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Infectious gastro–enteritis — opportunities for dose response modelling

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Summary

When pathogenic microorganisms enter the human body via ingestion with food or drinking water, they encounter a system of barriers mounted by the host. In order to reach parts of the intestinal tract that are suitable for growth and attachment, each of the barriers must be overcome successfully. The present view on infection states that at least one of the ingested pathogens must survive to start colonization. This is the basis for dose response models, used for quantitative risk assessment.
Defense mechanisms against microbial infection and invasion may be immunological or non–immunological. The intestinal tract has its own immunological system, the gut–associated lymphoid tissue (GALT), which plays an important role in host defenses against pathogens, but cells of the intestinal lymphoid tissue also appear to constitute a preferred site of translocation for many pathogens. In addition to this, a whole range of non–immunological barriers is present in the healthy host: excretions (digestive juices) and the low pH in the stomach, shedding of epithelial layers, peristaltic movement, and the resistance to colonization exerted by a normal intestinal microbial flora.
Many pathogens invade host cells, and take part in often complicated interactions with host cells. Many microorganisms have developed mechanisms to evade host defenses, and may excrete substances that disrupt the host cell metabolism in subtle ways. Recently even two–way communication (type III intercellular signalling) has been discovered between certain pathogenic bacteria and the host cells they attack. These insights can be used to adapt or improve dose response models, or even develop new models for certain aspects of the host response to gastroenteric pathogens. In this report, the validity of the Beta Poisson model for multiple barriers is demonstrated and some attention is given to the single hit principle. An approximation that is usually neglected receives some attention, and it is shown that for certain parameter values the approximation leads to results different from the exact formula. Finally, onsets are given for new models, incorporating extra information about infection and illness.

Samenvatting

Pathogene micro–organismen die het menselijk lichaam binnendringen via voeding of door het drinken van besmet water, krijgen te maken met een door de gastheer opgeworpen systeem van barrières. Teneinde delen van het spijsverteringskanaal te bereiken die geschikt zijn voor groei en hechting, moet elk van de tussenliggende barrières overwonnen worden. De gangbare visie op infectie gaat ervan uit dat minstens één van de ingeslikte pathogenen moet overleven om te kunnen koloniseren. Dit is de basis voor dosis–responsmodellen zoals toegepast bij de microbiologische risico–analyse.

Afweermechanismen tegen infectie en invasie door micro–organismen kunnen immunologisch zijn of non–immunologisch. Het spijsverteringskanaal heeft een eigen immuunsysteem, het 'gut–associated lymphoid tissue' (GALT), dat een belangrijke rol speelt bij de verdediging tegen pathogene micro–organismen. Cellen van dit systeem blijken echter tevens een voorkeurspositie in te nemen bij translocatie van verscheidene pathogenen. Daarnaast bestaat er in een gezonde gastheer nog een reeks niet–immunologische barrières: excreties (spijsverteringssappen) en de maagzuurbarrière, afstoten van de bovenste laag van het darmepitheel, peristaltische bewegingen en de resistentie tegen kolonisatie die wordt opgebracht door een normale darmflora.

Vele pathogenen dringen binnen in cellen van de gastheer en gaan een vaak complexe wisselwerking aan met de gastheercel. Vele micro–organismen hebben mechanismen ontwikkeld om de afweer van de gastheer te ontwijken, en kunnen stoffen uitscheiden die de stofwisseling van de gastheercel op soms subtiele wijze beïnvloeden. Recentelijk is zelfs een systeem ontdekt voor communicatie in twee richtingen (type III intercellulaire communicatie) tussen bepaalde pathogene bacteriën en de cellen die ze aanvallen.

Al deze inzichten kunnen worden gebruikt voor verbetering van bestaande dosis–responsmodellen, of voor de ontwikkeling van nieuwe modellen voor specifieke aspecten van de respons op pathogene micro–organismen. In dit rapport wordt de geldigheid van het Beta–Poissonmodel voor meer dan een barrière gedemonstreerd en wordt enige aandacht besteed aan het 'single–hit' principe. Ook besproken wordt een benadering in de afleiding van het Beta–Poissonmodel, en aangetoond wordt dat deze benadering voor bepaalde parameterwaarden resultaten oplevert die verschillen van de exacte formule. Tenslotte worden enkele aanzetten gedaan tot modellen, waarbij extra informatie over infectie en ziekte kan worden gebruikt.

Chapter 1

Introduction

The consequences of exposure to pathogenic micro–organisms are important for public health. In the Netherlands, the yearly incidence of gastro–enteritis is about 6 million cases, a substantial part of which possibly caused by an infective agent (Hoogenboom-Verdegaal, 1993).

Governments and public health officials urgently need a rational basis for setting standards to control the microbiological properties of foods, drinking water, and other vectors for pathogenic micro–organisms. This requires insight into the relation between intensity of exposure to pathogens and the health effects this may cause.

In many cases, it is not possible to directly assess the consequences of microbial contamination of foods or drinking water with epidemiological methods. This may be due to a very low dose, as is often the case with drinking water. The size of the exposed population may then still lead to non-negligible effects (Teunis et al., 1997b). In foods, illness cases may be difficult to find, because the causative agent cannot be traced in retrospect, or contamination of a particular portion of food may be a rare event. In addition to this, it is often of interest to estimate potential effects, at a stage when no actual illnesses have occurred yet. This is also important when the consequences of intervention measures are to be evaluated. In such cases, quantitative risk assessment may provide a viable alternative to epidemiological methods (Teunis et al., 1994).

In order to judge the significance of exposure to a certain pathogen, insight into dose response relations is indispensable. For instance, once a dose response relation has been established, this may be used for extrapolation, to predict effects after exposure to extremely low doses (Teunis et al., 1997b).

Gastro–enteric pathogens may cause many different effects, dependent on properties of both host and the pathogenic organism. Globally, three stages may be defined: infection, acute gastrointestinal illness, and chronic illness or complications. When the pathogen reproduces within the host's digestive tract, often accompanied by excretion of the newly formed organisms, infection is manifest (Last, 1995; Sengers et al., 1991; Finlay and Falkow, 1989). This may be accompanied by symp-

toms of acute gastro–enteritis: diarrhea, vomiting, fever, etcetera, lasting for some time (days, a few weeks at most). In a small number of cases the acute stage is followed by more serious, chronic symptoms, associated with systemic infection (liver malfunction, chronic fatigue, paralysis (autoimmune diseases, like Guillain–Barré syndrome). Eventually, even death may occur after systemic illness in weak patients, or after inadequate treatment of acute illness.

Infection occurs when a microorganism has been swallowed, has successfully passed the stomach, has succeeded in reaching a site suitable for colonization, and has reproduced, so that excretion and detection are possible. Hence, infection precedes illness: when a sufficiently high number of pathogens lives within a portion of the intestinal tract, this may lead to symptoms of acute gastro–enteritis. The growth rate leading to such an elevated concentration of pathogenic organisms possibly is an important factor for the development of illness symptoms.

The stages used here to describe the processes of infection and illness appear to be useful for the formulation of a mathematical model, but they represent a highly simplified view of the pathogenesis of gastro–enteritis. Many intermediate forms may occur, with no separate stages to be discerned. For many pathogenic organisms, descriptions of the effects may have to be specified separately, according to specific properties of the pathogen in question.

Dose response experiments with human volunteers exposed to controlled doses of pathogenic micro–organisms have been published for various gastroenteric pathogens. The results are usually fitted well with a single hit model (Haas, 1983; Teunis et al., 1996b), and the data have been successfully used for quantitative risk assessment (Haas et al., 1993; Teunis et al., 1997b, 1996a). Some serious shortcomings were also noted, however: the factor time is absent from the calculations (latency, or incubation period); the probability of illness changes differently with dose than the probability of infection (not monotonously increasing, but in some cases a decrease at very high doses is seen (Teunis et al., 1996b)); no opportunities for generalization with regard to microorganism (strain, prior conditions, related species, similar pathogenesis), host (general condition, immune status, conditions within the intestinal tract), and vector (water, properties of the food vehicle).

The question we would like to answer now is: which physiological variables (of both the host and the microorganism) are correlated with the symptoms that are relevant to us (to personal or public health), and are suitable as illness indicators? This report is an attempt to summarize and list information pertaining to this question, with special reference to experimental evidence from animal studies. Where possible, two aspects are studied separately: the growth of micro–organisms in the gastrointestinal tract; and the effects caused by the presence (or metabolism) of these micro–organisms.

The present literature study attempts to list available information on the sites within the host intestinal tract where growth or destruction of pathogenic micro–organisms takes place, and on the expected rates of growth or destruction. This involves knowledge on the influence of host defenses on the occurrence of infection: role of the immune system, motor patterns in the gut, role of the (bio-)chemical environ-

ment, and protective role of the normal intestinal microflora against colonization by foreign microbes. For quantitative analysis, interdependencies between these factors may also be important.
Knowledge about the numbers of pathogens living in various compartments of the digestive tract may then serve as a basis for describing the occurrence of acute symptoms. To do this, specific knowledge about interactions between the pathogenic microorganism and host tissues is required. In order to narrow the number of different symptoms to keep track of, classifications of diarrheal disorders may be used (Rijntjes, 1987):
Based on mechanism:

secretory diarrhea due to disturbed electrolyte metabolism of enterocytes

exudative diarrhea inflammatory reaction to tissue damage

osmotic diarrhea due to lowered resorption of low molecular weight substances

increased motility shortened contact time leading to decrease in resorption of water and dissolved substances

or, alternatively, based on pathogenesis:

enterotoxic diarrhea functional disturbance, without cell or tissue damage

invasive diarrhea cell damage by cytotoxic toxins or bacterial invasion

viral diarrhea decreased resorption as a result of enterocyte death and secretory reaction to inflammation

Terms from either of these two classifications may be used whenever they are suited for a particular application. They merely serve as shorthand descriptions for the multitude of different symptoms that may occur with gastro–enteritis.
Finally, the present state of affairs regarding dose response models for gastroenteric pathogens is described, and outlines are given for model descriptions of relevant aspects of gastro–enteritis.

Chapter 2

Defense mechanisms

The intestinal mucosa acts as a barrier against the translocation of infectious micro–organisms, both mechanically and immunologically (Table 2.1). In normal circumstances, this barrier is somewhat "leaky" and low numbers of bacteria may pass though the the mucosal lamina propria. Translocation appears to proceed via the intracellular or transcellular pathway, rather than extracellularly (by disrupting tight junctions between mucosal cells) (Berg, 1992). Hence, low numbers of non-pathogenic or weakly pathogenic organisms may be found in lymphatic and blood vessels of the intestinal tract. Normally, these low numbers of bacteria are killed by the host immune system.
The gastrointestinal tract has the ability to distinguish between 'acceptable' and 'non–acceptable' micro–organisms. Thereby, the ability to mount a defensive response against pathogens is preserved, while at the same time, the production of an inappropriate response against normal bacterial flora is avoided (Duncan and Edberg, 1995).

2.1 Immunological

The intestinal immune system is in constant contact with antigenic material, and should not react to every microorganism that passes the digestive tract. Immune tolerance is an important distinctive feature of the mucosal immune system, which still remains incompletely understood.

2.1.1 Humoral immunity

The gut–associated lymphoid tissue (GALT) includes lymphocytes and reticuloendothelial system in the lamina propria underlying the mucosal epithelium, organized aggregates in the mucosa, lymphoid follicles and Peyer's patches in the small intestine.
Peyer's patches are separated from the lumen by a single layer of columnar epithelium that includes microfold or M cells. M cells appear to be specialized antigen

Site	Major defenses
Oropharynx	Lysozyme
	Production of liquids
	Normal flora (attachment and bacteriocins)
	IgA antibody
	Proteolytic enzymes in saliva
Esophagus	Peristalsis
Stomach	Acid pH
	Proteolytic enzymes
Small intestine	Peristalsis
	Mucin production
	Bile acids
	IgA antibody
	Primary lymphoid system (Peyer's patches)
	Epithelial shedding
	Normal flora
Large intestine	Peristalsis
	Normal flora
	Epithelial shedding
	Mucin production

Table 2.1: The host defenses of the gastrointestinal tract (Duncan and Edberg, 1995).

uptake cells. They are covered with less extensive microvilli than normal villus cells, probably to render adhesion molecules on their luminal surface more accessible. Absorbed molecules are not lysed, but are transported to the basolateral cell surface, where there are macrophages and other antigen–presenting cells. Unlike M cells, intestinal villus cells have MHC class II receptors, possibly representing an additional antigen–presenting capacity to T cells in the lamina propria (Duncan and Edberg, 1995).

Antigen–presenting cells couple antigen with MHC class II receptors and activate CD4+ T cells, which then produce various lymphokines (IL–2, IL–4, IL–6, INFγ, etc.). Interleukin(IL)–6 appears to be highly important for the production of mucosal IgA *in vivo* (Lewis and E, 1995). These lymphokines stimulate B cells within Peyer's patches to commit to IgA production. These B cells exit via efferent lymph nodes and enter the portal circulation where they meet Kupffer cells. Kupffer cells can act as yet another blockade by clearing microbes and antigen–antibody complexes, thereby avoiding systemic immunological stimulation. Finally, these B cells return to the lamina propria to differentiate and start secreting IgA from the crypts of Lieberkühn. Secretory component (SC) is made by the epithelial enterocytes, is upregulated by T cell–released INFγ and TNFα, associates with the J chain of dimeric and polymeric IgA and IgM on the basolateral surface, and transports the antibodies to the luminal surface, where they are cleaved and released (Duncan and Edberg, 1995).

Recent studies have shown that differences exist between mucosal and systemic B–cell responses: mucosally derived B–cells are phenotypically more homogeneous,

express more advanced maturation markers, and lack L–selectin (associated with homing to peripheral lymph nodes). Memory T cells in the lamina propria also appear to be different from other memory cells: low CD45RA and high CD45RO expression, low CD29 expression. Integrin $\alpha 4\beta 7$, expressed on mucosal T and B cells, appears to play a key role as mucosal adressin (Lewis and E, 1995).
The most important function of secretory IgA is antigen exclusion and bacterial agglutination, to prevent antigen uptake by the epithelium. In addition, IgA seems able to neutralize micro–organisms and their toxins and inhibit their motility and growth. On the other hand, IgA does not fix complement efficiently and resists phagocytosis. IgA also prevents other immunoglobulins from interacting with the antigen. Thereby, it may play an important role in providing immune tolerance of the intestinal immune system (Duncan and Edberg, 1995).
IgG and IgM are also present in the GALT, in lower concentrations. Small amounts of IgE are associated with mast cells in the lamina propria.
Secretory IgA (S–IgA) from the intestinal lumen of infected mice appeared to have protective effects against infection of mice with *Vibrio cholerae* (Fubara and Freter, 1973). Application of S–IgA reduced the fraction of the total *Vibrio* population that was attached to the mucosa, but not the total number of pathogenic bacteria.
In addition to its primary function of providing an immune barrier against penetration of the intestinal mucosa by pathogens and their excretions, IgA may exert its action at other levels. Before crossing the mucosa to reach the intestinal lumen, IgA antibodies pass through epithelial cells themselves. There, they may interact with intracellular pathogens (e.g. viruses) and form complexes to enhance their excretion into the lumen (Lamm et al., 1995).

2.1.2 Cellular immunity

In the lamina propria, granulocytes and monocytes are found. The T cells in the lamina propria are mostly of the memory CD4+ type, with the ability to express high levels of cytokine mRNA, but lacking the expression of a lymph node homing receptor, unlike circulating CD4+ T cells. T cells, dependent on the T–cell subtype, are involved in the regulation of certain aspects of the humoral immune system. Besides this regulatory capacity, T cells play a crucial role in the resistance to many viruses, bacteria and parasites. In addition to the usual CD3+ T cells an intraepithelial T cell subset is present in the epithelium of the gut. These intraepithelial lymphocytes (IEL) have some unusual properties[1]. Upon activation, these IEL cells are cytotoxic against virally infected villus cells and secrete IFNγ, TNFα, and IL–2, which may help protect neighbour cells. According to theory, most of

[1] A large proportion of these CD3+ T cells are CD8+ or cytotoxic/suppressor T cells. However, 30 – 50% of these CD8+ T cells lack the mature T cell marker CD5 and many express an unusual CD8+ marker. About 12% exhibit the unusual T cell receptor $\gamma\delta$, instead of the usual $\alpha\beta$. Studies in mice indicate that these T cells differentiate independently of the thymus. A minor subset expresses only CD7, and not CD3, markers, which are usually seen on T cell precursors before entrance and development in the thymus.

these T cells would exhibit suppressor activity, thus contributing to the tolerance of the intestinal immune system to constant nonself antigen exposure (Duncan and Edberg, 1995).

2.1.3 Age dependency

The mucosal immune system represents a main line of defense against the entry of pathogens from the environment. This system is structurally mature by the time of birth, but functional maturity is reached after a period in which environmental and behavioural factors exert their influence (up to 12 months in duration). The mucosal immune system matures at a slower rate than its systemic counterpart (Husband and Gleeson, 1996). During the first year of life transient periods occur, in which IgA is absent in saliva, and there is increased susceptibility to bronchial hyperreactivity in later life. These hypoimmune periods seem to be associated with deficiencies in antigen processing, rather than deficient lymphocyte responsiveness (Husband and Gleeson, 1996). Initial intestinal inoculation via food appears to be associated with long–term maturation of the mucosal immune system. Breast feeding may increase the rate of mucosal maturation, thereby aiding in the protection against microbial pathogens. Immediately after birth, there is an increased intestinal permeability to macromolecules. In humans, this represents a potential hazard, because the immune system is in a tolerant state at this developmental stage[2]
The rate of maturation of the mucosal immune system may be depressed by several factors (Table 2.2) (Husband and Gleeson, 1996):

- Exposure to maternal antibody (transplacental, or with colostrum)
- Endogenous production of stress hormones
- Presence of external stress factors, like nutritional deficits, exposure to allergens, pollutants, or toxins.

2.2 Non–immunological

The non–immunological defense system represents the first line of defense against enteric pathogens. In humans living in hygienic circumstances, non–immunological defense probably offers sufficient protection. In patients with humoral and cell mediated immune deficiency, bacterial gastro–enteritis is not found more frequently than in immunocompetent persons (Sarker and Gyr, 1992).

2.2.1 Gastric acid

Gastric acid plays an important role in normal digestion, by facilitating breakdown of food material into digestible components, and augmenting dietary iron and cal-

[2]In humans, this may be an evolutionary legacy without clear benefits. In ruminants, for example, absorption of colostrum is essential for the acquisition of passive immunity.

Influencing factor	Result
Prenatal antigen exposure	Increase in IgA–containing cells at mucosal sites and IgA antibodies in secretions
Stress hormones	Corticosteroid immunosuppression at birth
	Prolonged period of mucosal membrane permeability
	Delayed onset of IgA production by GALT
Feeding regimes	Different colonization of gastrointestinal tract resulting in non–breast–fed infants having an accelerated mucosal immune response
Colostrum	Accelerated maturation of mucosal epithelium
	Reduced incidence of infection
	Reduced incidence of atopy
	Suppressed mucosal immune response
	Improved responses to vaccines
Nutritional deficits	Severe protein malnutrition results in secondary immune deficiency
	Vitamin and mineral deficiency results in poor immune responses
	Excessive protein or fat intake is immune suppressive
Infection	Accelerates mucosal immune responses in infants
	Associated with disturbance of immune regulation
Allergen exposure	Adverse reactions reduced by: prolonged breast feeding, maternal elimination diets, and high levels of colostral IgA
Smoking	Alters nasal mucosa and gastrointestinal function
	Increases respiratory morbidity and atopy
	Increases infection rates
Alcohol	Fetal exposure results in impaired cellular immune development
	Reduction in milk cytokines impairs vertical transmission of immunity to offspring and reduces immune protection

Table 2.2: Environmental factors influencing the postnatal maturation of the mucosal immune system (Husband and Gleeson, 1996).

cium absorption. The low pH in the human stomach also acts as a major barrier against pathogenic micro–organisms, and it suppresses colonization of the proximal bowel by oropharyngeal and faecal flora (Sarker and Gyr, 1992; Gorden and Small, 1993). In addition to this, the production of pepsin helps in denaturation and digestion of antigens and viable micro–organisms (Duncan and Edberg, 1995). The bactericidal action of gastric contents is effective at pH below 4.0 . Reduction of the acidity, either due to endogenous causes (e.g. aging, certain diseases like pernicious anemia, possibly infection by *Helicobacter pylori*) or as a result of malnutrition, or therapeutic inhibition, increases the susceptibility to infection by pathogenic organisms. Increased gastric pH may also lead to bacterial overgrowth: establishment of components of the oral or large bowel microflora in the stomach and the small intestine (Sarker and Gyr, 1992; Larner and Hamilton, 1994).
Achlorhydria or hypochlorhydria appears to increase the infectivity of *Vibrio cholerae* and *Campylobacter jejuni* in human feeding studies (Cash et al., 1974; Black et al., 1988; Teunis et al., 1996b). Increased gastric pH has also been observed to predispose for salmonellosis (typhoid and non–typhoid), shigellosis, giardiasis, and cholera. *Clostridium difficile* infection (pseudomembranous colitis) may also be associated with hypochlorhydria (Sarker and Gyr, 1992).
Conversely, bacterial, viral and parasitic infections suppress gastric acid production in man and animals. Bacterial infections like salmonellosis are associated with suppression of histamine stimulated acid secretion. Generally, the acid suppression mechanism is not well understood. Alterations of the mucosal morphology are often thought to be involved. Suppression appears to be stronger when there is fever, indicating that fever, rather than infection itself, may be the cause of suppression. An increase in stress induced endogenous prostaglandins (PGE_2) may further modulate hypochlorhydria associated with infection (Sarker and Gyr, 1992).

2.2.2 Epithelial cell turnover and mucus

The intestinal epithelium serves as a barrier against pathogen entry, with tight junctions preventing the passage of large molecules. The rapid turnover of epithelial cells (3 –6 days) removes damaged cells as a site for infection and minimizes opportunities for attachment of pathogenic micro–organisms (Duncan and Edberg, 1995).
Infection by a pathogenic microorganism often leads to an acceleration of the renewal rate of the epithelial surface. Epithelial cells, connective tissue stroma,and lymphocytes are disposed with exuded fluids. Pathogens adhering to these cells, or between epithelial cells, are washed away, as are infected epithelial cells. Together with peristaltic movements this acts as a removal mechanism against colonization. Aside from its function as a lubricant, protecting the epithelial surface from digestive juices and physical damage, the mucus layer also functions as a trap for micro–organisms, to prevent their attachment to epithelial cells. Mucus consists of a glycoprotein core surrounded by oligosaccharide units that protect it from proteolytic attack. With this composition, mucus traps bacteria by mimicking epithelial

receptors for bacteria, and acting as a physical sieve (Duncan and Edberg, 1995). Physical damage to the mucosa increases bacterial translocation to the mesenteric lymph nodes, liver, and spleen. Such conditions may be brought about by experimental chemical treatment, but also after hemorrhagic shock, thermal injury, or exposure to endotoxins, as produced by enteric pathogens (Berg, 1992).
Some organisms have succeeded in utilizing the extracellular matrix as a substrate for adherence. In the gastro–intestinal tract, this is a mode of action for *Helicobacter pylori* in the gastric mucosa (Ljungh et al., 1996).

2.2.3 Intestinal motility

In humans, the phase III activity of the interdigestive motor complex (MMC) occurs every 84 – 112 minutes and travels down the upper intestinal tract at a velocity of 6 – 8 cm per minute (Sarker and Gyr, 1992). The intense contraction within this motor pattern is also called the "intestinal housekeeper". This activity rapidly moves the contents of the digestive tract down the small intestine. This prevents stagnation and bacterial overgrowth, and counteracts colonization by pathogenic micro–organisms. Intestinal motility also redistributes the endogenous enteric microflora, and prevents migration of colonic organisms to the upper digestive tract.
In germfree animals, the intestinal propulsion rate appears to be decreased compared to that in animals with a normal intestinal flora. The presence of a normal microflora may thus be an important factor in the maintenance of a normal rate of intestinal emptying (Abrams and Bishop, 1967).
Changes in normal motility may adversely affect the intestinal ecology. Increased motility may lead to a reduction in normal microflora. A decrease in intestinal motor activity may lead to stasis and bacterial overgrowth.
There is not much information concerning the effects of enteric infection on intestinal motility. Bacterial enterotoxins may produce abnormal motor patterns in animals. In rabbits, cholera toxin produces abnormal neural activity, the "migrating action potential complex" (MAPC), with bursts of intense activity. In the same animal model, invasive bacteria elicited repetitive bursts of action potentials. Hence, different motor patterns may be associated with different types of pathogenesis in infectious diarrhea (Sarker and Gyr, 1992).

2.2.4 Pancreatic juice and bile

Patients with pancreatic exocrine insufficiency have more severe and prolonged episodes of acute diarrheal illness. Animal experiments have shown that pancreatic excretions are involved in the local defense against cholera under conditions of protein deficiency (Sarker and Gyr, 1992). Lactoferrin, present in pancreatic secretions, can have bacteriostatic effects by competing for iron (Duncan and Edberg, 1995). Pancreatic lipase appears to have bactericidal activity *in vitro*. Canine pancreatic fluid shows antibacterial activity against *E. coli*, *Shigella* spp, *Salmonella* spp, and *Klebsiella pneumoniae*. Bacteriostatic properties of pancreatic fluid have

		Mean no. of bacteria, $\log_{10}$/ml at different incubation times*				
		0h	2h	3h	6h	24h
Escherichia coli	PF	4.1	3.5	0	0	0
(24 strains)	C	4.0	4.8	7.2	8.6	9.2
Shigella spp	PF	4.2		3.4	2.6	1.1
	C	4.1		4.3	3.4	2.2
Salmonella typhimurium	PF	4.1		4.1	3.9	0
(6 strains)	C	4.1		4.7	7.5	9.0
Klebsiella pneumoniae	PF	4.1		4.1	3.9	0
(12 strains)	C	4.1		6.0	8.2	9.1
Pseudomonas aeruginosa	PF	4.1		4.2	4.2	4.3
(16 strains)	C	4.1		5.7	8.1	8.9
Staphylococcus faecalis	PF	4.0		5.7	6.3	7.1
(9 strains)	C	4.1		5.2	7.4	8.2
Staphylococcus, coagulase+	PF	4.1		3.7	3.6	3.2
(12 strains)	C	4.2		5.3	6.5	9.4
Staphylococcus, coagulase+	PF	3.5		3.2	3.7	4.3
(9 strains)	C	3.4		3.6	4.8	8.2
Bacterioides fragilis	PF	4.2		4.7	7.3	9.7
(2 strains)	C	4.1		4.5	7.3	9.3

Table 2.3: *In vitro* activity of canine pancreatic fluid against various human faecal and urinary isolates (Sarker and Gyr, 1992). PF = pancreatic fluid; C = control. Standard deviation in all instances $\leq 6.4\times10^2$ and therefore not included in table. * Difference of more than 2 log units is considered significant.

been demonstrated, and human pancreatic fluid enhances the bactericidal activity of a number of drugs. Exocrine pancreatic insufficiency is also associated with bacterial overgrowth in the proximal digestive tract (Sarker and Gyr, 1992).

Deconjugated bile acids have inhibitory effects on micro–organisms *in vitro*, support from *in vivo* studies is lacking, however. IgA from bile supposedly mediates removal of pathogens by the disposal of IgA antigen complexes into the gut lumen (Sarker and Gyr, 1992). Absence of bile in the intestine appears to promote mucosal injury and bacterial translocation (Slocum et al., 1992).

2.2.5 Lysozyme

Lysozyme is present in the digestive system in the ductal cells of salivary glands, in the absorbing epithelium of the intestinal tract [3], and in pancreatic fluid. Various infections have been reported to increase this enzyme. Lysozyme might play a role in the mucosal defense against invading organisms, by means of its bacteriolytic activity. Specific information on the action mechanism and the influence of lysozyme on gastroenteric pathogens is lacking (Sarker and Gyr, 1992).

[3] Produced by paneth cells in the crypts of the villi (Duncan and Edberg, 1995)

2.2.6 Colonization resistance

The contribution of the autochthonous intestinal microflora to the resistance against infections by pathogenic organisms has been demonstrated in a series of experiments (Abrams and Bishop, 1966; van der Waaij et al., 1972; van der Waaij and Berghuis, 1974; Maier et al., 1972).
Competition for carbon substrate has been implied as a possible basis for the protective effects of intestinal flora against foreign invaders. Under the anaerobic conditions in the caecum and colon, the production of volatile fatty acids combined with low pH appears to effectively depress the multiplication of *Shigella* in mice (Maier et al., 1972; Baskett and Hentges, 1973). The structure of the intestinal mucosa is also affected by the presence of normal microflora. Translocation of pathogenic organisms is also affected, via changes in the rate of intestinal emptying (Abrams and Bishop, 1966).
The colonization resistance of the digestive tract has been defined by van der Waaij et al. (1972) as the logarithm of the oral dose of bacteria that is needed for colonization of the digestive tract for longer than two weeks in 50% (in a group of 20) of the exposed animals. The magnitude of the CR appears to be correlated with the presence of several anaerobic bacterial species in the intestinal flora.

Luminal flora

The intestinal microflora forms an extensive and very complex ecosystem with both aerobic and anaerobic micro–organisms. Bacteria from the oral cavity are washed with saliva into the stomach. In humans, most of these bacteria are destroyed by gastric juices. In the human stomach, the bacterial concentration usually is less than 10^3 colony forming units (cfu) per ml, mostly aerobic species (Simon and Gorbach, 1984). In small rodents (mice and rats, the pH in the stomach is higher, allowing the growth of indigenous yeasts (Artwohl and Savage, 1979).
The human small intestinal flora is relatively sparse, with probably less than 10^5cfu per ml. Both aerobic and anaerobic species are present, the majority of the species presumably are anaerobic (Sarker and Gyr, 1992; Simon and Gorbach, 1984). Distal to the iliocaecal sphincter, bacterial concentrations increase sharply. In the colon, anaerobic species far outnumber aerobic species (by a factor 10^2–10^4). Nearly a third of the faecal dry weight consists of viable bacteria. Table 2.4 list the major species in the various parts of the intestinal tract, and their abundance.
The normal intestinal flora may be very important as a host defense mechanism. In normal situations, an individual's intestinal flora is highly effective in resisting colonization by potentially pathogenic invaders (Hentges, 1993). The indigenous flora produces a variety of antimicrobial substances, including colicins and short chain fatty acids, which are potentially bactericidal and bacteriostatic and are therefore considered to inhibit the growth of invading organisms.
A reduced bacterial flora increases the susceptibility to infection. Diarrhea associated with the use of antibiotics is common, and presumably caused by the alter-

	stomach	jejunum	ileum	faeces
total count	$0–10^3$	$0–10^5$	$10^3–10^7$	$10^{10}–10^{12}$
aerobic or facultative anaerobic species				
enterobacteria	$0–10^2$	$0–10^3$	$10^2–10^6$	$10^4–10^{10}$
streptococci	$0–10^3$	$0–10^4$	$10^2–10^6$	$10^5–10^{10}$
staphylococci	$0–10^2$	$0–10^3$	$10^2–10^5$	$10^4–10^7$
lactobacilli	$0–10^3$	$0–10^4$	$10^2–10^5$	$10^6–10^{10}$
fungi	$0–10^2$	$0–10^2$	$10^2–10^3$	$10^2–10^6$
anaerobic species				
Bacteroides	rare	$0–10^2$	$10^3–10^7$	$10^{10}–10^{12}$
bifidobacteria	rare	$0–10^3$	$10^3–10^5$	$10^8–10^{12}$
Gram–positive cocci*	rare	$0–10^3$	$10^2–10^5$	$10^8–10^{11}$
clostridia	rare	rare	$10^2–10^4$	$10^6–10^{11}$
eubacteria	rare	rare	rare	$10^9–10^{12}$

Table 2.4: Bacterial species in the human gastrointestinal tract (Simon and Gorbach, 1984). * including *Peptostreptococcus* and *Peptococcus*.

ations to the normal indigenous flora. Germfree animals are highly susceptible to colonization by pathogenic micro–organisms, compared to animals with an intact normal intestinal microflora (Hentges, 1993).
The gut microflora also plays an important role in the metabolization of drugs and other foreign compounds. Many drugs are neutralized due to the metabolizing activity of enteric bacteria. The enterohepatic circulation of steroid hormones also constitutes an important metabolic pathway that requires the presence of a physiologically active microflora (Simon and Gorbach, 1984).

Epithelial flora

In the intestinal epithelium, a unique population of bacteria exists, with organisms different from those inhabiting the lumen. Electron microscopy shows that they are firmly adherent to the mucus covering the microvilli of the crypts in the distal small bowel. Knowledge about the composition of this flora is incomplete, as is its role in defense. It seems possible that they interfere with the colonization and multiplication of pathogenic bacteria by competing for nutrients (Savage, 1987; Sarker and Gyr, 1992). It has also been suggested that these epithelium associated bacteria are important for maintaining the stability of the intestinal flora (Savage, 1987).
The association of micro–organisms with the intestinal epithelium requires specific abilities, discussed in short here.
Adherence to the epithelial surface, with a certain degree of specificity (as with enteropathogenic *E. coli*, for instance). Filamentous segmented Gram–positive bacteria that adhere to columnar epithelial cells of the small bowel also are highly host–specific. Mechanisms for adherence are not well understood, both proteins and polysaccharides may be involved (Savage, 1987). Adherence of enteropathogenic *E. coli* probably depends on their expressing pili on their surface membranes (Sav-

age, 1987).
Motility and chemotaxis. Many bacteria in the intestines are motile. Motile bacteria may be attracted chemotactically into the mucous gel covering the epithelium. This includes pathogenic bacteria like *Vibrio cholerae*, but also many indigenous species (Savage, 1987).
Organisms associated with epithelia must be able to find nutrients in this habitat. Many species can hydrolyze mucus, some are also able to hydrolyze proteins made by the host. The resident bacteria also compete with the host for nutrients from the lumen, mainly in the stomach and the proximal parts of the small intestine. In the lower parts of the bowel, these nutrients are largely removed by the host's digestion mechanisms. Compounds secreted by the surface associated bacteria may also alter the local microenvironment, and be taken up by the host (Savage, 1987).
The mucus layer covering the intestinal epithelium allows for a pH gradient to exist, so that, in the stomach for instance, a suitable environment for survival and even reproduction of micro–organisms (e.g. yeasts like *Candida pintolopesii* in mice) may be kept up. In addition to pH, main factors for survival of micro–organisms in the epithelial environment are viscosity and host resistance.
Viscosity may be an important factor for motile bacteria. Aside from acting as a passive resistance, motile organisms may also react to increased viscosity by increasing their motor activity (Savage, 1987).
The host also reacts to the presence of microflora, for instance the peristaltic rate and renewal of mucosal cells are under the influence of the intestinal flora.
Theoretically, epithelial bacteria could reproduce in the lumen, and not at the epithelial surface at all, followed by motion to the epithelium and attachment. There is not much experimental evidence on the modes of reproduction in bacterial communities of the intestinal epithelium.
In rodents, different types of mucosal epithelium appear to be inhabited by different bacterial communities. In the stomach of these animals, there are two compartments with different epithelial lining. The adherent flora of the stratified squamous epithelium mainly consists of lactobacilli and streptococci. The columnar epithelium of the rodent stomach often hosts adherent yeasts. The columnar epithelium of the lower small intestine has a attached to it filamentous bacteria, tightly fixed at one end to the microvillous membranes. The columnar epithelium of the caecum and colon is covered with a complex community of anaerobic and facultative anaerobic species, as well as filamentous bacteria (Savage, 1987).
Lactic acid bacteria reproduce via binary fission, and colonize the epithelia by growing laterally. Yeasts multiply by budding, and form a continuous layer, even deep into the crypts. Most of the bacteria of the caecum and colon also reproduce via binary fission. In addition to this, many are motile, which may be used to move onto newly formed epithelial surfaces.
The filamentous bacteria are "anchored" quite firmly into the luminal membranes of the epithelial cells, possibly to stay in place during the migration of a newly formed epithelial cell. New epithelial cells are produced by mitosis in the crypts of Lieberkühn and migrate outward to the villi, until they are extruded at the top of

the villi, often within a few days from their formation. Reproduction in a filament takes place via three processes (Savage, 1987): binary fission leading to chain elongation, narrowing and breakage to produce new filaments, and production of two prokaryotic bodies from one "mother" cell that move to the epithelium and establish the start of a new filament (Savage, 1987). Some filamentous species are able to survive adverse conditions by forming endospores.
In the stomach and upper small intestine, the contents may move at rates too high for non–attached species to maintain their presence by luminal multiplication. In these conditions, the epithelial flora may serve as a source of new bacteria, to maintain a stable composition of the functional microflora. In the caecum and colon where contents move at a much slower rate, the role of the epithelial flora is less clear. They metabolize endogenous compounds (e.g. short chain fatty acids), and may have a role in providing an inoculum after a period of famine or illness, in which the normal luminal flora is lost (Savage, 1987).

Variation in gut microflora

There appears to be considerable variation in composition of the gut flora between individuals, but within a single individual, the flora is quite stable for long periods of time (Simon and Gorbach, 1984).
Maintenance of a normal gut microflora is considered to be controlled by host factors, including motility, immunological factors, and intestinal secretions. In optimal conditions, coliform bacteria are able to divide every 20 minutes, in the intestinal tract generation times amount to a few divisions per day. This inhibition is achieved by a range of regulatory mechanisms. The most important are intestinal motility, secretion of gastric acid (but not other digestive juices), and interactions of bacterial species with one another (Simon and Gorbach, 1984). Dietary factors appear to have little effect on the composition of the faecal flora. On the other hand, the metabolic activity of the flora does show marked changes with diet composition (Simon and Gorbach, 1984).
The endogenous gut flora also is important in maintaining the histological structure of the gut. In germ–free animals, it has been demonstrated that the 'normal' histology of the gut mucosa is determined by the presence of the bacterial flora. Without a resident flora, the intestinal wall has been found to be thinner and to contain only few lymphocytes (Simon and Gorbach, 1984; Sarker and Gyr, 1992). In gnotobiotic mice inoculated intragastrically with complete caecal flora from a donor SPF mouse bacterial translocation to the mesenteric lymph node complex (MLN) only occurs in the first week after inoculation. When the intestinal flora is allowed to stabilize, translocation ceases (Berg, 1992). Similarly, disturbances in the intestinal flora by oral antibiotics may increase colonization and translocation by exogenous bacteria.
When germfree animals are exposed to normal flora, the intestinal mucosa rapidly acquires a normal morphology, with infiltration by lymphocytes macrophages and plasma cells, reminiscent of a chronic inflammatory response (Simon and Gorbach,

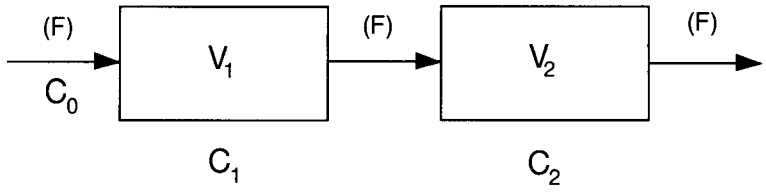
Dose	$\hat{K}$	$\hat{\lambda}_1$	$\hat{\lambda}_2$
10^{11}	5.47	0.510	9.45
10^{9}	4.61	0.510	3.92
10^{7}	1.83	0.238	12.68
10^{5}	1.58	0.244	11.99
10^{3}	0.87	0.472	12.80

Table 2.5: Maximum likelihood parameter values for the two–compartment model fitted to the data of van der Waaij and Berghuis (1974).

1984). In germfree rodents, the caecum is enlarged, the intraluminal pH is higher, and intestinal motility, intestinal transit and gastric emptying are decreased. There is also increased carbohydrate absorption in germfree animals. The enhancement of nutrition uptake by adding antibiotics to animal feed is supposedly caused by suppression of the microflora (Simon and Gorbach, 1984).
During acute diarrheal illness, the normal microbial flora may be eclipsed by a pathogen, e.g. with *Vibrio cholerae*. In patients with nonspecific or viral diarrhea, the small intestine may contain large numbers of coliforms, and high concentrations of *Klebsiella*, *Proteus*, and *Pseudomonas* spp. in faecal cultures. After rapid passage of diarrheal stool, numbers of anaerobic bacteria are reduced in the colon (by 5–6 log units) (Simon and Gorbach, 1984).

Quantitative aspects

The resistance to colonization has been studied quantitatively by van der Waaij et al. (1972). They observed a difference in survival time between normal and antibiotic–treated mice after oral challenge with various species of Enterobacteriaceae. All of the antibiotic–treated mice developed bacteraemia, whereas this response in mice with normal intestinal contents appeared to be dose–dependent in a gradual manner. In the first 3–4 days after oral challenge with high doses of "potentially pathogenic" bacteria, "abnormal" colonization of the digestive tract was found, with high numbers of bacteria of the inoculated species in the oral cavity, stomach, duodenum, jejunum, ileum, caecum, and colon. Translocation to the mesenteric lymph nodes and spleen also occurred often in these animals. In animals carrying a normal intestinal flora, this situation only lasted for a few days, and then returned to normal. When the normal intestinal flora was suppressed by using antibiotics, this initial colonization phase appeared to persist, for as long as the suppression lasted. Irradiation of the animals (van der Waaij and Berghuis, 1974) with high doses (X rays, 700 rad) produced responses similar to those of animals treated with antibiotics.
The colonization resistance appeared to vary gradually with the applied dose of foreign bacteria (a streptomycin resistant strain of *E. coli*). In figure 2.2 the faecal concentration of the inoculated strain at various intervals after administration is reproduced from (van der Waaij and Berghuis, 1974).
In animals treated with antibiotics to kill off the normal intestinal flora, the fae-



$$\frac{dC_1}{dt} = -\frac{F}{V_1}C_1, \frac{dC_2}{dt} = \frac{F}{V_2}C_1 - \frac{F}{V_2}C_2$$

$$C_1(0) = C_0, C_2 = 0$$

$$C_1(t) = C_0 e^{-\frac{F}{V_1}t}, C_2(t) = \frac{C_0 V_1}{V_2 - V_1}\left(e^{-\frac{F}{V_2}t} - e^{-\frac{F}{V_1}t}\right)$$

Figure 2.1: Two compartment model for the intestinal tract. F = flow rate, V_1, V_2 = compartment volumes, C_0 = input concentration, C_1, C_2 = compartment concentrations.

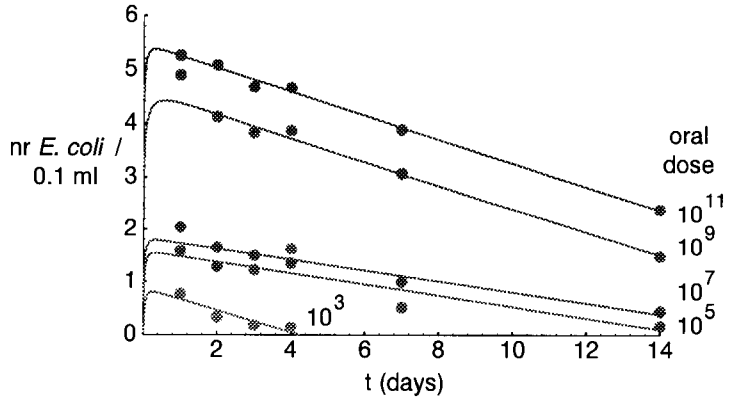


Figure 2.2: Variation in faecal concentration of *E. coli* in untreated mice, with time after oral inoculation with various doses (van der Waaij and Berghuis, 1974). Curves fitted for a two–compartment model as described in text.

cal concentration of the inoculated bacteria quickly rose to very high levels (above 10^8 per 0.1 g faeces), and remained at this level. Assuming that in normal untreated animals, after the initial colonization phase, net bacterial growth returns to approximately zero, with reproduction and death rates approximately equal in magnitude, the decline in faecal concentration may be represented with a simple two–compartment model (Figure 2.1). Colonization has occurred in a prior compartment, which serves as input for the two (distal) compartments we consider here. Also shown in Figure 2.2 are curves for the output of a two–compartment model of the transfer of bacteria through the distal parts of the intestinal tract:

$$n_{\text{out}} = K\left(e^{-\lambda_1 t} - e^{-\lambda_2 t}\right)$$

Fitted by maximum likelihood, assuming lognormally distributed errors (Haas and Jacangelo, 1993). The resulting parameter values, in Table 2.5 show little variation in rate parameters (λ_1 and λ_2). This would be in agreement with a more or less constant transport rate of the intestinal tract. The parameter K, indicating the magnitude of the input concentration (and some volume fractions, see Figure 2.1), increases steadily with the size of the inoculum. This may indicate a dose dependent depression in colonization resistance. This could also be caused, however, by some defense mechanism which depresses pathogen growth, but only after a certain reaction time. A high initial concentration (a large inoculum) would then allow the pathogens to reach higher numbers within a given amount of time. This line of reasoning is elaborated somewhat in section 4.6.

Chapter 3

Interactions at the cellular level

3.1 Invasion

Once a pathogenic microorganism has succeeded in attaching itself to cells of the intestinal epithelium, it may penetrate these cells, and spread to neighbouring cells in the epithelium, or proceed to other internal organs, to cause systemic infection.

3.1.1 Penetration

The process of penetration of a host cell by a bacterium can be divided into four steps (Miller et al., 1988):

- approach of the bacteria to the host cell and initial interactions (adherence, attachment)
- internalization and entry of the host cell (invasion)
- intracellular survival and replication
- exit from the host cell

Initial interactions

Bacterial cells may be motile, using flagella or other means of locomotion. This may help them to find susceptible host cells, possibly via chemotaxis. This has been found for *Campylobacter* and *Helicobacter*. In many pathogenic bacteria, the contribution of chemotaxis to their virulence has not been studied, and the significance of locomotion remains uncertain. For instance, some highly virulent pathogens, like *Shigella* spp., are nonmotile.
In *Vibrio cholerae*, motility enhances their association with intestinal mucosa. Chemotaxis may also contribute to the rate with which *Salmonella* spp. enter mucosal cells (Finlay and Falkow, 1989).
Many pathogenic bacteria can adhere to the surface of host cells, without internalization. In *Yersinia* species, adherence depends on genes essential for host cell

penetration (Miller et al., 1988). Bacterial adhesins may be expressed for specific components of host cell surface, dependent on structural proteins like microfilaments, that are essential for internalization.
Much is known about the factors involved in bacterial adherence to host cells. In its simplest form, there is a receptor on the host cell surface –usually a specific carbohydrate residue– and a bacterial adhesin –a protein structure on the bacterial surface which interacts with the host cell receptor. Many *Enterobacteriaceae* have fimbriae or pili on their outer surface, which act as adhesins. Several different types of pili may be present in a single strain of bacteria, encoded in different regions on the chromosome or plasmids. Common or type 1 pili may be involved in infection, but their presence is not strictly linked to infection. Cells expressing pili may not be infectious, and nonfimbriated strains of *Salmonella typhimurium* are as virulent as fimbriated strains (Finlay and Falkow, 1989). N-methylphenylalanine pili are found in a number of pathogenic bacteria localized at mucosal surfaces, and have been identified as virulence determinants. There are also nonfimbrial adhesins: hemagglutinins, found in *Salmonella typhimurium*, different fibronectin binding proteins[1], and invasion proteins. The latter are not only involved in attachment of bacteria to the host surface, but also play a role in entry into the host cell.
For an organism like *Salmonella enterica*, attachment and penetration are presumed to be two distinct stages (Saarinen et al., 1996). Attachment of *Salmonella enterica* Serotype Typhimurium induces changes in the morphology of the host cell: ruffling of the plasma membrane, facilitating the uptake of particles into the host cells.

Internalization

After establishing intimate contact with a (non–phagocyte) host cell, bacteria may be ingested via a process similar to phagocytosis. This results in an internalized bacterium, surrounded by a vacuolar membrane[2]. Most invasive pathogens exploit existing eucaryotic endocytosis pathways (Finlay and Falkow, 1989). Endocytosis is usually accompanied by cytoskeletal rearrangement, associated with microfilament, but not microtubule function. Invasive strategies of a few important intestinal pathogens are compared in table 3.1.
Enteroinvasive *E. coli* and *Shigella* typically invade the mucosal epithelial cells in the lower bowel. Lateral spreading to other cells in superficial layers of the mucosal epithelium causes much tissue damage. Host cells are entered by a process of induced endocytosis, leaving the bacteria within a host cell membrane vesicle. This membrane is lysed after a short while (15 min.), releasing the organism into

[1]Group A streptococci (*Streptococcus pyogenes*) express lipoteichoic acid as fibronectin binding adhesin, while *Staphylococcus aureus* binds to the same receptor group, with a distinctly different adhesin. The causative agent of syphilis, *Treponema pallidum*, also binds fibronectin, at a different site.

[2]Coated pits are cell surface structures involved in receptor–mediated endocytosis, and often form part of the membrane surrounding a vacuolized bacterium.

Species	*Salmonella* (non *typhi*)	*E. coli*, *Shigella*	*Yersinia*
Cell type entered	Epithelial/Peyer's patches (M cells)	Mucosal epithelial	Peyer's patches
Host microfilaments required for entry	yes	yes	yes
Endosome acidification required for entry or intracellular replication	no	no	no
Intracellular location	vacuole	cytoplasm	vacuole
Vacuoles with bacteria coalesce	yes		no
Intracellular replication	yes	yes	slow, varies with cell line
Bacterial metabolic activity required for entry	yes	yes	no
Adherence to epithelial cell surfaces at 4 °C	no	?	yes
Plasmid required for entry	no	yes	no

Table 3.1: Comparison of invasion strategies for a few intestinal pathogens (Finlay and Falkow, 1989).

the cytoplasm. Lysis is mediated by contact hemolysin (virulence plasmid mediated). Intracellular replication cannot take place within an intact endocytic vacuole (Finlay and Falkow, 1989). Upon release into the host cell cytoplasm, host protein synthesis is inhibited, and the invading bacteria multiply rapidly. Eventually, the host cell is lysed and the bacteria are released to infect neighbouring epithelial cells.

For *Salmonella typhimurium*, invasion is an essential step in pathogenesis. Most *Salmonella* species migrate through the superficial layers of the intestinal mucosa, into deeper tissue, often reticuloendothelial cells. As *Salmonella* cells approach the mucosal surface, the epithelial microvilli start to degenerate (section 3.1.1). After entry, the bacteria remain enclosed within a vacuole. When multiple infection occurs, each pathogen first sits within its own vacuole; these vacuoles then merge, and most organisms then are found within a single large vacuole. Ileal M cells may be the primary entry site for *Salmonella typhi*, other salmonellae also seem to prefer the terminal ileum, both M cells and normal epithelial cells (Finlay and Falkow, 1989). Upon interaction with epithelial surfaces, *Salmonella* cells synthesize several new polypeptides required for adherence and invasion (see also section 3.2 on type III secretion and intercellular signalling).

Like *Salmonella*, *Yersinia* spp. remain enclosed in membranous vacuoles within infected host cells. Intracellular replication is much slower than that of *Salmonella* or *Shigella*. The kinetics of bacterial uptake into host cells have been studied in host cell cultures. *Yersinia* spp. (*Y. enterocolitica*, *Y. pseudotuberculosis*) are taken up into HeLa cells at a constant rate for 2 hrs, after an initial lag of 0.5 hrs. The percentage of infected host cells and the multiplicity of infection depend on the number of bacteria added to the cell culture (Miller et al., 1988).

Survival within host cells

Yersinia pseudotuberculosis remained confined within vacuoles inside human fibroblasts for 7 days (Miller et al., 1988). Metabolically inactive *Yersinia* spp. can adhere to and be internalized by eucaryotic cells. *Salmonella* spp. must be viable and actively synthesizing RNA and proteins.
Inside the host cells that are specialized in ingesting foreign particles, the invading bacteria are also enclosed into a phagocytic vacuole, to be killed by low pH and digested. Pathogenic species like *Salmonella* spp. or *Yersinia* spp. may survive prolonged periods of time within such a vacuole, however. They may be able in achieving this by using an alternative pathway for internalization, the same as used to enter nonprofessional phagocytes ('normal' epithelial cells), thus avoiding phagolysosomal fusion, and subsequent digestion.
In cultured intestinal cells, *Salmonella enterica* st. Enteritidis survived for as many as 14 days or more, with a gradual decline in numbers of living bacteria per infected cell (Saarinen et al., 1996). Upon infection, cultured cells began to secrete NO, in amounts peaking at (post infection–) day 2, and then gradually declining to background levels (at day 6 p.i.).
In response to invasion by pathogens, many cell types produce NO[3]. A primary role for NO may be to aid in killing off vacuolized bacteria.
Invasion of host cells and surviving there for prolonged periods may provide pathogens with a means to reinfect neighbour host cells and establish themselves within an epithelium with a high turnover rate.
When pathogenic micro–organisms have entered a specialized phagocytic cell, they must survive any attacks made by the antibacterial apparatus of these cells. Possible adaptations include (Finlay and Falkow, 1989):

- avoid entering the macrophage via a pathway leading to fusion with a lysosome
- inhibit endosome acidification and phagolysosomal fusion (e.g. *Legionella pneumophila*, *Toxoplasma gondii*, *Nocardia asteroides*)
- resist or neutralize the antibacterial agents delivered by phagolysosomal fusion (*Salmonella*, *Yersinia*)

Other adaptations may help pathogenic bacteria in successfully establishing themselves in a host organism, and multiplying to numbers sufficient for transmission to other susceptible hosts. Among those are bacterial toxins, mechanisms to avoid actions of the host immune system (antiphagocytic activity, antigenic variation, development of IgA proteases, and serum resistance[4]). See also section 3.2.

[3]Increased NO synthesis is also a response to many other stimuli, such as TNF, IFN-α, IFN-β, IFN-γ, IL-1, IL-6, and various endotoxins.

[4]Serum resistance: the prevention of lysis by complement. *Salmonella* spp. have O antigens in their lipopolysaccharide, which renders them more resistant to complement than isogenic strains lacking LPS. Other pathogens may employ other mechanisms, by shielding of complementary chains, or developing aberrant configurations of LPS (Finlay and Falkow, 1989).

Bacteria that have succeeded in entering a host cell, often pass through that cell to deeper tissues, or the blood stream: translocation.

3.1.2 Translocation

Under suboptimal conditions, bacteria may pass through the intestinal epithelium and be carried via the lymph to the mesenteric lymph node complex (MLN), and further[5]. This process has been called bacterial translocation. It has been suggested that translocation of indigenous bacteria also takes place continually, at very low rates, in healthy immunocompetent hosts (Berg, 1995). These low numbers of bacteria are killed by the host immune system, leaving the MLN complex sterile.
Under very adverse conditions, of a major inflammatory insult, translocation directly into the portal blood stream may be the main pathway for systemic infection (Mainous et al., 1991).
When they are killed in the process, bacteria crossing the intestinal mucosal barrier cannot be cultured from the mesenteric lymph node complex, so that, literally speaking, no translocation takes place.
Mechanisms promoting translocation in laboratory animals: intestinal bacterial overgrowth (sections 2.2.1, 2.2.3, 2.2.4), deficiencies in host immune defenses, and increased permeability or damage to the intestinal mucosal barrier (Berg, 1995; Deitch et al., 1992).
Deficiencies in the host immune system may lead to bacterial translocation and systemic infection. In pathogenic situations (e.g. when bacterial overgrowth occurs) indigenous bacteria translocate to the MLN, but do not spread to other organs, and systemic infection does not develop. Translocation of virulent pathogenic organisms, however, may result in systemic spreading and infection of liver, spleen, kidneys, or other organs. Eventually, spreading may proceed to the peritoneal cavity or the blood stream, and lethal sepsis may occur.
Immunostimulating agents appear to decrease translocation in mice, even when the translocation process is underway. Also in mice, promotion of bacterial translocation is found after administration of immunosuppressive agents like prednisone, or thymectomy (Berg, 1992).
The MLN may be divided into three segments (Gautreaux et al., 1994), each draining lymph from a different part of the associated gastrointestinal tract[6]. In antibiotic treated mice, indigenous bacterial species like *Escherichia coli* and *Proteus mirabilis* appear to translocate mainly to the caecum, ileum, and jejunum, reflecting the distribution of their population levels along the intestinal tract. A pathogen like *Salmonella typhimurium*, however, translocates to all segments, without regional differences (Gautreaux et al., 1994).

[5] In neonate mammals, ingested immunoglobulins and other macromolecules easily pass the intestinal epithelium. In adult life, small amounts of macromolecules may still pass through the intact intestinal epithelium (Berg, 1980).

[6] MLN segment 1 drains lymph from the distal ileum, caecum, and ascending colon. MLN segment 2 drains lymph from the proximal ileum. MLN segment 3 drains lymph from the jejunum

The rate at which translocation occurs appears to differ between bacterial species. Organisms like *E. coli*, *Proteus*, and *Enterobacter* translocate more efficiently than other indigenous bacteria, especially obligate anaerobes (Steffen et al., 1988).
Translocation is also promoted by physical damage to the intestinal epithelium. Bacterial endotoxins increase translocation by this mechanism (Deitch et al., 1989). Oxygen free radicals may play a role here, inhibition of xanthine oxidase reduces the incidence of endotoxin–induced bacterial translocation (Deitch et al., 1989; Parks et al., 1982).
Berg (1995) proposes a three stage view upon the pathogenesis of translocation:

1. spreading to the MLN (result of intestinal bacterial overgrowth)
2. spleen/liver (intestinal mucosal injury)
3. blood (compromised immune defenses)

Whereas combination of two or more of these stages may lead to fatal complications.

3.2 Evasion mechanisms

The common link in microbial strategies to successfully infect the host, is the close association or attachment to the intestinal epithelium, as an essential first step in pathogenesis. In order to achieve this, the microorganism must survive and evade the defense mechanisms of the host. Many virulence factors are controlled by a regulator, to enable adaptation to both external environments before reaching a host, and the internal environments within the host.
In recent years, the existence of two–way biochemical interaction between bacterial and host cells has been discovered for many pathogens. Extracellular protein secretion by pathogenic bacteria may involve various pathways. In *Neisseria gonorrhoeae*, extracellular secretion of IgA protease is autonomous, without the involvement of accessory proteins. The so–called Type II secretion pathway employs the *sec*–dependent pathway for transport across the inner membrane, and additional accessory proteins for secretion through the outer membrane. The Type I pathway does not employ the *sec* machinery, but requires at least three other accessory proteins. This pathway is used for many bacterial toxins, for example *E. coli* hemolysin. The recently discovered Type III (or contact–dependent) secretion system bears resemblance to the flagellar assembly apparatus, and involves a large number of accessory proteins. This secretion system requires extracellular signals for activation (Galan and Bliska, 1996; Mecsas and Strauss, 1996). Components of this secretion system have been found in *Shigella*, *Yersinia*, *Salmonella*, and enteropathogenic *E. coli*. Activating extracellular signals include the presence of serum, Congo red, low calcium levels, conditions associated with growth in tissue culture (Galan and Bliska, 1996). Host cell responses to Type III protein secretion include bacterial internalization, host cell cytoskeleton modification

Virulence factor	Specific examples	Activity
Specific attachment to intestinal epithelium	Poliovirus, rotavirus, *V. cholerae*	Epithelial association prevents washing out
Motility	*V. cholerae*, certain *E. coli* strains	Ability to penetrate mucus to reach target cell
Production of mucinase (neuraminidase)	*V. cholerae*	Assists in target cell attachment
Acid resistance	*M. tuberculosis*, *H. pylori*, parasite cysts, enteroviruses (hepatitis A, poliovirus, coxsackieviruses, echoviruses)	Passage through stomach
Bile resistance	*Salmonella*, *Shigella*, enteroviruses, *Enterococcus faecalis*, *E. coli*	Survival to reach large intestine
Resistance to proteolytic enzymes	Enteroviruses, parasites	Survival to reach large intestine
Anaerobic growth	*Bacteroides fragilis*, *Clostridium difficile*	Growth advantage
Local toxin production	*V. cholerae*, *Campylobacter*, ETEC	Outflow of water, hyperperistalsis, pseudomembrane
Systemic toxin production	*S. dysenteriae*	Distant target organ affected
Ingestion of preformed toxin	*C. perfringens*, *S. aureus*, *B. cereus*, *C. botulinum*	Outflow of water, systemic malfunction
Perforation of the mucosal epithelium	*E. histolytica*	Abscess
Epithelial cell invasion	*Cryptosporidium*, *Salmonella*, viruses	Outflow of water, hyperperistalsis
Attachment	*Giardia*, microspora, cyclospora	prevention of nutrient absorption

Table 3.2: Major microbial virulence factors active in the gastrointestinal tract (Duncan and Edberg, 1995).

Bacterium	Environmental signal
Escherichia coli	Iron, temperature, carbon source
Salmonella typhimurium	Osmolarity, starvation, stress, pH, growth phase
Shigella spp.	Temperature
Vibrio cholerae	Osmolarity, pH, temperature, amino acids, CO_2, iron
Yersinia spp.	Temperature, ionized calcium

Table 3.3: Signals in the gastrointestinal tract that regulate virulence expression (Duncan and Edberg, 1995).

(facilitating spreading of the bacterium to neighbouring cells), second messenger modulation, and the formation of attaching and effacing lesions (enteropathogenic *E. coli*) (Galan and Bliska, 1996).
Some of the most important virulence factors are listed in table 3.2. Signals regulating the expression of some of these factors are given in table 3.3.

Vibrio cholerae Gram–negative rod, can cause large volumes of watery diarrhea. Not very infectious, except when ingested in pH–buffering medium. Motile, chemotactic attraction to the mucus, secretes mucinase and proteases to penetrate the mucus layer. Attachment to the epithelium (hemagglutinins and pili) is necessary for multiplication and toxin production. No invasion of the epithelium. Cholera toxin disrupts the cAMP control cycle and the increase in cAMP causes villus cells to stop absorbing electrolytes and crypt cells to secrete more electrolytes, leading to watery diarrhea. The production of toxin is coregulated with pili assembly and other colonization factors, controlled by environmental signals (table 3.3).

Host IgA binds to the toxin to prevent entry into host cells.

Pathogenic *Vibrio* species produce exocellular proteases enhancing the permeability of the epithelium (Shinoda et al., 1996).

Escherichia coli Many different types of pathogenic *E. coli*, including

- Enterotoxigenic *E. coli* (ETEC) are not highly infectious (like *V. cholerae* and produce two enterotoxins causing watery diarrhea. One toxin is very similar to cholera toxin, the other (ST) mimics the guanylate cyclase activator of the host.
- Enteropathogenic *E. coli* (EPEC) more infectious than ETEC, no toxins produced, but damages microvilli upon attachment, leading to loss of absorption and diarrhea.
- Enteroinvasive *E. coli* (EIEC) causes acute inflammation with bloody diarrhea. Clinically similar to *Shigella*, but less infectious.
- Enterohemorrhagic *E. coli* (EHEC) damages microvilli after attachment, acute inflammation and bloody diarrhea. Produces Shiga–like

toxin type I and II. *E. coli* O157:H7 is acid resistant, particularly virulent, and can produce the hemolytic uremic syndrome (HUS): acute renal failure, anemia, and thrombocytopenia.

All types require pili for attachment to the epithelium. Regulation is under the influence of environmental signals, listed in table 3.3.

Shigella Highly infectious, invasive. Must enter and multiply within the colonic epithelium. Chromosomal and plasmid–encoded virulence genes. Inhibition of these genes is released at temperatures near 37°C. Prevention of attachment via intestinal motility is an important host defense against *Shigella*. Initial watery diarrhea with *S dysenteriae* is caused by shiga toxin, which interrupts host cell protein synthesis. Subsequent invasion causes diarrhea with blood and mucus. Invasion proceeds laterally within the lamina propria, causing ulcerations and bleeding (Theriot, 1995). *Shigella* may use the M cell antigen uptake system as entry point.

Yersinia enterocolitica Invades mucosal epithelium, most damaging is the inflammatory response of the host immune system. Enterotoxin similar to *E. coli* ST. Strong trophism for lymphoid tissue (M cell entry site).

Clostridium difficile May cause antibiotic–associated pseudomembranous colitis (the presence of normal colonic flora prevents colonization). Produces enterotoxins, not invasive, must bind a specific brush border receptor before releasing toxin A. Toxin A causes cytoskeletal disruption in host cells, increased permeability causes an acute inflammatory response. Toxin B acts as a synergist to toxin A. IgA inactivates both toxins, but the main host defense is the intact colonic microflora.

Protozoan parasites *Cryptosporidium* penetrates the epithelium, inflicts damage to villus cells and causes diarrhea. The trophozoite attaches to the brush border. Upon maturation it loses its two innermost membranes and the remaining outer membrane fuses with the host membrane below the terminal web. The host membrane dissolves, so that there is direct contact between the host cell cytoplasm and the trophozoite membrane. In villus cells the parasite remains near the luminal surface, in M cells it can be found deep within the cytoplasm.

Giardia Only limited tissue invasion, causes an acute inflammatory response, marked eosinophilia and humoral response in the lamina propria, and foul–smelling diarrhea.

Entamoeba histolytica trophozoites invade colonic crypts and penetrate the submucosa to form ulcerative abscesses. Can also be taken up systemically and attack the liver. Adherence seems to require microfilament function.

Viruses Rotavirus (reoviridae, double–stranded segmented RNA) is extremely infectious. Infects and lyses mature (i.e. villus) enterocytes, absorption losses

lead to diarrhea. CD8+ cytotoxic T cells are important in host defense. VP4 is an important virulence factor, a viral hemagglutinin required for entry into enterocytes. Host immune responses are evaded by gene reassortment during mixed infections.

Norwalk and Norwalk–like viruses (SRSV, single stranded positive–sense RNA) cause histopathological lesions, such as shortened villi and crypt hypertrophy in the mucosa of the small bowel. The host immune system normally is capable of clearing the virus.

Reovirus is considered a model for astrovirus, parvovirus, and enteric viruses causing systemic disease. In mice, the virus uses the M cell uptake system to invade the mucosa, and binds to the basolateral surface of crypt enterocytes to infect them. Infection may be a homing signal for lymphocytes to move towards the epithelium and become IEL cells.

Not all strains of a virulent bacterial species are equally pathogenic. Most natural bacterial population appear to consist of several discrete clonal lineages, indicating that the rate of recombination between different strains or species is low. In many pathogenic bacterial species, diseases are caused by a small proportion of the total number of clones that exist (Finlay and Falkow, 1989). This may be the subpopulation that happens to possess all of the necessary virulence determinants. Bacteria may acquire virulence factors via various methods, including conjugation, transposition, and transduction. These factors are often encoded on mobile genetic elements like plasmids, transposons, and bacteriophages. Virulence determinants are often flanked by a transposon, so that they may be easily transferred to another strain (pathogenicity islands, see e.g. Mecsas and Strauss (1996)).

Chapter 4

Dose response studies

Dose response relations for pathogenic micro–organisms causing acute gastro–enteritis can be described adequately by the Beta Poisson model (Furumoto and Mickey, 1967; Haas, 1983; Teunis et al., 1996b). This model is based on the assumption that exposure, infection, and eventually illness and death are conditional events: without exposure to one or more pathogenic organisms infection cannot develop. Without infection, i.e. when there are no pathogenic organisms colonizing the intestines, illness cannot occur, etcetera. In spite of its apparent simplicity, this model appears to perform quite well with many (as good as all that are available) experimental data. This may be explained by the following considerations:

- it is assumed, that pathogens are distributed randomly within the ingested medium. A second assumption, that a single surviving microorganism is sufficient to start infection (the "single–hit" hypothesis), causes the dose response relation to be highly insensitive to the actual distribution of the organisms in the medium.
- any microorganism which succeeds in entering the digestive system of the host, only has a minute chance of surviving to reach a site suitable for colonization, and multiplying to attain sufficient numbers to be detected after excretion (in other words, achieving apparent infection). This may be interpreted as a barrier against infection. For the shape of the dose response relation, it is completely irrelevant, if the pathogen has to deal with a single barrier, or with a chain of successive barriers, that all have to be overcome.

4.1 Multiple barriers

In chapter 2, several mechanisms have been presented, that a host may employ to prevent infection. As a conceptually simple first approach, we consider each of these defenses as a barrier for the pathogenic micro–organisms. Any microorganism that encounters such a barrier, is assumed to cross this barrier (i.e. succeeds in arriving at the next barrier in a viable state) with a certain probability. Conversely,

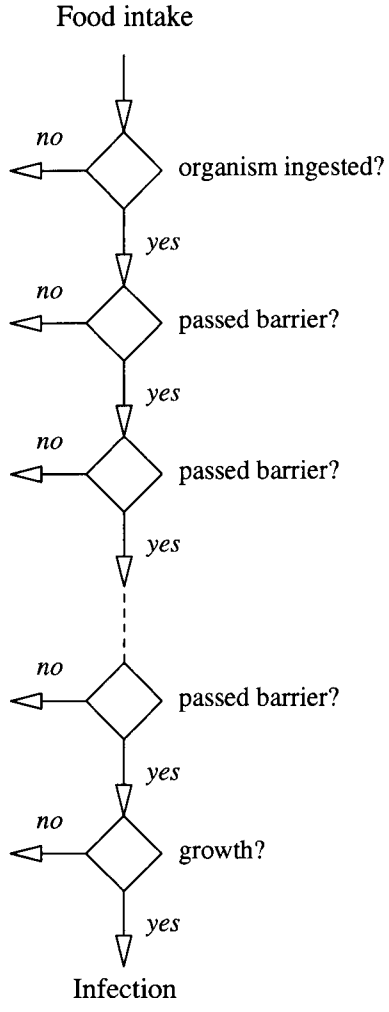


Figure 4.1: Multiple barriers imposed by the host on an ingested pathogenic microorganism, to minimize the probability of their succeeding to infect the host.

when a microorganism fails to cross a barrier, it cannot proceed to cause infection, and is lost. This causal chain (Figure 4.1, see also Haas (1983)) may be translated into quantitative statements:
Probability of j out of n passing a barrier (binomial distribution, probability π):

$$\text{Prob}(j; n, \pi) = B(j; n, \pi) = \binom{n}{j} \pi^j (1-\pi)^{n-j}$$

When there are m barriers (probabilities $\pi_1 \ldots \pi_m$):

$$\text{Prob}(j; n, \pi_1, \ldots, \pi_m) = B(j; n, p_m) \quad ; p_m = \prod_{i=1}^{m} \pi_i$$

Probability of 1 or more out of n passing these barriers:

$$\text{Prob}(j \geq 1; n, \pi_1, \ldots, \pi_m) = 1 - (1 - p_m)^n$$

When the probability of ingesting n organisms is Poisson distributed (mean dose D):

$$\text{Prob}(n; \text{D}) = P(n; \text{D}) = \frac{e^{-\text{D}} \cdot \text{D}^n}{n!}$$

then the probability that at least one organism survives all barriers becomes:

$$\text{Prob}(\text{infection}) = \sum_{n=1}^{\infty} \text{Prob}(j \geq 1; n, \pi_1, \ldots, \pi_m) \cdot \text{Prob}(n; \text{D})$$

Which may be simplified to:

$$\text{Prob}(\text{infection}) = 1 - e^{-\text{D}p_m}$$

Which may be translated (Haas, 1983; Teunis et al., 1996b) into the familiar expression of the exponential model:

$$P_{\text{inf}}(\text{D}; r) = 1 - e^{-r\text{D}} \approx r \cdot \text{D}$$

(if $r.\text{D} \ll 1$).
Based on the assumption that the probabilities of surviving all barriers and succeeding in growth within the host's intestine are equal for any host and any individual microorganism in the inoculum. This does not seem to be a very realistic simplification, as has been pointed out in several publications (Furumoto and Mickey, 1967; Haas, 1983; Haas et al., 1993; Teunis et al., 1996b). (Furumoto and Mickey, 1967) therefore proposed to use a Beta distribution to describe the variation in these probabilities within both pathogen and host populations. This of course leads to the well established Beta–Poisson model:

$$P_{\text{inf}}(\text{D}; \alpha, \beta) \approx 1 - \left(1 + \frac{\text{D}}{\beta}\right)^{-\alpha} \approx \frac{\alpha}{\beta} \cdot \text{D}$$

(if $\alpha \cdot \text{D} \ll \beta$)

4.2 Single or multiple hits?

These dose response relations may be fitted to experimental data, by means of maximum likelihood methods (Haas et al., 1993; Teunis et al., 1996b). An example is given in the left part of Figure 4.2, with an exponential dose response curve fitted to data from a human feeding study.

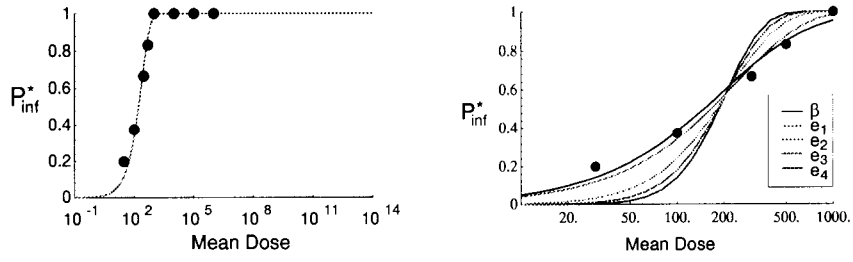


Figure 4.2: Dose response data for infection of human volunteers with *Cryptosporidium parvum* (DuPont et al., 1995). **Left:** best fitting (exponential) dose response curve (Teunis et al., 1996b), **Right:** Single and multiple hit dose response curves fitted to the same data set.

Now we may explore the changes in shape of the exponential DR–curve when infection is assumed to result not from a single surviving pathogen (single hit), but from two survivors, or three, or more. As demonstrated by Gifford and Koch (1969), allowing for a threshold of two, three, or more organisms leads to an ever increasingly steep dose response curve. In the right part of Figure 4.2, DR curves for thresholds of 1 (exponential relation), 2, 3, and 4 are shown, fitted by maximum likelihood[1] to the same data set (as in the left part of Figure 4.2). Also shown is a Beta–Poisson curve, demonstrating that this allows for a DR relation which is even less steep than that of the single hit (exponential) curve. As a matter of fact, the occurrence of very flat DR curves historically was an important motivation for using mixtures of DR relations, like the Beta–Poisson model.

4.3 Approximation

In the original paper by Furumoto and Mickey (1967), they were able to derive the simple, attractive dose response formula, now referred to as the "Beta Poisson" dose response model. It is less well known, that in order to arrive at such a simple relation, they had to make a few approximations, which are only valid for certain parameter values.
The exact solution for the case of Poisson distributed organisms and Beta distributed probability of infection given ingestion, is:

$$P_{\text{inf}}(\mathrm{D};\alpha,\beta) = 1 - {}_1F_1(\alpha, \alpha+\beta, -\mathrm{D})$$

in which ${}_1F_1()$ is the Kummer confluent hypergeometric function (Abramowitz and Stegun, 1984). When $\beta \gg 1$, and $\alpha \ll \beta$, the simple relation given in section 4.1 holds.

[1]Resulting in rejection of the 2–, 3–, and 4–hit models by means of the goodness of fit criteria for the deviance from the likelihood of a constraints–free model: 0.50, 4.00, 8.61, and 13.50 for the 1–, 2–, 3–, and 4–hit model, respectively. Compare this to $\chi^2_{1,0.95}$=3.841.

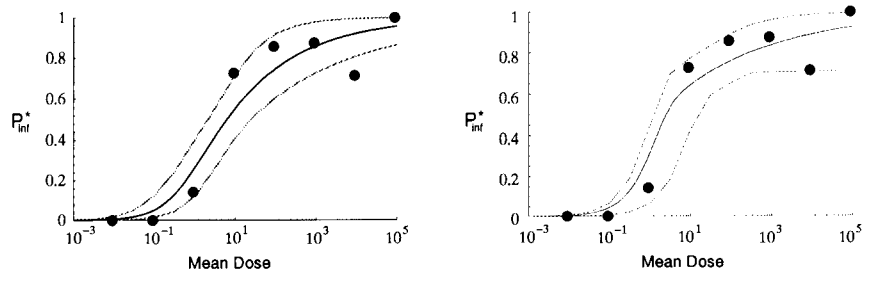


Figure 4.3: Dose response relation for infection of human hosts with rotavirus (Ward et al., 1986), with **left:** dose response curve of the Beta Poisson model (MLE: $\hat{\alpha} = 0.26$, $\hat{\beta} = 0.42$) and **right:** best fitting exact model, without the approximations made by Furumoto and Mickey (1967). Parameter values for this curve (MLE) $\hat{\alpha} = 0.167$, $\hat{\beta} = 0.192$). In both figures, a 95% confidence range is given.

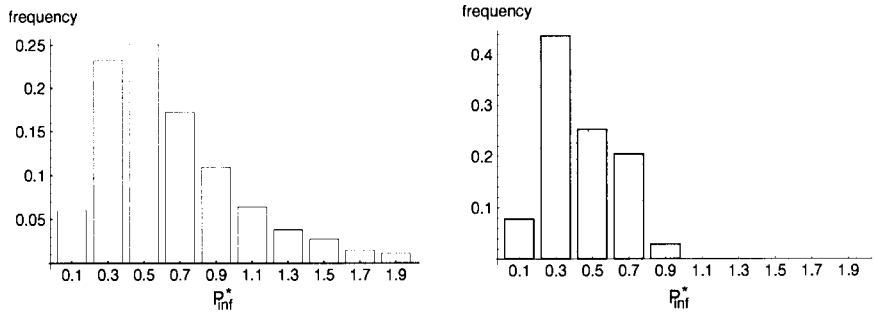


Figure 4.4: Estimated distribution of the dose response slope at low doses obtained by bootstrapping data from the experiment by Ward et al. (1986), for the Beta Poisson model **left** and the exact model (Kummer confluent hypergeometric function) **right**.

Figure 4.3 shows a comparison between the exact solution and the familiar formula, for data leading to parameter values that do not match the requirements for approximation. It may be concluded that the shape of the two functions is quite similar, but at somewhat different values of the parameters.

At low doses, the hypergeometric function appears to be approximately linear, as is seen from the first few terms in a power series expansion:

$$1 - {}_1F_1(\alpha, \alpha+\beta, -\mathrm{D}) \approx \frac{\alpha}{\alpha+\beta}\mathrm{D} - \frac{\alpha(1+\alpha)}{2(\alpha+\beta)(1+\alpha+\beta)}\mathrm{D}^2 +$$

$$\frac{\alpha(1+\alpha)(2+\alpha)}{6(\alpha+\beta)(1+\alpha+\beta)(2+\alpha+\beta)}\mathrm{D}^3 + \ldots$$

For the Beta Poisson formula, a similar result is obtained:

$$1-\left(1+\frac{\mathrm{D}}{\beta}\right)^{-\alpha} \approx \frac{\alpha}{\beta}\mathrm{D} - \frac{\alpha(1+\alpha)}{2\beta^2}\mathrm{D}^2 + \frac{\alpha(1+\alpha)(2+\alpha)}{6\beta^3}\mathrm{D}^3 + \ldots$$

It is easily checked that when $\beta \gg \alpha$ and $\beta \gg 1$, both series tend to coincide. For the rotavirus example shown in Figure 4.3, linear low dose approximation yields

$$\frac{0.167}{0.167+0.192} = 0.465$$

for the hypergeometric formula, and

$$\frac{0.26}{0.42} = 0.619$$

for the Beta Poisson formula. A difference in shape at high doses may thus implicate a difference in slope at low doses. This may also be appreciated by inspecting Figure 4.4. Note however, that in most of the published cases (summarized in Teunis et al. (1996b)), the conditions for approximation by the simple Beta Poisson formula are fulfilled.

4.4 Random distribution

We may investigate the influence of clustered occurrence of pathogenic micro–organisms by assuming that they are distributed according to a negative binomial distribution. The probability of having n micro–organisms in the inoculum then is

$$\mathrm{Prob}(n;w,r) = \binom{n+r-1}{r-1} w^r (1-w)^n$$

with mean

$$\mathrm{D} = \frac{r(1-w)}{w}$$

As discussed earlier, the probability that at least a single organism survives and causes infection can be represented as

$$\mathrm{Prob}(j \geq 1; n, p) = 1-(1-p)^n$$

where p is the probability of any organism surviving to infect the host. The probability of infection now becomes

$$\mathrm{Prob}(\text{infection}) = \sum_{n=1}^{\infty} [1-(1-p)^n] \binom{n+r-1}{r-1} w^r (1-w)^n$$

Note that the term with $n = 0$ may be included without any effect on the result. This summation may be rearranged into

$$\begin{aligned}\text{Prob(infection)} &= 1 - \sum_{n=0}^{\infty} \binom{n+r-1}{r-1} w^r \left[(1-w)(1-p)\right]^n \\ &= 1 - \sum_{n=0}^{\infty} \binom{n+r-1}{r-1} w^r \left[1-(p+w-pw)\right]^n\end{aligned}$$

and, by substituting $u = p + w - pw$

$$\text{Prob(infection)} = 1 - \left(\frac{w}{p+w-pw}\right)^r \sum_{n=0}^{\infty} \binom{n+r-1}{r-1} u^r (1-u)^n$$

The latter part sums to unity, which leaves

$$\text{Prob(infection)} = 1 - \left(\frac{w}{p+w-pw}\right)^r$$

By substituting $w = r/(r+D)$, the probability of infection with inoculum negative binomially distributed becomes

$$\begin{aligned}\text{Prob(infection)} &= 1 - \left(\frac{p}{w} + 1 - p\right)^{-r} \\ &= 1 - \left(\frac{p(r+D)}{r} + 1 - p\right)^{-r} \\ &= 1 - \left(1 - \frac{pr - pD - pr}{r}\right)^{-r} \\ &= 1 - \left(1 + \frac{pD}{r}\right)^{-r}\end{aligned}$$

With this we have shown that when the pathogenic organisms are negative binomially distributed, the dose response relation is identical to the Beta Poisson formula. Not only does this further validate the use of this formula in quantitative risk analysis, but it also allows a different interpretation for the parameters. The shape parameter r here represents the clustering parameter of the pathogen distribution. The scale parameter equals r/p, so that r divided by the scale parameter would represent the probability of a single organism surviving to cause infection.
If we would also take into account variation in p, by assuming this probability Beta distributed, the resulting relation can be calculated (see e.g. Furumoto and Mickey (1967))

$$\text{Prob(infection)} = \int_{\pi=0}^{1} 1 - \left(1 + \frac{\pi D}{r}\right)^{-r} \frac{\Gamma(\alpha+\beta)}{\Gamma(\alpha)\Gamma(\beta)} \pi^{\alpha-1}(1-\pi)^{\beta-1} d\pi$$

If r is large, it is easy to see that

$$\left(1+\frac{\pi D}{r}\right)^{r} \rightarrow \mathrm{e}^{\pi D}$$

so that the above expression for the probability of infection once again leads to the familiar Beta Poisson formula, following the arguments given by Furumoto and Mickey (1967). If, on the other hand, clustering is substantial ($r \approx 1$ or even smaller), this simplification does not hold, and

$$\text{Prob(infection)} = 1 - {}_2F_1(\alpha, r; \alpha+\beta; -D/r)$$

with ${}_2F_1$ another hypergeometric function. Unfortunately enough, a simple approximating expression is not easily found. Limiting cases may be studied, however:
Adopting the approach of Furumoto and Mickey (1967): substitute $\pi = y/\beta$

$$\text{Prob(infection)} = f(D;\alpha,\beta,r)$$

$$f(D;\alpha,\beta,r) = \int_{\pi=0}^{\beta} 1-\left(1+\frac{yD}{\beta r}\right)^{-r} \frac{\Gamma(\alpha+\beta)}{\Gamma(\alpha)\Gamma(\beta)\beta^{\alpha}} y^{\alpha-1}(1-\frac{y}{\beta})^{\beta-1}dy$$

For large β, this simplifies to

$$f(D;\alpha,\beta,r) = 1-\frac{1}{\Gamma(\alpha)}\int_{\pi=0}^{\beta}\left(1+\frac{yD}{\beta r}\right)^{-r} y^{\alpha-1}\mathrm{e}^{-y}dy$$

If $\frac{Dy}{\beta r} \ll 1$ then the first part of the integrand $\left(1+\frac{Dy}{\beta r}\right)^{-r}$ may be approximated by $\mathrm{e}^{-D/\beta}$, so that

$$f(D;\alpha,\beta) \approx 1-\frac{1}{\Gamma(\alpha)}\int_{\pi=0}^{\beta} y^{\alpha-1}\mathrm{e}^{-y(1+D/\beta)}dy$$

$$f(D;\alpha,\beta) \approx 1-\left(1+\frac{D}{\beta}\right)^{-\alpha}$$

At low doses, r disappears from the dose response relation.

If $\frac{Dy}{\beta r} \gg 1$ simplifies the dose response relation to

$$f(D;\alpha,\beta,r) \approx 1-\frac{1}{\Gamma(\alpha)}\int_{\pi=0}^{\beta}\left(\frac{Dy}{\beta r}\right)^{r} y^{\alpha-1}\mathrm{e}^{-y}dy$$

$$f(D;\alpha,\beta,r) \approx 1-\left(\frac{\beta r}{D}\right)^{r}\frac{\Gamma(\alpha-r)}{\Gamma(\alpha)}$$

Which provides a "high dose" approximation.

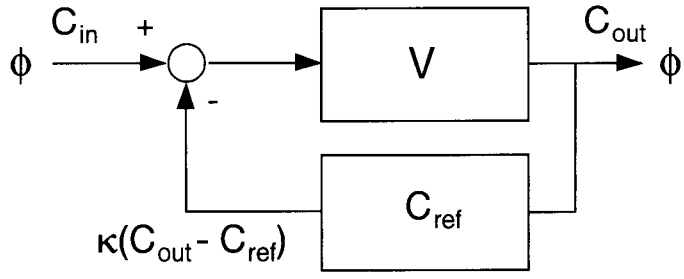


Figure 4.5: Example of a possible feedback control loop: regulation of the concentration of a dissolved compound in a metabolic compartment.

4.5 Homeostasis

In a normally functioning biological system, many physiological variables have to lie within narrow limits, in order to keep the animal (or plant, or cell, for that matter) in a good condition: a stable metabolic state. On the other hand, animals (or any living system) are exposed to varying environments, more or often less friendly to their, regarding temperature, moisture, chemical composition, and indeed microbial population.
In order to achieve internal stability within such a strongly fluctuating environment, all living organisms employ regulatory mechanisms. To biologists, these are known as homeostatic mechanisms, technicians are used to calling them control systems (Doucet and Sloep, 1993).
The system presented in the block diagram of Figure 4.5 may be translated into a differential equation:

$$V\frac{dC_{\text{out}}}{dt} = -\Phi C_{\text{out}} + \Phi C_{\text{in}} - \kappa V\left(C_{\text{out}} - C_{\text{ref}}\right)$$

with

V volume of the compartment

Φ flow rate

κ rate constant for the control signal

C_{in} concentration of the compound at the input

C_{out} concentration of the compound within the compartment,

which may be written as:

$$\frac{dC_{\text{out}}}{dt} = \frac{\Phi}{V}\left(C_{\text{in}} - C_{\text{out}}\right) - \kappa\left(C_{\text{out}} - C_{\text{ref}}\right)$$

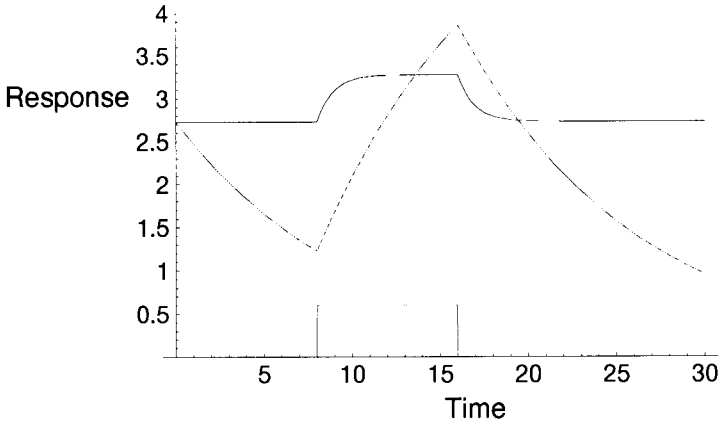


Figure 4.6: Step response for a hypothetical first order system, without feedback control (strongly fluctuating 'sawtooth' response) and with feedback control, showing the decrease in amplitude of fluctuations in the regulated variable, and the tendency to keep the output at a constant level.

Typical solutions to a step change in input concentration are given in Figure 4.6. Note that without feedback control, the output tends to drift, here towards zero. Doucet and Sloep (1993) point out that a 'sharp' set point may be rarely found in living systems. Nevertheless, the general concept seems a satisfyingly simple representation of a very common mechanism.
Whenever interactions between pathogenic micro–organisms and host defenses are to be represented in a model, the possibility that control loops are involved, should be considered. As demonstrated above, this means that relations between variables may involve "covert" system components whose actions cannot be studied separately. Control loops may involve strongly nonlinear components: the feedback signal in the example given above could also be activated only when the deviation from the reference level exceeds a certain threshold. Finally, the existence of feedback loops raises the issue of system stability. An extensive body of literature exists on these issues.

4.6 Temporal effects

When pathogenic micro–organisms enter a host, an immune response is mounted to inactivate the entered pathogens and to prevent them from colonizing the host. Viewed from the position of the invading pathogens, we may elaborate as follows: Suppose, a pathogen enters the host. It may be killed by the hosts defense systems (a.o. the immune system). Suppose, now, that this latter process is described by a hazard function

$$h(j) = aj$$

where j is the number of organisms that has entered (to be detected by the defense systems), and a some proportionality constant.
The hazard function may be seen as representing the probability of death (of the pathogen) between time t and $t + dt$, provided it was still living at time t. In formula:

$$h(t) = \frac{f(t)}{1 - F(t)}$$

The survivor function $F(t)$ gives the probability that the organism has died by time t. The derivative of the survivor function $f(t)$ then represents a probability density, of dying between time t and $t + dt$.
A constant hazard function (with respect to time) leads to an exponential survivor function:

$$F(t) = 1 - e^{-ajt}$$

When, at $t = 0$, j organisms have entered the host, the probability of survival at time τ is:

$$e^{-aj\tau}$$

The probability that not one of those j organisms survives at time τ then becomes

$$\left(1 - e^{-aj\tau}\right)^j$$

and the probability of at least one surviving organism is

$$1 - \left(1 - e^{-aj\tau}\right)^j$$

When organisms are sampled from a Poisson distribution (Furumoto and Mickey, 1967)

$$\text{prob}(j;\mu) = \frac{\mu^j e^{-\mu}}{j!}$$

For any j the probability of survival of at least one organism becomes

$$\sum_{j=1}^{\infty} \left[1 - \left(1 - e^{-aj\tau}\right)^j\right] \frac{\mu^j e^{-\mu}}{j!}$$

This may be simplified somewhat by noting that the term with $j = 0$ may be included without contribution to the result

$$\sum_{j=0}^{\infty} \left[1 - \left(1 - e^{-aj\tau}\right)^j\right] \frac{\mu^j e^{-\mu}}{j!} = \sum_{j=0}^{\infty} \frac{\mu^j e^{-\mu}}{j!} - \sum_{j=0}^{\infty} \left(1 - e^{-aj\tau}\right)^j \frac{\mu^j e^{-\mu}}{j!}$$

$$= 1 - \sum_{j=0}^{\infty} \left(1 - e^{-aj\tau}\right)^j \frac{\mu^j e^{-\mu}}{j!}$$

Unfortunately enough, this expression cannot be easily simplified to a formula suitable for fitting to dose response data. If we assume, however, that the hazard of being killed by the host defenses is proportional to the mean dose

$$h(\mu) = a\mu$$

the expression

$$1 - \sum_{j=0}^{\infty} \left(1 - e^{-a\mu\tau}\right)^j \frac{\mu^j e^{-\mu}}{j!}$$

may be simplified

$$= 1 - \sum_{j=0}^{\infty} \frac{e^{-\left(\mu - \mu e^{-a\mu\tau}\right)}}{e^{-\left(\mu - \mu e^{-a\mu\tau}\right)}} \frac{e^{-\mu}}{j!} \left(\mu - \mu e^{-a\mu\tau}\right)^j$$

$$= 1 - e^{-\mu} e^{\left(\mu - \mu e^{-a\mu\tau}\right)} = 1 - e^{-\mu e^{-a\mu\tau}}$$

If the probability that a surviving organism causes infection is not 1 but smaller (relative infectivity b), then, analogous to Furumoto and Mickey (1967) and Haas (1983), this relation becomes

$$g(\mu, \tau; a, b) = 1 - e^{-b\mu e^{-a\mu\tau}}$$

How should this relation be interpreted?
At the time of exposure, $\tau = 0$ we have the familiar exponential dose response formula:

$$g(\mu, 0; a, b) = 1 - e^{-b\mu}$$

After a long period of time, we get zero:

$$\lim_{\tau \to \infty} g(\mu, \tau; a, b) = 0$$

For $\tau \neq 0$ there is a maximum at $\mu = 1/a\tau$.
If infection takes place immediately after exposure (ingestion of a contaminated sample), without delay, all of the ingested organisms may take part, and the normal exponential dose response relation is valid. If however there is some delay between exposure and infection (i.e. actual attachment, growth, colonization only starts after a certain period of time), the above double exponential relation would apply.
In order to be useful for practical purposes, this model needs working out in some more detail:

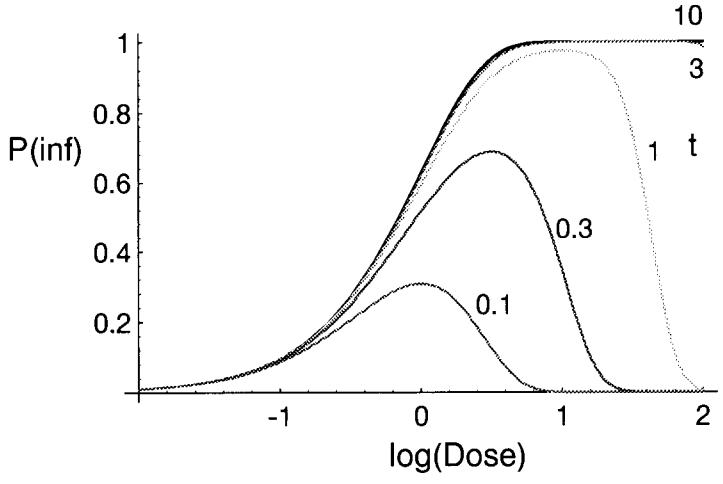


Figure 4.7: Set of dose response functions for the 'pathogen hazard' model. a = 0.1, b=1.0, τ 0.1 to 10, increasing with a factor $\sqrt{10}$.

- implement variation in infectivity, analogous to the Beta Poisson model
- implement variation in time of infection, for instance by using a lognormal distribution, since many reports indicate lognormally distributed incubation periods
- working out the first case, where the actual ingested number of organisms is used, instead of their mean number

Chapter 5

Concluding remarks: experiments and modelling

In this survey, the barriers to infection and illness caused by gastroenteric pathogens have been identified. Furthermore, several opportunities for dose response modelling have been presented. In chapter 4 onsets have been given for carrying on from the Beta Poisson model. Many things have been left out: improvement of fitting methods for small data sets where the standard likelihood based methods are invalid, methods for generalization across strain or even species limits, use of predator–prey models for the interaction between pathogens and host defense, etcetera.
The Beta Poisson model is based on a fairly general set of assumptions, and appears to be well suited to describe the majority of known results from human feeding studies (Haas, 1983; Teunis et al., 1996b). If we want to proceed beyond this model, by extending it or by formulating completely new alternatives, we need experimental data as a basis.

5.1 Animal

In addition to the knowledge on human responses to gastro–enteric pathogens, experimental data from animals may provide valuable information for the improvement of dose response models. Animal experiments offer a number of advantages compared to experiments with human volunteers.
Firstly, for the development of models based on physiological characteristics of gastro–enteritis, detailed information on the changes occurring upon and after infection is necessary. These may be studied in an animal, where biopsies or autopsies may provide insight into qualitative but also quantitative effects elicited by pathogenic micro–organisms in the intestinal epithelium.
Secondly, limits on the numbers of experimental subjects are less stringent in animal experiments, than in those with human volunteers. Therefore, analysis may

involve less uncertainty, and greater discriminative power against mismatch between the model and the experimental data.
Thirdly, comparison with the existing human dose response models may give interesting insights into the influence of host factors on the dose response relation.
A potential problem to be solved is that of how to translate animal dose response relations to humans. This is where the great value of the existing data base of human dose response relations lies: in contrast to many problems in toxicology, we have at our disposal a set of reference parameters, to compare with the results from animal experiments. By considering only relative effects, for instance the shift in dose response parameters in aged animals compared to young adult animals, a plausible estimation of similar effects in humans may be found. In immunotoxicity research, a methodology has been developed to extrapolate results from animal experiments to humans, based on similar reasoning (Selgrade et al., 1995). The so–called parallelogram approach may aid in the extrapolation of animal data to man (Garssen et al., 1996; van Loveren et al., 1997).

5.2 Human

There clearly is great need for human dose response data, and it is equally clear that we will not have them at our disposal, since such experiments are so hard to perform, and so expensive. Only the pathogens deemed most significant (by the scientific medical community or public opinion) have a chance of receiving this kind of attention. That is, if their morbidity is not too high, so that the risks of the exposed volunteers are not unacceptably high.
Whenever there are new insights, existing dose response data may be studied again. In addition to the actual dose response data, experimental literature often contains lots of extra observations on duration and severity of symptoms, incubation period, excretion patterns, etcetera. All these may be interpreted with the right model, should this become available.
Another aspect that requires some attention is the statistical analysis of variation in dose response relation (parameters) among both micro–organisms and hosts. Preliminary tests indicate that some generalization is possible, at the level of bacterial strains, and serotypes (Fazil, 1996; Teunis et al., 1996b, 1997a). These studies indicate that data from different experiments may be pooled to yield a single dose response relation. Practical problems usually concern assessment of the risk of untested batches, or new strains, or even unknown variants of a pathogenic species. Often, one would like to make a statement on the dose response properties of such an organism, when the only information available consists of a single incident. For instance, a (small) number of persons becoming ill after eating contaminated food. Usually, even the exposure is poorly characterized in such cases. In such cases, where frequentist statistics do not offer much perspective, a Bayesian approach may be useful, by opening the opportunity to use both existing knowledge on related species and data from the incident as priors. Some practical problems still

need to be solved, but it is hoped that this area of research will receive attention in the future, so that formalisms on how to deal with such complicated problems will become available.

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