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THE DEVELOPMENT OF COLONISATION RESISTANCE IN THE INFANT

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SUMMARY

The animal host and its intestinal microbial flora constitute and enormously complex ecosystems which interact to regulate the course of successional events in the development of both the intestinal flora and colonisation resistance. Colonisation resistance included all factors that hamper colonisation of the intestinal tract with exogenous microorganisms, including pathogens. Some of the regulatory factors are exerted by the animal host (e.g., pH, oxidation-reduction potential, antibody, diet, antimicrobial agents, bile acids, peristalsis), while other regulatory factors are exerted by the microorganisms themselves (e.g., modification of bile acids, depletion of essential substrates from the environment, competition for attachment sites, creation of a restrictive physiologic environment and elaboration of antibiotic-like substances). Mechanisms regulating the indigenous flora are often redundant such that two or more of these inhibitory mechanisms usually function synergistically in controlling the growth of microorganisms. The dramatic quantitative and qualitative fluctuations in the bacterial populations of the normal intestinal flora which occur immediately after birth up until the time the animal begins to sample solid food indicate that the normal flora are not well balances. It has been suggested that this may contribute to some of the intestinal diseases seen in young children since the protective mechanisms of the normal flora are probably diminished or absent. The objective of this paper is to review the various mechanisms, both host related and microbial flora related, that may be involved in the development of colonisation resistance in the infant. In addition, the role of the intestinal flora and volatile fatty acids in the development of colonisation resistance in hamsters to *Clostridium difficile* intestinal colonisation will be reviewed.

INTRODUCTION

The intestinal microbial flora is an enormously complex ecosystem which significantly impacts on the early development, quality of life, and ageing of the animal host. Perhaps the most important function of the indigenous intestinal microflora to the host is its ability to interfere with colonisation of the intestinal tract with exogenous microorganisms, including pathogens. Terms such as bacterial antagonism (*Freter*, 1956), competitive exclusion

(*Lloyd* et al., 1977), bacterial interference (*Aly* and *Shinefield*, 1982) and colonisation resistance (*van der Waaij* et al., 1971) have been used to describe this function. It is has become evident during the last few years that the mechanisms which control bacterial populations in the intestinal tract are complex and multifactorial. The objectives of this paper are to: 1) Describe in general terms the development of the intestinal flora in the infant intestinal tract; 2) Give an overview of the mechanisms that are involved in colonisation resistance and the role they may play in the infant intestinal tract; and 3) Examine the development of colonisation resistance in the hamster intestinal tract against *Clostridium difficile*.

DEVELOPMENT OF INTESTINAL FLORA

Microbial colonisation of the infant intestinal tract is a complex process lasting throughout the first year of life and is dependent upon complex regulatory mechanisms which involves the animal, its environment and diet, and the microbes themselves. However, despite the regulatory complexity and the wide variety of bacteria constantly infiltrating the intestinal tract of infants, the sequential accession of intestinal bacteria during the neonatal period occurs in characteristic and orderly patterns (*Harris* et al., 1976; *Lee* and *Gemmell*, 1972; *Long* and *Swenson*, 1977; *Salanitro* et al., 1977).

Microbial colonisation of the newborn infant begins immediately after birth; infants are colonised by microorganisms originating from both the maternal microbial flora and other human contacts as well as from an increasing array of inanimate objects. Initial colonisation is fortuitous, depending on the first suitable organisms to arrive at a particular site as well as such factors as the route of delivery, the type of nourishment received (breast milk or formula), and the degree of exposure to the hospital environment. Many of the microbes are not able to colonise habitats in the neonatal intestinal tract and disappear from it soon after birth. In general, the new-born is first colonised with non-fastidious organisms such as enteric Gram-negative bacilli and streptococci (*Lee* and *Gemmell*, 1972; *Long* and *Swenson*, 1977). These organisms are followed by predominantly anaerobic species, which slightly suppress the population sizes of the initial colonisers. Thus, one group of organisms after another become dominant only later to be suppressed by organisms which in turn are suppressed (*Lee* and *Gemmell*, 1972; *Long* and *Swenson*, 1977). This process continues through stages until a stable, climax flora develops which resembles the intestinal flora of adults and is dominated by many species of anaerobic bacteria. This generally occurs at the time the animals are weaned from a predominate milk diet. The typical adult microbial intestinal flora consists of over 400 different species of aerobic, facultative and anaerobic bacteria (*Moore* and *Holdeman*, 1974). Within a given individual, the composition of the colonic flora remains remarkably stable once the ecosystem has reached maturity (*Caugant* et al., 1981; *Gorbach* et al., 1967; *Tannock* and *Savage*, 1974).

The dramatic qualitative and quantitative fluctuations in the bacterial populations of the normal intestinal flora which occurs immediately after birth until the time the animal begins to sample solid food indicates that the

Table 1: Mechanisms of colonization resistance

Host regulatory mechanisms	
Receptor specificity	Antibody
Receptor analogues	Cellular immunity
Peristalsis	Antimicrobial agents
pH	Epithelial cell turnover
Hormones	Electrolyte composition
Diet	
Bacterial regulatory mechanisms	
Modification of bile acids	
Induction of immunologic processes	
Depletion of or competition for essential substrates	
Competition for bacterial receptor sites	
Creation of restrictive physiologic environments	
Elaboration of antibiotic-like substances	

normal flora is not well balanced at this time. It has been suggested that this may contribute to some of the intestinal diseases seen in young children since the protec-tive mechanisms of the normal flora are probably diminished or absent (*Cooperstock* and *Zedd*, 1983). For example, infant botulism is a disease in which *Clostridium botulinum* multiplies and produces its potent neurotoxin in the intestinal tract of infants up to about one year of age (*Arnon*, 1980). A similar type of intestinal infection with *C. botulinum* is extremely rare in adults. There is experimental evidence in animals which suggest that variations between the normal flora of infants and adults may account for differences in their susceptibility to *C. botulinum* intestinal colonisation (*Sugiyama*, 1979; *Sullivan* et al., 1988; *Wang* and *Sugiyama*, 1984). Additional examples of the susceptibility of the infant intestine to colonisation by pathogenic clostridia include *C. difficile* (*Rolfe*, 1988), in which up to 90% of infants less than one year of age are asymptomatically colonised while asymptomatic adults seldom have *C. difficile* in their intestinal tracts, *Clostridium spiroforme* (*Carman* and *Borriello*, 1984), a cause of colitis in baby rabbits, and *Clostridium perfringens* type A (*Dabard* et al., 1979), which, when combined with *C. difficile*, causes synergistic infection in young hares.

MECHANISMS OF COLONISATION RESISTANCE

The population levels and types of microbes in many climax communities of the gastrointestinal tract, and the succession of these communities, are regulated by multifactorial processes (*Savage*, 1977b; *Rolfe*, 1984b). Colonisation resistance includes all processes that hamper colonisation of the intestinal tract by exogenous microorganisms and are listed in Table 1. Some of these regulatory processes are exerted by the animal host, its diet and environment. Some are exerted by the microbes themselves. Mechanisms regulating the indigenous microflora are often redundant such that two or

more of these inhibitory mechanisms frequently function synergistically in controlling the growth of microorganisms. This section of the paper will describe a few mechanisms of colonisation resistance and the role they may have in the development of the infant intestinal microbial flora.

Influence of host on microbial flora

The animal host influences the composition and activities of the normal intestinal microbial flora by a variety of mechanisms. Some of these mechanisms are described below and discussed in relation to the development of colonisation resistance in the neonate intestinal tract.

Receptors on intestinal mucosa

The adherence of bacteria to intestinal epithelial cells undoubtedly influences the sequential development of the neonatal intestinal microflora which in turn influences the development of colonisation resistance. Furthermore, in many species the intestinal epithelial cells undergo marked developmental changes within the first few weeks of life. For example, *Cheney* and *Boedeker* (1984) demonstrated that an enteropathogenic strain of *Escherichia coli* does not adhere to brush borders prepared from rabbits 15 days of age or younger. Brush border receptors for this strain of *E. coli* were first detected in rabbits 21 days of age and by 35 days of age the brush border receptor activity had reached adult levels. Examination of brush border lactase activity revealed that the emergence of *E. coli* receptors correlated with the onset of developmental changes associated with weaning.

Antimicrobial agents

Ingestion of antimicrobial agents by the animal host can have dramatic influences on the sequential development of the neonatal intestinal flora and thereby influence the development of colonisation resistance (*Bennet* et al., 1984). Aside from their ability to destroy microorganisms outright, antimicrobial agents impair the adherence of microorganisms to epithelial cells, even when present in sub-inhibitory concentrations, and therefore their ability to colonise the host (*Vosbeck* et al., 1979).

Diet

Several investigators have observed major differences in the infant intestinal microflora that correlates to the type of milk diet received by the infant (*Benno* et al., 1984; *Stark* and *Lee*, 1982). For example, bifidobacteria are usually identified as the predominant flora in breast-fed infants, while enterobacteria, *Bacteroides*, and clostridia are found in low numbers. In contrast, bacterial populations in the faeces of formula-fed infants are more diverse with higher numbers of enterobacteria and lower numbers of bifidobacteria. Also, faecal *Bacteroides* and clostridia occur in larger numbers and in higher percentages of formula-fed infants. The effect of infant diet on the development of colonisation resistant is evident when studying *C. difficile* intestinal colonisation. Investigators have isolated *C. difficile* significantly more often from the intestinal tracts of formula-fed than breast-fed infants (*Cooperstock* et al., 1982; *Tullus* et al., 1989).

Immunologic factors

There is considerable controversy as to whether an immunological response by the host influences the development of the composition of the indigenous flora. Nonetheless, there are a number of possible mechanisms by which local intestinal antibody or breast milk antibody might influence the development

of the intestinal flora as well as colonisation resistance. These include direct interference with bacterial attachment to epithelium, or interactions with other defence mechanisms including macrophages, the lactoperoxidase system, lactoferrin, lysozyme or complement. In addition, synergistic interactions between the immunologic response of the host and the direct antagonistic effects of indigenous microorganisms may be important in normal flora development and colonisation resistance (*Shedlofsky* and *Freter*, 1974).

Bacterial antagonism

In addition to host regulated factors, bacterial antagonism is another important component in the development of the infant intestinal flora and colonisation resistance. Bacterial antagonism is the inhibition of growth or reduction in number of one bacterial species by one or more other bacterial species. *In vivo* and *in vitro* studies have defined several mechanisms by which one bacterium may inhibit the multiplication of another (Table 1). Although the importance of these mechanisms in regulating the composition of the normal flora is unclear, there is strong evidence that strictly anaerobic bacteria play an essential role in each of the mechanisms (*Freter* and *Abrams*, 1972). Some of these mechanisms are described below.

Bile acids

The same bile acids which are essential for digestion and absorption of dietary fats in the intestinal tract, as well as the metabolism of cholesterol, may play an important role in regulating the composition of the normal intestinal microflora (*Savage*, 1977a). The primary bile acids, cholic acid and chenodeoxycholic acid, are synthesised by the liver and are conjugated to either taurine or glycine. Human bile also contains conjugates of a secondary bile acid, deoxycholic acid, which is formed by the dehydroxylation of cholic acid. Conjugated bile acids are poor inhibitors of bacterial growth (*Savage*, 1977a). However, in the large intestine, the bile acid conjugates are hydrolysed to release free acids by a variety of bacteria, particularly anaerobes (*Drasar* and *Hill*, 1974). Investigators have demonstrated that both Gram-positive and Gram-negative intestinal bacteria are inhibited by free bile acids (*Floch* et al., 1972; *Williams* et al., 1975). Since many pathogenic bacteria are destroyed *in vitro* by deconjugated bile acids, it has been proposed that an indirect means by which the normal gastrointestinal flora may contribute to resistance to intestinal pathogens is by deconjugating bile acids. For example, the *in vitro* growth of *C. botulinum* is inhibited by low concentrations of the secondary bile acid lithocholic acid (*Huhtanen*, 1979). Higher concentrations of lithocholic acid are found in the intestinal contents of human adults, who are resistant to intestinal infection with *C. botulinum*, whereas lithocholic acid is not found in the intestinal contents of infants, who are susceptible to intestinal infection with *C. botulinum* (*Bonglovanni*, 1965).

Depletion of or competition for essential substrates

Competition for carbon and energy sources has been proposed as the major mechanism controlling bacterial populations in static and continuous flow cultures of mouse caecal flora (*Freter* et al., 1983). Investigators have postulated that a "protective" normal bacterial flora consists of diverse group of indigenous microorganisms capable of using all the potential carbon and energy sources in a highly reduced environment (*Freter* et al., 1983). An exogenous organism attempting to en-

ter such an environment would not have an available energy source and would be unable to colonise the particular environment. However, if bacterial strains are absent or removed from the normal adult indigenous microflora, such as in the infant or through the use of antimicrobial agents, the limiting nutrient(s) that normally supported these strains will then increase in concentration and, at this higher concentration, will be able to support the growth of other bacteria, including enteric pathogens.

Competition for bacterial receptor sites

Mucosal attachment is a prerequisite for successful colonisation of the intestinal tract by both the indigenous microflora and pathogens (*Freter*, 1980). Adherence to epithelial cells not only prevents their expulsion but may also stimulate their growth, since nutrients tend to concentrate at solid-liquid interfaces. The adherence or lack of adherence of indigenous bacteria to intestinal epithelial cells has not been adequately investigated in human infants. However, studies have shown that bacterial competition for attachment sites in the small intestine can prevent adherence and subsequent pathogenicity of exogenous pathogenic microorganisms (*Savage*, 1980). For example, *Davidson* and *Hirsch* (1975) showed that mice and pigs orally inoculated with a non-toxigenic strain of *E. coli* possessing the K88 antigen were protected from subsequent oral challenge with an toxigenic strain of *E. coli* possessing the K88 antigen. The K88 antigen mediates attachment of *E. coli* to intestinal epithelium. No protection is observed when a non-toxigenic, K88 negative strain of *E. coli* is first introduced. This protection is attributed to the blocking of the K88 antigen receptor site on the intestinal epithelium by the non-toxigenic strain of *E. coli*.

Creation of a restrictive physiologic environment

Another means of bacterial antagonism is the creation of a physiologic environment by one microorganism which is inhibitory to another. By-products of bacterial metabolism which can contribute to the creation of a restrictive physiologic environment include hydrogen ion concentration, oxidation-reduction potential, and volatile fatty acids.

Hydrogen ion concentration

The pH of stool obtained from breast-fed neonates stabilises at a mean of about 5.0 to 5.5 after the first week of life and remains at this level as long as the infant receives a diet of only breast milk (*Bullen* et al., 1977). The poor buffering capacity of breast milk and the high counts of bifidobacteria undoubtedly account for the low pH of the intestinal contents of breast-fed infants. In contrast, the mean pH of stool obtained from formula-fed neonates may reach values as high as 8.5, suggesting that the intraluminal metabolic events in formula-fed infants differ dramatically from breast-fed infants. The low pH of intestinal contents from breast-fed infants undoubtedly influences the type of bacteria which can colonise the intestinal mucosa. Low pH is considered to be the major mechanism by which lactic acid producing bacteria (primarily *Lactobacillus*, *Bifidobacterium* and *Streptococcus*) inhibit the *in vivo* and *in vitro* growth of various facultative and anaerobic bacteria (*Tannock*, 1984).

Oxidation-reduction potential

The oxidation-reduction potential of the gastrointestinal tract immediately after birth is positive (*Grutte* et al., 1965). However, within the first few days of life facultative bacteria colonise the intestinal tract and create a re-

duced environment favourable to the subsequent appearance of anaerobic bacteria. The oxidation-reduction potential of the intestinal tract then continues to decline to the extremely reduced levels characteristic of adults. A role for low oxidation-reduction potential in protection against enteric infection has been proposed by *Meynell* (1963). He suggested that resistance of conventional mice to enteric infections is due to the combined effects of low pH, inhibitory concentrations of volatile fatty acids and low oxidation-reduction potential.

Volatile fatty acids

Volatile fatty acids (VFAs) are present throughout the intestinal tract as end-products of the fermentation of soluble carbohydrates and other nutrients by components of the intestinal flora. Several investigators have shown VFAs to be inhibitory to indigenous and non-indigenous bacterial components of the intestinal tract and have postulated that VFAs play a role in the sequential development of the neonatal intestinal microflora and colonisation resistance (*Byrne* and *Dankert*, 1979; *Freter* et al., 1983; *Lee* and *Gemmell*, 1972; *Meynell*, 1963; *Pongpech* and *Hentges*, 1989). Acetic acid is the major, and often the only, fatty acid in stools of breast-fed neonates during the first few days of life (*Bullen* et al., 1977). The presence of acetic acid is presumably due to the predominance to *Bifidobacterium*, a major acetic acid producer. Later in life, some breast-fed infants may also have low levels of propionic and butyric acids in their intestine. On the other hand, a variety of volatile fatty acids are usually present in the intestines of formula-fed infants, including acetic, butyric and propionic acids. Interestingly, it is during the period in which the concentrations of volatile fatty acids are increasing that there is a marked decline in the number of intestinal *E. coli* and streptococci. The pH of the intestinal environment is extremely important in the inhibitory activity of VFAs. At pH levels above 7.0, the VFAs are primarily in the dissociated state and unable to inhibit microbial growth (*Hentges*, 1983). On the other hand, as the pH is lowered, the proportion of undissociated acid molecules increases and the acids are then able to enter the bacterial cell resulting in inhibition of metabolism.

Elaboration of an antibiotic-like substance

Another mechanism of bacterial antagonism is the production of an antibiotic-like substance by one microorganism which inhibits the multiplication of another. The chemical nature and mode of action of these inhibitory substances are quite diverse and include ammonia, hydrogen peroxide, haemolysins, lysostaphins, bacterial enzymes, bacteriophage tails, defective bacteriophage and bacteriocins.

The most extensively studied of the antibiotic-like compounds are the bacteriocins. Practically every genera of bacteria have been shown to produce bacteriocins or bacteriocin-like compounds. A bacteriocin is defined as a diffusable substance produced by a microorganism which possesses an essential biologically active protein moiety and has a bactericidal mode of action against other bacterial strains but not against the producing microorganism. The significance of bacteriocins as regulators of bacterial populations is unclear. It was suspected for many years that the stability of the intestinal flora and its resistance to colonisation by exogenous bacteria was the result of elaboration of bacteriocins by resident intestinal microorganisms. However, although *in vitro* production of bacteri-

ocins by intestinal flora components has been demonstrated, results from *in vivo* studies indicate that these substances are of little ecological significance in the intestinal tract (*Ikari* et al., 1969).

DEVELOPMENT OF COLONISATION RESISTANCE AGAINST *CLOSTRIDIUM DIFFICILE*

Toxigenic *C. difficile* is the major aetiologic agent of pseudomembranous colitis associated with antimicrobial administration in humans and of antibiotic-induced ileocaecitis in Syrian hamsters, the latter serving as an animal model of this disease (*George*, 1988; *Onderdonk*, 1988). It has also been shown that *C. difficile* is an aetiologic agent in approximately 30% of cases of antimicrobial agent-associated non-specific colitis and in approximately 20% of cases of antimicrobial agent-associated diarrhoea without colitis (*George*, 1988). Although the mechanisms by which *C. difficile* causes diarrhoea and mucosal injury are not entirely understood, at least two potential virulence factors have been reported; an enterotoxin (toxin A) and a cytotoxin (toxin B) (*Donta*, 1988; *Lyerly* and *Wilkins*, 1988).

An extremely interesting observation is the high percentage of healthy neonates which harbour both intestinal *C. difficile* and cytotoxin. Up to 90% of healthy infants less than one year of age are asymptomatically colonised with toxigenic *C. difficile* (*Rolfe*, 1988). Intestinal carrier rates for *C. difficile* in healthy infants decline to approximately 30% during the second year of life, a carrier rate still higher than the 0 to 4% reported to occur in the healthy adult population (*Rolfe*, 1988). It is unclear why healthy, non-antibiotic treated infants are susceptible to *C. difficile* colonisation whereas healthy, non-antibiotic treated adults are usually resistant. However, the above data suggests that a developmental change in resistance to *C. difficile* intestinal colonisation occurs somewhere between infancy and adulthood. This section of the paper will review the *in vivo* and *in vitro* experiments which suggest that the variations between the intestinal microflora of infants and adults may account for the differences in their susceptibility to *C. difficile* intestinal colonisation.

EVIDENCE THAT THE INTESTINAL FLORA IS IMPORTANT IN PROTECTING THE HOST AGAINST *CLOSTRIDIUM DIFFICILE* INTESTINAL COLONISATION

Why *C. difficile* readily colonises the intestinal tracts of non-antibiotic treated infants and is relatively rare in healthy adults is unknown. However, a majority of the theories proposed to explain the mechanisms by which *C. difficile* overgrows in the intestinal tract consider the inhibitory interactions which undoubtedly exist between *C. difficile* and the normal intestinal bacterial flora. There is considerable experimental evidence that the intact gastrointestinal bacterial flora is important in protecting the host against *C. difficile*-associated intestinal disease. This experimental evidence is briefly described below.

Antibiotic treatment

The administration of an antimicrobial agent to adult hamsters, guinea pigs, mice and rats increases their susceptibility to intestinal colonisation with toxigenic *C. difficile* (*Wilson* et al., 1986). In the absence of antibiotics these adult animals are resistant to intestinal colonisation by *C. difficile*. The most obvious explanation for this association between antibiotic use and *C. difficile* intestinal overgrowth is that antimicrobial agents eliminate or suppress key normal flora components which normally inhibit *C. difficile* intestinal overgrowth. Studies have been performed to evaluate the qualitative and quantitative changes in the intestinal flora permitting colonisation by *C. difficile* (*Mulligan* et al., 1984; *Onderdonk* et al., 1977). These studies can probably be summarised best by stating that most antibiotics which commonly induce *C. difficile* intestinal overgrowth cause such massive changes in the colonic flora that it is impossible to determine which changes are important in allowing *C. difficile* to colonise in numbers large enough to cause disease.

Infant colonisation

As mentioned above, differences between the bowel flora of infants and that of adults appears to be sufficient to influence colonisation by *C. difficile*. In neonates, *C. difficile* flourishes before the normal intestinal flora has the opportunity to become established (*Rolfe*, 1988). Presumably, the intestinal tracts of human infants lack essential components present in adult flora that prevent colonisation of the intestine by *C. difficile*. Human infants are not unique in their colonisation by *C. difficile*. *C. difficile* is the causal agent of neonatal diarrhoea in conventional and gnotobiotic young hares and other strains of *Clostridium*, especially *C. perfringens*, enhances its pathogenic effect (*Dabard* et al., 1979). As will be described in greater detail later, infant hamsters are also readily colonised with *C. difficile*.

Germfree animals

The importance of the indigenous microbial flora in protecting against colonisation by *C. difficile* has also been demonstrated in gnotobiotic animals. The intestinal tracts of germfree mice (*Onderdonk* et al., 1980), rats (*Czuprynski* et al., 1983) and hares (*Dabard* et al., 1979) are readily colonised with *C. difficile*. On the other hand, adult mice, rats and hares with a conventional microflora are resistant to *C. difficile* intestinal colonisation even when large numbers are inoculated orally. Furthermore, when the intestinal flora of healthy humans, mice, hamsters or hares is introduced into mice previously monoassociated with *C. difficile*, the pathogen is suppressed to undetectable levels within 3 weeks (*Itoh* et al., 1987; *Jin* et al., 1984; *Raibaud* et al., 1980; *Wilson* and *Freter*, 1986; *Wilson* et al., 1986).

Similar observations were made in continuous flow cultures in which hamster caecal microbial flora markedly suppressed the growth of *C. difficile* (*Wilson* and *Freter*, 1986; *Wilson* and *Perini*, 1988).

***In vitro* inhibition**

A number of faecal bacterial have been shown to be antagonistic to the *in vitro* growth of *C. difficile* (*Barclay* and *Borriello*, 1982; *Malamou-Ladas* and *Tabaqchali*, 1982; *Rolfe* et al., 1981). For example, *Rolfe* et al. (1981) examined 23 representative anaerobic and aerobic genera for antagonism against *C. difficile* growth using two *in vitro* procedures. Strains of bacteria in six of the genera inhibited the multiplication of *C. difficile*, with

lactobacilli and group D enterococci displaying the most antagonistic activity.

An *in vitro* method for measuring colonisation resistance against *C. difficile* has been developed which is based on the growth of *C. difficile* in faecal emulsions prepared from the faeces of different patient groups and subjects of different ages (*Borriello* et al., 1988). Generally, faecal emulsions derived from infants, children, and geriatric patients are less inhibitory than those of healthy adults. It has also been reported that the faeces of breast-fed infants are significantly more inhibitory to growth of *C. difficile* than were the faeces of formula-fed infants.

In vivo inhibition by faecal homogenates

The importance of the normal intestinal flora in protection against *C. difficile* colonisation is further supported by the work of *Wilson* et al. (1981). These investigators demonstrated that vancomycin-induced ileocaecitis can be prevented in hamsters by daily enema and orogastric administration of homogenised caecal contents prepared from healthy hamsters not receiving antimicrobial agents. The protective effect of the caecal homogenates is eliminated by heating at 100°C for 20 minutes, by filtration through a 0.22 mm membrane filter, or by exposure to clindamycin but was not eliminated by exposure to gentamicin or vancomycin. These studies indicate that specific bacterial components present in the homogenates are responsible for preventing the establishment of *C. difficile* intestinal colonisation.

Similar approaches to the restoration of faecal homeostasis have been shown to be clinically effective in patients suffering from relapsing intestinal disease due to *C. difficile* (*Bowden* et al., 1978; *Schwan* et al., 1984; *Tvede* and *Rask-Madsen*, 1989).

Inhibition by non-toxogenic strains of *Clostridium difficile*

Wilson and *Sheagren* (1983) and *Borriello* and *Barclay* (1985) have demonstrated that prior colonisation of clindamycin-treated hamsters with non-toxigenic strains of *C. difficile* protects them from subsequent colonisation with pathogenic strains. The protection observed requires the presence of viable, non-toxigenic *C. difficile*. The protection noted in these experiments was specific in that other species of clostridia would not protect the animals against disease (*Borriello* and *Barclay*, 1985). Colonisation with non-toxigenic strains of *C. difficile* has also been effective treatment for recurrent *C. difficile*-associated colitis in humans (*Seal* et al., 1987).

In summary, there is a wealth of data to show that components of the normal intestinal flora are involved in resistance to colonisation by *C. difficile*. However, we are still a long way from delineating the microbial components (or their metabolic products) that are responsible for this effect in humans.

CLOSTRIDIUM DIFFICILE COLONISATION OF INFANT HAMSTERS

The asymptomatic intestinal colonisation of infant humans by *C. difficile* undoubtedly reflects fundamental differences in the composition and physicochemical milieu in different age groups. Investigations examining the ability of *C. difficile* to colonise the intestinal tracts of infant hamsters and

Table 2: Colonization of Infant Hamsters with *Clostridium difficile*[a]

Age (days) of hamsters at time of challenge[b]	Number of hamsters in each age group	Percent of hamsters colonised[c]	*C. difficile* CFU/gram (wet weight)[d]	Cytotoxin titer[e]
1	18	0	0	0
2	26	0	0	0
3	22	0	0	0
4	24	58	6.5±1.7	1.7
5	17	73	6.6±2.3	2.2
6	19	77	7.1±0.6	3.4
7	20	100	7.3±0.8	4.8
8	22	82	7.2±1.1	4.2
9	22	50	6.7±1.9	1.8
10	16	25	5.1±1.7	0.9
11	24	25	4.9±2.5	0.5
12	23	0	0	0
13	19	0	0	0
14	20	0	0	0
15	25	0	0	0
16	17	0	0	0
>70	10	0	0	0

[a]Adapted from *Rolfe* (1984a)
[b]Hamsters were challenged orogastically with 10^7 viable cells of *C. difficile*
[c]Hamsters were sacrificed 72 hours after *C. difficile* challenge and their intestinal tracts were examined for the presence of *C. difficile*
[d]Mean (log 10) ± standard deviation per gram (wet weight) of intestine. Only hamsters colonized with *C. difficile* are included.
[e]Cytotoxin titer expressed as the log 10 of the reciprocal of the highest dilution that produced actinomorphic changes of at least 50% of the cells in the monolayer. Only hamsters with detectable levels of cytotoxin are included.

the feasibility of using this animal model to study parameters associated with the asymptomatic colonisation of the infant are described below. In particular, the role of the intestinal flora and short-chain volatile fatty acids in colonisation resistance against *C. difficile* was examined in infant and adult hamsters (*Iaconis* and *Rolfe*, 1986; *Rolfe* and *Iaconis*, 1983; *Rolfe*, 1984a; *Rolfe* and *Iaconis*, 1985; *Rolfe* et al., 1986).

Asymptomatic colonisation

Using infant hamsters as a model, experiments were performed to determine whether infant hamsters are colonised with *C. difficile* and, if colonised, what effect colonisation has on the health of the animals. Non-antibiotic treated hamsters of different ages received 10^7 viable cells of a toxigenic strain of *C. difficile* orogastrically and 72 hours later were sacrificed and their intestinal tracts examined for the presence of *C. difficile* (*Rolfe* and *Iaconis*, 1983). The results, summarised in Table 2, show that hamsters have an age dependent susceptibility to non-lethal enteric *C. difficile* colonisation similar to human infants. *C. difficile* only colonised the intestinal tracts of hamsters between 4 and 11 days of age. Hamsters younger and older were resistant to *C. difficile* intestinal colonisation. In animals colonised with *C. difficile*, maximum population levels around 4 x 10^7 occurred in animals 7 days of age,

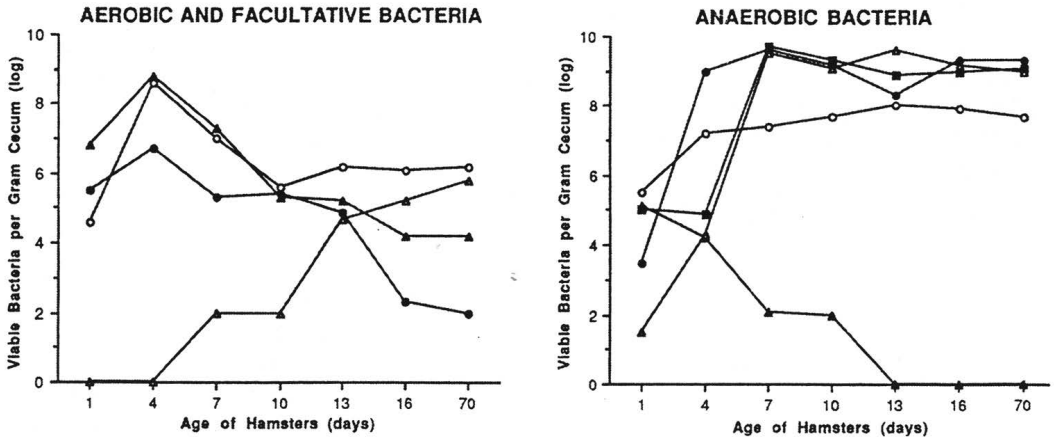

Figure 1: Development of the aerobic, facultative and anaerobic caecal flora of hamsters. (Adapted from *Rolfe*, 1984).
Three hamsters at each age were sacrificed, and the aerobic, facultative and anaerobic caecal flora was determined with selective and non-selective media. The results are depicted as the average concentration (log 10) of bacteria per gram of caecum (wet weight).
Symbols for aerobic and facultative bacteria: ¤, Gram-negative bacilli; ❍, *Streptococcus* sp.; ●, *Staphylococcus* sp.; ɋ, Gram-positive bacilli.
Symbols for anaerobic bacteria: ●, *Bacteroides* sp.; ❍, *Lactobacillus* sp.; ■, Gram-positive cocci; ▲, *Veillonella* sp.; △, *Clostridium* sp.

after which the population decreased progressively becoming undetectable by 12 days of age. The colonised infant hamsters do not display evidence of toxicity despite numbers of *C. difficile* and titres of cytotoxin comparable to those found in the intestinal tracts of adult hamsters with *C. difficile*-associated intestinal disease. Colonisation of the infant hamsters intestinal tract with *C. difficile* was persistent in that *C. difficile* could be recovered from hamsters up to 8 days after intragastric challenge of 4 day old hamsters with this microorganism. The number of *C. difficile* required to colonise 50% of 7 day old hamsters was 18 viable cells whereas 10^8 viable cells of *C. difficile* failed to colonise the intestinal tracts of non-antibiotic treated adult hamsters.

Development of normal intestinal flora

The infant hamster model of asymptomatic *C. difficile* intestinal colonisation was next used to examine the mechanisms responsible for differences in susceptibility of infant and adult hamsters to *C. difficile* intestinal colonisation. The development of the intestinal flora in hamsters was first examined to determine if the differing susceptibilities of hamsters to enteric *C. difficile* colonisation could be related to differences in the composition of the intestinal flora (*Rolfe*, 1984a). Figure 1 depicts the progression of ecological succession in hamsters from 1 day of age through weaning and adulthood. Gram-negative aerobic bacilli and streptococci were the predominant microorganisms in the caeca of 1 day old animals, whereas the concentrations of aerobic and anaerobic bacteria were approximately the same in 4 day old hamsters. After 4 days of age there was a steady decline in the counts of aerobic bacteria, with the exception of aerobic Gram-positive bacilli. These microorganism were first detected in

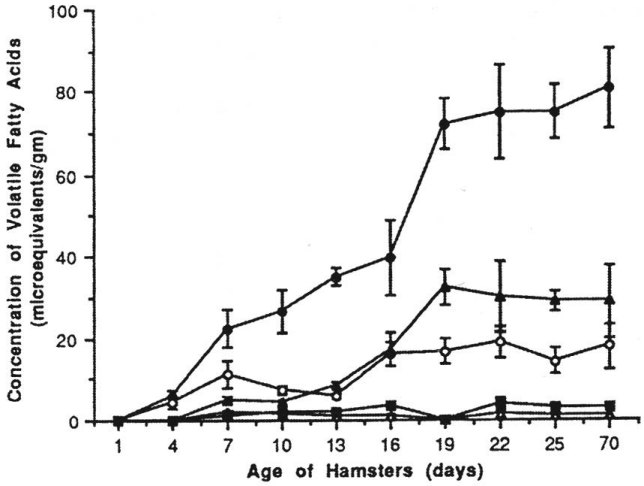

Figure 2: Concentrations of volatile fatty acids in the caeca of hamsters as determined by gas-liquid chromatography. (Adapted from *Rolfe*, 1984).
The values are mean concentrations (micro-equivalents per gram of caecal content [wet weight]) + standard deviation of volatile fatty acids for five pools of five hamsters each at ages 1 and 4 days and five individual animals at all other ages. Symbols: ●, acetic acid; ▲, butyric acid; ❍, propionic acid; ■, valeric acid; △, isovaleric acid.

animals 7 days old; Thereafter, their population levels increased with the age of the hamsters. The total caecal population of anaerobic bacteria continued to increase from the time of birth until the animals were approximately 7 days old, after which counts fluctuated between 10^9 and 10^{10} bacteria per g of caecum. The anaerobic bacteria remained the predominant members of the caecal flora in hamsters more than 4 days old, exceeding the caecal concentrations of aerobic bacteria by as much as 10,000 fold. There was a close similarity in the manner in which the intestinal flora developed in hamsters and the development of the intestinal flora reported for other species of animals, including humans (*Smith* and *Crabb*, 1961; *Stark* and *Lee*, 1982). Therefore, although the precise composition of the colonic flora of hamsters is different from man, the overall structure of the ecosystem and presumably the principles which govern bacterial interactions are similar. Unfortunately, it was not possible to directly correlate these changes in the composition of the intestinal flora to the development of *C. difficile* colonisation resistance. The majority of changes in the hamsters intestinal flora occurred before the age at which hamsters become resistant to *C. difficile* enteric colonisation.

Role of volatile fatty acids

The development of colonisation resistance to *C. difficile* intestinal colonisation correlated with the time at which the hamsters began to sample solid food. The changing diet of infant hamsters may have resulted in alterations of the metabolic activities of the intestinal flora, leading to the creation of a restrictive physiologic environment in the intestinal tract. Diet has been shown to influence the concentrations of VFAs in the intestinal tracts of adult and infant animals (*Byrne* and *Dankert*, 1979). In addition, several investigators have presented experimental evidence that VFAs are important regulators of bacteria populations in the intestine

Table 3: *In vitro* inhibitory activity of volatile fatty acids against *Clostridium difficile* at different pH values[a]

Acid	MIC (μeq/ml) at pH:		
	7.0	6.6	6.0
Acetic	100	100	50
Propionic	50	50	25
Butyric	25	25	6.2
Isovaleric	12.5	12.5	3.1
Valeric	6.2	6.2	1.5

[a]Adapted from *Rolfe* (1984a)

(*Bohnhoff* et al., 1964; *Freter* et al., 1983; *Meynell*, 1963). Therefore, the concentrations of VFAs and pH values were measured in the intestinal tracts of infant and adult hamsters to determine whether they could account for observed differences in colonisation resistance against *C. difficile* (*Rolfe*, 1984a).

The concentrations of short chain VFAs in the caecal contents of infant and adult hamsters are shown in Figure 2. As expected, the concentrations of caecal VFAs increased with the age of the hamster. No VFAs were present in the caeca of 1 day old hamsters. Thereafter, the concentrations of acetic and butyric acids increased and attained maximum concentrations at approximately 19 days of age. The other major VFA, propionic acid, also increased in concentration with the age of the hamsters. However, the caecal concentrations of propionic acid displayed more fluctuation than was observed for acetic and butyric acids. In addition, propionic acid attained maximum concentrations when the hamsters were approximately 16 days old. Low and variable concentrations of isovaleric and valeric acids were present in hamsters 7 days of age or older. These two acids accounted for less than 9% of the VFAs in any caecal sample. The average pH of the caecal contents in hamsters of all ages ranged from 6.6 to 7.0.

The *in vitro* inhibitory activities of acetic, propionic, butyric, isovaleric, and valeric acids against *C. difficile* were assayed individually in broth at different pH levels (Table 3) (*Rolfe*, 1984a). All five VFAs became more inhibitory as the pH of the medium became more acidic. However, the inhibitory activity of the VFAs remained constant at pH levels between 6.6 and 7.0. Although acetic acid attained the highest concentration in the caeca of hamsters, only butyric acid reached concentrations inhibitory to the in vitro multiplication of *C. difficile*. This is probably due to the fact that a greater proportion of butyric acid than acetic acid is in the undissociated state at the normal pH of the caecum (*Maier* et al., 1972). However, an inhibitory concentration of butyric acid was not attained until the hamsters were approximately 19 days of age, several days past the age at which they became resistant to enteric *C. difficile* colonisation.

C. difficile was inoculated next into broth containing mixtures of VFAs which were prepared to correspond to the concentrations found in the caeca of hamsters of different ages (*Rolfe*, 1984a). In addition, the pH of the VFA mixtures was adjusted to correspond to the average pH found in the caeca of hamsters at each particular age. The

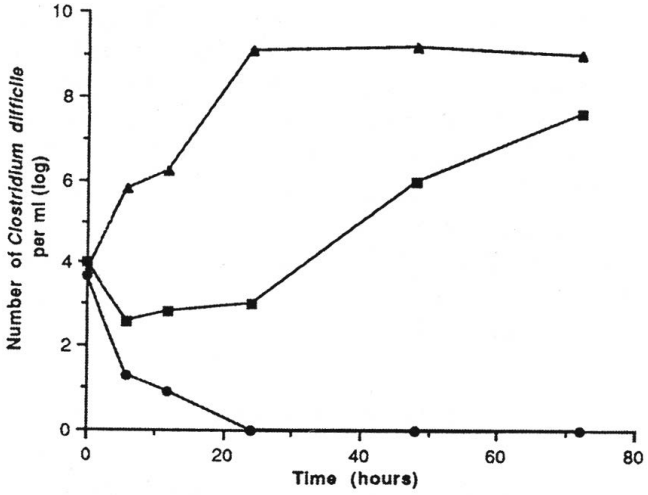

Figure 3: Growth of *Clostridium difficile* in broth containing mixtures of volatile fatty acids at average concentrations present in the caecal of hamsters at different ages. (Adapted from *Rolfe*, 1984).
The pH of the volatile fatty acid mixtures was adjusted to correspond to the average pH measure in the caeca of hamsters at each particular age. The following symbols indicate age (in days): ▲, 1 to 13 days of age; ■, 16 days of age; ●, 19 days of age and older.

results of these experiments, depicted in Figure 3, demonstrated a direct correlation between the *in vitro* inhibitory activity of the VFA mixtures and the susceptibility of hamsters 4 days of age or older to *C. difficile* intestinal colonisation. The concentrations of VFAs in the caeca of hamsters between 1 and 13 days of age did not inhibit the multiplication of *C. difficile*. The growth of *C. difficile* in these concentrations of VFAs was similar to the growth observed in broth without VFAs adjusted to the same pH. The VFAs present in the caeca of 16 day old hamsters, on the other hand, caused an initial 1 to 2 log 10 decrease in the number of viable *C. difficile*. This loss in viability was followed by an 18 h stationary phase, after which multiplication of *C. difficile* resumed but at a slower rate than observed in broth without VFAs. The concentrations of VFAs present in the caeca of hamsters older than 16 days of age were immediately bactericidal for *C. difficile*. These results suggest that VFAs are a contributing factor which accounts for the *in vivo* inhibition of *C. difficile* multiplication. Sixteen days is approximately the age at which hamsters become resistant to intestinal colonisation with *C. difficile*. The depression of *C. difficile* multiplication by concentrations of VFAs present in the caecal contents of 16 day old hamsters may be sufficient to permit other antibacterial mechanisms (e.g., peristalsis, bile acids, competition for nutrients) to completely eliminate *C. difficile* from the intestinal tract. Given the complexity of the colonic flora, there is not likely one simple explanation for the suppression of *C. difficile*. Furthermore, the colonic microflora is known for its redundancy of control mechanisms by which the population size of a given bacterial species is controlled (*Freter*, 1975).

Interestingly, the concentration of VFAs increased in hamsters 7 days of age and older even though the counts

of anaerobic bacteria remained approximately constant in these animals. However, since the caecum of rodents regularly harbour around 10^{11} bacteria per gram of wet weight, it would appear that on day 16 of succession less than 10% of the cells present were actually isolated and identified. Thus, the predominant flora very likely did continue to change after day 7, and this points out the extreme difficulty of cultivating the predominant anaerobic flora of the colon.

The bacterial components in the caecum responsible for the production of inhibitory concentrations of VFAs are unknown. Investigators have postulated that the anaerobic bacterial flora is the principal producer of VFAs and the most important constituent of colonisation resistance in the gastrointestinal tract (*Hentges*, 1979; *van der Waaij* et al., 1971).

The resistance of hamsters less than 4 days of age to *C. difficile* intestinal colonisation is apparently due to factors other than the presence of VFAs. Caecal microbial flora studies have shown that anaerobic bacteria are not present in high concentrations in the caeca of hamsters less than 4 days of age (*Rolfe*, 1984a). Furthermore, investigations have shown that the oxidation-reduction potential in the caeca of 1 day old hamsters is sufficiently high to prevent multiplication of *C. difficile* (*Rolfe*, 1984a). However, the oxidation reduction potential decreases to a level permitting *C. difficile* multiplication within 4 days after birth.

CONCLUSION

There is convincing evidence that the indigenous intestinal flora provides natural protection against infection by a number of pathogenic bacteria. The protective mechanisms are impaired, however, when the normal flora is disturbed, such as through the use of antimicrobial agents, or before the normal flora has a chance to fully develop, as in new-borns and infants. A completely satisfactory understanding of the succession of the normal flora in neonates and infants and the development of colonisation resistance may not soon be achieved because of the complexity of the ecosystem. However, this is basic information which must be understood if we ever hope to control intestinal diseases of infants.

There is considerable evidence that a variety of mechanisms operate to exclude pathogens from the intestinal tract. The mechanisms involved in colonisation resistance against infection by opportunistic and pathogenic organisms are complex, as are the interactions between the hundreds of bacterial species present in the intestine that are responsible for the protective mechanisms. It is unlikely that a single species is responsible for the inhibitory effect of the normal flora on potential pathogens. Instead, synergistic relations between members of the normal flora appear to be important in suppressing the intestinal colonisation by potential pathogens. Further work will be necessary to reveal the relative contributions of the various factors in maintaining the integrity of the intestinal flora. However, possibilities exist for the controlled manipulation of the normal microflora so that health promoting activities of the microbes are emphasised.

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