Gut, 1971, **12**, 230-235

Progress report

Gastrointestinal structure and function in germ-free or gnotobiotic animals

A truly germ-free animal harbours no associated forms of life, including viruses. Occasionally the term 'germ-free' has been used in a more restricted sense to denote animals that are free of pathogens only. For this reason it is preferable to use the term 'axenic' or the more general term 'gnotobiotic', when referring to animals in which the composition of any associated fauna or flora (biota), if present, is fully defined. Gnotobiotic animals, unless deliberately contaminated, are bacteria free but may harbour congenitally transmitted agents such as the leukaemogenic virus, found in most strains of what are usually referred to as 'germ-free' mice.

Some of the advantages of investigating animals free of associated microbes were recognized even during the pioneering days of microbiology, as shown by the first report of the rearing of such animals[1]. Primarily because of technical difficulties, their use was greatly restricted until simple inexpensive apparatus made of plastic film was developed[2]. At present the most commonly used species in germ-free work are mice, rats, and chickens. Germ-free rats and mice are available in large numbers from both institutional and commercial colonies, some of which have been maintained continuously since 1954.

Recently, methods for obtaining and using germ-free animals have been simplified further by the development of disposable isolation apparatus which can be used for the larger animals, including pigs and even calves[3], as illustrated in Figures 1 and 2. These isolators can be used to contain animals that are infected with highly contagious pathogens as well as to exclude all microorganisms. Clinically, on an experimental basis, isolators have been used for surgical procedures and in the treatment of patients with extensive burns or those who have been immunosuppressed[4]. It seems likely that apparatus which so effectively controls cross-infection in the laboratory can also be used to solve similar problems in the hospital.

This brief review does not attempt to cover all the physiological differences between conventional and germ-free animals but focuses on those relating to gastrointestinal morphology and function. A comprehensive treatise on the germ-free animal in research has already been published[5].

The Germ-free Gastrointestinal Tract

MORPHOLOGY OF THE SMALL INTESTINE

The small intestine of germ-free animals differs from the conventional in several important respects. The germ-free intestinal wall looks and is thinner, not only because it is less cellular, but also because it is less well hydrated. The villi of germ-free dogs are the same length as those in conventional

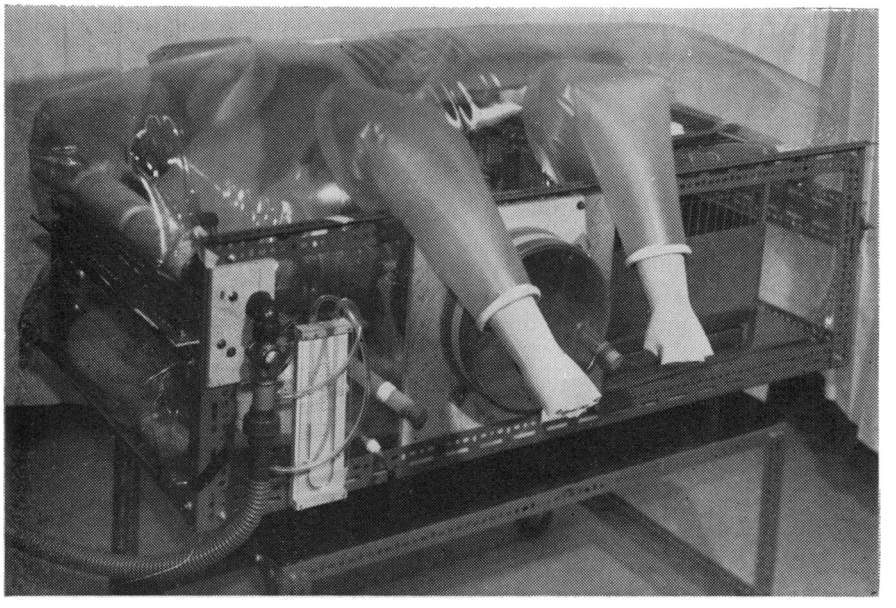

Fig. 1 *A flexible film isolator containing a cage for rearing a piglet. The isolator has a pair of gloves on both sides of the chamber. Air filters are located within the chamber at each end, the intake to the left and the extract to the right; note the orifice flow meter on the air supply line. The double-doored entry port is on the front of the chamber below the gloves. The animal cage and all heat-resistant materials are sterilized in an autoclave before being placed within the chamber. Surface sterilization is accomplished by means of a 2% solution of peracetic acid.*

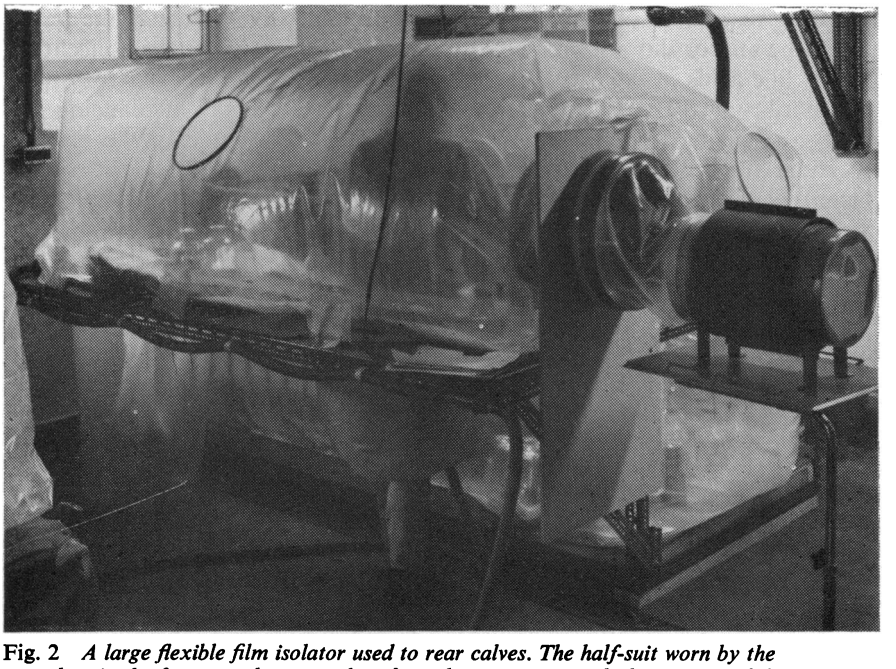

Fig. 2 *A large flexible film isolator used to rear calves. The half-suit worn by the attendant in the foreground protects him from direct contact with the interior of the isolator without interfering with his manipulations. A steam-sterilized drum is shown attached to the entry port on the extreme right.*

animals but are thinner and more pointed at the tip. There is an associated reduction in both the amount of lamina propria and in mucosal surface area[6]. In germ-free mice, histological examination of the lamina propria shows a sparse stroma, with few lymphocytes and macrophages; Peyer's patches are smaller. The epithelial cells are very uniform in shape and size and their microvillous brush borders appear wider than normal. The total mucosal thickness is less than in conventional mice, mainly because the villous crypts are shallower. The mitotic count in crypt cells is lower and the time taken for ^{3}-thymidine-labelled crypt cells to migrate to the villous tip is twice as long as in conventional animals[7]. This slow rate of cell turnover means that each cell exists in a mature state for a longer period and may explain why germ-free rats have higher levels of disaccharidase activity than their conventional counterparts[8].

These features of germ-free small intestinal morphology are due both to lack of the immunological stimulus provided by ingested bacterial antigens and to absence of the accelerating effect exerted by bacteria on the rate of cell extrusion from the villous tips. The morphological characteristics of the conventional intestine can, to some extent, be reverted to those of the germ-free state by the addition of low levels of antibiotics to the diet[6].

THE MEGACAECUM OF GERM-FREE RODENTS

A peculiar characteristic of germ-free mice and rats is the possession of an enormously distended caecum, weighing over 10 times as much as normal[9]. The contents are liquid and hypotonic, with a low concentration of Cl^- and HCO^{3-}. The *p*H is higher than in conventional animals and the reducing capacity is decreased[10]. Asano[11] has shown that feeding an anion exchange resin in the chloride form tends to restore the germ-free caecum to normal size, and has suggested that this effect is due to enhanced water transport following restitution of the Cl^- concentration. One other effect of the resin, which the author did not comment upon, would be to lower the concentration of bile acids in the caecum; these are known to inhibit water transport and motility in the large intestine[12]. The most effective way of reducing the enlarged caecum to a normal size, however, is by colonizing the gastrointestinal tract with anaerobic bacteria, certain species of clostridia and bacteroides being best in this respect[13].

INTESTINAL ABSORPTION

Before considering absorption it is important to point out that the motility of the gastrointestinal tract differs from normal in germ-free animals. Both gastric emptying and speed of transit through the small intestine are slower in germ-free mice[14]. This may be one of the reasons why glucose and d-xylose are absorbed more efficiently than in conventional mice[6].

A considerable amount of work has been done on the absorption and excretion of cholesterol and its metabolic degradation products in germ-free animals. Conventional rats excrete larger quantities of neutral sterols, predominantly in the form of coprostanol and coprostanone[15]. These two compounds are formed by the bacterial modification of cholesterol. Germ-free rats excrete a smaller amount of neutral sterol, over 90% of which is unchanged cholesterol. The higher excretion of endogenous neutral sterols in conventional animals was considered to be due to the higher rate of sloughing of mucosal cells rather than to any inhibitory effect that bacterial

modification of cholesterol might have on its reabsorption. In the same study it was shown that bile acid excretion in germ-free rats differs from normal. Bile acids are excreted in smaller amounts and remain in the conjugated form in germ-free animals, whereas bile acids undergo extensive modification in conventional animals, including deconjugation and 7 α-dehydroxylation. Deconjugation of bile acids does not explain their increased excretion in conventional animals[16] but 7 α-dehydroxylation could be responsible by potentiating their adsorption to insoluble dietary fibre[17] and thus reducing their reabsorption from the colon.

The overall absorption of protein is probably reduced in germ-free rodents since faecal excretion of nitrogen is higher than normal, presumably due to absence of proteolytic enzymes of bacterial origin. Unabsorbed, partly digested peptides accumulate in the caecum and their osmotic effect has been suggested as contributing to its distension[18].

Germ-free guinea pigs are said to be less susceptible than normal to the dietary induction of scurvy, presumably because vitamin C is utilized by bacteria in conventional animals[19]. In contrast, germ-free rats develop vitamin K deficiency on vitamin K-deficient diets but conventional rats do not. This can be prevented by inoculating the germ-free animals with *E. coli*[20], which presumably synthesize vitamin K in a form in which it can be absorbed. Germ-free rats have a high incidence of urinary calculi and this is associated with an increased intestinal absorption of calcium, although whether this is related to enhanced absorption of vitamin D is not known[21].

Immunology

Germ-free colostrum-deprived piglets kept on a synthetic diet have no detectable immunoglobulins in the serum[22]. Similarly germ-free mice, maintained on a highly purified synthetic diet from which all molecules with a molecular weight of $>10{,}000$ had been filtered out, were found to have low white cell counts and no detectable serum IgG[23]. However, if the germ-free rat is inoculated with a virulent strain of *S. typhimurium*, this results not only in the appearance in the serum of specific agglutinins, but also in a rise of IgG to normal levels[24].

Turning to cellular immunity, the lymphoid tissue of the gut of germ-free animals is dormant and hypoplastic but is capable of responding to antigenic stimulation[25]. However, this response may be inadequate in the face of a severe challenge. Germ-free guinea pigs die if exposed to *Shigella flexneri* but not if they have been previously exposed to *E. coli*. This protective effect is probably not simply the result of bacterial antagonism but may also reflect the maturation of lymphoid tissue that takes place in the intestine after the preliminary stimulus by *E. coli*[26]. Exposure of colostrum-deprived germ-free piglets to a pathogenic strain of *E. coli* resulted in an increased number of plasma cells in the lamina propria[27]. However, this was accompanied by only a slight rise in serum IgA, although serum IgG levels increased quite markedly[28].

Use of Germ-free Animals in Clinical Research

Amundsen and Gustafsson[29] used germ-free rats to study experimentally induced intestinal strangulation. Germ-free animals not only survived longer but the fluid which exuded from the serosa of their obstructed loop of

intestine was shown to be not toxic to mice when injected intraperitoneally. In contrast the fluid from conventional animals was found to be lethal, due to its high bacterial content. In an earlier study of the same problem, Cohn, Floyd, Dresden, and Bornside[30] pointed out that germ-free dogs and rats tolerated anaesthesia less well than their conventional counterparts. Since germ-free animals have smaller livers than normal it is possible that decreased hepatic microsomal enzyme activity might have contributed to their high incidence of anaesthetic deaths, through a failure to metabolize anaesthetic agents as efficiently as conventional animals. The relationship between hepatic microsomal enzymes and their possible induction by intestinal bacterial metabolites might well prove a fruitful field of study.

Extracts of germ-free rat colon have been used to detect the presence of haemagglutinating antibodies in the sera of patients with ulcerative colitis. The antigens from rat colons appeared to be similar to those from human tissue but the reaction could only be demonstrated with germ-free animals on account of the masking effect of antibacterial antibodies present in tissue from human subjects or conventional rats[31].

Germ-free animals have also proved useful in studying the mechanism of the hypocholesterolaemic effect of the polybasic antibiotic neomycin. Eyssen, Evrard, and van den Bosch[32] showed that neomycin lowered the serum cholesterol of germ-free chicks and increased their faecal bile acid excretion. This suggested that the hypocholesterolaemic effect of neomycin was not due to its antibiotic action but to its polybasic properties. More recently Thompson, Henry, Edington, and Trexler[33] have shown that neomycin increases the faecal excretion of neutral sterols and fatty acids in germ-free pigs without causing significant damage to the intestinal mucosa. These findings support observations made *in vitro* and in conventional rats and in human subjects, which suggest that ionic interaction between neomycin and fatty acids and bile acids results in precipitation of micellar lipids, including cholesterol, within the intestinal lumen[34,35].

Finally, Nance and Kline[36] have stated that hepatic encephalopathy and hyperammonaemia occur in germ-free dogs with portocaval shunts. The fact that they were also able to demonstrate a prompt rise in blood ammonia after a protein meal or an oral load of urea suggests that mucosal ureases may be important in the pathogenesis of hepatic encephalopathy. The beneficial effect of neomycin in patients with this problem, which can be observed long after the re-emergence of a resistant colonic flora[37], might be due, perhaps, to its known toxicity to the intestinal mucosa when given in high doses. These hypotheses need further investigation, however, especially in view of earlier work demonstrating that germ-free rats fail to catabolise urea to any significant extent[38].

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