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ROLE OF INTESTINAL MICROFLORA AND THE IMMUNE SYSTEM OF MOTHER MICE IN THE DEVELOPMENT OF A "WASTING SYNDROME" IN THEIR CONGENITALLY THYMUSLESS OFFSPRING

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THE IMPORTANCE OF THE COLONISATION RESISTANCE AND THE IMMUNE SYSTEM FOR THE DEVELOPMENT OF NORMAL DEFENCE

Results of several of our studies performed in last two decades regard the development and value of the intestinal colonisation resistance (CR) for baby mice as well as its 'clinical' consequences in this animal species. Before weaning the CR has been found to be low in comparison with adult animal but still of great importance to the health and survival of neonatal mice. The interaction between the intestinal microflora (IMF) and the host's immune system may start right after birth. At the site of the immune system, particularly in the first weeks, the thymus may play an important role in the development of the IMF. The role of the thymus in the maintenance of the IMF was also studied directly (*van der Waaij*, 1986) as well as indirectly in mice (*van der Waaij*, 1984).

In sequence, the following items involved in intestinal colonisation since birth will be discussed:

1. The development of:
   a. an IMF in adult ex-germfree and new-born mice,
   b. the CR (for *E. coli*) after birth in conventional mice,
   c. the CR in congenitally athymic mice after birth to illustrate the role of the thymus.
2. The broad spectrum of influences of a (foster-)mother on the development of an IMF in offspring involving:
   a. the importance of a functioning thymus in the dam,
   b. the quality of the dam's IMF regarding the CR for opportunistic/pathogenic bacteria.
3. The possible role of the B1-cell derived polyspecific IgM system
   a. idiotype directed interactions with other immune cells,
   b. apparent role of anti-idiotype in the clearance of translocated bacteria and cell debris.

At the end of this review, three working-hypothesis concerning the development of an IMF in baby mice and the apparent role of their immune system, modulated by their mother's immune system, will be presented. These hypothesis are meant as a lead to further study and understanding of this subject which forms the basis to our response to environmental antigens; a Research Priority of the International Study Group on New Antimicrobial Strategies (http:www//isgnas.org/).

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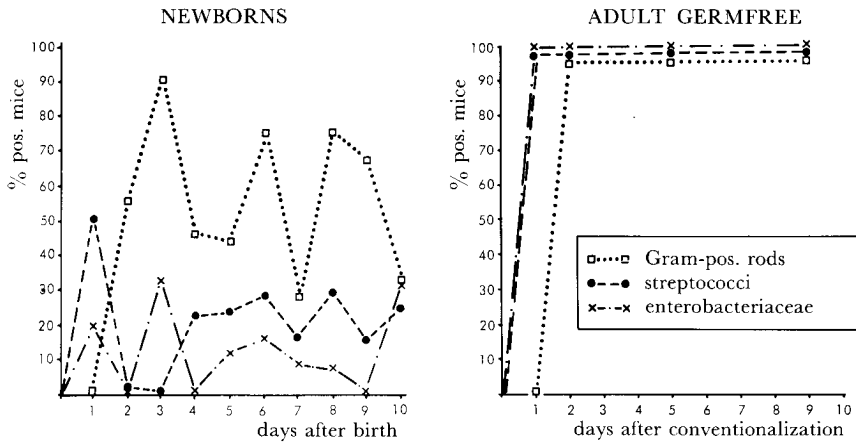


Figure 1: Development of faecal microflora upon conventionalisation of new-born and adult euthymic mice (*van der Waaij*, 1968).

THE INTESTINAL TRACT ENVIRONMENT SHOWING DIFFERENCE BETWEEN NEW-BORN AND ADULT GERMFREE MICE

At birth, baby mice are - like the adult ex-germfree (ex-GF) counterparts - entering into a conventional environment coming from a germfree location. The colonisation pattern of 'pre-weaning' and 'adult' ex-GF mice upon conventionalisation, has been studied in the past and may be of importance to our insight in the 'normal' development of an intestinal microflora in mice since birth (*van der Waaij*, 1968).

The effect of such *physiologic* (post-birth) and *experimental* conventionalisation (ex-GF) of mice regarding faecal flora development, are shown in Figure 1.

Relevant conclusion:

The CR of baby mice, as far as the bacterial groups studied in the faecal flora (see insert in the figure) are concerned, was found significantly higher than that of adult ex-germfree mice. The results show that at conventionalisation the IMF develops very different in both age groups. This difference could partly be ascribed to the low transit time in the adult GF-mouse which exists until an intestinal microflora has colonised the gut (*van der Waaij* et al., 1974). Another, possibly more important, difference between the two 'kinds of originally germfree mice', however, concerns the difference between:

a. contamination source
b. the 'nutritional environment' for bacteria in the gut

The contamination source:

Baby mice are physiologically (also in this experiment) predominantly contaminated by their mothers and get mother milk as the only (selective?) food source, while the adult ex-GF animals in this experiment received conventional food pellets and will have become contaminated by all kinds of sources in their conventional environment.

The nutritional environment:

It is likely, that the intestinal (meconium) contents at birth, as well as the mother-milk diet, both provide

physiologically a 'selective environment' to bacteria which come in contact with the new-borns and are ingested.

EXPERIMENTAL STUDY OF COLONISATION RESISTANCE FOR AN *ESCHERICHIA COLI* STRAIN IN BABY MICE

New-born mice have been orally contaminated with a streptomycin resistant strain of *Escherichia coli* (SR-*E. coli*) shortly after birth. A dose of about 10^6 SR-*E. coli* (a loopful of 10^9 bacteria/ml) was given orally to 20 litters of ND2 mice. Every other day, two litters (15-21 animals) were sacrificed to determine their SR-*E. coli* concentration in their colon (*Heidt* and *van der Waaij*, 1979). The 95% confidence limits of the mean concentrations found are presented in Figure 2.

Although not exactly following the formula proposed for adult animals (i.e. faeces of 50% of the mice negative by day 14), the curve shows significantly lower CR-values in the first three weeks than after four weeks and at 42 days. By day 42 the CR appeared comparable to what was found in earlier studies in adult conventional ND2 mice (*van der Waaij* et al., 1971).

Relevant conclusions:

1. In the first 3 to 4 weeks after birth, the CR for *E. coli* was low compared to adult values. During this 'low CR period', colonisation by environmental bacteria, predominantly coming from the dam, may have been enhanced compared to later on.
2. If 'intestinal tolerance', developing before and after birth (*Strobel* and *Ferguson*, 1984; *Fazekas de St. Groth* et al., 1984; *Zöller*, 1988) plays a role in the maintenance/stability of the IMF, it may develop in the first three to four weeks; i.e. in the "instruction phase" of the intestinal immune system.

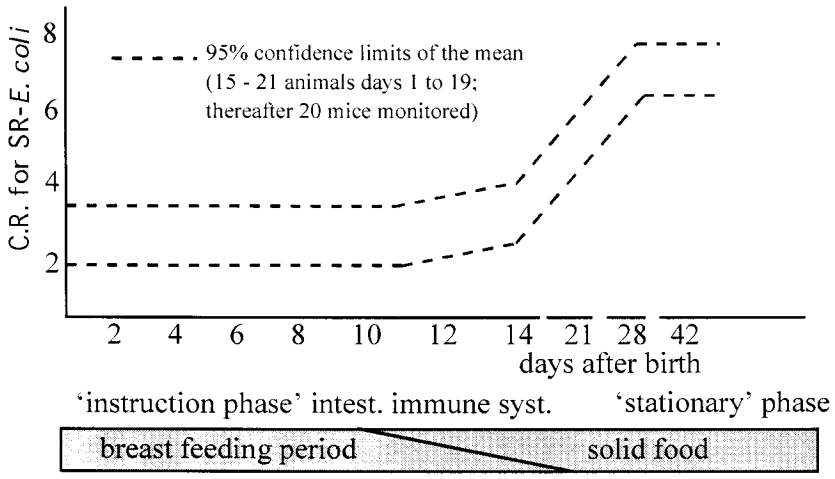


Figure 2: The development of the colonisation resistance for SR-E.coli of the digestive tract since birth (*Heidt* and *van der Waaij*, 1979).

3. If antibodies (IgG?; IgA?) play a role in the selection of intestinal bacteria ('rejection') in the first three weeks, it may occur predominantly by *passively acquired* milk-antibodies. Later, in the "stationary phase", the immune system of the young animal may take over this IMF-controlling function.

THE DEVELOPMENT OF AN INTESTINAL MICROFLORA IN CONGENITALLY ATHYMIC MICE

Both studies reviewed above make likely that one or more a factors in the intestinal environment/diet largely determine the colonisation pattern of the gut at conventionalisation; the microflora of mice differs strongly in composition during their first week of life and at weaning. This difference in microflora between (euthymic) baby and adult mice has been described by various authors but perhaps first by *Schaedler* and co-authors (1965). The development of the IMF of congenitally athymic mice maintained under different hygienic circumstances (conventional and SPF[1]) has unfortunately not yet been studied in greater detail. However, in mice, indirect evidence is available that the presence or absence of a thymus at birth is not of great importance to the developing IMF. Before weaning, the IMF of the athymic mouse may not differ significantly from its euthymic counterpart. This assumption is based on a study by our group performed in the late seventies, in which the CR of congenitally athymic baby mice was measured by biotyping of *Enterobacteriaceae* species (*van der Waaij,* 1981).

When congenitally athymic *nude* (*nu/nu*) Balb/c mice were maintained conventionally, they developed signs and symptoms of 'wasting disease' (weight loss, diarrhoea and hunched back) from about the sixth week of the experiment when they were 9-10 weeks of age. Results of biotyping of *Enterobacteriaceae* species revealed in those first weeks a quite normal 'turnover' of biotypes (a mean of 2 different types per mouse per week). After six weeks, however, the number of different biotypes per mouse increased to four and later even to six different biotypes per sample in weeks 8-12 of the experiment. All mice died between day 94 and 203 of life. However, in case *nude* (*nu/nu*) mice were born and maintained under strict hygienic SPF-conditions, no evidence of wasting disease was seen and 100% of the animals were still healthy at day 203; i.e. when the last conventionally maintained *nude* mice had died. The *Enterobacteriaceae* species present in their IMF remained constant in concentration and biotype during the subsequent approximately 200 days of observation. In the faecal samples, which were initially collected weekly, the same two *E. coli* biotypes were isolated in 'normal' concentrations and no new *Enterobacteriaceae* biotypes were seen. These results are depicted schematically in Figure 3.

Relevant conclusions:

1. The microflora at weaning in the athymic offspring of euthymic mice, maintained in the 'conventional environment', will have consisted of many different bacterial species which contributed to their CR. The

[1] Specific pathogen free

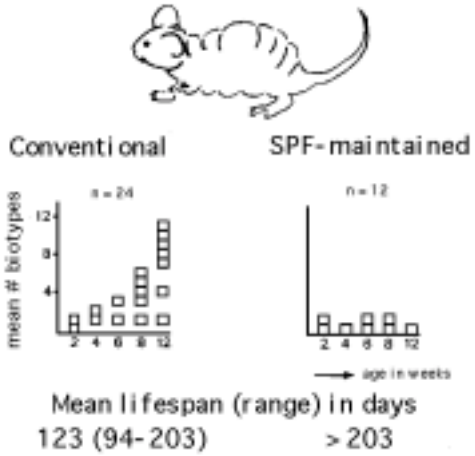


Figure 3: Development of intestinal CR in congenitally athymic mice (*van der Waaij*, 1981).

bacteria in the offspring originated from the dam's IMF. After weaning, however, the control by the mother-milk factor (IgG?, IgA?) stopped. Some of the newly ingested bacteria, coming from exogenous environmental sources other than the dam, may from then on have found in the *nude* offspring a good niche in the intestinal tract for colonisation (no effective control by immune system). Some of those 'new bacteria', may gradually have taken the place of previously installed 'protective strain(s)' in the sense of the CR. They thus may have caused the strong decrease of the CR found in these animals by about the sixth week post weaning.

2. A decreased CR in the athymic offspring, still maintained in a conventional environment, may have led to high concentrations of newly ingested opportunistic bacteria such as *Enterobacteriaceae* species. These opportunistic microbes may have translocated; a deficient (poly-specific) IgM spectrum (see point 7) may have enhanced lectino-phagocytosis and complement activation and thus caused inflammatory responses in the submucosa and other places (multi-focal) where these translocating bacteria (or parts of them) landed. Such multi-focal (chronic) inflammation may have caused the 'wasting disease' (diarrhoea, and weight loss).

EVIDENCE SHOWING THAT THE *SEVERITY* OF AN 'EARLY FORM' OF WASTING DISEASE IS DETERMINED BY THE IMMUNE SYSTEM OF THE LACTATING DAM

Croy and *Osoba* (1973) have described wasting disease in congenitally athymic mice which were obtained by mating *nude* (*nu/nu*) mice in various different combinations of athymic and euthymic males and females. No information is available about the IMF. However, it is very likely that these mice, being maintained conventionally, did have a decreased CR.

Table 1: Wasting disease following mating of homozygotic and heterozygotic *nude* (*nu/nu*) mice; offspring 50% *nude* (*nu/nu*) (*Croy* and *Osoba*, 1973)

Male	Female	Fostering by	Survival at weaning
nu/+	*nu/nu*	*nu/nu* mother	high mortality
nu/+	*nu/nu*	*nu/+* foster mother[1]	low mortality
nu/nu	*nu/+*	*nu/+* mother	low mortality

[1] Humeral and cellular immune factors in the milk (*Nepommaschy* et al., 1988).

The results of their study, shown in Table 1, make likely that (an) immune factor(s) in the milk (antibodies in the euthymic *nu/+* dams?) may have been responsible for the outcome of the experiment. It is conceivable namely, that opportunistic bacteria ingested by the baby mice during the lactation period, came predominantly from the dam (*Nepommaschy* et al., 1988):

In the *nu/+* fostered mice, these bacteria may have been controlled by (IgG/IgA) antibodies from their mothers and thus controlled translocation of opportunistic bacteria.

In the *nu/nu* fostered athymic offspring, the high 'early wasting' and mortality may have been caused by:

1. A deficient IMF coming from the dam (low CR permitting opportunistic bacteria to 'take' and 'overgrow'), or/and
2. Absence of absorbable antibodies (IgM?) in the milk, which are normally associated with a rapid tissue clearance. This is assumed because the dam may have had 'gaps' in her poly-specific B1-cell system as she most likely originated from a *nu/+* mother.

Perhaps both factors were involved as:

1. A low CR may have caused 'intestinal overgrowth' by opportunistic bacteria and thus their translocation,
2. In the absence of relevant (anti the corresponding Id polyspecific IgM) antibody, *lectino-phagocytosis associated with complement activation*, may have been responsible for an acute intestinal inflammatory response, diarrhoea and the wasting. The response being directed anti-translocating bacteria and/or bacterial fragments or other translocating antigens.

BREEDING WITH EUTHYMIC EX-GERMFREE MICE ASSOCIATED WITH A COW-IMF CAUSING A LOW CR

To study the importance of IMF-controlling antibodies in the milk, *Geertsema* et al. (1990) have attempted to produce mice with a long lasting low CR. To this end, these authors associated C3H ex-germfree mice with microfloras of six different animal species. For this purpose the ex-germfree mice were maintained in six different isolators. It was hoped to obtain mice with a low CR to components of their microflora, such as *E. coli*. In adult ex-germfree mice, an uncontrolled intestinal colonisation might cause translocation of 'overgrowing' bacteria and induction of immunity rather than immunologic intestinal tolerance. After mating, the offspring might also have got the low CR of their mother's IMF. If factors in the milk, such as antibodies, play an essential role in the control of the composition of the IMF in baby mice, this

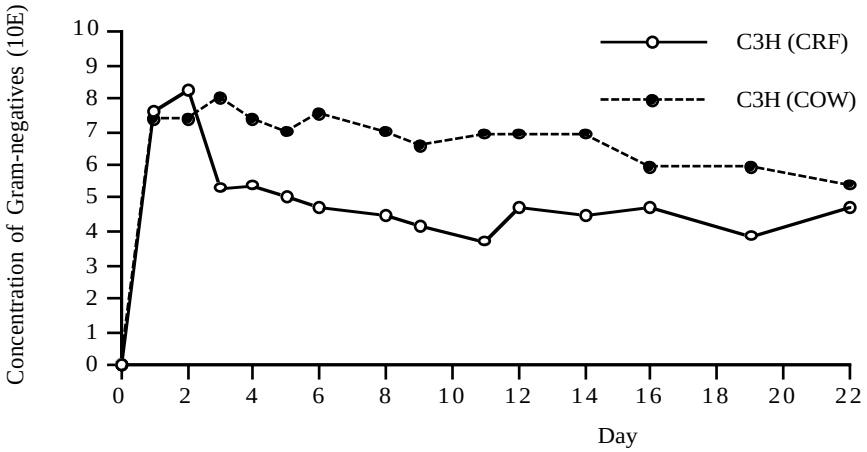


Figure 5: Concentration of *E. coli* in the faeces following oral association with cow faeces in adult ex-germfree mice (*Geertsema* et al., 1990).

might become apparent in these baby mice.

It was found, that the faecal flora of a cow fulfilled their requirement; e.g. that the ex-germfree should have a low CR for *E. coli* before, during and after pregnancy (Figure 5). A low CR in these cow-IMF mice was in accordance with the low morphologic diversity of bacteria in their faeces. Serum antibody titres to faecal bacteria or *in vivo* coating were (unfortunately) not determined.

Five female cow-microflora mice, maintained inside a germfree isolator, were mated with cow-IMF males three weeks after association. All mice became pregnant: one litter died soon after delivery and four out of four died in the second and third week of life; i.e. before weaning. At death, these young animals had diarrhoea and strong growth retardation. At autopsy, their *E. coli* concentration in the colon was 10^8/g; e.g. about four logs higher than in the mice with a normal IMF such as those employed in the study depicted in Figure 2. In the thymus, microscopically a thin cortex was found with areas showing destruction by dendritic cells, in the colon signs of inflammation were seen. The dams as well as the control mice which were not mated but remained clinically healthy.

Relevant conclusions:

1. In this experiment, the cow faecal microflora was clearly not 'thriving' in the mouse gut. Upon association, a poor protective IMF (low CR) developed in comparison with the control group associated with a faecal mouse flora (CRF) (*van der Waaij* et al., 1977). The low CR caused by the cow faecal flora, may have permitted (significant?) translocation of the *E. coli* strain (and other bacteria?) thereby inducing an immune response to this bacterium. The latter (IgG antibodies?) may have contributed to the decrease of the *E. coli* concentration of two logs found in the course of three weeks of observation (Figure 5).
2. A poor ecosystem of the gut in the pregnant mice may establish in the offspring causing an *extremely low* CR in such baby mice.
3. Euthymic dams, with an *E. coli* strain, but few bacteria in their IMF forming a (low) CR to *E. coli*, could

become pregnant and delivered an offspring. However, all these baby mice died well *before* weaning because of severe diarrhoea and wasting disease.

4. On the basis of results reported in Table 1 and those reported above about the cow microflora-associated mice, this *pre-weaning wasting disease* developed regardless the presence of a thymus in the nursing/fostering animal as well as in the offspring.
   Note the difference with *nu/+* mice, which had passive IgG-antibody protection and developed 'late' wasting disease. This may be due to the normal CR in these mice associated with a low translocation rate of potentially pathogenic bacteria if any.
5. A low CR to the *E. coli* strain may have given it the opportunity to colonise the offspring's intestines in high numbers and translocate. Strong translocation of the *E. coli* and (or other opportunistic bacteria), may have induced immunity and therewith an acute inflammatory response in the gut mucosa and wasting disease.
6. During foetal life, their Id-IgM producing B1-cell clone - mimicking *E. coli* (and other opportunistic bacteria?) - may have been suppressed/eliminated. As a result the polyspecific anti-*E. coli* IgM production may have stopped (see point 7) giving way to the complement activating IgG from the dam (and their own euthymic immune system?).

THE APPARENT ROLE OF THE POLYSPECIFIC IGM B1-CELL SYSTEM AND THE THYMUS DEPENDENT B2-CELL SYSTEM IN DETERMINING THE COMPOSITION OF THE IMF

The studies reviewed so far, indicate that clearance of bacteria, which in the presence of a normal CR translocate in low numbers (physiologic translocation), occurs without signs of inflammation. Even in the athymic mouse, when the turnover of new *Enterobacteriaceae* biotypes is maintained low, no inflammation associated with diarrhoea occurs. This implies that in the clearance of bacteria from tissues, their poly-reactive B1-cell system may play a role. However, the innate defence of the congenitally athymic animals, the lectino-phagocytosis, may involve complement activation and therewith inflammation; particularly when a 'gap' exists in the poly-reactive IgM. A brief review of publications relevant to our hypothesis follows.

Establishment of some of the B-cell clones, which constitute the adult bone marrow derived B-cell repertoire, appears facilitated and guided by *'idiotype-directed' interactions among complementary sets of B-cells early during ontogeny*. *In vivo* experiments, reported by *Elliott* and *Kearny* (1992) it has been shown that the program of B-cell development, involving so-called 'idiotypic interactions', may be obligatory in the development of certain B1 cells that provide opsonic activity against antigen (bacterial) translocation. This program of B-cell development is further modulated/facilitated in newborn mice during lactation. In adult mice, which have been transplanted with progenitor cells from adult bone marrow, it is absent. Thus the 'idiotype-directed selection' of the adult B-cell repertoire may be limited to foetal-neonatal stages of development.

The B1-cell system and the (T-cell

controlled) B2 cell system, both develop during foetal life. Interaction between both systems may have positive consequences in case a pathogenic micro-organism is involved: If a pregnant host experiences a serious infection by a pathogen, she normally develops high titres of specific IgG antibodies in response to the micro-organism involved. Because of its small size, IgG passes through the placenta and in the foetus, where it may suppress/eliminate the 'internal images' (idiotypic antibodies) of the microbial antigen(s) involved. The degree of 'internal image' suppression would be an IgG titre related process. Normally, the production of 'internal image' would be regulated by its IgM antibody counterpart: the anti-idiotype in the foetus and new-born. Id-producing B1-cell clones would disappear when strongly affected by IgG. As a result, anti-Id IgM production is no longer stimulated neither by the Id-IgM nor by the *original* (microbial) *antigen* when it disappears as it gets cleared from the foetal circulation and its tissues. In the absence of antigen and Id-antibody, the no longer stimulated anti-Id B1-cell clone may also disappear. The result of such clonal deletion will be a 'gap' ('functional opening') in the Id-network (*Fougereau* and *Schiff*, 1988; *Martinez-A* et al., 1983). This hypothetical condition, that a 'gap' in the "Id-network", would be of advantage to the new-born if a pathogen affects the mother and may contaminate them. A 'gap' in the Id-network would provide the possibility to an immediate response to the pathogen; i.e. in a *conventional T-cell/B-cell controlled fashion* including inflammation at the meeting place.

If the thymus-controlled part of immune system of the mother plays a role in the control of her own IMF and that her offspring, it may occur either by:

1. induction of intestinal tolerance (acceptance), or
2. production of specific antibodies (IgG/IgA?; rejection?).

Maternal modulation of the immune system of her offspring during pregnancy and/or lactation may occur, as mentioned before, by subsequent deletion of Id- and anti-Id B1- clones. Sufficiently high titred specific antibodies (IgG) produced by the mother to bacteria (components of her own IMF) could thus 'prepare' the immune system of the foetus/new-born for a specific (IgG) response.

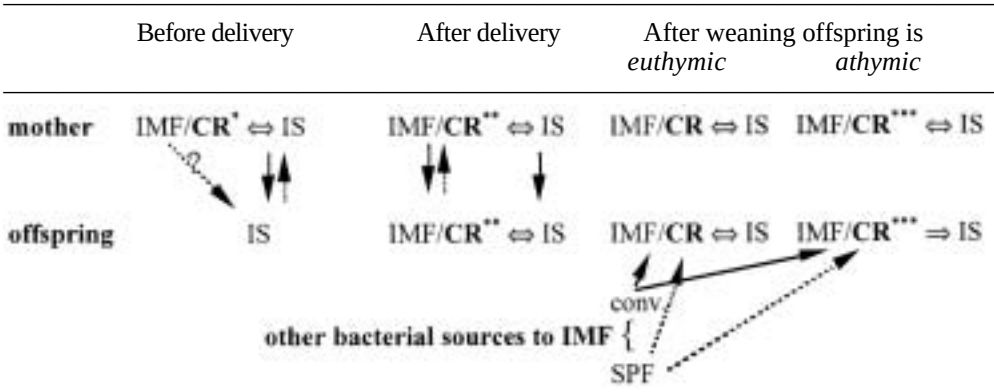
If IgG/IgA plays a role in the control of IMF, it may make survival of bacteria at the site difficult. The bacteria in question may get selectively 'suppressed' and thus in disadvantaged position in comparison with bacteria with no antibodies coating them.

Because naïve B1-cells that lack co-stimulatory molecules, could play a role in the development of 'intestinal tolerance' (*Brandtzaeg* et al., 1999), these cells may play an important role in the 'acceptance' of bacteria forming the IMF early in life. B1-cells would present antigens of Gram-negatives by binding to endotoxin and presenting the polysaccharide part to helper T-cells.

These hypothesised interactions, the role of the poly-specific IgM (B1-cell) system and that of the thymus, require confirmation and prospective studies. Interactions requiring further investigation, are summarised in Table 2.

On the basis of data presented and the assumption that idiotypic (Id) network by B1-cells exist to play a role in the modulation of the foetal/new-born immune system, the two different 'forms' of wasting disease, the "early" and the "late" form, caused by different mechanisms, could be understood:. The pathogenesis of both forms occurring respectively before weaning (acute early form) or after weaning (chronic late form) is hypothesised in Table 3.

Table 2: A hypothesis concerning the influence of mother mice on the defence (CR + immunity) of her offspring



* : If the CR is high the anti-IMF IS-reactivity is low.

** : If the CR is significantly decreased, opportunist. microbes may establish in high numbers, translocate, induce antibody response and cause diarrhoea and wasting.

*** : Mothers with a decreased CR do not or poorly reproduce. Mothers with a normal CR give rise to a litter with a normal CR at weaning. After weaning however, the CR may decrease and over growth by opportunistic bacteria may occur associated with diarrhoea and wasting disease.

IS : Immune system

PROPOSED WORKING HYPOTHESIS

Generally known and information in the previous sections is used to formulate relevant facts forming the basis of working hypothesis:

1. In conventional mice, contaminations from environmental sources may vary between different locations and differ from time to time in severity. Such oral contaminations can occur daily and may involve all kinds of micro-organisms; they may range from pathogenic (normally rare), via potentially pathogenic to non-pathogenic. The newly ingested microbes may well or not be able to use nutritional sources available inside the gut, settle and 'thrive' when not hindered by other bacteria at the site or by the host (immune system?). In the first two weeks of life, a different set of bacteria 'thrives' and predominates in the gut environment than after weaning.

Hypothesis: *If newly ingested bacteria 'thrive' in the intestines, they may/or not contribute to the CR to subsequently ingested bacteria/yeasts.*

In case an individual gets colonised with bacteria which are of low or no value to the CR but are *not hindered by the CR-microflora* and thus 'thrive', they may stay for a long period (if not 'rejected by immune system?). If such bacteria with no contribution to the CR can take over positions of valuable (CR-active) others, this may indirectly become harmful to the host as it may decrease the CR.

In a conventional environment, an individual with a low CR can easily get colonised with quite a number of different opportunistic

Table 3: Schematic presentation of development of "early" and "late" wasting disease

	Early wasting (before weaning)	Late wasting (after weaning)
During pregnancy + lactation anti-Id:	Deletion Id-B-cell clone? thereby anti-Id IgM decreased?	
Factors involved:	1. Low CR + *high anti-Id IgG* in dam and offspring	1. In athymic mice low CR (in *conv*. maintained)
	2. Complement activating anti-Id IgG antibodies bind to translocating opportunistic micro-organisms	2. High concentration and translocation of opportunistic micro-organisms cause lectino-phagocytosis + complement activation

(and pathogenic?) micro-organisms, colonising the gut in high concentrations. Translocation (in high numbers?) of such 'overgrowing' opportunists, is to be expected. If such individuals fail to clear these translocating micro-organisms in the normal (physiologic) way, i.e. without inflammation, they may develop acute/chronic infection in the gut wall as well as in more remote organs.

2. Particularly in the first weeks of life, 'thriving' bacteria may induce '*intestinal immunologic tolerance*'. This tolerance induction may most likely occur in the Peyer's patches following attachment and translocation during their transit through the (small) intestines.
   Hypothesis: Intestinal tolerance could imply that the bacteria involved get 'accepted' by their host so that they can stay if they 'thrive' in the intestines. Else, if immunity is induced, regardless the fact that they can 'thrive', specific antibodies may bring them in a disadvantaged position to other bacteria as they get gradually 'rejected' from the gut (see point 3).
3. In euthymic mother mice with a normal IMF (normal CR), the CR in their offspring, although initially low (but higher than ex-GF), is sufficient to guarantee development of a 'normal' IMF with a normal CR.
   Hypothesis: Antibodies (IgG) circulating in the dam during pregnancy and after delivery (such as IgG and IgA? secreted with the milk), may:
   1. Modulate the B1-cell population producing Id and anti-Id antibodies.
   2. Control the composition of the intestinal population in the offspring.

   Only CR-associated components of IMF of the dam, for which she is immunologically tolerant, may be able to take and 'thrive' sooner or later in the young during the preweaning period. Antibodies present in the intestines since birth and directed to (immunogenic) bacteria which are passing through the intestinal tract of the dam, may function as a 'sieve'. Such antibodies though initially originating from the dam may later, when intestinal tolerance is no longer the major type of response, be formed by the immune system of the offspring.

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