolism, development of intestinal mucin, and changes in pancreatic enzymes. These studies lead to the comment that the intestinal microflora "represent an entity with a weight comparable to one of the larger organs of the body" and therefore deserve further study, some of which is only possible in the gnotobiotic animal [*30*].

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