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Human Flora-associated (HFA) Animals as a Model for Studying the Role of Intestinal Flora in Human Health and Disease

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Abstract

Although the intestinal flora in animals plays an important role in health and disease, there is little direct information regarding the role of the human intestinal flora. By inoculating germfree animals with human faeces, the major components of the human flora can be transferred into the ex-germfree animals, i.e. human flora-associated (HFA) animals. HFA animals therefore provide a stable model for studying the ecosystem and metabolism of the human intestinal flora. Results with HFA animals suggest the role of the human intestinal flora is somewhat different from the role of the animal flora in conventional experimental animals. Studies using HFA animals, therefore, will provide much needed information on the precise role of the intestinal flora in relation to humans. HFA animals also can be used as models to investigate the interactions between the human intestinal flora, host factors, dietary manipulations, and therapeutics, such as probiotics, prebiotics, and antibiotics.

Introduction

Although animal studies demonstrate a well established role for the intestinal flora in health and disease, it is often difficult to determine the precise role of the human intestinal flora. This is largely due to the problems of studying the human intestinal flora in humans due to the difficulties in controlling genetics and environmental and dietary conditions. Thus, the results of studies with human volunteers are often not clear. There also are ethical problems associated with utilising pathogens, carcinogens or toxic substances in human volunteers. In addition, it must be kept in mind that the composition and metabolic activities of the intestinal flora of experimental animals are significantly different from the human intestinal flora (Hirayama *et al.*, 1995a), making questionable the extrapolation of results from animal studies to the human system. In an attempt to solve these concerns, germfree animals associated with the human faecal flora, i.e. human flora-associated (HFA) animals, provide a better model for studying the ecology and metabolism of the intestinal bacteria of humans (Hirayama, 1999).

Production of human flora-associated (HFA) animals

Investigators have reported that the faecal bacteria of human donors can be transferred to germfree mice and rats (Raibaud *et al.*, 1980; Hazenberg *et al.*, 1981; Debure *et al.*, 1989; Hirayama *et al.*, 1991). We subsequently inoculated germfree mice with faecal suspensions from six different healthy human adults and confirmed that the major components of the human flora could be successfully transferred into these HFA mice. Furthermore, we demonstrated that the dominant bacterial groups remained constant in these HFA mice. However, bifidobacteria were sometimes eliminated from some of the HFA mouse groups (Hirayama *et al.*, 1995a). Interestingly, the elimination of bifidobacteria seemed to be dependent on the composition of the flora in the inoculated samples.

Mallett *et al.* (1987) demonstrated that certain cancer related enzymic activities of human faecal flora can be simulated in rats associated with human intestinal bacteria. Hirayama *et al.* (1995a) reported that the activities of β -glucosidase and β -glucuronidase in HFA mice were similar to those in humans and different from those in conventional mice, while nitroreductase activity in HFA mice was intermediate between those of humans or conventional mice, and nitrate reductase in HFA mice showed higher activity than that in humans or conventional mice. Although the concentrations of putrefactive products and short chain fatty acids in the faeces of HFA mice were significantly lower than those in human faeces and similar to those in conventional mice, the composition of the metabolites in HFA mice were more similar to that in humans (Hirayama *et al.*, 1995a). *p*-Cresol, which is detected in human faeces but not in conventional mice, was detected in most of the HFA mouse groups and the concentration of faecal indole in some of the HFA mouse groups was significantly higher than that in conventional mice. The composition of short chain fatty acids in HFA mice was closer to that in humans than to that found in conventional mice. Thus, bacterial metabolism in the intestine of HFA mice reflected that of humans with respect to some metabolic activities but not others, even though the bacterial compositions of the faeces of HFA mice were similar to that of the human inocula.

The composition and metabolic activities of the human intestinal flora established in the intestines of HFA mice can be reproduced in the intestines of the offspring of the HFA mice without any remarkable changes (Hirayama *et al.*, 1995a; 1995b). The intestinal flora of the HFA mice was also reproduced in ex-germfree mice by transferring germfree mice into the cages of HFA mice and allowing them to be colonized with the human intestinal flora (Hirayama *et al.*, 1994). These studies demonstrated that the human intestinal flora once established in HFA mice can be maintained for a long period.

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Application of HFA animals to studies on the intestinal flora of humans

Effects of dietary supplements and components on the human intestinal flora

Probably the most direct application of HFA animals is in studies of the effects of dietary factors on the human intestinal flora. Indeed, HFA animals have often been used to investigate the effects of dietary supplements on the composition and metabolism of the human intestinal flora *in vivo*, especially fermented dairy products (Djouzi *et al.*, 1997) and indigestible oligosaccharides, i.e. prebiotics (Fujiwara *et al.*, 1991; Rowland and Tanaka, 1993; Hirayama *et al.*, 1994; Kikuchi *et al.*, 1996; Djouzi and Andrieux, 1997; Kleessen *et al.*, 2001), and changes in dietary macrocomponents (Rumney *et al.*, 1993a; Hirayama *et al.*, 1994; Hambly *et al.*, 1997b). For example, Djouzi *et al.* (1997) demonstrated that milk fermented with *Lactobacillus casei* significantly increased the amount of indigenous bifidobacteria in the faeces of HFA rats. Similar results have been described in humans. They also reported a decrease in β -glucuronidase activity in both rats and humans given *L. casei*. Rowland and Tanaka (1993) reported an increase in the caecal concentration of total bacteria, lactobacilli and bifidobacteria by feeding transgalactosylated oligosaccharides to HFA rats. Their results were consistent with those obtained in a human volunteer study (Ito *et al.*, 1990) although there was no change in total bacteria. Djouzi and Andrieux (1997) found that feeding of β -galacto-oligosaccharides and β -fructo-oligosaccharides significantly increased the numbers of *Bifidobacterium* in faeces but did not change the total numbers of anaerobic bacteria in HFA rats. These effects also have been observed previously in human subjects (Ito *et al.*, 1990; 1993; Gibson *et al.*, 1995). They then compared the effects of three different oligosaccharides in HFA rats and found differences among the different oligosaccharides in their effects on the intestinal flora.

Hambly *et al.* (1997b) reported an increase of putative metabolic biomarkers of colorectal cancer risk in the intestinal flora of HFA rats fed a diet similar to that consumed in the West and associated with high risk of colon cancer. We previously also demonstrated significant changes in the composition of the intestinal flora of HFA mice fed a high-meat and high-bran diet (Hirayama *et al.*, 1994). Although some studies utilizing human volunteers report significant changes in the faecal flora with changes in dietary composition (Reddy *et al.*, 1975), others (Drasar *et al.*, 1976; Hentges *et al.*, 1977) report only minor effects. Thus, influences of different dietary components on flora composition may be detected more clearly with HFA animals rather than with human volunteers. Furthermore, our study with HFA mice suggested the beneficial effects of fructo-oligosaccharides on the intestinal flora at doses equivalent to those usually given to in humans, which are far lower than the doses used in animal experiments (Hirayama *et al.*, 1994).

HFA animals have also been used to compare, under controlled conditions, responses to dietary components of human intestinal floras of differing compositions or from human floras from different sources (Ducluzeau *et al.*, 1984; Andrieux *et al.*, 1998; Silvi *et al.*, 1999). Silvi *et al.*

(1999) investigated the effects of resistant starch, which is a portion of starch undigested by pancreatic amylase in the small intestine, on the intestinal flora of HFA rats inoculated with faeces from Italian or United Kingdom donors and indicated that different human floras may respond in different ways to dietary change. Andrieux *et al.* (1998) established HFA rat groups inoculated with human flora with different levels of methane production and showed that the effects of dietary seaweed on some of the intestinal floral metabolism were different among the HFA groups.

Metabolism of dietary components by human intestinal flora

The metabolism of dietary components by the human intestinal flora also can be investigated using HFA animals. Rouzaud *et al.* (2003), using rats harbouring the whole human faecal flora, found that the human intestinal flora participated in the *in vivo* breakdown of plant glucosinolates. Using HFA rats, Roland *et al.* (1995) also reported that consumption of different types of fibre is related to the differences in production of fermentation metabolites, i.e. short chain fatty acids, D- and L-lactic acids, H₂ and CH₄.

HFA rats have also been employed as a model for studying cholesterol metabolism in the intestine (Gérard *et al.*, 2004). Bowey *et al.* (2003) investigated the metabolism of isoflavones and lignans in HFA rats associated with intestinal flora from different human subjects exhibiting differing metabolic characteristics to these phytoestrogens. The metabolic patterns of isoflavones and lignans of each subject could be transferred to the rat intestine, suggesting the inability of some subjects to produce equol, a metabolite of daidzein, is due to the lack of specific components of the intestinal flora.

Roles of human intestinal flora in the effects of dietary components on host physiology

The human intestinal flora plays a critical role in the modification of the effects of dietary components on the physiological and morphological properties of the host. For example, utilizing HFA animals, an important role of human intestinal flora has been reported in the effects of dietary components on mucosal architecture and the biosynthesis, secretion, and degradation of mucin (Sharma and Schumacher, 1995b; Fontaine *et al.*, 1996; Meslin *et al.*, 1999; Kleessen *et al.*, 2003).

Hepatic metabolic activities against dietary mutagens/carcinogens are thought to play an important role in human carcinogenesis. Studies using HFA animals also have demonstrated that the human intestinal flora influences the effects of dietary components on some hepatic xenobiotic metabolising enzymes (Lhoste *et al.*, 2003; Humblot *et al.*, 2004). Roland *et al.* (1994) indicated the different effects of different types of dietary fibre on hepatic and intestinal drug-metabolising enzymes using rats inoculated with the human whole faecal flora. Rumney *et al.* (1993a) demonstrated that hepatic S9 fraction from HFA rats fed diet with different cancer risk-associated macrocomponents have different activities in *in vitro* activation of dietary mutagens. The authors further suggested that risk-related dietary components

interact with the human intestinal flora to modulate the production of endogenous DNA-adducting and cross-linking substances.

In addition, the human intestinal flora colonized in HFA animals has been demonstrated to have an important role in the generation of mutagenic or carcinogenic compounds from dietary substances. Rumney *et al.* (1993b) reported metabolism of dietary mutagen by the intestinal flora to 7-keto derivative, which is a direct-acting mutagen in the *Salmonella* mutagenicity test, while the mother compound requires S9 activation. They also demonstrated that the use of HFA rats produces results particular relevant to humans, especially when the animals are fed human diets. This conversion also was enhanced in HFA rats fed a high-risk diet for colon cancer (Hambly *et al.*, 1997b) and decreased by ingestion of an indigestible sugar (Rowland *et al.*, 1996). Ward *et al.* (1990) compared the effect of dietary fat on the production of carcinogenic *N*-nitrosoamines in rats harbouring different intestinal floras as well as in HFA rats. Utilizing HFA mice and gnotobiotic techniques, Narushima *et al.* (2000) demonstrated the human intestinal flora plays a critical role in conversion of conjugated primary bile acids to secondary bile acids, which have been implicated in human carcinogenesis.

Sandré *et al.* (2001) demonstrated the immunomodulatory activity of a peptide derived from bovine casein on mouse macrophages obtained from HFA or germfree mice and the cells from HFA mice were more susceptible than those from germfree mice to the peptide effects. Sudo *et al.* (2000), using HFA mice and confirmed in specific pathogen-free mice, reported an important role of dietary nucleic acids in promoting a shift in Th1/Th2 balance toward Th1-dominant immunity.

HFA animals have also been used in nutritional studies (Dufour-Lescoat *et al.*, 1991; Grolier *et al.*, 1998). Grolier *et al.* (1998) demonstrated that the presence of the human intestinal flora decreases the bioavailability of carotene in HFA rats, although intestinal bacteria have no direct effect on the bioavailability of these pigments.

Effects of human intestinal flora on host physiology

The intestinal flora also has direct effects on the host. Thus, HFA animals can be a useful tool for studying *in vivo* the effects of the human intestinal flora on the host. The architecture of the colonic epithelial mucus layer was affected by the colonization of a human flora in rats (Kleessen *et al.*, 2003). Production and distribution of mucin were significantly different from those of germfree rats when the germfree rats were inoculated with a human flora (Sharma and Schumacher, 1995a; Fontaine *et al.*, 1996). Human flora associated in rat intestine significantly altered the number of the goblet cells and goblet cell glycoconjugates (Sharma and Schumacher, 1995b; Kleessen *et al.*, 2003). An HFA animal study also demonstrated that microbial-diet interactions can affect the numbers of enteroendocrine cells (Sharma and Schumacher, 1996).

Recently, immunological studies have employed HFA animals as an experimental tool. Gaboriau-Routhiau *et al.* (2003) reported that the establishment of a human intestinal flora in a murine model plays a protective role against *Escherichia coli* toxin-mediated abrogation of oral

tolerance to an unrelated co-ingested protein. Imaoka *et al.* (2004) found that the addition of segmented filamentous bacteria indigenous to mice to the inoculated human intestinal flora given to HFA mice established normal immunological responses in the GI tracts of the HFA mice. These segmented filamentous bacteria normally adhere strongly to the ileal epithelial cells in mice.

Simulation of conditions in the human intestinal tract

HFA animals also have been used to investigate the effects of therapeutic doses of antibiotics on the human intestinal flora (Tancredi *et al.*, 1981; Andremont *et al.*, 1983; Gismondo *et al.*, 1995; Perrin-Guyomard *et al.*, 2001; Barc *et al.*, 2004). Furthermore, HFA animals have been employed as a model for evaluating the impact of drug residues in food on the human intestinal flora (Corpet, 1993; Cerniglia and Kotarski, 1999; Perrin-Guyomard *et al.*, 2001).

Tuohy *et al.* (2002) investigated the transfer of plasmids from genetically modified microorganisms *in vivo* and demonstrated that the HFA rat model provides data of real relevance to the purported risks of DNA transfer from food-borne genetically modified microorganisms. Nijsten *et al.* (1995) investigated the *in vivo* transfer of antibiotic resistance plasmids using a rat model associated with the intestinal flora of various origins including humans and showed that the *in vivo* transfer of resistance plasmids is possible among the rat or human intestinal flora. Interestingly, the human intestinal flora appeared to permit better transfer of antibiotic resistance via plasmids than an intestinal flora derived from either pigs or rats.

Using HFA mice, Oozeer *et al.* (2002) demonstrated that an inoculated lactic acid bacterial strain could survive and synthesize proteins in the intestinal environment.

HFA animals as a model for studies which are impossible with human volunteers

HFA animals are useful models for studies that are impossible or difficult to perform with human volunteers. For example, there are ethical issues associated with human studies of colonization resistance against pathogenic bacteria or the effects of inoculated toxic chemicals and carcinogens.

Edwards *et al.* (2003) consequently developed a rat model associated with human breast-fed infant flora maintained on a modified infant formula to investigate the ability of the human flora to inhibit adhesion of pathogens to mucosal cells, as well as effects on the indigenous bacterial populations and bacterial metabolism. HFA animals also have been used for studying the influence of diet on colonization resistance against *Salmonella typhimurium* (Hentges *et al.*, 1992; 1995), *Clostridium perfringens*, *Staphylococcus aureus*, *Candida albicans* and *Pseudomonas aeruginosa* (Ducluzeau *et al.*, 1984).

HFA animals have been used as a new model to investigate the influence of the human intestinal flora on mutagenic/carcinogenic substances *in vivo*. Hirayama *et al.* (2000) found the capacity of human faeces to increase or decrease mutagenic activities of chemicals could be transferred into HFA mice. The presence of the intestinal flora was essential for the activity of faeces against the mutagens. HFA mice were then administered dietary and

environmental mutagens orally and DNA adduct formation was investigated as an *in vivo* biomarker of cancer risk. The process of chemical carcinogenesis is thought to be initiated by DNA adduct, the covalent binding of genotoxic agents to DNA (Gordon and Pesti, 1971). These results with HFA mice clearly showed that the human intestinal flora has an active role in DNA adduct formation. Scheepers *et al.* (1994a; 1994b) also demonstrated with HFA rats that the metabolic activity of the human intestinal flora is an essential step in haemoglobin and DNA adduct formation.

The strong impact of human intestinal flora on the genotoxic effects of dietary mutagens measured by Comet assay also has been demonstrated using HFA rats (Kassie *et al.*, 2001; Knasmüller *et al.*, 2001). Furthermore, the protective effects of dietary factors against the genotoxic effects of carcinogens in the Comet assay have been demonstrated in HFA animal studies (Rowland *et al.*, 1996; Humblot *et al.*, 2004). Kassie *et al.* (2004), by comparing HFA mice inoculated with faeces from vegetarians and meat eaters, showed that diet related differences in the intestinal flora have a strong impact on the genotoxic effects of food carcinogens. The formation of aberrant crypt foci, preneoplastic precursors for colon tumours, and chemically induced colon cancer have also been investigated in HFA animals (Hambly *et al.*, 1997; Tache *et al.*, 2000; Knasmüller *et al.*, 2001).

Conclusions

HFA mice and rats provide stable models for studying the ecosystem and metabolism of the human intestinal flora in conditions similar to those found in humans. It has been reported that the composition and metabolic activities of the human intestinal flora differs from that of experimental animals. Also, studies have demonstrated that the role of the intestinal flora suggested in experiments with HFA animals is significantly different from that obtained from conventional animal experiments (Ward *et al.*, 1990; Rumney *et al.*, 1993b; Sharma and Schumacher, 1995b; Hirayama *et al.*, 2000; Sudo *et al.*, 2000; Tache *et al.*, 2000; Kassie *et al.*, 2001).

HFA animals also are a useful substitute for human volunteers. It is possible to control the experimental conditions of HFA animals including genetic, environmental, and dietary conditions, which are often quite difficult to control in human studies. Furthermore, HFA animal experiments can be performed with a sufficient number of animals for statistical analysis and, if necessary, be repeated under the same conditions. HFA animals can be challenged with pathogens or toxic substances that cannot be used ethically in human studies. Also, intestinal contents, except for faeces or tissue samples, cannot be obtained easily from healthy human subjects without application of invasive techniques that cannot be justified for ethical reasons.

HFA animals, however, have some of the same limitations as other animal models. For example, bacterial metabolism in the intestine of HFA mice reflects that of human faeces with respect to some metabolic activities but not to others. Some bacterial groups are occasionally eliminated from HFA animals while other dominant bacterial groups remain constant. We have reported

that bifidobacteria are eliminated from some HFA mouse groups, probably due to the composition of flora in the inoculated sample (Hirayama *et al.*, 1995a). These HFA animals without bifidobacteria therefore cannot be used to study the effects of bifidogenic dietary factors. On the other hand, such bifidobacteria-free HFA animals might be useful for studying the importance of bifidobacteria in the human intestinal flora.

Caution also should be exercised in extrapolating the results from HFA animal experiments when a group of HFA animals is given the intestinal flora from a just one individual. Further studies should be conducted to "standardise" the human intestinal flora of HFA animals as an "average" human flora. For example, Silvi *et al.* (1999) chose to inoculate germfree rats with pooled faecal suspensions rather than utilizing individual faecal samples to provide results of more general applicability.

Laboratory rodent diet is different from the normal human diet. Rumney *et al.* (1993b) reported that the rate of conversion of potential mutagens to direct-acting mutagenic derivatives by intestinal bacteria is dependent on diets, and that the results obtained with HFA rats were particularly relevant to humans when the animals were fed a human diet. Thus, development of a special diet for HFA animals is required in order to establish HFA animals as a suitable model for studying the human intestinal flora.

In spite of the above limitations, studies using HFA animals provide much needed information of relevance concerning the role of the human intestinal flora. HFA animals will undoubtedly continue to contribute to investigations concerning the effects of drugs, antibiotics, probiotics, diet, and prebiotics on the human intestinal flora and its relationships with the human host.

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