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HOW IT STARTED - AND WHAT IS MAS?

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SUMMARY

The purpose of this article is to review the historical aspects of gnotobiology as it relates to gastro-intestinal and vascular smooth muscle motility. The idea of the germfree experiment was first expressed by *Pasteur* in 1885 however, he did not believe that normal germfree life was possible. About ten years elapsed before *Nuttall* and *Thierfelder* (1895a, 1895b) did their now classical experiments on germfree mammals and although few animals were derived, they noted that in the absence of the microflora "the caecum was strongly puffed up and filled to overflowing with a brown liquid which contained cheese-like coagula". This was the first reported distension of intestinal smooth muscle associated with the absence of the microflora. However, systematic studies with germfree animals did not truly begin until the late 1940s and early 1950s when Reyniers and his colleagues at the University of Notre Dame (*Reyniers* et al, 1946), Gustaffson at the University of Lund (*Gustafsson*, 1947) and Miyakawa at the University of Nagoya (*Miyakawa* et al., 1951) established academic organisations devoted to the study of germfree life. It became obvious based on these early observations that the germfree animal compared to its microflora laden counterpart exhibited numerous physiological and biochemical anomalies. The idea of a musculoactive substance (MAS) derived from the intestinal contents of germfree rats which could contribute to the observed decreased intestinal motility was first reported by *Gordon* in 1967; the events leading up to this observation and subsequent studies related to microflora associated MAS are the subject of this review.

INTRODUCTION

The early studies described nearly a century ago noted that the caecum of germfree animals was markedly enlarged and that the contents contained " a brown liquid which contained cheese-like coagula" (*Nuttall* and *Thierfelder*, 1895). Systematic studies with germfree animals did not truly begin until the late 1940s and early 1950s when Reyniers and his colleagues at the University of Notre Dame (*Reyniers* et al, 1946), Gustaffson at the University of Lund (*Gustafsson*, 1947) and Miyakawa at the University of Nagoya (*Miyakawa* et al., 1951) established academic organisations devoted to the study of germfree life. It became evident that the germfree animal exhibited numerous biochemical and physiological anomalies compared to its microflora

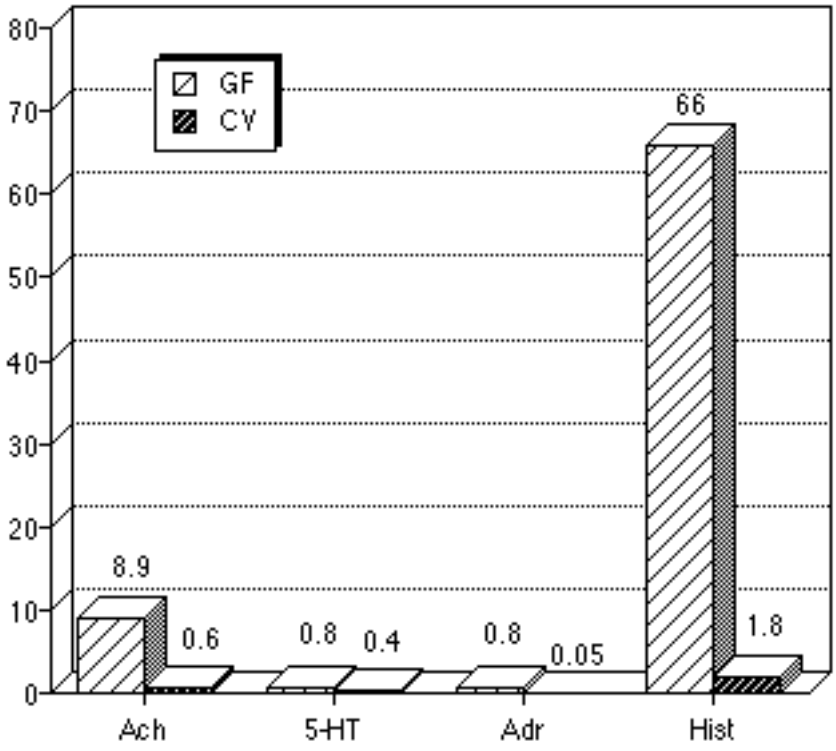

Figure 1: Caecal reactivity of germfree (GF) and conventional (CV) rats to biologically active amines (threshold dose - estimated average mg/ml bath). Modified from *Strandberg* et al., 1966.

laden counterpart. The accumulation of mucinous material in the distended lower bowels and the chronic mild diarrhoea became hallmarks of the germfree state. Although the diet may influence slightly the size of the caecum in the absence of the microflora, the germfree host has 5-10 times the caecal volume relative to the conventional counterpart. Maintaining an animal in the absence of microflora elements precipitates numerous gastro-intestinal and cardiovascular anomalies (*Gordon* and *Bruckner*, 1984). In addition to the "mild chronic diarrhoea" some of the more frequently cited physiological anomalies are 1) decreased oxygen consumption, 2) decreased cardiac output, 3) decreased regional blood flow to numerous organs and 4) decreased intestinal and vascular smooth muscle contractility.

MUSCULOACTIVE SUBSTANCE (MAS)

It was reported in the mid-nineteen sixties that the caecal contents of germfree mice and rats contained bioactive substances that were toxic when administered intraperitoneally and altered smooth muscle contractility in a number of smooth muscle preparations (*Gordon*, 1965). The amount of toxic substance(s) found in the germfree caecum was calculated to be 5-10 fold greater in amount but not in concentration compared to the microflora bearing

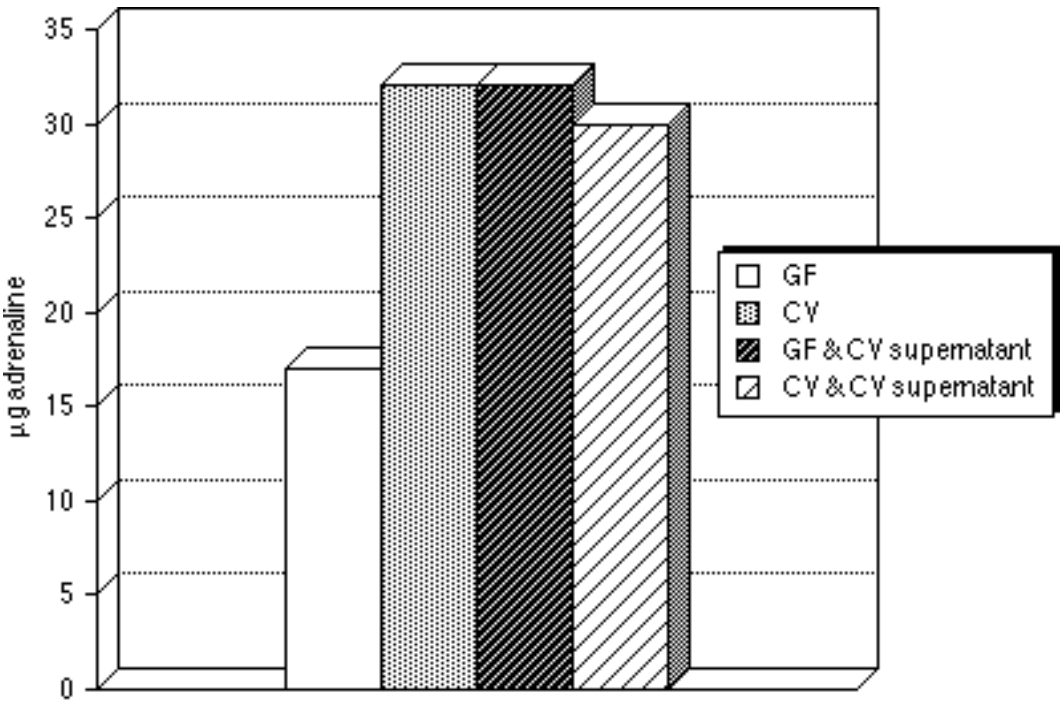

Figure 2: Adrenaline relaxation of caecal strips (GF=germfree; CV=conventional). Modified from *Staley*, 1968.

animals. These early observations suggested that these bioactive substances are endogenous to the host and that the normal intestinal microflora inactivate or reduce the amounts of these substances. The first clear indication that a reduction of caecal muscle tone occurred in the germfree animal was noted by *Strandberg* et al. (1966). It was found that the normal spontaneous caecal muscle contractions, in vitro, of conventional rats were not observed in the germfree specimens. Additionally when these caecal strips were challenged with a number of agonists (acetylcholine - Ach, serotonine - 5-HT, adrenaline - Adr, or histamine - Hist) the germfree preparations were 2-100 fold less sensitive than the muscle strips from conventional animals (Figure 1). Furthermore, *Staley* (1968) observed that a standard depressant dose of adrenaline relaxed caecal strips from germfree animals less than conventional controls (Figure 2). When the germfree caecal strips were exposed to the bacterium free conventional caecal content filtrate, they exhibited marked contractions and if further exposed to adrenaline responded with the same degree of relaxation as the conventional preparations. These observations suggested that the microflora may convert musculodepressant substances which are endogenous to the host into substances which may increase vascular tone. It appeared most likely that the hypo-responsiveness of the germfree caecum to these bioactive substances was most likely due to effects of the luminal contents, since the concentrations of bioactive amines in the caecal wall of germfree and conventional rats were similar (*Strandberg*, et al. 1966). There-

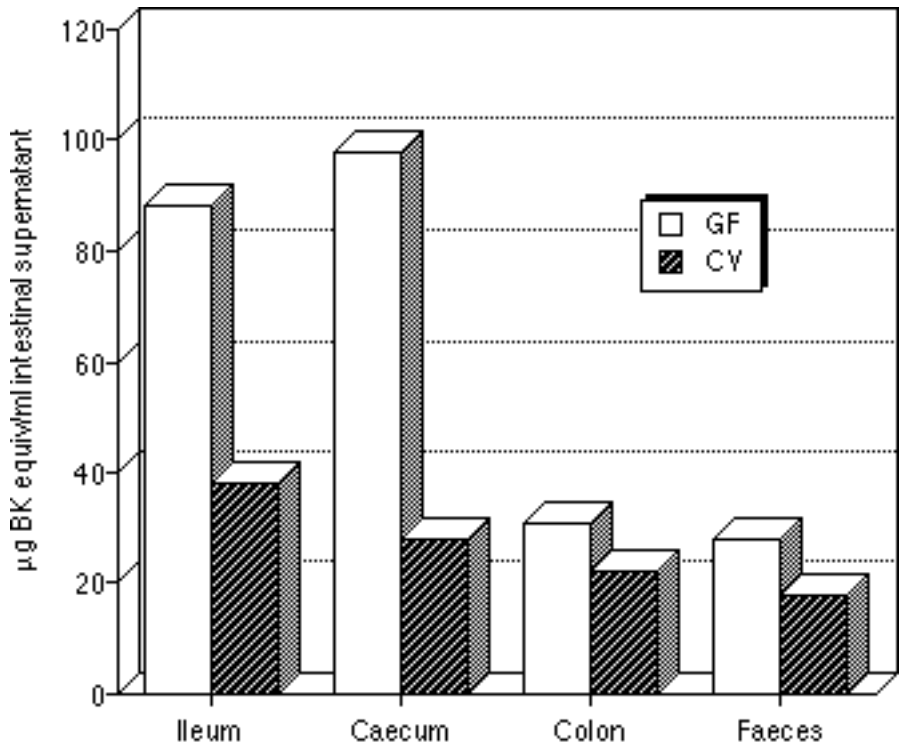

Figure 3: MAS relative concentration in the mouse gastro-intestinal tract (GF=germfree; CV=conventional). Modified from *Gordon*, 1967.

fore, the now classical studies reported by Gordon showed clearly that the toxic substances and the MAS were separate entities (*Gordon* et al., 1965). These substances were separated by ion-exchange chromatography into two pooled fractions. A fraction designated as the "terminal pool" was tested on rat uterus and guinea pig ileum in muscle bath preparations as well as cardiac output and regional blood flow to various organs in the rat. The bioactivity of the MAS as determined by uterine contractility was expressed as bradykinin equivalent units/ml of intestinal contents (Figure 3). It is evident that the germfree mouse harbours 1-3 fold more MAS in its intestinal contents than the conventional rodent; in rat caecal contents this difference is almost five fold. Mice fed various diets showed essentially the same amount of MAS in all segments of the gastro-intestinal tract. On intravenous administration of the germfree terminal pool fraction to rats a marked hypotensive effect was noted which was not observed with the conventional animal preparation (*Gordon*, 1967). These direct effects of MAS on vascular and intestinal smooth muscle appear to be blocked by specific protease inhibitors. However, an indirect effect noted by using subthreshold concentrations of MAS, which in presence of blood plasma can induce uterine contractions, cannot be blocked by protease inhibitors. The indirect effect could be inactivated by kinin degrading enzymes, indicating that the indirect effect may be related to bradykinin like substances. From these observations it was suggested that the direct MAS ef-

Table 1: Bioactivity of germfree caecal content fractions (MAS)

	Arteriole epi. refractory	Villi contraction	Blood pressure	Uterus contraction	Ileal contraction
Caecal contents	refractory	slight increase	hypo	increased	increased
Terminal pool	refractory	increased	hypo	increased	increased
Initial pool	no effect	decreased	no effect	not tested	not tested
a-pigment	refractory	increased	not tested	not tested	not tested
Amino acid	not tested	decreased	not tested	not tested	not tested

fect was due to a faecal kallikrein like substance which was capable of releasing kinin(s). Further fractionation of the "terminal pool" using anion exchange chromatography and sephadex molecular sizing yielded a compound termed a-pigment (MW 4000; *Wostmann* et al., 1973). The biologic effects of a-pigment include the following: 1) increased spontaneous contractions of the dog intestinal villi, 2) inhibition of adrenaline induced hypertension and 3) inhibition of adrenaline mediated contraction of mesenteric-precapillary arterioles. Alpha-pigment was tentatively identified as a ferritin degradation product most likely originating from desquamated epithelial cells which is known to impart adrenaline refractoriness to smooth muscles (*Gordon* and *Kokas*, 1968). Whether these MAS derived from intestinal lumenal contents can be absorbed has not been resolved and therefore the physiological regulatory role of these substances remains uncertain.

Table 1 summarizes the physiological effects of MAS.

INTESTINAL MOTILITY

The impairment of the lower bowel muscle function of germfree rodents is well established but its mechanism is insufficiently understood. Direct and indirect agents in the lumen which are capable of transferring a musculo-depressant action appear to be involved (*Dupont* et al., 1965; *Strandberg* et al., 1966; *Gordon* and *Kokas*, 1968). The impairment of gastro-intestinal function presents a serious impediment to the germfree animal's vitality ultimately resulting in cessation of peristalsis. The increased intestinal transit time can be noted early in life in both rodents and dogs (*Abrams* and *Bishop*, 1967; *Gustafsson* and *Norman*, 1969; *Heneghan* and *Mittelbronn*, 1981; *Van Elder* et al., 1988). Following caecectomy, the germfree rodent showed a slightly higher rate of intestinal transit than the caecectomized control. Furthermore the removal of the caecum has been shown to redress many of the cardiovascular anomalies of the germfree rodent. The cardiac output and refractoriness of the micro-vessel to norepinephrine approached values noted for the microflora laden counterparts (*Gordon* and *Bruckner*, 1984). The influence of the gut microflora on intestinal myoelectric activity has recently

been studied. It was found that the frequency of myoelectric complex migration was less in the germfree vs. the conventional rat. If the germfree animal was conventionalized, a microflora re-established, the frequency of the myoelectric complex migration increased within a week (*Caenepeel*, et al. 1989; *Husebey* et al, 1992). The pattern of propagation was more regular after conventionalisation but the slow wave frequency in the proximal jejunum was not increased nor the frequency of spike potentials following jejunal infusion of glucose (*Husebey* et al., 1994). The mechanism(s) responsible for the altered intestinal motility in the absence of the microflora are still not well understood. Microbial alterations of the gut milieu which may alter motility are 1) production of short chain and/or volatile fatty acids (acetic, butyric, propionic), 2) conversion of primary to secondary bile acids, 3) degradation of kalliekreins and/or kinins, 4) alterations of intestinal fatty acids which are eicosanoid precursors, 5) eicosanoid production or degradation and 5) other MAS alteration, e.g., NO, motilin. By introducing a microflora or by surgical removal of the caecum in the germfree rodent many of the physiological aberrations can be eliminated thus indicating that the large caecal pool of these vaso-active, musculo-depressant substances accumulate in the germfree state and contribute to the above described phenomenon.

CIRCULATORY ANOMALIES

Circulatory and metabolic anomalies such as reduced cardiac output, decreased regional blood flow and lower metabolic rates (oxygen consumption) have been observed in germfree animals relative to their conventional counterparts. It appears that these physiological alterations are associated with the intestinal contents since the removal of the caecum all but eliminates the differences between the germfree and conventional state (*Bruckner-Kardoss* and *Wostmann*, 1967; *Gordon* et. al., 1966; *Baez* et. al. 1973). This suggests that bioactive substances which originate from the intestinal lumen may be available for absorption and thus impart changes in vascular smooth muscle tone. It was found that in germfree or antibiotic treated animals the precapillary arterioles exhibited decreased vasomotor action and were refractory to noradrenaline compared to the conventional controls (*Baez* and *Gordon*, 1971; *Bruckner*, 1981). A peptide (α-pigment) was isolated from germfree caecal contents which on topical application to mesenteric micro-vessels imparted norepinephrine refractoriness and this same substance isolated from conventional animals showed markedly reduced activity. Furthermore, the norepinephrine refractoriness could be markedly reduced by treating antibiotic animals with Na salicylate, a prostaglandin inhibitor (*Bruckner*, 1973; 1981; See Figure 4). These substances are not eliminated by caecectomy, which normalizes many of these parameters, but seem only to be reduced in quantity and thus the systemic load appears to be diminished. Although it is not clear exactly what these substances might be the "α -pigment" which has been shown to impart epinephrine refractoriness is most likely derived from ferritin (*Gordon* and *Kokas*, 1968).

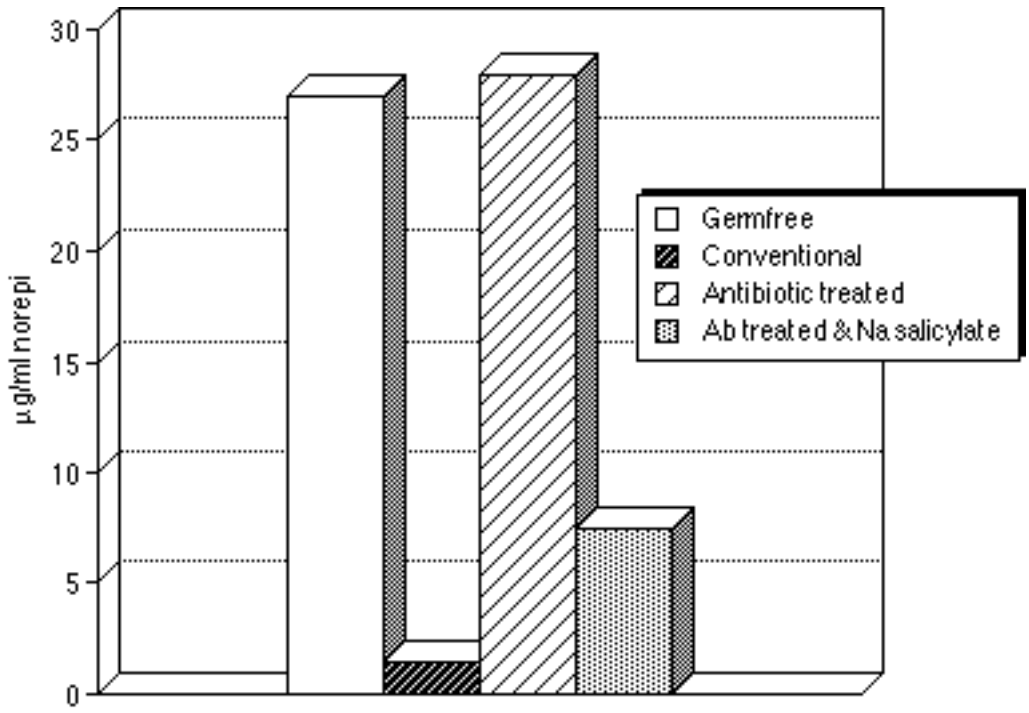

Figure 4: Arteriole norepinephrine sensitivity (mg/ml norepinephrine to elicit approximately 50% constriction). Modified from *Baez* and *Gordon*, 1971, and from *Bruckner*, 1981.

OTHER MICROFLORA RELATED MAS

Short Chain Fatty Acids (SCFA)

The intestinal microflora is capable of producing SCFA or volatile fatty acids from carbohydrate and protein substrates. The metabolism of carbohydrates generally leads to production of acetic, butyric and propionic acids while protein degradation yields primarily isobutyric or isovaleric acids (*Bugaut* and *Bentejac*, 1993). The concentration of these SCFA ranges from 1-13 mM/kg intestinal contents in the ileum-jejunum up to 50-300 mM/kg in the caecum and colon of monogastric animals. It is well known that SCFA can increase the ileal and colonic motility but appear not to alter duodeno-jejunal contractility (*Masliah* et al., 1992). Germfree animals are practically devoid of SCFA, however, small amounts were detected in the small intestine and caecum and these levels were attributed to dietary origin (*Hoverstad* and *Midtvedt*, 1986). Other physiologic effects of SCFA include stimulation of colonic blood flow (vasodilation), increasing pancreatic enzyme secretion, promoting Na and water absorption in the colon, potentiating intestinal mucosal growth, providing a preferential fuel source for the colonic mucosa and possibly lowering serum cholesterol via regulation of HMG-CoA reductase activity (*Bugaut* and *Bentejac*, 1993).

Eicosanoids

The sensitivity of gastro-intestinal smooth muscle to eicosanoids (prosta-

glandins, leukotrienes, thromboxane, prostacyclin) has been utilized for bioassay of these compounds. However, the effects of these bioactive lipids on GI smooth muscle show considerable variability depending on the type of eicosanoid, the dose, the species of the animal studied and even the muscle layer being used. For example $PGF_{2\alpha}$ contracts both longitudinal and circular gastric smooth muscle while PGE_2 relaxes circular muscle and has a variable effect on longitudinal. Prostanoids interact directly with the receptors on the membranes of gut smooth muscle cells and these receptors are not blocked by atropine, hexamethonium, mepyramine, methsergide, propranolol or phentolamine suggesting that the effects of prostanoids on gut motility are not mediated by acetylcholine, histamine, serotonin or catecholamines. These prostanoid characteristics are similar to the effects noted for the smooth muscle preparations and the effects of the caecal contents from germfree animals. As a general rule, $PGF_{2\alpha}$, prostaglandin endoperoxides and PGE_2 analogues appear to stimulate motility of most parts of the gut in most species, while the naturally occurring eicosanoids of the E series and PGI_2 are primarily inhibitory (*Moore*, 1988). Prostacyclin and PGE_2 are quantitatively the most important metabolites of arachidonic acid formed in the gut. These eicosanoids have been shown to reduce the amplitude and duration of gut slow waves, alter ion and water transport, and play a role in mucosal cytoprotection. Little is known about the effects of the microflora on eicosanoid production, however, there are reported elevations of eicosanoids associated with diarrhoea and inflammatory bowel disease which may be directly or indirectly related to microflora alterations. The absence of the gut microflora has been shown to alter dramatically essential and non-essential fatty acid metabolism (*Evrard* et al., 1964; *Demarne* et al., 1979; *Bruckner*, 1987). It has been noted that the germfree animals have 3-5 times the amounts of mono- and polyunsaturated (PUFA) endogenous intestinal and faecal fatty acids compared to their conventional counterparts. These fatty acids are normally hydrogenated or degraded by the intestinal microflora and may thus alter the availability of these fatty acids for eicosanoid synthesis. As previously mentioned the "norepinephrine refractoriness" of mesenteric micro-vessels associated with antibiotic decontamination of the gut microflora could be partly redressed by feeding the rats a prostanoid synthesis inhibitor (Figure 4). Although the mechanisms are not clear regarding the eicosanoid and microflora interactions the following events may be involved: 1) microbial degradation of PUFAs in the gut lumen thus decreasing substrate availability for eicosanoid formation, 2) microbial alteration of endogenously produced eicosanoids, e.g., PGI_2 and/or 3) kinins or α-pigment which are altered by the microflora may stimulate specific membrane phospholipase and thus influence eicosanoid homeostasis.

Motilin and Nitric Oxide

Motilin is a 22 amino acid polypeptide which has potent stimulatory effects on gastro-intestinal smooth *muscle in vivo* and *in vitro* perhaps via alteration of cytosolic calcium levels (*Higuchi* et al., 1994). Little is known about the influence of the microflora on motilin gut concentrations. Nitric oxide (NO) has been shown to be a potent vasodilator and to be involved in sodium choleate-induced fluid secretion and diarrhoea in rats (*Mascolo* et al., 1994). Although the microbial status of the rat does not seem to alter the levels of expired NO (*Persson* et al., 1994), there is virtually no information on the levels of NO in

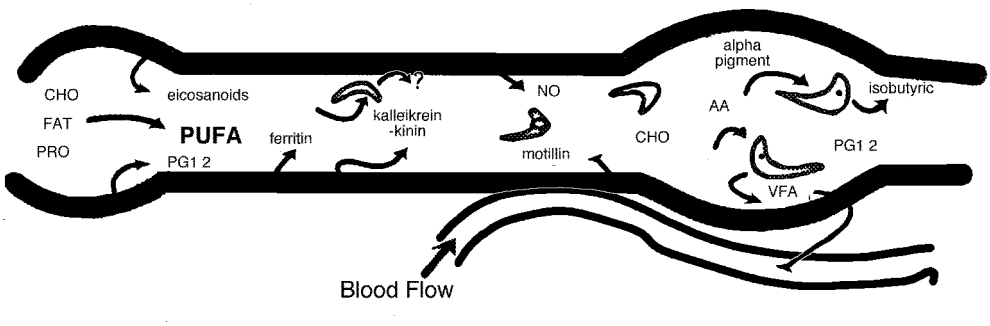

Figure 5: Microflora and intestinal bioactive substances (MAS).

the gut relative to microbial status of the host. It is evident that the gut microflora alters gastro-intestinal motility, water transport and vascular smooth muscle reactivity of the host, however, the mechanisms are far from being elucidated. Some possible interactions are depicted in Figure 5.

CONCLUSION

Gnotobiotic animals have been a valuable tool to elucidate the influence of the microflora on the physiology and biochemistry of the host animal. It is apparent that the gut microorganisms contribute to the maintenance of water and electrolyte balance, intestinal and vascular smooth muscle contractility, and to the normal physiological integrity of the GI tract. It appears that a number of the germfree anomalies are associated with the ability of the gut microflora to inactivate and/or metabolize endogenously produced substances which, if unaltered, impart vaso-depressant characteristics to intestinal and vascular smooth muscle. In the rodent these bioactive substances, e.g., kinins, α-pigment, eicosanoids, bile acids, SCFA, can produce "mild chronic diarrhoea," distension of the caecum and colon, biogenic amine refractoriness of smooth muscle and a decreased metabolic rate. By removing these MAS substances through caecectomy or by establishing a conventional gut microflora many of these anomalies can be reduced or eliminated. It is clear that many of the interactions between the intestinal microorganisms and MAS are not well understood and since these substances appear to be involved with the aetiology of many gastro-intestinal diseases, e.g., inflammatory bowel disease, diarrhoea, intestinal stasis, it is hoped that investigative efforts will be continued to elucidate the mechanisms involved.

ACKNOWLEDGEMENTS

This review of MAS is dedicated to Helmut Gordon whose pioneering efforts in the field of gnotobiology helped us to appreciate the complex interactions of the gut microflora with its symbiotic host.

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